α-Hederin, but Not Hederacoside C and Hederagenin from *Hedera helix*, Affects the Binding Behavior, Dynamics, and Regulation of β_2-Adrenergic Receptors[†]

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ABSTRACT: Hederacoside C, α-hederin, and hederagenin are saponins of dry extracts obtained from the leaves of ivy (*Hedera helix* L.). Internalization of β_2-adrenergic receptor−GFP fusion proteins after stimulation with 1 μM terbutaline was inhibited by preincubation of stably transfected HEK293 cells with 1 μM α-hederin for 24 h, whereas neither hederacoside C nor hederagenin (1 μM each) influenced this receptor regulation. After incubation of A549 cells with 5 nM Alexa532-NA, two different diffusion time constants were found for β_2AR−Alexa532-NA complexes by fluorescence correlation spectroscopy. Evaluation of the autocorrelation curve revealed diffusion time constants: $\tau_{bound1} = 1.4 \pm 1.1$ ms ($n = 6$) found for receptor−ligand complexes with unrestricted lateral mobility, and $\tau_{bound2} = 34.7 \pm 14.1$ ms ($n = 6$) for receptor−ligand complexes with hindered mobility. The distribution of diffusion time constants was 24.3 ± 2.5% for τ_{bound1} and 8.7 ± 4.3% for τ_{bound2} ($n = 6$). A549 cells pretreated with 1 μM α-hederin for 24 h showed dose-dependent alterations in this distribution with 37.1 ± 5.5% for τ_{bound1} and 4.1 ± 1.1% for τ_{bound2}. Simultaneously, the level of Alexa532-NA binding was significantly increased from 33.0 ± 6.8 to 41.2 ± 4.6%. In saturation experiments, α-hederin did not influence the β_2-adrenergic receptor density (B_{max}), whereas the K_D value for Alexa532-NA binding decreased from 36.1 ± 9.2 to 24.3 ± 11.1 nM. Pretreatment of HASM cells with α-hederin (1 μM, 24 h) revealed an increased intracellular cAMP level of 13.5 ± 7.0% under stimulating conditions. Remarkably, structure-related saponins like hederacoside C and hederagenin did not influence either the binding behavior of β_2AR or the intracellular cAMP level.

Extracts obtained from the leaves of the common ivy *Hedera helix* L. are often used in the treatment of upper respiratory tract conditions characterized by hypersecretion of a viscous mucus (i.e., catarrh) and coughing. These extracts have also been applied as adjuvants for the treatment of inflammatory bronchial disease. Clinical improvements in lung function have been observed, on the basis of spirometric and plethysmographic measurements, as well as for the accompanying signs of coughing and expectoration (*1*−*4*). Moreover, ivy leaf extracts are spasmolytic, reducing smooth muscle spasm, as well as bronchodilatory and antibacterial, which is mainly due to their triterpene saponin content (*5*−*7*). Saponins are considered as the active substances and have been used for the standardization of herbal medicines (*6, 8*). Approximately 10% of the ethanolic ivy extract consists of a complex mixture of saponins with the bis-desmoside hederacoside C as the main component, together with smaller amounts of the monodesmoside α-hederin and the aglycon hederagenin. The expectorant activity of saponins is thought to be mediated by the gastric mucosa, with reflex stimulation of the bronchial mucous glands via parasympathic pathways. This mode of action, however, cannot explain the bronchiolytic effects, as shown by dose-dependent inhibition of PAF-induced bronchoconstriction in guinea pigs by an ethanolic ivy extract (*9*), or by in vitro inhibition of acetylcholine-induced spasm in guinea pig ileum by α-hederin and hederagenin (*6*). These findings suggest that the ivy leaf extract has β_2-adrenergic mimetic activity, and this could explain both secretolytic and bronchiolytic effects.

In this paper, we describe the influence of the major saponins from *H. helix* on the regulation of β_2-adrenergic receptor activity using live cell imaging and fluorescence correlation spectroscopy and on the intracellular cAMP[1] level.

EXPERIMENTAL PROCEDURES

Substances. α-Hederin, hederacoside C, and hederagenin were obtained from HWI-Analytics (Rülzheim, Germany).

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[1] Abbreviations: Alexa-NA, Alexa532-arterenol; cAMP, cyclic adenosine 3′,5′-monophosphate; HASM, human airway smooth muscle; FCS, fluorescence correlation spectroscopy; β_2AR, β_2-adrenergic receptor; β_2AR−GFP, β_2-adrenergic receptor−green fluorescent protein fusion.

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Terbutaline hemisulfate was purchased from Sigma (Taufkirchen, Germany). All other reagents were obtained from Merck (Darmstadt, Germany), if not otherwise stated.

