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Inhibition by fenamates of calcium influx and proliferation of human lymphocytes

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1 Flufenamic and tolfenamic acids have recently been shown to inhibit receptor-mediated calcium influx in human neutrophils. The present work was designed to study the effects of these two nonsteroidal anti-inflammatory drugs on human peripheral blood lymphocyte activation.

2 Peripheral blood mononuclear cells (PBMNCs; containing 90% lymphocytes) were stimulated by mitogen concanavalin A (Con A) or by a combination of an inhibitor of microsomal Ca^{2+}-adenosine triphosphatase thapsigargin (TG) and phorbol myristate acetate (PMA). The effects of the two fenamates on cell proliferation were compared with respective changes in calcium metabolism.

3 Flufenamic and tolfenamic acids (10-100 μM) inhibited both Con A and TG+PMA-induced [^{3}H]-thymidine incorporation in a dose-dependent manner. At the same concentration range, the two fenamates inhibited the increase in intracellular free calcium concentration induced by Con A or TG+PMA. This effect was due to inhibition of calcium influx whereas calcium release from intracellular stores remained unaltered.

4 The inhibition of divalent cation influx was confirmed by showing that fenamates inhibited TG+PMA-induced Mn^{2+} influx.

5 The inhibitory effects of fenamates on PBMNC proliferation and Ca^{2+} influx were qualitatively similar with those of SK&F 96365, an earlier known inhibitor of receptor-mediated calcium entry. Ketoprofen, a chemically different prostaglandin synthetase inhibitor did not show similar suppressive effects on PBMNCs.

6 The data suggest that flufenamic and tolfenamic acids suppress proliferation of human peripheral blood lymphocytes by a mechanism which involves inhibition of Ca^{2+} influx and is not related to inhibition of prostanoid synthesis.

Keywords: Calcium; cellular proliferation; flufenamic acid; mitogens; non-steroidal anti-inflammatory drugs; second messengers; lymphocytes; tolfenamic acid

Introduction

The mechanism by which mitogens induce proliferation of lymphocytes is widely studied but not yet completely understood. T cell activation in response to mitogen has been shown to be related to activation of a complex intracellular messenger cascade. Cell activation leads to hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate, yielding inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from intracellular stores and DAG activates protein kinase C. Activation of T cells by mitogens has been shown to induce a rise in cytoplasmic free calcium ($[Ca^{2+}]_i$) within minutes. The increase in $[Ca^{2+}]_i$ consists of two phases, a transient release from intracellular stores and a sustained influx across plasma membrane (Gardner, 1989; Gallin, 1991; Gallin & Grinstein, 1992; Lewis & Cahalan, 1995; Szamel & Resch, 1995). Ca^{2+} has been suggested to be critical for full cell cycle progression, since proliferation is inhibited by treatments that reduce $[Ca^{2+}]_i$ or antagonize the calcium/calmodulin cascade (Cardenas & Heitman, 1995; Whitaker, 1995). Calcium and its primary receptor protein calmodulin have been implicated in the re-entry of quiescent cells into the proliferative cycle as well as for traversing the G_1/S, G_2/M and metaphase/anaphase boundaries of the cell cycle (Lu & Means, 1993; Means, 1994). Early patch clamp studies have ruled out the existence of voltage-gated Ca^{2+} channels in the T cell membrane. Instead, non-voltage gated 'capacitative' store-operated calcium entry has been characterized. Patch clamp studies suggest that the Ca^{2+} influx flows through a highly Ca^{2+} selective, inwardly rectifying, Cd^{2+}- and Ni^{2+} sensitive channel (Gallin, 1991; Gallin & Grinstein, 1992; Lewis & Cahalan, 1995).

Inhibition of prostanoid synthesis by non-steroidal anti-inflammatory drugs (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 (Brooks & Day, 1991; Abramson, 1992). We and others have shown that fenamates differ from other NSAIDs in their ability to inhibit several neutrophil functions (Perez *et al.*, 1987; Kankaanranta *et al.*, 1994; Moilanen & Kankaanranta, 1994). Recently, we found that two fenamates, flufenamic and tolfenamic acids inhibited receptor-mediated calcium influx in human neutrophils which was associated with inhibitory action on neutrophil function (Kankaanranta *et al.*, 1995; Kankaanranta & Moilanen, 1995). Furthermore, in patch clamp studies flufenamic acid has been shown to antagonize nonselective cation channels in rat exocrine pancreas (Gögelein *et al.*, 1990) and mouse fibroblasts (Jung *et al.*, 1992). Inhibition of the nonselective cation channel in mouse fibroblasts was found to be concomitant with arrest of cell growth (Jung *et al.*, 1992). Similarly, other antagonists of nonselective cation channels or store-operated calcium entry have been shown to inhibit cell proliferation (Hupe *et al.*, 1991; Chung *et al.*, 1994; Benzaquen *et al.*, 1995).

As calcium, especially extracellular calcium and its sustained influx into the cells is considered crucial for cell proliferation, then inevitably, whether fenamates affect calcium influx in lymphocytes, they also should reduce proliferation. The aim of the present study was to test the above hypothesis. Thus we studied the effects of fenamates (flufenamic and tolfenamic acids) on human peripheral blood mononuclear cell (PBMNC) proliferation and especially whether the inhibition of proliferation is related to inhibition of Ca^{2+} influx in the same cells. For comparison we used (1) SK&F 96365, an experimental

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antagonist of receptor-mediated calcium entry as described in several cell types including lymphocytes (Merritt *et al.*, 1990; Chung *et al.*, 1994), and (2) ketoprofen, a chemically different cyclo-oxygenase inhibitor (Veyes, 1991). The present results show that the two fenamates and SK&F 96365, but not ketoprofen, inhibit human lymphocyte proliferation and Ca^{2+} influx. Inhibition of lymphocyte proliferation associated with inhibition of Ca^{2+} influx is thus presented as a new mode of action of NSAIDs with fenamate structure.

