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THE RATIO OF MACROPHAGE PROSTAGLANDIN AND LEUKOTRIENE SYNTHESIS IS DETERMINED BY THE INTRACELLULAR FREE CALCIUM LEVEL

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Abstract—The induction of eicosanoid synthesis in various cell types by different physiological stimuli is dependent on an increase in the intracellular calcium level and stimulation of the protein kinase C (PKC). In a model system this can be mimicked by using calcium ionophores and direct PKC activators. In mouse peritoneal macrophages calcium ionophores induced the formation of prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4). A synergistic enhancement of both eicosanoids could be achieved by simultaneous addition of the calcium ionophore A23187 together with a suboptimal dose of the direct protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Low concentrations of the ionophore, resulting in only marginally increased intracellular calcium levels, led to a more than additive prostaglandin E_2 production in combination with TPA. Higher concentrations of A23187 together with TPA favoured LTC_4 synthesis, whereas PGE_2 levels at the same time were even diminished. This observed shift from prostaglandin to leukotriene formation was amplified by simultaneous addition of indomethacin. Manganese as inhibitor of the A23187-induced calcium influx decreased PGE_2 synthesis. On the other hand, in the presence of manganese LTC_4 production was also inhibited at high concentrations of A23187 but elevated in the absence or at low doses of A23187. Our data provide evidence that in macrophages the ratio of cyclooxygenase and lipoxygenase products caused by mediators, acting via the phospholipase C or D/PKC signal transduction pathway, is regulated by the extent of the intracellular calcium increase.

Tissue macrophages exert potent regulatory functions within the immune system. In addition to their direct cytotoxic and phagocytic effects they are also involved in the regulation of the local homoeostasis. Several soluble and particulate stimuli induce the secretion of various enzymes and cytokines [1], and moreover cause the synthesis of different eicosanoids by these cells [2, 3]. Thereby, macrophages are thought to play a key role in the pathogenesis of chronic inflammatory disorders [4] and a functional antagonism between the secreted prostanoids and leukotrienes can often be observed. In mouse resident peritoneal macrophages prostaglandin E_2 (PGE_2†) and leukotriene C_4 (LTC_4) are the most prominent eicosanoid species formed [5]. It has previously been reported that the particulate stimulus zymosan as well as calcium ionophores induced the formation of PGE_2 and LTC_4 in these cells, whereas incubation with direct activators of the protein kinase C (PKC), such as the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or synthetic diacylglycerols, only led to the synthesis of PGE_2 [6–11]. From those data it can be concluded that, besides the availability of the common eicosanoid substrate arachidonic acid (AA), a distinct intracellular calcium increase is required for the activation of the 5′-lipoxygenase, the key enzyme of leukotriene syntheses [12]. Addition of TPA together with suboptimal concentrations of the calcium ionophore A23187 evoked a synergistic LTC_4 production [13–15].

In this report we demonstrate that PGE_2 synthesis could be synergistically enhanced, too, by the simultaneous addition of TPA and very low concentrations of A23187. At high intracellular calcium levels preferentially LTC_4 and less PGE_2 was produced by PKC activation. Furthermore, the effects of the cyclooxygenase inhibitor indomethacin as well as manganese, which prevents ionophore-induced calcium influx, on the calcium ionophore- and calcium ionophore/TPA-induced eicosanoid production in mouse resident peritoneal macrophages is described.

MATERIALS AND METHODS

Macrophage culture. Resident macrophages were collected by peritoneal lavage from untreated 6–8-week-old DBA/2 mice (Zentralinstitut für Versuchstierforschung, Hannover, F.R.G.) as described recently [10] and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with $NaHCO_3$ (2 g/L), glucose (4.5 g/L), penicillin (100 units/mL), streptomycin sulphate (100 μg/mL), sodium pyruvate (1 mM), L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M) and 1% non-essential amino acids (all from Gibco, Karlsruhe, F.R.G.) in 24-well plates (Nunc, Wiesbaden, F.R.G.) at a cell

