Biochemical Pharmacology, Vol. 39, No. 3, pp. 559–567, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INVOLVEMENT OF CALCIUM IN THE THIMEROSAL-STIMULATED FORMATION OF LEUKOTRIENE BY fMLP IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

ARMIN HATZELMANN, MICHAEL HAURAND* and VOLKER ULLRICH†
Faculty of Biology, University of Konstanz and *Grünenthal GmbH, Center of Research, Aachen, Federal Republic of Germany

(*Received* 17 *May* 1989; *accepted* 3 *September* 1989)

Abstract—Only small amounts of leukotrienes could be detected by reverse-phase HPLC analysis after stimulation of human polymorphonuclear leukocytes (PMN) by the receptor agonist *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). Preincubation of the cells with the organomercury compound thimerosal prior to fMLP-addition, however, resulted in the formation of significant amounts of 5-lipoxygenase derived metabolites. This effect was dose-dependent with respect both to fMLP and thimerosal. Thimerosal alone did neither lead to the formation of HPLC-detectable leukotrienes nor to the release of arachidonic acid in [1-^{14}C]arachidonic acid prelabelled cells. The formation of leukotrienes by fMLP/thimerosal required extracellular Ca^{2+}. Measurements of intracellular Ca^{2+}-levels revealed that (i) thimerosal alone is able to release Ca^{2+} from internal stores and (ii) thimerosal causes a persistent accumulation of Ca^{2+} within the cells after stimulation by fMLP. We conclude that by the synergistic action of fMLP and thimerosal the Ca^{2+}-levels exceed the threshold for phospholipase A_2 activation resulting in the liberation of arachidonic acid and subsequently in the formation of 5-lipoxygenase products. Our results suggest that thimerosal may provide a model for leukotriene formation under pathophysiological conditions when SH-group oxidation leads to increased intracellular Ca^{2+}-levels.

Human polymorphonuclear leukocytes (PMN‡) are not only involved in the host defense against bacterial infections but also play a pivotal role in several pathophysiological situations [1]. Stimulation of human PMN by soluble receptor agonists, like fMLP, induces chemotaxis, adherence, degranulation and stimulation of an "oxidative burst". It is generally accepted that the binding of fMLP to its receptor results in the activation of phospholipase C initiating the so-called "phosphatidylinositol-cycle" [2]. It follows the activation of proteinkinase C by diacylglycerol, release of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate (IP_3) and the influx of extracellular Ca^{2+} [3]. Subsequently, the intracellular Ca^{2+}-increase activates phospholipase A_2 leading to the liberation of arachidonic acid which in PMN is metabolized by 5-lipoxygenase both to 5-hydroperoxyicosatetraenoic acid and to leukotriene A_4 [4, 5]; in neutrophils LTA_4 is further converted to LTB_4 by a specific hydrolase [6].

In contrast to the stimulation with Ca^{2+}-ionophores (such as A23187 or ionomycin) activation of human PMN by fMLP does not lead to HPLC-detectable amounts of leukotrienes in spite of the increase of intracellular Ca^{2+}-levels. If exogenous arachidonate is added fMLP supports the formation of 5-lipoxygenase products [7, 8]. Obviously, the fMLP-stimulus does not allow to generate sufficient amounts of arachidonate.

The intracellular levels of this fatty acid in cells are controlled by two main parameters [9]: the action of phospholipase A_2 which requires Ca^{2+} for activation and the reincorporation of arachidonate into complex and neutral lipids. Thus, inhibition of reacylation and activation of phospholipase A_2 should lead to increased levels of arachidonate. Thimerosal, an organomercury compound, was reported to be an inhibitor of acyl-CoA:lysophosphatidyl-acyltransferase [10] and by this property the formation of prostanoids in rat peritoneal macrophages and human platelets [11, 12] and of prostacyclin in rabbit aorta strips [13] upon incubation with thimerosal has been explained. In this paper we provide evidence that in PMN thimerosal enhances the fMLP-induced formation of 5-lipoxygenase metabolites by modulating the Ca^{2+}-homeostasis of the cells.

MATERIALS AND METHODS

Chemicals. Thimerosal (sodium ethylmercurithiosalicylate), fMLP (*N*-formylmethionyl-leucyl-phenylalanine), TPA (12-*O*-tetradecanoyl phorbol 13-acetate), DTT (dithiothreitol), EGTA (ethyleneglycol-bis-[β-amino-ethylether]-*N*, *N*, *N'*, *N'*-tetraacetic acid) and digitonin were purchased from the Sigma Chemical Co. (Deisenhofen, F.R.G.).

Arachidonic acid was obtained from Larodan

† Correspondence to: V. Ullrich, Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Federal Republic of Germany.

‡ Abbreviations: PMN, polymorphonuclear leukocytes; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; DMSO, dimethylsulfoxide; HO-Δ_4-Ach, hydroxyicosatetraenoic acid; HPLC, high performance liquid chromatography; LT, leukotriene.

Enzymes: Acyl-CoA:lysophosphatidylacyltransferase (EC 2.3.1.23); Ca^{2+}-ATPase (EC 3.6.1.8); lactate dehydrogenase (EC 1.1.1.27); 5-lipoxygenase (EC 1.13.11.34); phospholipase A_2 (EC 3.1.1.4); phospholipase C (EC 3.1.4.3); proteinkinase C (EC 2.7.1.37).

(Malmö, Sweden), [1-^{14}C]arachidonic acid from New England Nuclear (Dreieich, F.R.G.) and [8-^{14}C]adenine from Amersham Buchler (Braunschweig, F.R.G.).

Ionomycin and fura-2/AM (pentaacetoxymethyl-ester) were obtained from Calbiochem (Frankfurt, F.R.G.).

All other solvents and chemicals utilized were of HPLC or analytical grade and were purchased from Merck (Darmstadt, F.R.G.).

Polymorphonuclear leukocytes. The preparation of human PMN was performed by dextran-sedimentation and subsequent centrifugation on ficoll-paque as described [14]. The purity of the PMN was about 95% and the viability, as measured by trypan blue exclusion and lactate dehydrogenase release was greater than 97%. After isolation the cells were stored at 4° in NaCl/P_i (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 280 mOsm).

