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CALCIUM IONOPHORE A23187 INDUCES RELEASE OF CHEMOKINETIC AND AGGREGATING FACTORS FROM POLYMORPHONUCLEAR LEUCOCYTES

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1 Rat and human polymorphonuclear leucocytes (PMNs) when exposed to calcium ionophore A23187 10 μM release products which cause aggregation of rat PMNs and chemokinesis of human PMNs.

2 Aggregating and chemokinetic activities are rapidly generated; maximal release occurs after 4 min, and can be detected in dilutions of the supernatant of up to 1:1000.

3 Generation of aggregating and chemokinetic activities is inhibited by nordihydroguaiaretic acid 10^{-4} to 10^{-7} M, 5,8,11,14-eicosatetraynoic acid 10^{-4} and 10^{-5} M, BW 755C 10^{-4} M and benoxaprofen 10^{-4} M, all compounds known to inhibit lipoxygenase pathways of arachidonic acid (AA) metabolism.

4 Conventional non-steroidal anti-inflammatory agents, such as aspirin and indomethacin, inhibited little or not at all the generation of these activities.

5 We conclude that the aggregating and chemokinetic activities induced by A23187 represent generation of biologically active products of lipoxygenase pathways of AA metabolism.

Introduction

Calcium ionophore, A23187, has been shown to stimulate responses of polymorphonuclear leucocytes (PMNs) associated with phagocytosis. These include oxidative metabolism (Schell-Frederick, 1974; Romeo, Zabucchi, Miani & Rossi, 1975), lysosomal enzyme release (Zabucchi, Soranzo, Rossi & Romeo, 1975; Smith & Ignarro, 1975) and chemiluminescence (Weidemann, Peskar, Wrogemann, Rietschel, Staudinger & Fischer, 1978). The ionophore also induces the release of chemotactic factors (Czarnetzki, König & Lichtenstein, 1975), causes PMN aggregation (O'Flaherty, Kreutzer, Showell, Becker & Ward, 1978) and stimulates the release from PMNs of prostaglandins and thromboxane B_2 (Knapp, Oelz, Roberts, Sweetman, Oates & Reed, 1977; Weidemann *et al.*, 1978; Wentzell & Epand, 1978). The results of Borgeat & Samuelsson (1979) demonstrate that, in addition to causing release of arachidonic acid (AA), ionophore A23187 activates enzyme systems involved in the further tranformation of AA which results in the release of products of the lipoxygenase pathways of AA metabolism (Stenson & Parker, 1979).

The present study investigates whether biologically active products of lipoxygenase pathways of AA metabolism are released following exposure to ionophore A23187 and determines whether these products could be assayed by PMN aggregation and chemokinesis assays.

Methods

Generation of aggregatory and chemokinetic activities

Cell suspensions (>85% PMNs) were prepared from peritoneal exudates obtained 24 h after the injection of 12% sodium caseinate into 200 to 300 g male Wistar rats (Cunningham, Smith, Ford-Hutchinson & Walker, 1979). Human PMNs were prepared by dextran sedimentation followed by a one stage purification on a Ficoll-Hypaque density gradient (Cunningham, Ford-Hutchinson, Oliver, Smith & Walker, 1978). Rat and human cells were washed once in Eagle's minimum essential medium buffered to pH 7.4 with 30 mM N'-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES) and resuspended to a final concentration of 1×10^7 cells/ml. Stock solutions of drugs (100 mM) were made up in dimethyl sulphoxide (DMSO) and appropriate dilutions were made in medium. Calcium ionophore, A23187, was made up as a 10 mM stock solution in DMSO. The final concentration of DMSO in the cell suspensions was

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always <0.2%. Cell suspensions (500 µl aliquots) were incubated in the presence and absence of drugs in a stirred aggregometer cell (37°C, 800 rev/min) for 5 min. Ionophore A23187 was then added (0.5 µl) to give a final concentration of 10 µM and the cells were incubated for a further 4 min. The reaction was terminated by rapid centrifugation of the cells followed by removal of the supernatants. No release of lactate dehydrogenase occurred during the 4 min incubation demonstrating that there was no non-specific cell damage. For the time course experiments the reaction was terminated at varying times after the addition of ionophore. The zero time sample was centrifuged immediately following addition of ionophore and the supernatant was removed within 45 s. The supernatants in these experiments were diluted 1:500 for the aggregation assay and 1:100 for the chemokinesis assay. Supernatants were either assayed the same day or stored overnight at −20°C.

