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Flufenamic and Tolfenamic Acids Inhibit Calcium Influx in Human Polymorphonuclear Leukocytes

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SUMMARY

Fenamates, a subgroup of nonsteroidal anti-inflammatory drugs, inhibit several functions of human polymorphonuclear leukocytes (PMNs) *in vitro*, by a thus far unknown mechanism. To determine the mechanism behind this action, we studied the effects of two fenamates (flufenamic and tolfenamic acids) on Ca^{2+} metabolism in human PMNs. The two fenamates inhibited the increases in intracellular free calcium concentration induced by either the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine or the calcium ionophore A23187 in fura-2-labeled PMNs. This inhibition was concluded to be due to blocking of the cation influx, as evidenced by measurement of Mn^{2+} influx and the influx of radioactive calcium. In addition, the actions of flufenamic and tolfenamic acids were similar to those of an experimental blocker of nonselective cation channels (SK&F 96365). The two other control compounds, an antagonist of voltage-dependent calcium channels (nifedipine) and an inhibitor of prostanoid synthesis (ketoprofen), were ineffective. In conclusion, inhibition of calcium influx in PMNs is introduced as a novel prostanoid-independent mode of action of two nonsteroidal anti-inflammatory drugs with fenamate structure, flufenamic and tolfenamic acids, which could explain their earlier documented inhibitory effects on PMN functions.

Inhibition of prostanoid synthesis by NSAIDs is proposed to be an insufficient explanation for their anti-inflammatory efficacy at therapeutic doses. Inhibition of leukocyte functions by NSAIDs has been presented as an additional and prostanoid-independent mechanism of their action (1–4). We and others have shown that fenamates differ from other NSAIDs in their ability to inhibit several PMN functions (degranulation, migration, leukotriene B_4 release, and platelet-activating factor production) at micromolar drug concentrations (5–12). Recently we showed that the actions of flufenamic and tolfenamic acids on PMN degranulation resemble those of an experimental blocker of NSCs, SK&F 96365 (13, 14). Subsequently, our preliminary data showed that tolfenamic acid inhibits the increase in $[Ca^{2+}]_i$ in isolated human PMNs.[1] Furthermore, flufenamic acid has been reported to inhibit NSCs in rat exocrine pancreas (15) and mouse fibroblasts (16). These findings prompted us to study in detail the effects of fenamates on calcium metabolism in human PMNs. The effects of flufenamic and tolfenamic acids were compared with those of 1) DCDPC, an inhibitor of NSCs in rat exocrine pancreas that is structurally related to fenamates (17), 2) SK&F 96365, an experimental blocker of receptor-mediated calcium entry in human PMNs (13), 3) nifedipine, a classical antagonist of L-type voltage-dependent calcium channels (18), and 4) ketoprofen, a chemically different cyclooxygenase inhibitor (19).

The present results show that two fenamates (flufenamic and tolfenamic acids) inhibit Ca^{2+} influx in human PMNs. Inhibition of Ca^{2+} influx in PMNs is thus presented as a new mode of action of NSAIDs with fenamate structure.

Experimental Procedures

Materials. A23187, EGTA, flufenamic acid, fMLP, fura-2/AM, ketoprofen, and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Tolfenamic acid was obtained from GEA Ltd. (Copenhagen, Denmark). DCDPC and SK&F 96365 were generous gifts from Prof. R. Greger (Albert Ludwigs University, Freiburg, Germany) and SmithKline Beecham Pharmaceuticals (Surrey, UK), respectively. Other reagents were obtained as follows: Ficoll-Paque (Pharmacia Fine Chemicals AB, Uppsala, Sweden), LumaSolve (Lumac LSC, Groningen, The Netherlands), $MnCl_2$ (Merck, Darmstadt, Germany), $NiCl_2$ (J.T. Baker, Deventer, Holland), nifedipine (Orion Pharmaceutical Co., Espoo, Finland), silicone DC 200 fluid (Serva

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[1] H. Kankaanranta, H. Wuorela, E. Siltaloppi, P. Vuorinen, H. Vapaatalo, and E. Moilanen. Intracellular mediators of polymorphonuclear leucocyte degranulation: effects of tolfenamic acid. Submitted for publication.

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; NSC, nonselective cation channel; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DCDPC, 3′,5-dichlorodiphenylamine-2-carboxylic acid; DPBS, Dulbecco's phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; AM, acetoxymethyl ester; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte; SK&F 96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride.

