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Short communication

Determination of the cell lytic properties of amphiphilic inhibitors of the cytosolic phospholipase A_2 against human platelets by measuring the liberation of serotonin with high-performance liquid chromatography and fluorescence detection

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Abstract

A procedure for the determination of the influence of detergents on the cell integrity of human platelets by measuring the release of serotonin with high-performance liquid chromatography and fluorescence detection is presented. After exposure of the platelets to the test compounds the cells and cell fragments, respectively, are centrifuged off. In the supernatants the liberated serotonin is determined directly without a further sample clean up. The amphiphilic inhibitors of cytosolic phospholipase A_2 ($cPLA_2$), arachidonyltrifluoromethyl ketone ($AACOCF_3$), palmityltrifluoromethyl ketone ($PACOCF_3$) and methyl arachidonylfluorophosphonate (MAFP), and the polyoxyethylene detergent Brij 58 were investigated for their cell lytic properties with this method. All compounds lysed the platelets liberating serotonin at a concentration of 33 μM. $AACOCF_3$ and Brij 58 even caused cell lysis at lower concentrations. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cell lysis; Cytosolic phospholipase A_2; Serotonin

1. Introduction

The cytosolic phospholipase A_2 ($cPLA_2$) catalyzes the first step of the biosynthesis of several pro-inflammatory lipid mediators such as prostaglandins, leukotrienes, lysophospholipids and the platelet activating factor (PAF) by hydrolyzing arachidonoyl-containing membrane phospholipids. Thus, inhibition of $cPLA_2$ is considered as an attractive target for the design of new anti-inflammatory drugs [1–3].

Regarding the structure of known $cPLA_2$ inhibitors it is noticeable that a greater lipophilic substituent must be present in the molecule to achieve good inhibitory potency [3]. This lipophilic region may be necessary to bind the inhibitor to the enzyme or to enrich the inhibitor in the lipophilic cell membrane, to which the $cPLA_2$ binds before abstracting a single membrane phospholipid for cleavage.

As a consequence of this characteristic, many of the known $cPLA_2$ inhibitors, such as the fluoroderivatives arachidonyltrifluoromethyl ketone ($AACOCF_3$) (1), methyl arachidonylfluorophosphonate (MAFP) (2) and palmityltrifluoromethyl ketone ($PACOCF_3$) (3) [4–6], have a detergent-like struc-

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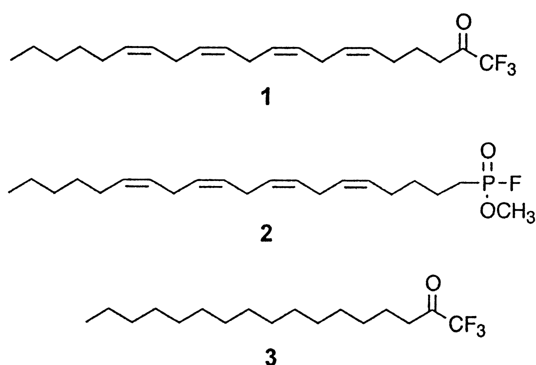

Fig. 1. Structure of the $cPLA_2$ inhibitors $AACOCF_3$ (1), MAFP (2) and $PACOCF_3$ (3).

ture (Fig. 1). Because of surface-activity they may be cytotoxic and lead to cell lysis.

For developing $cPLA_2$ inhibitors we use a test system applying intact platelets [7]. To determine the cell lytic properties of the test compounds we have established two assays. According to published procedures [8–10] the first measures the alteration of the platelet suspension turbidity [11] and the second the release of lactate dehydrogenase (LDH) from the cells [12]. Here we describe a further very simple and reliable procedure for the determination of the influence of an amphiphilic test compound on the integrity of human platelets by measuring the release of serotonin (5-hydroxytryptamine, 5-HT) with high-performance liquid chromatography (HPLC) and fluorescence detection. For the evaluation of the assay the commercially available $cPLA_2$ inhibitors $AACOCF_3$, MAFP and $PACOCF_3$, which have been widely used to determine the biochemical functions of $cPLA_2$, and the polyoxyethylene detergent Brij 58, have been selected as test compounds.

2. Experimental

2.1. Materials

Materials were obtained from the following sources. $AACOCF_3$, MAFP and $PACOCF_3$ (Biomol); Brij 58, 5,8,11,14-eicosatetraynoic acid (ETYA), phosphate-buffered saline tablets, serotonin creatinine sulfate complex, 5-methoxytryptamine (Sigma); 1-octanesulfonic acid sodium salt hydrate (Acros Organics); EDTA–Na_2, DMSO, acetonitrile and methanol HPLC grade (Merck); phosphoric acid 85% (Baker); buffy coats of human blood containing a citrate buffer as anticoagulant (Institute of Transfusion Medicine, University of Münster).