Aggregation assays

PMN aggregation assays were carried out in a Payton aggregometer using rat peritoneal cells. This cell population was used because peritoneal PMNs aggregate more readily than peripheral cells and because rat peritoneal cells can be readily obtained in large numbers (Cunningham, Shipley & Smith, 1980). Rat peritoneal cells (5×10^6) were suspended in 500 µl of Eagle's minimum essential medium buffered to pH 7.4 with 30 mM HEPES. Cell suspensions were preincubated for 5 min in a stirred aggregometer cell (37°C, 800 rev/min) prior to addition of 2 µl of ionophore generated supernatant (1:250 dilution). Supernatants obtained from human PMNs were diluted 1:200 for assay on rat cells. The results are expressed as the change in transmittance as measured in mm on the recorder trace. To assess the effects of drugs on the aggregation response itself, cell suspensions were incubated in the aggregometer cell in the presence and absence of drugs for 5 min before the addition of 2 µl of a control ionophore-generated supernatant.

Chemokinesis assay

Human PMNs for the chemokinesis assay were prepared and assayed by the agarose micro-droplet assay (Smith & Walker, 1980). Rat peritoneal cells do not migrate readily with this technique. In each experiment six replicates of each sample were randomly distributed within a 96 well microtitre plate and migration was assessed after 4 h incubation at 37°C in a humidified incubator. Supernatants obtained from PMNs exposed to ionophore were diluted 1:100 in medium before assay except where stated. Chemokinetic responses were calculated by subtracting the migration in the presence of medium containing ionophore (100 nM final concentration) (b) from the migration in the presence of supernatants from ionophore treated PMNs (a). The effects of drugs were expressed as % inhibition of this response calculated according to the formula $(c - b)/(a - b) \times 100$ where (c) is the migration response to supernatants from PMNs stimulated with ionophore in the presence of drugs.

Materials

Drugs used were: calcium ionophore A23187 (Calbiochem); nordihydroguaiaretic acid (Sigma); 5,8,11,14-eicosatetraynoic acid (Roche); indomethacin (Merck, Sharpe and Dohme); 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (BW 755C: Wellcome Research Laboratories); benoxaprofen (Lilly Research); phenylbutazone (Geigy); naproxen (Syntex); acetylsalicylic acid and sodium salicylate (BDH Chemicals); flurbiprofen (Boots).

Results

Release of aggregating and chemokinetic activities from rat polymorphonuclear leucocytes

Exposure of rat PMNs to 10 µM ionophore A23187 in an aggregometer cell caused a biphasic aggregation response. The first peak was maximal at 1.5 min. A second irreversible response began at 4 min and was still increasing after 15 min. When supernatants, obtained after 4 min incubation with ionophore, were added to fresh suspensions of rat PMNs at dilutions ranging from 100 to 1000 fold, a rapid reversible aggregating response was observed peaking at 45 s. Maximal responses were obtained with a dilution of 1:200; no further increase in aggregation was seen with higher concentrations. The same dilutions of the supernatant also possessed significant chemokinetic activity towards human PMNs when assayed in the agarose micro-droplet assay. Equivalent dilutions of medium containing 10 µM ionophore A23187 without cells showed no significant biological activity in either assay.

Figure 1 shows the time course of release of aggregating and chemokinetic activity from rat PMNs. Maximal release of both activities occurred after 4 min, no significant differences being observed between samples incubated for 4 min and 10 min.