Methods

Cell isolation and proliferation assay

PBMNCs were isolated by Ficoll-Paque gradient centrifugation (Bøyum, 1976) from venous blood obtained from healthy volunteers who had abstained from any drugs for at least one week before sampling. The PBMNC suspension consisted of lymphocytes (90.0±1.7%), monocytes (7.4±1.5%) and polymorphonuclear leukocytes (2.6±0.5%) (mean±s.e.mean, $n=7$). PBMNCs were suspended in RPMI 1640 Glutamax-1 supplemented with 10% foetal bovine serum (FBS), penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and amphotericin B (250 ng ml^{-1}). PBMNCs were cultured in 96-well plates (2×10^5 cells/200 μl/well). Lymphocyte (T helper) proliferation was induced by concanavalin A (Con A; 1 $\mu g\ ml^{-1}$). Thapsigargin (TG; 100 nM) combined with phorbol myristate acetate (PMA; 1 nM) induced proliferation comparable to that described previously (Scharff *et al.*, 1988). The compounds tested were added to the incubation just before the proliferative stimulus. The cells were incubated for 2 days at 37°C (in 5% CO_2) and then pulsed for 20 h with 0.1 μCi [^{3}H]-thymidine. The cells were harvested and the incorporated radioactivity measured in a β-counter. To evaluate a direct cytotoxicity of the compounds studied, Trypan blue exclusion test was performed at the end of the incubations. At the concentrations used none of the compounds affected cell viability.

Calcium measurements

$[Ca^{2+}]_i$ concentrations and Ca^{2+} influx were measured according to Grynkiewicz *et al.* (1985) as previously described (Kankaanranta *et al.*, 1995; Kankaanranta & Moilanen, 1995). Briefly: isolated PBMNCs ($40 \times 10^6\ ml^{-1}$ in 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid] (HEPES) buffer with 1.5 mM Ca^{2+}) were loaded with the acetoxymethyl ester of the fluorescent probe fura-2 (5 μM) for 30 min at 37°C in a shaking waterbath. PBMNCs were diluted with HEPES-buffer 1:3 and kept at room temperature for 10 min to allow them to re-equilibrate. Thereafter PBMNCs were washed twice and finally suspended in HEPES-buffer to obtain a cell suspension containing 5×10^6 PBMNCs ml^{-1} of buffer. The changes in fluorescence were recorded with a Shimadzu RF-5000 spectrofluorometer (Shimadzu Corp., Kyoto, Japan) in thermostatted (37°C) quartz cuvettes with continuous stirring. The excitation wavelengths were set at 340 nm and 380 nm and emission at 500 nm when changes in $[Ca^{2+}]_i$ were measured. Fenamates were not used at concentrations higher than 60 μM due to their possible interference with fura-2 fluorescence.

The increases in $[Ca^{2+}]_i$ were stimulated either by adding Con A (50 $\mu g\ ml^{-1}$) or TG (100 nM) with PMA (1 nM). EGTA (2.5 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 from the extracellular media an 'add-back' modification was used (Andersson *et al.*, 1986; Kankaanranta & Moilanen, 1995). The cells were suspended in 10 mM HEPES-buffer supplemented with EGTA (1 mM) without Ca^{2+} and with or without the drug studied. PBMNCs were incubated for 1 min with the drug before stimulation. The cells were stimulated by adding TG+PMA, and the calcium release from intracellular stores was followed for 60 s. Thereafter Ca^{2+} (2.5 mM) was added to overcome the action of EGTA and to obtain extracellular free calcium concentration of about 1.5 mM. This resulted in an influx of extracellular Ca^{2+} into activated PBMNCs. After the addition of Ca^{2+} into the incubate, that proportion of the fluorescence which was reversed in the presence of an inorganic blocker of calcium entry, Ni^{2+} (5 mM) was interpreted to represent Ca^{2+} influx.

Calibration of the signal was performed basically according to the method described by Grynkiewicz *et al.* (1985). The maximal fluorescence (F_{max}) was measured after adding 2 μM ionomycin and the minimum fluorescence (f_{min}) in the presence of 25 mM EGTA (pH 8.6) and 0.1% Triton X-100. The intracellular free calcium concentrations were calculated from the equation: $[Ca^{2+}]_i$ (nM) $= R^*224^*(F-F_{min})/(F_{max}-F)$, 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_{max}/F_{min} at 380 nm.

The resting $[Ca^{2+}]_i$ of PBMNCs suspended in HEPES-buffer was 138±13 nM ($n=15$). After stimulation with Con A (50 $\mu g\ ml^{-1}$) and TG+PMA the maximal $[Ca^{2+}]_i$s were 329±57 nM ($n=4$) and 860±110 nM ($n=11$), respectively.

Measurement of Mn^{2+} influx

Measurement of Mn^{2+} influx was performed as previously described (Kankaanranta *et al.*, 1995; Kankaanranta & Moilanen, 1995). PBMNCs were loaded with fura-2/AM (1 μM) for 30 min at 37°C as above. The cells were finally suspended in Ca^{2+}-free HEPES-buffer (5×10^6 cells ml^{-1}). The excitation wavelengths were set at 340 and 360 nm and emission at 500 nm. At an excitation wavelength of 340 nm, fura-2 fluorescence increases with increasing $[Ca^{2+}]_i$, while 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 60 s after stimulation of the cells with thapsigargin and PMA. Ni^{2+} (5 mM) was used as a control compound known to block calcium channels and thus both Ca^{2+} and Mn^{2+} influx induced by thapsigargin in PBMNCs.

Materials

EGTA, flufenamic acid, fura-2/AM, HEPES, ionomycin, ketoprofen, PMA, thapsigargin and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO). Tolfenamic acid was obtained from GEA Ltd., Copenhagen, Denmark. SK&F 96365 (1-(β-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl)-1H-imidazole hydrochloride, was a generous gift from SmithKline Beecham Pharmaceuticals, Surrey, U.K.. Other reagents were obtained as indicated: Con A and Ficoll-Paque (Pharmacia AB, Uppsala, Sweden), $MnCl_2$ (Merck, Darmstadt, Germany), $NiCl_2$ (J.T. Baker, Deventer, Holland), RPMI 1640 with Glutamax-I and FBS (Gibco BRL, Paisley, U.K.), and [methyl-^{3}H]-thymidine (Amersham International plc, Buckinghamshire, U.K.).

