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Suppression of receptor-mediated Ca^{2+} mobilization and functional leukocyte responses by hyperforin

Christian Feißt, Oliver Werz*

Institute of Pharmaceutical Chemistry, University of Frankfurt, D-60439 Frankfurt, Germany

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Abstract

We have recently identified hyperforin, a lipophilic constituent of the herb *Hypericum perforatum* (St. John's wort), as a dual inhibitor of the proinflammatory enzymes cyclooxygenase-1 and 5-lipoxygenase. The aim of the present study was to further elucidate antiinflammatory properties and respective targets of hyperforin. We found that hyperforin inhibited the generation of reactive oxygen species (ROS) as well as the release of leukocyte elastase (degranulation) in human isolated polymorphonuclear leukocytes (PMNL), challenged by the G protein-coupled receptor (GPCR) ligand *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) with an $IC_{50} \approx 0.3$ μM. When PMNL were stimulated with phorbol-12-myristate-13-acetate (PMA) or ionomycin, hyperforin (up to 10 μM) failed to inhibit ROS production and elastase release, respectively. Moreover, hyperforin blocked receptor-mediated Ca^{2+} mobilization ($IC_{50} \approx 0.4$ and 4 μM, respectively) in PMNL and monocytic cells, and caused a rapid decline of the intracellular Ca^{2+} concentration in resting cells. In contrast, the Ca^{2+} influx induced by ionomycin or thapsigargin was not suppressed. Comparative studies with the specific phospholipase C inhibitor U-73122 and hyperforin revealed similarities between both compounds. Thus, U-73122 and hyperforin blocked fMLP- and PAF-induced Ca^{2+} mobilization, ROS formation, and elastase release, but failed to suppress these responses when cells were stimulated by PMA or ionomycin. Also, both compounds rapidly decreased basal Ca^{2+} levels in resting cells and led to a rapid decline of the Ca^{2+} elevations evoked by fMLP or PAF. Our data suggest that hyperforin targets component(s) within G protein signaling cascades that regulate Ca^{2+} homeostasis, coupled to proinflammatory leukocyte functions.

Keywords: Calcium; G protein-coupled receptors; Hyperforin; *Hypericum perforatum*; Reactive oxygen species; Phospholipase C

1. Introduction

Hyperforin is assumed as one of the main active constituents of *Hypericum perforatum*, a traditional herbal medicine effective for the treatment of mild to moderate depressive disorders. Hyperforin has been shown to inhibit the reuptake of several neurotransmitters and clinical effects of *H. perforatum* extracts on depression correlate with its hyperforin content [1–3]. However, the knowledge about the molecular target(s) of hyperforin are unknown and the precise mode of action of how hyperforin exerts its antidepressive effects is incompletely understood.

In addition to its antidepressive properties, hyperforin possesses also antiinflammatory activity, by inhibiting the proliferation and induction of apoptosis of peripheral blood mononuclear cells [4,5]. Moreover, hyperforin blocks 5-LO and COX-1, two crucial enzymes in the biosynthesis of proinflammatory eicosanoids [6], and clinical studies revealed significant benefit of hyperforin in the topical treatment of mild to moderate atopic dermatitis [7]. Thus, hyperforin may act as an antiinflammatory agent, presumably at multiple levels.

Many inflammatory cells respond to signals transduced via GPCR, which directly impact several functional cellular responses related to inflammation and allergy [8]. Upon ligation of GPCRs by proinflammatory agonists,

* Corresponding author. Tel.: +49-69-798-29337; fax: +49-69-798-29323.
E-mail address: o.werz@pharmchem.uni-frankfurt.de (O. Werz).

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DAG, diacylglycerol; DCF-DA, 2′,7′-dichlorofluorescein diacetate; ER, endoplasmatic reticulum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GPCR, G protein-coupled receptor; 13(*S*)-HPODE, 13(*S*)-hydroperoxyoctadecadienoic acid; InsP3, inositol 1,4,5-trisphosphate; LO, lipoxygenase; MM6, Mono Mac 6; PAF, platelet-activating factor; PBS, phosphate buffered saline pH 7.4; PGC buffer, PBS containing 1 mg/mL glucose and 1 mM $CaCl_2$; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PMNL, polymorphonuclear leukocytes; ROS, reactive oxygen species; SR, sarcoplasmatic reticulum; TGFβ, transforming growth factor beta.

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such as PAF, fMLP, or leukotriene B_4, the Gα subunit of the G protein releases GDP which is then replaced by GTP, resulting in a separation of the Gα− from the Gβγ subunit. Both subunits may activate PLC isoenzymes, which catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate, leading to the production of two intracellular second messengers, InsP3 and DAG [9]. InsP3 binds to specific InsP3 receptors mediating the release of Ca^{2+} from internal stores that, together with DAG, activates PKC isoenzymes [10]. These signaling molecules regulate numerous functional cellular responses of leukocytes including chemotaxis, degranulation, formation of ROS, and the release of AA and its transformation to eicosanoids.