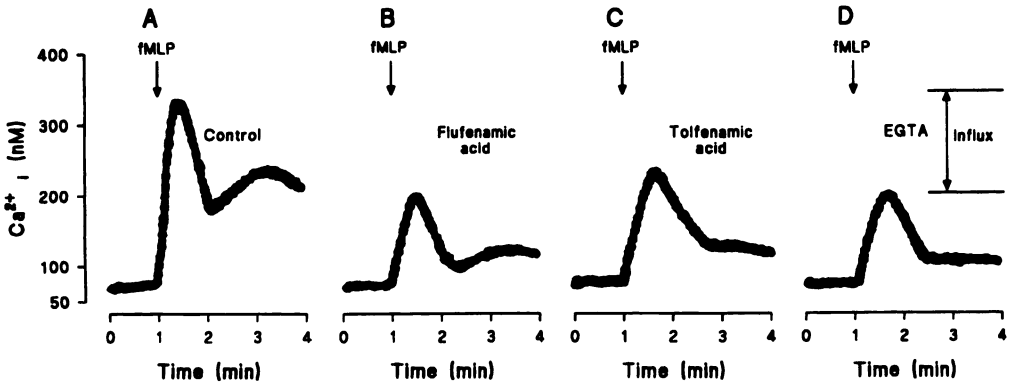

Fig. 1. Effects of flufenamic acid (30 μM) (B), tolfenamic acid (30 μM) (C), and EGTA (2 mM) (D) on fMLP (100 nM)-induced increases in $[Ca^{2+}]_i$ in fura-2-loaded human PMNs (suspended in DPBS) in the presence of extracellular Ca^{2+} (0.9 mM) and the control experiment without any drug (A). The cells were incubated with the drug for 5 min before addition of the stimulus. Typical traces from at least four similar experiments are shown.

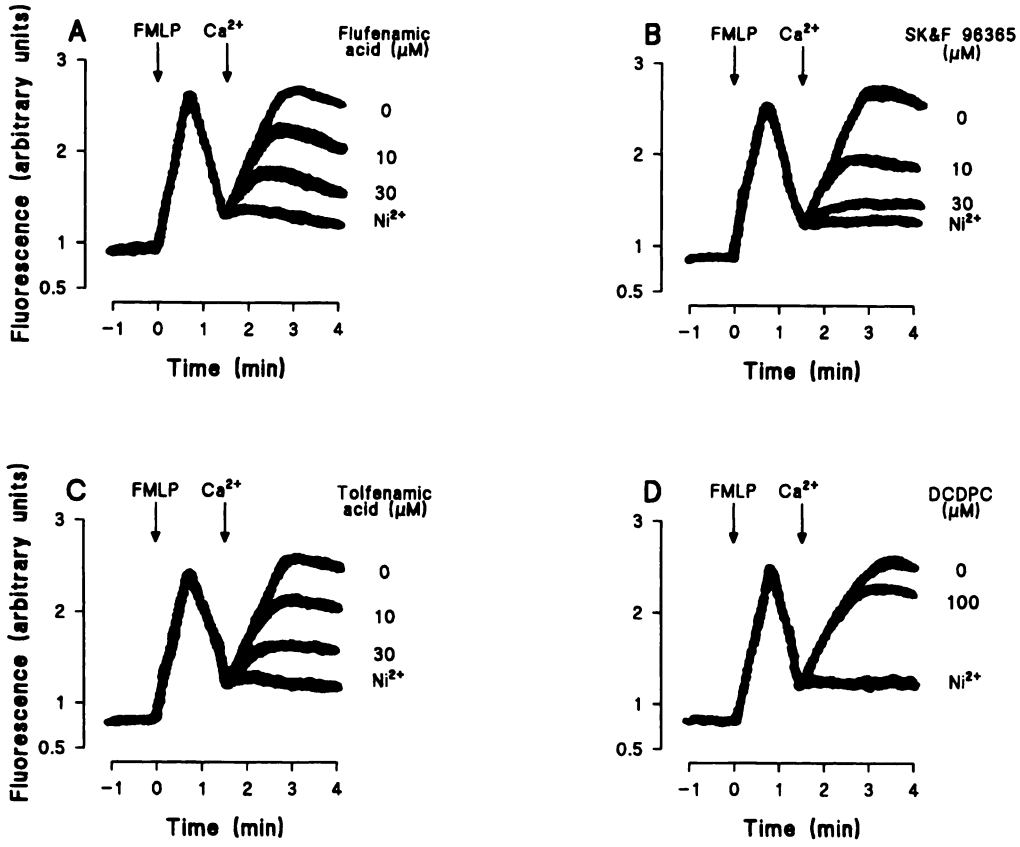

Fig. 2. Effects of flufenamic acid (A), SK&F 96365 (B), tolfenamic acid (C), and DCDPC (D) on fMLP (100 nM)-induced Ca^{2+} release from intracellular stores (first peak) and Ca^{2+} influx (second peak). Fura-2-loaded PMNs were suspended in PBS supplemented with 1 mM EGTA. PMNs were incubated with the drug for 5 min before addition of the stimulus. fMLP and Ca^{2+} (2 mM) were added as indicated. Ni^{2+} (5 mM) was used as a control that completely blocks NSCs. Traces are superimposed for clarity. Typical traces from at least three similar experiments are shown. For additional details, see Experimental Procedures.

Feinbiochemica GmbH & Co, Heidelberg, Germany), and $^{45}Ca^{2+}$ (Amersham International, Buckinghamshire, UK).

Isolation of human PMNs. PMNs were isolated as follows (7). Blood was collected by venipuncture from healthy volunteers who had abstained from any drugs for at least 1 week before sampling. A buffy-coat preparation of citrated blood was layered on Ficoll-Paque and centrifuged according to the method of Bøyum (20). Red cells were removed by dextran sedimentation, followed by lysis of the remaining erythrocytes with Tris-buffered 0.15 M NH_4Cl. PMNs were washed twice with DPBS (0.9 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, 137 mM NaCl, 8 mM Na_2HPO_4). After the isolation procedure, the viability of the cells was >97%, as determined by trypan blue exclusion, and the PMN suspension contained <2% mononuclear leukocytes.

