Melittin modulates keratinocyte function through P2-receptor-dependent ADAM activation*

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Background: Melittin is an antimicrobial peptide that is also being considered as anti-inflammatory and anti-cancer agent.

Results: Melittin provokes P2 receptor activation which leads to ADAM-dependent transactivation of the EGFR and augments keratinocyte proliferation and migration.

Conclusion: Melittin modulates cellular functions through activation of ADAM-mediated shedding.

Significance: The use of melittin may elicit unexpected and unwanted effects via ADAM activation.

SUMMARY

Melittin, the major component of the bee venom, is an amphipathic, cationic peptide with a wide spectrum of biological properties that is being considered as an anti-inflammatory and anti-cancer agent. It modulates multiple cellular functions but the underlying mechanisms are not clearly understood. Here, we report that melittin activates disintegrin-like metalloproteases (ADAMs) and that downstream events likely contribute to the biological effects evoked by the peptide. Melittin stimulated the proteolysis of ADAM10 and ADAM17 substrates in human neutrophil granulocytes, endothelial cells and murine fibroblasts. In human HaCaT keratinocytes, melittin induced shedding of the adhesion molecule E-cadherin and release of TGF-alpha, which was accompanied by transactivation of the EGF-receptor and ERK1/2 phosphorylation. This was followed by functional consequences such as increased keratinocyte proliferation and enhanced cell migration. Evidence is provided that ATP release and activation of purinergic P2 receptors are involved in melittin-induced ADAM activation. E-cadherin shedding and EGFR phosphorylation were dose-dependently reduced in the presence of ATPases or P2 receptor antagonists. The involvement of P2 receptors was underscored in experiments with HEK cells, which lack the P2X7 receptor and showed strikingly increased response to melittin stimulation after transfection with this receptor. Our study provides new insight into the mechanism of melittin function which should be of interest particularly in the...
context of its potential use as an anti-inflammatory or anti-cancer agent.

Bee venom produced by the honey bee (Apis mellifera) contains a large number of bioactive peptides including melittin, apamin, adolapin, and mast cell-degranulating peptide. Melittin is the most abundant component, constituting 50% of whole bee venom (1). This amphipathic peptide of 26 residues contains a hydrophobic stretch of 19 amino acids followed by a cluster of 4 positively charged residues at the COOH-terminus. Melittin is able to intercalate into cell membranes, causing changes in biophysical membrane properties (2-5). It belongs to the family of antimicrobial peptides (AMPs) that have become the subject of increasing discussion as promising anti-cancer drugs and substitutes for conventional antibiotics (6, 7). Several cancer cells including renal, lung, breast and bladder cells can be selectively destroyed by melittin in vitro (8). Its potential use as an agent to treat hepatocellular carcinoma, breast cancer and prostate cancer has been tested in animal models, with positive outcome (9-11).

Moreover, melittin has been described to exert anti-inflammatory, anti-rheumatoid, anti-arthritis and pain-relieving effects (8), but the mode of action is still largely unknown (12). Besides biophysical membrane interaction, melittin might directly influence cellular function by activating downstream signaling. It is discussed as a potent activator of phospholipase A2 (PLA2) thereby also promoting arachidonic acid synthesis (13). PLA2-dependent cytotoxic effects and activation of caspase-3 are reported to contribute to anti-cancer cytotoxicity (14). Melittin has also been suggested to reduce inflammatory responses by inhibiting the DNA-binding activity of NF-κB (15), but this concept remains controversial (12).

Metalloproteases play important roles in inflammatory diseases and cancer progression. It has been proposed that melittin could contribute to anti-rheumatoid effects by inhibiting matrix metalloprotease (MMP)-3 production (16). In another study, MMP-9 expression in MCF-7 cells was abolished by melittin treatment (17). Besides MMPs, disintegrin like metalloproteases (ADAMs) play important roles in health and disease (18). They control diverse cellular functions through the release of transmembrane molecules from the cell surface (19). ADAM10 and ADAM17 are the best characterized members of this family. ADAM10 is critically involved in Notch receptor signaling and ADAM17 activity is essential for epidermal growth factor receptor (EGFR) activation. Deletion of both genes leads to embryonic death in knockout mouse models (20, 21). Several substrates have been identified for both proteases. ADAM10 is the major protease involved in the cleavage of cell adhesion molecules such as neuronal (N)-cadherin (22), epithelial (E)-cadherin (23) or vascular-endothelial (VE)-cadherin (24), but also releases the EGFR ligands betacellulin and EGF (25). ADAM17, also known as TACE (TNF-alpha converting enzyme), was identified as the enzyme releasing soluble TNF-alpha from its transmembrane precursor form. Due to the release of this pro-inflammatory cytokine and other cell surface molecules that modulate inflammation, ADAM17 is being discussed as a potential drug target for several inflammatory pathologies.

