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SIGNAL RESPONSE TRANSDUCTION IN RABBIT NEUTROPHIL LEUCOCYTES

THE EFFECTS OF EXOGENOUS PHOSPHOLIPASE A2 SUGGEST TWO PATHWAYS EXIST

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Abstract—Rabbit neutrophils stimulated by chemotactic peptide (fMLP) or phorbol ester (PMA) respond with a metabolic burst which can be assayed by following luminol-enhanced chemiluminescence. Depending upon the agonist used, exogenous bee-venom phospholipase A2 (PLA2) will enhance or inhibit the response. Neutrophil activation by fMLP is enhanced by PLA2 or by the addition of arachidonic acid, but unaffected by lysophosphatide. The cellular response to PMA is markedly inhibited by PLA2 or by lysophosphatide, though not completely abrogated, but is enhanced by arachidonic acid. The lysophosphatide inhibition overrides the arachidonic acid potentiation of the PMA-induced response. Neither PLA2 nor arachidonic acid alone will activate the cells; it seems that agonist is essential.

We interpret these results to mean that at least two signal-response transduction systems are involved in agonist-induced metabolic activation of rabbit neutrophil leucocytes.

Neutrophil leucocytes respond to a variety of signals during an acute inflammatory episode, and their responses vary depending upon the signal substance and its concentration. Two commonly used agents which activate neutrophil metabolism are the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP),* and phorbol myristate acetate (PMA)[1]. These two substances induce a variety of responses (reviewed in ref. 2). The chemotactic peptide stimulates movement optimally at 10^{-8} M, and secretion at 10^{-6} M. At high concentration it also makes the cells very adhesive [3], and their movement is poor, possibly as a result of the increased adhesion. The phorbol ester induces secretion, but only of the specific granules, and inhibits movement [4]. Given the diversity of signals and responses, it might be reasonable to expect that more than one transduction pathway exists, and it seems that PMA can act to induce secretion without altering intracellular calcium levels, whereas fMLP induces a change in cytosolic calcium levels [5]. In the present paper we show that the transduction pathways used by the cell in response to fMLP and PMA can be separated by the use of exogenous phospholipase A2 (PLA2), which stimulates the former and inhibits the latter. The mechanism for this can partly be explained on the basis of the effects of products of PLA2 activity. The effect of exogenous PLA2 has particular interest because an endogenous PLA2 has been implicated in signal–response transduction.

* Abbreviations used: Arach., arachidonic acid; BSA, bovine serum albumin; fMLP, formyl-methionyl-leucyl-phenylalanine; OAG, oleoyl acyl glycerol; PLA2, phospholipase A2; PMA, phorbol myristate acetate; PI, phosphatidyl inositol.

MATERIALS AND METHODS

Cells. Peritoneal exudate neutrophils were prepared by the intra-peritoneal injection of 0.9% (w/v) NaCl solution with 0.1% (w/v) oyster glycogen (Sigma, U.K.) into female NZW rabbits: the exudate was drained after 4 hr. Cells were stored in the exudate solution at 4° for up to two days; this did not seem to alter the ability of the cells to respond with a metabolic burst in response to agonists.

Chemiluminescence assay. Metabolic activation of neutrophils was assessed by luminol-enhanced chemiluminescence [6–8] using an LKB 1251 luminometer. The response has been quantified on the basis of the maximal chemiluminescence. Except where explicitly stated otherwise, cells were resuspended in the exudate fluid at 2×10^6 cells/ml without washing: each tube normally contained 0.5 ml cell suspension, 10^{-5} M luminol, agonist solution(s), and enough phenol red-free buffered salts solution (BSS: NaCl 8 g/l, KCl 0.4 g/l, $CaCl_2$ 140 mg/l, $MgCl_2.6H_2O$ 200 mg/l, glucose 1 g/l, HEPES 2.388 g/l) to bring the final volume to 1 ml. Although the use of unwashed cells may seem curious, activation of the cells was minimised by omitting the resuspension and centrifugation steps used in washing.

Chemicals. Phospholipase A2 was prepared from bee venom by chromatography on CM cellulose and Biogel P30 columns, a modification of the method of Shipolini [9], as described by Diaz *et al.* [10].