Our previous report showed that hyperforin potently inhibits the biosynthesis of proinflammatory eicosanoids [6]. In the present study, we sought to investigate if hyperforin may also interfere with other proinflammatory responses of leukocytes such as the oxidative burst and degranulation (e.g. elastase release). Our data show that hyperforin potently inhibits the formation of ROS and the release of elastase in fMLP-stimulated PMNL, apparently by interference with G protein signaling cascades leading to a suppression of receptor-mediated Ca^{2+} mobilization.

2. Materials and methods

2.1. Materials

Hyperforin sodium salt was kindly provided by Dr. S.S. Chatterjee. The compound was dissolved in dimethylsulfoxide (DMSO) and kept in the dark at −20°. Freezing thawing cycles were kept to a minimum. Since the results obtained with hyperforin from a certain stock were consistent over time, the compound appeared stable during storage and handling. TGFβ was purified from outdated platelets as described [11], calcitriol was a gift from Schering AG. Materials used: Nycoprep, PAA Laboratories; ionomycin, thapsigargin, AA, PMA, MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanailide, cytochalasin B, and fMLP, Sigma; PAF and 13(*S*)-HPODE, Cayman; Fura-2/AM and U-73122, Alexis; DCF-DA, Molecular Probes.

2.2. Cells and incubations

MM6 cells were cultured and differentiated with TGFβ and calcitriol as described [12]. Human PMNL were freshly isolated from leukocyte concentrates obtained at St. Markus Hospital. In brief, venous blood was taken from healthy adult donors, leukocyte concentrates were prepared by centrifugation and PMNL were immediately isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories), and hypotonic lysis of erythrocytes as described previously [13]. PMNL (purity > 96–97%) were finally resuspended in PBS containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated. For inhibitor studies, hyperforin or U-73122, dissolved in DMSO, or vehicle (control) were added to the cells, which were kept in the dark for 20 min at RT prior cell stimulation. To exclude toxic effects of hyperforin during these preincubation periods, control experiments with PMNL and MM6 cells were performed to investigate cell viability by trypan blue exclusion. Preincubation with hyperforin (for up to 60 min) caused no significant change in cell viability and there were no differences in the inhibitory effects of hyperforin that may depend on the length (20 or 60 min) of the preincubation periods.

2.3. Determination of cellular peroxide formation

Measurement of peroxides in PMNL was conducted using the peroxide-sensitive fluorescence dye DCF-DA, that reacts with hydrogen peroxide, but also with nitric oxide [14]. Freshly isolated PMNL (1×10^7 in 1 mL PGC buffer) were preincubated with hyperforin, U-73122, or vehicle (DMSO (control)) for 20 min at RT in the dark, and then treated with DCF-DA (1 μg/mL) for 2 min at 37° prior addition of the stimuli. The fluorescence emission at 530 nm was measured after excitation at 480 nm in a thermally controlled (37°) fluorimeter cuvette with continuous stirring in a spectrofluorometer (Aminco-Bowman series 2). The mean fluorescence data measured 5 min after stimulus addition are expressed as arbitrary fluorescence units.

2.4. Determination of leukocyte elastase release

PMNL (5×10^7), resuspended in 1 mL PGC buffer, were preincubated with hyperforin, U-73122, or vehicle (DMSO (control)) for 20 min at RT in the dark. For stimulation with 1 μM fMLP, cells were preincubated with cytochalasin B (10 μM) for 5 min at 37°, ionomycin was used as stimulus without pre-treatment with cytochalasin B. The reaction was terminated after 10 min at 37° by placing the samples on ice for 2 min. After centrifugation (1000 *g*, 5 min, 4°), the supernatants were incubated with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanailide (200 μM) for 5 min at 37°. The extent of *p*-nitrophenol release was measured at 405 nm. Direct effects of hyperforin on elastase activity were determined by addition of hyperforin to supernatants of cytochalasin B + fMLP-stimulated PMNL and subsequent incubation with substrate for 5 min at 37°.

2.5. Measurement of intracellular Ca^{2+} levels

PMNL (1×10^7) or differentiated MM6 cells (3×10^6) in 1 mL PGC buffer were incubated with 2 μM Fura-2/AM for 30 min at 37°, washed, resuspended in 1 mL PGC

buffer and preincubated with hyperforin, U-73122, or vehicle (DMSO (control)) for 20 min at RT in the dark. Then, cells were transferred into a thermally controlled (37°) fluorimeter cuvette in a spectrofluorometer (Aminco-Bowman series 2) with continuous stirring and stimuli were added. The fluorescence emission at 510 nm was measured after excitation at 340 and 380 nm, respectively. Intracellular Ca^{2+} levels were calculated according to the method of Grynkiewicz *et al.* [15]. F_{max} (maximal fluorescence) was obtained by lysing the cells with 1% Triton-X 100 and F_{min} by chelating Ca^{2+} with 10 mM EDTA.