Statistical analysis

Results are expressed as mean±s.e.mean. The drug concentration causing 50% inhibition (IC_{50}) was estimated in each experiment on the basis of a semi-logarithmic dose-response curve. Statistical significance was calculated by analysis of variance for repeated measures supported by Dunnett's multiple comparisons test. Differences were considered significant when $P<0.05$.

Results

Effects of fenamates on Con A-induced PBMNC proliferation and intracellular calcium concentration

Flufenamic and tolfenamic acids inhibited Con A (1 $\mu g\ ml^{-1}$)-induced human PBMNC proliferation (IC_{50} values 48±11 and

52±8 μM, respectively) (Figure 1). Ketoprofen, a non-fenamate non-steroidal antiinflammatory drug (NSAID), reduced proliferation approximately by 20% at 30 and 100 μM drug concentrations. As proteins are known to bind NSAIDs effectively, we studied the effects of the abovementioned NSAIDs in serum-free system. The absence of FBS did not significantly alter Con A (1 μg ml^{-1})-induced proliferation (1995±585 and 1823±346 c.p.m. in the presence and absence of FBS, respectively). In serum free media both fenamates were more potent inhibitors of PBMNC proliferation (IC_{50} values 12±4 and 11±2 μM for flufenamic and tolfenamic acids, respectively) than in a medium supplemented with 10% FBS. Ketoprofen (up to 30 μM) was not able to reduce PBMNC proliferation in FBS-free medium (Figure 1).

To evaluate the mechanism behind the inhibitory action of fenamates on lymphocyte proliferation, we studied the effects of fenamates on Con A (50 μg ml^{-1}) induced increases in $[Ca^{2+}]_i$ in isolated PBMNCs. Flufenamic and tolfenamic acids (30 μM) significantly reduced Con A-induced increase in $[Ca^{2+}]_i$ (Figure 2). Ketoprofen, at high drug concentrations (100 μM) reduced the increase in $[Ca^{2+}]_i$ marginally.

Effects of fenamates on thapsigargin-induced PBMNC proliferation and $[Ca^{2+}]_i$

We employed thapsigargin, an inhibitor of sarcoplasmic Ca^{2+}-adenosine triphosphatase (Ca^{2+}-ATPase), which induces store-operated calcium influx similar, if not identical, to that induced by receptor agonists. Thapsigargin (100 nM) alone did not induce proliferation, but when combined with otherwise nonstimulating concentrations of PMA (1 nM), it produced a significant and reproducible proliferation of human PBMNCs (109±9 and 1590±340 c.p.m. in the absence and presence of TG+PMA). Both fenamates, but not ketoprofen reduced TG+PMA-induced proliferation in a dose-dependent manner (Figure 3). The IC_{50} values for flufenamic and tolfenamic acids were 72±9 and 51±7 μM, respectively.

TG (100 nM)+PMA (1 nM) induced an increase in $[Ca^{2+}]_i$ to levels about two times as high as found after Con A (50 μg ml^{-1}). This increase in $[Ca^{2+}]_i$ was antagonized by flufenamic and tolfenamic acids in a dose-dependent manner. Flufenamic and tolfenamic acids at 60 μM, but not at lower drug concentrations artefactually slightly reduced fura-2 fluorescence which is seen as a minor decrease in the baseline in Figure 4. However, this small decrease in baseline cannot explain the vast decrease in fura-2 fluorescence by fenamates after TG+PMA-stimulation. To see whether the fenamates affect the release of Ca^{2+} from intracellular stores induced by TG+PMA, we studied their effects on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Ca^{2+} chelated by 2.5 mM EGTA). In the absence of extracellular calcium both fenamates (60 μM) did

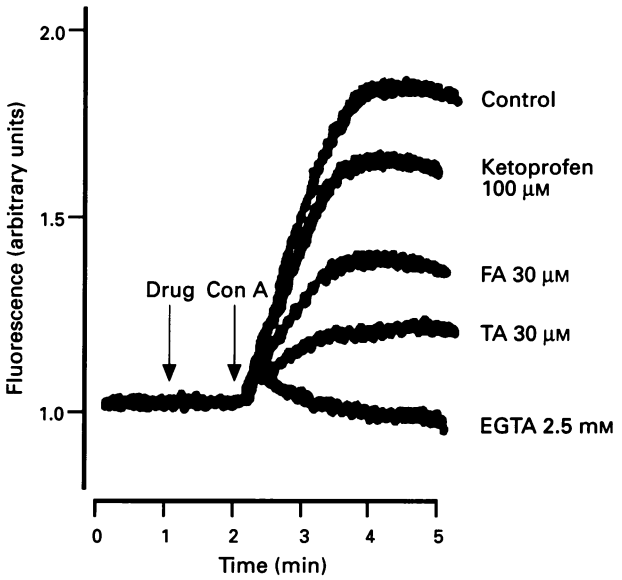

Figure 2 Effects of flufenamic acid (FA), ketoprofen and tolfenamic acid (TA) on concanavalin A (Con A; 50 μg ml^{-1})-induced increase in free intracellular calcium concentration in human mononuclear cells. Fluorescence (arbitrary units) in Fura-2 loaded peripheral blood mononuclear cells (PBMNCs) was measured by use of 340 and 380 nm excitation and 500 nm emission wavelengths. The drugs and Con A were added as indicated. EGTA (2.5 mM) was used to chelate the extracellular calcium. Traces are superimposed for clarity. This series of experiments has been repeated four times with similar results. For actual free intracellular calcium values see Methods.

