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Release of anandamide from blood cells

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Abstract

Background: Endogenous ligands of cannabinoid receptors (endocannabinoids), in particular anandamide (arachidonylethanolamide), have been recognized as being of crucial importance in a variety of physiological functions. Plasma concentrations of anandamide have been measured in a number of investigations; however, discrepant data on ''normal'' anandamide plasma concentrations were reported. Since this might be caused by pre-analytical variables, we investigated the impact of different sample handling conditions on measured plasma anandamide concentrations.

Methods: Blood samples were taken from healthy volunteers in EDTA- or heparin-containing tubes; whole blood samples were kept at +4°C, room temperature, or 37°C, respectively, for up to 120 min before obtaining plasma by centrifugation. Plasma anandamide concentrations were measured by an isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method.

Results: A marked time- and temperature-dependent increase in plasma anandamide concentrations ex vivo was observed in both EDTA- and heparin-containing tubes. Mean anandamide concentrations approximately doubled when EDTA samples were kept at 4°C for 60 min before centrifugation [immediately centrifuged, 1.3 μg/L (SD 0.3 μg/L); 2.8 μg/L (SD 0.5 μg/L) after storage for 60 min; n=12). After storage of heparinized whole-blood samples for 120 min at 37°C, a mean plasma anandamide concentration of 11.9 μg/L (SD 1.8 μg/L) was found. In cell-free plasma, no increase in anandamide concentrations was found.

Conclusion: Anandamide is released from blood cells ex vivo at a very high rate; therefore, strictly standardized pre-analytical protocols have to be applied for plasma anandamide determination.

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Introduction

In the early 1990s, two G-protein-coupled receptors binding Δ-9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana, were characterized (CB1 and CB2) (1–3). Subsequently, amides and esters of long-chain polyunsaturated fatty acids have been identified as endogenous ligands of cannabinoid receptors; among these ''endocannabinoids'', arachidonylethanolamide (synonym anandamide) seems to be the most important (4–6).

During recent years, it has become evident that the interaction of endocannabinoids and CB receptors is part of fundamental regulatory mechanisms in numerous physiological processes.

CB_1 receptors have been found in most areas of the brain, generally with a presynaptic localization, where they play important roles in controlling neurotransmitter release (7, 8). There is strong evidence that central endocannabinoid effects are involved in cognition, pain processing, mood, memory, control of food intake and energy balance, and in problem drug use (9–11). Recently, rimonabant, a CB_1 receptor antagonist, has been introduced for the treatment of drug addiction and obesity-related disorders (12).

CB_2 receptors have been found primarily in peripheral locations, especially on immune and endothelial cells (3). CB_2 receptor-based peripheral endocannabinoid signaling is involved in regulation of the immune system (13–15), apoptosis (16, 17) and vasomotor activity (18–20). Recently, a link between common CB_2 receptor gene polymorphisms and autoimmune diseases was suggested (21).

Endocannabinoid-inactivating enzymes, particularly fatty acid amide hydrolase (FAAH), have been proposed as therapeutic targets for a variety of diseases (22, 23).

Plasma concentrations of anandamide have been studied in a number of investigations (24–29); interestingly, wide variations of mean plasma anandamide concentrations were reported in cohorts of healthy volunteers [e.g., Fernandez-Rodriguez et al. (27), mean anandamide concentration 0.13 μg/L; De Marchi et al. (24), mean concentration 0.9 μg/L] (conversion of units for anandamide: [nmol/L]×0.35→[μg/L]; [μg/L]×2.87→[nmol/L]). In these studies, reliable and accurate isotope dilution mass spectrometry methods were applied, but the authors gave no detailed information on preanalytical sample handling. Since nucleated blood cells are known to be capable of both the release and metabolization of anandamide (30), preanalytical variables may account for the discrepant observations on ''normal'' plasma anandamide con-

centrations. Therefore, we decided to study the impact of these variables on plasma anandamide concentrations systematically.