Measurement of $[Ca^{2+}]_i$ and Ca^{2+} influx. Isolated PMNs (40 × 10^6/ml of DPBS) were loaded with the AM form of the fluorescent probe fura-2 (5 μM) for 30 min at 37°, in a shaking water-bath. PMNs were diluted with DPBS (1/3) and kept at room temperature for 10 min to allow reequilibration. PMNs were then washed twice and finally suspended in DPBS to yield a cell suspension containing 5 × 10^6 PMNs/ml of buffer. The fura-2 loading in the absence of albumin resulted a relatively light loading of the PMNs, thus allowing us to accurately monitor the changes in $[Ca^{2+}]_i$ without marked buffering of Ca^{2+} inside the cell. The changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) in thermostatted (37°) quartz cuvettes, with continuous stirring. The excitation wavelengths were set at 340 nm and 380 nm and emission was at 500 nm when changes in $[Ca^{2+}]_i$ were measured. Fenamates were not used at concentrations higher than 30 μM, due to their possible interference with fura-2 fluorescence.

The increases in $[Ca^{2+}]_i$ were stimulated by addition of the chemotactic peptide fMLP (100 nM) or the calcium ionophore A23187 (100 nM). EGTA (2 mM) was used to chelate the extracellular calcium when calcium release from intracellular stores was measured. To assess Ca^{2+} release from intracellular stores and influx, a modification of the method described by Andersson *et al.* (21) was used. The cells were suspended in EGTA (1 mM)-supplemented PBS (DPBS but containing no calcium) with or without the studied drug. PMNs were

TABLE 1

Effects of DCDPC, flufenamic acid, ketoprofen, nifedipine, SK&F 96365, and tolfenamic acid on fMLP (100 nM)- and A23187 (100 nM)-induced Ca^{2+} influx in fura-2-labeled human PMNs

The data represent the means ± standard errors of single experiments. The number of determinations is shown in parentheses. The results are percentage of control values, i.e., in each batch of cells the increase in fura-2 fluorescence in fMLP- or A23187-activated cells in control experiments (without the drug) was set as 100%. The values obtained in the presence of Ni^{2+} (fMLP-induced influx) or EGTA (A23187-induced influx) were subtracted from the results before calculation of the percentage of control values. For the analysis, the maximal responses were measured. For typical traces, see Figs. 2 and 3.

	Ca^{2+} influx	
	fMLP	A23187
	% of control	
DCDPC		
30 μM	88 ± 8 (4)	97 ± 7 (3)
100 μM	66 ± 7 (4)	81 ± 9 (3)
Flufenamic acid		
3 μM	115 ± 17 (4)	92 ± 9 (6)
10 μM	63 ± 3 (4)	89 ± 14 (6)
30 μM	14 ± 2 (4)	20 ± 6 (6)
Ketoprofen		
30 μM	96 ± 7 (4)	112 ± 4 (3)
100 μM	95 ± 4 (4)	118 ± 7 (3)
Nifedipine		
1 μM	112 ± 18 (3)	95 ± 1 (3)
10 μM	103 ± 17 (3)	90 ± 3 (3)
SK&F 96365		
3 μM	98 ± 13 (4)	72 ± 5 (5)
10 μM	38 ± 3 (4)	40 ± 5 (5)
30 μM	9 ± 5 (4)	23 ± 4 (5)
Tolfenamic acid		
3 μM	88 ± 12 (4)	105 ± 10 (5)
10 μM	54 ± 6 (4)	80 ± 16 (5)
30 μM	15 ± 3 (4)	28 ± 12 (5)

incubated with the studied drug for 5 min before stimulation. The cells were stimulated by addition of fMLP, and calcium release from intracellular stores was monitored for 90 sec. Thereafter Ca^{2+} (2 mM) was added to overcome the action of EGTA and to yield an extracellular free calcium concentration of about 1 mM. This results in an influx of extracellular Ca^{2+} into activated PMNs (21). The time point (90 sec after the stimulus) at which Ca^{2+} was added to the cell suspension was chosen because it represents the maximal influx of Ca^{2+} (21).

After the addition of Ca^{2+} to the incubation, the proportion of the fluorescence that was reversed in the presence of an inorganic blocker of NSCs, Ni^{2+} (5 mM), was interpreted to represent Ca^{2+} influx. When the calcium ionophore A23187 was used as the stimulus to increase $[Ca^{2+}]_i$, the fluorescence measured in cells suspended in EGTA (1 mM)-containing PBS was interpreted to be due to calcium release from intracellular stores. To obtain the value of Ca^{2+} influx, the fluorescence measured in the presence of EGTA was subtracted from the total fluorescence (13).

Calibration of the signal was performed basically according to the method described by Grynkiewicz *et al.* (22). The maximal fluorescence (F_{max}) was measured after addition of 2 μM ionomycin, and the minimum fluorescence (F_{min}) was measured in the presence of 25 mM EGTA, pH 8.6, and 0.1% Triton X-100. The $[Ca^{2+}]_i$ values were calculated from the equation $[Ca^{2+}]_i$ (nM) = $R \cdot 224 \cdot (F - F_{min})/(F_{max} - F)$, where 224 represents the dissociation constant for fura-2, F is the fluorescence of the intact cell suspension, and R is the ratio of F_{min}/F_{max} at 380 nm.

The resting $[Ca^{2+}]_i$ of PMNs suspended in DPBS was 60 ± 5 nM (n = 38). After stimulation with 100 nM fMLP and 100 nM A23187, the maximal $[Ca^{2+}]_i$ values were 309 ± 7 nM (n = 12) and 536 ± 33 nM (n = 22), respectively.