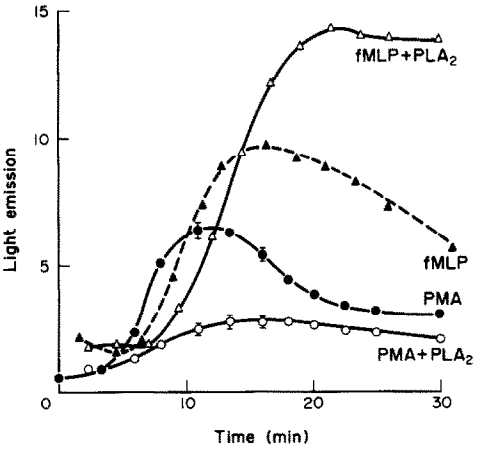

Fig. 1. The effect of PLA2 on fMLP- and PMA-induced chemiluminescence. Stimulation of cells with 10^{-7} M fMLP (△) or 1 μg/ml PMA (○) caused an enhanced chemiluminescence. Phospholipase A2 1 μg/ml, added at the same time as the agonist (open symbols), either enhanced or inhibited the response of the cells. The PMA curves are based on three replicates and the standard error bars are shown (except when smaller than the symbol). The fMLP curves are based on single samples, but the effect is similar to that seen in many experiments (Table 1).

Activation was by the procedure described by Drainas and Lawrence [11], inactivation by treatment with *p*-bromophenacyl bromide, according to the method of Roberts *et al.* [12]: activation and inactivation were assessed by conductimetric assays [10]. All other chemicals were obtained from Sigma (U.K.). PMA, oleoyl acyl glycerol and lysolecithin were dissolved in dimethyl sulphoxide (DMSO) at 1 mg/ml or higher, and diluted in BSS.

Presentation of results. Combining data from different experiments poses problems because of the large variance generated by between-experiment differences. The source of this variation remains obscure, and standard normalisation procedures do not work. The effects of different treatments were always qualitatively the same, and we have tested everything on at least three batches of cells from different animals, although data is shown only for experiments in which agonists or treatments were matched and could legitimately be combined.

RESULTS

Both fMLP and PMA induced a chemiluminescence response in the cells, a result in accord with much other published work [13, 14 *inter alia*]. The two agonists do, however, seem to act rather differently.

Effect of exogenous PLA2

Exogenous PLA2 itself did not induce chemiluminescence, but it dramatically altered the response of the cells to the two agonists. Activity induced by fMLP was markedly enhanced by PLA2, whereas PMA-induced chemiluminescence was strongly inhibited (Fig. 1 and Table 1). To test whether the effect was mediated by phospholipase activity, we inhibited the exogenous enzyme with *p*-bromophenacyl bromide [12, 15] and could show that its activity was reduced to approximately 3% of that shown by the untreated enzyme. This was consistent with the activity as judged by conductimetric assays which showed the treated enzyme to have approximately 5% residual activity. Activation of the PLA2, according to the method of Drainas and Lawrence [11], did not, however, alter its enhancement of agonist-induced chemiluminescence (96.4 ± 15% of the activity of unactivated enzyme). The effect of PLA2 was greatest at the concentration of fMLP which was most effective in metabolically activating the cells (10^{-6} M), and the PLA2 response was dose-dependent (Fig. 2), rapid (preincubation with the enzyme made no appreciable difference), and long-lasting (the effects were still obvious 1 hr after agonist addition).

When PMA was the agonist, the response, although an inhibition and not an enhancement in this case, was similarly dependent on the amount of enzyme (Fig. 2), but the inhibition was apparent at all PMA doses (Fig. 3). The inhibition was not total, and even with the highest doses of PLA2, the cells gave a greater response than in the complete absence of agonist. As with fMLP, the preincubation time with PLA2 made no difference, the effect occurred during the period between agonist addition and development of the response, some 10–12 min.

Effects of PLA2 products

fMLP-induced responses. Since PLA2 activity generates both a lysophospholipid and a fatty acid, we surmised that the judicious addition of the products alone might have a similar effect. Further, serum albumin, which sequestrates both fatty acid and lysophosphatide might inhibit the effect of PLA2 itself. The addition of bovine serum albumin (BSA) to the incubation mixture enhanced the response to fMLP very slightly, but diminished the enhancing effect of PLA2 (Fig. 4). Although PLA2 was less effective in enhancing the fMLP-induced response when BSA was present, there was still a significant increase: since the products of PLA2 will be generated in close proximity to the cell, BSA in the aqueous medium will probably not be completely effective at removing them. The addition of arachidonic acid had an effect similar to that brought about by PLA2 (Fig. 4 and Table 2), and like PLA2 had its maximal effect at 10^{-6} M fMLP: arachidonic acid alone had no effect. The effect of a variety of other fatty acids and of lysolecithin was also tested (Table 2). Provided the concentration of lysolecithin was no greater than 10 μg/ml it had no effect on the fMLP-induced response and was ineffective on its own.