Materials and methods

Analytical method

For anandamide quantification, we used a novel, semi-automated, isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method. A Waters Alliance 2795 HPLC module coupled to a Waters Micromass Quattro Ultima Pt MS/MS system (Waters, Milford, MA, USA) was used. For calibration, pure solutions of anandamide in methanol/water were used (1–50 μg/L). As an internal standard, fourfold deuterium-labeled anandamide was synthesized as previously described (31) (Figure 1); the purity of this material was 97.2%.

For protein precipitation, 200 μL of acetonitrile (containing 50 μg/L labeled anandamide) was added to 100 μL of plasma. After vortex mixing and centrifugation at 16,000×*g* for 15 min, a clear supernatant was obtained and transferred into HPLC vials. Aliquots of 30 μL of this preparation were injected onto an extraction column (Waters Oasis HLB® column; 25 μm, 2.1×20 mm). The column was washed for 1 min with water/methanol (95:5 v/v) at a flow rate of 4 mL/min. Using column switching with a six-port high-pressure switching valve, the extraction column was then eluted in back-flush with methanol/1 mM ammonium acetate (90:10 v/v at 0.8 mL/min) and the extract was transferred onto the analytical column (LiCrospher® 100 RP-18, 125×4 mm, 5 μm; Merck, Darmstadt, Germany) kept at 37°C. The extraction column was subsequently washed with acetonitrile/methanol (50:50 v/v; 1 min at 4 mL/min). The eluate from the analytical column was transferred at a split ratio of approximately 1:10 to the LC-MS/MS system. Electrospray atmospheric-pressure ionization in the positive mode was used. The source parameters were set to obtain the protonated quasi-molecular ions of anandamide and the labeled internal standard compound (capillary voltage, 2.3 kV; cone voltage, 50 V; source temperature, 120°C; desolvation temperature, 350°C; nitrogen flow, approx. 550 L/h; collision energy, 10 V). The following transitions were recorded in multiple-reaction monitoring: anandamide, 348→62; labeled anandamide, 352→66. The retention time of anandamide was approximately 5.4 min. The method was linear from 0.8 to 50 μg/L and was reproducible, with inter-assay coefficients of variation of 6.8% and 5.3% for mean anandamide concentrations of 2.1 and 12.8 μg/L, respectively (n=12). No significant differences in the peak areas of deuterated anandamide were observed when pure solutions or plasma samples were spiked, ruling out relevant ion-suppression effects. The lower limit of detection of the method (defined as a signal/noise ratio ≥4:1) was 0.5 μg/L. A representative chromatogram is shown in Figure 2.

Experiments

In a first study, 12 healthy volunteers were included after written informed consent. Blood was collected by venipuncture into five EDTA-containing tubes (S-Monovette® EDTA; Sarstedt, Numbrecht, Germany). One of the tubes was centrifuged within 5 min after venipuncture and plasma was immediately frozen (−20°C). Two tubes were kept at +4°C and were centrifuged after 60 or 120 min, respectively, to obtain plasma, which was then frozen immediately. Two further tubes were kept at room temperature (22–25°C) for 60 or 120 min before centrifugation.

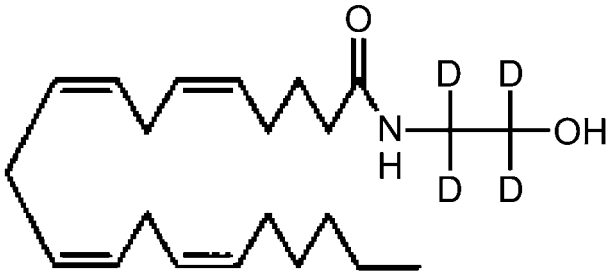

Figure 1 Chemical structure of deuterated anandamide used for internal standardization.

