Leukotriene synthesis in calcium-depleted human neutrophils: arachidonic acid release correlates with calcium influx

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The relationship between intracellular calcium concentration ($[Ca^{2+}]_i$), the release of arachidonic acid and the synthesis of leukotriene B_4 (LTB_4) was investigated using Ca^{2+} -depleted human polymorphonuclear leucocytes (PMNs) in which $[Ca^{2+}]_i$ can be manipulated by varying the concentration of exogenous Ca^{2+} added with agonists. In this model, Ca^{2+} , platelet-activating factor (PAF) and *N*-formyl-Met-Leu-Phe (FMLP), added alone, were unable to induce arachidonic acid release or LTB_4 synthesis, as assessed by measurements of the products by MS and HPLC, respectively. However, the simultaneous addition of Ca^{2+} and either PAF or FMLP to these Ca^{2+} -depleted PMNs resulted in an influx of Ca^{2+} proportional to the extracellular concentration of Ca^{2+} and caused a substantial release of arachidonic acid and synthesis of LTB_4 . The $[Ca^{2+}]_i$ values for threshold and maximal arachidonic acid release were found to be

150 nM and 350 nM respectively, suggesting the involvement of cytosolic phospholipase A_2 (cPLA $_2$). Under stimulatory conditions resulting in similar $[Ca^{2+}]_i$, Ca^{2+} -depleted PMNs released significant amounts of arachidonic acid but normal (Ca^{2+} -repleted) PMNs did not, indicating that Ca^{2+} depletion of PMNs altered the normal regulation of arachidonic acid release and facilitated the release of the fatty acid upon stimulation with agonists. cPLA $_2$ and mitogen-activated protein kinase (MAP kinase) phosphorylation, as assessed by changes of electrophoretic mobility, occurred in both Ca^{2+} -repleted and Ca^{2+} -depleted PMNs upon addition of agonist. These data demonstrate that in Ca^{2+} -depleted PMNs stimulated with agonists, arachidonic acid release and LTB_4 synthesis correlated with extracellular Ca^{2+} influx.

INTRODUCTION

Leukotriene B_4 (LTB_4) is a potent chemotactic factor and activator of inflammatory cell functions, such as degranulation and adhesion [1,2]. Human polymorphonuclear leucocytes (PMNs) synthesize LTB_4 in response to diverse stimuli, such as phagocytosis, chemoattractant receptor stimulation and calcium ionophores. One common feature of agents that induce LTB_4 synthesis is their ability to elevate the intracellular concentration of free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$).

Binding of the soluble agonists platelet-activating factor (PAF) and *N*-formyl-Met-Leu-Phe (FMLP) to specific receptors causes the phospholipase C-mediated release of diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$) [3,4]. In turn, binding of $InsP_3$ to intracellular receptors stimulates the release of Ca^{2+} from internal stores [3], followed by an influx of extracellular calcium [5–8]. The resulting mobilization of Ca^{2+} is believed to be essential for LTB_4 synthesis, as key enzymes involved in this process, namely, phospholipase A_2 (PLA $_2$) and 5-lipoxygenase (5-LO), are Ca^{2+} -dependent. The release of arachidonic acid from phospholipids depends on the activation of a PLA $_2$. While the 14 kDa secreted PLA $_2$ (sPLA $_2$) [9,10] and the 85 kDa cytosolic PLA $_2$ (cPLA $_2$) [11,12] have the ability to release arachidonic acid from membrane phospholipids in various cell types, the exact nature of the enzyme involved in the LTB_4 biosynthetic pathway of human PMNs remains to be ascertained. Previous studies have shown that sPLA $_2$ requires Ca^{2+} in the millimolar range for catalytic activity [13], whereas cPLA $_2$ is dependent on Ca^{2+} in the submicromolar range for binding to membrane structures [14–17]

where its activity is reported to be modulated by G-proteins [18,19] and phosphorylation by a mitogen-activated protein kinase (MAP kinase) [20,21]. Free arachidonic acid is then metabolized to LTB_4 via 5-LO, which is dependent on Ca^{2+} for translocation and activity [22]. However, although PMN soluble agonists elevate $[Ca^{2+}]_i$, they do not stimulate, to a significant extent, the synthesis of LTB_4 [23]; we and others showed that 5-LO is activated by soluble agonists and that, under these conditions, arachidonic acid availability limits LTB_4 synthesis [24–26]. Thus, the roles of intracellular or extracellular Ca^{2+} in the regulation of LTB_4 synthesis in human neutrophils remains to be clarified.

In the present study, we used an original model, Ca^{2+} -depleted human PMNs, to assess the relationship between $[Ca^{2+}]_i$, arachidonic acid release and LTB_4 synthesis. Indeed, Ca^{2+} -depleted PMNs show increased permeability to extracellular Ca^{2+} [8,27]; thus, $[Ca^{2+}]_i$ can be easily controlled in the cells by manipulating the concentration of Ca^{2+} in the incubation medium. Our data demonstrate that arachidonic acid release in Ca^{2+} -depleted PMNs correlates with extracellular Ca^{2+} influx and is compatible with a possible involvement of cPLA $_2$ in arachidonic acid release under these experimental conditions.

