Sulphasalazine inhibition of human granulocyte activation by inhibition of second messenger compounds

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Abstract

The effects of sulphasalazine on the production of second messenger compounds in human granulocytes have been characterised by various stimuli. The increases in cytosolic calcium, inositol trisphosphate, diacylglycerol, and phosphatidic acid (all important mediators of intracellular signal transduction) triggered by stimulation were inhibited by sulphasalazine. The metabolites 5-amino-salicylic acid and sulphapyridine were less potent inhibitors than the mother compound.

It is concluded that sulphasalazine inhibits the synthesis of phosphoinositide derived second messenger compounds at the level of phospholipase C or its regulatory guanosine 5'-triphosphate (GTP) binding protein. Inhibition of phosphatidic acid synthesis was either due to the same mechanism, or to interaction with a phospholipase D regulating GTP binding protein.

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Sulphasalazine, a drug used for the treatment of inflammatory bowel disease,¹ has during the last decade also been shown to treat effectively other diseases of a presumably autoimmune nature, such as rheumatoid arthritis, ankylosing spondylitis,² and psoriasis.³

The aetiology of tissue injury in rheumatoid arthritis is unclear. A variety of cells, including granulocytes, have been suggested to contribute to the initiation and propagation of the immune response in the rheumatoid synovium. Kitsis and Weissmann⁴ have reviewed the role of the neutrophil granulocyte as a key player in the articular and extra-articular manifestations of rheumatoid arthritis. They compared rheumatoid joint inflammation to the Arthus reaction, a neutrophil dependent interaction in which repeated exposure to an antigen results in the formation of immune complexes and activation of complement, causing an influx of granulocytes and tissue destruction.

Sulphasalazine has been found to inhibit granulocyte activation, assessed as superoxide production by cells activated with serum treated zymosan,⁵ chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)^{6 7} and immune complexes.⁸ Sulphasalazine has also been found to inhibit, for example, granulocyte chemotaxis,⁹ lymphocyte proliferation,¹⁰ and interleukin 2 production¹¹ in addition to monocyte production of interleukin 1.¹²

We have previously found by using the granulocyte as a model system that sulphasalazine inhibits cellular activation before activation of protein kinase C by interference with the synthesis of phosphoinositide derived second messenger compounds.⁶ As several cell types utilise phosphoinositide dependent signal transduction, sulphasalazine may modify a principal common mechanism for the regulation of cell activity, which may explain the beneficial effects of the drug in a variety of diseases.

Many of the stimuli used in the earlier studies trigger an increase in cytosolic calcium as part of the activation sequence. The increase in calcium has, in a variety of cell systems, been found to depend on remodelling of phosphoinositides¹³ (see fig 1). Ligation of a cell receptor leads to the phospholipase C dependent hydrolysis of phosphatidylinositol-4,5-bisphosphate generating two triggers of cell responses, namely diacylglycerol, an activator of protein kinase C,¹⁴ and inositol-1,4,5-trisphosphate, which triggers the release of cell associated calcium.¹³ The link between the receptor and phospholipase C is in many cells a guanosine 5'-triphosphate (GTP) binding protein.¹⁵

Several drugs have been shown to modulate granulocyte responses such as superoxide production,¹⁶ and a few studies have indirectly associated the drug action with an interaction with the synthesis of phosphoinositide derived second messenger molecules^{17 18} and the GTP binding protein.¹⁹

The purpose of this study was to evaluate whether the inhibitory effects of sulphasalazine on cellular activation were related to the production of second messenger compounds. We found that sulphasalazine inhibited the production of inositol-1,4,5-trisphosphate and consequently the increase in cytosolic calcium, which indicated an interaction with either the plasma membrane receptor, the associated GTP binding protein, or the phospholipase C. Sulphasalazine still inhibited activation of cells stimulated with guanosine 5'-[γ-thio]triphosphate (GTP[S]), a stimulus which bypasses the plasma membrane receptor.

The production of phosphatidic acid, a metabolite of diacylglycerol, the other product of phospholipase C activity, was also inhibited by sulphasalazine in cells unspecifically labelled in the lipid glyceride backbone. Phosphatidic acid is alternatively formed by hydrolysis of phosphatidylcholine; we found that sulphasalazine also inhibited the production of radioactive phosphatidic acid in cells specifically labelled in phosphatidylcholine.

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Figure 1 Schematic illustration of the part played by second messenger compounds in the activation of cells. The binding of a ligand (L) to a membrane receptor (R) activates a phospholipase C (PLC) via the action of a guanine 5'-triphosphate (GTP) binding protein (G). The activity of the GTP binding protein is regulated by its intrinsic GTPase activity, which hydrolyses GTP and thereby inactivates the protein. By hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂), PLC generates diacylglycerol (DAG), an activator of protein kinase C (PKC), and inositol-1,4,5-trisphosphate (IP₃), which triggers the release of cell associated calcium and the subsequent activation of a protein kinase (CaPK). Diacylglycerol is further phosphorylated to phosphatidic acid (PA). Phosphatidic acid is also formed by a phospholipase D mediated (PLD) hydrolysis of phosphatidylcholine (PC). Phosphatidic acid is subsequently dephosphorylated to DAG.