Up to now a large number of ADAM17 substrates have been identified. Inter alia, the protease controls the function of cell adhesion molecules such as L-selectin on neutrophil granulocytes (21) and the release of the EGFR ligands amphiregulin, epiregulin, TGF-alpha or heparin-binding EGF (HB-EGF) (26). ADAM10 as well as ADAM17 appear to promote cancer progression by EGFR activation and release of cell adhesion molecules (18).

Recently, we demonstrated that biophysical alterations of cell membrane properties modulate ADAM10 and ADAM17-mediated substrate cleavage (27). Application of free unsaturated fatty acids induced ADAM-mediated shedding by increasing cell membrane fluidity and augmenting the mobility of enzyme and substrate in the membrane. From these findings, the suspicion arose that other membrane active agents such as melittin might also augment the function of ADAMs.

In this communication, we report that melittin indeed provokes rapid substrate cleavage by ADAMS in diverse cell types. We found, however, that the increase in ADAM-mediated shedding was not due to changes in membrane fluidity. Instead, evidence is presented that exposure of cells to sublethal concentrations of melittin results in P2 receptor activation. This in turn is responsible for augmentation of ADAM activity and downstream EGFR transactivation in HaCaT keratinocytes.

**EXPERIMENTAL PROCEDURES**

**Reagents and antibodies -** Melittin was synthesized with an amidated C-terminus by the Fmoc solid-phase peptide synthesis technique on
an automatic peptide synthesizer (model 433 A; Applied Biosystems) as described (28). Monoclonal antibodies against the cytoplasmic domain of E-cadherin (C36) and N-cadherin were purchased from BD Bioscience. ADAM10 was detected using a polyclonal antiserum described previously (20). Rabbit polyclonal anti-ADAM17 cytotail antibody was kindly provided by Carl Blobel (NY) (29). Monoclonal antibody against VE-cadherin and peroxidase-conjugated immunoglobulins to mouse or rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. The EGFR-function blocking antibody Cetuximab (C225) was from Merck. CD62L antibodies were were from ebioscience. Pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), suramin, hexokinase, apyrase, BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, sodium), EGTA, TAPI-0 and MTT (3-(4,5-Dimethyloxazol-2-yl)-2,5 diphenyltetrazolium-bromide) were obtained from Sigma. Evans blue and marimastat were purchased from Tocris. Hydroxamate-based inhibitors GW280264 ((2R,3S)-3-(formyl-hydroxyamino)-2-(2-methyl-1-propyl) hexanoic acid [(1S)-5-benzylxoycarbamoylamo-1-(1,3-thiazol-2-ylcarbamoyl)-1-pentyl] amide) and GI254023 ((2R,3S)-3-(formyl-hydroxyamino)-2-(3-phenyl-1-propyl) butanoic acid [(1S)-2,2-dimethyl-1-methylcarbamoyl-1-propyl] amide) were synthesized as described in US patents US 6 172 064, US 6 191 150. and US 6 329 400. They were kindly provided by Dr. A. Ludwig, UK Aachen, Germany) and are described in detail elsewhere (30). The human P2X7R cDNA was synthesized as described in US patents US 6 191 150. and US 6 329 400. They were kindly provided by Dr. A. Ludwig, UK Aachen, Germany) and are described in detail elsewhere (30). The human P2X7R cDNA was synthesized as described in US patents US 6 172 064, US 6 191 150. and US 6 329 400. They were kindly provided by Dr. A. Ludwig, UK Aachen, Germany) and are described in detail elsewhere (30). The human P2X7R cDNA was kindly provided by Carl Blobel (31).

Cell culture - HUVECs were obtained from Provitro and cultured in Endothelial Cell Growth Medium (PromoCell). All experiments were performed with HUVECs from passages 3-7. HaCaT keratinocytes were provided by Dr. N.E. Fusenig (DKFZ, Heidelberg, Germany) (32). Mouse embryonic fibroblast (MEF) cell lines from ADAM10<sup>−/−</sup> mice and respective wild-type animals were generated and characterized as described elsewhere (20, 33). MEFs, HaCaT keratinocytes and HEK-293T cells (ATCC) were grown in high glucose DMEM (PAA, Linz, Austria) supplemented with 10% FCS and 1% penicillin/streptomycin.