Reverse-phase HPLC analysis and Ca^{2+}-measurements. Extraction and HPLC analysis of total arachidonate metabolites as well as the measurements of intracellular Ca^{2+}-levels were performed as described [14].

Measurement of phospholipase A_2 activity. Cells prelabelled with [1-^{14}C]arachidonic acid were used [14]. To determine the effect of thimerosal 10^7 cells/mL in NaCl/P_i/1 mM Ca^{2+} were preincubated at 37° with the indicated concentrations of this substance. Five minutes after stimulation with fMLP (0.1 μM) the cells were spun down for 10–15 sec in an Eppendorf centrifuge at 10,000 *g* and 0.95 mL of the supernatant was collected. Aliquots of the supernatants were counted for radioactivity in a liquid scintillation counter (PN 4700, Isomess, Straubenhardt, F.R.G.) and the values taken as a parameter for phospholipase A_2 activity. Additionally the supernatant was extracted by 4 mL ethylacetate at pH 3, the ethylacetate phase evaporated to dryness under nitrogen and dissolved in 50 μL chloroform/methanol (9:1, v/v) for thin-layer chromatography (aluminium plates 20 × 20 cm, silica gel 60, Merck) essentially as described by Smith and Waite [15].

Briefly the plates were chromatographed first over a distance of 6.5 cm in a solvent system containing chloroform/methanol/acetic acid/water (75:50:10:6, v/v/v/v), taken out, and allowed to airdry. The plates were then completely rechromatographed in a solvent system containing hexane/diethylether/formic acid (90:60:6, v/v/v). This system allows the separation of polar 5-lipoxygenase derived metabolites (LTB_4 and its isomers ω-OH- and ω-COOH-LTB_4), hydroxyicosatetraenoic acids (15*S*)-5-OH- and (15*S*)-15-OH-Δ_4Ach) and free arachidonic acid. Calculation of the peak areas was performed on a thin-layer chromatography linear analyzer (Isomess, Staubenhardt, F.R.G.).

Lactate dehydrogenase and adenine nucleotide release. Lactate dehydrogenase activity was measured as described by Bergmeyer [16] and adenine nucleotide release as described by Shirhatti and Krishna [17] with some modifications. In brief, cells (2×10^7/mL in NaCl/P_i/1 mM Ca^{2+}/5 mM glucose) were incubated for 2 hr at 25° with 4 μM (0.2 μCi/mL) [8-^{14}C]adenine; afterwards the cells were washed two times. The release of radioactivity into the supernatant of the different assays were referred to 100% radioactivity of a cell suspension treated with 0.1% Triton X-100. Compared to control incubations (without thimerosal) neither lactate dehydrogenase nor adenine nucleotide release into the supernatant were enhanced by thimerosal (tested up to 100 μM), suggesting that it did not affect the viability of the cells under the conditions used in our assays.

Statistical analyses. Results are expressed as the means ± SE of the number of experiments indicated.

RESULTS

Effect of thimerosal on leukotriene B_4 formation

After stimulation of human PMN with fMLP no (5*S*)-5-OH-Δ_4Ach or LTB_4 and only trace amounts of ω-COOH- and ω-OH-LTB_4 could be detected (Fig. 1A). Preincubation of the cells with thimerosal, however, resulted in the formation of the primary 5-lipoxygenase metabolites (5*S*)-5-OH-Δ_4Ach, LTB_4 and its isomers (Fig. 1B). Also small amounts of (12*S*)-12-OH-Δ_4Ach were detectable most probably due to contamination by platelets. The amount and the pattern of metabolites was very similar to that obtainable after stimulation of human PMN with Ca^{2+}-ionophores, e.g. ionomycin, as demonstrated in Fig. 1C. This chromatogram also shows the peaks for ω-COOH- and ω-OH-LTB_4 which could not be monitored in the presence of thimerosal because of an interference with this compound.

The stimulatory effect of thimerosal was dose-dependent with respect to both fMLP and thimerosal (Fig. 2A and B). At 50 μM of the organomercurial compound a maximum was obtained with a sharp decline at 100 μM where obviously other inhibitory actions became dominant. This dose–response curve was dependent on the cell number and shifted to the left with decreasing cell concentrations. For fMLP the optimum was at 1 μM in the presence of 50 μM thimerosal. Subsequent assays were performed at suboptimal concentrations of both agents in order to exclude possible unspecific or deleterious effects. No leukotriene formation could be detected with thimerosal alone. The stimulation by thimerosal in the presence of fMLP was abolished when extracellular Ca^{2+} was omitted.

Effect of thimerosal on intracellular Ca^{2+}-levels

Fura-2 loaded cells in the response of 0.5 mM extracellular Ca^{2+} responded to thimerosal by a slow rise in Ca_i^{2+} and between 5 and 20 μM approached a steady state level within 2–3 min while at 50 μM the Ca^{2+}-level continuously increased (Fig. 3A). When extracellular Ca^{2+} was omitted and EGTA was present the initial rise was unchanged but after several minutes the steady state declined again and after 10–15 min almost reached the starting Ca^{2+} level again (Fig. 3B).

When fMLP was added 3 min after thimerosal a characteristic biphasic Ca^{2+}-signal appeared (Fig. 4A). It is obvious that the first Ca^{2+}-transient was always identical irrespective of the previous thimerosal effect but that the second phase response was greatly increased by thimerosal. The latter effect