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† Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; fura-2 AM, fura-2 acetoxymethyl ester; H-7, 1-(isoquinolinesulphonyl)-2-methylpiperazine; LTC_4, leukotriene C_4; PBS, phosphate-buffered saline (Dulbecco); PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

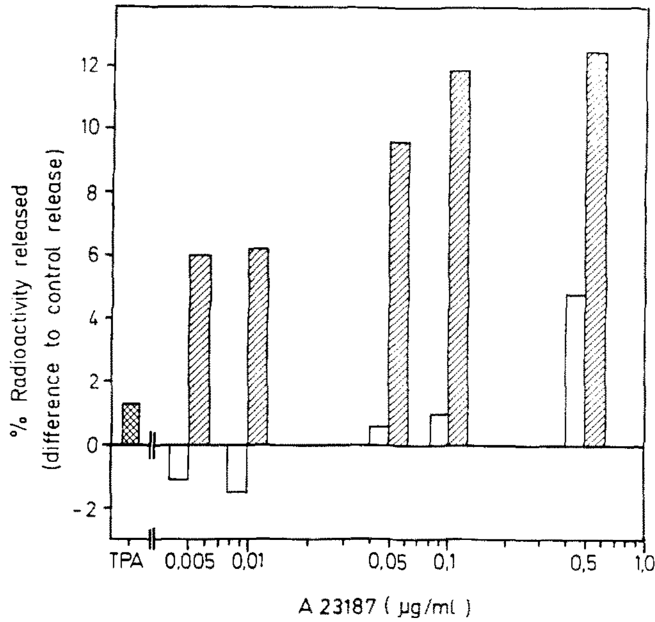

Fig. 1. [^{3}H]AA release induced by calcium ionophore in the absence or presence of the PKC activator TPA. Adherent macrophages were prelabelled with [^{3}H]AA and washed free of non-incorporated label as described in Materials and Methods. After incubation for 30 min in DMEM/0.5% BSA with A23187 in the indicated concentrations in the absence (open bars) or presence (shaded bars) of TPA (10^{-8} M) released radioactivity was determined in the supernatants by liquid scintillation counting. Values are expressed as means from two experiments performed in duplicates as difference to the control release (9.2% of total incorporated radioactivity).

density of 5×10^5 cells/mL. After a preincubation time of 2 hr at 37° in a humidified atmosphere of 5% CO_2 in air non-adherent cells were removed by washing with phosphate-buffered saline (PBS, without Ca^{2+} and Mg^{2+}), and the adherent cells (about 2.5×10^5 cells/well) were cultured in DMEM under the conditions specified in the individual experiments. As determined by morphologic criteria and Pappenheim staining the cultures always consisted of more than 95% macrophages. Cell viability, as judged by Trypan Blue exclusion, was never below 90%.

Release of AA from labelled cells. Adherent macropoages were cultured for 2 hr in DMEM containing 0.5 μCi/mL [^{3}H]AA (New England Nuclear, Dreieich, F.R.G., sp. act. 240 Ci/mmol) and then washed twice with PBS containing 0.5% bovine serum albumin (BSA) in order to remove non-incorporated label. The cells were provided with fresh DMEM/0.5% BSA and incubated with the indicated stimuli for 30 min at 37° in a humidified atmosphere of 5% CO_2 in air. Radioactivity of the cell supernatants was determined by liquid scintillation counting.

Determination of eicosanoid synthesis. Adherent macrophages in DMEM were exposed to the indicated concentrations of A23187, TPA, indomethacin, and manganese chloride (all from the Sigma Chemical Co., Deisenhofen, F.R.G.), respectively, for 60 min. The supernatants were removed, centrifuged (1 min, 12,000 *g*), and the amounts of PGE_2 and LTC_4 were determined by specific radioimmunoassay as described elsewhere in detail [5]. PGE_2 was obtained from Paesel (Frankfurt, F.R.G.) and LTC_4 was kindly provided by Hoechst (Frankfurt, F.R.G.). [^{3}H]PGE_2 (sp. act. 185 Ci/mmol) and [^{3}H]LTC_4 (sp. act. 38.4 Ci/mmol) were purchased from New England Nuclear.