MATERIALS AND METHODS

Reagents

PAF, FMLP, Hepes, Fura-2/AM, and $CaCl_2 \cdot 3H_2O$ were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), monoclonal anti-phosphotyrosine antibody (UB 05-321) was

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; 5,15-DiHETE, 5(S),15(S)-dihydroxy-6,8,11,13-(*E,Z,Z,E*)-eicosatetraenoic acid; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salts solution; 5-HETE, 5-hydroxyeicosatetraenoic acid; 15-HpETE, 15(S)-hydroperoxy-5,8,11,13-(*Z,Z,Z,E*)-eicosatetraenoic acid; 5-LO, arachidonate 5-lipoxygenase; LTB_4 , leukotriene B_4 ; MAP kinase, mitogen-activated protein kinase or extracellular signal-regulated kinase (ERK); PAF, platelet-activating factor; cPLA $_2$, cytosolic phospholipase A_2 ; sPLA $_2$, soluble PLA $_2$; PMNs, polymorphonuclear leucocytes; RP, reversed-phase.

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from UBI (Lake Placid, NY, U.S.A.), monoclonal anti-(MAP kinase) (clone MK12; which recognizes ERK1 and ERK2) was from Transduction Laboratories (Lexington, KY, U.S.A.), polyclonal anti-cPLA₂ (MF-140) was a generous gift from Dr. Philip Weech (Merck-Frosst, Pointe-Claire-Dorval, Qué., Canada), 15(*S*)-hydroperoxy-5,8,11,13-(*Z,Z,Z,E*)-eicosatetraenoic acid (15-HpETE) was synthesized as described previously [25], Ficoll-Paque was from Pharmacia (Dorval, Qué., Canada), Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution (HBSS) was from GIBCO (Burlington, Ont., Canada), octadeceno-arachidonic acid ([³H]₈arachidonic acid) was from Cayman Chemical (Ann Arbor, MI, U.S.A.), and HPLC-grade solvents were purchased from Anachemia (Montréal, Qué., Canada).

Cell preparation procedures

Human peripheral blood was collected by venipuncture into heparinized tubes and PMNs were isolated as previously described [25]. All solutions used for PMN isolation were nominally Ca²⁺-free. Final PMN preparations were more than 95% pure and viability exceeded 98%, as determined by Trypan Blue exclusion. The PMNs were resuspended in Ca²⁺-free HBSS containing 10 mM Hepes (pH 7.4) at a density of 5×10^6 cells/ml for incubation.

Cell incubations

PMNs isolated in the absence of Ca²⁺ were preincubated without Ca²⁺ (Ca²⁺-depleted PMNs) or in the presence of 1.6 mM Ca²⁺ for 10 min at 37 °C (Ca²⁺-repleted PMNs) before stimulation with the agonists. Incubations were terminated by adding 0.5 vol. of ice-cold methanol/acetonitrile (1:1, v/v) containing 12.5 ng of 19-hydroxy-prostaglandin B₂ and prostaglandin B₂ as internal standards, and the denatured cell suspensions were stored at -20 °C until analysis by reversed-phase (RP) HPLC.

5-LO product analysis

Denatured samples were centrifuged at 2000 *g* for 10 min and supernatants were subjected to RP-HPLC without further treatment using on-line extraction procedures as previously described [28]. LTB₄, 6-*trans*-LTB₄ isomers, ω -oxidation metabolites of LTB₄, and 5-hydroxyeicosatetraenoic acid (5-HETE) were measured by photometry at 280 nm and 229 nm using fixed-wavelength UV detectors. The limits of detection were 0.5 ng at 280 nm and 1 ng at 229 nm.

5-LO assay

The activation of 5-LO was measured by the enzyme-catalysed transformation of exogenous 15-HpETE into 5(*S*),15(*S*)-dihydroxy-6,8,11,13-(*E,Z,Z,E*)-eicosatetraenoic acid (5,15-DiHETE) as previously described [25]. Briefly, 3 μ M of 15-HpETE is added along with the agonist or its diluent (dimethyl sulphoxide, 0.1%) to all suspensions and then incubated for 5 min. The incubation was stopped and the samples were processed and analysed as described above for 5-LO products. The reaction product, 5,15-DiHETE, was detected at 229 nm; the limit of detection was 1 ng.

Arachidonic acid assay

[³H]₈Arachidonic acid was added (20 ng per sample) as an internal standard at the denaturation step. Samples were then subjected to RP-HPLC for 5-LO product analysis, and arachidonic acid-containing fractions were collected and analysed by liquid chromatography/mass spectrometry (LC/MS) (PE Sciex

API III, Thornhill, Ont., Canada) as previously described [29]. Arachidonic acid was quantified by extrapolating the measured [³H]₈arachidonic acid/[³H]₈arachidonic acid ratio on a calibration curve. The limit of detection was 25 pg.

