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PHARMACOLOGICAL CONTROL OF HUMAN POLYMORPHONUCLEAR LEUKOCYTE DEGRANULATION BY FENAMATES AND INHIBITORS OF RECEPTOR-MEDIATED CALCIUM ENTRY AND PROTEIN KINASE C

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Abstract—The present work was designed to study the mechanism of inhibitory action of flufenamic and tolfenamic acids on the degranulation response of human polymorphonuclear leukocytes (PMNs). We have recently shown that fenamates inhibit PMN degranulation as well as other PMN functions at micromolar drug concentrations. However, the mechanism of their action remains unknown. To clarify this mechanism, the degranulation response was induced by agents known to activate different steps in the activation cascade in PMNs: the receptor-mediated activator fMLP (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine); a calcium ionophore (A23187); an inhibitor of calcium-ATPase (thapsigargin); and an activator of protein kinase C (phorbol myristate acetate, PMA). For comparison, SK&F 96365 (an inhibitor of receptor-mediated calcium entry), Ro 31-8220 (an inhibitor of protein kinase C) and ketoprofen (another cyclooxygenase inhibitor) were used. Flufenamic and tolfenamic acids inhibited A23187- and fMLP-induced degranulation in a dose-dependent manner. The thapsigargin-triggered response was reduced only slightly and that induced by PMA remained unaltered. The pattern of the inhibitory action of fenamates differed from those of Ro 31-8220 and ketoprofen. The action of fenamates resembled that of the inhibitor of receptor-mediated calcium entry, SK&F 96365, especially when A23187, fMLP or PMA were used to stimulate the cells. This prompted us to measure the effects of flufenamic and tolfenamic acids on receptor-mediated calcium entry. The two fenamates inhibited the fMLP-induced increase in intracellular free calcium in fura-2 loaded PMNs in the presence but not in the absence of extracellular calcium. The results suggest that the suppressive actions of fenamates on PMN degranulation are neither related to the activity of cyclooxygenase nor PMA-activated protein kinase C. In contrast, fenamates resemble the antagonist of receptor-mediated calcium entry, SK&F 96365, in their antagonistic action on PMN degranulation.

Key words: calcium; non-steroidal anti-inflammatory drugs; neutrophil; degranulation; Ro 31-8220; SK&F 96365

Suppression of the chemoattractant-induced activation of PMNs§ has been implicated in the mechanisms of the anti-inflammatory action of certain non-steroidal anti-inflammatory drugs (NSAIDs) at antirheumatic doses [1, 2]. We and others have recently shown that fenamates, e.g. flufenamic, meclofenamic, mefenamic and tolfenamic acids, inhibit human PMN degranulation, leukotriene B_4 release, platelet-activating factor production and migration by a so-far unknown mechanism [3–8].

The wide range of PMN functions inhibitable by fenamates led us to suggest that fenamates might inhibit some steps in the PMN activation cascade common to all these functions. To test this hypothesis, we measured the effects of flufenamic and tolfenamic acids on PMN degranulation induced by the chemoattractant fMLP (*n*-formyl-L-methionyl-L-leucyl-L-phenylalanine) and three other stimuli known to activate different steps in the intracellular second messenger cascade. These were A23187 (a calcium ionophore), thapsigargin (an inhibitor of calcium-ATPase, which increases intracellular calcium independent of inositolphosphates) [9] and PMA (an activator of protein kinase C). The effects of the fenamates were compared to ketoprofen (a chemically different cyclooxygenase inhibitor), SK&F 96365, an inhibitor of receptor-mediated calcium entry [10, 11] and Ro 31-8220, an inhibitor of protein kinase C [12, 13].

MATERIALS AND METHODS

Isolation of human PMNs. PMNs were isolated as

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§ Abbreviations: PMN, polymorphonuclear leukocytes; NSAID, non-steroidal anti-inflammatory drugs; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA, phorbol myristate acetate; SK&F 96365, 1-(β-(3-(4-methoxyphenyl) propoxy) - 4 - methoxyphenethyl) - 1*H* - imidazole hydrochloride; Ro 31-8220, 3-(1-(3-(amidinothio)propyl)-3-indolyl)-4-(1-methyl-3-indolyl)-1*H*-pyrrole-2,5-dione; DPBS, Dulbecco's phosphate buffered saline; IP_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol.