Measurement of intracellular calcium levels. Intracellular free calcium was determined as previously described [5]. Briefly, mouse peritoneal exudate cells were suspended in phosphate-buffered saline (PBS) at a cell density of 1×10^5 cells/mL in a polypropylene vial and incubated with 5 μM fura-2 AM (Calbiochem, Frankfurt, F.R.G.) for 40 min at 37°. After washing by centrifugation (10 min at 600 *g*) the cells were resuspended in PBS supplemented with Ca^{2+} (1 mM), Mg^{2+} (1 mM) and glucose (1 g/L). Changes in intracellular calcium levels after addition of ionomycin (Calbiochem) and/or TPA were determined by fluorescence measurement (excitation 340 nm, emission 492 nm) at 37° under continuous stirring [16].

RESULTS

Synthesis of eicosanoids is initiated by the release of their common substrate non-esterified AA from phospholipid stores. We therefore determined the effect of the calcium ionophore A23187 on AA release from [^{3}H]AA prelabelled macrophages. Compared to control cells A23187 induced a measurable increase in free AA within 30 min at A23187 concentrations $\geq$0.05 μg/mL (Fig. 1). Addition of the phorbol ester TPA (10^{-8} M), a direct activator of the PKC, itself led to a moderate AA release, but a synergistic increase in free AA became obvious when TPA and A23187 were added in combination

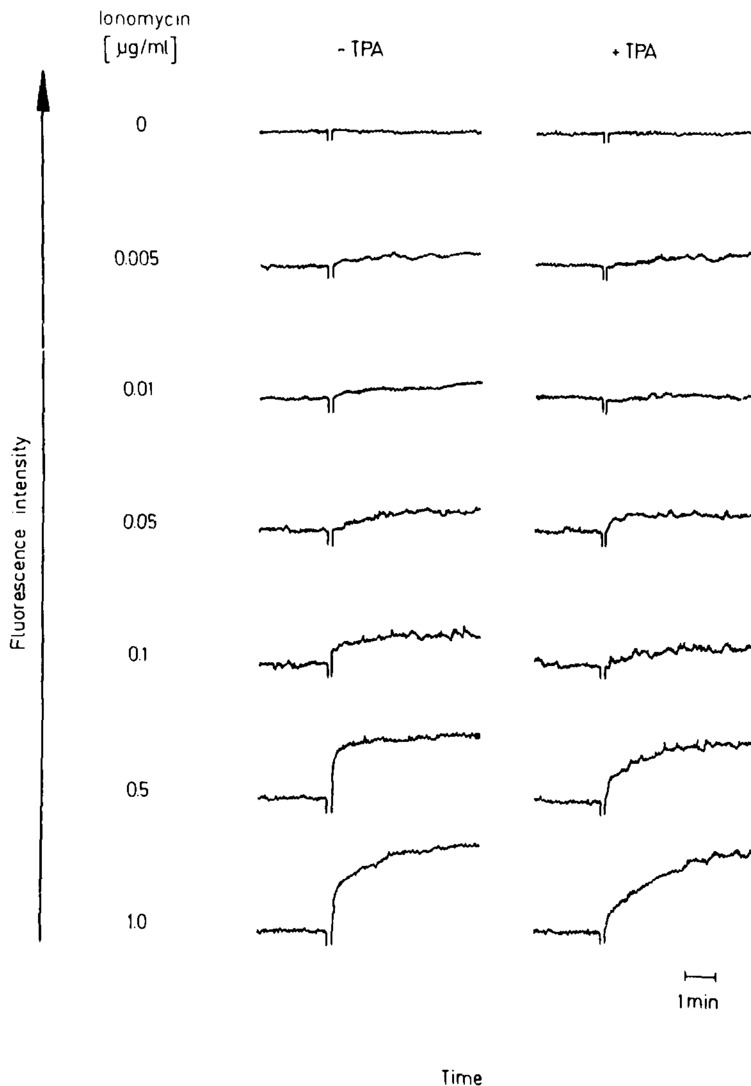

Fig. 2. Effect of the calcium ionophore ionomycin on the intracellular calcium level in the absence or presence of the PKC activator TPA. Mouse peritoneal exudate cells were prelabelled with fura 2-AM and fluorescence intensities were measured as described in Materials and Methods. Ionomycin in the indicated concentrations was added to the cell suspension after 2 min of preincubation with or without TPA (10^{-8} M). Original recordings from one representative experiment out of three similar ones are shown.