Measurement of [Ca²⁺]_i

Fura-2 fluorescence was monitored as previously described [30]. Briefly, 1×10^7 PMNs/ml were incubated with 1 μ M Fura-2/AM for 30 min at 37 °C. The cells were then washed twice, resuspended at 5×10^6 PMNs/ml, and transferred to the thermally (37 °C) controlled and magnetically stirred cuvette compartment of the spectrofluorimeter (Aminco-Bowman series 2, SLM-Aminco, Urbana, IL, U.S.A.). The excitation and emission wavelengths for [Ca²⁺]_i measurements were 340 and 510 nm respectively, whereas the excitation and emission wavelengths for Mn²⁺ influx measurements were 360 and 510 nm respectively. [Ca²⁺]_i was calculated as previously described [31].

SDS/PAGE of proteins and immunoblotting

Tyrosine-phosphorylated proteins were separated, blotted and revealed as described previously [32] with slight modifications. Briefly, PMN suspensions (5×10^6 /ml) were stimulated for 1 min then centrifuged rapidly in a microfuge. Cell pellets were resuspended in 100 μ l of lysis buffer (Hepes-buffered HBSS, pH 7.4, 0.5% Triton X-100, 10 mM *p*-nitrophenyl phosphate, 10 μ g/ml PMSF, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 2 mM Na₃VO₄). An aliquot (100 μ l) of 2 $\times$ sample buffer (0.125 M Trizma base, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol) was added to the samples, which were then boiled for 10 min. Proteins were separated by electrophoresis on 7.5–20% or 5–15% polyacrylamide gradient gels, transferred on to poly(vinylidene difluoride) (PVDF) membranes, and blotted with an anti-phosphotyrosine antibody. Membranes blotted with anti-phosphotyrosine antibodies were treated with 15% H₂O₂ for 1 h and reblotted with a monoclonal anti-ERK1/ERK2 antibody (diluted 1:2500).

For cPLA₂ analysis, PMN suspensions (1×10^7 /ml) were stimulated for 1 min, proteins were precipitated by adding 2 vol. of ice-cold acidified methanol (0.01 M HCl in methanol), and the mixtures were kept overnight at -80 °C. It was noted that the method of cPLA₂ precipitation with acidified methanol improved the resolution of the cPLA₂ bands and thus facilitated the assessment of the electrophoretic mobility shift of the enzyme. Samples were centrifuged and precipitated protein pellets were resuspended in 1 $\times$ sample buffer and boiled for 10 min. Proteins were separated by SDS/PAGE and transferred as above. cPLA₂ was blotted with a rabbit polyclonal anti-cPLA₂ serum and revealed using a horseradish peroxidase-coupled monoclonal antibody and DuPont's Western blot chemiluminescence reagent (DuPont-NEN, Boston, MA, U.S.A.).

Statistical analysis

Where mentioned, statistical significance was assessed using Student's paired *t*-test.

RESULTS

Effects of agonists on [Ca²⁺]_i in Ca²⁺-repleted and Ca²⁺-depleted PMNs

Preparation and incubation of PMNs under Ca²⁺-free conditions resulted in a basal level of [Ca²⁺]_i of 75 ± 9 nM (mean $\pm$ S.E.M., *n* = 9). The responsiveness of these Ca²⁺-depleted PMNs was assessed by stimulation with soluble agonists: FMLP and PAF increased [Ca²⁺]_i by 30 ± 5 nM and 40 ± 6 nM (mean $\pm$ S.E.M., *n*

Figure 1 Effect of PAF and FMLP on $[Ca^{2+}]_i$ in Ca^{2+} -depleted and Ca^{2+} -repleted PMNs

Ca^{2+} -depleted and Fura-2-loaded PMNs were incubated in the absence or presence of 1.6 mM Ca^{2+} (Ca^{2+} -repleted PMNs) for 10 min at 37 °C. At $t = 0$ min, 0, 0.2, 1.0 or 1.6 mM Ca^{2+} was added to Ca^{2+} -depleted PMNs (broken lines), alone (a), or with 100 nM PAF (b) and 100 nM FMLP (c). Ca^{2+} -repleted PMNs (solid lines) were stimulated with 100 nM PAF or 100 nM FMLP at $t = 0$ min. Results shown are from one experiment representative of three separate experiments.

= 4) over basal levels respectively (Figure 1). Preincubation of Ca^{2+} -depleted PMNs for 10 min at 37 °C in the presence of 1.6 mM Ca^{2+} repleted the internal Ca^{2+} stores and resulted in a normal biphasic elevation of $[Ca^{2+}]_i$ upon PAF or FMLP receptor stimulation. In contrast, the simultaneous addition of 0, 0.2, 1.0 and 1.6 mM Ca^{2+} and 100 nM FMLP or PAF to a suspension of Ca^{2+} -depleted PMNs resulted in an elevation of $[Ca^{2+}]_i$ that was proportional to the amount of exogenous Ca^{2+} and characterized by the absence of the rapid initial phase of $[Ca^{2+}]_i$ increase. In the