Measurement of Mn^{2+} influx. Measurement of Mn^{2+} influx was performed basically according to the method of Merritt *et al.* (23). PMNs were loaded with fura-2/AM (1 μM) for 30 min at 37° as described above. The cells were finally suspended in PBS (5×10^6 cells/ml). The excitation wavelengths were set at 340 and 360 nm, with emission at 500 nm. At an excitation wavelength of 340 nm, fura-2 fluorescence increases with increasing $[Ca^{2+}]_i$, whereas the fluorescence at 360 nm is insensitive to changes in $[Ca^{2+}]_i$. However, the fluorescence at both excitation wavelengths is quenched by Mn^{2+}. Mn^{2+} (final concentration, 100 μM) was added 90 sec after stimulation of the cells with fMLP. Ni^{2+} (5 mM) was used as a control compound known to block NSCs and thus both Ca^{2+} and Mn^{2+} influx induced by fMLP in PMNs (23).

Measurement of $^{45}Ca^{2+}$ influx. $^{45}Ca^{2+}$ influx was measured basically according to the method of Naccache *et al.* (23, 24). PMNs (in DPBS) were preincubated in the presence or absence of cytochalasin B (10 μM) and the drug for 5 min. $^{45}CaCl_2$ (1 μCi/5 × 10^6 PMNs, corresponding to 1.2 nmol of $^{45}Ca^{2+}$/5 × 10^6 PMNs) in buffer (for control cells) or $^{45}CaCl_2$ and the appropriate stimulus were added. Incubations (10 min) were terminated by addition of EGTA (final concentration, 10 mM). Immediately after addition of EGTA, the cells were centrifuged (10,000 × g, for 15 sec) and the EGTA-containing supernatant was removed. The cell pellet was resuspended in physiological saline solution and layered on top of silicone DC 200 oil and a 50-μl cushion of 12% sucrose. Cells were separated from the medium by centrifugation (10,000 × g, for 20 sec). The cell pellet was solubilized in 300 μl of LumaSolve and its radioactivity was counted in an LKB Wallac 1219 RacBeta liquid squintillation counter (LKB Wallac, Turku, Finland). The concentrations of the two stimuli (fMLP and A23187) were the same as in fura-2 studies (100 nM), and the incubation time was chosen to be optimal on the basis of a time-response curve made with cells from four different donors.

Results

Flufenamic and tolfenamic acids (30 μM) inhibited fMLP-induced increases in $[Ca^{2+}]_i$ in PMNs (suspended in calcium-containing medium) to levels seen after the addition of EGTA (Fig. 1). The inhibitory action was evident independently of the presence of cytochalasin B or the concentration of fMLP (10–1000 nM). This inhibitory effect on $[Ca^{2+}]_i$ was not due to chelation of calcium by fenamates. In the presence of EGTA, flufenamic and tolfenamic acids (30 μM) could not markedly affect the release of intracellular calcium, and thus an effect on calcium influx was suggested.

To further characterize the inhibition of fMLP-induced $[Ca^{2+}]_i$ increases by fenamates, a modification of the method described by Andersson *et al.* (21) was used. After incubation of the fura-2-loaded PMNs in calcium-free medium (PBS with 1 mM EGTA), the cells were stimulated with fMLP and the Ca^{2+} release from intracellular stores was monitored for 90 sec. Thereafter extracellular Ca^{2+} (2 mM) was added to overcome the action of EGTA and to induce the influx of extracellular calcium (Fig. 2). To confirm that the second peak in the fura-2 fluorescence, which occurred after addition of extracellular calcium, was due to Ca^{2+} influx into the cell, an inorganic blocker of NSCs, Ni^{2+} (5 mM) (23), was used to prevent the increase in fura-2 fluorescence (Fig. 2). This technique allowed us to differentiate between the release of intracellular Ca^{2+} and the influx of extracellular Ca^{2+} in the same cell preparation.

Flufenamic acid, tolfenamic acid, and SK&F 96365 (3–30 μM) dose-dependently inhibited fMLP-induced influx of extracellular Ca^{2+} into PMNs (Fig. 2; Table 1), but they did not markedly affect the release of Ca^{2+} from intracellular stores (Fig. 2). The two fenamates were about as potent as SK&F

TABLE 2

IC_{50} values for DCDPC, flufenamic acid, ketoprofen, nifedipine, SK&F 96365, and tolfenamic acid effects on fMLP (100 nM)- and A23187 (100 nM)-induced Ca^{2+} influx in human PMNs, as measured by using fura-2-labeled cells or the influx of $^{45}Ca^{2+}$

The data represent the means ± standard errors of single (fura-2 method) or duplicate ($^{45}Ca^{2+}$ influx) experiments. The number of determinations is shown in parentheses.

	IC_{50}			
	Fura-2 method		$^{45}Ca^{2+}$ influx	
	fMLP	A23187	fMLP	A23187
	μM			
DCDPC	>100 (4)	>100 (3)	NT[a]	NT
Flufenamic acid	13 ± 1 (4)	18 ± 3 (6)	29 ± 3 (4)	14 ± 3 (5)
Ketoprofen	NE[b] (4)	NE[b] (3)	>100 (4)	NE[b] (4)
Nifedipine	NE[c] (3)	NE[c] (3)	NT	NT
SK&F 96365	8 ± 1 (4)	7 ± 1 (5)	20 ± 4 (4)	3 ± 1 (4)
Tolfenamic acid	11 ± 2 (4)	22 ± 6 (5)	24 ± 9 (4)	17 ± 3 (4)

[a] NT, not tested.
[b] NE, no effect (highest concentration tested, 100 μM).
[c] NE, no effect (highest concentration tested, 10 μM).