(Fig. 1). Under this condition AA was released in a more than additive manner even at extremely low concentrations of A23187 (0.005 μg/mL, which is equivalent to a concentration of 10 nM). Analysis by high performance liquid chromatography revealed that under the chosen experimental design (i.e. presence of 0.5% BSA as fatty acid trap) liberated AA was found nearly completely non-metabolized in the cellular supernatants (data not shown).

As fatty acid deacylating enzyme systems (phospholipase A_2, phospholipase C or D/diacylglycerol lipase) are calcium-dependent we tested the hypothesis that TPA might further enhance the intracellular calcium increase induced by A23187. In order to investigate the ionophore-mediated calcium influx by fura-2 fluorescence technique the non-fluorescent ionomycin instead of A23187 was used. Previous experiments had shown that the effects of ionomycin and A23187 on AA release and eicosanoid synthesis were almost identical (data not shown). As can be seen in Fig. 2 ionomycin dose-dependently caused increases in the intracellular calcium amounts due to a sustained influx from the surrounding medium. TPA did not alter the steady-state calcium level achieved by ionomycin, but the maximal increase occurred somewhat delayed (Fig. 2).

We next investigated the metabolic routes of AA liberated by A23187/TPA action in serum- and BSA-free macrophage cultures. Synthesis of PGE_2 and LTC_4, as the main metabolites produced by mouse peritoneal macrophages, was induced by the calcium ionophore A23187 in a dose-dependent manner (Fig. 3A). Whereas low concentrations of A23187 resulted in comparable amounts of PGE_2 and LTC_4, higher intracellular calcium levels favoured the LTC_4 production. The sole application of the phorbol ester

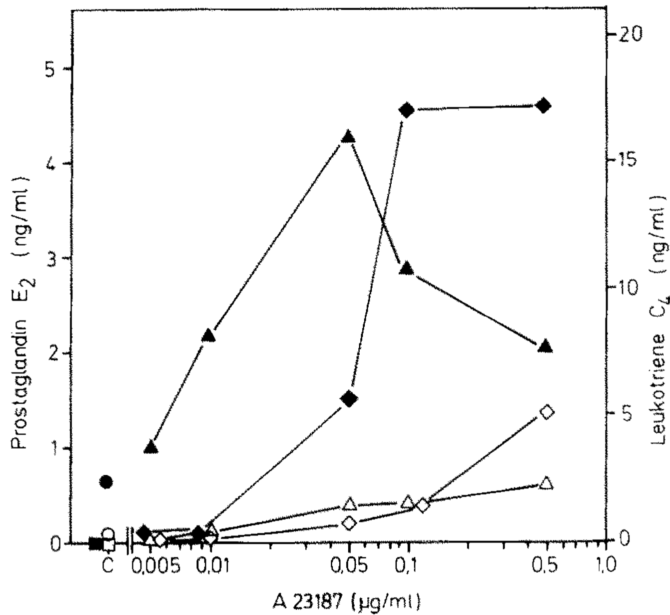

Fig. 3. Enhancement of eicosanoid synthesis by calcium ionophore A23187 in the absence (open symbols) or presence (closed symbols) of the PKC activator TPA. Adherent macrophages were incubated for 60 min with A23187 in the indicated concentrations in the absence or presence of TPA (10^{-8} M). PGE_2 and LTC_4 were determined in the supernatants by specific radioimmunoassay. Values are expressed as means (with deviations always less than 10%) from duplicate cell incubations and quadruplicate eicosanoid determinations from one representative experiment out of three similar ones. (c) Control; (○) control PGE_2; (□) control LTC_4; (△) PGE_2 induced by A23187; (◇) LTC_4 induced by A23187; (●) PGE_2 induced by TPA; (■) LTC_4 induced by TPA; (▲) PGE_2 induced by A23187 + TPA; (◆) LTC_4 induced by A23187 + TPA.