Transfection - HEK cells were seeded in six-well plates and transfected on the next day with 1 µg/well plasmid DNA encoding for P2X7. Transfection was performed using TurboFect™ in vitro Transfection Reagent (Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s instructions. Cells were grown for 48 h prior to stimulation and protein extraction for western blot analysis.

MTT assay - Metabolic activity was determined in 96-well plates in triplicates by quantifying the reduction of MTT to formazan. Briefly, cells were incubated with the indicated amounts of melittin in 100 µl serum-free medium for 30 min. Untreated and 2.5% Triton-X treated cells served as controls. Afterwards medium was replaced by 100 µl of 0.5 mg/ml MTT in 10% standard cell culture medium and 90% PBS and incubated for 2 h at 37 °C. Formed formazan crystals were dissolved by adding 100 µl of 10% Triton-X in isopropanol. Extinction was measured at 590 nm and absorbance values were normalized to untreated and Triton-X treated controls.

Bioluminescence assay for determination of cellular ATP - CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to determine the release of soluble ATP. The assay was performed according to manufacturer’s instructions.

Membrane fluidity - HaCaT keratinocytes (1 ml, 5 x 10<sup>6</sup> cells) were incubated for 30 min at 32°C with diphenyl hexatriene (DPH, 4 µM). To remove unbound DPH, the cells were centrifuged for 5 min at 3000xg, were washed twice with PBS and were resuspended in 10 ml PBS. The cells were then transferred to a thermostated cuvette under continuous stirring. Anisotropy was measured in a Quantamaster (PTI, Canada) fluorimeter using Glan-Thompson polarizers at excitation and emission wavelengths of 360 ± 4 nm and 430 ± 4 nm, respectively, including a GG390 cut-off filter at the emission site. Steady-state fluorescence anisotropy (r) was determined according to r = (I<sub>VV</sub>-I<sub>VH</sub>)/(I<sub>VV</sub>+2 I<sub>VH</sub>), where I<sub>VV</sub> and I<sub>VH</sub> are the fluorescence intensities observed with the excitation polarizer in the vertical position, and the analyzing emission polarizer in both the vertical (I<sub>VV</sub>) and horizontal (I<sub>VH</sub>) configuration. Factor G corrects for the unequal transmission of differently polarized light.

Calcium influx - Peptide mediated calcium influx in HaCaT keratinocytes was monitored over time by fluorescence spectroscopy using the Ca<sup>2+</sup>-sensitive dye Indo-1. The assay and all washing steps were performed either in PBS, pH 7.4 containing 1% FCS (buffer A) or in the same buffer supplemented with 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (buffer B). Cells were washed in buffer A or B and adjusted to 10<sup>5</sup> cells/ml, preloaded with 1 µM Indo-1-AM (Invitrogen, Molecular Probes) for 45 min at 37°C, washed...
again, and adjusted to $10^5$ cells/ml in the respective buffer. The assay was performed at room temperature in 2 ml glass cuvettes using a spectrofluorometer (Fluorolog-3, Horiba, USA) with an excitation wavelength of 355 nm as well as emission wavelengths of 405 and 485 nm to detect the Ca$^{2+}$-bound and the Ca$^{2+}$-free form of the dye. Fluorescence of the cell suspension was monitored for 1 min before the addition of peptide without any measurable change in fluorescence. Then, melittin was added in indicated concentrations and the fluorescence was monitored for additional 4 min. Data are represented as the quotient ($F_{405}/F_{485}$) of the normalized emission intensities ($F_t/F_0$) of the two wavelengths, i.e. the fluorescence intensity at a certain time point ($F_t$) divided by the mean fluorescence intensity before addition of peptide ($F_0$). At least two independent experiments were performed and one representative experiment is shown.

**L-selectin FACS analysis** - PMN were isolated from heparinized peripheral blood of healthy donors using Biocoll Separating Solution (Biochrom AG) according to the manufacturer’s recommendations. Residual erythrocytes were removed using lysis buffer containing NH$_4$Cl. PMN purity was >90% as determined by flow cytometric analysis and by counting in a hemocytometer. Stock suspensions of the cells (20-30 x $10^6$ per ml) were kept in PBS on ice and used within 2 h. Experiments were conducted in HBSS using 3 x $10^6$ cells/ml.

**Immunoblotting** - Immunoblots for the analysis of VE-cadherin, N-cadherin and E-cadherin were performed as described elsewhere (22-24). Signals were recorded by a luminescent image analyzer (Fuji image reader LAS1000) and analyzed with image analyzer software (GEL-PROANALYSER, Media Cybernetics, Silver Spring, MD). To generate the control blots for expression of ERK1/2 and EGFR, western blots were incubated in stripping reagent (100 mM 2-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7) at 55 °C for 30 min and then re-probed with anti ERK1/2 or EGFR antibody, respectively.