2.6. Determination of PLC activity in vitro

PLC activity in 100,000 *g* supernatants of differentiated HL60 cells and isolated PMNL from human blood was assayed by measuring the hydrolysis of [^{3}H]-phosphadtidylinositol 4,5-bisphosphate into inositol phosphates as described [16].

2.7. Statistics

The statistic program "GraphPad PRISM 3.0" was used for statistical comparisons.

3. Results

3.1. Hyperforin suppresses G protein-mediated formation of reactive oxygen species and leukocyte elastase release in PMNL

Freshly isolated PMNL were preincubated with hyperforin for 20 min at RT, challenged with natural occurring agonists that involve G protein signaling such as fMLP (1 μM), PAF (100 nM), or AA (40 μM), as well as with the PKC activator PMA (100 nM), and the formation of ROS was detected using the peroxide-sensitive dye DCF-DA. Hyperforin dose-dependently suppressed fMLP-induced ROS production with an IC_{50} value of approximately 0.3 μM (Fig. 1). Similarly, stimulation with 40 μM AA, that caused prominent ROS production, was clearly inhibited by hyperforin ($IC_{50} \approx 0.3$ μM, not shown). In agreement with others [17,18], PAF failed in marked ROS formation in PMNL, thus an exact determination of the inhibitory potency of hyperforin appeared difficult and was not further examined. In contrast to fMLP and AA, ROS formation induced by PMA was not significantly affected by hyperforin up to 10 μM (Fig. 1). Moreover, hyperforin (3 or 10 μM) did not alter the oxidation of DCF-DA by exogenously added 13(*S*)-HPODE (3 μM) in a cell-free system (not shown). Thus, hyperforin appears to block ROS formation induced by natural agonists, by interfering with G protein signaling pathways, rather than by acting as an antioxidative agent or as a direct inhibitor of ROS-producing enzymes such as NADPH oxidase.

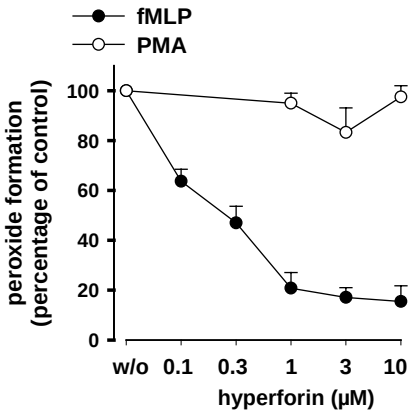

Fig. 1. Effects of hyperforin on agonist-induced formation of reactive oxygen species in PMNL. Freshly isolated PMNL (10^7 in 1 mL PGC buffer) were preincubated with the indicated concentrations of hyperforin for 20 min at RT prior addition of DCF-DA (1 μg/mL) for 2 min. Then, fMLP (1 μM) or PMA (100 nM) were added and the generation of ROS was measured as described in Section 2. Data determined 5 min after addition of stimuli are expressed as percentage of the positive control ± SE, N = 4. The fluorescence of unstimulated, fMLP-challenged, and PMA-treated cells was 80 ± 20, 315 ± 72, and 808 ± 171 arbitrary fluorescence units, respectively.

In accordance with the results from above, hyperforin also suppresses elastase release, presumably by interference with G protein signaling pathways. Thus, treatment of PMNL with hyperforin for 20 min at RT, dose-dependently inhibited the release of leukocyte elastase after subsequent stimulation with cytochalasin B plus fMLP (10 and 1 μM, respectively) with an $IC_{50} \approx 0.4$ μM (Fig. 2). No such inhibitory properties of hyperforin were apparent for PMNL stimulated with ionomycin, and hyperforin up to 3 μM caused only weak inhibition of elastase itself.

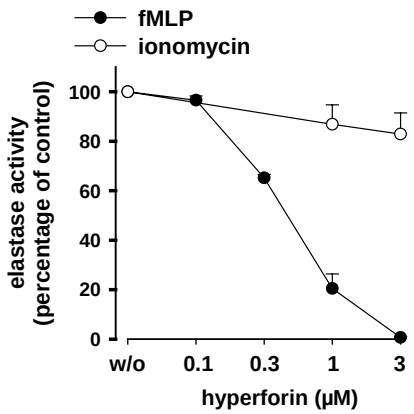

Fig. 2. Effects of hyperforin on agonist-induced elastase release from PMNL. Freshly isolated PMNL (10^7 in 1 mL PGC buffer) were preincubated with hyperforin for 20 min at RT. For stimulation with 1 μM fMLP, cells were preincubated with cytochalasin B (10 μM) for 5 min, ionomycin (1 μM) was used as stimulus without pre-treatment with cytochalasin B. After 10 min at 37°, cells were centrifuged and the supernatants were assayed for elastase activity as described in Section 2. Data determined are expressed as percentage of the control (stimulated cells without inhibitor) ± SE, N = 4.