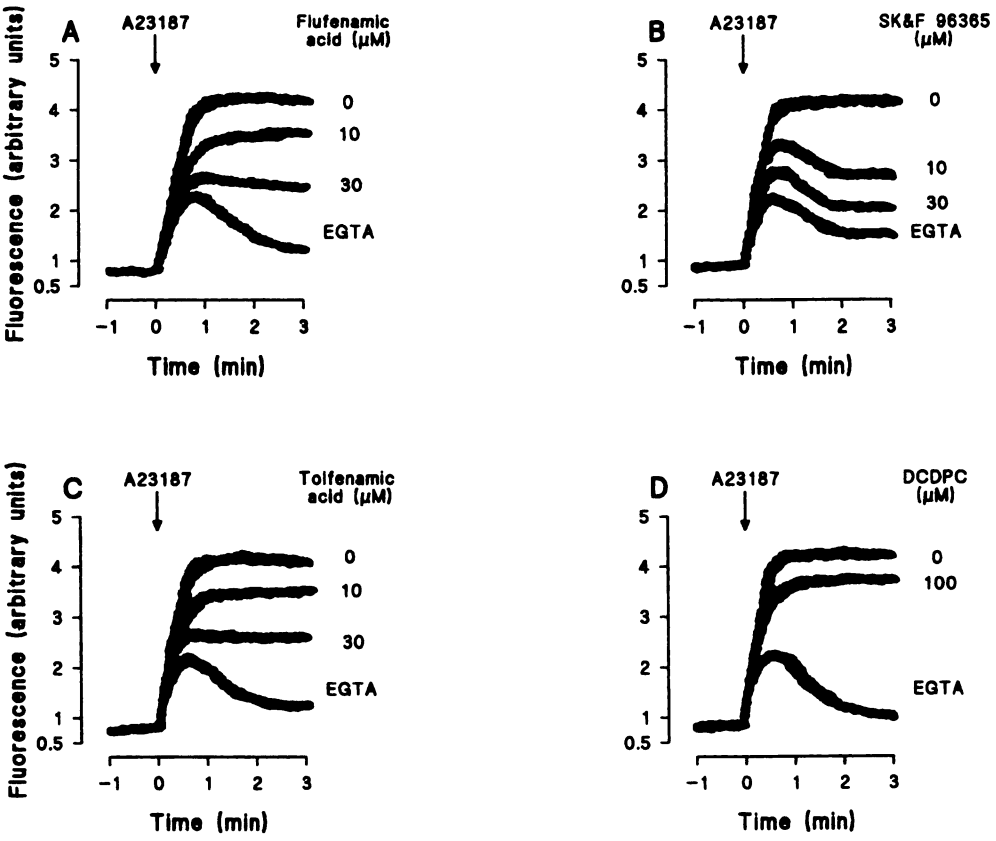

Fig. 3. Effects of flufenamic acid (A), SK&F 96365 (B), tolfenamic acid (C), and DCDPC (D) on A23187 (100 nM)-induced Ca^{2+} influx. Fura-2-loaded PMNs were suspended in DPBS. PMNs were incubated with the drug for 5 min before addition of the stimulus. A23187 was added as indicated. EGTA (2 mM) was used to assess the release of Ca^{2+} from intracellular stores. Traces are superimposed for clarity. Typical traces from at least three similar experiments are shown. For additional details, see Experimental Procedures.

96365, an earlier known organic NSC antagonist (Table 2). In contrast, DCDPC (100 μM) (Fig. 2), ketoprofen (100 μM), and nifedipine (10 μM) did not significantly affect Ca^{2+} release (data not shown) or influx in PMNs (Table 1).

To study whether the aforementioned inhibition of Ca^{2+} influx was dependent on receptor-mediated activation of the PMN, the calcium ionophore A23187 was used as a stimulant. In calcium-containing medium, A23187 (100 nM) evoked a fast and long-lasting increase in $[Ca^{2+}]_i$, which was mainly due to influx of extracellular Ca^{2+} and was diminished in the presence of EGTA (Fig. 3). Flufenamic acid, tolfenamic acid, and SK&F 96365 inhibited A23187-induced influx of Ca^{2+} in a concentration-dependent manner (Fig. 3; Table 1). These compounds did not inhibit the release of Ca^{2+} from intracellular stores, as measured in the presence of EGTA (data not shown). Again, DCDPC (100 μM) (Fig. 3), ketoprofen (100 μM), and nifedipine (10 μM) were not effective in inhibiting Ca^{2+} influx (Table 1).

