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Mechanism of calcium ionophore A23187-induced priming of bone marrow-derived macrophages for tumor cell killing: Relationship to priming by interferon

(calcium mobilization/phospholipids)

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ABSTRACT **Interferon primes macrophages for tumor cell killing by rendering them sensitive to triggering agents such as lipopolysaccharide. In an attempt to determine the nature of the priming signal, we tested phorbol 12-myristate 13-acetate, diacylglycerol, platelet-activating factor, arachidonic acid, leukotriene B_4, and the calcium ionophore A23187 for their ability to prime mouse bone marrow-derived macrophages for activation to kill P815 mastocytoma target cells. The ionophore A23187 was the only substance that was able to replace the interferon priming signal. A23187 priming appeared to be due in part to induction of interferon α/β in the macrophage cultures, since its effect was partially but specifically blocked by antibody to interferon α/β. Consistent with this was the observation that A23187 induced interferon α/β production in macrophage cultures. The fact that A23187 priming was not completely reversed by antibody to interferon would suggest that factors unrelated to interferon induction also played a role in macrophage priming. The failure of phorbol myristate acetate or diacylglycerol to prime macrophages for tumor cell killing would suggest that activation of protein kinase C is not sufficient for priming. Thus, A23187 appears to provide the priming signal for macrophage killing through the combination of interferon- and non-interferon-induced mechanisms.**

Activation of macrophages for killing of tumor cells is a two-signal process. The first signal, called priming, is usually induced by interferon (IFN), which renders the macrophages sensitive to second-signal triggering agents such as lipopolysaccharide (1–3). The biochemical basis for the IFN-induced priming signal is not known but could possibly involve calcium mobilization and/or production of phospholipid-derived second messengers. IFN-γ activation of macrophages for superoxide production and its down-regulation of transferrin receptors on macrophages, for example, are mimicked by the calcium ionophore A23187 and the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) (4–6). The phospholipid-derived natural mobilizer of calcium within the cell is inositol 1,4,5-trisphosphate, whereas the phospholipid-derived natural activator of protein kinase C in the cell is diacylglycerol (7, 8). Recently, IFN-γ has been shown to activate a protein kinase C-like activity in cells (9, 10). For these reasons, therefore, we were interested in determining the ability of A23187, PMA, and diacylglycerol and several other phospholipid-derived messengers to replace IFN in the priming phase of the activation of macrophages for tumor-cell killing.

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MATERIALS AND METHODS

Reagents. The calcium ionophore A23187 and arachidonic acid were obtained from Calbiochem. The tumor promoter PMA and platelet-activating factor (PAF) 1-*O*-alkyl-2-acetyl-*sn*-glycero(3)phosphocholine were obtained from Sigma. Leukotriene B_4 was generously provided by Upjohn. The synthetic diacylglycerol 1-oleoyl-2-acetylglycerol was obtained from Avanti Polar Lipids. Absolute ethanol stock solutions of these lipids and A23187 were further diluted in Hepes-buffered modified Eagle's minimal essential medium (Hepes/MEM) containing 10% fetal bovine serum prior to addition to cultures. Recombinant mouse IFN-γ was kindly provided by Genentech (South San Francisco, CA). Bacterial lipopolysaccharide (LPS) was the gift of D. Morrison (Emory University, Atlanta). The immunoglobulin fraction of rabbit antiserum to mouse IFN-α/β was provided by D. Murasko (Medical College of Pennsylvania, Philadelphia). One milliliter of antibody was capable of neutralizing $\approx 2 \times 10^6$ units of IFN-α/β. All reagents were free of detectable endotoxin by the *Limulus* amebocyte lysate assay.

Bone Marrow-Derived Macrophages. Bone marrow cells were aspirated from the femurs of 8–12-week-old male C3H/HeN mice and cultured at 37°C in 5% CO_2 in bone marrow medium, consisting of Hepes/MEM supplemented with 2 mM glutamine, 15% L-cell conditioned medium, 10% fetal bovine serum, and 5% horse serum. Pure macrophages were obtained after 12–14 days of culture and were used in priming/activation studies with macrophages.