Alexa532-Arterenol. The synthesis, identity, and binding behavior of Alexa532-arterenol (Alexa-NA) at β_2-adrenergic receptors were reported by our group (*10*).

Cell Culture. Human cancer cell line A549 was obtained from DSMZ (No. ACC 107) (Braunschweig, Germany). The cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum (all from Gibco, Invitrogen, Karlsruhe, Germany). For fluorescence correlation spectroscopy experiments, cells were seeded at a density of $1.7-3.4 \times 10^4$ cells/cm^2 on heat-sterilized glass coverslips (#1, ∅18 mm, Marienfeld, Lauda-Königshofen, Germany) and cultured in 12-well chambers (Nunc, Langenselbold, Germany) at 37 °C and 5% CO_2. Cells were used for experiments after being in culture for 3−4 days at 80−90% confluency.

Human kidney cells (HEK293) obtained from DSMZ (No. ACC 305) were cultivated in DMEM-F12 medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% fetal calf serum. For live cell imaging, cells were plated onto heat-sterilized glass coverslips (#1, ∅ 18 mm, Marienfeld) at a density of $2.5-5 \times 10^4$ cells/cm^2 in 12-well chambers (Nunc) and cultivated for 2−4 days at 37 °C and 5% CO_2. After reaching 70−80% confluency, cells were used for experiments.

Human airway smooth muscle cells (HASM) were obtained from I. Hall (Institute of Pharmaceutical Science and Experimental Therapeutics, University of Nottingham, Nottingham, U.K.). They were grown in DMEM-F12 medium supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. For the cAMP assay, 1×10^3 cells per well were seeded in a 96-well plate (Nunc) and cultivated at 37 °C and 5% CO_2.

Tranfection of HEK293 Cells. HEK293 cells were stably transfected with a plasmid encoding the β_2-adrenergic receptor fused to green fluorescent protein (β_2AR−GFP). The cloning procedure was described previously (*11*). The β_2AR−GFP construct was a kind donation of M. J. Lohse (Department of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany). For transfection, cells were seeded at a density of 2.5×10^3 cells/cm^2 in cell culture dishes (3.5 cm diameter, Sarstedt, Nümbrecht, Germany). Two micrograms of β_2AR−GFP plasmid was mixed with 6.6 μL of ExGen 500 reagent (Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions, and cells were incubated with the transfection solution for 3 h.

To establish a stable cell line, transfected cells were selected by adding 500 μg/mL G418-sulfate (PAA Laboratories GmbH, Linz, Austria) to the culture medium 72 h post-transfection. Clones expressing β_2AR−GFP were identified by fluorescence microscopy and separated for further growth 2 weeks after transfection.

Fluorescence Correlation Spectroscopy (FCS). FCS setup and instrumentation were described previously (*10*). Briefly, measurements were performed with a ConfoCor instrument (Zeiss, Jena, Germany) equipped with an argon laser (LGK 7812 ML 2, Lasos, Jena, Germany). For excitation, the 514 nm line of the laser was separated by an appropriate filter (515 FS 10-25, Andover, Salem, MA) and focused through a water immersion objective (C-Apochromat, 63×, NA 1.2) into the sample (laser power $p_{514} = 2.4$ kW/cm^2). The emitted fluorescence was separated from the excitation light with a dichroic filter (FT540, Andover) and from the background noise with a bandpass filter (EF530-600, Andover). After passing a variable pinhole (40 μm), the intensity fluctuations were detected by an avalanche single-photon counting module (SPCM-AG Series, PerkinElmer Optoelectronics, Fremont, ON). Online data processing and recording were realized by a digital hardware correlator (ALV-5000, ALV, Langen, Germany). Using FCS Access Control Software (version 1.2, Zeiss), data displaying was possible simultaneously.

After incubation of the cells with Alexa-NA, the focus was positioned to the upper membrane of the cell by motor-aided scanning of the cell in the z-direction. At the position of the half-maximal fluorescence of the upper membrane, the focus took in fast-diffusing free molecules and slow-diffusing ligand bound to the receptor as well. Additionally, the lower part of the focus was localized in the cytoplasm below the plasma membrane, allowing the detection of internalized receptor-ligand complex.