**In Vitro Wound Healing** - HaCaT keratinocytes were seeded in 12-well plates (Sarstedt) and grown until they reached confluence (48 h). A cell-free area was introduced by scraping the monolayer with a pipette tip (10 µl, Sarstedt). To avoid a proliferative effect, cells were treated with 2 mM hydroxyurea (Sigma-Aldrich) for 24 h. For stimulation experiments, FCS-free medium containing metalloprotease inhibitors or DMSO was added. After 15 min pre-incubation, the cells were treated with melittin (0.5 µM). Cells were photographed before stimulation and 24 h after stimulation by using an inverted phase-contrast microscope (Zeiss). The cell free area was quantified using AxioVision (Zeiss) before and after stimulation, respectively.

**Cell Proliferation Assays** - HaCaT keratinocytes were cultured over night in medium containing FCS before starvation for 48 h. Subsequently, medium containing various agents was added and cells were incubated for 24 h. PI cell cycle analysis was performed as previously described (34).

**Quantitative real-time-PCR** - mRNA was isolated with peqGOLDTriFast (Peqlab) and reverse transcribed by Maxima Reverse Transcriptase (Thermofisher Scientific) as described by the manufacturer’s protocol. The quantitative real-time-PCR was performed in a Lightcycler II (Roche). The reaction mixture consisted of 10 ng cDNA, 0.5 µM of each primer (hb2M, forward: ATGAGTATGCTTGGTGTGA, reverse: GGCACTTCAACCTCCATG; hADAM10, forward: AGTGACCATCACAATTGTG, reverse: CCAGGCATAGCATGACAG; hADAM17, forward: AGACAGAGAACCACCTGAAAG, reverse: CCCATGAGTGTTCCGATAG) and 2 µl My budget 5x EvaGreen QPCR Mix Capillary (BioBudget) per 10 µl mixture. Water was used as a control. Each mRNA expression was normalized against beta-2 microglobulin mRNA ($\Delta\Delta$CT-method) and all data are presented as fold change against the unstimulated control. Each experiment was performed in duplicate and the standard error of mean (s.e.m) has been calculated on the basis of the two experiments.

**Statistical analysis of CTF generation** - All values are expressed as means ± standard error of the mean (s.e.m) or as otherwise indicated. Variance analysis was performed with one-way ANOVA (SigmaStat 3.1 software; Erkrath, SYSTAT, Germany). Pair wise and multiple comparison procedures were performed with Bonferroni test. P-values < 0.05 were classified as statistically significant.

**RESULTS**

Melittin effects on cellular viability are cell type dependent - High concentrations of melittin are known to induce necrosis and apoptosis, while low concentrations have been shown to enhance cell proliferation (35). To exclude that potential cytotoxic effects would affect substrate
processing and to define sublethal concentrations of melittin in our experiments, MTT reduction was measured and taken as a parameter of cell viability. The yellow tetrazolium MTT is reduced by metabolically active cells leading to generation of intracellular purple formazan which can be quantified. As shown in Fig. 1, all cells tested responded to melittin treatment with a decrease in MTT reduction. Human monocytes were most sensitive and were not used in the following experiments (Fig. 1A). Murine embryonic fibroblasts (MEFs) and human umbilical vein endothelial cells (HUVECs) were least sensitive and significant reduction in cellular viability occurred at 2 and 4 µM melittin, respectively (Fig. 1B,C). Melittin concentrations ≥ 0.5 µM led to a significant decrease of MTT reduction in human neutrophil granulocytes (Fig. 1D). HaCaT keratinocytes tolerated melittin effects up to a threshold concentration of 1 µM (Fig. 1E).

The same results for melittin-induced cytotoxicity in HaCaT keratinocytes have been previously reported (36). We concluded that concentrations between 0.5 µM and 1 µM melittin were tolerable and they were usually employed, except in experiments with MEFs where higher doses were required to induce a significant increase in shedding events.

To discern whether melittin treatment would also lead to ATP release, we additionally measured ATP in the supernatant of melittin-treated HaCaT keratinocytes. As shown in Fig. 1F, melittin evoked ATP release dose-dependently. Nanomolar concentrations became detectable in the supernatants also when nontoxic melittin concentrations were applied.