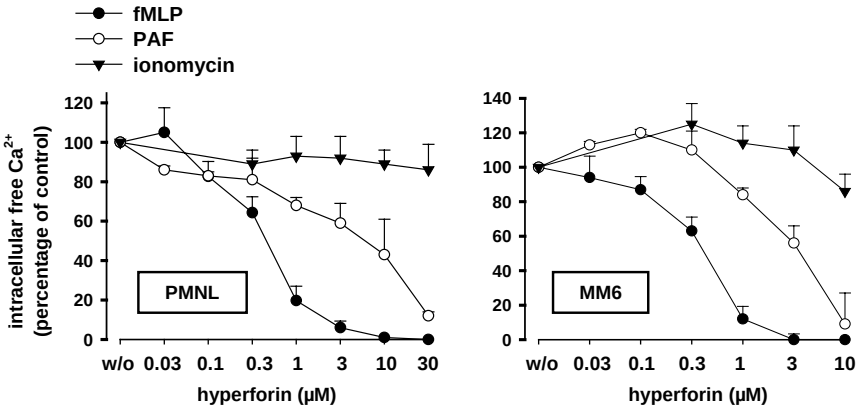

Fig. 3. Hyperforin inhibits receptor-coupled Ca^{2+} mobilization in leukocytes. Freshly isolated PMNL (10^7) or differentiated MM6 cells (3×10^6) in 1 mL PGC buffer were loaded with 2 μM Fura-2/AM. Cells were preincubated in the absence (control) or presence of hyperforin at the indicated concentrations for 20 min at RT and stimulated with 100 nM fMLP, 100 nM PAF or 1 μM ionomycin at 37°. The fluorescence was measured and intracellular free Ca^{2+} was calculated as described. Results are expressed as the percentage of the Ca^{2+} level (mean ± SE, N = 3–4) vs. control (100%). Stimulation with fMLP, PAF, or ionomycin elevated the intracellular Ca^{2+} levels from 145 ± 10 nM to 289 ± 10, 257 ± 27, or 840 ± 63 nM in MM6 cells and from 60 ± 8 nM to 266 ± 41, 257 ± 66, or 403±11 nM in PMNL, respectively.

3.2. Effects of hyperforin on intracellular Ca^{2+} levels in PMNL and MM6 cells

Ca^{2+} is considered important for agonist-induced leukocyte activation [19] and we investigated if hyperforin could affect the mobilization of Ca^{2+}, thereby inhibiting the agonist-induced ROS production and elastase release. In accordance with others [20,21], removal of Ca^{2+} by chelation with 1 mM EDTA and 30 μM BAPTA/AM abolished fMLP-induced ROS formation and elastase release in PMNL (not shown). Preincubation with hyperforin for 20 min at RT, dose-dependently prevented Ca^{2+} mobilization in PMNL challenged by fMLP or PAF (Fig. 3), or AA (not shown). Interestingly, the IC_{50} values were approximately 0.4–0.6 μM hyperforin when fMLP or AA was used as agonist, whereas 10-fold higher concentrations of hyperforin were required when cells had been stimulated with PAF ($IC_{50} \approx 5$ μM). Similarly, also in the monocytic cell line MM6, pretreatment with hyperforin for 20 min at RT, potently blocked the Ca^{2+} release induced by fMLP with an $IC_{50} \approx 0.4$ μM, whereas also 10-fold higher concentrations of hyperforin were required to suppress the Ca^{2+} influx induced by PAF ($IC_{50} \approx 4$ μM, Fig. 3). In control experiments, hyperforin up to 10 μM failed to block Ca^{2+} mobilization in PMNL or MM6 cells evoked by ionomycin (Fig. 3) or thapsigargin (not shown), that circumvent G protein signaling.

Variation of the preincubation period (1 up to 20 min) with hyperforin prior agonist addition had no significant influence on the potency of hyperforin. Also, hyperforin was capable to immediately (within seconds) abrogate the fMLP- or PAF-induced elevation of Ca^{2+} (Fig. 4A). Moreover, we observed a rapid and dose-dependent decline of the basal intracellular Ca^{2+} levels in resting cells within about 5–10 s after addition of hyperforin (Fig. 4B).

Stimulation of leukocytes with fMLP or PAF causes Ca^{2+} release from internal stores such as the ER/SR, followed by an entry of Ca^{2+} from the external medium. Accordingly, chelation of external Ca^{2+} by 1 mM EDTA impaired the fMLP- or PAF-induced Ca^{2+} response in PMNL (Fig. 4C). When hyperforin was added to EDTA-treated PMNL for 20 min at RT, elevation of agonist-induced intracellular Ca^{2+} mobilisation was blocked (Fig. 4C), indicating that hyperforin affects the Ca^{2+} release from internal stores.