The effects of varying ionophore concentrations (100 nM to 100 µM) were also studied and optimal release of both biological activities was observed at 10 µM. No differences were observed in the release of both biological activities when the peritoneal exudate was further purified by centrifugation on Ficoll-Hypaque density gradients to remove contaminating

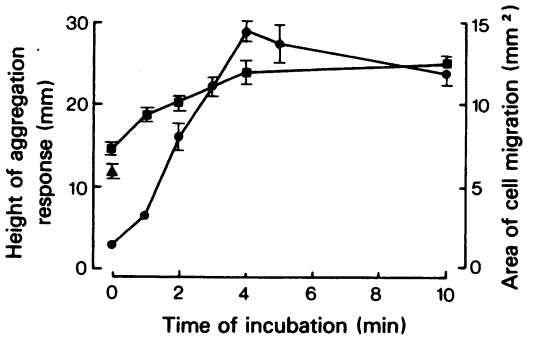

Figure 1 Time course of generation of aggregating and chemokinetic activities from rat polymorphonuclear leucocytes (PMNs) in the presence of 10 μM ionophore A23187. Supernatants were separated from the generating rat PMNs (10^7 cells/ml) at intervals (abscissa scale) and added to rat PMNs for aggregation assays (●), and to human PMNs for chemokinesis assays (■). Cell migration in the presence of 100 nM ionophore alone (▲). Each point represents the mean ($n = 6$); vertical lines show s.e. mean.

lymphocytes and macrophages, confirming the fact that the biological activities were generated by PMNs.

Effects of drugs on rat polymorphonuclear leucocytes

Table 1 shows the effects of a number of known inhibitors on the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism on the generation of chemokinetic or aggregating activities from rat PMNs stimulated with the ionophore A23187. Results from the aggregation experiments were pooled as control responses did not significantly vary from day to day. The migration areas observed in the chemokinesis assays varied from day to day and the results are expressed as percentage inhibition of the migration response calculated according to the equation described in the methods section. The migration areas for a number of pooled experiments expressed as means ± s.e. mean as determined by planimetry were as follows: buffer alone 8.7 ± 0.6 mm^2 (range 4.6 to 11.9, $n = 17$), buffer plus 100 nM ionophore A23187

Table 1 Effects of drugs on the generation of aggregating and chemokinetic activities from ionophore A23187-stimulated rat polymorphonuclear leucocytes

Treatment	*Concentration* (M)	n	*Mean height of aggregation response*	% *inhibition*	n	% *inhibition of chemokinetic response*
Control		36	41 ± 1	—	—	—
Nordihydroguaiaretic acid	10^{-4}	6	0 ± 1	100**	6	100 ± 5**
	10^{-5}	12	0 ± 1	99**	12	99 ± 1**
	10^{-6}	12	4 ± 1	90**	6	90 ± 6**
	10^{-7}	12	21 ± 3	49**	6	50 ± 7*
5,8,11,14-Eicosatetraynoic acid	10^{-4}	6	1 ± 1	98**	4	95 ± 6**
	10^{-5}	12	1 ± 1	97**	4	95 ± 6**
	10^{-6}	12	36 ± 2	NS	6	54 ± 4*
	10^{-7}	6	41 ± 2	NS	6	18 ± 6
Indomethacin	10^{-4}	6	12 ± 2	71**	6	51 ± 13*
	10^{-5}	6	42 ± 4	NS	6	9 ± 10
	10^{-6}	6	40 ± 2	NS	6	11 ± 8
BW755C	10^{-4}	6	3 ± 1	94**	6	68 ± 10*
	10^{-5}	6	35 ± 1	NS	6	5 ± 4
	10^{-6}	6	38 ± 3	NS	6	7 ± 7
Benoxaprofen	10^{-4}	6	1 ± 3	97**	6	100 ± 8**
	10^{-5}	6	41 ± 4	NS	6	16 ± 6
Acetylsalicyclic acid	5×10^{-4}	6	42 ± 5	NS	6	36 ± 6*
Sodium salicylate	5×10^{-4}	6	41 ± 2	NS	6	23 ± 3*
Phenylbutazone	10^{-4}	6	40 ± 2	NS	6	24 ± 6
Naproxen	10^{-4}	6	37 ± 2	NS	6	19 ± 5
Flurbiprofen	10^{-4}	6	36 ± 3	NS	6	21 ± 4

Results expressed ± s.e. mean; ** $P < 0.001$, * $P < 0.05$, Student's t test; NS = no significant change from control.