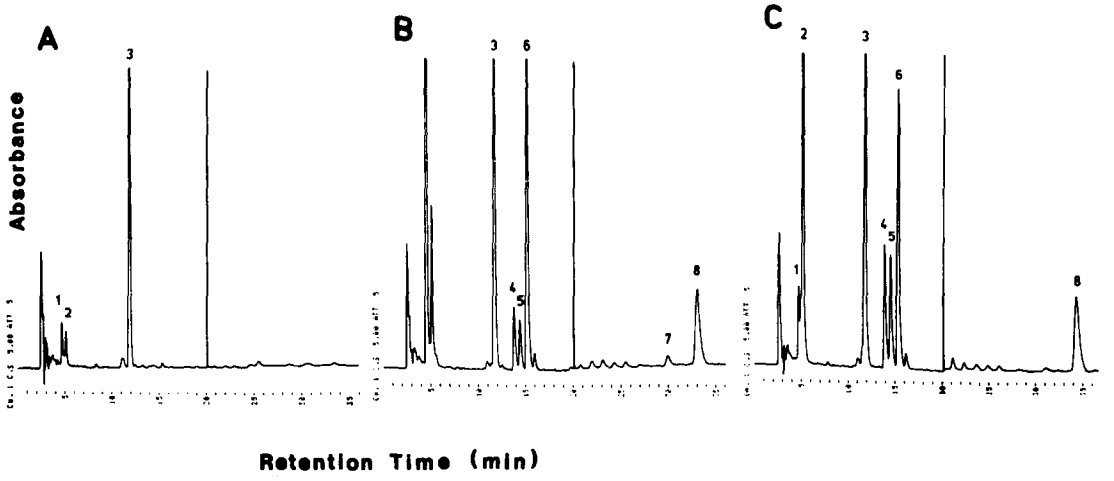

Fig. 1. Reverse-phase HPLC chromatograms of 5-lipoxygenase metabolites from human PMN (10^7 cells/mL in NaCl/P_i/1 mM Ca^{2+}) stimulated with (A) 100 nM fMLP; (B) 100 nM fMLP after preincubation with 20 μM thimerosal; and (C) 0.5 μM ionomycin for 5 min at 37°. At a retention time of 20 min the detection wavelength was switched from 280 nm to 237 nm. ω-COOH-LTB_4 (1); ω-OH-LTB_4 (2); prostaglandin B_2, internal standard (3): Δ^6-trans-LTB_4, 2 isomers (4,5); LTB_4 (6); (12*S*)-12-OH-Δ_4Ach (7); (5*S*)-5-OH-Δ_4Ach (8).

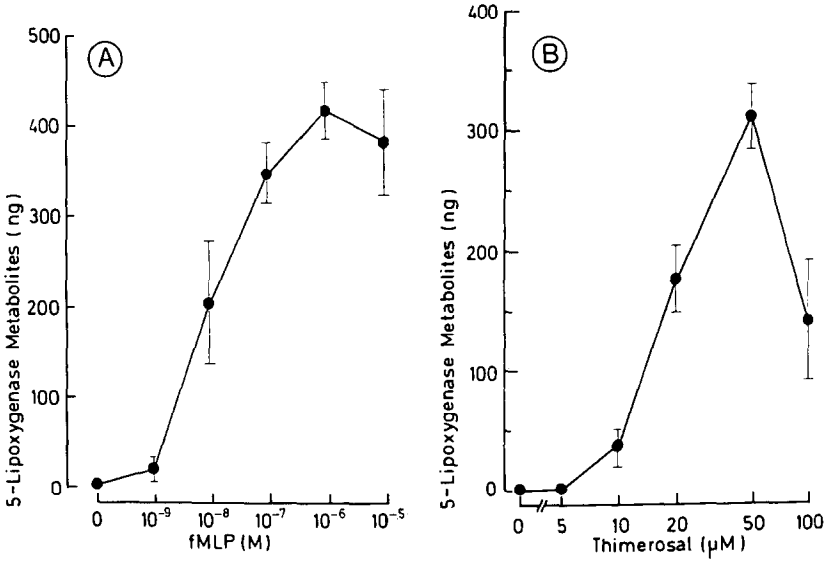

Fig. 2. Concentration-dependence of the fMLP/thimerosal stimulated formation of leukotrienes in human PMN. Cells (1.5×10^7/mL in NaCl/P_i/1 mM Ca^{2+}) were preincubated for 5 min with thimerosal (0.5% DMSO) at 37° and then stimulated with fMLP for a further 5 min. The amounts of 5-lipoxygenase metabolites were analysed by reverse-phase HPLC. (A) thimerosal 50 μM; (B) fMLP 100 nM. Values are given as mean ± SE from three (A) or four (B) independent experiments.

could be suppressed by DTT. Also the proteinkinase C stimulant TPA decreased the thimerosal-dependent rise in Ca_i^{2+} and lowered the second phase, too, but had no influence on the fMLP-induced first phase (Fig. 4B). The traces in Fig. 4C were obtained with different concentrations of ionomycin also used in assays for 5-lipoxygenase products (Table 1). This table allows a comparison of the Ca^{2+}-levels and the 5-lipoxygenase metabolites obtained under identical conditions. Evidently Ca^{2+}-threshold levels exist below which no 5-lipoxygenase activity can be found.

Characteristics of the thimerosal-induced Ca^{2+}*-effects upon subsequent stimulation with fMLP*

In a cell-free system thimerosal did not influence the Ca^{2+}-dependent fura-2 fluorescence. It can therefore be excluded that thimerosal has any unspecific effect on the Ca^{2+} monitoring system. Figure 5A gives evidence that thimerosal exerts its action only in the presence of extracellular Ca^{2+} since in the presence of EGTA the second phase of the Ca^{2+}-transients is almost completely abolished and also no 5-lipoxygenase metabolites are formed. If, however,

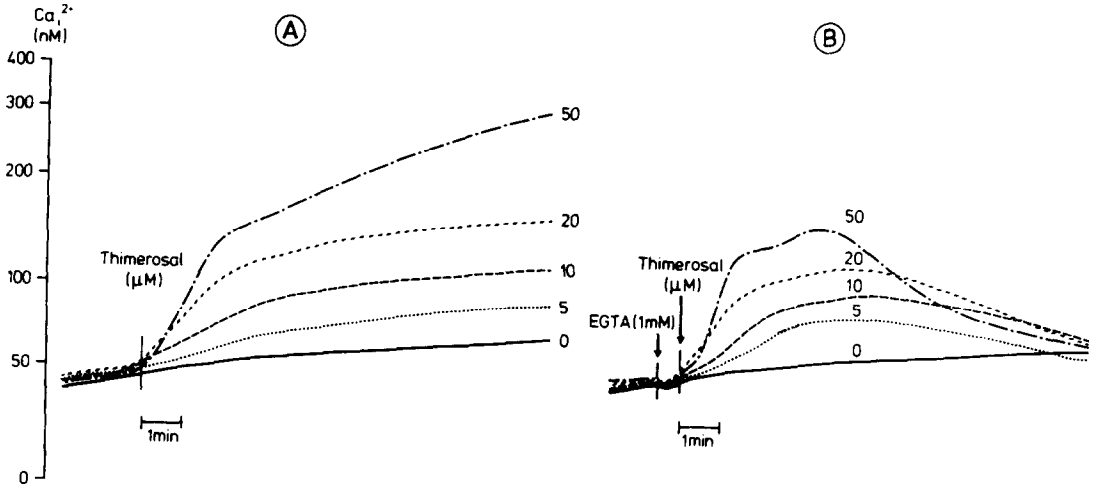

Fig. 3. Influence of extracellular Ca^{2+} on the thimerosal-induced rise in cytosolic free Ca^{2+}. Fura-2 loaded cells (10^7/mL in NaCl/P_i/0.5 mM Ca^{2+}/2 mM glucose) were equilibrated at 37°. (A) Incubation with increasing concentrations of thimerosal. (B) Preincubation of the cells with 1 mM EGTA prior to addition of increasing concentrations of thimerosal.

