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EFFECTS OF TENIDAP ON Ca^{2+}- AND PROTEIN KINASE C-MEDIATED PROTEIN PHOSPHORYLATION, ACTIVATION OF THE ARACHIDONATE-MOBILIZING PHOSPHOLIPASE A_2 AND SUBSEQUENT EICOSANOID FORMATION IN MACROPHAGES

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Abstract—Tenidap is a novel antirheumatic drug which combines non-steroidal antiinflammatory drug-like cyclooxygenase inhibition with cytokine modulating qualities in rheumatoid arthritis. We show herein that tenidap (5–20 μM) inhibited protein kinase C-mediated signalling leading to release of arachidonate in mouse macrophages by interfering with the up-regulation of the 85 kDa arachidonate-mobilizing phospholipase A_2, although it did not inhibit this enzyme directly. The Ca^{2+}-mediated activation of arachidonate mobilization was inhibited only at higher concentrations (20–40 μM). Studies of protein phosphorylation indicated that tenidap in itself was capable of inducing the phosphorylation of several protein bands through interaction with intracellular protein kinases and/or phosphatases. Importantly, tenidap inhibited both arachidonate release and the increase in intracellular protein phosphorylation when the cells were stimulated with zymosan. We propose that the main inhibitory influence of tenidap on the macrophage signalling investigated here is exerted at some level between protein kinase C and the 85 kDa phospholipase A_2 and quite possibly also at the receptor-linked activation of phospholipase C.

Key words: 85 kDa phospholipase A_2; arachidonate; tenidap

Tenidap is a 3-substituted 2-oxindole with a cytokine modulatory effect. It is under clinical investigation for the treatment of patients with rheumatoid arthritis. Tenidap strongly inhibits the cyclo-oxygenase pathway of eicosanoid formation. Unlike other NSAIDs†, it decreases the levels of acute phase proteins [1, 2] and *in vitro* inhibits the production of IL-1 and IL-6, as well as tumor necrosis factor [3–5]. It has also been claimed to block the synthesis of eicosanoid products *in vitro* via the 5-lipoxygenase pathway [6–8].

Most earlier *in vitro* studies of tenidap have used polymorphonuclear leukocytes. In this paper, the actions of tenidap on the signal transduction pathway involving calcium and protein kinase C and leading to mobilization of arachidonic acid and formation of eicosanoids have been investigated using mouse macrophages. Different agents have been used to stimulate at various points in this signal transduction pathway: while phorbol diester acts as a direct protein kinase C agonist, the calcium ionophore A23187 elevates intracellular calcium levels and zymosan particles cause activation through receptor-mediated interactions via G proteins. Furthermore, we have monitored the pattern of protein phosphorylation and assessed the turnover of inositol phospholipids via inositol phosphates and glycerophosphinositol. The latter gives important hints regarding the activation of phospholipase A and phospholipase C pathways, respectively.

The macrophage is prominent in rheumatoid synovial inflammation and is likely to play a critical role in the initiation and maintenance of chronic rheumatoid synovitis. Macrophages are important generators of cytokines and eicosanoids, and the signal transduction pathways that regulate the formation of the latter are at least partially known. The mobilization of arachidonic acid is controlled by a recently discovered intracellular 85 kDa phospholipase A_2 [9–12], which is regulated by cytosolic Ca^{2+}-concentration as well as signalling via protein kinase C (Fig. 1).

The aims of the present study have been to assess whether tenidap affects the above-mentioned mechanisms, and, if so, to pin-point as closely as possible its intracellular target(s), apart from the obvious inhibition of the cyclo-oxygenase pathway. Particular attention has been given to correlating the tenidap-induced changes in the phosphorylation pattern of intracellular proteins to the effects of the drug on the phospholipase A and phospholipase C pathways.

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† Abbreviations: IL, interleukin; M 199, medium 199; NSAID, non-steroidal antiinflammatory drug; PMA, 4β-phorbol 12-myristate 13-acetate; PGE_2, prostaglandin E_2; HETE, 5-hydroxyeicostetraenoic acid; LTC_4, leukotriene C_4.