Figure 2 Effect of PAF and FMLP on Mn^{2+} influx in Ca^{2+} -depleted and Ca^{2+} -repleted PMNs

Ca^{2+} -depleted and Fura-2-loaded PMNs were preincubated in the absence or presence of 1.6 mM Ca^{2+} (Ca^{2+} -repleted PMNs) for 10 min at 37 °C, then washed, and resuspended in Ca^{2+} -free buffer immediately before stimulation. At $t = 0$ min, 0.1 mM Mn^{2+} was added to Ca^{2+} -repleted PMNs (solid line) or Ca^{2+} -depleted PMNs (broken line), alone, or in combination with 100 nM PAF or 100 nM FMLP. Results shown are from one experiment representative of four separate experiments.

absence of the agonists, the addition of 0.2, 1.0 and 1.6 mM Ca^{2+} to Ca^{2+} -depleted PMNs induced a slow elevation in $[Ca^{2+}]_i$ which plateaued at a level proportional to the concentration of exogenous Ca^{2+} .

Previous studies showed that Ca^{2+} -depleted PMNs exhibit increased permeability to extracellular cations [8,27]. This increase in plasma membrane permeability can be monitored by the quenching of intracellular Fura-2 fluorescence by the influx of extracellular Mn^{2+} through bivalent-cation channels [7]. The excitation wavelength was fixed at 360 nm, where the Fura-2 fluorescence is largely independent of Ca^{2+} binding but is quenched by Mn^{2+} . Figure 2 shows that the addition of 0.1 mM Mn^{2+} to Ca^{2+} -depleted PMNs resulted in a rapid influx of Mn^{2+} as assessed by the decreasing fluorescence. In contrast, when 0.1 mM Mn^{2+} was added to Ca^{2+} -repleted PMNs, the plasma membrane was impermeable to the cation. The permeability of Ca^{2+} -depleted and Ca^{2+} -repleted PMNs to extracellular cations was next examined under conditions of stimulation by agonists. The addition of PAF to Ca^{2+} -depleted PMNs resulted in an immediate influx of Mn^{2+} that was slightly greater than in the absence of the agonist. However, the addition of FMLP failed to enhance the influx of Mn^{2+} . In Ca^{2+} -repleted PMNs, the addition

Figure 3 Effect of PAF and FMLP on arachidonic acid release and 5-LO product synthesis in Ca^{2+} -depleted and Ca^{2+} -repleted PMNs

Ca^{2+} -depleted PMNs ($5 \times 10^6/\text{ml}$) were incubated in the absence or presence of 1.6 mM Ca^{2+} (Ca^{2+} -repleted PMNs) for 10 min at 37 °C. At $t = 0$ min, 1.6 mM Ca^{2+} was added to Ca^{2+} -depleted PMNs (open symbols) along with 100 nM PAF and 100 nM FMLP. At $t = 0$, Ca^{2+} -repleted PMNs (closed symbols) were stimulated with 100 nM PAF or 100 nM FMLP. Aliquots of PMN suspension were taken at the indicated time points and arachidonic acid and 5-LO products were measured as described in the Materials and methods section. Values given for 5-LO products account for the sum of LTB_4 , 20-OH- LTB_4 , 20-COOH- LTB_4 , 6-*trans*- LTB_4 isomers, and 5-HETE. Results shown are from one experiment representative of three separate experiments.

of FMLP or PAF resulted in an influx of extracellular Mn^{2+} that was clearly delayed (and of lower magnitude) and occurred after the emptying of the internal Ca^{2+} stores, as monitored by the initial spike of Ca^{2+} mobilization in Ca^{2+} -repleted cells (Figure 1). These results are consistent with the interpretation that the increase in $[\text{Ca}^{2+}]_i$ in Ca^{2+} -repleted PMNs is the result of Ca^{2+} released from internal stores and of the influx of extracellular Ca^{2+} , while in Ca^{2+} -depleted PMNs the increase in $[\text{Ca}^{2+}]_i$ is predominantly the consequence of Ca^{2+} influx from the extracellular milieu.

Arachidonic acid release and 5-LO product synthesis in Ca^{2+} -repleted and Ca^{2+} -depleted PMNs

Stimulation of Ca^{2+} -repleted PMNs with either PAF or FMLP did not result in detectable 5-LO product synthesis as detected by RP-HPLC (Figure 3). However, when 1.6 mM Ca^{2+} was added along with either FMLP or PAF to Ca^{2+} -depleted PMNs, a significant synthesis of 5-LO product was detected after a short lag time of about 30 s. The addition of PAF consistently resulted in greater stimulation of synthesis of 5-LO products than the

addition of FMLP. The 5-LO products detected during PAF stimulation consisted of LTB_4 (and its oxidation metabolites) and 5-HETE in similar amounts; those detected during FMLP stimulation consisted of LTB_4 and its oxidation metabolites only. The addition of 1.6 mM Ca^{2+} , PAF or FMLP alone to Ca^{2+} -depleted PMNs failed to induce LTB_4 synthesis (results not shown).