To confirm that flufenamic and tolfenamic acids inhibited divalent cation influx, their effects on fMLP-induced Mn^{2+} influx in PMNs were studied. In contrast to Ca^{2+}, Mn^{2+} quenches fura-2 fluorescence and this quenching is not affected by simultanous changes in $[Ca^{2+}]_i$ when fura-2 fluorescence is measured using excitation and emission wavelengths of 360 nm and 500 nm, respectively. Fluorescence at 340 nm was simultaneously recorded to confirm the accurate

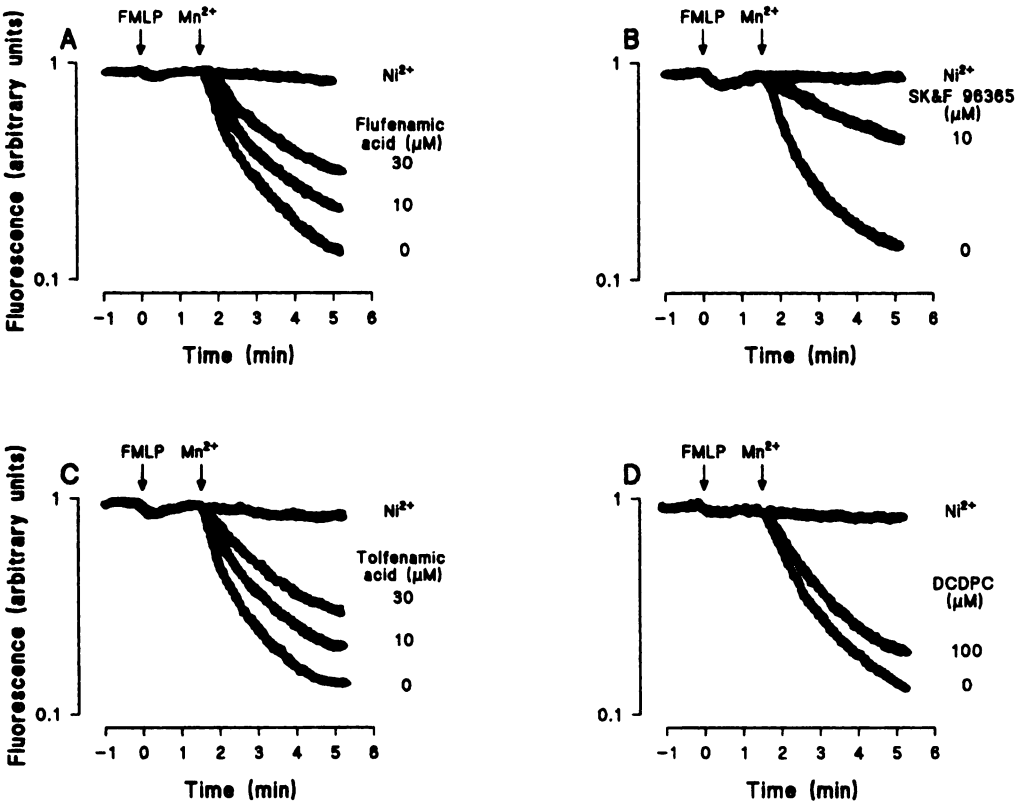

Fig. 4. Effects of flufenamic acid (A), SK&F 96365 (B), tolfenamic acid (C), and DCDPC (D) on fMLP (100 nM)-induced Mn^{2+} influx. Fura-2-loaded PMNs were suspended in PBS. PMNs were incubated with the drug for 5 min before addition of the stimulus. fMLP and Mn^{2+} (100 μM) were added as indicated. Traces are superimposed for clarity. Typical traces from at least three similar experiments, at excitation and emission wavelengths of 360 and 500 nm, respectively, are shown. For additional details, see Experimental Procedures.

release of Ca^{2+} from intracellular stores, to exclude the possibility that inhibition of Mn^{2+} influx was due to effects on intracellular Ca^{2+} metabolism. However, this was not the case (data not shown). Flufenamic acid, tolfenamic acid, and SK&F 96365 dose-dependently inhibited fMLP-induced Mn^{2+} influx (Fig. 4). Ni^{2+} (5 mM) completely blocked the Mn^{2+} influx (Fig. 4). DCDPC (100 μM) (Fig. 4), ketoprofen (100 μM) (data not shown), and nifedipine (10 μM) (data not shown) were ineffective in inhibiting Mn^{2+} influx.

The fura-2 fluorescence method contains some possible hazards and thus measurement of $^{45}Ca^{2+}$ influx was used to confirm that flufenamic acid, tolfenamic acid, and the reference compound SK&F 96365 were able to inhibit Ca^{2+} influx into PMNs. The fMLP-induced $^{45}Ca^{2+}$ influx was measured in the presence of cytochalasin B, which amplifies the response to a more reproducible level, as shown earlier by Naccache *et al.* (24). fMLP and A23187 induced an influx of $^{45}Ca^{2+}$ corresponding to 7,540 ± 1,500 and 25,700 ± 3,200 cpm/10^7 PMNs (16 experiments), respectively, whereas the basal uptake of $^{45}Ca^{2+}$ in the same experiments was 794 ± 88 cpm/10^7 PMNs (16 experiments).

The results (Fig. 5; Table 2) showed that flufenamic acid, tolfenamic acid, and SK&F 96365 all inhibited, in a concentration-dependent manner, both fMLP- and A23187-induced $^{45}Ca^{2+}$ influx into the PMNs, whereas ketoprofen was ineffective. These results are well in keeping with those achieved by using fura-2-labeled PMNs and give additional proof of the inhibitory action of fenamates on Ca^{2+} influx in human PMNs.