Live Cell Imaging. Pictures of live HEK293 cells stably expressing β_2AR−GFP were taken with an Axiovert 100 M fluorescence microscope equipped with an oil immersion objective (Plan Neofluar 100×, NA 1.3), an Axiocam HRm camera, a HBO 50 lamp, and Axiovision 3.1 (Zeiss). Pictures shown are representative of three independently performed experiments. Cells were preincubated for 24 h with addition of 1 μM α-hederin, hederacoside C, or hederagenin to the culture medium. A maximum of 0.1% ethanol was added as vehicle to control cells and treated cells. Prior to measurements, cells were washed three times with Locke's solution [154.0 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$ dihydrate, 1.0 mM $MgCl_2$ hexahydrate, 3.6 mM $NaHCO_3$, 5.0 mM HEPES, and 2.0 mM D-(+)-glucose monohydrate] at pH 7.3. The coverslips were mounted on a coverslip carrier above the microscope objective with an incubation volume of 300 μL. For internalization experiments, cells were incubated for 20 min with 1 μM terbutaline hemisulfate prior to measurements. All measurements were performed at 20 °C, and cells were used for at most 60 min.

cAMP Assay. The cAMP assay was performed using the HitHunter cAMP assay kit for adherent cells (Amersham Bioscience, Freiburg, Germany) following the manufacturer's instructions. One day after being seeded, cells were incubated for 24 h with 1 μM α-hederin, hederacoside C, or hederagenin. A maximum of 0.1% ethanol was added as a vehicle to control cells and treated cells. Before the cAMP concentration was examined, cells were further pretreated with 10 μM forskolin/terbutaline or with 10 mM phosphate buffer solution (1.9 mM KH_2PO_4, 18.1 mM Na_2HPO_4, and 149 mM NaCl) at pH 7.4 for 10 min at 37 °C. The fluorescence was measured using a GENios microplate reader (Tecan, Crailsheim, Germany) applying an excitation wavelength of 530 nm and an emission wavelength of 610 nm. Data processing was conducted with Magellan 3.0 (Tecan).

Data Analysis. The following autocorrelation function which describes the three-dimensional diffusion behavior of receptor−ligand complexes was used for the data evaluation of all FCS binding experiments:

$$G(\tau) = 1 + \frac{\sum_{j=1}^{n} Q_j^2 N_j}{(\sum_{j=1}^{n} Q_j N_j)^2} \frac{1}{1 + \tau/\tau_{D_j}} \sqrt{\frac{1}{1 + (\omega_0/z_0)^2 \tau/\tau_{Dj}}}$$

with

$$\tau_{D_j} = \frac{\omega_0^2}{4D_j}$$

and

$$Q_j = \sigma_j \eta_j g_j$$

where N_j is the average number of molecules of species j in the volume element, τ_{D_j} is the diffusion time constant of species j, τ is the correlation time, ω_0 is the radius of the observation volume in the focal plane, z_0 is the radius of the observation volume in the z-direction, D_j is the translational diffusion coefficient of species j, Q_j is the quantum yield factor, σ_j is the absorption coefficient, η_j is the fluorescence quantum yield, and g_j is the fluorescence detection efficiency of species j.

Statistcal Data Evaluation. All data points from FCS experiments represent mean values and standard deviations of six independent experiments. The statistical significance of results was proven with one factorial analysis of variance (ANOVA). The results were considered to be significant for p values of ≤ 0.05.

Ligand Binding Studies. A549 cells were treated as described for the live cell imaging procedure with additional 5 nM Alexa532-NA for 15 min in Locke's solution prior to FCS measurements. For saturation experiments, concentrations between 2 and 200 nM were preincubated with A549 cells for 15 min prior to FCS measurements. Data of all experiments were further analyzed with Origin 7.5 (OriginLab Corp., Northampton, MA).

RESULTS

The influence of α-hederin, hederacoside C, and hederagenin on cellular regulation was investigated by internalization studies of β_2-adrenergic receptor−GFP fusion proteins (β_2AR−GFP) in stably transfected HEK293 cells. Stimulation with 1 μM terbutaline, a specific β_2-agonist, led to a pronounced endocytotic internalization of occupied receptors that occurred after 20 min. Compared to the control cells, those stimulated showed large intracellular vesicles [early endosomes positive for the receptor−ligand complex (Figure 1B)]. Preincubation with 1 μM α-hederin for 24 h clearly led to an inhibition of receptor internalization even after stimulation with 1 μM terbutaline for 20 min. Compared to the positive control (Figure 1B), similar intracellular vesicles were not observed in α-hederin-treated cells (Figure 1D). Although hederacoside C and hederagenin are structurally related to α-hederin (Figure 2), preincubation with each compound at 1 μM for 24 h did not influence internalization of occupied β_2-adrenergic receptors (β_2AR) (Figure 1F,H).