8.1 ± 0.3 mm^2 (range 6.6 to 9.4, $n = 10$) and 1:100 dilution of supernatant from cells treated with 10 μM ionophore A23187 19.9 ± 0.5 mm^2 (range 17.6 to 24.8, $n = 6$). The effects of the drugs on the two biological activities were very similar. Thus both biological activities were inhibited by 10^{-4} to 10^{-7} M nordihydroguaiaretic acid (NDGA), 10^{-4} and 10^{-5} M 5,8,11,14-eicosatetraynoic acid (ETA), 10^{-4} M BW 755C and 10^{-4} M benoxaprofen. None of the conventional non-steroidal anti-inflammatory agents inhibited by more than 75%, even at the high doses used. The most effective was indomethacin which significantly inhibited both systems at 10^{-4} M. To exclude the possibility that any of the inhibitory actions could be due to effects of the drugs themselves on either the aggregating or chemokinetic processes, drugs were added directly to both systems before addition of supernatants from PMNs exposed to ionophore A23187. All the drugs used were tested at 10^{-6} M (allowing for the dilution effect in the original experiments) except acetylsalicylic acid and sodium salicylate which were tested at 5×10^{-6} M. None of the drugs had a significant effect on either assay at these concentrations.

Release of aggregating and chemokinetic activities from human polymorphonuclear leucocytes

Human peripheral PMNs exposed to ionophore A23187 also released aggregating and chemokinetic activities with an identical time course. The optimal ionophore concentration was again 10 μM. The effects on the production of these activities of 10^{-5} and 10^{-6} M NDGA, ETA and indomethacin were also similar to those observed with rat PMNs (Table 2).

Discussion

Calcium ionophore (A23187) has been shown to induce the release of products of the lipoxygenase pathways of AA metabolism from human PMNs (Borgeat & Samuelsson, 1979; Stenson & Parker, 1979). We have used PMN chemokinesis and aggregation to determine the release of these products under similar conditions from rat and human PMNs on the basis of reports that (1) hydroxy fatty acids formed from AA by lipoxygenase pathways are potent chemotactic and chemokinetic agents for PMNs (Goetzl & Sun, 1979); (2) other chemotactic factors, such as the complement derived peptide C5a and synthetic peptides cause PMN aggregation (Craddock, Hammerschmidt, White, Dalmasso & Jacob, 1977; O'Flaherty & Ward, 1979); (3) products of the lipoxygenase pathways of AA metabolism may cause PMN aggregation (Ford-Hutchinson, Bray & Smith, 1979).

The present results on the release of the aggregating and chemokinetic activities from rat and human PMNs support the concept that these activities are caused by products of the lipoxygenase pathways of AA metabolism. Thus NDGA, an inhibitor of lipoxygenase activity (Tappel, Lundberg & Boyer, 1953), was a potent inhibitor of the release of these biological activities. ETA was also a good inhibitor. This compound inhibits cyclo-oxygenase and lipoxygenase pathways of AA metabolism (Tobias & Hamilton, 1979) although in human PMNs ETA inhibits the production of 12-hydroxyeicosatetraenoic acid more than 5-hydroxyeicosatetraenoic acid (Stenson, Atkinson, Kulczycki & Parker, 1978). In contrast, indomethacin which is a powerful inhibitor of cyclo-oxygenase activity and a weak inhibitor of lipoxygenase activity (Hamberg & Samuelsson, 1974) was only weakly active. The inhibition of aggregation and chemokinesis by NDGA, ETA and indomethacin was not correlated with their effects on thromboxane B_2 biosynthesis by rat peritoneal PMNs (Ford-Hutchinson, Bray & Smith, 1979). Two other drugs which have been reported to be inhibitors of both lipoxygenase and cyclo-oxygenase pathways, BW 755C and benoxaprofen (Higgs, Flower & Vane,