Priming/Activation of Macrophages for Tumor Cell Killing. C3H/HeN mouse bone marrow macrophages were seeded at 5×10^5 cells in 0.1-ml volumes (in Hepes/MEM supplemented with 5% fetal bovine serum) into flat-bottomed 96-well tissue culture plates (Costar). Killing mediated by the bone marrow macrophages was measured by a 16-hr ^{51}Cr-release assay (11). In general, macrophages were preincubated with 0.1 ml of medium containing various concentrations of A23187, IFN, lipids, and/or LPS for 4 hr. ^{51}Cr-labeled (Amersham; 333 mCi/mg of Cr; 1 Ci = 37 GBq) P815 mastocytoma cells (2×10^4) were then added as targets to each well in 0.1 ml of medium. Samples were assayed in duplicate for killing as reflected by ^{51}Cr release as determined in a scintillation counter. Results are expressed as percent of maximum ^{51}Cr release. In general, standard deviations for various determinations were <10% of the mean.

IFN Assay. IFN was assayed on mouse L cells by plaque reduction, with vesicular stomatitis virus as the indicator virus, as described (12). One unit of IFN caused a 50% reduction in plaque formation.

Abbreviations: IFN, interferon; PMA, phorbol 12-myristate 13-acetate; PAF, platelet-activating factor; LPS, lipopolysaccharide.

RESULTS AND DISCUSSION

IFN primes macrophages for tumor cell killing by rendering them sensitive to triggering by LPS (1–3). The data in Fig. 1 show that the calcium ionophore A23187 can similarly prime bone marrow-derived macrophages for induction of killing of P815 mastocytoma cells. Both A23187 and LPS increased macrophage killing in a dose-dependent manner, with 3 μM ionophore and 3 ng of LPS/ml inducing killing comparable to that of 3 units of IFN-γ/ml with the same LPS concentration. Thus the calcium ionophore A23187 was as effective as IFN-γ in priming bone marrow macrophages for tumor cell killing.

The relatively low but significant release of ^{51}Cr by cultures treated with A23187 alone was apparently due to a direct effect on the target cells, since similar release also occurred in the absence of macrophages. Morphologically, the ionophore-treated macrophages had a spread appearance, similar to that of IFN-γ-treated cells, and were viable, based on their ability to exclude trypan blue dye. More convincing evidence that macrophages were not adversely affected by the A23187 treatment was obtained by incubating the ionophore with macrophages for 4 hr followed by removal and washing prior to target cell addition. This sequence of treatment resulted in killing comparable to that of similar treatment with IFN-γ but did not result in significant ^{51}Cr release in the absence of LPS (data not shown). Thus, macrophages treated with the calcium ionophore A23187 did not express cytotoxicity toward tumor cell targets until subsequent exposure to LPS. A23187, therefore, resembles IFN-γ in priming macrophages.

If the calcium ionophore truly mimicked IFN-γ in priming macrophages, then the two substances should not be capable of activating macrophages for killing when added to cultures together in the absence of LPS. This was the case (Fig. 2); both A23187 and IFN-γ primed macrophages for triggering by LPS for killing but failed to cooperate with each other for significant induction of killing in the absence of LPS.

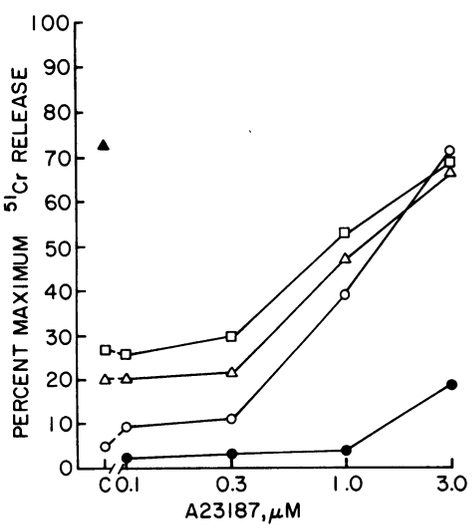

FIG. 1. Macrophage-priming dose–response curves for the calcium ionophore A23187. A23187 was preincubated with bone marrow macrophages for 4 hr in the presence of the following concentrations of LPS: 3 ng/ml (○), 10 ng/ml (△), 30 ng/ml (□), no LPS (●). The "no LPS" pattern of ^{51}Cr release was the same in the absence of macrophages. For comparison, macrophages were also primed by 3 units of IFN-γ/ml in the presence of 3 ng of LPS/ml (▲). Cytotoxicity was measured as ^{51}Cr released from prelabeled P815 mastocytoma cells after 16-hr incubation. C, controls without A23187.