PMA-stimulated responses. The PMA-induced response was also enhanced by the addition of arachidonic acid (Table 2, Fig. 3). As with the fMLP response, arachidonic acid was much more effective than other fatty acids. In contrast, however, lysolecithin markedly inhibited the PMA effect, in a dose dependent manner (Table 2). The presence of BSA in the medium enhanced the PMA-stimulated luminescence rather more than it did the fMLP-induced response, and the inhibitory effect of lysolecithin was lost if BSA was present (Table 2). Substitution of

Table 1. The effect of adding exogenous PLA2 to rabbit neutrophils treated with PMA or fMLP

Agonist	Chemiluminescence (as % of value for agonist alone ± SEM) +1 μg/ml PLA2	N	e
None	98 ± 13	6	5
10^{-7} M fMLP	218 ± 12	29	15
10^{-7} M fMLP + 1 mg/ml BSA	137 ± 7	7	3
0.1 μg/ml PMA	61 ± 14	4	2
1 μg/ml PMA	29 ± 14	10	7

N = number of observations.
e = number of separate experiments.
In order to compare different experiments the values quoted are for the percentage change brought about by adding 1 μg/ml PLA2. The PLA2 alone induced a chemiluminescence of 1.2 ± 0.2 (N = 5) units, whereas 10^{-7} M fMLP induced a response of 75–80 units (see Table 2).

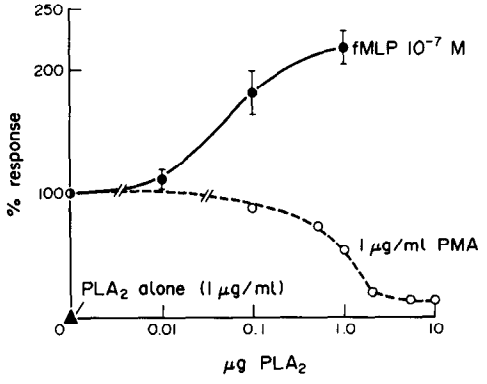

Fig. 2. The effect of varying the amount of PLA2 added on the response to PMA or fMLP. Cells were stimulated either with 10^{-7} M fMLP or 1 μg/ml PMA and various concentrations of PLA2 were added. The effect of PLA2 without agonist is also shown (▲). The effect of the PLA2 is shown as a percentage of the result with fMLP or PMA alone.

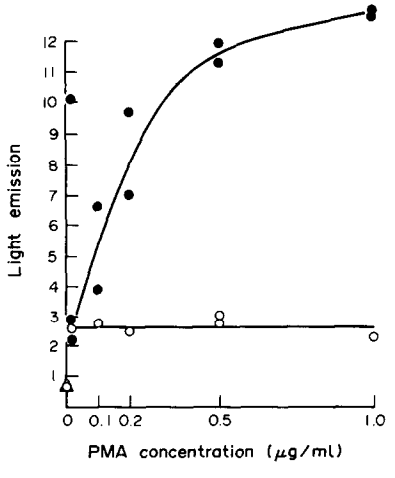

Fig. 3. The inhibitory effect of PLA2 as the concentration of PMA is varied. Phorbol ester stimulated cells in a dose dependent fashion (●), with 1 μg/ml giving almost maximal effect. The addition of PLA2 (1 μg/ml) reduced the light emission (○), but to a level significantly higher than that for cells (△) (or cells + PLA2 (△)) alone. Each point represents the result from a single tube; additional data is presented in Table 1.

oleoyl acyl glycerol (OAG) for PMA as the agonist gave comparable results, although considerably higher concentrations of OAG were required (data not shown).

These experiments indicate that the two products of PLA2 activity have opposite effects on the PMA-mediated response when present individually: if the PLA2 inhibition was due to its products then lysolecithin should be inhibitory in the presence of arachidonic acid, and this was the case (Table 3). The interaction of the two is complex, as can be seen by comparing the effect at different times.