**Melittin induces ADAM-dependent substrate cleavage** - Shedding of certain substrates is preferentially elicited by specific members of the ADAM family. ADAM10 is critically involved in the release of the adhesion molecule N-cadherin in fibroblasts and neuronal cells (22, 37, 38). To discern whether melittin would stimulate ADAM10-dependent shedding, we analyzed N-cadherin processing in MEFs using monoclonal antibodies against the C-terminus of the adhesion molecule. The generation of the N-cadherin C-terminal fragment (CTF) in wild-type cells was compared with ADAM10-deficient fibroblasts. As shown in Fig. 2A, melittin dose-dependently induced N-cadherin shedding in wild-type cells while the CTF was not generated in ADAM10-deficient cells.

Analogously, shedding of VE-cadherin was found to occur in response to 0.5-1 µM melittin. Shredding was inhibited by the broadspectrum metalloprotease inhibitor marimastat (Fig. 2B), and also by GI254023X, a preferential inhibitor of ADAM10 (data not shown).

In order to analyze the stimulated cleavage of a preferential ADAM17 substrate, the release of soluble L-selectin (CD62L) was determined in neutrophil granulocytes using FACS analysis (21, 31, 39). L-selectin was rapidly shed from cells following treatment with 0.5 µM melittin. This effect was abrogated in the presence of the broad-spectrum metalloprotease inhibitor marimastat (Fig. 2C).

**Melittin induces ADAM-dependent E-cadherin shedding and transactivation of the EGFR in HaCaT keratinocytes** - The effects of melittin on HaCaT keratinocytes were then investigated. In these cells, E-cadherin is preferentially cleaved by ADAM10 (23), while shedding of TGF-alpha is dependent mainly on ADAM17 (21). Melittin dose-dependently augmented proteolysis of E-cadherin (Fig. 3A), and this effect could be significantly reduced by the use of broad-spectrum-metalloprotease inhibitor marimastat but also by the preferential ADAM10 inhibitor GI254023X and GW280264, an inhibitor of ADAM10 and ADAM17 (Fig. 3B). Melittin simultaneously provoked release of TGF-alpha into the supernatant. As to be expected, this event was strongly reduced by the mixed ADAM10/ADAM17 inhibitor GW280264 and marimastat, but only slightly affected by the preferential ADAM10 inhibitor GI254023X (Fig. 3C).

TGF-alpha is a potent ligand of the EGFR which critically participates in the control of cell differentiation, proliferation and cell survival. In addition to TGF-alpha, six other ligands are known to activate the EGFR via EGF-like motifs, and all are shed by ADAM10 and/or ADAM17 (25, 40). It followed that treatment of cells with melittin might lead to EGFR activation. Indeed, exposure of HaCaT keratinocytes to melittin induced EGFR phosphorylation with downstream activation of ERK1/2, which were prone to inhibition by marimastat (Fig. 3D).

**Melittin induces metalloprotease-dependent HaCaT keratinocyte cell proliferation and migration** - The results suggested that functional consequences might follow the shedding processes (23, 24, 41).

Incubation of HaCaT keratinocytes with melittin indeed resulted in increased cell proliferation, as determined by propidium iodide (PI) cell cycle analysis (Fig. 4A). This effect was essentially abrogated in the presence of the broad-spectrum metalloprotease inhibitor TAPI-
0. To address the role of melittin for HaCaT keratinocyte migration, we used an in vitro model for wound healing. In this assay, scrape wounds were generated in confluent HaCaT keratinocyte cultures and cells were allowed to migrate for 24 h. The scratch assays were performed in the presence of 2 mM hydroxyurea to prevent HaCaT keratinocyte proliferation (42). While mock treated cells just started to cover the denuded area, melittin stimulation led to nearly complete wound closure. This effect was abolished in the presence of the broad-spectrum metalloprotease inhibitor marimastat (Fig. 4B). Application of the monoclonal antibody Cetuximab, which blocks access of ligands to the EGF-receptor, significantly reduced HaCaT keratinocyte migration (Fig. 4C), indicating that EGFR activation indeed plays a key role in the observed cellular reaction.

Cell membrane fluidity and ADAM10/ADAM17 expression are not changed by melittin - We have presented evidence that alterations in membrane fluidity have a direct impact on ADAM function, and found that application of unsaturated fatty acids to cells augmented cleavage of ADAM substrates in a manner that now seemed to be mimicked by melittin (27). Because the peptide modulates the biophysical properties of membranes (43), the diffusion of fatty acyl chains in HaCaT keratinocyte membranes was determined using steady-state fluorescence anisotropy with diphenyl hexatriene (DPH) as a probe. Melittin at concentrations that induced ADAM substrate processing exerted no effects on membrane fluidity. Even much higher concentrations remained without effect (supplemental Fig. S1). In contrast, rapid decrease in anisotropy was observed in response to oleic acid, which was used as positive control.