Materials and methods

TEST COMPOUNDS

Sulphasalazine, sulphapyridine, and 5-amino-salicylic acid were from Kabi Pharmacia (Uppsala, Sweden).

BUFFER

Dulbecco's phosphate buffered saline (PBS) with, or when specifically stated without, 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺ was from Statens Veterinärmedicinska Anstalt (Uppsala, Sweden).

ISOLATION OF GRANULOCYTES

Granulocytes were isolated from heparinised blood from healthy volunteers, not using anti-inflammatory drugs, by the method of Böyum²⁰ with minor modifications.⁶

INOSITOL-1,4,5-TRISPHOSPHATE SYNTHESIS

Granulocytes, 150×10⁶/ml, were labelled with 1110 kBq/ml of [³H]myoinositol (TRK.856, Amersham, Solna, Sweden), for two hours at

37°C as described previously.²¹ Labelled granulocytes were washed and resuspended in Hanks's balanced salt solution (HBSS, Gibco, Paisley, UK) containing 10 mM LiCl. Each sample, containing 25×10⁶ cells in 1 ml HBSS with LiCl, was preincubated with the test compound for 10 minutes at 37°C before challenge with 0.1 μM fMLP (Calbiochem, La Jolla, CA, USA). The reaction was terminated by addition of 7.5% trichloroacetic acid. Inositol-1,4,5-trisphosphate was isolated by ion exchange chromatography as described previously.²¹

CYTOSOLIC CALCIUM

The granulocytes, 1×10⁸/ml, were incubated with 10 μM FURA-2AM (Molecular Probes Inc., Eugene, OR, USA) as described by Korchak *et al.*²² The FURA-2 loaded cells were suspended to 25×10⁶ cells/ml in ice cold PBS without Ca²⁺ and Mg²⁺ until used.

The cells, 2.5×10⁶/ml were preincubated in PBS either with or without Ca²⁺ and Mg²⁺ (in the latter case the buffer was supplemented with 1 mM EGTA) and test compound for two minutes before the addition of stimulus. Fluorescence changes were monitored in a Shimadzu RF-5000 spectrophotometer equipped with a continuous magnetic stirring device at the dual excitation wavelengths of 340 and 380 nm, and emission at 510 nm. The concentration of cytosolic calcium was calculated by the ratio method of Grynkiewicz *et al.*²³

Sulphasalazine at 10 μmol/l had considerable absorbances at 340 and 380 nm. As the reduction of the emitted light was about 25% at the two wavelengths, however, sulphasalazine had only a negligible effect on the calculations of [Ca²⁺] using the ratio method.

GTP INDUCED SUPEROXIDE PRODUCTION

The granulocytes were electroporated essentially as described by Nasmith *et al.*²⁴ Cells were resuspended in permeabilisation buffer to 10⁷ cells/ml and permeabilised with two discharges of 5 kV/cm from a 25 μF capacitor by using a Bio-Rad gene pulser apparatus equipped with a 0.8 ml cuvette. Permeabilised cells were used immediately (within a few minutes). The permeabilisation buffer contained 10 mM HEPES, 140 mM KCl, 1 mM EGTA, and 0.193 mM CaCl₂ (giving a free [Ca²⁺] concentration of 100 nmol/l), 1 mM MgCl₂, and 10 mM glucose (pH 7.0, 295 mosM).

A 133 μM solution of cytochrome *c* (type VI from horse heart, Sigma Chemical, St. Louis, MO, USA) in 750 μl permeabilisation buffer containing the test compound was prewarmed to 37°C. Thereafter 100 μl of a 20 mM NADPH solution was added immediately followed by 50 μl of the cell suspension and 100 μl of GTP[S]. Prolonged exposure of NADPH to cytochrome *c* will result in the reduction of the latter and was thus avoided. After incubation at 37°C the absorbances at 550 and 540 nm were recorded spectrophotometrically.⁶ The superoxide production was expressed as the difference in absorbance at these wavelengths.⁶ All values

given are the mean of at least duplicate parallel experiments.

GTPASE ACTIVITY

Granulocytes were suspended in 0.25 M sucrose, 10 mM HEPES, and 1 mM EGTA (pH 7.5) to about 10^7 cells/ml. The cell suspension was sonicated with a 10 second pulse of a Branson B15 cell disruptor. Intact cells and nuclei were removed by 10 minutes of centrifugation at 400 g, and membranes were sedimented by centrifugation for 30 minutes at 16 000 g. Membranes were either used immediately or frozen.