Discussion

Prostanoid-independent anti-inflammatory actions of NSAIDs have achieved increased attention (1–4). Fenamates seem to differ from other NSAIDs in that they inhibit several PMN functions relevant in inflammation (5–12). Recently, we found that flufenamic and tolfenamic acids inhibit PMN degranulation similarly to SK&F 96365, an experimental blocker of NSCs, but differently from Ro 31–8220, a specific inhibitor of protein kinase C (14). Furthermore, in our preliminary experiments tolfenamic acid was found to affect the chemotactic peptide-induced increase in $[Ca^{2+}]_i$.[1] This prompted us to study further the mechanism of action of fenamates. The results show that the two fenamates studied (flufenamic and tolfenamic acids), but not another cyclooxygenase inhibitor, ketoprofen, inhibit chemoattractant- and calcium ionophore-induced Ca^{2+} influx in human PMNs at micromolar drug concentrations.

Upon receptor-mediated activation of the PMN, the receptor-agonist complex is coupled to G proteins, which leads to the activation of phosphatidylinositol-specific phospholipase C. Phospholipase C induces the degradation of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate and diacylglycerol. Inositol-1,4,5-trisphosphate induces a release of Ca^{2+} from intracellular stores, and diacylglycerol activates protein kinase C. Upon activation of PMN by a receptor-mediated stimulus, phosphatidylcholine-specific phospholipase D is also activated. These complex events together lead to the cellular responses of the PMN in an inflammatory focus (for review, see Ref. 25).

The increase of $[Ca^{2+}]_i$ in activated PMNs is the sum of Ca^{2+} released from intracellular stores and Ca^{2+} influx from the extracellular space. Human PMNs do not have voltage-operated calcium channels but do possess NSCs (26, 27). This is also evidenced by our result showing that SK&F 96365 but not nifedipine inhibited fMLP- and A23187-induced increases in $[Ca^{2+}]_i$ in human PMNs. The concentration of

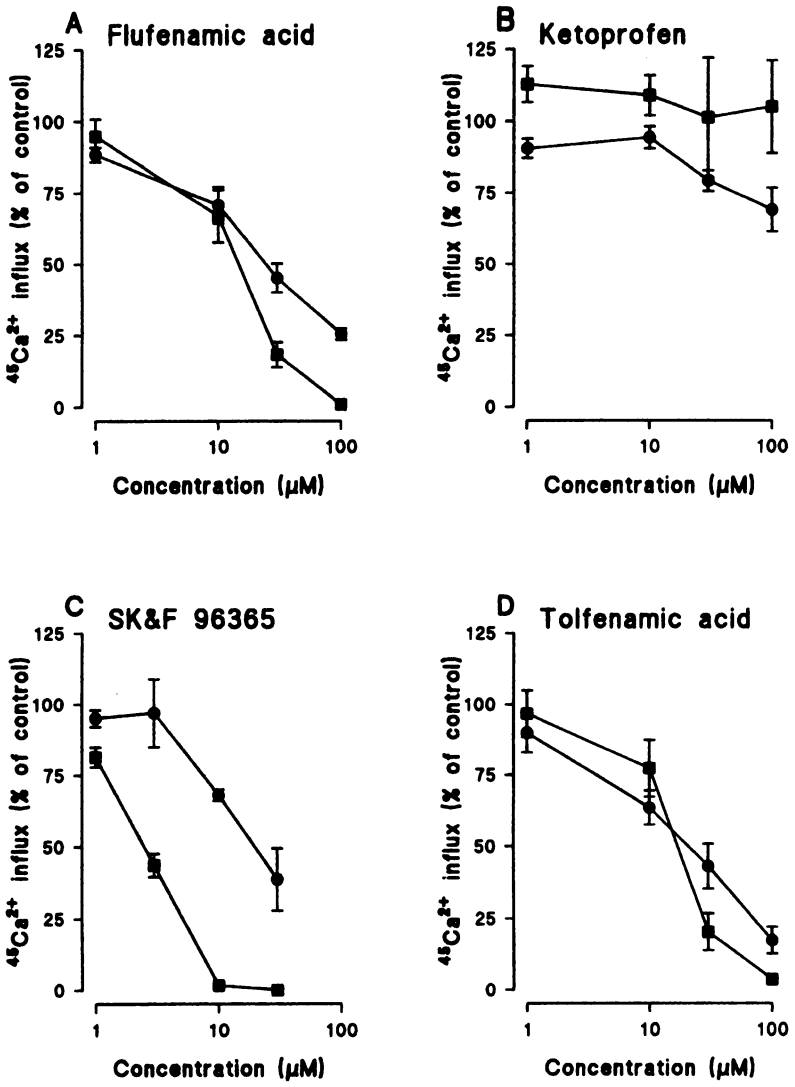

Fig. 5. Effects of flufenamic acid (A), ketoprofen (B), SK&F 96365 (C), and tolfenamic acid (D) on $^{45}Ca^{2+}$ influx induced by fMLP (100 nM) (●) or A23187 (100 nM) (■). fMLP-induced influx was measured in the presence of cytochalasin B (10 μM). PMNs were suspended in DPBS and incubated with the drug for 5 min before $^{45}Ca^{2+}$ and the appropriate stimulus were added. Incubations (10 min at 37°) were stopped by addition of EGTA (10 mM). The data represent the means ± standard errors of duplicate experiments with cells from four different donors. For IC_{50} values see Table 2, and for additional details see Experimental Procedures.

nifedipine used was chosen to be several orders of magnitude higher than that known to suppress voltage-dependent channel functions in myocardium and several other cell types (18). However, it did not affect $[Ca^{2+}]_i$ in PMNs. The open/closed state of NSCs in PMNs seems to be regulated by the filling state of the intracellular calcium stores (28), possibly through a secondary mediator (29). The most important physiological ions transported through NSCs are Ca^{2+}, Na^+, and K^+. As a surrogate for Ca^{2+}, Mn^{2+} has been used in several studies as a divalent cation able to enter the cells through NSCs (23). In this study, the pharmacological control of Ca^{2+} release and influx was studied in chemotactic peptide- and calcium ionophore-activated PMNs by using a technique to separate Ca^{2+} release and influx and by using Mn^{2+} as a surrogate. The results were confirmed by measuring influx of radioactive calcium.