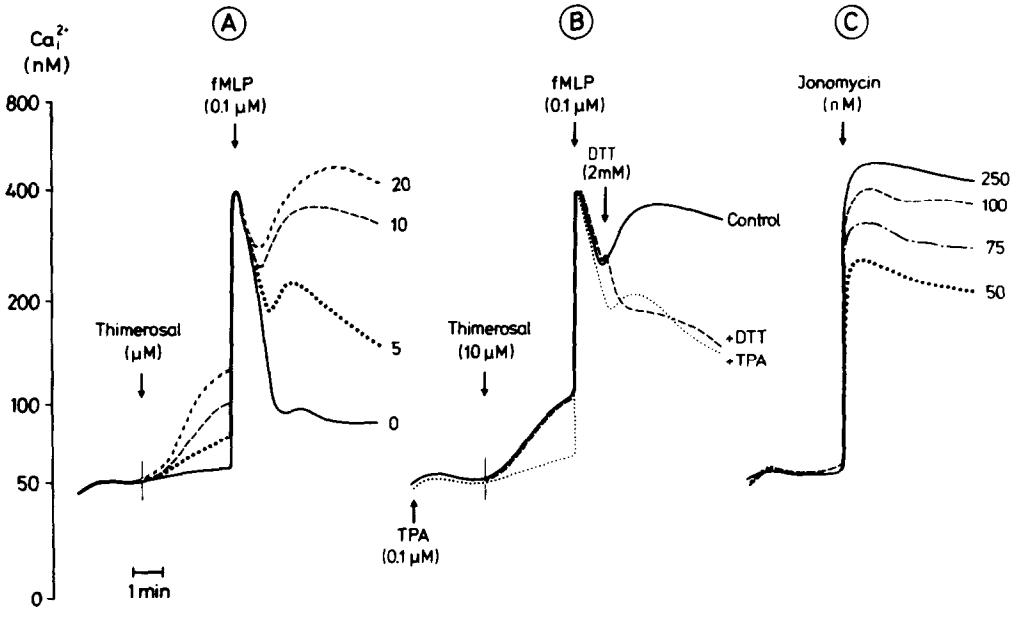

Fig. 4. Influence of thimerosal on the intracellular Ca^{2+}-levels of human PMN after stimulation with fMLP. Fura-2 loaded cells (10^7/mL in NaCl/P_i/1 mM Ca^{2+}/2 mM glucose) were equilibrated at 37°. (A) Preincubation of the cells with increasing concentrations of thimerosal prior to stimulation with fMLP. (B) Inhibition of the fMLP/thimerosal elicited enhanced Ca^{2+}-level by either preincubation of the cells with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) or addition of dithiothreitol (DTT) subsequent (1 min) to fMLP-stimulation. (C) Generation of comparable intracellular Ca^{2+}-levels with increasing concentrations of ionomycin. Five minutes after stimulation with either fMLP or ionomycin, respectively, 1 mL-aliquots of the assays were taken out of the cuvette and analysed for leukotrienes by reverse-phase HPLC analysis. The corresponding values are summarized in Table 1.

an equivalent amount of Ca^{2+} is reintroduced the second phase appears again and is much more pronounced when thimerosal is present.

The essential role of extracellular Ca^{2+} is supported when EGTA is added as the second phase develops (Fig. 5B). A time-dependent decrease to near basal levels occurs which could be reversed by Ca^{2+}-addition. These traces implicate that the activity of the Ca^{2+}-ATPases could not have been dramatically altered by thimerosal. A similar conclusion is reached when the fMLP/thimerosal experiment is carried out with 0.1 mM Ca^{2+} and in addition

Table 1. Influence of fMLP/thimerosal and ionomycin on intracellular Ca^{2+}-levels and formation of 5-lipoxygenase metabolites

System	Internal Ca^{2+}-level (nM)		5-Lipoxygenase metabolites (ng/10^7 cells)
	3 min after addition of thimerosal	3 min after addition of fMLP or ionomycin	
fMLP control	59 ± 4*	107 ± 5	ND
+ thimerosal, 5 μM	91 ± 7	217 ± 23	12 ± 3
10 μM	111 ± 6	337 ± 28	117 ± 25
20 μM	132 ± 6	500 ± 25	234 ± 27
fMLP/20 μM thimerosal control	116 ± 10	388 ± 33	153 ± 21
+ 2.0 mM DDT	116 ± 10	197 ± 19	17 ± 4
+ 0.1 μM TPA	71 ± 3	198 ± 30	56 ± 29
Ionomycin, 50 nM	—	236 ± 10	ND
75 nM	—	289 ± 13	ND
100 nM	—	391 ± 17	44 ± 5
250 nM	—	536 ± 34	332 ± 54

For experimental conditions see legend to Fig. 3. The initial Ca^{2+}-level subsequent to fMLP-stimulation was not changed under the described conditions and was determined to be 392 ± 25 nM. Values are given as mean ± SE from four independent experiments.

* Basal internal Ca^{2+}-level. ND, not detectable; DTT, dithiothreitol; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate.