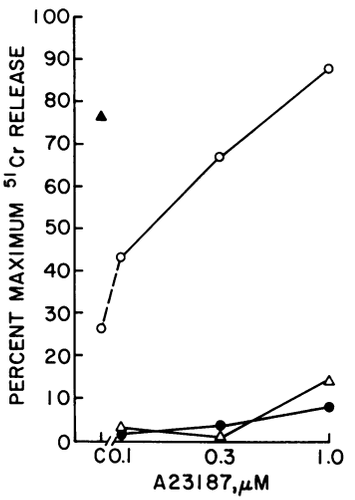

FIG. 2. Macrophage-priming/activation dose–response curves for the calcium ionophore A23187 in combination with LPS or IFN-γ. A23187 was preincubated for 4 hr with bone marrow macrophages in the presence of the following: 3 ng of LPS/ml (○), 3 units of IFN-γ/ml (△), medium with no other additions (●). For comparison, macrophages were also primed by 3 units of IFN-γ/ml in the presence of 3 ng of LPS/ml (▲). Cytotoxicity was measured as described in the legend for Fig. 1. C, controls without A23187.

Since macrophages are capable of producing IFN-α/β (13, 14), it is possible that the priming effect of the ionophore A23187 was due to the induction of IFN in the macrophage cultures. Accordingly, we determined the ability of A23187 to prime macrophages in the presence of antibodies to IFN-α/β and IFN-γ. As shown in Fig. 3, a concentration of rabbit anti-IFN-α/β that was capable of neutralizing greater than 10^5 units of IFN-α/β partially blocked the priming effect of A23187, with significant priming still remaining in cultures treated with 3 μM A23187. In some experiments, the priming by 3 μM A23187 that was refractory to neutralization by anti-IFN-α/β resulted in up to 80% killing of targets. Consistent with this was the observation that anti-IFN-α/β

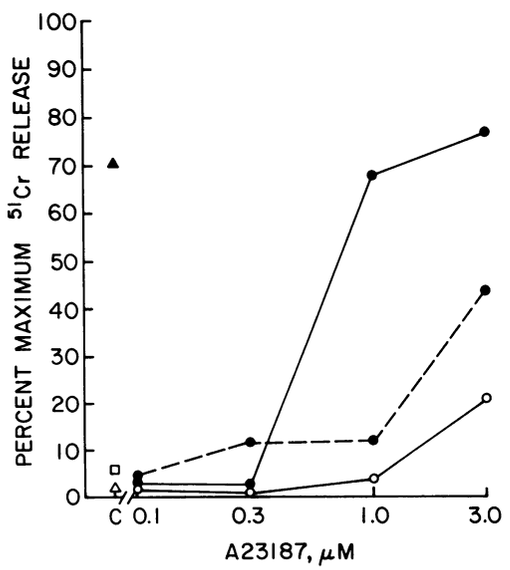

FIG. 3. Effect of antibody to mouse IFN-α/β on A23187 priming of macrophages. Bone marrow macrophages were preincubated for 4 hr with A23187 (○—○), A23187 plus LPS (●—●), A23187 plus LPS plus anti-IFN-α/β (●---●), IFN-α/β plus LPS (▲), IFN-α/β plus LPS plus anti-IFN-α/β (△), or LPS (□). LPS was used at 3 ng/ml; IFN-α/β, at 3000 units/ml; and anti-IFN-α/β, at a concentration sufficient to neutralize at least 10^5 units of IFN-α/β. Cytotoxicity was measured as described for Fig. 1. C, controls without A23187.