Our results clearly show that the two fenamates, i.e., flufenamic and tolfenamic acids, were able to inhibit Ca^{2+} influx induced by fMLP or A23187, whereas intracellular release was unaffected. The mode of action of the two fenamates was found to mimic that of an experimental blocker of NSCs, SK&F 96365 (13) (for review, see Ref. 30). The results are in accordance with previous reports that flufenamic acid blocks the NSCs in rat exocrine pancreas (15), mouse fibroblasts (16), rat isolated myocardium (31), and *Xenopus* oocytes (32). The effects of fenamates on Ca^{2+} metabolism may be complicated by the finding that flufenamic acid itself releases Ca^{2+} from intracellular stores in a neonatal mouse mandibular cell line (33). DCDPC is structurally related to fenamates and has been reported to block NSCs in rat exocrine pancreas (17). In the present work DCDPC had only a slight inhibitory action on divalent cation influx in human PMNs. This suggests that the potency of the fenamate compounds to block NSCs varies according to the cell type studied. Furthermore, in both PMNs and exocrine gland cells NSCs with different conductances and permeabilities have been described (for reviews, see Refs. 26, 27, and 34).

To bypass the receptor-mediated increase in $[Ca^{2+}]_i$, the calcium ionophore A23187 was used. The current concept regarding the mode of action of A23187 is that it transports Ca^{2+} across the membrane down its concentration gradient (35), but the detailed mechanism of this action remains unknown. Recently, another calcium ionophore, ionomycin, has been reported to enhance Ca^{2+} influx by stimulation of store-regulated cation entry but not by a direct action at the plasma membrane in endothelial cells (36). It is tempting to speculate that A23187 could activate a similar store-operated calcium influx in PMNs, but currently there is no evidence to

prove that. The present finding that chelation of extracellular calcium by EGTA did not completely inhibit A23187-induced increases in $[Ca^{2+}]_i$ is consistent with this idea. Fenamates and SK&F 96365 did not affect A23187-induced increases in $[Ca^{2+}]_i$ in the presence of EGTA but significantly reduced the rise in $[Ca^{2+}]_i$ in Ca^{2+}-containing buffer. This suggests that these three compounds inhibit A23187-induced Ca^{2+} influx rather than Ca^{2+} release from cellular stores. These results also argue against a chemical interaction between A23187 and fenamates as an explanation for the action of these drugs. In conclusion, the two fenamates as well as SK&F 96365 inhibited both receptor-mediated and calcium ionophore A23187-induced Ca^{2+} influx. At the moment it is not known whether these effects could be explained by a common mechanism involving Ca^{2+} transport.

PMN functions that are relevant in inflammation include migration, phagocytosis, degranulation, superoxide anion production, and release of lipid class inflammatory mediators. All of these functions are at least partly dependent on the rise in $[Ca^{2+}]_i$, and they can be inhibited by removing extracellular calcium, blocking its influx, preventing the release of Ca^{2+} from intracellular stores, or antagonizing calmodulin or Ca^{2+}/calmodulin-dependent protein kinase (25, 35, 37–40). SK&F 96365, an earlier known inhibitor of NSCs in PMNs, has been reported to inhibit PMN adhesion, migration (13), and degranulation (14). Inorganic blockers of NSCs, such as Ni^{2+} and La^{3+} ions, also act as suppressors of activated PMN functions such as migration (39) and degranulation (14).[1] The inhibition of Ca^{2+} influx by fenamates occurred in the same concentration range and could well explain their earlier reported inhibitory effects on PMN functions, i.e., degranulation, leukotriene B_4 release, platelet-activating factor synthesis, and migration (5–12).

The main mode of action of NSAIDs is inhibition of prostanoid synthesis. To determine whether inhibition of Ca^{2+} influx could be linked to cyclooxygenase inhibition, ketoprofen, a propionic acid-derived cyclooxygenase inhibitor, was used as a control compound. Ketoprofen did not affect Ca^{2+} influx even at drug concentrations of 100 μM, although the IC_{50} for inhibition of prostanoid synthesis is <1 μM (19). Indomethacin has been shown not to inhibit NSC activity in mouse fibroblasts, whereas flufenamic acid was effective (16). Furthermore, the IC_{50} of tolfenamic acid for prostanoid synthesis in human PMNs under experimental conditions comparable to those used in the present work was 23 nM (7). Therefore, we conclude that fenamates inhibit divalent cation influx by a prostanoid-independent mechanism.

In conclusion, the data presented in this paper show that two fenamates, flufenamic and tolfenamic acids, block divalent cation influx into PMNs. Inhibition of Ca^{2+} influx is introduced as a novel mode of action of flufenamic and tolfenamic acids in human PMNs and could explain their earlier reported inhibitory action on the functions of activated PMNs.

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