GTPase activity was calculated by measuring the liberation of phosphate-32 from [γ - 32 P]-GTP as described by Pelz *et al.*²⁵ The reaction mixture contained 10 μ l cell membrane suspension (membranes from the granulocytes in about 3 ml blood), 100 μ l buffer (100 mM NaCl, 20 mM TRIS-HCl, 5 mM MgCl₂, 0.1 mM EGTA, 1.14 mM ATP, 500 μ M adenylylimidodiphosphate, 0.25 mM ouabain, pH 7.5) with about 50 nmol/l added [γ - 32 P]-GTP (NEG-004, New England Nuclear, Stockholm, Sweden; 1.22 TBq/mmol radioactivity). The reaction was terminated after 10 minutes by addition of 0.5 ml of a 5% charcoal mixture containing 0.1% dextran 500 (Kabi Pharmacia) and 0.5% bovine serum albumin in Dulbecco's phosphate buffer. The tubes were vortexed and centrifuged for five minutes at 11 000 g, whereafter the phosphate-32 activity in the supernatant was measured.

SYNTHESIS OF PHOSPHATIDIC ACID IN GLYCEROL LABELLED CELLS

Granulocytes from 70 ml of blood were suspended in 2 ml of HBSS containing 0.5 mM CaCl₂²⁶ and incubated for one hour at 37°C with 740 kBq [3 H]glycerol (NET 022, New England Nuclear).

The cells were washed twice and resuspended in HBSS. Cells from two different donors were combined immediately before the experiment.

The cell suspension, 15×10^6 cells in 500 μ l containing 5 μ g/ml of cytochalasin B (Calbiochem) and the test compound as indicated were preincubated for 10 minutes at 37°C. Thereafter the cells were stimulated by addition of 0.1 or 1 μ M fMLP. The reaction was terminated by addition of 1.875 ml chloroform/methanol (1:2, v/v) after 60 seconds. To obtain phase separation, 625 μ l of chloroform and 625 μ l of water were added. The lower phase containing the phospholipids was collected and evaporated.

For the phosphatidic acid determinations the phospholipids were dissolved in 30 μ l chloroform/methanol/water (75:25:2, v/v). Exogenous phosphatidic acid, 20 μ g, and phosphatidylinositol, 10 μ g, were added as carriers making the total volume 50 μ l. The phospholipids were separated by thin layer chromatography on Silicagel 60 plates (5715, E. Merck, Darmstadt, Germany) essentially as described previously.²⁶ The mobile phase consisted of methyl acetate/propan-1-ol/chloroform/methanol/0.25 M KCl/acetic acid (25:25:25:12:9:0.062, v/v). After the chromatographic separation the phospholipid

spots were stained with iodine vapour. The spots corresponding to phosphatidic acid, phosphatidylinositol, and phosphatidylcholine were scraped and counted in a scintillation counter.

PHOSPHOLIPASE D ACTIVITY

Radiolabelling of granulocytes with [3 H]alkyllysophosphatidylcholine (octadecyl-lyso platelet activating factor, TRK.745, Amersham, Solna, Sweden), cell activation and analysis were performed essentially as described by Billah *et al.*²⁷ Briefly, 20×10^6 cells/ml were suspended in 25 mM HEPES (pH 7.4) with 125 mM NaCl, 0.7 mM MgCl₂, 0.5 mM EGTA, 10 mM glucose, and 1 mg/ml albumin. Radiolabel, 185 kBq/ml was added and the suspension was incubated for 75 minutes at 37°C. After washing, the cells were resuspended to 5×10^6 cells/0.45 ml in HEPES buffer without albumin.

The cells were preincubated with cytochalasin B (5 μ g/ml) and test compound for five minutes at 37°C, after which stimulus was added, either 0.1 μ M fMLP, or 20 mM NaF plus 30 μ M AlCl₃ or 0.1 μ M phorbol-12-myristate-13-acetate (PMA, Sigma Chemical). The final sample volume was 500 μ l, containing 5×10^6 cells. The reaction was terminated by addition of 1.875 ml chloroform/methanol/acetic acid (100:200:4, v/v). Chloroform and water (625 μ l of each) were then added to obtain phase separation. The lower phase was collected and evaporated. For the determination of phosphatidic acid and diacylglycerol the phospholipids were dissolved in 30 μ l chloroform/methanol/water (75:25:2, v/v). Phosphatidic acid, 20 μ g, was added as carrier making the total volume 50 μ l. The phospholipids were separated by thin layer chromatography as described by Billah *et al.*²⁷ their solvent systems I and III. After the chromatographic separation the phospholipid spots were stained with iodine vapour. The spots corresponding to phosphatidic acid and diacylglycerol were scraped and counted in a scintillation counter after addition of 0.25 ml methanol and 10 ml Instagel. Counts were corrected for quenching by sulphasalazine.