The kinetics of arachidonic acid release were assessed in Ca^{2+} -depleted and Ca^{2+} -repleted PMNs. Figure 3 shows that the soluble agonists failed to stimulate significant release of arachidonic acid (over basal levels) in Ca^{2+} -repleted PMNs. In contrast, the simultaneous addition of soluble agonists and 1.6 mM Ca^{2+} to Ca^{2+} -depleted PMNs induced the release of arachidonic acid, which peaked at 1 to 2 min. The addition of Ca^{2+} or the soluble agonists alone to Ca^{2+} -depleted PMNs did not stimulate the release of arachidonic acid (results not shown).

Activation of 5-LO in Ca^{2+} -repleted and Ca^{2+} -depleted PMNs

5-LO activation was assessed using an assay that measures 5-LO-catalysed formation of 5,15-DiHETE from exogenous 15-

Table 1 Effect of PAF and FMLP on 5-LO activation in Ca²⁺-depleted and Ca²⁺-repleted PMNs

Ca²⁺-depleted PMNs (5×10^6 /ml) were incubated in the absence or presence of 1.6 mM Ca²⁺ (Ca²⁺-repleted PMNs) for 10 min at 37 °C. Then 1.6 mM Ca²⁺ was added along with 3 μ M 15-HpETE and 100 nM PAF or 100 nM FMLP to Ca²⁺-depleted PMNs. Ca²⁺-repleted PMNs were stimulated with either 100 nM PAF or 100 nM FMLP in the presence of 3 μ M 15-HpETE. After 5 min, incubations were stopped and 5,15-DiHETE synthesis was measured as described in the Materials and methods section. Results are expressed as the mean $\pm$ S.E.M. of eight experiments each performed in triplicate.

	5,15-DiHETE synthesis (ng/5 $\times 10^6$ PMNs)		
	Diluent	PAF	FMLP
Ca ²⁺ -depleted PMNs	2.8 $\pm$ 0.3	52.3 $\pm$ 9.2	22.1 $\pm$ 4.7
Ca ²⁺ -repleted PMNs	4.1 $\pm$ 0.6	36.0 $\pm$ 4.8	19.5 $\pm$ 2.7

HpETE [25]. Table 1 shows that both PAF and FMLP induced the activation of 5-LO in Ca²⁺-repleted PMNs. The simultaneous addition of Ca²⁺ and soluble agonists to Ca²⁺-depleted PMNs also induced the activation of 5-LO at levels not different from those measured in Ca²⁺-repleted PMNs ($P = 0.057$ for PAF and $P = 0.304$ for FMLP, $n = 8$). The addition of Ca²⁺ alone to Ca²⁺-depleted PMNs failed to activate 5-LO, whereas PAF and FMLP, by themselves, induced a minor activation of the enzyme in these cells (results not shown). The addition of 15-HpETE alone to either Ca²⁺-depleted or Ca²⁺-repleted PMNs resulted in 5,15-DiHETE synthesis that did not exceed 3 ng/5 $\times 10^6$ PMNs.

Taken together, these results show that 5-LO activation in Ca²⁺-depleted and Ca²⁺-repleted PMNs did not correlate with the drastic changes in LTB₄ synthesis observed under similar conditions (Figure 3).

Correlation of arachidonic acid release with [Ca²⁺]_i in Ca²⁺-depleted PMNs

The relationship between arachidonic acid release and the concentrations of exogenous Ca²⁺ added to Ca²⁺-depleted PMNs was examined (Figure 4, left-hand panels). Free arachidonic acid levels progressively increased in response to the addition of soluble agonists and 0.1 mM to 0.3 mM extracellular Ca²⁺, at which concentration maximum release occurred. In these experiments, [Ca²⁺]_i also increased in the same range of extracellular Ca²⁺ concentration, but did not plateau (Figure 4, middle panels). [Ca²⁺]_i was next plotted against the levels of free arachidonic acid (Figure 4, right-hand panels). The resulting curves were best fitted using a sigmoidal function for both PAF ($r^2 = 0.957$) and FMLP ($r^2 = 0.998$). These results show that the release of arachidonic acid occurs between 150 nM and 350 nM [Ca²⁺]_i, at which concentration maximum release is reached.

cPLA₂ electrophoretic mobility shift in Ca²⁺-repleted and Ca²⁺-depleted PMNs

The phosphorylation of cPLA₂ on the Ser-505 residue by MAP kinase was recently shown to activate the enzyme and to lead to a decreased electrophoretic mobility [20]. The observation that arachidonic acid is released in Ca²⁺-depleted PMNs but not in Ca²⁺-repleted PMNs led us to examine the phosphorylation

Figure 4 Correlations between arachidonic acid release, [Ca²⁺]_i, and extracellular Ca²⁺ concentration in Ca²⁺-depleted PMNs stimulated with PAF and FMLP

Ca²⁺ was added along with 100 nM PAF or 100 nM FMLP to Ca²⁺-depleted PMNs (5×10^6 /ml) at 37 °C. [Ca²⁺]_i and arachidonic acid were measured after 1 min as described in the Materials and methods section. Results are expressed as the mean $\pm$ S.E.M. of three (arachidonic acid) or four ([Ca²⁺]_i) separate experiments. Data shown in the right-hand panels were generated from the data used in the left-hand and middle panels.