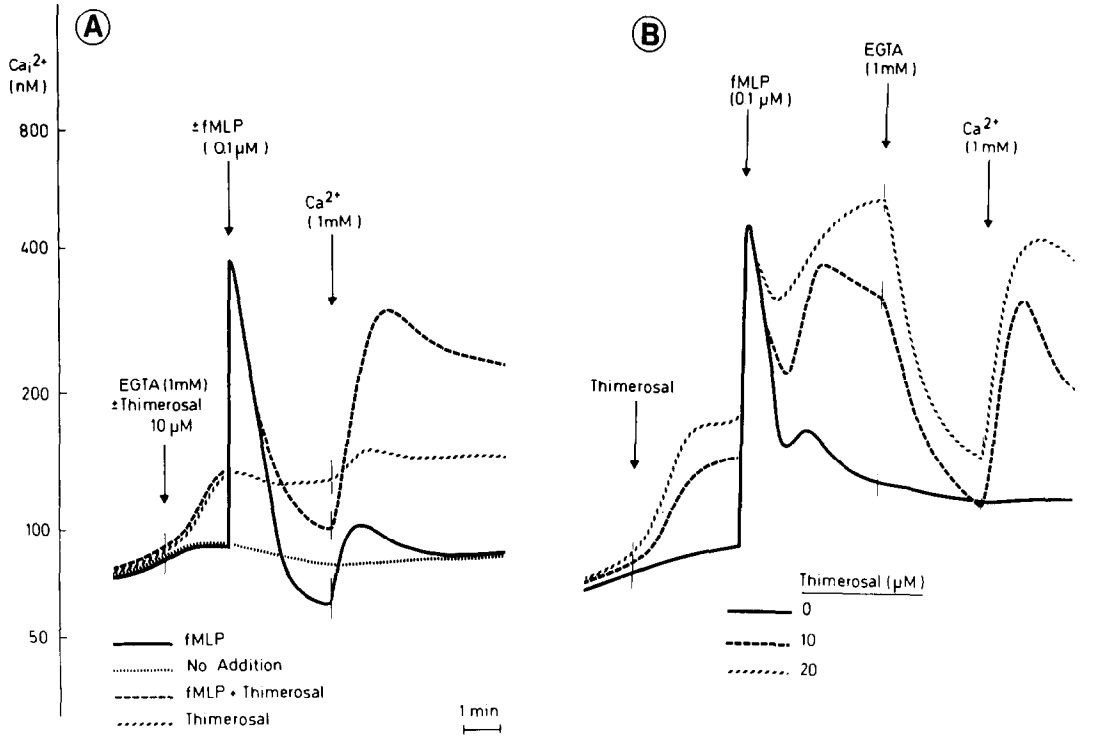

Fig. 5. Influence of thimerosal on the fMLP-stimulated Ca^{2+}-influx. Fura-2 loaded cells (10^7/mL) were equilibrated at 37° in NaCl/P_i/0.5 mM Ca^{2+}/1 mM glucose. (A) Extracellular Ca^{2+} was complexed by the addition of 1 mM EGTA prior to the stimulation by fMLP. The extracellular Ca^{2+}-concentration of about 0.5 mM was again liberated by the addition of 1 mM Ca^{2+} 2 min after fMLP-stimulation. (B) Preincubation with increasing concentrations of thimerosal and subsequent stimulation by fMLP were performed in Ca^{2+}-containing buffer. Three minutes after fMLP-stimulation extracellular Ca^{2+} was complexed by the addition of 1 mM EGTA and 2 min later about 0.5 mM extracellular Ca^{2+} were liberated again by the addition of 1 mM Ca^{2+}.

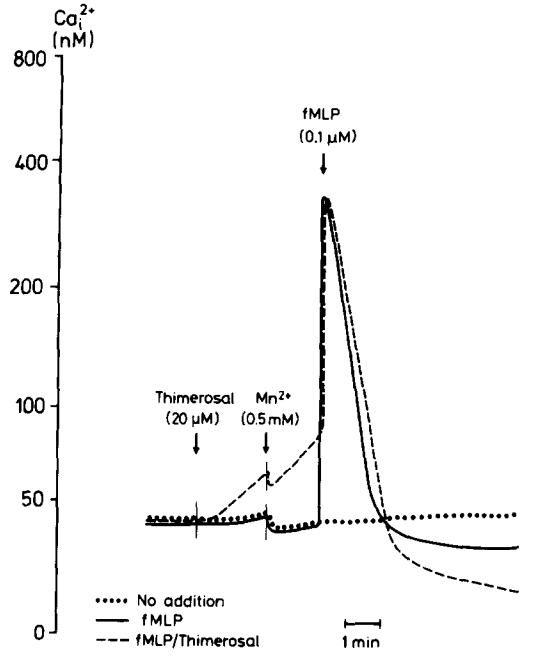

Fig. 6. Influence of extracellular Mn^{2+} on the intracellular fura-2 signal after stimulation with fMLP/thimerosal. Fura-2 loaded cells (10^7/mL in $NaCl/P_i$/0.1 mM Ca^{2+}/1 mM glucose) were equilibrated at 37°.

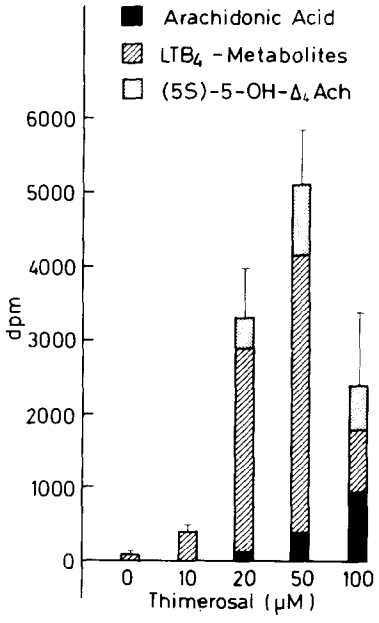

Fig. 7. Influence of thimerosal on the fMLP-stimulated liberation of [1-^{14}C]arachidonic acid. Cells (10^7/mL in $NaCl/P_i$/1 mM Ca^{2+}), prelabelled with [1-^{14}C]arachidonic acid, were preincubated with increasing concentrations of thimerosal (0.5% DMSO) for 5 min at 37°. After stimulation with 100 nM fMLP for 5 min the cells were spun down and the supernatants were assayed for radioactivity (dpm) and for the metabolization pattern as described in Materials and Methods. The dpm-values of the blank (no addition) were subtracted. The resulting values are given as mean ± SE from four independent experiments; 1000 dpm correspond to 10 pmol radioactivity. Under identical conditions, but without fMLP-stimulation, the released radioactivity never exceeded the blanks.