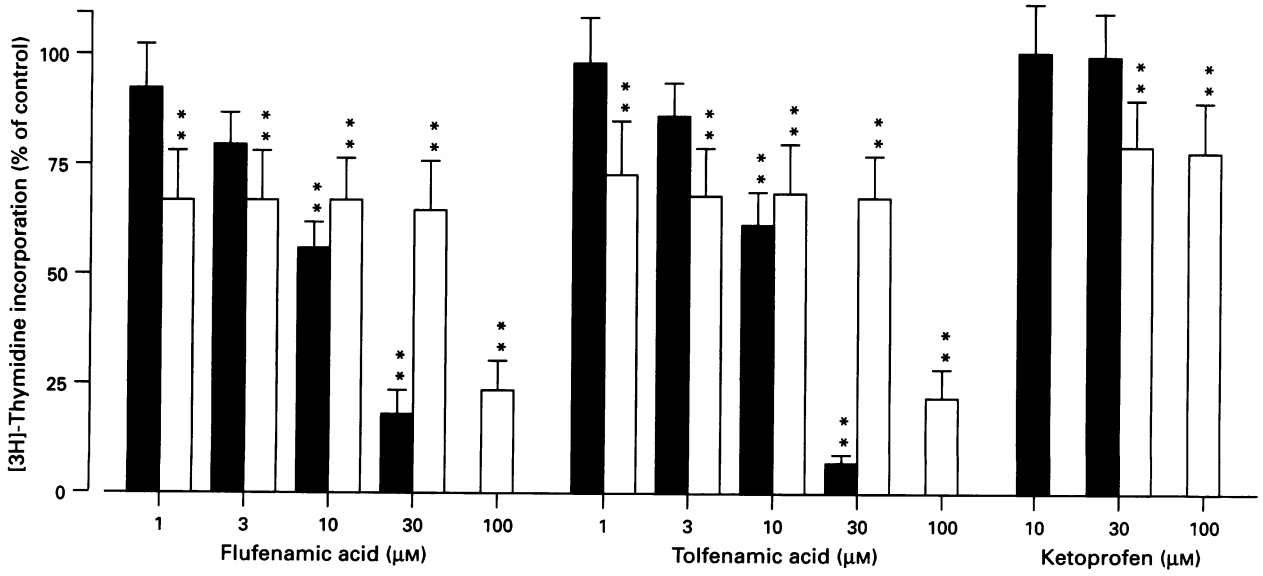

Figure 1 Effects of flufenamic acid, tolfenamic acid and ketoprofenon concanavalin A (Con A) (1 μg ml^{-1})-induced proliferation of human peripheral blood mononuclear cells (PBMNC) in RPMI 1640 medium with (open columns) or without (solid columns) foetal bovine serum. Results are expressed as percentage of control, i.e. cells incubated without the drug. The control values with Con A were 1995±585 and 1828±346 c.p.m. and without Con A 177±25 and 99±5 c.p.m. in the presence and absence of serum, respectively. Results are mean±s.e.mean, $n=6$. Differences from corresponding control values are denoted by $^{**}P<0.01$.

not significantly reduce the increase in $[Ca^{2+}]_i$ induced by TG + PMA or ionomycin (1 μM) (Figure 4). This suggests that the attenuation of $[Ca^{2+}]_i$ increase by fenamates is due to inhibition of Ca^{2+} influx. This result also excludes the possibility that the decrease in $[Ca^{2+}]_i$ by fenamates after Con A- or TG + PMA-stimulation was due to an artefactual interference of fenamates with fura-2 fluorescence. Ketoprofen did not affect significantly TG + PMA-induced increase in $[Ca^{2+}]_i$.

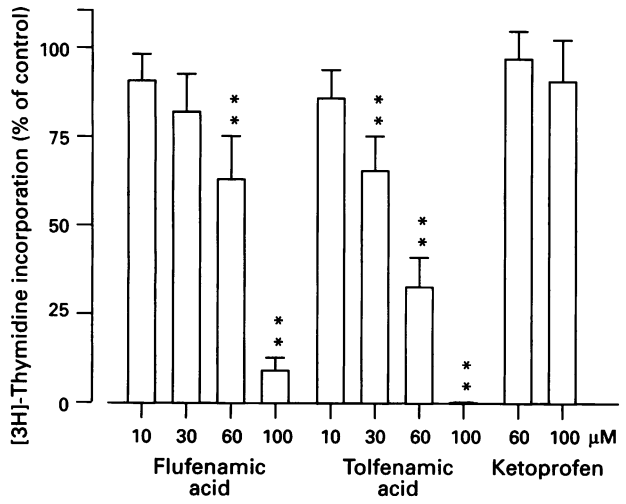

Figure 3 Effects of flufenamic acid, tolfenamic acid and ketoprofen on thapsigargin (TG, 100 nM) + phorbol myristate acetate (PMA, 1 nM)-induced human peripheral blood monoclear cell (PBMNC) proliferation. Results are expressed as percentage of control, i.e. cells incubated without the drug. The control values were 1590 ± 340 and 105 ± 9 c.p.m. in the presence and absence of TG + PMA, respectively. Results are mean ± s.e.mean, $n = 6$. Differences from corresponding control value are denoted by $^{**}P < 0.01$.

Effects of fenamates on Ca^{2+} and Mn^{2+} influx in PBMNCs

Estimation of the inhibitory activity of a drug on Ca^{2+} influx based on a single trace representing both the release of calcium from the intracellular stores and influx of calcium from extracellular media is not satisfactory. For this reason we used an 'add-back' modification of the fura-2 fluorescence method as described earlier (Kankaanranta & Moilanen, 1995). The fura-2 loaded cells were first stimulated with TG + PMA in the absence of extracellular Ca^{2+} to record the release of Ca^{2+} from intracellular stores. Thereafter, extracellular Ca^{2+} (final concentration of free Ca^{2+} 1.5 mM) was added and influx of Ca^{2+} into the cells was seen as an increase in fura-2 fluorescence (Figure 5). This method allowed us to differentiate between the release of calcium from intracellular stores and influx from extracellular media in the same cells. To exclude the possibility that the increase in fluorescence after addition of extracellular Ca^{2+} is due to the binding of Ca^{2+} to extracellular fura-2, we used routinely $NiCl_2$ (5 mM) to block the cation flux. As can be seen in Figure 5 the increase in fura-2 fluorescence in the presence of Ni^{2+} was minimal as compared to the respective control. Thapsigargin induces a store-operated Ca^{2+} influx in several human cell types including lymphocytes. To further test our system, we included SK&F 96365, an antagonist of store-operated calcium entry, in the