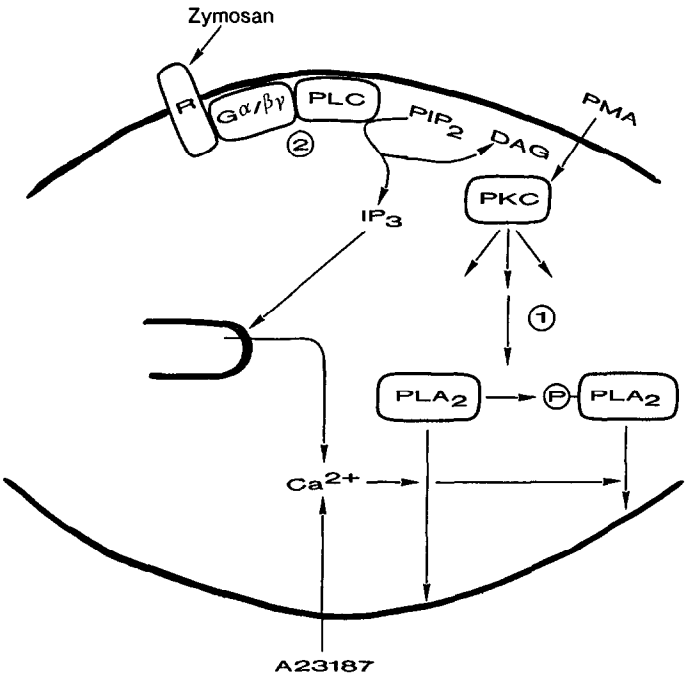

Fig. 1. A schematic view of macrophage signalling via Ca^{2+} and protein kinase C to the mobilization of arachidonate. $G\alpha\beta\gamma$, GTP-binding, heterotrimeric protein; PLC, phosphoinositide phospholipase C (isoform β); PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PLA_2, 85 kDa intracellular phospholipase A_2.

MATERIALS AND METHODS

Preparation and stimulation of macrophages. Resident peritoneal cells were harvested in 4 mL of M 199 (Flow Laboratories, Irvine, Ayrshire, Scotland) containing 1% heat-inactivated fetal calf serum and heparin (20 units/mL) from outbred female albino mice (Antimex, Stockholm, Sweden). The cells were plated onto plastic twelve-well Linbro tissue culture dishes and incubated in an atmosphere of 5% CO_2 in air. Non-adherent cells were removed 2 hr after plating. To each dish was added 1.0 mL M 199 containing 10% fetal calf serum. The cells were labeled for 22 hr with 2 μCi of [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]arachidonic acid (Amersham, Little Chalfont, UK; sp. act. 100–135 Ci/mmol). In some experiments, tenidap was added during the labeling period.

Tenidap ((Z)-5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1*H*-indole-1-carbamide, sodium salt) was a gift from Pfizer Inc. (Sandwich, UK), 4β-phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St Louis, MO, U.S.A.), and the ionophore A23187 from Boehringer (Mannheim, Germany). These chemicals were added in 2–10 μL of a dimethyl sulphoxide solution. Control experiments were performed to exclude interference from the solvent on arachidonic acid mobilization. Zymosan was purchased from Sigma, and 12 μL of a 50 mg/mL suspension was added in each experiment. Essentially fatty-acid-free bovine serum albumin was purchased from Sigma.

Before stimulation, the cells were washed with Dulbecco's PBS. They were then allowed to equilibrate in 1.0 mL M 199 for 30 min, with or without addition of tenidap 15 min before challenge with PMA or A23187 for 30 min. The culture medium was then removed and the cells scraped off the dish in 1 mL ice-cold 0.1% Triton X-100. Aliquots of both the medium and the cell solution were collected and the radioactivity determined in a Packard model 4530 scintillation spectrometer after the addition of 10 mL Beckman Ready Safe liquid scintillation cocktail.

Analysis of eicosanoids. Macrophage cultures were labeled with 10 μCi/mL of radioactive arachidonic acid. After the experiment, the culture medium was prepared for HPLC by adjusting the pH to 3.0 with formic acid. An aliquot of 450 mL of each medium was taken to analysis. Metabolites of arachidonic acid were separated by HPLC (Spectra-Physics, San Jose, CA, USA; model SP-8700) using a 4 × 250 mm column of 5 μm LiChrosorb RP-18 (E. Merck, Darmstadt, Germany). At starting point, the mobile phase was methanol/0.1% acetic acid in water (1:1, v/v), where the water phase had been brought to pH 5.4 by ammonia. Na_2EDTA was added to the eluent at 1 mM concentration. Separation was performed by running a 1 hr linear gradient to 100% methanol at a flow rate of 0.5 mL/min. Fractions of 0.7 mL were collected and analysed by scintillation spectrometry. The PGE_2, thromboxane B_2, prostaglandin $F_{2\alpha}$, 5-HETE, 15-hydroxyeicosatetraenoic acid, 6-keto-prostaglandin $F_{1\alpha}$, leukotriene B_4 and LTC_4 had all previously been identified as to their retention times [13–14].

Determination of phospholipase A_2 activity. Mouse macrophages were isolated and cultured on six-well Linbro tissue culture dishes without radioactive labeling, but otherwise as described above. The cells were scraped off the dishes and homogenized in a buffer consisting of 80 mM KCl, 5 mM dithioerythritol, 1 mM EDTA and 10 mM HEPES, pH 7.4. The homogenate from three or six dishes was centrifuged at 700 *g* for 5 min and the resulting supernatant was further centrifuged at 10^5 *g* for 60 min to obtain a cytosolic fraction and a membrane pellet. An aliquot (5–20 μL) of the cytosol fraction was then taken to assay. The assay mixture contained 100 pmol phosphatidylcholine with added 1-stearoyl-2-[^{3}H]arachidonoyl-phosphatidylcholine solubilized by sonication, $CaCl_2$ (620 nmol total, 290 μM free Ca^{2+}) and fatty-acid-depleted BSA (100 μg), all dissolved in a total volume of 525 μL of the buffer described above, but without dithioerythritol. After incubation for 30 min at 37° the reaction was stopped by the addition of 1.5 mL of a solution containing chloroform/methanol/HCl (2:1:0.01) as well as carrier lipids. The lipid phase was taken to chromatography on a column containing 100 mg silic acid equilibrated in chloroform. The fatty-acids were eluted with 2 × 0.5 mL chloroform and the phospholipids with 2.1 mL methanol, and the radioactivity of these samples determined as described above.