Table 2 Effects of drugs on the generation of aggregating and chemokinetic activities from ionophore A23187-stimulated human polymorphonuclear leucocytes

Treatment	*Concentration* (M)	n	*Mean height of aggregation response*	% *inhibition*	n	*% inhibition of chemokinetic response*
Control		6	41 ± 1	—	—	—
Nordihydroguaiaretic acid	10^{-5}	6	6 ± 1	86**	11	47 ± 4**
	10^{-6}	6	36 ± 1	13*	6	22 ± 5
5,8,11,14-Eicosatetraynoic acid	10^{-5}	6	10 ± 1	75**	11	72 ± 11**
	10^{-6}	6	35 ± 1	15*	6	-20 ± 4
Indomethacin	10^{-5}	6	33 ± 1	21**	11	14 ± 7
	10^{-6}	6	43 ± 1	NS	6	-17 ± 5

Results expressed ± s.e. mean; ** $P < 0.005$, * $P < 0.05$, Student's t test; NS = no significant change from control.

1979; Walker & Dawson, 1979) also showed inhibitory activity. Five other conventional non-steroidal anti-inflammatory agents were tested and none showed any appreciable activity. These results suggest that inhibition of lipoxygenase pathways does not significantly contribute to the anti-inflammatory actions of conventional non-steroidal anti-inflammatory drugs.

We conclude, therefore, that ionophore A23187 stimulates release of biologically active products of the lipoxygenase pathway of AA metabolism from both rat and human PMNs. These products may be epoxide or hydroperoxy intermediates or they may be hydroxy fatty acids such as 5-hydroxyeicosatetraenoic acid. Such AA metabolites may contribute to the biological effects resulting from fatty acid metabolism in PMNs. It will be of some interest to study the potential role of these substances in the recruitment of neutrophils to inflammatory sites. The production of aggregating and chemokinetic responses by these products provides a useful bioassay for such materials.

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References

BORGEAT, P. & SAMUELSSON, B. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. natn. Acad. Sci., U.S.A.*, **76**, 2148–2152.

CRADDOCK, P.R., HAMMERSCHMIDT, D., WHITE, J.G., DALMASSO, A.P. & JACOB, H.S. (1977). Complement (C5a)-induced granulocyte aggregation *in vitro*. *J. clin. Invest.*, **60**, 260–264.

CUNNINGHAM, F.M., FORD-HUTCHINSON, A.W., OLIVER, A.M., SMITH, M.J.H. & WALKER, J.R. (1978). The effects of D-penicillamine and levamisole on leucocyte chemotaxis in the rat. *Br. J. Pharmac.*, **63**, 119–123.

CUNNINGHAM, F.M., SHIPLEY, M.E. & SMITH, M.J.H. (1980) Aggregation of rat polymorphonuclear leucocytes *in vitro*. *J. Pharm. Pharmac.* (in press).

CUNNINGHAM, F.M., SMITH, M.J.H., FORD-HUTCHINSON, A.W. & WALKER, J.R. (1979). Migration of peritoneal leucocytes in the rat. *J. Path.*, **128**, 15–20.

CZARNETZKI, B.M., KONIG, W. & LICHTENSTEIN, L.M. (1975). Release of eosinophil chemotactic factor from human polymorphonuclear neutrophils by calcium ionophore A23187 and phagocytosis. *Nature*, **258**, 725–726.

FORD-HUTCHINSON, A.W., BRAY, M.A. & SMITH, M.J.H. (1979). The aggregation of rat neutrophils by arachidonic acid: a possible bioassay for lipoxygenase activity. *J. Pharm. Pharmac.*, **12**, 868–869.

GOETZL, E.J. & SUN, F.F. (1979). Generation of unique mono-hydroxyeicosatetraenoic acids from arachidonic acid by human neutrophils. *J. exp. Med.*, **150**, 406–411.