TPA at a suboptimal concentration (10^{-8} M), on the other hand, led to an unchanged LTC_4 production, but significantly increased the PGE_2 amount (Fig. 3B). The combined action of various concentrations of A23187 and 10^{-8} M TPA resulted in a synergistic augmentation of both eicosanoid levels. Whereas the synergistically enhanced PGE_2 synthesis occurred at the lower concentrations of A23187 used, higher concentrations of the calcium ionophore (>0.01 μg/mL) were needed to achieve a similar effect with LTC_4. Noteworthy, high production of LTC_4 was accompanied by a diminished synthesis of PGE_2 (Fig. 3B).

The observed shift in eicosanoid synthesis from PGE_2 to LTC_4 by the simultaneous addition of high concentrations of A23187 together with TPA could also be achieved at lower A23187 concentrations by inclusion of the cyclooxygenase inhibitor indomethacin (Table 1). Whereas indomethacin (10^{-6} M) effectively decreased A23187/TPA-induced PGE_2 levels, LTC_4 production by A23187/TPA was clearly increased under this condition.

PKC inhibitors such as H-7, sphingosine, tamoxifen, or staurosporine, at concentrations which largely abolished the cytosolic PKC activity in mouse peritoneal macrophages, extensively diminished both PGE_2 and LTC_4 formation induced by A23187 plus TPA (data not shown).

Calcium influx stimulated by A23187 can be effectively blocked by manganese (Mn^{2+}), due to its high affinity to this particular ionophore [17]. When macrophages were incubated with A23187 and/or TPA in the presence of manganese (40 μM) differential effects on PGE_2 or LTC_4 synthesis were obvious (Table 2). PGE_2 synthesis induced either by A23187 alone or by combined action of A23187/TPA was inhibited by manganese. On the other hand, manganese revealed a biphasic effect on LTC_4 production. In the absence or at low concentrations of A23187 manganese significantly increased LTC_4 amounts. At higher concentrations of A23187 with or without TPA, resulting in a marked elevation of LTC_4 amounts, manganese led to a pronounced inhibition of the LTC_4 synthesis (Table 2).

DISCUSSION

Macrophages represent a group of cells that widely differ in their functional capacities. The explicit spectrum of eicosanoids produced by a defined macrophage subpopulation additionally depends on the tissue origin of the cells, the nature of the stimulus, and the state of differentiation [1, 18]. In this report we present data concerning the role of PKC and the intracellular calcium level in the regulation of rapid eicosanoid synthesis in resident peritoneal macrophages, not addressing their function in the control of macrophage priming and activation, for example by lipopolysaccharide or gamma-interferon [19].

Although the formation of different prostanoids and leukotrienes depends on the coordinate interaction of several enzymes and cofactors in a complex manner liberation of AA from its lipid stores has

Table 1. Effect of the cyclooxygenase inhibitor indomethacin on the calcium ionophore/phorbol ester-induced eicosanoid synthesis

	PGE_2 (ng/mL)			LTC_4 (ng/mL)		
A23187 in μg/mL	—	TPA	TPA + indo	—	TPA	TPA + indo
0.005	0.06	1.1	0.18	0.21	0.38	0.54
0.01	0.06	1.5	0.27	0.27	0.57	1.3
0.05	0.09	2.4	0.21	0.40	1.6	6.4
0.1	0.10	1.3	0.11	0.90	4.6	9.5
0.5	0.16	1.7	0.11	1.8	11.9	12.0

Macrophages were incubated for 60 min with calcium ionophore A23187 in the indicated concentrations in the absence or presence of the phorbol ester TPA (10^{-8} M) or TPA + indomethacin (10^{-6} M). PGE_2 and LTC_4 were determined in the supernatants by specific radioimmunoassay. Control values were 0.05 ng/mL PGE_2 and 0.21 ng/mL LTC_4, respectively. Values are expressed as means from duplicate cell incubations and quadruplicate eicosanoid determinations from one representative experiment out of three.
indo, indomethacin.

generally been considered as a necessary initial step [20]. There is good evidence that in macrophages AA is deacylated mainly from phosphatidylcholine and phosphatidylinositol by specific PLA_2 [7], although other enzyme systems such as phosphatidylcholine-specific PLC or PLD/diacylglycerol lipase [21] may also be operative.