Table 1. Concentration- and time-dependent stimulatory effects of A23187, fMLP, PMA and thapsigargin on human PMN degranulation*

	Concentration† (μM or μg/mL)				
	0	0.01	0.1	0.3	1
A23187	0.7 ± 0.1	NT‡	0.9 ± 0.1	1.5 ± 0.2	3.6 ± 0.5
fMLP	0.7 ± 0.1	1.1 ± 0.1	2.4 ± 0.2	NT	3.3 ± 0.3
PMA	0.7 ± 0.1	1.2 ± 0.3	2.7 ± 0.4	NT	3.0 ± 0.5
Thapsigargin	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	NT	1.3 ± 0.2

	Time (min)					
	0.2	2	5	10	20	30
A23187 (1 μM)	0.9 ± 0.1	1.4 ± 0.1	2.3 ± 0.2	3.2 ± 0.1	NT	4.2 ± 0.3
fMLP (1 μM)	1.6 ± 0.2	2.8 ± 0.3	3.1 ± 0.4	3.2 ± 0.3	NT	3.2 ± 0.3
PMA (0.1 μg/mL)	0.6 ± 0.1	1.5 ± 0.2	1.7 ± 0.2	1.9 ± 0.1	2.4 ± 0.3	2.4 ± 0.2
Thapsigargin (1 μM)	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.2 ± 0.2

* fMLP-, PMA- and thapsigargin-induced degranulation was measured in the presence of cytochalasin B (5 μg/mL). The enzyme activity released by 10^6 PMNs is expressed as μg of phenolphthalein formed from phenolphthalein-β-(D)-glucuronide. Mean ± SEM, four to six duplicate experiments.

† μM in the case of A23187, FMLP and thapsigargin and μg/mL in the case of PMA.

‡ NT, not tested.

follows: blood was collected by venipuncture from healthy volunteers who had abstained from any drugs for at least one week before sampling. A buffy-coat preparation of citrated blood was layered on Ficoll-Paque and centrifuged according to Bøyum [14]. 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. After the isolation procedure the viability of the cells was > 97% as determined by trypan blue exclusion, and the PMN suspension contained less than 2% mononuclear leukocytes.

Degranulation in isolated PMNs. The cell suspensions (10×10^6 PMNs/mL of DPBS) were first incubated for 10 min at 37° with either flufenamic acid, tolfenamic acid, ketoprofen, Ro 31-8220 or SK&F 96365. Thereafter the cells were activated by calcium ionophore A23187 (1 μM, 10 min), chemotactic peptide fMLP (1 μM, 10 min), PMA (0.1 μg/mL, 20 min) or thapsigargin (1 μM, 20 min). fMLP-, PMA- and thapsigargin-induced degranulations were measured in the presence of cytochalasin B (5 μg/mL). The incubation conditions were chosen to be optimal on the basis of the concentration- and time–response curves shown in Table 1. Under these incubation conditions, the β-glucuronidase (EC 3.2.1.31) release was 15–30% of the total β-glucuronidase activity released from Triton X-100-treated cells. After the indicated time of incubation, the cells were spun down (10 000 *g*, 30 sec, 20°) and the cell-free supernatant carefully removed. β-Glucuronidase activity in the supernatant was assayed spectrophotometrically by measuring the formation of phenolphthalein from phenolphthalein-β-(D)-glucuronide substrate [15, 16]. The enzyme activity released from 1×10^6 PMNs is calculated on the basis of a phenolphthalein standard curve and is expressed as μg phenolphthalein formed from the substrate. The direct effects of the compounds at the highest drug concentration were tested on the enzyme activity in β-glucuronidase-containing samples of Triton X-100 lysed cells and were found to be negligible. At the concentrations used none of the compounds affected cell viability as assessed by trypan blue exclusion.