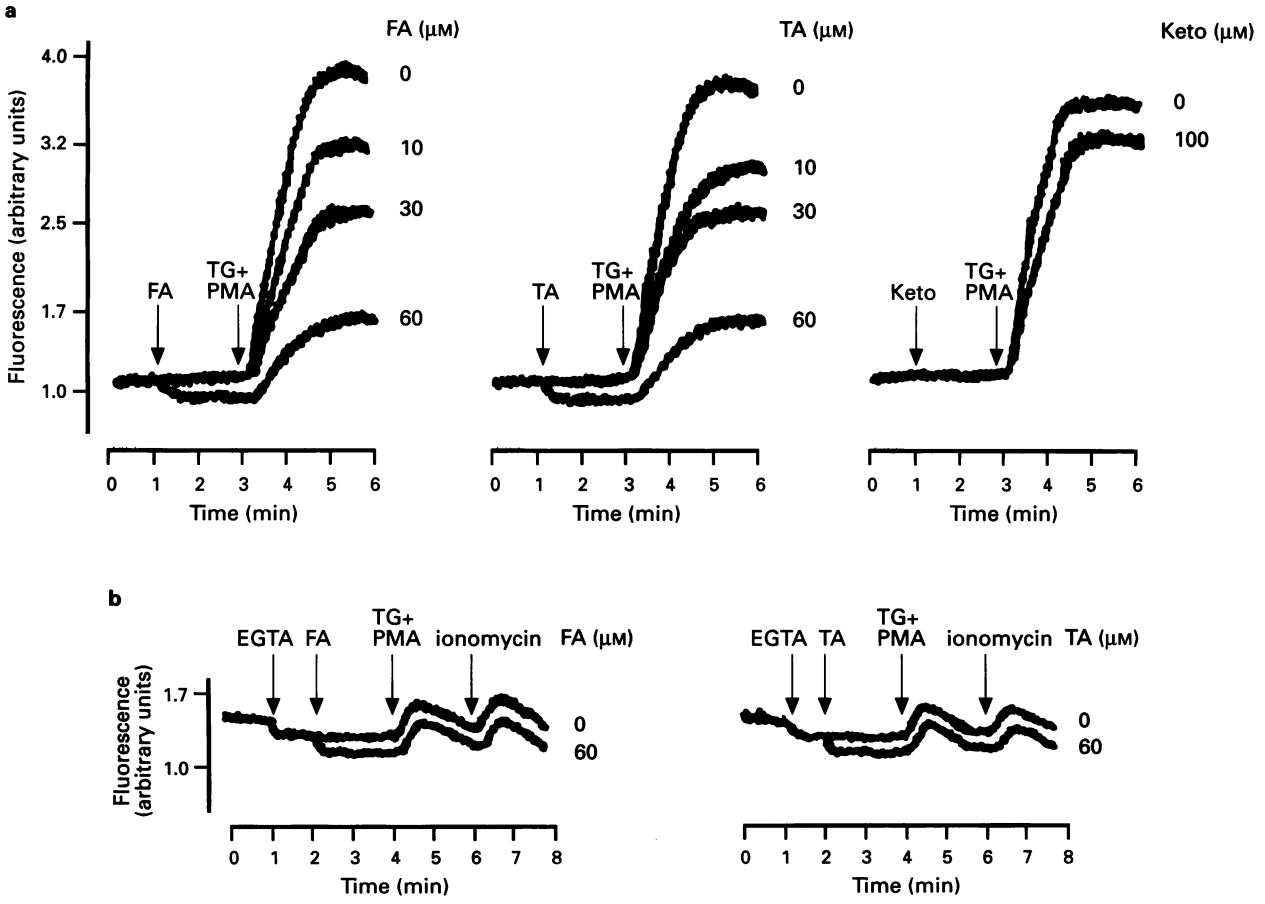

Figure 4 (a) Effects of flufenamic acid (FA), tolfenamic acid (TA) and ketoprofen (keto) on thapsigargin (TG, 100 nM) + phorbol myristate acetate (PMA, 1 nM)-induced increase in free intracellular calcium concentration in human mononuclear cells suspended in 10 mM HEPES-buffer with 1.5 mM Ca^{2+}. (b) Effects of FA and TA on TG + PMA-induced release of calcium from intracellular stores. EGTA (2.5 mM) was used to chelate the extracellular calcium. EGTA, drugs, TG + PMA and ionomycin (1 μM) were added as indicated. Fluorescence (arbitrary units) in Fura-2 loaded PBMNCs was measured with 340 and 380 nm excitation and 500 nm emission wavelengths. Traces are superimposed for clarity. This series of experiments has been repeated four times with similar results. For further details, see methods.

test pattern. SK&F 96365 reduced in a dose-dependent manner the increase in fura-2 fluorescence after addition of extracellular calcium (Figure 5) thus confirming that the increase in fura-2 fluorescence was due to influx of Ca^{2+}.

In the absence of extracellular Ca^{2+}, TG+PMA induced a transient rise in $[Ca^{2+}]_i$, which was not affected either by the fenamates (up to 60 μM), ketoprofen (100 μM) or SK&F 96365 (30 μM). After addition of extracellular Ca^{2+} (final concentration of free Ca^{2+} 1.5 mM), the fenamates, but not ketoprofen, reduced the increase in fura-2 fluorescence in a dose-dependent manner. On a molar basis the fenamates were less effective than SK&F 96365 (Figure 5). At 60 μM drug concentrations both fenamates decreased the baseline in a few seconds after addition.

The inhibition of cation influx by fenamates was confirmed by using Mn^{2+} as a surrogate for Ca^{2+}. At the excitation and emission wavelengths of 360 nm and 500 nm, respectively, the quenching of fura-2 fluorescence by Mn^{2+} is not affected by changes in [Ca2+]$_i$. Fluorescence at 340 nm was simultaneously recorded to confirm the accurate release of Ca^{2+} from intracellular stores. $NiCl_2$ (5 mM) was used to block completely the channels and no quenching of the fluorescence was seen in its presence. Flufenamic and tolfenamic acids (60 μM) as well as SK&F 96365 (10 μM), but not ketoprofen (100 μM), clearly reduced the quenching of fura-2 fluorescence at 360 nm excitation wavelength (Figure 6), but did not affect the release of intracellular Ca^{2+} as seen at 340 nm wavelength (data not shown). This confirms the ability of fenamates to inhibit cation influx in human PBMNCs.