STATISTICS

Results are expressed as the mean (SD). Statistical significance was determined using Student's *t* test. A *p* value of less than 0.05 was considered significant.

Results

SYNTHESIS OF INOSITOL-1,4,5-TRISPHOSPHATE

Initial experiments showed that the intracellular concentration of inositol-1,4,5-trisphosphate concentration was at a maximum about 30 seconds after activation with 0.1 μ M fMLP. The radioactivity in the inositol-1,4,5-trisphosphate fraction was 79 counts/min before activation and 317, 374, and 175 counts/min 15, 30, and 60 seconds after activation in a representative experiment. A reaction time of 30 seconds was therefore chosen for studies of the effects of the drug.

Table 1 Inhibition of human granulocyte inositol-1,4,5-trisphosphate synthesis by sulphasalazine and its metabolites. The granulocytes were stimulated with 0.1 μ M *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine for 30 seconds. Results given as mean (SD)

Test compound	Concentration (μ mol/l)	Inhibition (%)
Sulphasalazine	100	45.6 (11.9) (n=8)
	10	28.5 (6.4) (n=2)
5-Aminosalicylic acid	100	13.8 (4.6) (n=4)
Sulphapyridine	100	7.5 (8.2) (n=4)

Table 1 shows that sulphasalazine (10 and 100 μ mol/l) inhibited the synthesis of inositol-1,4,5-trisphosphate. The metabolites sulphapyridine and 5-aminosalicylic acid at 100 μ mol/l inhibited the reaction significantly less than 10 μ mol/l of the mother molecule.

CYTOSOLIC CALCIUM

Figure 2 shows that a transient increase in cytosolic calcium occurred after stimulation with fMLP. The peak value was reached after about eight to 10 seconds (including time for stirring), and was followed by a less rapid decline. The increase in cytosolic calcium varied between cells from different donors and was 85 (25) nmol/l for 1 nM fMLP in the presence of extracellular calcium (12 subjects). In the absence of extracellular calcium it was 70 (17)

Figure 2 Changes in cytosolic calcium in granulocytes stimulated with 10 nM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). Effect of 120 seconds preincubation with 10 μ M sulphasalazine. The arrow indicates the addition of fMLP.

Table 2 Effect of sulphasalazine and sulphapyridine on the increase of cytosolic calcium in human granulocytes. The results are mean (SD) percentage inhibition of the peak value, which was reached about 10 seconds after addition of the stimulus

Stimulus*	Test compound	Concentration (μ mol/l)	Inhibition (%)
Medium with extracellular Ca 1 nM fMLP	Sulphasalazine	5	34.2 (6.3) (n=6)
	Sulphasalazine	10	61.1 (12.1) (n=5)
	Sulphapyridine	10	12.2 (7.5) (n=4)
Medium with EGTA, without Ca 1 nM fMLP	Sulphasalazine	5	47.8 (13.7) (n=4)
	Sulphasalazine	10	67.7 (7.9) (n=3)
	Sulphapyridine	10	10.5 (6.0) (n=3)

*fMLP = *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine.

nmol/l (six subjects) and 126 (69) nmol/l (nine subjects) respectively for 1 and 10 nM fMLP.

Table 2 shows that sulphasalazine inhibited the increase in cytosolic calcium. At a concentration of only 5 μ mol/l almost 50% of the response to 1 nM fMLP was inhibited in the absence of extracellular calcium. Sulphapyridine was considerably less potent. 5-Aminosalicylic acid was not studied due to its substantial intrinsic fluorescence at the excitation wavelengths applied.

GTP INDUCED SUPEROXIDE PRODUCTION

Electropermeabilised granulocytes stimulated with GTP[S] (tetralithium salt, 50 μ mol/l) produced enough superoxide to reduce 8.1 nmol cytochrome *c* for each 500 000 cells (table 3). The production of superoxide after stimulation with 10 μ M GTP[S] was slightly less, or 6.8 nmol for each 500 000 cells.

The superoxide production was inhibited in the presence of sulphasalazine (100 μ mol/l). The inhibition varied between blood donors and was more pronounced after stimulation with 10 μ M GTP[S] (43–87% inhibition) than with 50 μ M GTP[S] (23–39% inhibition). GTP[S] did not stimulate superoxide production in intact (not electropermeabilised) cells (not shown).

In the original method,²⁴ 1 mM ATP was added to the reaction mixture. Exogenous ATP is, however, known to activate²⁸ granulocytes leading to formation of inositol phosphates and an increase in Ca^{2+} , and to prime granulocytes for enhanced superoxide production after fMLP stimulation.²⁹ Favourably, in contrast to Nasmith *et al*²⁴ and in agreement with Hartfield and Robinson,³⁰ we found that the superoxide production did not require exogenously added ATP.