Determination of the intracellular free calcium ($[Ca^{2+}]_i$). Isolated PMNs (40×10^6/mL DPBS) were loaded with the acetoxymethyl ester of the fluorescent probe fura-2 (5 μM) for 30 min at 37° in a shaking waterbath. PMNs were washed twice and suspended in DPBS to obtain a cell suspension containing 5×10^6 PMNs/mL of buffer. The changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) in thermostated (+37°) quartz cuvettes with continuous stirring. The excitation wavelengths were set at 340 nm, 380 nm and emission at 500 nm. The increases in $[Ca^{2+}]_i$ were stimulated with fMLP (0.1 μM). EGTA (2 mM) was used to chelate the extracellular calcium. Use of EGTA allowed measurement of calcium release from intracellular stores only. The cells were incubated for 10 min with fenamates before stimulation. Calibration of the signal was performed according to Grynkiewicz *et al.* [17]. The maximal fluorescence (F_{max}) was measured in the presence of 25 mM EGTA (pH 8.6) and 0.1% Triton X-100. $[Ca^{2+}]_i$ was calculated from the equation: $[Ca^{2+}]_i$ (nM) = $R \times 224 \times (F - F_{min})/(F_{max} - F)$, where 224 represents the dissociation constant for fura-2, F is the fluorescence of intact cell suspension and R is the ratio of F_{min}/F_{max} at 380 nm. The unstimulated and stimulated $[Ca^{2+}]_i$ levels were 79 ± 11 and 302 ± 10 nM (mean ± SEM of 10 experiments), respectively.

Materials. A23187, cytochalasin B, EGTA,

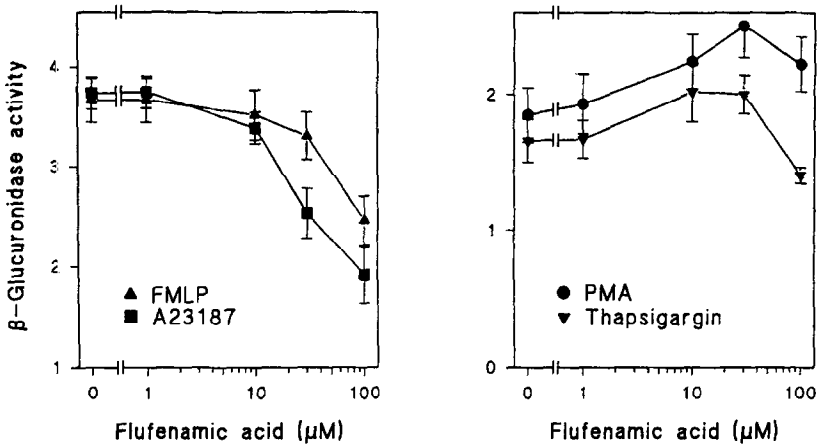

Fig. 1. The concentration–response curves of the effects of flufenamic acid on human PMN degranulation induced by A23187 (1 μM, 10 min), fMLP (1 μM, 10 min), PMA (100 ng/mL, 20 min) and thapsigargin (1 μM, 20 min). β-Glucuronidase release was measured as a marker of degranulation. The activity of the β-glucuronidase released from 10^6 PMNs is expressed as μg of phenolphthalein formed from phenolphthalein-β-(D)-glucuronide as calculated on the basis of a phenolphthalein standard curve. Mean ± SEM, five to six duplicate experiments.

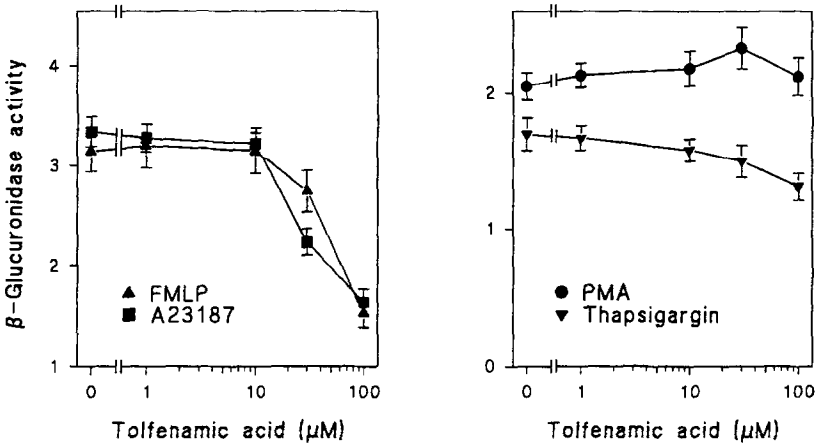

Fig. 2. The concentration–response curves of the effects of tolfenamic acid on human PMN degranulation induced by A23187 (1 μM, 10 min), fMLP (1 μM, 10 min), PMA (100 ng/mL, 20 min) and thapsigargin (1 μM, 20 min). β-Glucuronidase release is expressed as in Fig. 1. Mean ± SEM, six to 10 duplicate experiments.

flufenamic acid, fMLP, fura-2, ketoprofen, phenolphthalein-β-(D)-glucuronide, PMA, thapsigargin and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Tolfenamic acid was obtained from Gea Ltd (Copenhagen, Denmark). Ro 31-8220 and SK&F 96365 were generous gifts from Dr T.J. Hallam (Roche Products Ltd, Hertfordshire, U.K.) and SmithKline Beecham Pharmaceuticals (Surrey, U.K.), respectively. Ficoll-Paque was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden).