Effects of SK&F 96365, an antagonist of store-operated calcium entry on PBMNC proliferation and $[Ca^{2+}]_i$

SK&F 96365, an inhibitor of store-operated calcium entry, inhibited both TG+PMA- (Figure 5) and Con A (data not shown)-induced increases in $[Ca^{2+}]_i$. To find out whether it

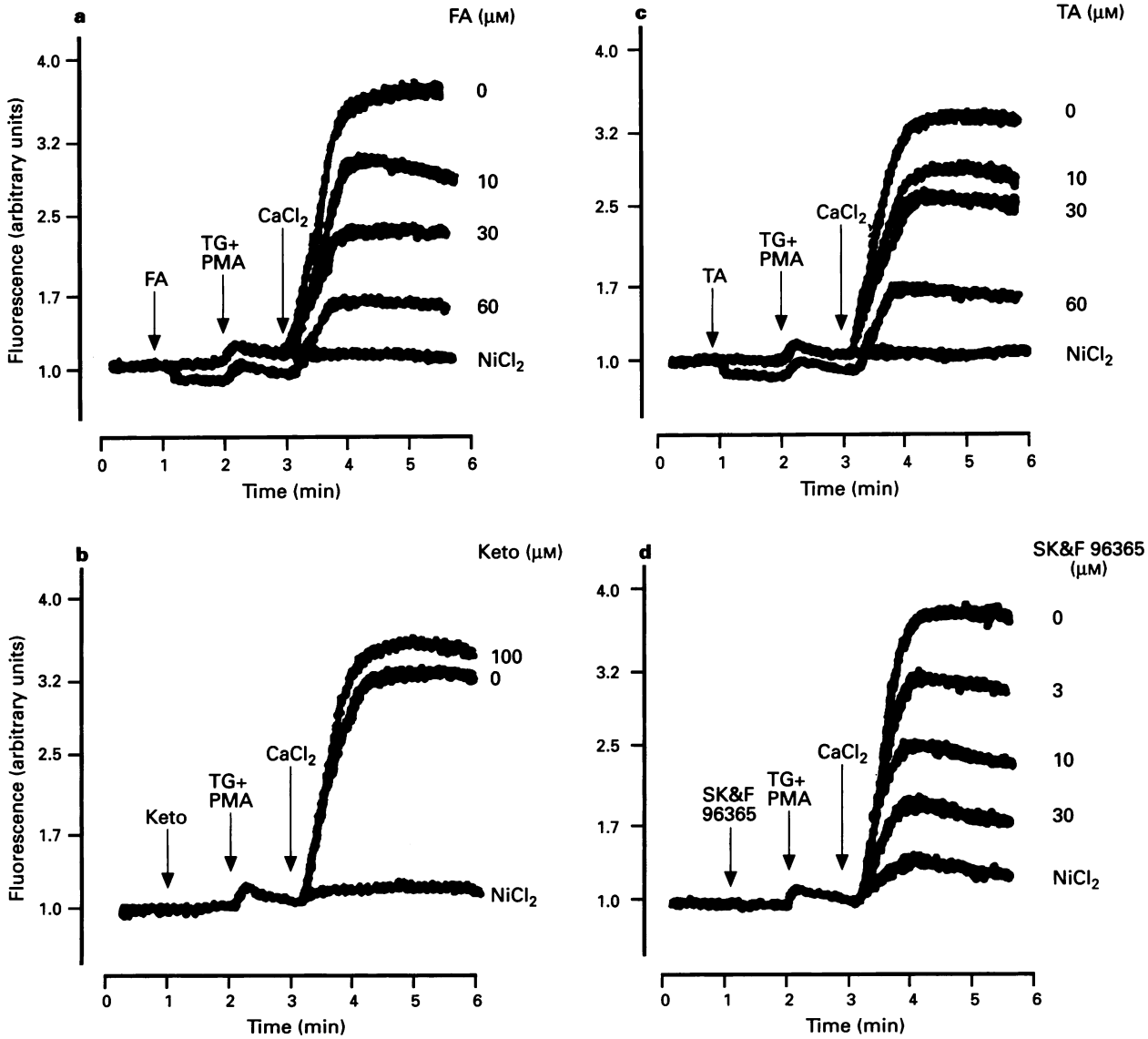

Figure 5 Effects of (a) flufenamic acid (FA), (b) ketoprofen (keto), (c) tolfenamic acid (TA) and (d) SK&F 96365 on thapsigargin (TG, 100 nM)+phorbol myristate acetate (PMA, 1 nM)-induced Ca^{2+} release from intracellular stores (first peak) and Ca^{2+} influx (second peak). Fura-2 loaded human mononuclear cells were suspended in 10 mM HEPES-buffer supplemented with 1 mM EGTA. Drugs, TG+PMA and $CaCl_2$ (2.5 mM) were added as indicated. $NiCl_2$ (5 mM) was used as a control compound that completely blocks calcium influx. Traces are superimposed for clarity. This series of experiments has been repeated four times with similar results. For further details, see Methods.

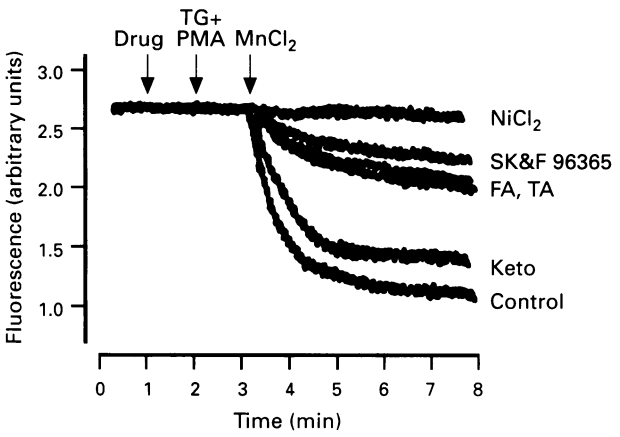

Figure 6 Effects of flufenamic acid (FA; 60 mM), ketoprofen (keto; 100 μM), SK&F 69365 (10 μM) and tolfenamic acid (TA; 60 μM) on thapsigargin (TG, 100 nM) + phorbol myristate acetate (PMA, 1 nM)-induced Mn^{2+} influx. Fura-2 loaded human mononuclear cells were suspended in 10 mM HEPES-buffer. Drugs, TG + PMA and $MnCl_2$ (100 μM) were added as indicated. $NiCl_2$ (5 mM) was used as a control compound that completely blocks Mn^{2+} influx. Typical traces at excitation and emission wavelengths of 360 and 500 nm are shown. Traces are superimposed for clarity. This series of experiments was repeated four times with similar results. For further details, see Methods.