3.3. Hyperforin shares properties with the PLC inhibitor U-73122

Release of Ca^{2+} from internal stores by agonists that ligate GPCR involves the stimulation of PLC isoenzymes that generate InsP3, which in turn releases stored Ca^{2+} by binding to InsP3 receptors [9]. U-73122 selectively inhibits PLC-dependent processes [22,23] and blocks receptor-coupled Ca^{2+} increases [24]. It appeared possible that, in analogy to U-73122, hyperforin could block Ca^{2+} mobilization by inhibiting PLC isoenzymes. Although hyperforin and U-73122 possess rather distinct chemical structures (Fig. 5A), U-73122 showed comparable effects as hyperforin with respect to Ca^{2+} homeostasis. Thus, the fMLP- or PAF-induced Ca^{2+} increases in PMNL or MM6 cells were prevented by U-73122 (IC_{50} around 2–5 μM, Fig. 5B), but U-73122 failed to suppress Ca^{2+} mobilization evoked by ionomycin. Notably, in contrast to hyperforin, there was no marked difference in the potency of U-73122 for cells challenged with fMLP or PAF. Also, addition of U-73122 caused a significant and rapid decrease of the basal intracellular Ca^{2+} levels in resting cells, as observed with hyperforin (Fig. 5C), and led to a rapid decline of the Ca^{2+} elevations evoked by fMLP (Fig. 5D) or PAF (not shown). Finally, U-73122 suppressed fMLP-induced ROS