HAMBERG, M. & SAMUELSSON, B. (1974) Prostaglandin endoperoxides: novel transformation of arachidonic acid in human platelets. *Proc. natn. Acad. Sci., U.S.A.*, **71**, 3400–3404.

HIGGS, G.A., FLOWER, R.J. & VANE, J.R. (1979). A new approach to anti-inflammatory drugs. *Biochem. Pharmac.*, **28**, 1959–1961.

KNAPP, H.R., OELZ, O.O., ROBERTS, J., SWEETMAN, B.J., OATES, J. A. & REED, P.W. (1977). Ionophores stimulate prostaglandin and thromboxane biosynthesis. *Proc. natn. Acad. Sci. U.S.A.*, **74**, 4251–4253.

O'FLAHERTY, J.T., KREUTZER, D.L., SHOWELL, H.S., BECKER, E.L. & WARD, P.A. (1978). Desensitisation of the neutrophil aggregation response to chemotactic factors. *Am. J. Path.*, **93**, 693–705.

O'FLAHERTY, J.T. & WARD, P.A. (1978). Leucocyte aggregation induced by chemotactic factors. *Inflammation*, **3**, 177–194.

ROMEO, D., ZABUCCHI, G., MIANI, N. & ROSSI, E. (1975). Ion movement across leucocyte plasma membrane and excitation of metabolism. *Nature*, **253**, 542–544.

SCHELL-FREDERICK, E. (1974). Stimulation of the oxidative metabolism of polymorphonuclear leucocytes by the calcium ionophore A23187. *FEBS Lett.*, **48**, 37–40.

SMITH, M.J.H. & WALKER, J.R. (1980). The effects of some antirheumatic drugs on an *in vitro* model of human polymorphonuclear leucocyte chemokinesis. *Br. J. Pharmac.* (In press).

SMITH, R.J. & IGNARRO, L.J. (1975). Bioregulation of lysosomal enzyme secretion from human neutrophils: roles of guanosine 3':5'-monophosphate and calcium in stimulus-secretion coupling. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 108–112.

STENSON, W.F., ATKINSON, J.P., KULCZYCKI, A. & PARKER, C.W. (1978). Stimulation of hydroxylated fatty acid and thromboxane synthesis in human neutrophiles by phagocytic and other stimuli. *Fedn. Proc.*, **37**, 1818.

STENSON, W.F. & PARKER, C.W. (1979). Metabolism of arachidonic acid in ionophore-stimulated neutrophils. *J. clin. Invest.*, **64**, 1457–1465.

TAPELL, A.L., LUNDBERG, W. & BOYER, P.D. (1953). Effects of temperature and anti-oxidants upon the lipoxidase-catalysed oxidation of sodium linoleate. *Archs Biochem. Biophys.*, **42**, 293–304.

TOBIAS, L.D. & HAMILTON, J.G. (1979). The effect of 5,8,11,14-eicosatetraynoic acid on lipid metabolism. *Lipids*, **14**, 181–193.

WALKER, J.R. & DAWSON, W. (1979). Inhibition of rabbit PMN lipoxygenase activity by benoxaprofen. *J. Pharm. Pharmac.*, **11**, 778–780.

WEIDEMANN, M.J., PESKAR, B.A., WROGEMANN, K., RIETZCHEL, E.T., STAUDINGER, H. & FISCHER, H. (1978). Prostaglandin and thromboxane synthesis in a pure macrophage population and the inhibition, by E-type prostaglandins, of chemiluminescence. *FEBS Lett.*, **89**, 136–140.

WENTZELL, B. & EPAND, R.M. (1978). Stimulation of the release of prostaglandins from polymorphonuclear leucocytes by the calcium ionophore A23187. *FEBS Lett.*, **86**, 255–258.

ZABUCCHI, G., SORANZO, M.R., ROSSI, F. & ROMEO, D. (1975). Exocytosis in human polymorphonuclear leucocytes induced by A23187 and calcium. *FEBS Lett.*, **54**, 44–48.

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