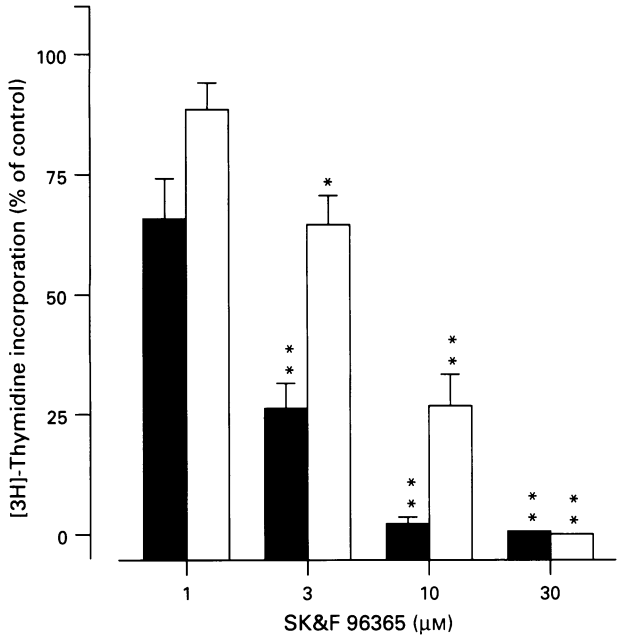

Figure 7 The effects of SK&F 96365 on thapsigargin (100 nM) + phorbol myristate acetate (PMA, 1 nM) (open columns) and concanavalin (Con A (1 $\mu g\,ml^{-1}$; solid columns)-induced proliferation of human peripheral blood mononuclear cell (PBMNC) in RPMI 1640 medium supplemented with foetal bovine serum. For control values see Figures 1 and 3. Results are expressed as percentage of control, i.e. cells incubated without the drug. Results are mean ± s.e.mean, $n = 6$. Differences from corresponding control values are denoted by $^*P < 0.05$ and $^{**}P < 0.01$.

affects lymphocyte growth, its effects on both Con A- and TG + PMA-induced PBMNC proliferation were tested in FBS-containing medium. SK&F 96365 reduced both Con A- and TG + PMA-triggered proliferation in a dose-dependent manner (IC_{50} values 1.5 ± 0.5 and 5.5 ± 1.1 μM for Con A and TG + PMA-induced proliferation, respectively; Figure 7).

Discussion

Mitogen-induced Ca^{2+} influx in lymphocytes is considered to be mediated through non-voltage-dependent Ca^{2+} channels because (1) voltage-gated calcium channels have not been found in lymphocytes by patch clamp techniques; (2) classical antagonists of voltage-dependent calcium channels do not reduce agonist-induced increases in $[Ca^{2+}]_i$; (3) depolarization by high K^+ concentration does not increase $[Ca^{2+}]_i$ (Gallin, 1991; Gallin & Grinstein, 1992; Lewis & Cahalan, 1995). Successively, patch clamp studies have revealed more than one different non-voltage-gated Ca^{2+} channels in lymphocytes (Kuno *et al.*, 1986; Kuno & Gardner, 1987; Lewis & Cahalan, 1989; McDonald *et al.*, 1993). Whole cell studies suggest that the main agonist-induced Ca^{2+} inflow is through a highly Ca^{2+} selective, inwardly rectifying channel, which is sensitive to Cd^{2+} and Ni^{2+} (Zweifach & Lewis, 1993; Premack *et al.*, 1994). This channel is activated either by IP_3 (McDonald *et al.*, 1993) or by the 'capacitative' (Putney, 1990) mechanism through depletion of intracellular Ca^{2+} stores (Zweifach & Lewis, 1993; Premack *et al.*, 1994). In the present study, we used both lectin (Con A) and TG + PMA to stimulate PBMNCs. TG is an inhibitor of endoplasmic reticulum Ca^{2+} ATPase, which causes depletion of intracellular Ca^{2+} stores and thus induces a capacitative calcium influx (Thastrup *et al.*, 1990). In T lymphocytes, Ca^{2+} influx induced by thapsigargin has been shown to be identical with that induced by mitogens (Zweifach & Lewis, 1993; Premack *et al.*, 1994). Only a few antagonists of 'capacitative' or receptor-mediated calcium entry exist which have been described to be effective in lymphocytes. Recently, an experimental antagonist of receptor-mediated calcium entry, SK&F 96365 (Merritt *et al.*, 1990) was shown to inhibit the inward Ca^{2+} current induced by antibody, carbachol or thapsigargin in leukemic Jurkat T-cells. Concomitant inhibition of interleukin-2 (IL-2) synthesis and cell proliferation was also found (Chung *et al.*, 1994). In the present study SK&F 96365 was used as an earlier characterized antagonist of store-operated calcium entry. The present data confirmed the earlier described (Nordström *et al.*, 1992; Chung *et al.*, 1994) inhibitory action of SK&F 96365 on lymphocyte proliferation and store-operated Ca^{2+} influx.

Flufenamic and tolfenamic acids reduced Con A and TG + PMA-induced PBMNC proliferation and increase in $[Ca^{2+}]_i$ in a dose-dependent manner, whereas ketoprofen (at 100 μM) had only a marginal inhibitory effect on cell proliferation and increase in $[Ca^{2+}]_i$ induced by Con A but not by TG + PMA. Con A-induced increase in $[Ca^{2+}]_i$ is mostly due to Ca^{2+} influx from extracellular space and most of it was abolished after chelation of extracellular Ca^{2+} by EGTA. This indicated that fenamates may affect Ca^{2+} influx rather than Ca^{2+} release from intracellular stores. To test this hypothesis we utilized TG + PMA to increase $[Ca^{2+}]_i$ in PBMNCs. Fenamates, but not ketoprofen, reduced TG + PMA-induced increase in $[Ca^{2+}]_i$ in the presence, but not in the absence of extracellular Ca^{2+}. In addition, we measured separately the effects of fenamates on Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular media in the same experiment (the 'add-back' experiments). The results clearly showed that flufenamic and tolfenamic acids did not affect Ca^{2+} release from intracellular stores but significantly reduced Ca^{2+} influx from the extracellular media. Ketoprofen did not have corresponding effects on calcium metabolism.