Analysis of inositol compounds. Macrophages were cultured on six-well Linbro tissue culture dishes and labeled with 10 μCi *myo*-(2-^{3}H)inositol (Amersham; 15 Ci/mmol) overnight. Experimental conditions were as above. The cells were scraped off the dish in 1 mL of ice-cold 50 mM HCl and lipids extracted with 6 mL of chloroform/methanol (1:1, v/v) containing 0.05% 2,6-di-*t*-butyl-*p*-cresol as an antioxidant. Lipid standards were added to the extract as carriers and phase separation was effected by the addition of 2 mL 50 mM HCl. Aliquots of the culture medium and the upper and lower phases were taken to radioactivity determination. Water-soluble inositol compounds were analysed on columns containing AG 1-X8 in formate form (Bio-Rad, Richmond, CA, U.S.A.), by a stepwise gradient of ammonium formate, and the inositol-containing lipids were analysed using thin layer chromatography as previously described [15–16].

Assessment of the protein phosphorylation pattern. Macrophages were cultured as above, with or without antirheumatic drug. On the second day of culture, the cells were labeled for 45 min with 0.2 mCi carrier-free $^{32}PO_4$ (Amersham International, U.K.). After challenge of the cells for 15 min with the relevant drugs, the cells were scraped off the dish. The protein samples were boiled and an aliquot was taken to SDS–polyacrylamide gel electrophoresis according to Laemmli, using 1.5-mm slab gels. Separation and stacking gels contained 10 and 3% polyacrylamide, respectively. After electrophoresis, the gels were fixed, Coomassie stained, destained, dried under vacuum onto filter papers and exposed to X-ray film (Kodak X-omat S film 100). The apparent M_r of the phosphoproteins was determined using a standard mixture of reference proteins (MW-SDS-200, Sigma).

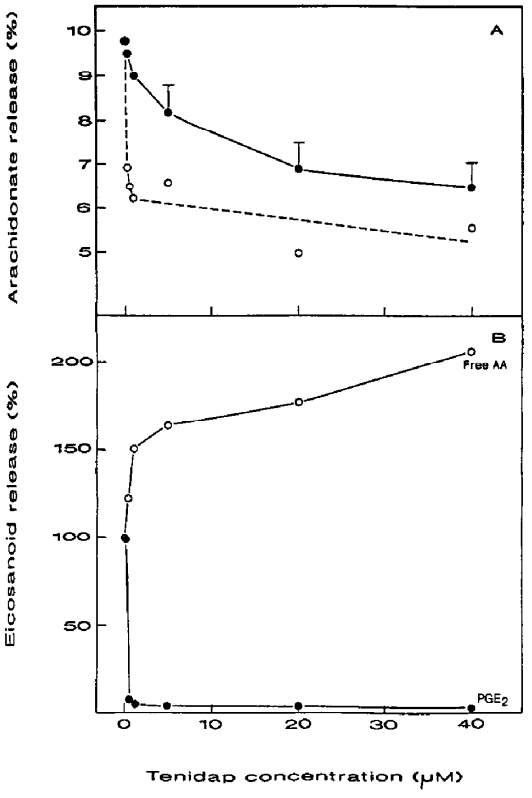

Fig. 2. The effects of tenidap on PMA-induced arachidonate mobilization using simultaneous addition of drugs (●) or a 22 hr pre-incubation with tenidap (○) (A), and the effects of tenidap (without preincubation) on PMA-induced formation of PGE_2 (●) and free arachidonate (○) (B). It should be noted that the release of free arachidonate induced by PMA was less than 2% of the amount of labeled PGE_2 produced. Results are given as mean ± SEM, (N = 5–9). Statistically significant inhibition of arachidonate release occurred at 5 μM tenidap ($P < 0.005$) and at 20 and 40 μM tenidap ($P < 0.0005$).

Statistical methods. In all statistical testing, the paired comparisons version of Student's *t*-test has been used.

RESULTS

Effect of tenidap on phorbol ester-induced eicosanoid formation

Initially, it was established that tenidap (0.1–100 μM) affected arachidonate release only marginally (with maximum release 150% of that of control cells at 5 μM tenidap) in the absence of other stimuli (not shown).