In plasma samples obtained immediately after venipuncture, a mean anandamide concentration of 1.3 μg/L was found (SD 0.3 μg/L). In plasma of samples kept at +4°C for 60 and 120 min, mean concentrations of 2.8 μg/L (SD 0.5 μg/L) and 3.8 μg/L (SD 0.4 μg/L), respectively, were measured ($p<0.05$; paired Wilcoxon test). In plasma of samples kept at room temperature, mean concentrations of 3.8 μg/L (SD 1.0 μg/L) after 60 min and 5.2 μg/L (SD 0.6 μg/L) after 120 min were found ($p<0.05$). Thus, a significant time- and temperature-dependent increase in plasma anandamide concentrations was observed. This may be explained by the release of anandamide from circulating immune cells (19, 30).

We suspected that this release might in part be the consequence of non-physiological stimulation of leukocytes due to calcium chelation by EDTA and a temperature below 37°C in the sample tubes. Therefore, in a second set of experiments, we used heparin-anticoagulated whole blood samples (S-Monovette® Lithium-Heparin; Sarstedt) kept at +4°C, room temperature, or 37°C for 30, 60 and 120 min. In samples from five healthy volunteers, a time- and temperature-dependent increase in anandamide concentrations was again observed (Figure 3). The most marked increase in anandamide concentrations was found in samples stored at 37°C; at this temperature, a mean ''Δ anandamide'' of 7.5 μg/L (SD 1.4 μg/L) was observed between 30 and 120 min after venipuncture. Lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), potassium, and glucose were analyzed in the plasma samples obtained immediately and after incubation at 37°C for 120 min, respectively. While glucose concentrations decreased by a mean of 19%, no significant changes in the concentrations of the other analytes were observed.

In a third set of experiments, whole blood samples from ten healthy volunteers were precipitated with a 4:1 mixture of methanol/zinc sulfate 50 g/L immediately after sampling (200 μL of whole blood and 200 μL of precipitation solution); after centrifugation, supernatants were analyzed for anandamide. In all cases the anandamide signal was below the predefined lower limit of detection (<0.5 μg/L).

To study the stability of anandamide in cell-free plasma, aliquots of six plasma samples obtained during the incubation experiments were kept at room temperature for 24 h prior to analysis (concentration range 2.0–14.0 μg/L). A decrease in mean anandamide concentration of 9.5% within 24 h was observed.

The investigation protocols for all experiments were approved by the institutional Review Board.

Results and discussion

We were able to demonstrate extensive release of anandamide from whole blood cells ex vivo. This