Statistics. Means and SEMs were calculated. The drug concentrations causing a 30% inhibition of the tested parameter (IC_{30}) were estimated on the basis of a semilogarithmic dose–response curve in each experiment.

RESULTS

Effects of flufenamic and tolfenamic acids, ketoprofen, SK&F 96365 and Ro 31-8220 on PMN degranulation

Flufenamic and tolfenamic acids (Figs 1 and 2) inhibited A23187- and fMLP-induced degranulation in a concentration-dependent manner. A23187-triggered degranulation was inhibited at somewhat lower drug concentrations (IC_{30} values 25 and 25 μM for flufenamic and tolfenamic acids, respectively) than fMLP-induced β-glucuronidase release (IC_{30} values 67 and 40 μM, respectively). At 100 μM drug concentrations flufenamic and tolfenamic acids also reduced thapsigargin-triggered degranulation slightly (by 18 and 33%, respectively). Neither of the fenamates inhibited PMA-induced degranulation.

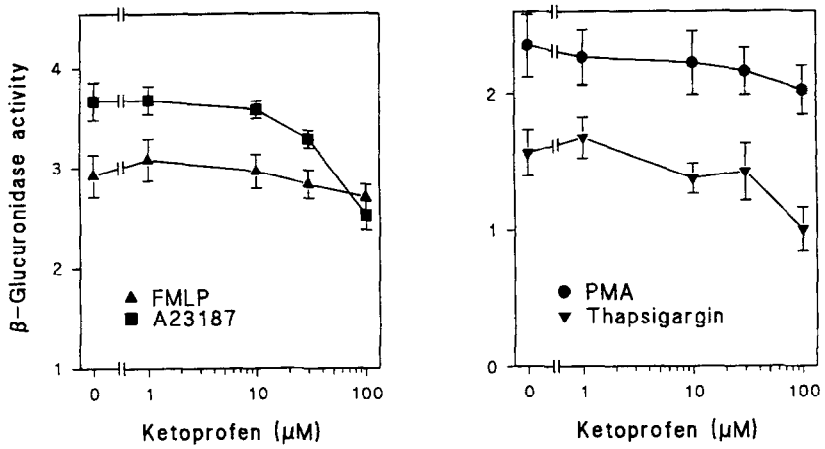

Fig. 3. The concentration–response curves of the effects of ketoprofen on human PMN degranulation induced by A23187 (1 μM, 10 min), fMLP (1 μM, 10 min), PMA (100 ng/mL, 20 min) and thapsigargin (1 μM, 20 min). β-Glucuronidase release is expressed as in Fig. 1. Mean ± SEM, six duplicate experiments.

A chemically different cyclooxygenase inhibitor, ketoprofen, suppressed A23187- and thapsigargin-induced degranulation (IC_{30} values 67 and 48 μM, respectively). fMLP- or PMA-stimulated response was not affected (Fig. 3). Fenamates were more potent inhibitors of A23187- or fMLP-induced degranulation than ketoprofen, whereas ketoprofen suppressed thapsigargin-induced degranulation more effectively.

SK&F 96365, an inhibitor of receptor-mediated calcium entry, inhibited PMN degranulation induced by A23187 or thapsigargin (IC_{30} values 3 and 3 μM, respectively) (Fig. 4). fMLP-induced degranulation was reduced less potently (IC_{30} 23 μM). SK&F 96365 inhibited PMA-induced degranulation only slightly.

An inhibitor of protein kinase C, Ro 31-8220, effectively antagonized the PMA-induced degranulation response (IC_{50} 0.5 μM) (Fig. 5). fMLP-stimulated β-glucuronidase release was also reduced at higher concentrations of Ro 31-8220 (IC_{30} 7 μM). Neither A23187- nor thapsigargin-induced degranulation was affected. These results suggest that the inhibitory action of the two fenamates on PMN degranulation resembles that of SK&F 96365, especially when the response was induced by fMLP, A23187 or PMA. This prompted us to measure the effects of flufenamic and tolfenamic acids on receptor-mediated calcium entry.