2.2. Preparation of the human platelet suspensions

About 50 ml of buffy coat was centrifuged in three 50 ml polypropylene tubes at 2000 *g* for 2 min at 4 °C and the platelet-rich supernatants were carefully separated by aspiration. In case of a substantial contamination by erythrocytes, the combined supernatants were centrifuged again at 2000 *g* for 1 to 2 min to remove the red blood cells. The obtained platelet-rich fraction was then centrifuged at 1000 *g* for 15 min at 4 °C. The pellet was gently resuspended in a mixture of phosphate-buffered saline and 3.7% aqueous EDTA–Na_2 (97+3, v/v). The volume of this mixture was equal to the volume of the platelet-rich supernatant obtained before. The suspension was centrifuged at 1000 *g* for 15 min at 4 °C and the platelets were resuspended in phosphate-buffered saline. The final cell concentration was adjusted to about 10^8 cells/ml (0.1 ml of this cell suspension diluted with 0.9 ml of phosphate-buffered saline gave an absorbance of 0.10 at 800 nm). The platelets were stored at 4 °C. The further experiments were carried out within 48 h.

2.3. Measurement of cell lysis by turbidimetry

A volume of 2.5 μl of a DMSO solution of ETYA (1.19 mg/ml), which was added to achieve the same conditions as in our assay for the evaluation of $cPLA_2$ inhibition [7], and 2.5 μl of a DMSO solution of the test compound (in the case of the control tests 2.5 μl of DMSO were taken instead) and 1.0 ml of the suspension of washed human platelets in phosphate-buffered saline were incubated in ground glass borosilicate tubes in a shaking water bath at 37 °C. After 10 min the glass tubes were taken out of the water bath and after further 5 min the absorbance was measured in semi-microcuvettes at 800 nm. For evaluation of cell lysis the absorbance in the presence of a test compound was compared with the mean level of the absorbance obtained in the absence of test compounds (control tests, $n=3$). Cell lysis led

to a decrease of absorbance. The absorbance of the control tests was about 1; linearity between cell concentration and absorbance (range 0–1) was proven. All data were analyzed by Student's *t*-test. A value of $P<0.05$ was considered significant.

2.4. Measurement of cell lysis by determination of the liberated serotonin

A volume of 2.5 μl of a DMSO solution of ETYA (1.19 mg/ml) and 2.5 μl of a DMSO solution of the test compound (in the control tests 2.5 μl of DMSO were taken instead) and 1.0 ml of the suspension of washed human platelets in phosphate-buffered saline were incubated in ground glass borosilicate tubes in a shaking water bath at 37 °C. After 10 min the glass tubes were placed into an ice-water mixture for about 5 min. Then the suspension was centrifuged for 10 min at 1000 *g* and 4 °C. A 0.5 ml aliquot of the supernatant was added to 0.5 ml of a 50 ng/ml internal standard (5-methoxytryptamine) solution in methanol–acetonitrile (1:1, v/v). The samples were stored at −80 °C until subjection to HPLC.

2.5. Instrumentation and HPLC conditions for the determination of serotonin

The HPLC system consisted of a Bischoff HPLC-pump model 2200, a Waters autosampler model Wisp 710 B, a Merck–Hitachi fluorescence detector model F-1050 and a Merck–Hitachi Chromato-Integrator model D-2000. Separation was achieved on a Waters HPLC column symmetry C_{18}, 4.6 mm (I.D.)×75 mm, particle size 3.5 μm. The mobile phase consisted of acetonitrile–water–phosphoric acid (85%) (20:80:0,1, v/v/v) containing 2.5 m*M* sodium octane-1-sulfonate (pH of the mobile phase: 2.2). The flow-rate was 0.7 ml/min and the injected sample volume was 20 μl. The fluorescence detector was set at an excitation wavelength of 285 nm and emission was monitored at 340 nm. For evaluation of cell lysis the serotonin concentration in the presence of a test compound was compared with the mean level of serotonin obtained in the absence of test compounds (control tests, $n=4$). Cell lysis led to an increase of serotonin concentration. All data were analyzed by Student's *t*-test. A value of $P<0.05$ was considered significant.