0.5 mM Mn^{2+}. Due to the influx of extracellular Mn^{2+} the signal from extracellular Ca^{2+} should be quenched which is indeed the case as shown in Fig. 6.

Effects of thimerosal on arachidonate metabolites

Although the results reported so far favour the increased Ca^{2+}-levels as the ultimate cause for arachidonate liberation through phospholipase A_2, the possibility still remained that thimerosal could elevate the concentration of free arachidonic acid due to inhibition of reacylation as proposed by others [11–13]. Measuring the inhibition of [1-^{14}C]arachidonate incorporation into neutral and complex lipids of PMN one can observe 15 and 25% inhibition at 10 and 20 μM thimerosal, respectively (data not shown).

We then studied the release of radioactivity in [1-^{14}C]arachidonate prelabelled cells (Fig. 7). Similar to the results in Fig. 2B we obtained a dose-dependent release of radioactivity into the supernatant of the cells after stimulation with fMLP in the presence of thimerosal. Analysis by thin-layer chromatography revealed that most of the liberated arachidonic acid had been metabolized by the action of 5-lipoxygenase. At higher thimerosal concentrations an inhibition of phospholipase A_2 and of 5-lipoxygenase occurred. The fMLP/thimerosal induced liberation of radioactivity could be almost totally inhibited by the addition (1 min after fMLP) of 2 mM dithiothreitol or 2-mercaptoethanol, respectively, while the same concentration of glutathione was ineffective (traces not shown). None of the three thiols did influence the liberation of [1-^{14}C]arachidonic acid by the Ca^{2+}-ionophore ionomycin. As expected thimerosal alone was not able to release any [1-^{14}C]arachidonic acid into the supernatant even after prolonged incubation up to 15 min.

DISCUSSION

Our results confirm previous findings that fMLP like other agonists of the "phosphatidylinositol-response" could not trigger the generation of HPLC-detectable amounts of LTB_4 in human PMN [7, 8, 18–20] although a sufficiently high Ca^{2+} signal was monitored with the fura-2 method. This signal, however, was of only short duration with a half-life time of about 1 min. When the organomercury compound thimerosal was preincubated with the cells a subsequent addition of fMLP caused the generation of significant amounts of 5-lipoxygenase products (Figs 1 and 2) although thimerosal alone was unable to elicit this response.

We were interested to clarify the underlying mechanism since leukotrienes are formed *in vivo* and are assumed to play an important role in inflammatory situations. Except the reaction with the terminal complement complex which causes a massive Ca^{2+}-influx through pore formation in the PMN plasma-

membrane [21], the physiological stimulus for 5-lipoxygenase activation is not known and therefore any system generating leukotrienes could be of potential value for the understanding of inflammation and its pharmacology.

As reported in literature, thimerosal was supposed to effectively inhibit the reacylation of arachidonate and hence increased levels of arachidonate could be responsible for the formation of prostanoids in human platelets and rat peritoneal macrophages [11, 12] or of prostacyclin in endothelial cells of rabbit aorta strips [13]. According to our results for PMN only 10 and 25% inhibition at the reacylation at 10 and 20 μM thimerosal, respectively, were observed [22]. Thimerosal alone even at higher concentrations ($\geq$50 μM) was not able to liberate arachidonate from prelabelled cells which is additional evidence against the reacylation hypothesis. We would like to conclude that, if at all, thimerosal may only slightly increase the level of liberated arachidonate and that rather the activity of the arachidonate releasing enzymes from lipids represents the crucial parameter in this system.

In human PMN most of the arachidonate release occurs through phospholipase A_2 [23–25]. Indeed, the thimerosal/fMLP-mediated liberation of [1-^{14}C]arachidonate in prelabelled cells could be inhibited by different inhibitors of this enzyme like chlorpromazine, quinacrine or 4-bromophenacyl bromide (data not shown) [22]. Phospholipase A_2 activity is strictly dependent on elevated intracellular Ca^{2+}-levels. We therefore measured Ca_i^{2+} and found that thimerosal was quantitatively and qualitatively changing the Ca^{2+}-levels and kinetics with and without fMLP.

Thimerosal alone caused an increase of the resting Ca^{2+}-levels until a new steady state was reached (Fig. 3, Table 1). This increase also occurred after complexing extracellular Ca^{2+} with EGTA suggesting that thimerosal is able to release Ca^{2+} from internal stores. It should be kept in mind that under these conditions no 5-lipoxygenase products were observed. If fMLP was added after preincubation with thimerosal the release of Ca^{2+} from the intracellular stores reached the same level as without thimerosal, but the normally rapid decrease back to the resting levels did not occur but rather high persisting Ca^{2+}-levels were attained over at least 5 min (Fig. 4). Interestingly this second phase of elevated levels was entirely due to extracellular Ca^{2+} (Fig. 5). Since the Ca^{2+}-response after fMLP alone also is biphasic and in its second phase is dependent on extracellular Ca^{2+}, one can postulate that thimerosal rather selectively increases the intracellular Ca^{2+} steady state levels on the expense of extracellular Ca^{2+}.

From the results with ionomycin (Fig. 4, Table 1) one can conclude that the dependence on intracellular Ca^{2+}-levels of leukotriene formation is not linear but rather seems to involve a threshold level. By a comparison with the absolute values of the Ca^{2+}-levels after fMLP plus thimerosal it is evident that no strict quantitative correlation exists concerning the formation of a certain amount of leukotrienes; additional modulating factors must therefore control the onset of arachidonate release in the case of receptor-agonist stimulation. Kinetic studies on arachidonate liberation by ionomycin (data not shown) demonstrate that within the first minute of stimulation only small amounts of arachidonic acid are released. We conclude that a persistently elevated internal Ca^{2+}-level seems to be a prerequisite for the formation of significant amounts of leukotrienes; this is the case for fMLP plus thimerosal but not for fMLP alone (see Fig. 4, Table 1).

The biochemical events leading to the increase of the second phase are difficult to access, since in principle both an inhibition of Ca^{2+} extruding mechanisms (most probably Ca^{2+}-ATPases) or an increased influx of extracellular Ca^{2+} could be involved.