Effects of flufenamic and tolfenamic acids on receptor-mediated calcium entry

Both flufenamic and tolfenamic acids inhibited fMLP-induced increase in $[Ca^{2+}]_i$ in human PMNs in calcium-containing buffer (Fig. 6). When the extracellular calcium was chelated by EGTA (2 mM), neither of the fenamates affected $[Ca^{2+}]_i$. This suggests that the two fenamates inhibit fMLP-induced calcium influx in isolated human PMNs.

Thapsigargin-induced human PMN degranulation

Thapsigargin is an experimental inhibitor of microsomal Ca^{2+}-ATPase. It increases intracellular calcium concentration, but its effects on PMN functions are mostly unknown. In the presence of cytochalasin B, thapsigargin was found to be a weak secretagogue (Table 1). To further characterize thapsigargin-induced degranulation, the effects of the calcium chelator EGTA and an inorganic cation channel blocker Ni^{2+} were studied. Both EGTA (2 mM) and Ni^{2+} (5 mM) effectively blocked thapsigargin-induced degranulation (94% and 97% inhibition, respectively). This suggests that thapsigargin-induced degranulation is dependent on the influx of extracellular calcium.

DISCUSSION

Since 1971 the inhibition of prostanoid synthesis has been conceived to be the main mode of action of NSAIDs. However, the inhibition of PMN functions by NSAIDs has gained increased attention as an additional mechanism of the anti-inflammatory action of certain NSAIDs [1, 2]. NSAIDs are not a homogenous group in this respect. The fenamates in particular seem to differ from other NSAIDs in their ability to inhibit PMN functions [3–8]. In the present study, flufenamic and tolfenamic acids were used to explore the mechanism of the inhibitory action of fenamates on PMN functions.

Four different stimuli were used to activate the degranulation response in PMNs. These included: (1) fMLP, as a receptor-mediated activator; (2) A23187, as a calcium ionophore; (3) thapsigargin, as a calcium ATP-ase inhibitor [9]; and (4) PMA, as an activator of PKC. Activation of PMNs by a receptor-mediated stimulus (fMLP) is related to coupling of the agonist–receptor complex with G-proteins and activation of phosphoinositide-specific phospholipase C. This results in the breakdown of phosphatidylinositol 4,5-bisphosphate into IP_3 and DAG. IP_3 induces an increase in $[Ca^{2+}]_i$ and DAG and phorbol esters activate protein kinase C [18, 19].

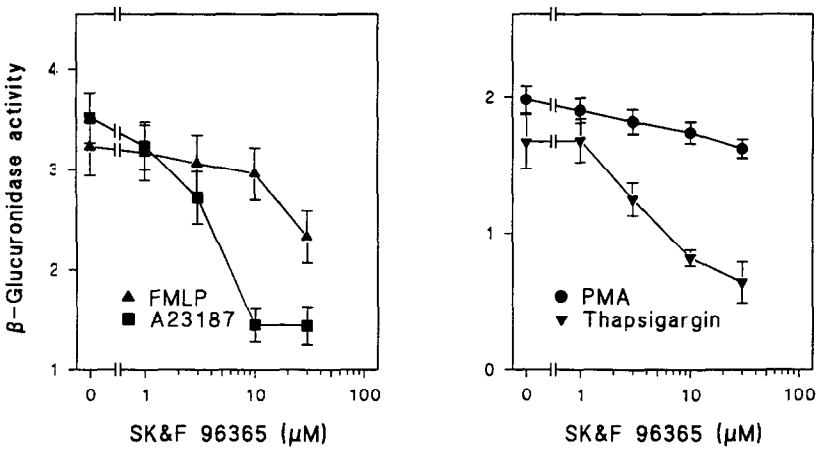

Fig. 4. The concentration–response curves of the effects of SK&F 96365 on human PMN degranulation induced by A23187 (1 μM, 10 min), fMLP (1 μM, 10 min), PMA (100 ng/mL, 20 min) and thapsigargin (1 μM, 20 min). β-Glucuronidase release is expressed as in Fig. 1. Mean ± SEM, five to six duplicate experiments.