Because of the inhibitory activity of α-hederin on internalization of the occupied β_2AR, the influence of the saponins on the receptor dynamics within the biomembrane of A549 cells was studied using fluorescence correlation spectroscopy

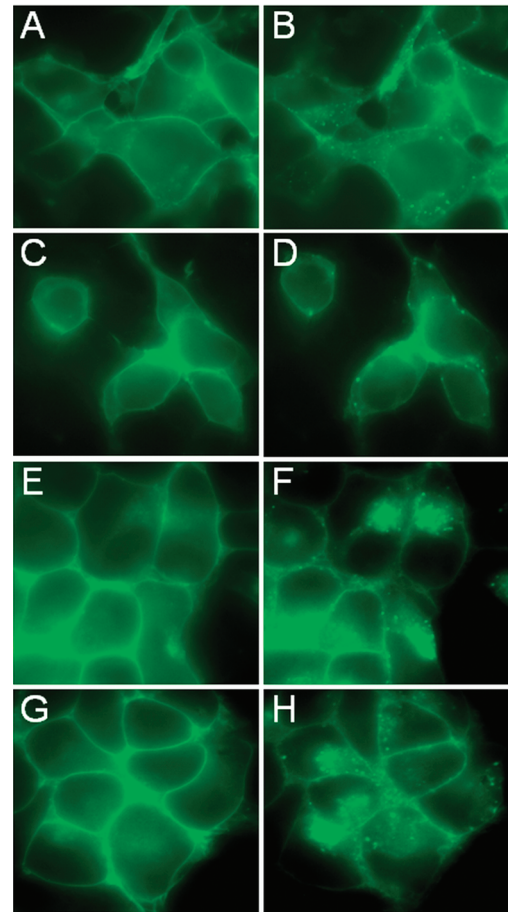
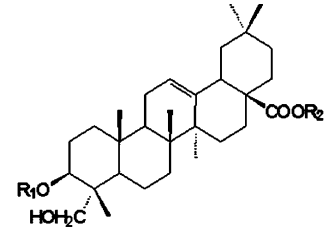


FIGURE 1: Live cell images of stably transfected HEK293 cells overexpressing β_2AR−GFP. Unstimulated control cells without pretreatment (A) and pretreated for 24 h with 1 μM α-hederin (C), hederacoside C (E), and hederagenin (G). Following incubation with 1 μM terbutaline for 20 min: stimulation of control cells (B), α-hederin-pretreated cells (D), hederacoside C-pretreated cells (F), and hederagenin-pretreated cells (H).



saponins	R_1	R_2
hederagenin	H-	H-
α-hederin	α-L-rha-(1→2)-α-L-ara-1-	H-
hederacoside C	α-L-rha-(1→2)-α-L-ara-1-	α-L-rha-(1→4)-β-D-glc-(1→6)-β-D-glc-1-

FIGURE 2: Chemical structures of saponins.

(FCS). After incubation of A549 cells with 5 nM Alexa-NA (arterenol, labeled with Alexa-532) for 15 min, the laser was focused on the upper membrane surface. Fluctuations in fluorescence intensity were measured for 60 s. Evaluation of the autocorrelation curve revealed an average fast diffusion time constant τ_{bound1} of 1.4 ± 1.1 ms and a slow diffusion time constant τ_{bound2} of 34.7 ± 14.1 ms ($n = 6$) found for receptor−ligand complexes with unrestricted and hindered lateral mobility, respectively (Figure 3, inset). By contrast, freely diffusing Alexa-NA showed a diffusion time constant τ_{free} of 0.060 ± 0.004 ms ($n = 6$). Diffusion time constants

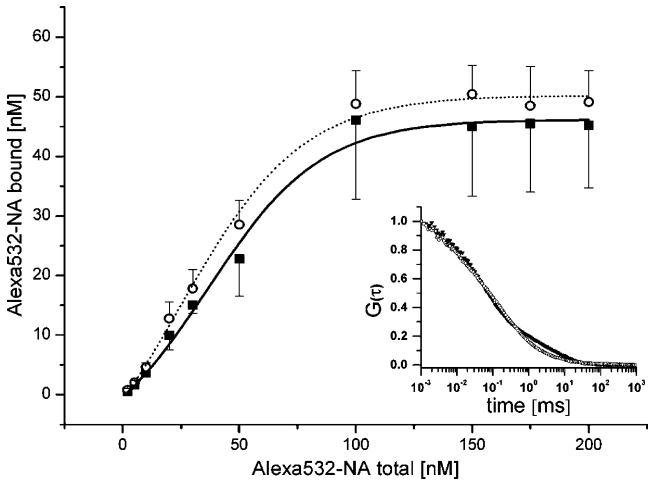


FIGURE 3: Alexa-NA binding to untreated (■) and 1 μM α-hederin-pretreated (○) A549 cells. Averaged bound Alexa-NA concentration vs the total Alexa-NA concentration. The bound Alexa-NA fraction was determined from the autocorrelation function for different Alexa-NA concentrations ($n = 6$). The inset shows autocorrelation curves for the binding of 5 nM Alexa-NA to untreated (▼) and 1 μM α-hederin-pretreated (○) A549 cells.