How effectively an increase of Ca^{2+}-ATPase activity influences the steady state level of Ca^{2+} can be seen from the addition of the phorbolester TPA, which was postulated to activate Ca^{2+}-ATPases [26, 27]. It not only completely prevented the thimerosal-induced rise in Ca^{2+} but also lowered the second phase after fMLP-stimulated (Fig. 3B). However, from the experiments in Fig. 5B we would like to conclude that thimerosal does not lead to a major inhibition of Ca^{2+}-extrusion since this would not be consistent with a rapid decrease in the Ca^{2+}-levels after EGTA-addition.

Therefore the possibility remains that an increased influx of external Ca^{2+} could have been the dominant factor for the permanently high Ca^{2+}-levels. This conclusion is supported by the experiments shown in Fig. 6 which indicate that thimerosal is enhancing the fMLP stimulated influx of extracellular Mn^{2+}. The increased influx of external Ca^{2+} may be simply the result of an unspecific effect of thimerosal to the plasmamembrane of the cells. However, the indicated concentrations of thimerosal did not cause an elevated [8-^{14}C]adenine nucleotide release which argues against this unselective mode of thimerosal action.

A suitable pathway would be a Ca^{2+}-influx through a plasmamembrane-located channel like the non-selective Ca^{2+}-activated ion channel described by von Tscharner *et al.* [3]. In contrast to Ca^{2+}-ATPases, for which essential SH-groups have been reported, not much is known about the class of non-voltage-gated receptor-operated Ca^{2+}-channels and their reactivity with SH-reagents. The almost complete and rapid reversibility of the thimerosal effect after dithiothreitol-addition (Fig. 4B) favors a very specific interaction with an SH-group participating directly or indirectly at the Ca^{2+}-influx mechanism. However, it is most important to realize that thimerosal can produce its elevating effect on the Ca^{2+}-level only after the "phosphatidylinositol-response" has conditioned the cells. Which event or factor could be involved in this gating process is unclear [28]. In sea urchin eggs the second messenger inositol 1,3,4,5-tetrakisphosphate (IP_4) [29] and inositol trisphosphate isomers in human T-lymphocytes [30] or *Xenopus laevis* oocytes [31] have recently been discussed in connection with opening of plasmamembrane Ca^{2+}-channels. One of these messengers or a related one could modulate the Ca^{2+}-channel opening time which then could be enforced or stabilized by a reaction with a SH-group leading to

an increased Ca^{2+}-influx. Keeping in mind the very similar kinetics of Ca^{2+}-increase in the presence and absence of extracellular Ca (Fig. 3) one can also postulate that thimerosal causes a leakage of Ca^{2+} from the IP_3-sensitive pool. This would be in agreement with the non-additive effect of fMLP on the first Ca^{2+}-transient. The hypothesis poses the difficulty that one has to assume a fMLP-mediated pathway of enhanced influx into the IP_3-sensitive compartment. Assuming a plasmamembrane-associated compartment one could even imagine that during the PI-response a facilitated uptake of extracellular Ca^{2+} into this IP_3 sensitive compartment could occur. This situation would fit the capacitative Ca^{2+} entry hypothesis, recently reviewed by Putney *et al.* [32].

If further experiments would varify such a mechanism the combined effects of "phosphatidylinositol-response" agonists and thimerosal could indeed be considered as a suitable model for a physiological mechanism of leukotriene formation, since the oxidation of SH-groups to disulfides or mixed disulfides with glutathione could be the physiological equivalent of thimerosal action. This hypothesis is under investigation. In addition it is noteworthy that the fMLP/thimerosal mediated leukotriene formation is modulated by several prostaglandins [33] elucidating the receptor-agonist coupled nature involved in the elevation of Ca^{2+}-levels in opposite to the effects of Ca^{2+}-ionophores.

Finally, the differences in the thimerosal action on PMN in comparison to platelets or endothelial cells should be mentioned. It was published that thromboxane formation and aggregation of platelets can occur by thimerosal alone without additional stimulation by other agonists [11, 12]. It is already known that in the case of platelets small amounts of arachidonate released will form thromboxane A_2 which acts as a complete agonist for aggregation and phospholipase A_2 activation. This is unlike the situation in PMN where LTB_4 causes only a "phosphatidylinositol-response" but not an activation of its own formation. In agreement with our results thimerosal is also enhancing intracellular Ca^{2+}-levels in platelets [34], macrophages [35] and endothelial cells [36]. For this reason we believe that the mechanisms of arachidonate liberation in platelets and the formation of prostacyclin and EDRF (endothelium derived relaxing factor) in endothelial cells by thimerosal have to be reevaluated with regard to the priming role of Ca^{2+}.

Acknowledgements—The present studies were supported by grants of the Deutsche Forschungsgemeinschaft. The authors thank Miss E. Schatz for expert technical assistance and Mr Stephan Forderkunz for performing additional experiments.