DISCUSSION

In this paper we have shown that exogenous PLA2 will produce a marked enhancement of fMLP-induced neutrophil chemiluminescence, but an inhibition with PMA as the stimulus. PLA2 on its own has no effect, yet the addition of exogenous PLA2 can double the response to fMLP, and halve the response to PMA. Both agonists are potentiated by arachidonic acid, suggesting that the transduction pathways converge at some point, but arachidonic acid on its own is ineffective. The inhibition of the PMA-induced response by PLA2 seems to be due to lysophosphatide, whereas the fMLP-induced chemiluminescence is insensitive to lysophospholipid. There are a variety of other pieces of evidence which point to the existence of multiple transduction pathways, in particular the effects of pertussis toxin, which inhibits fMLP-induced responses but not PMA effects [16], and the pathways can be partially dissociated in other ways [17, 18].

Recently, attention has tended to focus on the phosphatidyl inositol (PI) transduction system [19] and its link with protein kinase C activation [20] and calcium mobilization; there is some reason to suppose that this pathway may play a part in triggering the response of neutrophil leucocytes [21] (although this has been disputed [22]), especially since phorbol esters are potent agonists [23, 24]. On the other hand, the activity of endogenous PLA2 is enhanced by agonists [25], again disputed by others [26]. The PI system and the PLA2 system must be separate to an extent, since the activity of PLA2 will generate neither diacyl glycerol nor inositol trisphosphate, although the secondary activity of diacyl

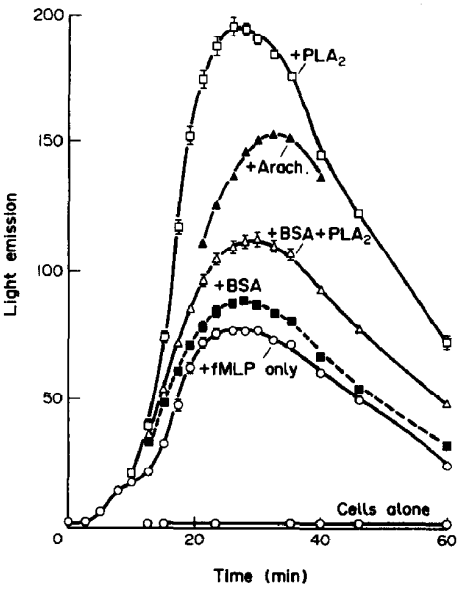

Fig. 4. The effect of BSA on fMLP-induced light emission. The time course of the response to fMLP (10^{-7} M), fMLP with BSA (1 mg/ml), fMLP with PLA2 (1 μg/ml), fMLP with BSA and PLA2, fMLP and arachidonic acid (10 μg/ml), is shown. BSA slightly enhances the effect of fMLP alone, but reduces the stimulation brought about by PLA2. Standard error bars are shown except when smaller than the symbol.

glycerol lipase could produce arachidonic acid from the PI system.

Two points in the present study require some additional comment. Firstly, it could be argued that the addition of exogenous PLA2 can bear no relationship to normal activation pathways. Since, however, the endogenous enzyme is inhibited by lipocortin, a secreted protein which is in the extracellular fluid phase (plasma), the endogenous enzyme must be located fairly superficially, and must be generating products on the outside of the membrane. Certainly the probable products of PLA2 are effective when added extracellularly, although the relatively high levels needed (when compared with the total cellular phospholipid) may indicate that the rate at with which these products become accessible to cytoplasmic enzymes is low. The protective effect of BSA also suggests that the products of the endogenous enzyme must be relatively accessible to the fluid phase. Since PLA2 is not particularly substrate specific (unlike phospholipase C), the products of PLA2 activity should be the same whether exogenous or endogenous enzyme is involved. In general too PLA2 will produce 5–10 times more lysophosphatide than arachidonic acid, since only a proportion of the fatty acids in the A2 position are of this species.