The possibility was considered that melittin might activate signaling pathways leading to modulation of ADAM expression, stability or maturation. Removal of the prodomain by furin-type proprotein convertases is necessary to convert the inactive pro-form to the mature active enzyme. However, even prolonged treatment of cells with melittin led to no changes in expression of ADAM10 or ADAM17, or to increased maturation of the enzymes (supplemental Fig. S1).

Increase in cytosolic calcium is not involved in ADAM activation - Melittin stimulation has been shown to induce calcium influx in different cell types. To determine whether extracellular calcium influx would contribute to ADAM activation, HaCaT keratinocytes were stimulated with increasing concentrations of this compound in calcium-containing and in calcium-free medium. A rapid dose-dependent increase in free intracellular Ca\(^{2+}\) was observed immediately after addition of melittin to HaCaT keratinocytes (supplemental Fig. S2, left panel). A pronounced effect was visible down to 0.2-0.4 µM melittin. The melittin-induced increase of intracellular Ca\(^{2+}\) in buffer lacking Ca\(^{2+}\) and Mg\(^{2+}\) (right panel) was reduced by 20 and 15% for 1 and 0.4 µM melittin compared to the Ca\(^{2+}\) increase in buffer containing Ca\(^{2+}\) and Mg\(^{2+}\). This indicated that melittin induced the influx of extracellular Ca\(^{2+}\) as well as the release of Ca\(^{2+}\) from intracellular stores.

Melittin effects depend on ATP release and P2 receptor activation - Purinergic P2 receptors participate in the regulation of several physiological processes. They are divided into two major families, the P2X receptors, which are ATP-gated ion channels, and the P2Y receptors, which are G-protein coupled receptors (45). ATP-gated ion channels, and the P2Y receptors, which are G-protein coupled receptors (45). ATP-gated ion channels, and the P2Y receptors, which are G-protein coupled receptors (45). HaCaT keratinocytes express both P2X and P2Y receptors (46) and can thus be activated by ATP. Since melittin stimulation led to ATP release and P2 receptors have been discussed in the context of ADAM activation (31, 47-54), we performed experiments using the broad-spectrum P2R inhibitor, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (55, 56). As shown in Figure 5A, PPADS abolished ERK1/2 activation dose-dependently (upper panel). Melittin-induced E-cadherin shedding was strongly reduced by PPADS to an extent comparable with marimastat effects (lower panel). To confirm these results, we additionally used the broad-spectrum P2 receptor antagonist, betacellulin (23, 31, 38, 44). However, the melittin-induced E-cadherin shedding did not rely on the increase of free calcium because neither chelation of extracellular calcium by 5 mM EGTA nor the use of the membrane-permeable calcium chelator BAPTA-AM affected the cellular response (supplemental Fig. S2). The same applied to the activation of EGFR signaling (supplemental Fig. S2).

Calcium can act in signal transduction and calcium ionophores are widely used to induce the shedding of several ADAM10 substrates such as N-cadherin, E-cadherin, CD44 or betacellulin (23, 31, 38, 44). However, the melittin-induced E-cadherin shedding did not rely on the increase of free calcium because neither chelation of extracellular calcium by 5 mM EGTA nor the use of the membrane-permeable calcium chelator BAPTA-AM affected the cellular response (supplemental Fig. S2). The same applied to the activation of EGFR signaling (supplemental Fig. S2).
hexokinase reduced E-cadherin shedding and ERK1/2 activation dose-dependently (Fig. 5D), although the effects were not completely abrogated. Endogenously released ATP therefore appears to represent one, but possibly not the sole trigger of P2 receptor-mediated ADAM activation.

HEK cells are widely used as models to study the role of purinergic receptors because they only express low amounts of certain P2Y receptors (57) and do not express P2X receptors. We compared the melittin response in HEK cells with HEK cells transiently transfected with the P2X7 receptor. As shown in Figure 7E, P2X7 transfection strikingly increased the response to melittin stimulation. The stimulation was abrogated by pretreatment with metalloprotease inhibitor marimastat and by treatment with the ATPase apyrase. Stimulation with the P2X7 agonist benzoyl-ATP (bzATP), which was used as positive control, elicited comparable effects.