GTPASE ACTIVITY OF GRANULOCYTE MEMBRANES

The GTPase activity in the presence of 50 nM GTP was 0.63 (0.10) nmol of released phosphate in the absence and 0.57 (0.14) nmol in the presence of 100 μ M sulphasalazine (four samples). No significant inhibition was thus seen in the presence of sulphasalazine.

SYNTHESIS OF PHOSPHATIDIC ACID IN GLYCEROL LABELLED CELLS

The response to stimulation with fMLP varied considerably between cells from different donors. The relative increase of radioactivity in the phosphatidic acid band 60 seconds after fMLP stimulation was 1.74 (0.22) fold (seven

Table 3 Effect of 100 μ M sulphasalazine on superoxide production by electropermeabilised human granulocytes stimulated with guanosine 5'-[γ -thio]triphosphate (GTP[S]). The superoxide production is expressed in nanomol reduction of cytochrome *c* per sample containing 500 000 cells. Results are given as mean (SD) (n=4)

Stimulus	Control	Sulphasalazine	Inhibition (%)
10 μ M GTP[S]	6.8 (1.7)	2.3 (1.1)	66 (19)
50 μ M GTP[S]	8.1 (1.3)	5.6 (1.4)	31 (8)

Table 4 Inhibition of human granulocyte phosphatidic acid synthesis by sulphasalazine and metabolites. The glycerol labelled granulocytes were stimulated with 0.1 µM N-formyl-L-methionyl-L-leucyl-L-phenylalanine for 60 seconds. The inhibition is expressed as mean (SD) percentage of the phosphatidic acid synthesis in the absence of test compound

Test compound	Concentration (µmol/l)	Inhibition (%)
Sulphasalazine	100	66.5 (18.6) (n=14)
	30	42.5 (27.6) (n=4)
	10	38.9 (19.0) (n=8)
	3	26.3 (8.4) (n=4)
5-Aminosalicylic acid	100	31.2 (15.3) (n=4)
Sulphapyridine	100	38.8 (18.2) (n=4)

samples) for 1 µM, and 1.73 (0.42) fold (29 samples) for 0.1 µM fMLP.

Sulphasalazine (100 µmol/l) inhibited the increase in phosphatidic acid after stimulation with fMLP (1 µmol/l) by 54 (14)% (seven samples). At 0.1 µM fMLP, the inhibition in the presence of 100 µM sulphasalazine was 67 (19)%, and the inhibition was dose dependent with respect to sulphasalazine (table 4). The metabolites sulphapyridine and 5-aminosalicylic acid inhibited phosphatidic acid synthesis only about one tenth as potently as sulphasalazine.

SYNTHESIS OF PHOSPHATIDIC ACID AND DIACYLGLYCEROL IN PHOSPHATIDYLCHOLINE LABELLED CELLS

The phospholipase D mediated synthesis of phosphatidic acid after stimulation with 0.1 µM fMLP varied considerably between cells from different donors (fig 3A). Initial experiments

Table 5 Effects of sulphasalazine and metabolites on phosphatidic acid and diacylglycerol synthesis by human granulocytes radiolabelled in phosphatidyl choline. Results are mean (SD) percentage inhibition

Stimulus	Reaction time (s)	Test compound	Concentration (µmol/l)	Inhibition (%)
Inhibition of phosphatidic acid				
100 nM fMLP*	30	Sulphasalazine	10	36.0 (1.7) (n=3)
	30	Sulphasalazine	100	89.6 (5.7) (n=5)
	120	Sulphasalazine	10	21.3 (12.9) (n=3)
	120	Sulphasalazine	100	83.3 (5.5) (n=3)
	30	Sulphapyridine	100	28.6 (11.4) (n=5)
	30	5-Aminosalicylic acid	100	-2.3 (14.5) (n=3)
100 nM PMA*	300	Sulphasalazine	100	0.3 (0.5) (n=2)
20 mM NaF	300	Sulphasalazine	100	56.0 (24.5) (n=3)
Inhibition of diacylglycerol				
100 nM fMLP	120	Sulphasalazine	10	43.3 (1.5) (n=3)
	120	Sulphasalazine	100	97.7 (13.6) (n=3)

*fMLP=N-formyl-L-methionyl-L-leucyl-L-phenylalanine; PMA=phorbol-12-myristate-13-acetate.

Figure 3 Changes in phosphatidic acid (A) and diacylglycerol (B) in human granulocytes stimulated with 100 nM N-formyl-L-methionyl-L-leucyl-L-phenylalanine. The cells were labelled with lysophosphatidylcholine. Results are of experiments using blood from three different donors.

showed that the maximum concentration of phosphatidic acid was obtained after 30–60 seconds, which agrees with earlier data.²⁷ Table 5 shows that sulphasalazine (100 µmol/l) was a potent inhibitor of phosphatidic acid formation after 30 and 120 seconds, also 10 µM sulphasalazine inhibited the reaction significantly. Sulphapyridine (100 µmol/l) inhibited the reaction slightly whereas 5-aminosalicylic acid was inactive.