REFERENCES

1. Malech HL and Gallin JI, Neutrophils in human diseases. *New Engl J Med* **317**: 687–694, 1987.
2. Snyderman CD, Smith CD and Verghese MW, Model for leukocyte regulation by chemoattractant receptors: roles of a guanine nucleotide regulatory protein and polyphosphoinositide metabolism. *J Leukoc Biol* **40**: 785–800, 1986.
3. von Tscharner V, Prod'hom B, Baggiolini M and Reuter H, Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature* **324**: 369–372, 1986.
4. Rouzer CA, Matsumoto T and Samuelsson B, Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A_4 synthase activities. *Proc Natl Acad Sci USA* **83**: 857–861, 1986.
5. Ueda N, Kaneko S, Yoshimoto T and Yamamoto S, Purification of arachidonate 5-lipoxygenase from porcine leukocytes and its reactivity with hydroperoxyeicosatetraenoic acids. *J Biol Chem* **261**: 7982–7988, 1986.
6. Radmark O, Shimizu T, Jörnvall H and Samuelsson B, Leukotriene A_4 hydrolase in human leukocytes. *J Biol Chem* **259**: 12339–12345, 1984.
7. Haines KA, Giedd KN, Rich AM, Korchak HM and Weissman G, The leukotriene B_4 paradox: neutrophils can, but will not, respond to ligand receptor interactions by forming leukotriene B_4 or its ω-metabolites. *Biochem J* **241**: 55–62, 1987.
8. Clancy RM, Dahinden CA and Hugli TE, Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by *N*-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. *Proc Natl Acad Sci USA* **80**: 7200–7204, 1983.
9. Irvine RF, How is the level of free arachidonic acid controlled in mammalian cells? *Biochem J* **204**: 3–16, 1982.
10. Hänsch GM, Gemsa D and Resch K, Induction of prostanoid synthesis in human platelets by the late complement components C5b-9 and channel forming antibiotic nystatin: inhibition of the reacylation of liberated arachidonic acid. *J Immunol* **135**: 1320–1324, 1985.
11. Körner C-F, Hausmann G, Gemsa D and Resch K, Rate of prostaglandin synthesis is not controlled by phospholipase A activity but by reincorporation of released fatty acids into phospholipids. *Agents Actions* **15**: 28–30, 1984.
12. Goppelt-Strübe M, Koerner C-F, Hausmann G, Gemsa D and Resch K, Control of prostanoid synthesis: role of reincorporation of released precursor fatty acids. *Prostaglandins* **32**: 373–385, 1986.
13. Förstermann U, Goppelt-Strübe M, Frölich JC and Busse R, Inhibitions of acyl-coenzyme A:lysolecithinacyltransferase activate the production of endothelium-derived vascular releasing factor. *J Pharmacol Exp Ther* **238**: 352–359, 1986.
14. Hatzelmann A and Ullrich V, Regulation of 5-lipoxygenase activity by the glutathione status in human polymorphonuclear leukocytes. *Eur J Biochem* **169**: 175–184, 1987.
15. Smith Jr DM and Waite M, Phospholipid metabolism in human neutrophil subfractions. *Arch Biochem Biophys* **246**: 263–273, 1986.
16. Bergmeyer HU. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), 3rd Edn, Vol. II, pp. 232–233. Verlag Chemie, Basel, 1983.
17. Shirhatti V and Krishna G, A simple and sensitive method for monitoring drug-induced cell injury in cultured cells. *Anal Biochem* **147**: 410–418, 1985.
18. Chandler DE and Kazilek CJ, Calcium signals in neutrophils can be divided into three distinct phases. *Biochim Biophys Acta* **931**: 175–179, 1987.
19. Tanizawa H and Tai H-H, Synergism between chemotactic peptide and platelet-activating factor in stimulating thromboxane B_2 and leukotriene B_4 biosynthesis in human neutrophils. *Biochem Pharmacol* **38**: 2559–2563, 1989.
20. Palmer RMJ and Salmon JA, Release of leukotriene B_4 from human neutrophils and its relationship to

degranulation induced by *N*-formyl-methionyl-leucyl-phenylalanin, serum-treated zymosan and the ionophore A23187. *Immunology* **50**: 65–73, 1983.
21. Seeger W, Suttorp N, Hellwig A and Bhakdi S, Non cytolytic terminal complement complexes may serve as calcium gates to elicit leukotriene B4 generation in human polymorphonuclear leukocytes. *J Immunol* **137**: 1286–1293, 1986.
22. Hatzelmann A, Arachidonsäuremetabolismus in menschlichen Granulozyten—Regulation der Leukotrienbiosynthese. In: *Konstanzer Dissertationen 236*, p. 64, Hartung-Gorre, Konstanz, 1988.
23. Walsh CE, Waite BM, Thomas MJ and DeChatelet LR, Release and metabolism of arachidonic acid in human neutrophils. *J Biol Chem* **256**: 7228–7234, 1981.
24. Takenawa T, Homma Y and Nagai Y, Role of Ca^{2+} in phosphatidylinositol response and arachidonic acid release in formylated tripeptide- or Ca^{2+} ionophore A23187-stimulated guinea pig neutrophils. *J Immunol* **130**: 2849–2855, 1983.
25. Meade CJ, Turner GA and Bateman PE, The role of polyphosphoinositides and their breakdown products in A23187-induced release of arachidonic acid from rabbit polymorphonuclear leukocytes. *Biochem J* **238**: 425–436, 1986.
26. Lagast H, Pozzan T, Waldvogel FA and Lew PD, Phorbol myristate acetate stimulates ATP-dependent calcium transport by the plasma membrane of neutrophils. *J Clin Invest* **73**: 878–883, 1984.
27. Rickard JE and Sheterline P, Evidence that phorbol ester interferes with stimulated Ca^{2+} redistribution by activating Ca^{2+} efflux in neutrophil leukocytes. *Biochem J* **231**: 623–628, 1985.
28. Nasmith PE and Grinstein S, Are Ca^{2+} channels in neutrophils activated by a rise in cytosolic free Ca^{2+}? *FEBS Lett* **221**: 95–100, 1986.
29. Irvine RF and Moore RM, Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}. *Biochem J* **240**: 917–920, 1986.
30. Kuno M and Gardner P, Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* **326**: 301–304, 1987.
31. Snyder PM, Krause K-H and Welsh MJ, Inositol trisphosphate isomers, but not inositol 1,3,4,5-tetrakisphosphate, induce calcium influx in *Xenopus laevis* oocytes. *J Biol Chem* **263**: 11048–11051, 1988.
32. Putney Jr JW, Tahemura H, Hughes AR, Horstman DA and Thastrup O, How do inositol phosphates regulate calcium signaling. *FASEB J* **3**: 1899–1905, 1989.
33. Haurand M and Flohé L, Leukotriene formation by human polymorphonuclear leukocytes from endogenous arachidonate. *Biochem Pharmacol* **38**: 2129–2137, 1989.
34. Hecker M, Brüne B, Decker K, Ullrich V, The sulfhydryl reagent thimerosal elicits human platelet aggregation by mobilization of intracellular calcium and secondary prostaglandin endoperoxide formation. *Biochem Biophys Res Commun* **159**: 961–968, 1989.
35. Kaever V, Goppelt-Strübe M and Resch K, Enhancement of eicosanoid synthesis in mouse peritoneal macrophages by the organic mercury compound thimerosal. *Prostaglandins* **35**: 885–902, 1988.
36. Lückhoff A, Release of prostacyclin and EDRF from endothelial cells is differentially controlled by extra- and intracellular calcium. *Eicosanoids* **1**: 5–11, 1988.