Secondly, it is perhaps worth remarking that the inhibition of PMA activation by PLA2 is only partial, and even with high levels of PLA2 and a preincubation period, the cells are showing considerably

Table 2. The effect of various additions on the chemiluminescence of rabbit neutrophils in response to PMA and fMLP

Agonist	Response	(no)	(% control)	Response	(no)	(% control)
	PMA (1 μg/ml)			fMLP (10^{-7} M)		
Set 1						
Agonist only	140 ± 1	(4)	(100)	77 ± 2	(5)	(100)
BSA 1 mg/ml	170 ± 2	(3)	(121)	89 ± 2	(4)	(116)
5 μg Lysolecithin	56	(2)	(40)	80	(2)	(104)*
+BSA + 5 μg Lysol	170	(2)	(121)	83	(1)	(108)
Set 2						
Agonist only	62 ± 2	(7)	(100)	75 ± 4	(9)	(100)
+10 μg Arach.†	180 ± 21	(6)	(290)	133 ± 3	(8)	(177)
+1 μg Arach.	83	(2)	(134)	104 ± 6	(7)	(139)
Set 3‡						
Agonist only	18 ± 1	(4)	(100)	80 ± 3	(8)	(100)
+1 μg C-20 acid:						
Alpha-linolenic	18	(2)	(102)	91 ± 7	(4)	(113)
Linoleic	18	(2)	(98)	89 ± 7	(4)	(111)
Gamma-linolenic	20	(2)	(112)	111 ± 10	(4)	(139)
Oleic acid	19	(2)	(104)	94	(2)	(118)

* In experiments with fMLP but unmatched for PMA this value was 107 ± 2% (97% for 10 μg, 102% for 1 μg).

† With arachidonic acid (Arach.) alone the peak value was 1.1 ± 0.1 (N = 6) cf cells alone 1.4 ± 0.1 (N = 13).

‡ In the experiments of set 3 the cells were much less responsive to PMA because they were preincubated at 37° (Lackie, in preparation).

Three separate sets of experiments are shown in this table; all experiments have been repeated on at least three separate preparations of cells, although often a full set of treatments was not used and the data have not therefore been included.

Table 3. The effect of the addition of both lysolecithin (lyso-PC) and arachidonic acid (Arach.) to PMA-stimulated rabbit neutrophils

Stimulus	(N)*	Chemiluminescence response (% of PMA value) 5 min†	Peak‡	19 min§
PMA alone	(5)	36.3 ± 0.7 (100)	65.7 ± 1.0 (100)	52.0 ± 0.9 (100)
PMA + Arach.	(5)	45.6 ± 1.0 (125)	68.5 ± 0.5 (104)	42.8 ± 0.7 (82)
PMA + Lyso-PC	(5)	5.1 ± 0.3 (14)	17.4 ± 0.9 (26)	16.0 ± 0.8 (31)
PMA + Arach. + Lyso-PC	(4)	5.1 ± 0.5 (14)	17.3 ± 0.9 (26)	16.9 ± 0.9 (33)
Arach.	(2)	1.2 (3)	2.3 (4)	2.1 (4)
Lyso-PC	(2)	1.6 (4)	2.4 (4)	1.8 (4)

PMA was used at 1 μg/ml, arachidonic acid at 10 μg/ml, and lysophosphatidyl choline (lyso-PC) at 5 μg/ml. In this experiment, the enhancing effect of arachidonic acid is most conspicuous at the early stages and the inhibition by lysolecithin becomes less marked at later stages. In similar experiments, the arachidonic acid effect was still evident at the time of peak response (Table 2). The response to arachidonic acid or lysolecithin in the absence of PMA was not significantly different from the background response of unstimulated cells.

* Number of replicate tubes.

† Measurements made between 4 and 6 min after adding cells.

‡ Peak value (around 11 min for PMA).

§ Measurements made between 18 and 20 min.

more than a background level of metabolic activation. This could be taken to imply that PMA acts in two ways, one susceptible to inhibition by PLA2 (probably through the action of lysophosphatide), the other route being insensitive. The nature of these two routes of neutrophil activation by PMA remains unclear.

If there are two distinct pathways of neutrophil activation, then we might ask how they differ, and why this duplication occurs. The fMLP system may act by stimulating endogenous PLA2; this would increase the production of arachidonic acid. Since arachidonic acid metabolites may themselves stimulate neutrophils, fMLP would trigger a positive feedback system, and suppress the PMA-activated system through lysophosphatide. Neutrophils stimulated with PMA only release specific granules [27, 28], and their responses are different from those seen with chemotactic peptides, particularly in respect of the behaviour of the motor machinery. It might therefore be that the PMA response is an exaggerated form of the response which neutrophils show during the early stages of an inflammatory response, and the fMLP-type response is that normally displayed upon arrival at the focus of inflammation.

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