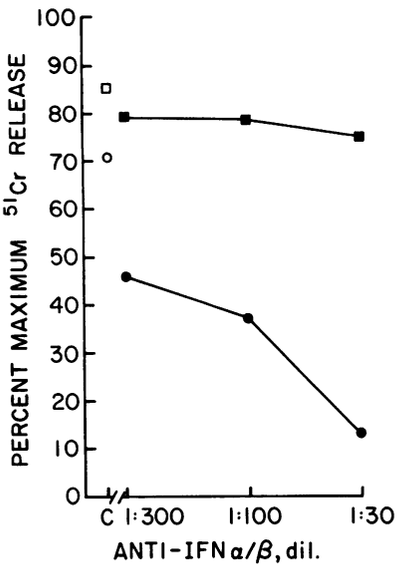

FIG. 4. Dose–response titration of effect of antibody to mouse IFN-α/β on A23187 priming of macrophages. A23187 [1 μM (●) or 3 μM (■)] was preincubated with bone marrow macrophages for 4 hr in the presence of 3 ng of LPS/ml and the indicated dilutions (dil.) of antibody. One milliliter of a 1:30 dilution of anti-IFN-α/β can neutralize at least 10^5 units of IFN-α/β. Cytotoxicity was measured as for Fig. 1. C, controls without antibody and with LPS and either 1 μM (○) or 3 μM (□) A23187.

antibody blockage of priming by 1 μM A23187 was proportional to the antibody concentration, whereas priming by 3 μM A23187 was relatively unaffected by the same antibody concentrations (Fig. 4). Rabbit anti-IFN-γ antibody that was capable of neutralizing 1000 units of IFN-γ had no effect on priming (data not shown) even in the presence of anti-IFN-α/β, which is consistent with the fact that macrophages produce IFN-α/β rather than IFN-γ (13, 14). Examination of the macrophage cultures revealed modest antiviral activity of similar potency for both 1 and 3 μM A23187 treatment, but only in the presence of LPS (Table 1). This antiviral activity was completely neutralized by the same concentration of antibody to IFN-α/β that was used to block priming. P815 targets did not produce IFN activity when treated with A23187 and LPS. That the priming effect of 1 μM A23187 was blocked more extensively by anti-IFN-α/β antibodies than was that of 3 μM A23187 would suggest that factors other than IFN production may play a role in priming by 3 μM

Table 1. Induction of IFN in bone marrow macrophage cultures by A23187 and LPS

Treatment	A23187 conc., μM	IFN activity, units/ml (n = 2)
None	–	<3
A23187	0.3	<3
	1.0	<3
	3.0	<3
LPS	–	3, 10
A23187 + LPS	0.3	<6
	1.0	30, 30
	3.0	30, 80
A23187 + LPS + anti-IFN-α/β	1.0	<5
	3.0	<3

Cultures were treated with A23187, LPS, and anti-IFN-α/β antibodies as for induction of killing, except that P815 targets were omitted. After 24 hr, the supernatants were tested for IFN activity. LPS was used at 3 ng/ml and anti-IFN-α/β at a final dilution sufficient to neutralize at least 10^5 units of IFN-α/β.

Table 2. Ability of various messengers to prime macrophages for tumor cell killing

Factor (conc.)	% ^{51}Cr release*
A23187 (1 μM)	103
PMA (10 ng/ml)	−3
Diacylglycerol† (3 μg/ml)	3
PAF‡ (3 μg/ml)	6
Arachidonic acid (10 ng/ml)	−16
Leukotriene B_4 (50 ng/ml)	1

*Relative to that obtained with IFN-γ (3 units/ml).
†1-Oleoyl-2-acetylglycerol.
‡1-*O*-Alkyl-2-acetyl-s*n*-glycero(3)phosphocholine.

A23187. Thus, A23187 priming of macrophages appears to involve IFN- and non-IFN-induced mechanisms.

A23187 is a potent mobilizer of calcium in the macrophage, both intracellularly and extracellularly (15). Additionally, A23187 has been shown to stimulate the production of the messengers diacylglycerol, PAF, arachidonic acid, and leukotrienes (16–20). We therefore determined whether PMA, diacylglycercol, PAF, arachidonic acid, or leukotriene B_4 could, like A23187, prime macrophages for tumor cell killing. As shown in Table 2, none of these substances was able to replace A23187 in priming of macrophages. Similar results were observed at several concentrations of the factors. Thus, the previously reported (5, 6, 21) common effects of A23187 and PMA in modulation of several macrophage functions do not apply to the priming for tumor cell killing as presented here. This also applies to the second messengers diacylglycerol, PAF, arachidonic acid, and leukotriene B_4. All of the factors presented in Table 2 are capable of providing the required helper signal for IFN-γ production by lymphocytes at the concentrations used here (22–24). Thus, their failure to induce priming was not due to their inaccessibility to the macrophages. PMA and diacylglycerol mediate their messenger signals via activation of protein kinase C (8), but their failure to prime macrophages for killing suggests that activation of protein kinase C is not sufficient in itself to prime macrophages for tumor cell killing.