Figure 5 Electrophoretic mobility of cPLA₂ in Ca²⁺-depleted and Ca²⁺-repleted PMNs

Ca²⁺-depleted PMNs (5×10^6 /ml) were incubated in the absence or presence of 1.6 mM Ca²⁺ (Ca²⁺-repleted PMNs) for 10 min at 37 °C. Dimethyl sulphoxide (0.1%; control), 1.6 mM Ca²⁺, 100 nM PAF or 100 nM FMLP, or 1.6 mM Ca²⁺ and 100 nM PAF or FMLP were added to Ca²⁺-depleted PMNs. Dimethyl sulphoxide, 100 nM PAF, or 100 nM FMLP was added to Ca²⁺-repleted PMNs. Incubations were stopped after 1 min by precipitation of proteins with acidified methanol, and the cPLA₂ mobility shift was analysed by SDS/PAGE (5–15% gradient gels) followed by immunoblotting with an anti-cPLA₂ antibody as described in the Materials and methods section. Results shown are from one experiment representative of three separate experiments.

status of cPLA₂ under both experimental conditions. SDS/PAGE separation of proteins of unstimulated Ca²⁺-depleted and Ca²⁺-repleted PMNs followed by blotting with an anti-cPLA₂ antibody revealed partial phosphorylation of the cPLA₂ as shown by the presence of a doublet, where the upper band corresponded to the phosphorylated form of the enzyme (Figure 5). PAF or FMLP stimulation of both Ca²⁺-depleted and Ca²⁺-repleted PMNs induced the phosphorylation of cPLA₂ as observed by the complete upward shift of the lower band. The addition of 1.6 mM Ca²⁺ and PAF or FMLP to Ca²⁺-depleted PMNs induced a similar shift of cPLA₂.

Tyrosine phosphorylation of proteins in Ca²⁺-repleted and Ca²⁺-depleted PMNs

Tyrosine phosphorylation of proteins in PMNs is thought to play an important role in the release of arachidonic acid [33]. We sought to examine whether the conditions tested in the present study for the release of arachidonic acid affected the tyrosine phosphorylation of PMN proteins, in particular the MAP kinase, the activity of which was shown to be stimulated by tyrosine phosphorylation [34–37] and related to the stimulation of cPLA₂ activity [20,21]. As for cPLA₂, the increased phosphorylation of MAP kinase can be monitored by the decreased electrophoretic mobility of the phosphorylated form of the protein [38,39]. Stimulation with either FMLP or PAF resulted in the tyrosine phosphorylation of several proteins in both Ca²⁺-depleted and Ca²⁺-repleted PMNs, as assessed by SDS/PAGE of proteins followed by blotting of cellular proteins with anti-phosphotyrosine antibodies (Figure 6a). The simultaneous addition of Ca²⁺ and the soluble agonists to Ca²⁺-depleted PMNs induced a significant increase in tyrosine phosphorylation of several proteins as compared with agonist stimulation of Ca²⁺-repleted PMNs. Densitometric analysis of the proteins that showed enhanced tyrosine phosphorylation in response to FMLP + Ca²⁺ in Ca²⁺-depleted PMNs revealed an enhancement of 1.4 ± 0.1 -fold (S.E.M., $n = 3$, $P = 0.034$) for the 120 kDa protein, 1.2 ± 0.1 -fold (S.E.M., $n = 3$, $P = 0.044$) for the 66 kDa protein, and 1.6 ± 0.2 -fold (S.E.M., $n = 3$, $P = 0.029$) for the 42 kDa protein. The enhancement of protein tyrosine phosphorylation in response to stimulation with PAF + Ca²⁺ in Ca²⁺-depleted PMNs was 3.2 ± 0.6 -fold (S.E.M., $n = 4$, $P = 0.017$) for the 80 kDa protein, 3.7 ± 0.9 -fold (S.E.M., $n = 4$, $P = 0.029$) for the 66 kDa

Figure 6 Tyrosine phosphorylation of proteins and electrophoretic mobility of MAP kinase in Ca²⁺-depleted and Ca²⁺-repleted PMNs

Ca²⁺-depleted PMNs (5×10^6 /ml) were incubated in the absence or presence of 1.6 mM Ca²⁺ (Ca²⁺-repleted PMNs) for 10 min at 37 °C. (a) Dimethyl sulphoxide (0.1%; control), 1.6 mM Ca²⁺, 100 nM PAF or 100 nM FMLP, or 1.6 mM Ca²⁺ and 100 nM PAF or FMLP were added to Ca²⁺-depleted PMNs. Dimethyl sulphoxide, 100 nM PAF, or 100 nM FMLP was added to Ca²⁺-repleted PMNs. After 1 min, incubations were stopped and cellular proteins were analysed by SDS/PAGE (7.5–20% gradient gels) followed by immunoblotting with anti-phosphotyrosine antibodies (a) as described in the Materials and methods section. Arrows indicate statistically significant enhancement of tyrosine-phosphorylated proteins by the agonist + Ca²⁺ (see text). (b) MAP kinase was analysed for mobility shifts by reblotting membranes shown in (a) with an anti-(MAP kinase) antibody which recognizes both ERK1 and ERK2. Results shown are from one experiment representative of three (FMLP) or four (PAF) separate experiments.