2.5.1. Measurement of cPLA$_2$ inhibitory potency

Inhibition of cPLA$_2$ was determined by measuring the calcium ionophore A23187-induced arachidonic acid release from human platelets with HPLC/UV-detection as previously described [7]. Briefly, to a solution of ETYA in DMSO, which inhibits formation of arachidonic acid metabolites in platelets, was added the DMSO solution of a test compound or DMSO alone (control tests) followed by the platelet suspension at 37 °C. Then cPLA$_2$ was activated by the calcium ionophore A23187. After termination of the enzyme reaction the produced arachidonic acid was cleaned up by solid-phase extraction and quantified with HPLC/UV-detection at 200 nm.

3. Results and discussion

Human platelets differ from other formed elements of blood in their ability to store high concentrations of serotonin. This mediator is localized in secretory granules of these cells. Thus, the release of serotonin has been used as an indicator for cytotoxicity of detergents against platelets. In these experiments the liberation of [^{14}C]serotonin from prelabeled platelets by liquid scintillation counting was measured [13,14]. Alternatively, the released endogenous serotonin was quantified by fluorometry after derivatization with *o*-phthaldialdehyde [9,15]. However, a direct detection of the endogenous serotonin as a marker for cell lysis using HPLC has not been described until now.

To determine the release of serotonin we incubated the platelets suspension in the absence and presence of test compounds under the conditions applied for measuring cPLA$_2$ inhibition [7]. However, instead of starting the cPLA$_2$ enzyme reaction by addition of calcium ionophore A23187, the cells were centrifuged at 1000 *g* for 10 min. An aliquot of the supernatant was added to a mixture of acetonitrile and methanol containing 5-methoxytryptamine as the internal standard. Then the samples were stored at −80 °C until subjection to HPLC analysis. Experiments concerning the stability of serotonin

showed that a change of the concentration of both serotonin and internal standard did not occur at this temperature in between 6 weeks. The HPLC was performed similar to a method described for the determination of serotonin in blood and urine [16] using a RP18 column and a mobile phase consisting of dilute phosphoric acid and acetonitrile containing the ion-pair reagent sodium octane-1-sulfonate. A fluorimetric detector was used applying 285 nm as the excitation wavelength and 340 nm as the emission wavelength. The calibration graphs were linear for the range investigated (1 to 225 ng/ml serotonin) yielding correlation coefficients higher than 0.998.

In the absence of test compounds the mean level of serotonin in the supernatant of the platelet preparations obtained from seven different blood buffy coats varied between 6 and 44 ng/ml (cell concentration: about 10^8 cells/ml). However, the variation of the control tests in between an incubation series was low (RSD <5%, n=4), so even relatively small elevations of the serotonin concentrations in the supernatant caused by a test compound could be considered as significant.

The effect of the $cPLA_2$ inhibitors $AACOCF_3$, MAFP and $PACOCF_3$ on the serotonin liberation of the platelets is shown in Table 1. The trifluoromethylketone $AACOCF_3$ led to the release of serotonin at 33 μ*M* (Fig. 2) and 10 μ*M*, whereas at 3.3 μ*M* no significant increase of serotonin in the extracellular fluid could be measured. The parallel determination of the cell lysis by turbidimetry led to a partly different result. With this method only at 33

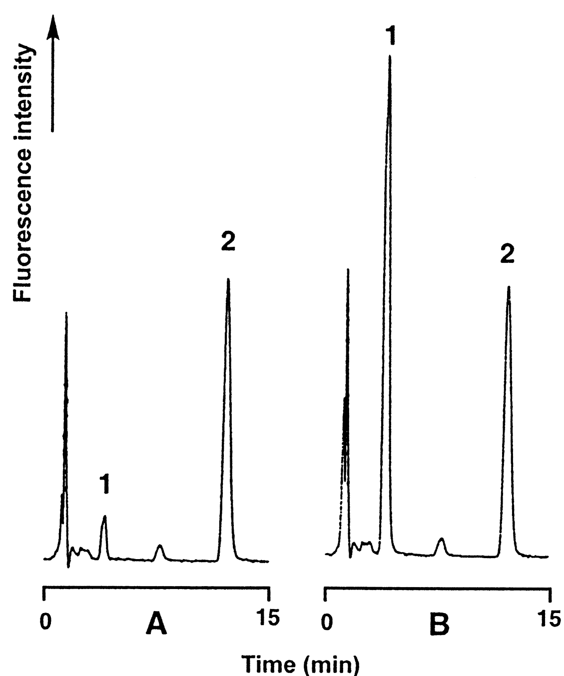

Fig. 2. HPLC chromatograms of serotonin in the supernatant of platelet suspensions: (A) Non-treated platelets. (B) Platelets treated with 33 μ*M* $AACOCF_3$. Peaks: 1=serotonin; 2=internal standard (5-methoxytryptamine).