Phosphatidic acid is metabolised to diacylglycerol²⁷ and the diacylglycerol synthesis consequently lagged behind the phosphatidic acid synthesis (fig 3B) in time, which agrees with earlier results.³¹ The synthesis of diacylglycerol was inhibited by sulphasalazine (table 5).

An accelerating increase in the concentration of phosphatidic acid after stimulation with 20 mM fluoride was seen for at least 10 minutes. Table 5 shows that 100 µM sulphasalazine inhibited the synthesis of phosphatidic acid by 56% after five minutes of stimulation.

Phosphatidic acid was produced in cells activated with PMA, essentially as described previously.³¹ No inhibitory effect of 100 µM sulphasalazine was found after five minutes (table 5).

Discussion

This study has shown that sulphasalazine inhibits the activation of human granulocytes through inhibition of the production of second messenger compounds derived from the GTP binding protein mediated activation of phospholipases C and D.

In the first set of experiments it was shown that sulphasalazine inhibited the phospholipase C dependent synthesis of inositol-1,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate as well as the increase in cytosolic calcium in granulocytes. The latter inhibition may be secondary to the inhibition of inositol-1,4,5-trisphosphate synthesis, as cytosolic calcium is released by the action of inositol-1,4,5-trisphosphate. When a low concentration of fMLP (1 nmol/l) was used to stimulate the release of intracellular calcium, the inhibitory potency of sulphasalazine was increased (table 2). A similar finding was reported by Carlin *et al*,⁶ where it was found

that sulphasalazine inhibited granulocyte superoxide production better at lower fMLP concentrations. Kanerud *et al*³² found, by using a single excitation wavelength, that 10 μ M sulphasalazine inhibited the increase in cytosolic calcium in granulocytes stimulated with 100 nM fMLP.

These inhibitory effects of sulphasalazine showed that the drug affected the intracellular signalling system either at the level of plasma membrane receptor, the GTP binding protein coupled to the receptor, or the enzymatic activity of phospholipase C.¹⁵

The first possibility (suggested by Stenson *et al*⁷) was addressed in experiments using electroporated granulocytes activated with the GTP analogue GTP[S], thus bypassing the plasma membrane receptors. Sulphasalazine did inhibit the granulocyte activation with GTP[S], which revealed that the drug effect was not due to interaction with the plasma membrane receptor for fMLP. Inhibition of the fMLP receptor as the major effect of sulphasalazine is unlikely, as earlier studies have shown that the drug inhibits cell activation by several receptor dependent stimuli other than fMLP, such as immune complexes⁸ and zymosan.⁵

Phospholipase C is considered to be activated by the α subunit of the GTP binding protein. This protein is active when associated with a molecule of GTP, and deactivation is accomplished by hydrolysis of the GTP by an intrinsic GTPase activity.¹⁵ It is thus possible that sulphasalazine inhibits granulocyte activation by enhancing the hydrolysis of GTP. No effect of sulphasalazine on the GTPase activity was noted in the experiments designed to study this function, however.

The two products of phospholipase C mediated degradation of phosphatidylinositol-4,5-bisphosphate, inositol-1,4,5-trisphosphate and diacylglycerol, are considered to be the major intracellular signalling substances produced by the cells as a response to fMLP stimulation.¹³ In addition, the importance of phosphatidic acid for cell activation has attracted attention. It is likely that hydrolysis of phosphatidylcholine to phosphatidic acid is of physiological importance as a signal transduction pathway in the activation of, for example, granulocytes,³³⁻³⁴ which emphasises that the phosphatidic acid is more than a precursor, or a metabolite, of diacylglycerol.

The synthesis of phosphatidic acid by granulocytes labelled with glycerol was inhibited by sulphasalazine, thus confirming our earlier results using cells labelled with phosphate-32.⁶ As in these earlier measurements, the recorded phosphatidic acid was mainly derived from diacylglycerol phosphorylated with phosphate-32 by the action of diacylglycerol kinase, the effect of sulphasalazine might have been due to inhibition of either the diacylglycerol kinase or to the synthesis of diacylglycerol.