On incubation of mouse macrophage cultures with the protein kinase C activator PMA (50 nM unless otherwise stated), a swift mobilization of arachidonate results. PGE_2 is the major eicosanoid formed, together with smaller amounts of 6-keto-prostaglandin $F_{1\alpha}$, which is the stable metabolite of prostaglandin I_2. Figure 2A shows that tenidap, in a dose-dependent manner caused a partial inhibition

Table 1. Dose–response for tenidap-induced inhibition of arachidonate release

Stimulus	Concentration	Tenidap (μM)	Arachidonate release (%)	P
Zymosan	0.1 mg/mL	0	17.5 ± 0.8	
		1	11.6 ± 1.3	<0.01
		5	8.2 ± 0.6	<0.001
		20	7.5 ± 0.5	<0.001
	0.3 mg/mL	0	18.7 ± 0.5	
		1	15.2 ± 1.0	<0.01
		5	11.8 ± 0.7	<0.01
		20	8.0 ± 0.4	<0.001
	0.6 mg/mL	0	21.8 ± 0.7	
		1	15.9 ± 1.2	<0.001
		5	12.9 ± 1.1	<0.001
		20	9.6 ± 0.9	<0.001
A23187	0.1 μM	0	8.2 ± 0.8	
		5	8.2 ± 0.8	NS
		20	7.0 ± 0.7	NS
	0.3 μM	0	10.6 ± 0.8	
		1	11.0 ± 1.4	NS
		5	10.0 ± 1.6	NS
		20	7.9 ± 0.5	<0.05
	1 μM	0	13.9 ± 0.8	
		1	13.3 ± 0.6	NS
		5	15.5 ± 1.0	NS
		20	11.2 ± 0.8	<0.05
PMA	50 nM	0	10.0 ± 0.9	
		1	10.2 ± 0.9	NS
		5	8.4 ± 1.4	NS
		20	7.6 ± 1.5	<0.05
	100 nM	0	12.0 ± 0.8	
		1	12.2 ± 0.7	NS
		5	9.7 ± 1.2	<0.05
		20	9.5 ± 0.6	<0.05

The influence of tenidap on arachidonate release (given as % of total incorporated [^{3}H]arachidonate) induced by various concentrations of the stimuli PMA, A23187 and zymosan. Results are given as means ± SEM, (N = 5 in all experiments). NS, not significant ($P > 0.05$)

of the PMA-induced mobilization of arachidonate. When PMA concentration was doubled, the degree of inhibition remained unchanged (Table 1). Interestingly, the inhibition was more pronounced after an overnight incubation with tenidap (22 hr), although the overnight incubation medium contained 10% fetal calf serum and tenidap is known to be strongly bound to serum proteins [17]. Tenidap was markedly atoxic to the macrophages: even a 22 hr incubation with 40 μM drug had no negative effect on cell viability, as indicated by determination of lactate dehydrogenase release as well as microscopy of the cells and measurements of arachidonate incorporation. As seen in Fig. 2B, tenidap exerted a powerful inhibition of PMA-induced PGE_2 formation, which far exceeded its effect on arachidonate mobilization. The formation of 6-keto-prostaglandin $F_{1\alpha}$ was similarly affected.

A series of separate experiments was performed with 0.1% BSA present in the medium as a fatty-acid acceptor, thus allowing a more direct assessment of arachidonate mobilization. The results showed that PMA-induced arachidonate mobilization was inhibited by tenidap irrespective of the presence or absence of BSA, although the net release was larger with BSA present (Table 2). At least a partial explanation of this might be that the concentration of tenidap in the medium was decreased due to its binding to the BSA.

Effect of tenidap on calcium ionophore-induced eicosanoid formation

A rise in intracellular calcium induced by the calcium ionophore A23187 (1 μM unless otherwise stated) leads to mobilization of arachidonic acid and formation of both PGE_2 and LTC_4 [18–19]. Coincubation with tenidap further increased this ionophore-induced mobilization of arachidonic acid at lower concentrations of the drug, while higher concentrations (20–40 μM) caused inhibition (Fig. 3A). This pattern was not changed by a reduction in ionophore concentration (Table 1). As in the case of PMA-induced activation of the macrophages, there was a downward shift of concentration dependence after overnight preincubation (22 hr) with tenidap, although less marked. The effect of tenidap on A23187-induced eicosanoid formation is shown in Fig. 3B. The formation of PGE_2 was again almost totally inhibited. Ionophore-induced formation of LTC_4 was also inhibited, but only at

Table 2. Effect of BSA on tenidap-induced inhibition of arachidonate release

Tenidap (μM)	Arachidonate release (%) PMA BSA	PMA No BSA	A23187 BSA	A23187 No BSA
0	13.8 ± 1.5	10.4 ± 0.6	17.6 ± 1.6	15.1 ± 1.0
3	15.0 ± 1.7	11.6 ± 0.8	22.0 ± 2.4	17.6 ± 1.1
10	7.5 ± 0.8	4.9 ± 0.4	29.0 ± 2.3	18.7 ± 1.4
40	7.3 ± 0.5	4.0 ± 0.4	13.5 ± 1.9	9.8 ± 1.0
100			10.0 ± 1.1	8.5 ± 1.0