**DISCUSSION**

The effects of melittin on cells are remarkably diverse and the functional outcomes essentially unpredictable (12, 58, 59). Reports on its anti-inflammatory and growth-suppressing action stand alongside with observations to the contrary. How the amphipathic peptide elicits these myriad reactions remains quite enigmatic. A cellular receptor is not known to exist. The cationic domain mediates binding, which is followed by membrane insertion of the hydrophobic sequence whose length suffices to span only half of the lipid bilayer. In analogy with pore-forming toxins, one possibility is that transmembrane pores are formed by melittin oligomers. While increases in membrane permeability for ions have been demonstrated and cell lysis occurs when critical concentrations are reached, data on the composition and structure of the putative pore are not available. Equally nebulous are the events that underlie triggering of the signaling pathways and the mediators that are involved in eliciting the sublethal effects of melittin.

This study adds another facet to the complex theme. We report that subcytotoxic concentrations of melittin cause rapid upregulation in function of ADAMs, the central shedding proteinases of nucleated cells. The roles fulfilled by the individual ADAMs are varied, their pattern of tissue expression diverse, and the signaling pathways leading to their activation heterogeneous (31).

We elected to focus our investigation on the effects of melittin on ADAM10 and ADAM17, two major members of the ADAM proteinase family. Our results indicate that melittin treatment leads to activation of both proteases, ADAM10 and ADAM17. Further analyses will reveal whether the agent does indeed represent a broad-spectrum ADAM activator as may be anticipated at this stage, and which would accord very satisfactorily with its propensity to evoke such a wide range of biological effects.

ADAM10 is the major sheddase of classical cadherins. We investigated the shedding of N-cadherin in murine fibroblasts, VE-cadherin in human endothelial cells, and E-cadherin in human HaCaT keratinocytes. Melittin rapidly stimulated cleavage of each substrate. Cleavage of VE-cadherin increases endothelial permeability. Cleavage of E-cadherin likely provokes loss of epithelial cell-cell contacts and may contribute to the genesis of eczematous dermatitis (41). Thus, an explanation retrospectively emerges for the recent finding that melittin enhances permeability of CaCo-2 epithelial cell monolayers (60). ADAM17 cleaves a large number of substrates including cytokines (e.g. TNF-alpha) and their receptors (e.g. IL6R), TGF-alpha and other EGFR ligands, and L-selectin. We investigated whether melittin would induce shedding of L-selectin in neutrophil granulocytes and TGF-alpha in HaCaT keratinocytes. The affirmative results obtained in both cases corroborate the contention that melittin truly can be viewed as an ADAM activator.

TGF-alpha can activate the EGFR signaling pathway to induce cell proliferation and migration (61). Since all EGFR ligands are released through proteolysis, it is quite likely that the shedding of additional ligands contributes to EGFR activation. In any case functional consequences would have to follow. The question whether cell growth would be promoted in our experiments was of particular interest since melittin is being considered as an anti-tumor agent. Subtoxic concentrations were indeed found to induce ERK1/2 phosphorylation. This process was metalloprotease and EGFR-dependent and vigorously stimulated proliferation and migration of human HaCaT keratinocytes. Detailed analyses using specific inhibitors or siRNA experiments will be required to differentiate the contribution of ADAM10, ADAM17 or even additional metalloproteases to these processes.
However, these proliferation promoting features are in line with and provide an explanation for the melittin-induced proliferation of gastrointestinal cells (35). With regard to the usefulness of melittin as anti-cancer agent (9), our results bring these approaches into question.

The pathway leading to ADAM activation by melittin is under investigation and has led to a number of findings that are of interest. The physical properties of membranes may participate in controlling ADAM function by influencing the velocity of molecular movement in the lipid bilayer. Enhancement of fluidity by unsaturated free fatty acids accelerates cleavage of membrane anchored substrates. The rapid kinetics of substrate cleavage provoked by melittin were reminiscent of those previously observed to be invoked by free fatty acids. We therefore examined the effect of melittin on membrane fluidity, but could observe no effects in the HaCaT keratinocytes.

Melittin is known to provoke Ca\(^{2+}\)-influx, which in turn would be a prime candidate for triggering ADAM activity. Dose-dependent increases of cytosolic Ca\(^{2+}\)-concentrations were indeed detected upon exposure to melittin. Surprisingly, however, ADAM upregulation by melittin displayed no requirement for Ca\(^{2+}\) and was observed in the presence of EGTA and in cells loaded with the intracellular calcium chelator BAPTA-AM. In the course of these investigations, we also employed conventional inhibitors of intra-cellular signaling pathways but could not identify any substance that effectively inhibited melittin-mediated ADAM activation (data not shown).