Whereas an elevated level of non-esterified AA is sufficient for an increased formation of cyclooxygenase products further cofactors are required for 5′-lipoxygenase activation and concomitantly LTC_4 synthesis. Besides ATP and several additional components, calcium is absolutely required for enzyme translocation to the membrane [22, 23]. Therefore, it has to be assumed that only stimuli simultaneously increasing the intracellular amounts of AA and calcium are able to augment leukotriene synthesis. This holds true for various hormonal mediators and phagocytic stimuli like zymosan or glucan, which are thought to induce eicosanoid synthesis via the breakdown of inositol lipids [24]. This leads to the generation of distinct inositol phosphates as messengers for calcium release from internal stores as well as calcium influx from the surrounding medium and of diacylglycerols as endogenous regulators of the PKC activity [25]. On the other hand, phosphatidylcholine-specific phospholipases [21] should only result in diacylglycerol formation followed by PKC activation, and solely prostanoids should be secreted. Unfortunately, agonists specifically effecting those enzymes in the macrophage system have not yet been described.

Those physiological situations were mimicked in a model system by using direct PKC activators or PKC activators together with calcium ionophores, respectively. AA deacylating enzyme systems are strictly

Table 2. Effect of manganese (Mn^{2+}) on calcium ionophore and calcium ionophore/phorbol ester-induced eicosanoid synthesis

A23187 in μg/mL in the *absence* of TPA	PGE_2 (ng/mL)		LTC_4 (ng/mL)	
	—	Mn^{2+}	—	Mn^{2+}
—	0.34	0.14	0.15	0.26
0.005	0.26	0.30	0.15	0.40
0.01	0.54	0.24	0.10	0.46
0.05	1.6	0.36	0.49	0.58
0.1	1.7	0.43	0.97	0.47
0.5	2.0	1.2	2.3	0.62
1.0	1.8	0.79	2.6	0.56
A23187 in μg/mL in the *presence* of TPA				
—	2.2	1.9	0.16	0.33
0.005	6.1	2.8	0.15	0.46
0.01	11.5	3.6	0.14	0.42
0.05	11.2	8.8	2.9	0.53
0.1	11.9	7.7	6.3	0.58
0.5	8.3	7.6	11.8	2.0
1.0	8.7	6.7	20.4	1.6

Macrophages were incubated for 60 min with calcium ionophore A23187 in the indicated concentrations in the absence or presence of the phorbol ester TPA (10^{-8} M) and/or Mn^{2+} (40 μM). PGE_2 and LTC_4 were determined in the supernatants by specific radioimmunoassay. Values are expressed as means from duplicate cell incubations and quadruplicate eicosanoid determinations.

calcium dependent [26]. The observed synergistic AA release induced by low concentrations of calcium ionophore/TPA could possibly be a result of an additional intracellular calcium increase as consequence of the phorbol ester addition. Measurement of intracellular calcium by fura-2 technique, however, revealed no influence of TPA on ionomycin-induced maximal calcium influx. These results are in good accordance to those reported by Lokesh and Kinsella [27], showing that chelation of intracellular calcium by quin-2 did not affect the phorbol ester-stimulated AA release. Therefore, a direct effect of the PKC, activated by TPA and translocated to the membrane [28], on AA liberating as well as AA reincorporating enzymes has to be assumed. We have previously reported that various PKC activators induced AA release from prelabelled cells and also inhibited incorporation of exogenously added AA into membrane phospholipids. Both effects were abrogated by different PKC inhibitors such as H-7 or sphingosine, used in concentrations sufficient for an effective inhibition of PKC activity but not showing any adverse effect on cell viability [9, 11]. The exact molecular mechanism underlying TPA action has not yet been defined, but both PLA_2 and lysophospholipid acyltransferase or separate regulatory proteins are candidates for a PKC-dependent phosphorylation. During the last years various PKC subspecies either calcium-dependent or calcium-independent have been described [29], which each might be involved in different regulatory processes. We are currently investigating the presence of PKC isozymes in murine macrophages by cytochemistry using subtype-specific monoclonal antibodies.