The influence of tenidap on arachidonate release induced by PMA (50 nM) or A23187 (1 μM), in the absence or presence of 0.1% BSA. Data are given as means ± SEM, (N = 5). In the case of PMA-stimulated cells, there was no significant potentiation of arachidonate release at 3 μM, but significant ($P < 0.001$) inhibition at 10 μM and 40 μM of the drug, both in the presence and absence of BSA. In the case of A23187-stimulated cells, in the presence of BSA, there was significant potentiation of arachidonate release both at 3 μM ($P < 0.05$) and 10 μM ($P < 0.0001$) tenidap, while there was significant ($P < 0.01$) inhibition at 40 μM. In the absence of BSA, there was no significant potentiation of arachidonate release at 3 μM, but significant ($P < 0.05$) potentiation at 10 μM and significant ($P < 0.05$) inhibition at 40 μM tenidap

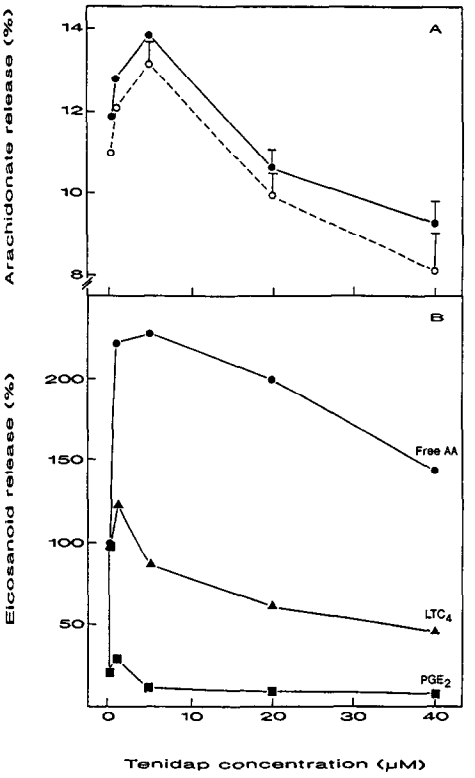

Fig. 3. In (A), the effects of tenidap on A23187-induced arachidonate release is plotted with (○) and without (●) previous 22 hr pretreatment, while in (B), the effects of simultaneously added tenidap on the eicosanoids formed is shown. The eicosanoids formed after challenge of the cells with calcium ionophore are LTC_4 (60%), PGE_2 (30%) as well as smaller amounts of 5-HETE and free arachidonate. In (A), results are given as means ± SEM, (N = 5–9). Statistically significant potentiation of arachidonate release ($P < 0.005$) was observed at 5 μM tenidap, no significant effect at 20 μM tenidap but significant inhibition ($P < 0.01$) at 40 μM tenidap.

higher concentrations of tenidap (20–40 μM), where there was also an inhibitory effect on ionophore-induced arachidonate mobilization. There was a considerable increase in the liberation of free arachidonic acid at lower concentrations, but this decreased at higher concentrations of tenidap as did the total release of arachidonate (Figs. 3A,B). When BSA was included in the medium, the tenidap-induced potentiation of arachidonate release at low concentrations of the drug appeared to be somewhat strengthened, but the inhibition at higher concentrations was still evident (Table 2).

Effects of tenidap on zymosan-induced eicosanoid formation

Stimulation of macrophages with zymosan (0.6 mg/mL unless otherwise stated) leads to release of arachidonate and formation of eicosanoids; predominantly PGE_2, but also LTC_4 and smaller amounts of 5-HETE [18]. As seen in Fig. 4A, tenidap caused a rather strong, dose-dependent inhibition of arachidonate mobilization, which was not significantly altered by a 22-hr preincubation with tenidap. This inhibition is unaltered by a reduction in zymosan concentration (Table 1). The presence of BSA (Fig. 4A) revealed, as with A23187-stimulated cells, a minor potentiation of arachidonate release at lower concentrations of tenidap, with significant inhibition occurring only at higher concentrations (20–40 μM); again, this might well be due to a decrease in available tenidap.

Even very low concentrations of tenidap led to a very strong inhibition of zymosan-induced PGE_2 synthesis, with a concomitant increase in free arachidonate most marked at low concentrations of the drug (Fig. 4B). The formation of LTC_4 was also diminished, more so than in the case of A23187-stimulated cells.

Effect of tenidap on phospholipase A_2 activity

In several preparations, the effect of tenidap on the *in vitro* activity of the 85 kDa arachidonate-mobilizing macrophage phospholipase A_2 was determined. The results showed that PMA induced an increase in the activity of this phospholipase. Coincubation with higher concentrations of tenidap significantly reduced this response (Table 3). This could not be explained by any direct effect of tenidap on the arachidonate-mobilizing 85 kDa phospholipase A_2, since the basal activity of this enzyme was unaffected even at high concentrations of the drug (Table 4). Nor did incubation of the cells with varying concentrations of tenidap in the absence of PMA affect the activity of this phospholipase (not shown).