Table 1
Cell lytic potency of the amphiphilic $cPLA_2$-inhibitors $AACOCF_3$, MAFP and $PACOCF_3$ and the polyoxyethylene detergent Brij 58 against human platelets

Compound	Concentration	Cell lytic potency[a]	
		Decrease of turbid (%)	Liberated serotonin (ng/ml cell suspension)
$AACOCF_3$	33 μ*M*	22±6	81±33
	10 μ*M*	n.s.	10±6
	3.3 μ*M*	n.s.	n.s.
MAFP	33 μ*M*	n.s.	64±10
	10 μ*M*	n.s.	n.s.
	3.3 μ*M*	n.s.	n.s.
$PACOCF_3$	33 μ*M*	3±0.4	9±6
	10 μ*M*	n.s.	n.s.
	3.3 μ*M*	n.s.	n.s.
Brij 58	33 μ*M*	84±5	129±53
	10 μ*M*	n.s.	15±9
	3.3 μ*M*	5±0.5	9±5

[a] Values are means±SD (n=3); n.s., not significantly different from control.

μ*M* cell damage was detected. At 10 μ*M*, however, $AACOCF_3$ did not cause any significant decrease in turbidity, probably because membrane lysis is being compensated by some kind of aggregation phenomenon [8]. Thus, the method measuring serotonin liberation detects cell lysis more sensitively than the turbidimetric assay in this case. The same result was obtained when evaluating the cell damaging properties of MAFP. While an affection of the cell integrity could not be seen with the turbidimetric method at 33 μ*M*, greater amounts of serotonin were liberated at this concentration by the compound. At 10 and 3.3 μ*M* cytotoxic effects of MAFP were detectable with neither method.

A slight but significant lysis of the cells by $PACOCF_3$ could be observed with the turbidimetric and the serotonin assay at a concentration of 33 μ*M*. At lower concentrations a decrease of turbidity and an increase of serotonin liberation, respectively, could not be measured any more.

To evaluate the margin between the $cPLA_2$-inhibitory potency and the cell lytic properties, we also determined the inhibition of $cPLA_2$ by the fluoroderivates 1–3 in intact human platelets monitoring the calcium ionophore A23187-induced arachidonic acid formation as previously described [7]. For $AACOCF_3$ and MAFP IC_{50}-values (inhibitor concentration, at which a 50% inhibition of the enzyme activity could be achieved) of about 3 μ*M* were obtained (Table 2). The IC_{50} of $PACOCF_3$ was greater than 10 μ*M* (32% inhibition at this concentration). The exact IC_{50} for this substance could not be determined, since at the next concentration stage examined (33 μ*M*) already cell lysis took place. Such a destruction of the cells, however, may erroneously indicate enzyme inhibition. This could be shown by measuring arachidonic acid liberation after disruption of the cells by sonication, or by freezing and thawing. In both cases, in the absence of an enzyme inhibitor, a significant decrease of arachidonic acid release was measured compared with the experiments performed with intact cells [11]. Comparing the results presented in Table 1 and 2 it may be said that the margin between enzyme inhibitory and cell lytic potency is greatest for MAFP.

Finally, we investigated the cell lytic effect of the polyoxyethylene detergent Brij 58. This detergent was reported to cause a release of $[^{14}C]$serotonin from preloaded washed human platelets at concentrations exceeding 40 μ*M* [13]. With our method we could detect significant liberation of endogenous serotonin already at a concentration of 3.3 μ*M* (Table 1). Determining the cell toxic properties of Brij 58 by the turbidimetric method afforded an interesting result. With this assay, a high cell lysis was seen at 33 μ*M*, while at 10 μ*M* the reduction of absorbance was no more significant. However, at the lower concentration of 3.3 μ*M* again a significant decrease of turbidity could be seen. This finding again shows that the turbidimetric assay does not indicate the destruction of cells in all cases and the determination of the serotonin release, which can be easily and reliably measured with HPLC applying fluorescence detection, more sensitively reflects cell lysis caused by detergent-like compounds.

Table 2
$cPLA_2$-inhibitory potency of the fluoroderivatives $AACOCF_3$, MAFP and $PACOCF_3$ in intact human platelets stimulated with calcium ionophore A23187

Compound	IC_{50} (μ*M*)[a]
$AACOCF_3$	3.3±0.5
MAFP	2.9±0.4
$PACOCF_3$	>10 μ*M* (32±7% inhibition at 10 μ*M*)

[a] Values are means±SD (n=3).

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