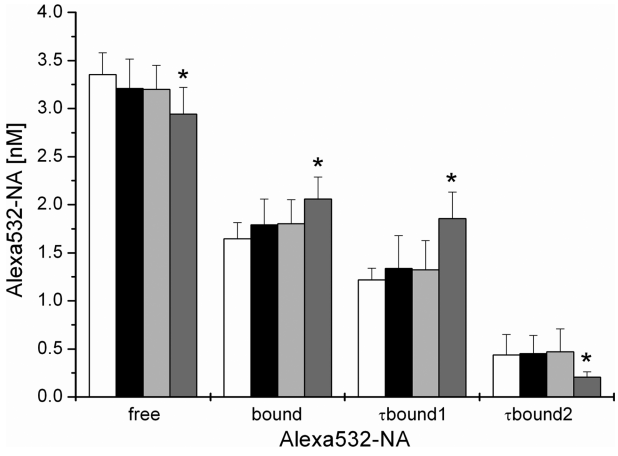


FIGURE 4: β_2AR binding studies on A549 cells. Occurrence of free and bound Alexa-NA and lateral mobility of β_2AR−ligand complexes with τ_{bound1} and τ_{bound2} in the presence of 5 nM Alexa-NA of control cells (white) and cells pretreated with 1 μM hederagenin (black), hederacoside C (light gray), and α-hederin (dark gray) ($p \leq 0.05$).

in these control experiments were distributed as follows: 67.0 ± 4.5% for τ_{free}, 24.3 ± 2.5% (equal to 1.21 ± 0.12 nM) for τ_{bound1}, and 8.7 ± 4.3% (equal to 0.43 ± 0.22 nM) for τ_{bound2}. The total binding was 33.0 ± 6.8% (equal to 1.65 ± 0.34 nM) (Figure 4). From τ_{bound1} and τ_{bound2}, corresponding diffusion coefficients of $D_1 = 9.09 \pm 5.09$ $\mu m^2/s$ and $D_2 = 0.28 \pm 0.07$ $\mu m^2/s$ were calculated for the different receptor−ligand complexes. Pretreatment of A549 cells with 1 μM α-hederin for 24 h clearly altered the distribution of diffusion time constants of Alexa-NA with values of 58.8 ± 5.6% for τ_{free}, 37.1 ± 5.5% (equal to 1.90 ± 0.27 nM) for τ_{bound1}, and 4.1 ± 1.1% (equal to 0.20 ± 0.06 nM) for τ_{bound2} (Figure 4 and Table 1; $n = 6$). A dose-dependent decrease in the level of receptor−ligand complexes with τ_{bound2} was found in FCS experiments (Figure 5). From an analysis of the autocorrelation curves, 0.01 μM α-hederin does not significantly alter the population with τ_{bound2}, whereas a decrease of 25% ($n = 6$) or 50% ($n = 6$) in τ_{bound2} was found for an α-hederin concentration of 0.1 or 1 μM,

Table 1: Influence of the Saponins on the Occurrence and Relative Distribution of the Diffusion Time Constants of β_2AR−Ligand Complexes

	τ_{bound1}		τ_{bound2}	
	τ (ms)	fraction (nM)	τ (ms)	fraction (nM)
control	1.40 ± 1.10	1.21 ± 0.12	34.7 ± 14.1	0.43 ± 0.22
α-hederin	0.93 ± 0.51	1.90 ± 0.27	49.2 ± 18.9	0.20 ± 0.06
hederacoside C	0.90 ± 0.51	1.32 ± 0.31	22.2 ± 11.3	0.47 ± 0.24
hederagenin	0.94 ± 0.46	1.34 ± 0.33	24.0 ± 10.3	0.45 ± 0.12

respectively. At the same time, τ_{bound1} showed a statistically significant dose-dependent increase of 58% ($n = 6$) in the presence of 1 μM α-hederin. Moreover, A549 cells pretreated with 1 μM α-hederin showed an increased level of Alexa-NA binding, i.e., from 1.65 ± 0.34 to 2.10 ± 0.20 nM ($n = 6$) compared to the control cells (Figure 4). The increase in the level of Alexa-NA binding was found to be statistically significant for concentrations between 2 and 50 nM (Table 2). Saturation experiments revealed a B_{max} value of 46.1 ± 1.4 nM and a K_D value of 36.1 ± 9.2 nM for control cells (Figure 3). α-Hederin-treated cells showed a similar value for B_{max} of 50.2 ± 1.1 nM, whereas the dissociation constant (K_D) decreased to 24.3 ± 11.1 nM. Thus, α-hederin did not influence the number of β_2ARs on the plasma membrane of A549 cells; however, an increased binding affinity for Alexa-NA was found. By contrast, pretreatment of the cells with 1 μM hederacoside C or hederagenin did not influence either the binding of Alexa-NA or the occurrence and relative distribution of τ_{free}, τ_{bound1}, and τ_{bound2} (Figure 4 and Table