Effects of tenidap on phosphoinositide turnover

In order to elucidate further the mechanisms

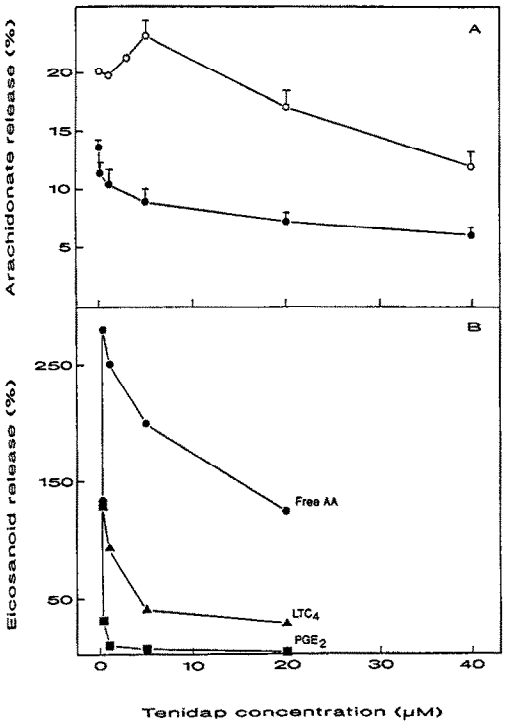

Fig. 4. In (A), the effect of simultaneously added tenidap on zymosan-induced arachidonate release, in the presence (○) or absence (●) of BSA, is shown. Data are given as means ± SEM, (N = 5–9). In the absence of BSA, statistically significant inhibition of zymosan-induced arachidonate release occurred at 0.1 and 1 μM tenidap ($P < 0.05$), at 5 μM ($P < 0.01$), as well as at 20 and 40 μM tenidap ($P < 0.001$). In the presence of 0.1% BSA statistically significant potentiation ($P < 0.05$) occurred at 5 μM tenidap and significant inhibition ($P < 0.05$) at 40 μM tenidap. In (B), the effect of simultaneously added tenidap on zymosan-induced eicosanoid formation is shown, with the data given as means of 3–5 experiments. The eicosanoids formed after challenge of the cells with zymosan are: PGE_2 (60%), LTC_4 (25%), 6-keto prostaglandin $F_{1\alpha}$ (5%), 5-HETE (less than 5%) as well as smaller amounts of free arachidonate.

Table 3. Inhibition by tenidap of the PMA-induced activation of cytosolic macrophage phospholipase A_2

Tenidap (μM)	Reduction of PMA-induced enzyme activation (%)	N	P
1	11 ± 9	3	NS
5	47 ± 18	5	<0.05
20	56 ± 19	4	<0.05

Macrophage cultures were treated with PMA (50 nM) for 30 min with or without pretreatment with tenidap for 10 min. The cultures were then harvested and phospholipase A_2 activity was determined. The effect of tenidap on PMA-induced activation is expressed as % reduction of the PMA-induced increase in enzyme activity (PMA + tenidap − control)/(PMA − control); mean ± SEM.

Table 4. The influence of tenidap on the basal activity of cytosolic macrophage phospholipase A_2

Tenidap (μM)	Effects on basal enzyme activity (%)	N
1	+3 ± 3	5
5	−7 ± 7	9
20	−7 ± 12	5
40	−13 ± 8	9

Using cytosolic fractions from control cells, the effect of tenidap on the basal activity of phospholipase A_2 was determined. Results are given as mean ± SEM. Statistical testing was performed as described in Materials and Methods, but no significant ($P < 0.05$) effect of tenidap on basal phospholipase activity could be detected.

involved in the tenidap-induced inhibition of the 85 kDa phospholipase A_2, [^{3}H]inositol-prelabeled cells were used. Tenidap did not in itself change the distribution of label among phosphoinositides, glycerophosphoinositol or inositol phosphates, compared with control cultures (Table 5). The formation of glycerophosphoinositol was markedly increased upon challenge of the cells with PMA as a result of activation of the phospholipase A pathway. There was a concomitant increase in inositol phosphates, mainly inositol monophosphate, most likely derived from glycerophosphoinositol [20]. Increasing concentrations of tenidap gradually inhibited both these effects, thus confirming the inhibition of phospholipase A_2.

Challenge of the cells with zymosan also resulted in direct activation of the phospholipase C pathway [15], with a considerable formation of inositol phosphates (Table 5). Tenidap inhibited this effect as well as glycerophosphoinositol formation. Furthermore, the calcium ionophore-induced formation of inositol phosphates was decreased by tenidap (Table 5).

Effects of tenidap on macrophage protein phosphorylation

Firstly, experiments were performed to assess the ability of tenidap in itself to induce protein phosphorylation. It was found that tenidap (1–5 μM but to a much lesser degree 20–40 μM) induced increased phosphorylation of several distinct protein bands, mainly those of 30, 34 and 43 kDa, but also two bands at 50 and 52 kDa (Fig. 5, lanes 1–3). This phosphorylation pattern is somewhat enhanced upon overnight preincubation with tenidap (not shown). The tenidap-induced phosphorylation of intracellular proteins was not related to its effects on the cyclooxygenase, since indomethacin (1–10 μM) did not affect the phosphorylation pattern in any way. The protein phosphorylation pattern induced by tenidap differs from that induced by zymosan or PMA in many respects (see Fig. 5, lane 1 vs lane 4). The major difference is that while tenidap induced phosphorylation of a 43 kDa protein, the other compounds caused phosphorylation of the 45 kDa

Table 5. The influence of tenidap on the generation of glycerophosphoinositol and inositol phosphates in macrophage cultures prelabeled with [^{3}H]inositol