The inhibition of the phosphatidic acid synthesis by sulphasalazine was similar in magnitude in cells with unspecifically (glycerol) labelled phospholipids and in cells specifically labelled in phosphatidylcholine. This similarity

agrees with some studies which have shown that almost all the diacylglycerol formed during cell activation emanates from dephosphorylation of phosphatidic acid produced by phospholipase D mediated hydrolysis of phosphatidylcholine.²⁷

The effects of sulphasalazine on phospholipase D mediated degradation of phosphatidylcholine were studied by specific labelling of this phospholipid. Sulphasalazine inhibited the synthesis of phosphatidic acid and the subsequent formation of diacylglycerol in cells activated with fMLP. Fluoride induced activation was also inhibited, again demonstrating that the effect of sulphasalazine was not interaction with a plasma membrane receptor. Sulphasalazine did not inhibit PMA induced phospholipase D activity, which clearly shows that the drug did not inhibit phospholipase D, but interfered with an earlier step in the fMLP and fluoride induced activation of this enzyme.

The mechanisms of hormone induced phosphatidylcholine hydrolysis are not known to the same extent as for phosphoinositide hydrolysis. Stimulation of granulocytes with fMLP or fluoride induces phospholipase D mediated phosphatidylcholine hydrolysis in addition to phospholipase C mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate. As cell stimulation with calcium ionophores and protein kinase C activating phorbol esters activates phospholipase D without phospholipase C involvement,³¹ it is possible that phospholipase D activation requires previous phospholipase C mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate, leading to protein kinase C activation by diacylglycerol and increased cytosolic Ca^{2+} by inositol-1,4,5-trisphosphate.³⁵ If so, the inhibition of sulphasalazine or the synthesis of phosphatidylcholine derived phosphatidic acid seen in this study is the consequence of a previous inhibition of phospholipase C. Alternatively, phospholipase D may not require previous phospholipase C activity but may be linked to a GTP binding protein, perhaps different from that linked to phospholipase C,³⁵⁻³⁶ with the possibility that sulphasalazine may also interact with this GTP binding protein.

It can be concluded from the results of this study that sulphasalazine inhibited cellular activation by interaction with the synthesis of phosphoinositide derived second messenger compounds at the level of phospholipase C or its regulatory GTP binding protein. Sulphasalazine also inhibited the synthesis of the putative second messenger phosphatidic acid, which may be due to either the same mechanism, or by interaction with a phospholipase D regulating GTP binding protein. The metabolites of sulphasalazine, 5-aminosalicylic acid and sulphapyridine, were less potent inhibitors than the mother compound.

- 1 Peppercorn M A. Sulfasalazine. Pharmacology, clinical use, toxicity, and related new drug development. *Ann Intern Med* 1984; **101**: 377-86.
- 2 Buxbaum J. Sulfasalazine in arthritis: current practice and future directions. *Intern Med Spec* 1990; **11**: 79-90.
- 3 Gupta A K, Ellis C N, Siegel M T, *et al*. Sulfasalazine improves psoriasis. *Arch Dermatol* 1991; **126**: 487-93.
- 4 Kitis E, Weissmann G. The role of the neutrophil in rheumatoid arthritis. *Clin Orthop* 1991; **265**: 63-72.