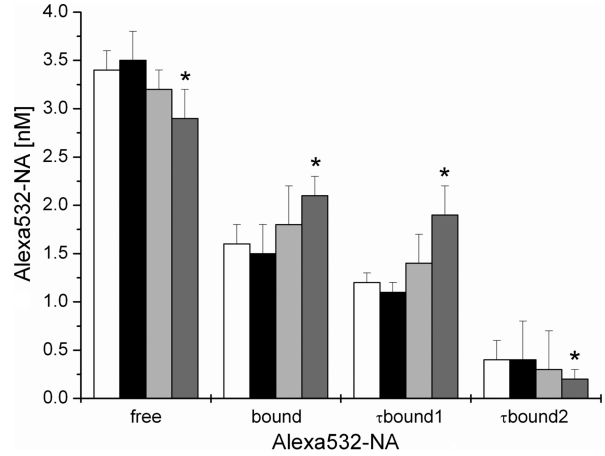


FIGURE 5: β_2AR binding studies on A549 cells. Occurrence of free and bound Alexa-NA and lateral mobility of β_2AR−ligand complexes with τ_{bound1} and τ_{bound2} in the presence of 5 nM Alexa-NA of control cells (white) and cells pretreated with 0.01 (black), 0.1 (light gray), and 1 μM α-hederin (dark gray) ($p \leq 0.05$).

Table 2: Saturation of the Alexa523-NA Binding at A549 Cells

Alexa523-NA total (nM)	Alexa523-NA bound to control cells (nM)	Alexa523-NA bound to α-hederin-pretreated cells (nM)
2	0.48 ± 0.07	0.76 ± 0.27[a]
5	1.65 ± 0.34	2.10 ± 0.20[a]
10	3.31 ± 0.76	4.59 ± 0.75[a]
20	10.00 ± 2.50	12.75 ± 2.86[a]
30	15.02 ± 1.39	17.82 ± 3.20[a]
50	22.80 ± 6.26	28.54 ± 4.10[a]
100	46.09 ± 13.31	48.83 ± 5.56
150	45.01 ± 11.70	50.44 ± 4.87
175	45.50 ± 11.50	48.53 ± 6.62
200	45.20 ± 10.50	49.80 ± 5.34

[a] $p \leq 0.05$.

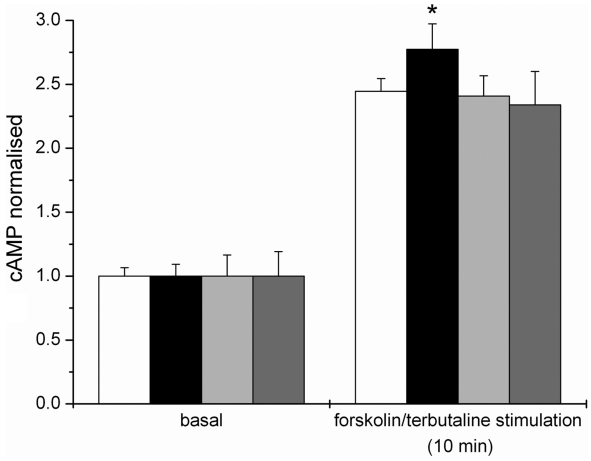


FIGURE 6: Accumulation of intracellular cAMP in HASM cells comparing control cell levels (basal) with cells incubated with 10 μM forskolin and 10 μM terbutaline for 10 min before (white) and after pretreatment for 24 h with 1 μM α-hederin (black), hederacoside C (light gray), and hederagenin (dark gray) ($p \leq 0.05$).

1). Intracellular cAMP levels of HASM cells were determined under stimulating conditions (10 μM foskolin and 10 μM terbutaline for 10 min) to confirm the increased level of β_2AR binding after pretreatment with 1 μM α-hederin for 24 h. Compared to control cells, a significantly increased cAMP level of 13.5 ± 7.0% ($n = 6$) was found for α-hederin-pretreated cells, whereas hederacoside C and hederagenin did not influence the intracellular concentration of the second messenger (Figure 6).