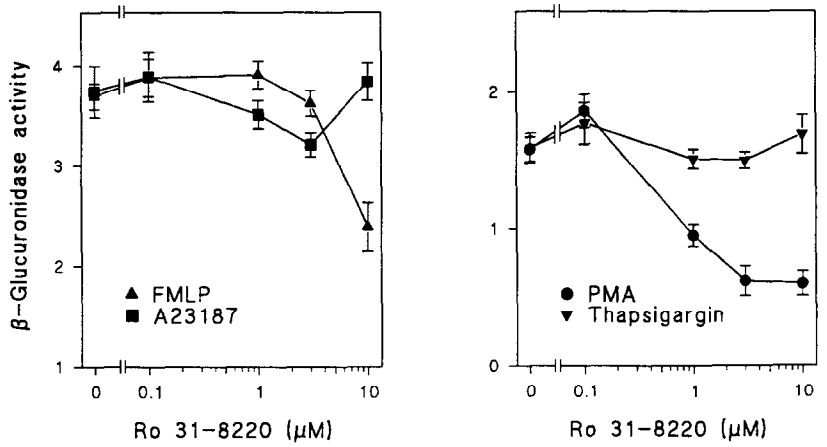

Fig. 5. The concentration–response curves of the effects of Ro 31-8220 on human PMN degranulation induced by A23187 (1 μM, 10 min), FMLP (1 μM, 10 min), PMA (100 ng/mL, 20 min) and thapsigargin (1 μM, 20 min). β-Glucuronidase release is expressed as in Fig. 1. Mean ± SEM, six duplicate experiments.

The current hypothesis on the mode of action of A23187 is that it transports Ca^{2+} across the membrane down its concentration gradient [20], but the detailed mechanism of this action remains unknown. Recently, another calcium ionophore, ionomycin, has been reported to enhance Ca^{2+} influx by stimulating store-regulated cation entry but not by a direct action at the plasma membrane in endothelial cells [21]. Both fMLP- and A23187-induced increases in $[Ca^{2+}]_i$ comprise two components, namely release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across plasma membrane [20]. PMA is a direct activator of protein kinase C but may also activate phospholipase D. It causes PMN degranulation and generation of toxic oxygen radicals without a concomitant increase in $[Ca^{2+}]_i$ [18–20].

Thapsigargin inhibits microsomal Ca^{2+}-ATPase and induces a slow but sustained rise in $[Ca^{2+}]_i$ [22]. In the present work, thapsigargin was used to increase $[Ca^{2+}]_i$ without concomitant receptor activation or ionophore use. Thapsigargin has been reported to induce calcium influx by emptying the intracellular calcium stores [22], but the details of its action remain unknown. We tested whether thapsigargin is a secretagogue in human PMNs. It was found to induce a weak but measurable secretory response of azurophilic granules in the presence of cytochalasin B. Thapsigargin-induced degranulation was inhibited by calcium chelator EGTA or the inorganic cation channel blocker Ni^{2+}. An inhibitor of receptor-mediated calcium entry, SK&F 96365, was a potent inhibitor of thapsigargin-induced

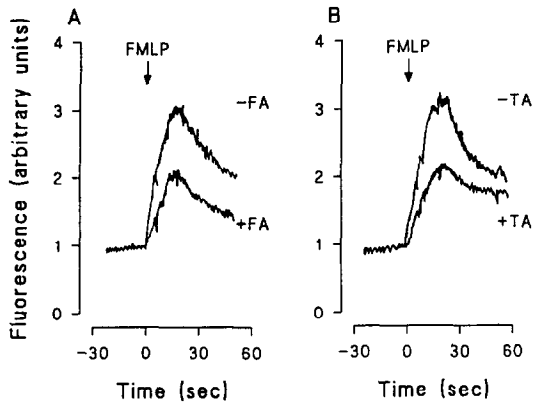

Fig. 6. The effects of (A) flufenamic (FA) and (B) tolfenamic (TA) acids (30 μM) on the fMLP (0.1 μM) -induced increase in $[Ca^{2+}]_i$ in fura-2 loaded human PMNs. The experiments were repeated five times with cells from different donors with similar results. Typical traces are shown. Traces are superimposed for clarity.

degranulation. It suppressed A23187- and thapsigargin-triggered degranulation at equimolar drug concentrations. The fMLP-induced response was inhibited only at higher concentrations of SK&F 96365. Flufenamic and tolfenamic acids also suppressed thapsigargin-induced degranulation. The two fenamates inhibited A23187- and fMLP-triggered β-glucuronidase release at lower drug concentrations than the thapsigargin-induced response. The above results suggest that the degranulation response induced by thapsigargin is dependent on the influx of extracellular calcium but utilizes different mechanisms than A23187 and fMLP.