To confirm that flufenamic and tolfenamic acids inhibit divalent cation influx, their effects on TG + PMA-induced Mn^{2+} influx were studied. In contrast to Ca^{2+}, Mn^{2+} quenches fura-2 fluorescence at Ca^{2+}-insensitive 360 nm excitation wavelength. This allows the determination of Mn^{2+} influx independently of simultaneous changes in $[Ca^{2+}]_i$. The fenamates and SK&F 96365, but not ketoprofen significantly reduced TG + PMA-induced Mn^{2+} influx in PBMNCs, thus confirming their inhibitory action on cation influx.

Fenamates are potent inhibitors of prostaglandin synthesis (McLean & Gluckman, 1983; Moilanen & Kankaanranta,

1994). From the theoretical point of view, the inhibition of prostaglandin synthesis is not the most likely mechanism responsible for the inhibition of Ca^{2+} influx in PBMNCs because prostaglandin E_2 (PGE_2) has been shown to inhibit Ca^{2+} influx (Chouaib *et al.*, 1987). Thus inhibition of prostaglandin synthesis should rather augment than inhibit Ca^{2+} influx. Ketoprofen, a chemically different prostaglandin synthesis inhibitor did not significantly affect Ca^{2+} influx in the present study. Some prostaglandin synthetase inhibitors, such as diclofenac and indomethacin have been found to enhance T cell proliferation by a mechanism involving an increase in $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+} into the cells. This enhancement of lymphocyte proliferation did not depend on inhibition of prostaglandin synthesis (Flescher *et al.*, 1991).

Our present result that fenamates inhibit calcium influx in human PBMNCs accords with our recent results that fenamates inhibit receptor-mediated Ca^{2+} influx in human neutrophils (Kankaanranta *et al.*, 1995; Kankaanranta & Moilanen, 1995). Previously, flufenamic acid has been shown to inhibit single nonselective cation channels in rat exocrine pancreas (Gögelein *et al.*, 1990). Furthermore, flufenamic acid has been shown to inhibit platelet-derived growth factor-induced 28 pS nonselective cation channel in a reversible manner with an equilibrium constant of approximately 10 μM in mouse fibroblasts. This channel block was related to inhibition of cell growth (Jung *et al.*, 1992). Whether Con A or thapsigargin-induced Ca^{2+} influx in lymphocytes utilizes similar nonselective cation channels that exist in exocrine pancreas, fibroblasts or in human neutrophils is not known. Based on the different conductances it seems that these channels are not similar (Von Tscharner *et al.*, 1986; Gögelein *et al.*, 1990; Jung *et al.*, 1992; Zweifach & Lewis, 1993). Recently, fenamates were also shown to regulate calcium activated chloride and potassium currents in rabbit portal vein smooth muscle (Greenwood & Large, 1995). Thus it seems that the fenamates are able to regulate several types of ion channels in different tissues and may not be specific antagonists of receptor-mediated or capacitative calcium entry.

Could the inhibition of Ca^{2+} influx by fenamates explain the reduction in proliferative activity? The expression of interleukin-2 (IL-2), induction of its receptor and subsequent IL-2 receptor mediated events are pivotal in T-cell activation (Waldmann, 1993). The requirement of the Ca^{2+} signal for IL-2 production has been demonstrated by several studies (Crabtree, 1989; Cardenas & Heitman, 1995). IL-2 gene expression is strictly dependent on a T-cell-specific protein complex, called nuclear factor of activated T cells (NF-AT). The functional NF-AT complex consists of two elements i.e. NF-ATp and NF-ATn. Calcium/calmodulin-dependent phosphatase calcineurin dephosphorylates the cytoplasmic phosphoprotein NF-ATp, which translocates to the nucleus where it combines with the newly synthesized nuclear component, NF-ATn to form a functionally active transcription factor. In this cascade elevated $[Ca^{2+}]_i$ functions as an activator of calcineurin thus allowing the cytoplasmic NF-ATp to dephosphorylate and translocate. Inhibitors of calcineurin, such as cyclosporin A and FK 506 prevent T cell activation (Bierer *et al.*, 1993; Liu, 1993; Cardenas & Heitman, 1995). Further support to this concept is that a reduced capacitative calcium entry in a mutant Jurkat T cell line is associated with a defect in NF-AT-dependent transcription (Fanger *et al.*, 1995). NF-κB, another transcription factor important in lymphocyte activation, is also partly regulated by Ca^{2+} and calcineurin (Bauerle & Henkel, 1994; Cardenas & Heitman, 1995). Thus Ca^{2+} seems to be an essential second mediator in several steps of the lymphocyte activation cascade and inhibition of Ca^{2+} influx could mediate the antiproliferative action of fenamates. This is also supported by the finding that SK&F 96365, an earlier known inhibitor of store-operated calcium influx, had an antiproliferative action on human PBMNCs comparable with that of fenamates.

Whether the antiproliferative activity of fenamates is present also in an inflammatory focus *in vivo* remains to be evaluated. That kind of effect could explain part of the anti-inflammatory activity of fenamates in diseases like rheumatoid arthritis or ankylosing spondylarthritis (Rejholec *et al.*, 1979; 1980). Our previous (Kankaanranta *et al.*, 1994; 1995; Kankaanranta & Moilanen, 1995) and present results suggest that antagonists of receptor-mediated/store-operated calcium entry inhibit neutrophil and lymphocyte activation processes. Receptor-mediated/store-operated calcium entry thus serves as a potential target for the development of anti-inflammatory drug therapy.

In conclusion, we have shown that two fenamates, flufenamic and tolfenamic acid inhibit human lymphocyte proliferation. Concomitantly with their antiproliferative action fenamates reduced both lectin and TG+PMA-induced Ca^{2+} influx in a prostaglandin-independent manner. Inhibition of proliferation and Ca^{2+} influx in human lymphocytes may explain part of the anti-inflammatory activity of these two NSAIDs. Flufenamic and tolfenamic acid are introduced as compounds able to inhibit human lymphocyte proliferation in a manner involving inhibition of Ca^{2+} influx.

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