DISCUSSION

The influence of the saponins hederagenin, hederacoside C, and α-hederin on the regulation of β_2-adrenergic receptor (β_2AR) activity in living cells was investigated by live cell imaging studies, fluorescence correlation spectroscopy, and intracellular cAMP detection assays. Dried ivy leaf extracts containing these saponins are used for the treatment of upper respiratory tract diseases. Therefore, alveolar type II cells (A549) and Alexa-532-labeled arterenol were used for studying β_2AR binding, β_2AR dynamics, and investigation of regulatory processes after ligand binding. These studies showed a β_2AR selectivity with a K_D of 36.1 ± 9.2 nM after dye coupling to the amine function and a B_{max} value of 46.1 ± 1.4 nM. Hegener et al. have recently published a report on the specific binding behavior of Alexa-NA toward β_2AR in hippocampal neurons and A549 cells (*10*). Overexpression of the β_2AR in A549 cells was not successful. Thus, β_2AR−GFP fusion protein expressed by stably transfected HEK293 cells was used for internalization studies. Live cell imaging investigations demonstrate clear internalization of β_2AR−GFP in HEK293 cells after treatment with 1 μM terbutaline for 20 min. The stimulated cells showed large intracellular vesicles compared to untreated control cells (Figure 1A,B). This finding is consistent with a decrease in the β_2AR density on cell membranes, mediated by a high agonist concentration. Phosphorylation of the agonist-occupied receptor protein by GRK2 is followed by binding of β-arrestin, c-Src, and AP-2, which finally leads to receptor internalization via clathrin-coated pits and formation of early endosomes (*11*−*14*). In HEK293 cells, β_2AR−GFP internalization after stimulation with 1 μM terbutaline was clearly inhibited by preincubation with 1 μM α-hederin for 24 h. In contrast to the positive control, α-hederin-pretreated cells did not show comparable vesicles in the cytosol, and therefore, only negligible internalization of β_2AR after terbutaline stimulation was found (Figure 1D). Remarkably, preincubation with hederagenin and hederacoside C, two saponins structurally related to α-hederin, did not influence this regulatory process at a concentration of 1 μM, as shown by live cell imaging studies (Figure 1F,H). These findings are notable because it is assumed that the compounds under investigation own membrane activity, this being true for the most of the saponins (*15, 16*). It is still unclear why internalization of β_2AR−ligand complexes was inhibited by α-hederin but not by hederagenin or hederacoside C. It is also not clear from the available data which mechanism is responsible for the inhibition of internalization. One can speculate that α-hederin is the most membrane-active due to its amphiphilic structure (Figure 2), influencing interactions between both phosphokinases (e.g., GRK2, c-Src), as well as certain other proteins (e.g., dynamin, β-arrestin), and the occupied β_2AR. This then decreases the desensitization and redistribution of receptor−ligand complexes from functional microdomains to coated pits and ultimately the level of internalization. The aglycon hederagenin and the bisdesmoside hederacoside C do not seem to have a α-hederin-like influence on β_2AR dynamics and regulation.

Our findings have clearly demonstrated that α-hederin is able to influence regulatory processes affecting the β_2AR activity. Therefore, the dynamics of β_2AR−ligand complexes in the biomembrane of A549 cells were investigated using fluorescence correlation spectroscopy (FCS). In control experiments, two different diffusion time constants were found with a τ_{bound1} of 1.4 ± 1.1 ms and a τ_{bound2} of 34.7 ± 14.1 ms. Free Alexa-NA exhibited a diffusion time constant τ_{free} of 0.060 ± 0.004 ms. Hegener et al. have been able to demonstrate that bound Alexa-NA with τ_{bound1} occurred immediately after the addition of the fluorescent ligand, whereas the appearance of receptor−ligand complexes with τ_{bound2} was delayed and observed at notable levels only after 10−20 min (*10*). Thus, these authors attributed the time constant τ_{bound2} to receptor−ligand complexes, which were subject to regulatory processes and were finally internalized. Fifteen minutes after incubation of A549 cells with 5 nM Alexa-NA, a distribution of the different diffusion time constants was found in the control experiments with 67.0 ± 4.5% for τ_{free}, 24.3 ± 2.5% (equal to 1.21 ± 0.12 nM) for τ_{bound1}, and 8.7 ± 4.3% (equal to 0.43 ± 0.22 nM) for τ_{bound2} (Figure 4). On the other hand, A549 cells pretreated with 1 μM α-hederin clearly showed differences in the total Alexa-NA binding and in the relative proportion of τ_{bound1} and τ_{bound2}. Whereas the total level of binding was increased from 1.65 ± 0.34 to 2.10 ± 0.20 nM, the contributions of τ_{bound1} and τ_{bound2} were altered from 1.21 ± 0.12 to 1.90 ± 0.27 nM and from 0.43 ± 0.22 to 0.20 ± 0.06 nM, respectively (Figure 4 and Table 1). These dose-dependent findings were statistically significant only for 1 μM α-hederin (Figure 5). In saturation experiments with α-hederin-pretreated cells, a higher binding affinity for Alexa-NA (K_D of 24.3 ± 11.1 nM) was found which clearly accounts for the increased level of ligand binding. Interestingly, neither hederacoside C nor hederagenin at concentrations up to 1 μM exhibited a significant influence on total Alexa-NA binding and receptor