TPA in suboptimal concentration slightly increased PGE_2 synthesis and had no effect on LTC_4 production. A synergistic production of PGE_2 was determined when TPA was added in combination with very low concentrations of A23187. At higher concentrations of the ionophore the 5′-lipoxygenase became activated and subsequently LTC_4 production was synergistically increased. Under this condition PGE_2 secretion was even diminished, which may be due to the limitation of the common substrate AA. Alternatively, elevation of the intracellular calcium level could induce liberation of AA from distinct compartments only accessible for the lipoxygenase. Inhibition of prostanoid synthesis by indomethacin, however, clearly increased the A23187/TPA-induced LTC_4 synthesis. It should be noted that indomethacin induced a shift in eicosanoid synthesis from PGE_2 to LTC_4 especially at low A23187 concentrations but did not affect the maximal ionophore-induced LTC_4 synthesis. At high intracellular calcium levels 5′-lipoxygenase activity is maximally enhanced and therefore inhibition of cyclooxygenase by indomethacin does not lead to an additional effect on LTC_4 production.

In a variety of pathophysiological situations a decrease in leukotriene secretion seems advantageous. Specific lipoxygenase inhibitors or substances preventing lipoxygenase translocation should influence eicosanoid production. In addition, drugs specifically lowering the intracellular calcium level could result in a shift from leukotriene to prostanoid synthesis. In a first attempt we investigated the effects of manganese, known to abolish A23187-induced calcium influx [17]. Manganese differentially affected the ratio of PGE_2 and LTC_4 synthesis, depending on the amount of calcium ionophore added. In the absence or at low concentrations of A23187 manganese led to an elevation of LTC_4 and at the same time to a decrease in PGE_2 synthesis. Thus a shift from PGE_2 to LTC_4 secretion similar as seen by inclusion of indomethacin was obvious under this condition. The reason for this finding is not clear, but manganese might probably directly influence cyclooxygenase or PGE_2-isomerase activity. At higher intracellular calcium levels, favouring LTC_4 production, manganese exhibited a more pronounced inhibitory effect on LTC_4 than PGE_2 synthesis, which resulted in a shift in eicosanoid production from LTC_4 to PGE_2. The discussion is complicated by the fact that calcium ionophores could additionally release calcium from internal storage sites or activate the plasmalemmal sodium/calcium exchanger but our results clearly demonstrate that in macrophages the ratio of prostanoid and leukotriene synthesis can be easily changed by various substances, either affecting AA metabolizing enzymes or the intracellular calcium level.

TPA has also been described to increase cyclooxygenase activity within hours by *de novo* synthesis of this enzyme [13, 30]. In macrophages we could not detect such an effect after 1 hr of incubation of cells with TPA and subsequent measurement of the cyclooxygenase/PGE_2-isomerase activity in a particulate cell fraction (data not shown). Rapid changes in macrophage eicosanoid synthesis obviously do not occur at the level of the cyclooxygenase and lipoxygenase system but by regulation of the fatty acid deacylation/reacylation cycle. The ratio of prostanoid and leukotriene production is thereby determined by the extent of the intracellular calcium increase [9, 12]. This finding bears important clinical implications. Drugs affecting the intracellular calcium level could be employed to specifically alter the amount of macrophage eicosanoid synthesis, thus generating a shift between the amounts of vasodilatory prostaglandins and vasoconstrictory cysteinyl-leukotrienes. For example in chronic obstructive lung diseases such a therapeutical attempt could probably result in a more successful outcome than the global inhibition of total eicosanoid synthesis.

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