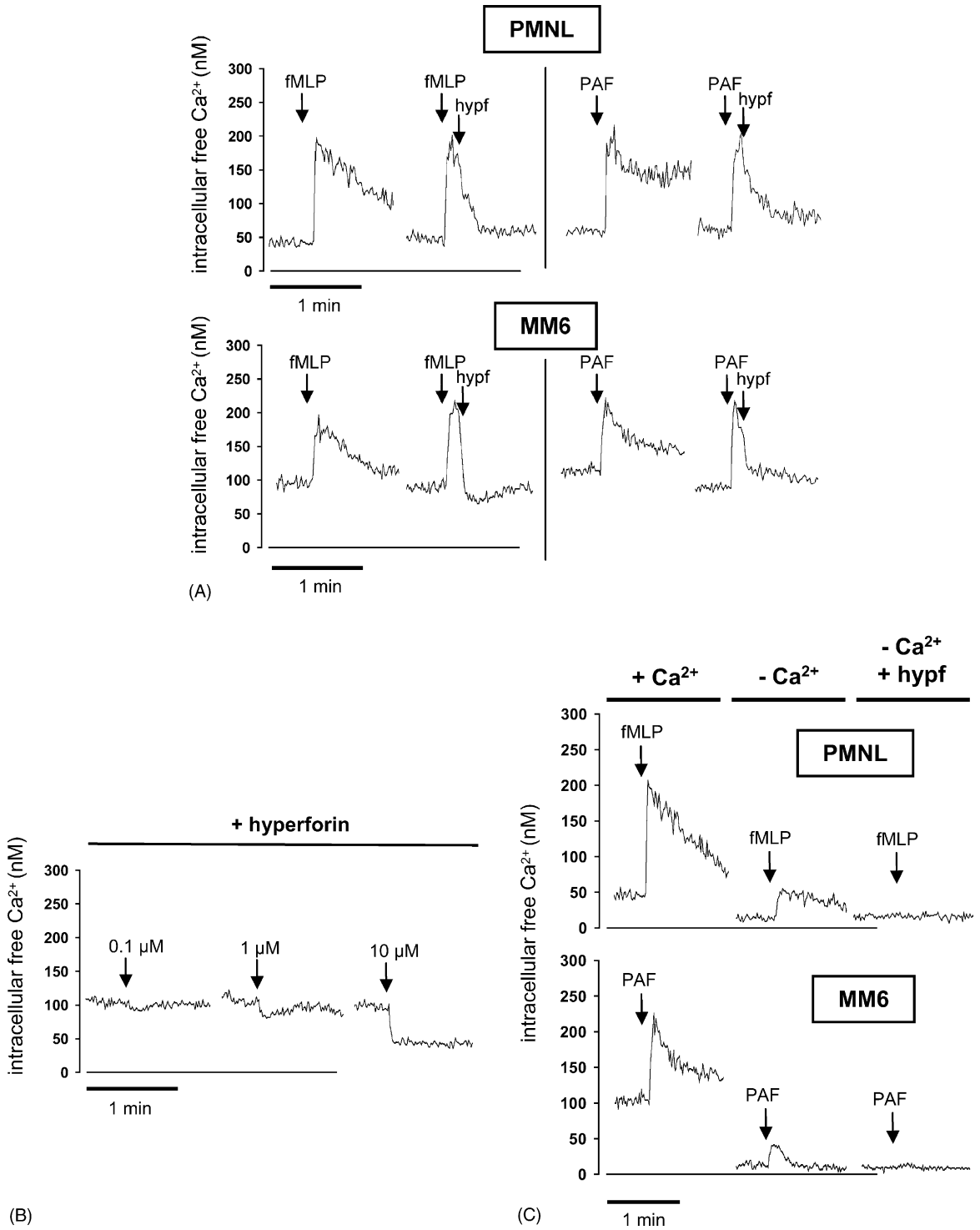

Fig. 4. Characteristics of hyperforin on Ca^{2+} homeostasis. (A) Hyperforin immediately abrogates agonist-induced Ca^{2+} elevations. PMNL (1×10^7, upper panel) or MM6 cells (3×10^6, lower panel) in 1 mL PGC buffer were loaded with 2 μM Fura-2/AM. Then, cells were stimulated with 100 nM fMLP or 100 nM PAF at 37°, and after 10 s, hyperforin (3 μM) was added. The fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of 3. (B) Decline of intracellular Ca^{2+} levels in resting cells. To Fura-2/AM-loaded MM6 cells the indicated concentrations of hyperforin were added, the fluorescence was recorded and intracellular free Ca^{2+} was calculated. The monitored curves show one typical experiment out of 3. (C) PMNL (1×10^7, upper panel) or MM6 cells (3×10^6, lower panel) in 1 mL PGC buffer were loaded with 2 μM Fura-2/AM and resuspended in either PGC buffer ($+Ca^{2+}$) or Ca^{2+}-free PG buffer containing 1 mM EDTA ($-Ca^{2+}$). Cells were preincubated with 3 μM hyperforin at RT, as indicated. After 20 min, cells were stimulated with 100 nM fMLP (PMNL) or 100 nM PAF (MM6 cells) at 37°, the fluorescence was recorded and intracellular free Ca^{2+} was calculated. The monitored curves show one typical experiment out of 3.