protein, and 3.0 ± 0.4 -fold (S.E.M., $n = 4$, $P = 0.009$) for the 42 kDa protein. While the identity of the tyrosine-phosphorylated proteins of 120, 80 and 66 kDa are currently unknown, the 42 kDa protein was identified as a member of the MAP kinase family on the basis of its immunoreactivity with an anti-(MAP kinase) antibody (Figure 6b). In the absence of added Ca²⁺, the agonists induced similar levels of tyrosine phosphorylation of MAP kinase in Ca²⁺-depleted and Ca²⁺-repleted PMNs.

DISCUSSION

Ca²⁺ plays a central role in the LTB₄ biosynthetic pathway because of its involvement in the activation of two key enzymes, 5-LO and PLA₂. Although the PLA₂-mediated release of arachi-

donic acid from phospholipids is dependent on Ca²⁺, this relationship is complex and not completely understood. Previous studies have shown that in a variety of cell types, agonist-induced arachidonic acid release can be prevented by removal of extracellular Ca²⁺ [40–46], suggesting that PLA₂ activation is dependent on the influx of extracellular Ca²⁺. In human PMNs, however, soluble agonists that cause an elevation of [Ca²⁺]_i do not induce significant arachidonic acid release even in the presence of extracellular Ca²⁺ [24–26]. To investigate the relationship between [Ca²⁺]_i, arachidonic acid release and LTB₄ synthesis in human PMNs, we used Ca²⁺-depleted PMNs, which show increased plasma membrane permeability to extracellular cations via non-specific channels [8,27]. This increased permeability to cations can be used to manipulate [Ca²⁺]_i by varying the extracellular concentration of Ca²⁺.

Our study measured a threshold of [Ca²⁺]_i for arachidonic acid release at ~150 nM, and maximal release was observed at ~350 nM under conditions of stimulation with either PAF or FMLP; the EC₅₀ values were 225 and 210 nM, respectively. These values are compatible with the EC₅₀ value of 300 nM measured for the activation of an arachidonoyl-selective high-molecular-mass cPLA₂ by Ca²⁺ from THP-1 monocytic cells [47]. Other studies on a U937 monocytic cell cPLA₂ have demonstrated activation with Ca²⁺ concentrations of 150–600 nM [48] and 100–1000 nM [49,50]. Thus, although a role for cPLA₂ in arachidonic acid release in human PMNs has not been established yet, the Ca²⁺ concentration ranges reported previously for cPLA₂ activation, and in the present study for arachidonic acid release in Ca²⁺-depleted PMNs, provide some support for the involvement of the cPLA₂ in the LTB₄ biosynthetic pathway in human PMNs, where such arachidonoyl-selective cPLA₂ was recently detected [51–53]. The sPLA₂ may also be present in PMNs and may participate in the release of arachidonic acid as suggested by pharmacological studies [54]; however, studies on this type of enzyme showed that its requirement for Ca²⁺ was in the millimolar range [13]. Such consideration does not rule out the potential participation of sPLA₂ in arachidonic acid release in PMNs, as the enzyme may act extracellularly (upon secretion) where the concentration of Ca²⁺ is in the millimolar range.

Our experiments with this model have enabled observations relevant to the regulation of LTB₄ synthesis to be made. Our data clearly demonstrate that the release of arachidonic acid (and LTB₄ synthesis) in Ca²⁺-depleted PMNs stimulated with agonists was closely related to the influx of extracellular Ca²⁺. However, the addition of Ca²⁺ alone to Ca²⁺-depleted PMNs induced a rise in [Ca²⁺]_i (150–225 nM at 1.6 mM extracellular Ca²⁺) that failed to induce the release of arachidonic acid, suggesting that the influx of Ca²⁺ alone is an insufficient signal; similarly the stimulation of these cells by the agonists alone, which resulted in cPLA₂ phosphorylation, did not induce detectable release of arachidonic acid. Moreover, in agreement with our observations, the treatment of PMNs with thapsigargin, an inhibitor of the endomembrane Ca²⁺-ATPase which causes an emptying of internal Ca²⁺ stores and a subsequent influx of extracellular Ca²⁺, does not induce arachidonic acid release in human PMNs [55]. However, thapsigargin efficiently primed the PMNs for arachidonic acid release induced by FMLP [55]. These data emphasized that Ca²⁺ influx in combination with agonist-stimulated events, i.e. phosphorylation, appear to be required for the induction of arachidonic acid release and LTB₄ synthesis. Accordingly, Ca²⁺ ionophores (such as A23187), which are recognized as potent inducers of LTB₄ synthesis, provoke a marked influx of extracellular Ca²⁺ but also the phosphorylation of several proteins, including MAP kinase [34] and cPLA₂ [50]. In the present study, we noted that the Ca²⁺-depletion of PMNs and stimulation with

1 μM A23187 (in the absence of extracellular Ca²⁺) resulted in a near complete suppression of LTB₄ synthesis as compared with similar stimulation of Ca²⁺-repleted PMNs (results not shown).