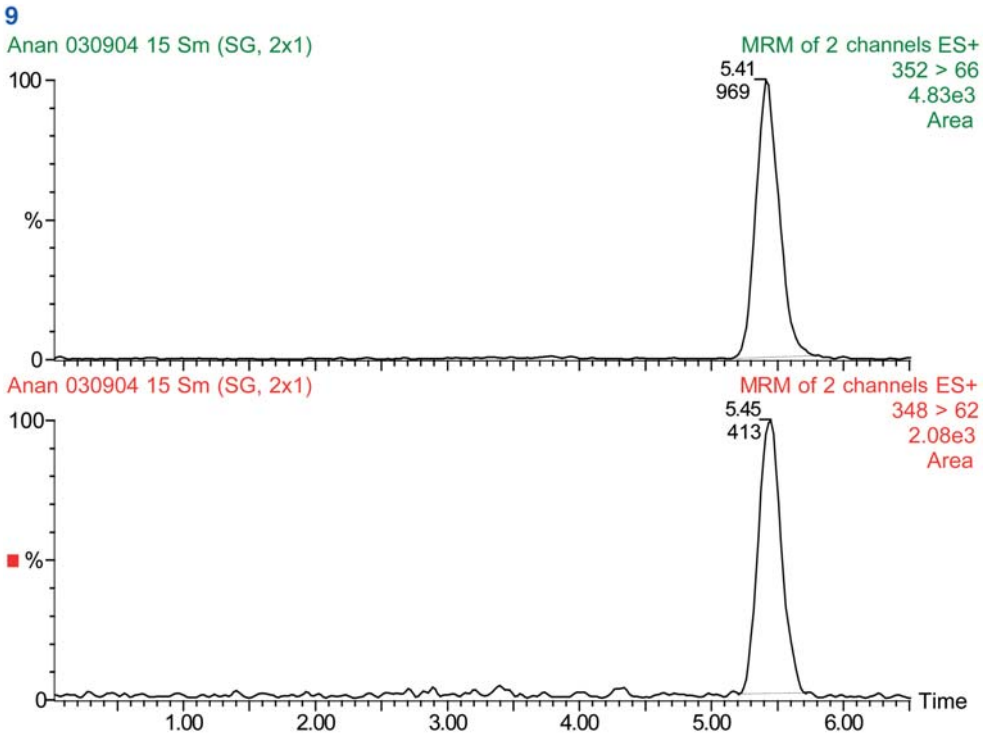

Figure 2 Representative chromatogram of anandamide and deuterated anandamide used as the internal standard obtained from a human whole-blood sample (concentration of anandamide, 4.3 μg/L; Y-axis represents the relative intensity of the signal with respect to the baseline of the chromatogram).

release may result from mobilization of pre-existing anandamide from cellular structures of blood cells, from de novo synthesis and exocytosis of anandamide ex vivo by blood cells, or from a combination of both.

To clarify this issue, we precipitated whole blood samples by adding methanol immediately after venipuncture under the assumption that this procedure would block all metabolic processes and disintegrate cell membranes. After centrifugation, we found only trace concentrations of anandamide in the supernatant. It cannot be completely ruled out that anandamide in part undergoes co-precipitation with proteins in this experiment; however, our observation rather suggests de novo synthesis of anandamide than release of pre-existing anandamide to cause ex vivo increases in plasma anandamide concentrations.

We conclude from our results that the determination of baseline plasma anandamide concentrations has to follow strictly standardized pre-analytical protocols with immediate sample centrifugation. Even if this protocol is followed, it cannot be completely ruled out that anandamide is released into plasma during centrifugation.

Stasis, hypoxia, and the foreign surface of a sample tube may trigger anandamide synthesis and release from blood cells; the potential of leukocytes to form anandamide has been demonstrated previously (19, 30). The in vivo baseline rate of anandamide synthesis in these cells under physiological conditions, however, remains uncertain. Besides preanalytical considerations, our data demonstrate that blood cells actually posses a very high anandamide release capacity in response to stressful stimuli; this may be of functional relevance for the peripheral CB_2 receptor-based endocannabinoid system (14–21).

Furthermore, our results demonstrate the feasibility of characterizing the anandamide release capacity of blood cells using a standardized ex vivo sequential plasma sampling protocol from incubated heparinized whole blood, providing a ''Δ anandamide'', e.g., between 30 and 120 min after venipuncture. During this time interval, we found a linear increase in anandamide concentrations in samples from healthy vol-

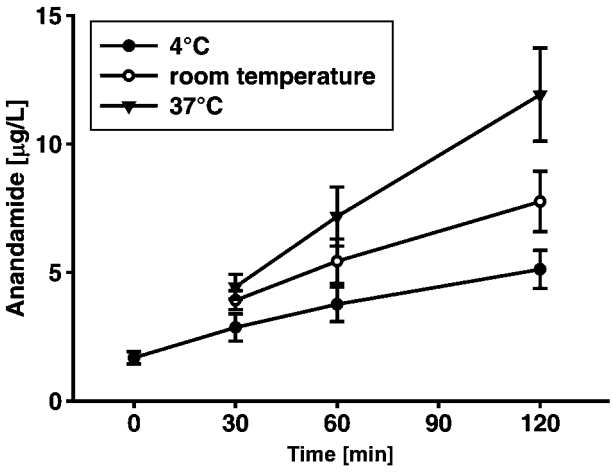

Figure 3 Anandamide concentrations found in plasma of heparinized whole-blood samples incubated at various temperatures for different time intervals (mean and SD; n = 5). Conversion of units for anandamide: [nmol/L] × 0.35 → [μg/L]; [μg/L] × 2.87 → [nmol/L].

unteers (Figure 3). We plan to use this sampling principle in further studies to investigate blood-cell anandamide synthesis capacity in health and different disease states.

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