- 5 Molin L, Stendahl O. The effect of sulfasalazine and its active components on human polymorphonuclear leucocyte function in relation to ulcerative colitis. *Acta Med Scand* 1979; **206**: 451-7.
- 6 Carlin G, Djursäter R, Smedegård G. Inhibitory effects of sulfasalazine and related compounds on superoxide production by human polymorphonuclear leukocytes. *Pharmacol Toxicol* 1989; **65**: 121-7.
- 7 Stenson W F, Mehta J, Spilberg I. Sulfasalazine inhibition of binding of N-formyl-methionyl-leucyl-phenylalanine (FMLP) to its receptor on human neutrophils. *Biochem Pharmacol* 1984; **33**: 407-12.
- 8 Neal T M, Vissers C M, Winterbourn C C. Inhibition by nonsteroidal anti-inflammatory drugs of superoxide production and granule enzyme release by polymorphonuclear leukocytes stimulated with immune complexes or formyl-methionyl-leucyl-phenylalanine. *Biochem Pharmacol* 1987; **36**: 2511-7.
- 9 Rhodes J M, Bartholomew T C, Jewell D P. Inhibition of leucocyte motility by drugs used in ulcerative colitis. *Gut* 1981; **22**: 642-7.
- 10 Comer S S, Jasin H E. In vitro immunomodulatory effects of sulfasalazine and its metabolites. *J Rheumatol* 1988; **15**: 580-6.
- 11 Fujiwara M, Mitsui K, Yamamoto I. Inhibition of proliferative responses and interleukin 2 productions by salazosulfapyridine and its metabolites. *Jpn J Pharmacol* 1990; **54**: 121-31.
- 12 Okamoto M, Sasano M, Goto M, et al. Suppressive effect of anti-rheumatic drugs on interleukin-1 β release from human peripheral blood monocytes. *Int J Immunopharmacol* 1991; **13**: 39-43.
- 13 Berridge M J, Irvine R F. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 1984; **312**: 315-21.
- 14 Nishizuka Y. Studies and perspectives of protein kinase C. *Science* 1986; **233**: 305-12.
- 15 Harnett M M, Klaus G G B. G protein regulation of receptor signalling. *Immunol Today* 1988; **9**: 315-20.
- 16 Cross A R. Inhibitors of the leukocyte superoxide generating oxidase: mechanism of action and methods for their elucidation. *Free Radic Biol Med* 1990; **8**: 71-93.
- 17 Wright C D, Kuipers P J, Hoffmann M D, Thuesen D O, Conroy M C. Inhibition of human neutrophil activation by the allergic mediator release inhibitor, CI-949. *Biochem Biophys Res Commun* 1990; **167**: 828-34.
- 18 Hurst N P, French J K, Gorjatschko L, Betts W H. Studies on the mechanism of inhibition of chemotactic tripeptide stimulated human neutrophil polymorphonuclear leucocyte superoxide production by chloroquine and hydroxychloroquine. *Ann Rheum Dis* 1987; **46**: 750-6.
- 19 Abramson S B, Leszczynska-Piziak J, Haines K, Reibman J. Non-steroidal anti-inflammatory drugs: effect on a GTP binding protein within the neutrophil plasma membrane. *Biochem Pharmacol* 1991; **41**: 1567-73.
- 20 Böyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968; **21** (suppl 97): 77-89.
- 21 Rossi F, Della Bianca V, Grzeskowiak M, Bazzoni F. Studies on molecular regulation of phagocytosis in neutrophils. *J Immunol* 1989; **142**: 1652-60.
- 22 Korchak H M, Vossall L B, Zagon G, Ljubich T, Rich A M, Weissmann G. Activation of the neutrophil by calcium-mobilizing ligands. *J Biol Chem* 1988; **263**: 11090-7.
- 23 Grynkiewicz G, Poenie M, Tsien R Y. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440-50.
- 24 Nasmith P E, Mills G B, Grinstein S. Guanine nucleotides induce tyrosine phosphorylation and activation of the respiratory burst in neutrophils. *Biochem J* 1989; **257**: 893-7.
- 25 Pelz C, Matsumoto T, Molski T F P, Becker E L, Sha'afi R. Characterization of the membrane-associated GTPase activity: effects of chemotactic factors and toxins. *J Cell Biochem* 1989; **39**: 197-206.
- 26 Della Bianca V, De Togni P, Grzeskowiak M, Vicentini L M, Di Virgilio F. Cyclic AMP inhibition of phosphoinositide turnover in human neutrophils. *Biochim Biophys Acta* 1986; **886**: 441-7.
- 27 Billah M M, Eckel S, Mullmann T J, Egan R W, Siegel M I. Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. *J Biol Chem* 1989; **264**: 17069-77.
- 28 Cockcroft S, Stutchfield J. ATP stimulates secretion in human neutrophils and HL60 cells via a pertussis toxin-sensitive guanine nucleotide-binding protein coupled to phospholipase C. *FEBS Lett* 1989; **245**: 25-9.
- 29 Ward P A, Cunningham T W, Walker B A M, Johnson K J. Differing calcium requirements for regulatory effects of ATP, ATPgammaS and adenosine on O₂-reponses of human neutrophils. *Biochem Biophys Res Commun* 1988; **154**: 746-51.
- 30 Hartfield P J, Robinson J M. Fluoride-mediated activation of the respiratory burst in electroporabilized neutrophils. *Biochim Biophys Acta* 1990; **1054**: 176-80.
- 31 Agwu D E, McPhail L C, Chabot M C, Daniel L W, Wykle R L, McCall C E. Choline-linked phosphoglycerides. A source of phosphatidic acid and diglycerides in stimulated neutrophils. *J Biol Chem* 1989; **264**: 1405-13.
- 32 Kanerud L, Hafström I, Ringertz B. Effect of sulphasalazine and sulphapyridine on neutrophil superoxide production: role of cytosolic free calcium. *Ann Rheum Dis* 1990; **49**: 296-300.
- 33 Korchak H M, Vossall L B, Haines K A, Wilkenfeld C, Lundquist K F, Weissmann G. Activation of the human neutrophil by calcium-mobilizing ligands II. *J Biol Chem* 1988; **263**: 11098-105.
- 34 Agwu D E, McPhail L C, Sozzani S, Bass D A, McCall C E. Phosphatidic acid as a second messenger in human polymorphonuclear leukocytes. *J Clin Invest* 1991; **88**: 531-9.
- 35 Billah M M, Anthes J C. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J* 1990; **269**: 281-91.
- 36 Anthes J C, Wang P, Siegel M I, Egan R W, Billah M. Granulocyte phospholipase D is activated by a guanine nucleotide dependent protein factor. *Biochem Biophys Res Commun* 1991; **175**: 236-43.