In the present study, the difference in the ability of Ca²⁺-repleted and Ca²⁺-depleted PMNs to release arachidonic acid and to form LTB₄ in response to agonist stimulation was striking, and indicated that intracellular Ca²⁺ depletion of PMNs strongly facilitates the release of free arachidonic acid. The major difference between the two experimental conditions tested is at the level of the internal Ca²⁺ stores, which are either normal or depleted. In view of recent studies that have shown that extracellular Ca²⁺ entry via the plasma membrane is regulated by the emptying of internal Ca²⁺ stores [8,56–58], it is tempting to speculate that in the Ca²⁺-repleted normal PMNs, insufficient emptying of internal Ca²⁺ stores by the agonists may limit the influx of Ca²⁺ required for arachidonic acid release. However, arachidonic acid release and LTB₄ synthesis were observed in Ca²⁺-depleted PMNs (upon simultaneous addition of Ca²⁺ and agonists) at [Ca²⁺]_i levels similar and lower than those observed in agonist-stimulated Ca²⁺-repleted PMNs, where arachidonic acid release did not occur, thus making this hypothesis unlikely.

cPLA₂ activity is increased by the phosphorylation of a serine residue by MAP kinase *in vitro* [20,21]; MAP kinase is itself activated by the phosphorylation of both serine and tyrosine residues [59,60]. Considering a possible role of cPLA₂ in the release of arachidonic acid, we compared the ability of the agonists to induce the phosphorylation of cPLA₂ and MAP kinase in Ca²⁺-depleted and Ca²⁺-repleted PMNs. The results show that phosphorylation of cPLA₂, as monitored by the shift in electrophoretic mobility, occurred in both Ca²⁺-depleted and Ca²⁺-repleted PMNs. With regard to MAP kinase phosphorylation experiments, it was noted that the data closely paralleled those of cPLA₂ phosphorylation. Indeed, MAP kinase phosphorylation, as assessed by the decreased mobility using an anti-(MAP kinase) antibody, was observed upon agonist stimulation in normal (Ca²⁺-repleted) PMNs, in agreement with other studies [34–37], and in Ca²⁺-depleted PMNs. While the role of these phosphorylation events in the regulation of arachidonic acid release in human PMNs remains to be established, the data demonstrated that there was no significant differences in the electrophoretic shift of cPLA₂ and MAP kinase between the agonist-stimulated Ca²⁺-depleted and Ca²⁺-repleted PMNs. However, a significant increase in the tyrosine phosphorylation of MAP kinase, as well as 120, 80, and 66 kDa proteins, was noted in Ca²⁺-depleted cells to which Ca²⁺ and the agonists were added simultaneously. While the physiological relevance of this observation is not known at present, one cannot but notice that it correlates with the increased liberation of arachidonic acid that is measured under the same conditions. Indeed, the greatest amount of 5-LO products synthesis occurred in the conditions where Ca²⁺ and PAF were added to Ca²⁺-depleted PMNs and where the strongest enhancement of tyrosine phosphorylation of 80, 66, and 42 kDa proteins was present. That the increased tyrosine phosphorylation of MAP kinase is not reflected in a parallel decrease in electrophoretic mobility (of MAP kinase and/or cPLA₂) may be due to the lower sensitivity of the latter parameter or to the existence of additional, and as yet unidentified, control pathways. Whether a causal relationship exists between the increased tyrosine phosphorylation of MAP kinase (and the other proteins) and the amounts of arachidonic acid liberated in Ca²⁺-depleted PMNs remains to be determined.

Interestingly, our [Ca²⁺]_i measurements and cPLA₂ phosphorylation data in Ca²⁺-depleted PMNs are fully compatible with the concept recently put forward that both enhanced [Ca²⁺]_i and cPLA₂ phosphorylation are necessary for stimulation of

cPLA₂ activity [52]. Indeed, while the addition of Ca²⁺ or agonists alone to Ca²⁺-depleted PMNs results in enhanced [Ca²⁺]_i and cPLA₂ phosphorylation respectively, arachidonic acid release by the Ca²⁺-depleted PMNs was observed only in the experimental conditions that resulted in the simultaneous enhancement of both of these events. Finally, we would like to emphasize that the Ca²⁺-depleted PMNs represent a useful model for studies of the pharmacological control of lipid mediator synthesis. Indeed, in contrast to normal PMNs, Ca²⁺-depleted PMNs yield 5-LO products upon stimulation with soluble agonists in amounts measurable by HPLC methods. For similar reasons, the model is also useful for the investigation of the mechanisms of regulation of LTB₄ synthesis in PMNs.

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