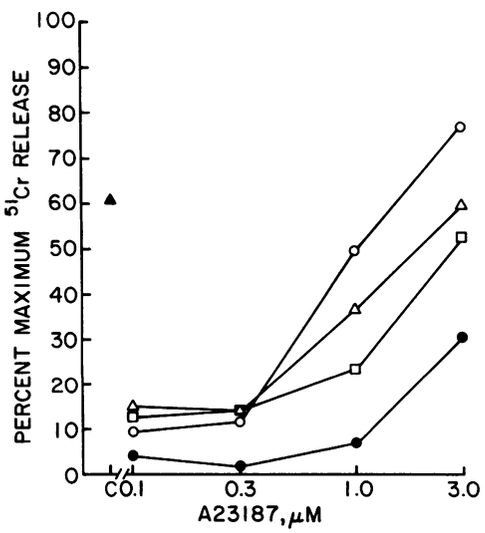

FIG. 5. Lack of synergism between A23187 and PMA in priming macrophages. A23187 was preincubated with bone marrow macrophages for 4 hr without other additions (●) or in the presence of 3 ng of LPS/ml plus the following concentrations of PMA: none (○), 1 ng/ml (△), 10 ng/ml (□). For comparison, macrophages were also primed by 10 units of IFN-γ/ml in the presence of 3 ng of LPS/ml (▲). Cytotoxicity was measured as for Fig. 1.

Having established that PMA cannot replace IFN-γ or A23187 in macrophage priming, we next determined whether it could potentiate the priming effect of A23187 or act as a trigger to cause the expression of cytolytic activity. As shown in Fig. 5, PMA at several concentrations did not potentiate priming by suboptimal concentrations of ionophore, nor did it trigger cytolytic activity. In fact, PMA exerted a suppressive effect on macrophage priming. Thus PMA neither primes nor potentiates priming of macrophages for killing in our system.

The question arises as to the biochemical basis for the direct (non-IFN) priming effect of A23187 on macrophages. Clearly, the effect of A23187 on cells is not just the mobilization of calcium but rather also the alteration of lipid metabolism (16–20). The major effects of A23187 on lipid metabolism in pituitary cells, for example, are hydrolysis of phosphatidylinositol 4-phosphate by a phospholipase C and of phosphatidylinositol 4,5-bisphosphate by a phospholipase A_2 (19). In platelets, A23187 activates a phospholipase A_2 and, in conjunction with cyclooxygenase products, a phospholipase C (20). These metabolic events result in significant production of messengers such as free arachidonic acid, diacylglycerol, phosphatidic acid, and lysophosphatidylinositol, but not inositol trisphosphate (19). Presence of such messengers as arachidonic acid, its lipoxygenase product leukotriene B_4, or diacylglycerol, however, is not sufficient for induction of macrophage priming, as we have shown here. Thus, the possible induction of several well-known second messengers by IFN via phospholipid metabolism would not be sufficient to explain IFN induction of priming in macrophages. Preliminary findings suggest that mobilization of calcium probably plays an important role in direct (non-IFN) priming by A23187, since a direct priming dose (3 μM) of the ionophore was 3–5 times more effective in raising intracellular calcium concentrations in the cell than was a relatively ineffective direct priming dose (1 μM) of the ionophore (unpublished data). It is also possible that a coordinate interaction of some of these A23187-mediated effects as well as the metabolites derived from some of the produced factors could provide the critical priming signal for macrophage activation. Future studies will focus, therefore, on the separate and combined effects of various A23187-induced lipid and nonlipid metabolites on priming, as well as focusing on the effects of IFN on calcium mobilization and lipid metabolism in macrophages. A determination of the similarities and differences between A23187- and IFN-induced metabolic changes in the cell should help identify the biochemical basis of priming.

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