Stimulus	Tenidap (μM)	Glycerophospho-inositol (%)	Inositol phosphates (%)	N	p1	p2
None	0	1.6 ± 0.2	12.2 ± 0.9	6		
	1	1.4 ± 0.2	13.3 ± 0.8	3	NS	NS
	5	1.6 ± 0.3	10.8 ± 0.3	3	NS	NS
	20	1.6 ± 0.2	11.8 ± 0.9	3	NS	NS
PMA	0	6.2 ± 1.0	18.4 ± 1.7	4		
	1	5.8 ± 1.1	16.9 ± 2.1	4	NS	NS
	5	5.1 ± 1.1	16.0 ± 2.2	4	$P < 0.05$	$P < 0.025$
	20	3.9 ± 0.7	13.1 ± 0.5	4	$P < 0.01$	$P < 0.005$
A23187	0	4.1 ± 0.5	48.4 ± 4.5	5		
	1	4.0 ± 0.5	46.8 ± 2.5	5	NS	NS
	5	3.6 ± 0.8	37.3 ± 2.2	5	NS	$P < 0.0005$
	20	2.7 ± 0.4	33.4 ± 1.8	6	$P < 0.005$	$P < 0.0005$
Zymosan	0	4.1 ± 0.5	58.2 ± 5.6	6		
	1	3.4 ± 0.2	50.0 ± 6.4	6	NS	$P < 0.0005$
	5	2.7 ± 0.2	44.0 ± 5.7	5	$P < 0.01$	$P < 0.0005$
	20	2.3 ± 0.2	36.4 ± 3.3	5	$P < 0.01$	$P < 0.0005$

Cultures were simultaneously exposed to tenidap and the stimulus (either 50 nM PMA, 1 μM A23187 or 0.6 mg/mL zymosan) used for 30 min. Results are expressed as percentage of total inositol radioactivity, mean ± SEM. Statistically significant effects of tenidap on the generation of glycerophosphoinositol (p1) or inositol phosphates (p2) are indicated.

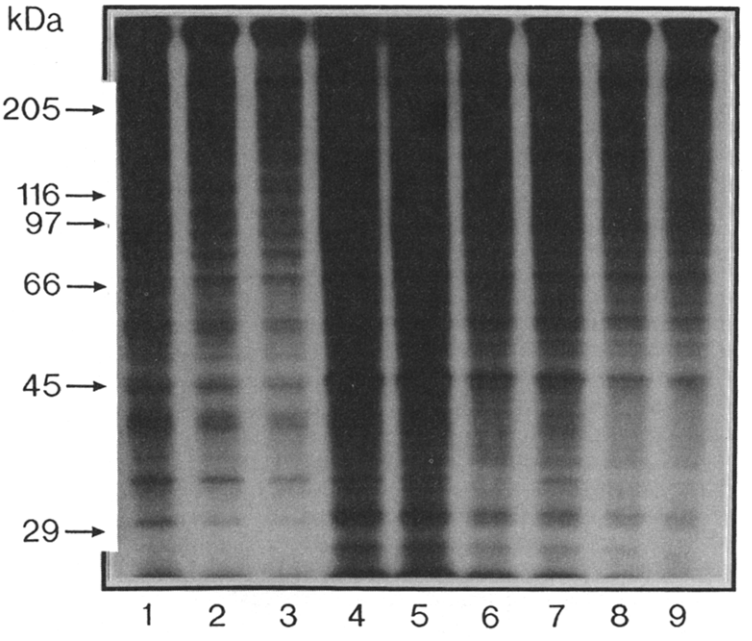

Fig. 5. Protein phosphorylation in response to tenidap and/or zymosan. Lanes 1–2: 5 and 1 μM tenidap; lane 3: control; lane 4: zymosan; lanes 5–6: 1 and 5 μM tenidap for 22 hr followed by zymosan; lanes 7–9: 5, 20 and 40 μM tenidap and zymosan added simultaneously.

protein. This protein was not phosphorylated by tenidap.

Having established that tenidap in itself can induce a change in the pattern of protein phosphorylation different from that caused by stimulation of protein kinase C (or a rise in intracellular calcium), attention was turned to examining whether tenidap affected phosphorylation induced by a protein kinase C agonist. Therapeutically relevant concentrations of tenidap (1–40 μM) did not significantly inhibit PMA-induced changes in protein phosphorylation (not shown). On the other hand, tenidap (5–40 μM) strongly inhibited zymosan-induced protein phosphorylation (Fig. 5, lanes 3–9), without any