dynamics distribution (Figure 4 and Table 1). Because of the increase in the total level of binding of Alexa-NA and the decreased number of receptor−ligand complexes with τ_{bound2}, one can conclude that the β_2AR density of α-hederin-treated cells is still high even under stimulating conditions and, therefore, that the β_2-adrenergic responsiveness of A549 cells is increased. After α-hederin incubation, an increased level of transmembrane signaling via the β_2AR was confirmed by augmented intracellular accumulation of the second messenger cAMP, leading to diverse downstream cell regulatory effects. For alveolar type II cells, this could cause an increased level of secretion of surfactant (*17*), which could thus explain the secretolytic effect of ivy extracts. In this manner, one could also expect both a decrease in the intracellular Ca^{2+} concentration and an increase in the level of phosphorylation of the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) in bronchial smooth muscle cells (*18*), which could itself explain the bronchospasmolytic effect of ivy extracts.

REFERENCES

1. Meyer-Wegener, J., Liebscher, K., Hettich, M., and Kastner, H. G. (1993) Efeu versus Ambroxol bei chronischer Bronchitis. Eine Doppelblindstudie zum Vergleich der klinischen Wirksamkeit und Verträglichkeit von Efeublättertrockenextrakt und Ambroxol. *ZFA 69*, 61–66.
2. Gulyas, A., Repges, R., and Dethlefsen, U. (1997) Konsequente Therapie chronisch-obsruktiver Atemwegserkrangungen bei Kindern. *Atemwegs- und Lungenkrankheiten 23*, 291–294.
3. Mansfeld, H. J., Höhre, H., Repges, R., and Dethlefsen, U. (1997) Sekretolyse und Bronchospasmolyse. Klinische Studie: Behandlung von Kindern mit chronisch obstruktiven Atemwegserkrankungen. *TW Pädiatrie 10*, 155–157.
4. Mansfeld, H. J., Höhre, H., Repges, R., and Dethlefsen, U. (1998) Therapie des Asthma bronchiale mit Efeublätter-Trockenextrakt. *MMW 140*, 26–30.
5. Cioaca, C., Margineanu, C., and Cucu, V. (1978) The saponins of *Hedera helix* with antibacterial activity. *Pharmazie 33*, 609–610.
6. Trute, A., Gross, J., Mutschler, E., and Nahrstedt, A. (1997) In vitro antispasmodic compounds of the dry extract obtained from *Hedera helix*. *Planta Med. 63*, 125–129.
7. Bedir, E., Kirmizipekmez, H., Sticher, O., and Calis, I. (2000) Triterpene saponins from the fruits of *Hedera helix*. *Phytochemistry 53*, 905–909.
8. *Hederae helicis folium*. In *ESCOP Monographs*, 2nd ed. pp 241−247 (2003) Thieme Press, Stuttgart, Germany.
9. Haen, E. (1996) Pharmacological activities of *Thymus vulgaris* and *Hedera helix*. *Phytomedicine 1*, 144.
10. Hegener, O., Prenner, L., Runkel, F., Baader, S. L., Kappler, J., and Häberlein, H. (2004) Dynamics of β2-adrenergic receptor-ligand complexes on living cells. *Biochemistry 43*, 6190–61999.
11. Krasel, C., Vilardaga, J. P., Bunemann, M., and Lohse, M. J. (2004) Kinetics of G-protein-coupled receptor signalling and desensitization. *Biochem. Soc. Trans. 32*, 1029–1031.
12. Ferguson, S. S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signalling. *Pharmacol. Rev. 53*, 1–24.
13. Tsao, P., and von Zastrow, M. (2000) Downregulation of G protein-coupled receptors. *Curr. Opin. Neurobiol. 10*, 365–369.
14. Claing, A., Laporte, S. A., Caron, M. G., and Lefkowitz, R. J. (2002) Endocytosis of G protein-coupled receptors: Roles of G protein-coupled receptor kinases and β-arrestin proteins. *Prog. Neurobiol. 66*, 61–79.
15. Rao, A. V., and Gurfinkel, D. M. (2000) The bioactivity of saponins: Triterpenoid and steroidal glycosides. *Drug Metab. Drug Interact. 17*, 211–235.
16. Hu, M., Konoki, K., and Tachibana, K. (1996) Cholesterol-independent membrane disruption caused by triterpenoid saponins. *Biochim. Biophys. Acta 1299*, 252–258.
17. Rooney, S. A. (2001) Regulation of surfactant secretion. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol. 129*, 233–243.
18. Garcia, J. G., Lazar, V., Gilbert-McClain, L. I., Gallagher, P. J., and Verin, A. D. (1997) Myosin light chain kinase in endothelium: Molecular cloning and regulation. *Am. J. Respir. Cell Mol. Biol. 16*, 489–494.

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