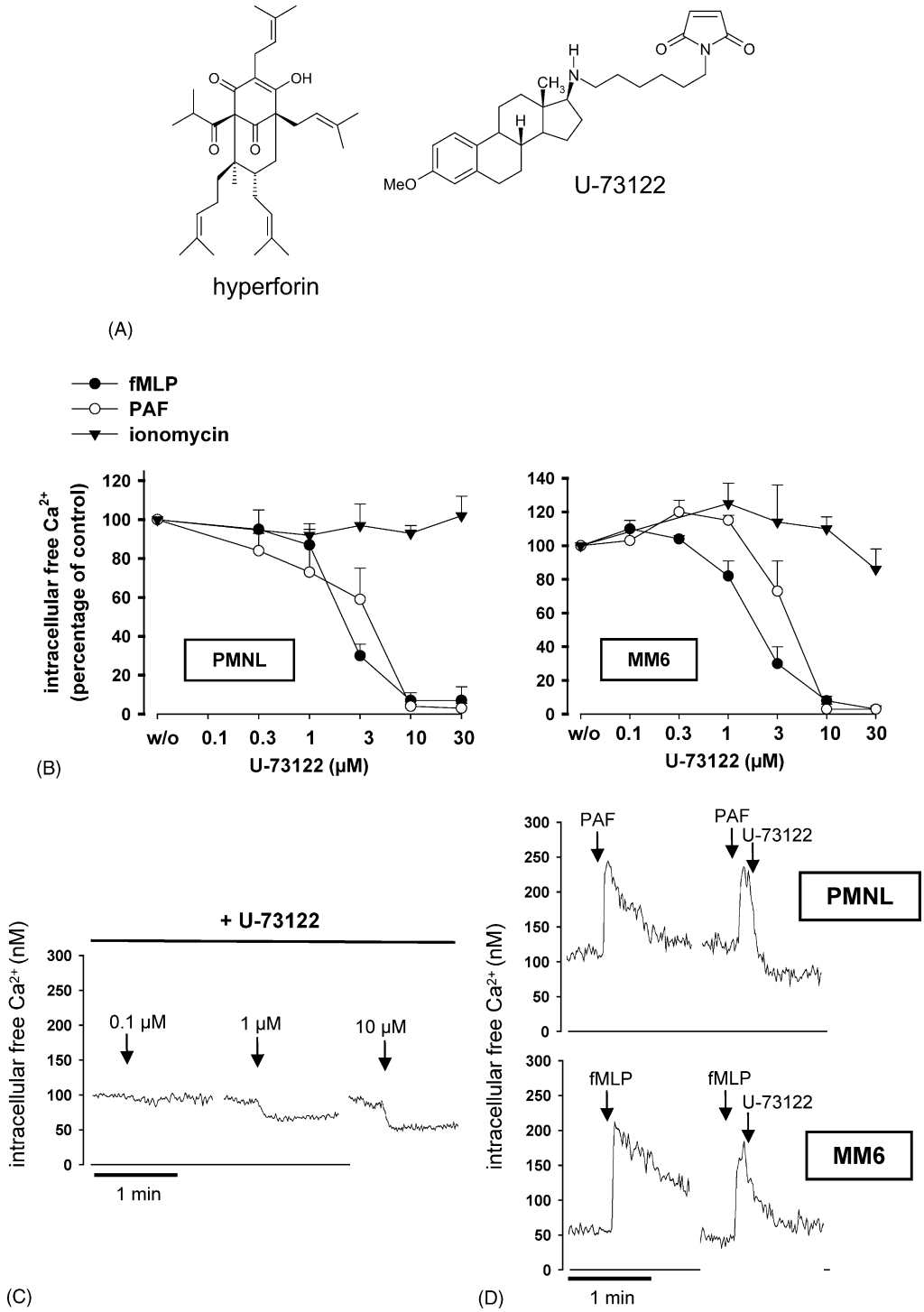

Fig. 5. Effects of U-73122 on Ca^{2+} homeostasis. (A) Chemical structures of hyperforin and U-73122. (B) U-73122 differentially inhibits agonist-induced Ca^{2+} mobilization. PMNL (1×10^7) or MM6 cells (3×10^6) in 1 mL PGC buffer were loaded with 2 μM Fura-2/AM, preincubated in the absence (control) or presence of U-73122 at the indicated concentrations for 20 min at RT and stimulated with 100 nM fMLP, 100 nM PAF or 1 μM ionomycin at 37°. The fluorescence was measured and intracellular free Ca^{2+} was calculated. Results are expressed as the percentage of the maximal Ca^{2+} level (mean ± SE, N = 3) vs. control (100%). (C) Decline of intracellular Ca^{2+} levels in resting cells. To Fura-2/AM-loaded MM6 cells the indicated concentrations of U-73122 were added, the fluorescence was recorded and intracellular free Ca^{2+} was calculated. The monitored curves show one typical experiment out of 3. (D) U-73122 immediately abrogates receptor-coupled Ca^{2+} elevations. PMNL (1×10^7) or MM6 cells (3×10^6) in 1 mL PGC buffer were loaded with 2 μM Fura-2/AM. Then, PMNL were stimulated with 100 nM PAF and MM6 cells were stimulated with 100 nM fMLP at 37°, and after 10 s, U-73122 (10 μM) was added. The fluorescence was measured and intracellular free Ca^{2+} was calculated as described. The monitored curves show one typical experiment out of 3–4.

generation and elastase release ($IC_{50} \approx 3$ μM, each) in PMNL, but failed to substantially inhibit ROS formation after stimulation with PMA or to suppress ionomycin-induced elastase release (not shown).

In order to test if, in analogy to U-73122, also hyperforin acts as a broad spectrum inhibitor of PLC enzymes, an *in vitro* PLC activity assay using 100,000 g supernatants of PMNL or differentiated HL60 cells as source of PLC isoenzymes was performed. Whereas U-73122 dose-dependently blocked PLC activity, hyperforin caused no significant inhibition of PLC activity up to 50 μM (not shown). Thus, hyperforin does not seem to function as a broad spectrum PLC inhibitor *in vitro*.

4. Discussion

We show here that in PMNL, hyperforin suppresses the formation of ROS and the release of leukocyte elastase in response to the proinflammatory GPCR ligand fMLP. Investigation of the signal transduction pathways suggests that hyperforin suppresses these leukocyte functions by its ability to block agonist-induced Ca^{2+} mobilization, presumably by interfering with components within G protein signaling cascades. Although comparative cellular studies with the PLC inhibitor U-73122 suggest that hyperforin could target PLC isoenzymes, direct inhibition of PLC activity *in vitro* by hyperforin could not be demonstrated, thus the precise mode of action of hyperforin remains unclear.

The formation of ROS in PMNL can be induced by naturally occurring agonists such as fMLP, PAF or AA that involve G protein signaling [20,25,26], but also by direct activation of PKC using PMA [27]. Hyperforin dose-dependently blocked ROS formation evoked by fMLP or AA, but not so in response to PMA that circumvents G protein signaling for ROS production. Accordingly, hyperforin does not directly interfere with ROS-producing enzymes and should also not act as an anti-oxidative compound that scavenges ROS (such as 13(*S*)-HPODE), but rather seems to interfere with proximal G protein signaling routes. These findings are in agreement with a recent study demonstrating that hyperforin suppresses the fMLP-induced oxidative burst in neutrophils, but fails to directly reduce oxygen radicals produced by horseradish peroxidase in an enzymatic assay [28]. In analogy to the effect of hyperforin on ROS formation, only (fMLP) receptor-mediated but not ionomycin-induced elastase release was inhibited by hyperforin. Since the IC_{50} values of hyperforin (approximately 0.3–0.4 μM) regarding fMLP-induced leukocyte functions and Ca^{2+} mobilization fit well to the plasma levels (0.2–0.38 μM) obtained after oral intake of *H. perforatum* extracts at standard dosage [29], these effects of hyperforin could be of pharmacological relevance in the therapy of inflammatory disorders.