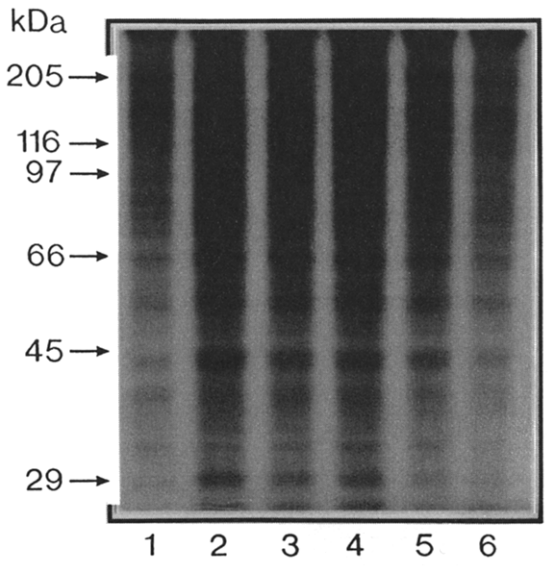

Fig. 6. Protein phosphorylation in response to calcium ionophore. Lane 1: control; lane 2: A23187 alone; lanes 3–6: A23187 and 1, 5, 20 and 40 μM tenidap (added simultaneously), respectively.

detectable changes in the pattern of whole cell proteins. Overnight preincubation strengthened this effect (Fig. 5, lanes 6 and 7). When calcium ionophore was used as a stimulant, the protein phosphorylation pattern was somewhat potentiated by tenidap at lower concentrations (1–5 μM), possibly as a result of the ability of tenidap in itself to phosphorylate proteins within this concentration range. At higher concentrations (20–40 μM), A23187-induced phosphorylation was gradually inhibited by tenidap (Fig. 6), but to a lesser extent than zymosan-induced phosphorylation changes.

DISCUSSION

Tenidap is known to be an efficient cyclooxygenase inhibitor, with an IC_{50} of <0.1 μM in rat basophilic leukaemia cells comparable to that for indomethacin. Our results from mouse macrophages agree well with these findings. But the actions of tenidap go beyond those of other NSAIDs. In rheumatoid arthritis, tenidap has a cytokine modulatory effect; tenidap treatment also results in a decrease in ESR and the plasma levels of C-reactive protein and serum amyloid A [1, 2].

In various experimental systems, evidence suggests that tenidap has an inhibitory influence on the formation of 5-lipoxygenase products, with IC_{50}-values in the range 2–25 μM [7, 8, 21, 22]. Our data from mouse macrophages do not support the hypothesis of a specific inhibitory effect of tenidap on the 5-lipoxygenase pathway. In fact, lower concentrations of tenidap further increase A23187-induced mobilization of arachidonic acid, while having only minimal effects on LTC_4 formation. At higher concentrations of tenidap (20–40 μM) there is a 30–50% inhibition of the A23187- or zymosan-induced formation of LTC_4. However, it must be noted that the tenidap-induced inhibition of arachidonic acid mobilization and its inhibition of the PMA-induced activation of the macrophage phospholipase A_2 are of comparable magnitude. Furthermore, the decrease in the liberation of free arachidonic acid shows a similar concentration dependence as the decrease in LTC_4 formation. Similar results, also in favour of an inhibitory effect on the release of arachidonic acid rather than on leukotriene synthesis, have been obtained with neutrophils stimulated with calcium ionophore [23].

It is remarkable that while tenidap strongly inhibits zymosan-induced arachidonate release, this is much less marked when either PMA or A23187 are used to stimulate the macrophages independent of the concentrations of the stimulants used (Table 1). A possible explanation for the stronger inhibitory effect of tenidap lies in its effects on the zymosan-induced formation of inositol phosphates. This could weaken signalling via both calcium and protein kinase C. It should be kept in mind that phagocytosis of zymosan particles also initiates an increase in cytoplasmic calcium [24]. In a recent study [25], tenidap has been shown to inhibit calcium influx as well as antigen-induced increase in intracellular calcium in a mast cell line. Tenidap also inhibited inositol phosphate formation in these cells.

Our results suggest that the effects of tenidap on signal transduction are more complex than previously assumed, and that the actions of the drug in mouse macrophages go beyond those of an inhibitor of either the cyclooxygenase or lipoxygenase pathways. Its effects on the mobilization of arachidonate and the activation of the macrophage 85 kDa phospholipase A_2 suggest that tenidap affects some earlier step in the signal transduction pathway, since it does not affect this phospholipase directly. Furthermore, the pattern of protein phosphorylation induced by zymosan is progressively inhibited by higher concentrations of tenidap. The phosphorylation of a 45 kDa protein is almost totally inhibited at 20–40 μM of the drug. In contrast, the phosphorylation pattern induced by PMA was only marginally affected by tenidap. When A23187 was used as a stimulant, 5 μM tenidap potentiated the phosphorylation pattern while higher concentrations (20–40 μM) inhibited it. These results on protein phosphorylation parallel those on arachidonate mobilization. The lack of inhibition of protein phosphorylation upon incubation with a direct stimulator of protein kinase C indicates that this enzyme is not a direct target for tenidap. Instead, the results indicate that a target for tenidap lies in the signal chain between protein kinase C and phospholipase A_2 (Fig. 1; site 1). Another target for tenidap may be receptor-linked phospholipase C activation (Fig. 1; site 2). Both these mechanisms might relate to the tenidap-induced inhibition of the induction of IL-1 and other proinflammatory cytokines in human blood monocytes [5] and mouse macrophages [4], and partially account for its effect on clinical and laboratory parameters in rheumatoid arthritis.

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