Human PMNs possess receptor-mediated calcium channels [23] or non-selective cation channels [24], which are opened due to the emptying of intracellular calcium stores [25]. SK&F 96365 is a potent inhibitor of receptor-mediated calcium entry in human PMNs and platelets [10] as well as in HL-60 cells [26]. SK&F 96365 inhibits human PMN adhesion and chemotaxis [10] and histamine-elicited secretory response in HL-60 cells [26]. The present results show that SK&F 96365 also inhibits human PMN degranulation induced by fMLP, A23187 or thapsigargin. The concentrations needed for half-maximal inhibition of the degranulation triggered by the two latter stimuli correspond very well to those reported to inhibit calcium influx [10]. However, fMLP-induced degranulation was markedly inhibited only at a 30 μM drug concentration, which has been reported to block calcium influx completely [10]. At drug concentrations of 30 μM or higher, SK&F 96365 also inhibits the release of intracellular calcium [10]. Smolen *et al.* [27] showed that fMLP-induced degranulation is only slightly inhibited in the absence of extracellular calcium. This suggests that SK&F 96365 inhibits fMLP-induced degranulation both by blocking Ca^{2+} influx and at higher drug concentrations by inhibiting the release of intracellular Ca^{2+}.

Ro 31-8220 is an inhibitor of protein kinase C in several tissues [12, 13]. It inhibits the respiratory burst [13], fluid pinocytosis and actin polymerization in PMNs [28]. We show here that Ro 31-8220 reduces phorbol ester-induced degranulation at concentrations comparable to those reported to inhibit superoxide anion production in PMNs [13]. fMLP-induced β-glucuronidase release was inhibited only at 10-fold higher concentrations. At concentrations above 10 μM Ro 31-8220 also inhibits Ca^{2+}/calmodulin-dependent kinase [12], which may be implicated in its effect on fMLP-induced degranulation.

At micromolar drug concentrations, flufenamic and tolfenamic acids reduced degranulation induced by fMLP, A23187 and thapsigargin but not that by PMA. A23187-induced degranulation was inhibited more effectively than that induced by fMLP. Thapsigargin-triggered response was reduced at highest drug concentrations. The pattern of the action fenamates differed from that of ketoprofen, suggesting a mechanism different from the inhibition of prostanoid synthesis. The inhibitory effects of fenamates on PMN degranulation resembled those of an antagonist of receptor-mediated calcium entry, SK&F 96365. This encouraged us to measure the effects of these two fenamates on fMLP-induced increase in $[Ca^{2+}]_i$. They inhibited fMLP-triggered rise in $[Ca^{2+}]_i$ in calcium-containing buffer but not in the presence of EGTA. EGTA chelates extracellular calcium and thus only the release of intracellular Ca^{2+} but the influx of extracellular Ca^{2+} does not occur in its presence. This suggests that flufenamic and tolfenamic acids inhibit fMLP-induced calcium influx. The fenamates were, however, less potent inhibitors of thapsigargin-induced degranulation than SK&F 96365, which may indicate a certain heterogeneity in calcium influx into PMNs. Our results are well in keeping with the recent finding that certain fenamates inhibit nonselective cation channels in the rat exocrine pancreas at concentrations comparable to those shown here to inhibit degranulation [29]. In addition, another fenamate, meclofenamic acid, has been reported to abolish the fMLP-induced $[Ca^{2+}]_i$ increase in PMNs [8]. Taken together, these data suggest that the inhibitory effects of flufenamic acid and tolfenamic acid on PMN degranulation could be partly due to inhibition of calcium influx. The clinical significance of the ability of fenamates to inhibit PMN functions has recently been discussed [5, 6].

In conclusion, the present results suggest that the suppressive actions of flufenamic and tolfenamic acids on PMN degranulation resemble that of SK&F 96365 (an antagonist of receptor-mediated calcium entry). Fenamates inhibit PMN degranulation in a different manner than Ro 31-8220 (an inhibitor of protein kinase C) and ketoprofen (a chemically different inhibitor of cyclooxygenase).

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