Upon receptor-coupled challenge, elevation of intracellular Ca^{2+} is an important signaling step for leukocyte activation [19,20], and in our study removal of intra- and extracellular Ca^{2+} by chelation with BAPTA/AM and EDTA completely prevented agonist-induced ROS formation and elastase release. In fact, hyperforin, at the same concentrations that were necessary to reduce ROS formation and elastase release, blocked the elevation of intracellular Ca^{2+} in PMNL and MM6 cells induced by various GPCR ligands, suggesting that depletion of intracellular Ca^{2+} could be the reason for reduced ROS formation and elastase release. However, hyperforin did not interfere with Ca^{2+} mobilization induced by ionomycin or thapsigargin, that circumvent G protein signaling. Thus, suppression of Ca^{2+} mobilization by hyperforin depends on the stimulus and the proximal signaling routes. Interestingly, it was found that in cortical synaptosomes, hyperforin elevates the intracellular Ca^{2+} levels [30], implying that the effect of hyperforin with respect to Ca^{2+} homeostasis may also depend on the cell type.

Cellular Ca^{2+} levels may increase by release from intracellular stores followed by an influx of extracellular Ca^{2+} [10]. Our results using EDTA to chelate extracellular Ca^{2+} demonstrate that hyperforin abolishes the fMLP-induced liberation of Ca^{2+} from intracellular stores, like the ER or the SR. Such Ca^{2+} mobilization is mediated by ligation of InsP3 receptors, located on the ER or SR [10]. Upon ligand binding to GPCR, InsP3 is produced via a pathway involving direct activation of certain PLC isoenzymes by Gα or Gβγ subunits of activated G proteins, depending on the type of subunit (for review, see [10]). U-73122 is a broad spectrum PLC inhibitor [22] that was found to suppress Ca^{2+} mobilization in many cell types, including platelets stimulated with either thrombin or thromboxane mimetics [23], as well as for agonist-stimulated PMNL [31]. Although the chemical structures of the compounds are rather distinct, hyperforin shared many properties with U-73122 regarding Ca^{2+} homeostasis. First, both compounds blocked fMLP- and PAF-induced intracellular Ca^{2+} release in PMNL or MM6 cells, but failed to suppress Ca^{2+} mobilization induced by thapsigargin or ionomycin. Second, U-73122 and hyperforin led to a rapid decline of the Ca^{2+} elevations evoked by fMLP or PAF. Third, both compounds rapidly decreased basal Ca^{2+} levels in resting cells. Based on these similar properties, it appears reasonable to speculate that hyperforin could act as a PLC inhibitor. However, hyperforin failed to suppress total PLC activity in an *in vitro* assay using 100,000 *g* supernatants of PMNL or HL60 cells as source for PLC isoenzymes, excluding that hyperforin acts as a broad spectrum PLC inhibitor. Nevertheless, hyperforin may be selective for distinct PLC isoenzymes, and those isoforms that might be potently inhibited, may account for only a small amount of the total PLC activity present in 100,000 *g* supernatants of PMNL or HL60 cells. Such speculations are favoured by the finding that hyperforin, in contrast to U-73122, differentially reduced Ca^{2+} increases in response to fMLP and PAF. Thus, 10-fold

higher concentrations of hyperforin were required to block the PAF response as compared to fMLP, whereas the potency of U-73122 was the same. At least 11 distinct isoforms of PLC isoenzymes, which are grouped into four subfamilies (β, γ, δ, and ε) have been identified in mammals [9]. These PLC isoenzymes are differentially recruited by distinct Gα subunits of G proteins ($G\alpha_i$, $G\alpha_q$, $G\alpha_s$ $G\alpha_0$, for review, see [32]) and distinct GPCRs associate with different G proteins [33]. For example, the fMLP receptor is coupled to a $G\alpha_i$ subunit that in turn preferentially activates PLC-β2, whereas the PAF receptor couples to $G\alpha_i$ as well as to $G\alpha_q$, the latter prefers PLC-β1 and PLC-β3 [9]. Thus, hyperforin may preferentially inhibit (fMLP receptor-coupled) PLC-β2 over (PAF receptor-coupled) PLC-β1 and PLC-β3. Alternatively, hyperforin may require an intact cellular environment or intracellular transformation into an active form to efficiently inhibit PLC. Finally, other components within the GPCR signaling cascades, such as certain G protein subunits ($G\alpha_i$ of the fMLP receptor vs. $G\alpha_q$ of the PAF receptor), regulators of G protein signaling (RGS), or InsP3 receptors must be taken into account as potential targets of hyperforin, which remain to be investigated.

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