The link between melittin and ADAMs emerged as attention then turned to a possible involvement of purinergic receptors. A few studies have demonstrated that, among their many functions, these receptors also play a role in regulating ADAM activity. ADAM-dependent cleavage of L-selectin from human leukemic B-cells is inducible by extracellular ATP (49, 52), and in mice, shedding of this adhesion molecule is dependent on P2\(X7\)R (62). The same receptor controls shedding of CD23 and CD27, two important mediators of the immune system (53, 54). In those studies, ADAM activation was provoked by application of ATP at supraphysiological concentrations. Here, we propose that purinergic receptor activation via membrane attack by an extrinsic agent can lead to upregulation of ADAM activation in the responding cell. The conclusion is derived from two lines of evidence. First, melittin-mediated ADAM activation was suppressed by PPADS, Evans blue, and suramin, three commonly employed inhibitors of purinergic receptors. Second, transfection of P2\(X7\) in HEK293 cells increased the ERK-activating capacity of melittin, which was again abrogated in the presence of apyrase, clearly indicating that ATP release represents a critical step for downstream transduction mechanisms. Given the complexity and redundancy of the large receptor family, no attempts were here undertaken to identify other members that might be involved in ADAM activation. As in previous studies (31), our report is limited to the description of the phenomenon. Elucidation of the molecular links between purinergic receptors and ADAMs remains a challenge for the future.

According to our data, melittin induced P2 receptor activation appears to involve ATP. However, how ATP is released and how sufficient concentrations of ATP should accumulate to trigger the receptors is not clear. Multiple mechanisms mediate the release of intracellular ATP in response to diverse stimuli, including mechanical stimulation, stretch, osmolarity change, oxidative stress, and microbial products (63). The MTT assays indicate that egress of ATP from cells occurs already at non-toxic melittin concentrations. Thus, we assume that this process represents a physiological cellular response. The human cathelicidin-derived antimicrobial peptide LL-37 has similarly been reported to induce transient release of ATP in the absence of overt cell cytotoxicity (64).

Even though melittin membrane interaction is quite likely to directly induce ATP release, additional mechanisms might be involved such as the generation of reactive oxygen species (ROS) (12). P2\(X7\) receptor activation leads to Pannexin-1 pore formation through which ATP release could occur, so that a self-amplifying activation loop may furthermore be created.

However, the melittin concentrations employed incurred little depletion of cellular ATP, and only nanomolar concentrations were measurable in the supernatants. Although local ATP-concentrations at the cell surface may be considerably higher, P2 receptor and ADAM activation might also be effected through more complex events. This would be in accord with the finding that the ATPase hexokinase reduced the finding that the ATPase hexokinase reduced P2X7 receptor activation leads to Pannexin-1 pore formation through which ATP release could occur, so that a self-amplifying activation loop may furthermore be created.

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Such a direct interaction with P2 receptors has recently been proposed for the antimicrobial peptide LL-37 (66), and reports on the P2R and EGFR-activation properties of this peptide are of particular interest here (67, 68). There is evidence that the peptide induces IL-1β release in lipopolysaccharide-primed monocytes through activation of P2X7 (64). Furthermore, the same receptor appears to mediate the mitogenic effect of the peptide on epithelial cells. The latter finding accorded with earlier studies in which ATP had been shown to stimulate cell proliferation (69, 70), most likely due to ADAM activation.

To sum, this study has revealed that ADAM activation is a major downstream event occurring in the wake of membrane attack by melittin (Fig. 6). Depending on the cell target, very diverse functional consequences are likely to follow that can hardly be predicted or controlled. This should be of interest particularly to investigators who are engaged in the development of melittin as a therapeutic agent.

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**COMPETING FINANCIAL INTEREST**
The authors declare that they have no competing financial interests.

**FIGURE LEGENDS**

**FIGURE 1. Low concentrations of melittin do not affect cell viability.** Primary human monocytes (A), murine embryonic fibroblasts (MEFs) (B), human umbilical vein endothelial cells (HUVECs) (C), human neutrophil granulocytes (D), and HaCaT keratinocytes (E, F) were incubated with different dosages of melittin for 30 min. Reduction of MTT was measured as readout for cell viability (A-E) and ATP release (F) was determined using a luminescent ATP-based assay. Data represent mean values ± s.e.m. of three independent experiments all measured in triplicates. * indicates significant difference compared to control (*P<0.05; ** P<0.01; ***P<0.001).

**FIGURE 2. Melittin increases ADAM-mediated proteolysis in different cell types.** (A) Murine embryonic fibroblasts (MEFs) were mock treated (control) or stimulated with increasing amounts of melittin for 30 min. Total cell extracts from wildtype-MEFs (Wt) and ADAM10-/- fibroblasts were subjected to immunoblot analysis and stained with a C-terminal anti-N-cadherin antibody. N-cadherin C-terminal fragment (CTF) generation in Wt-MEFs was calculated by densitometric analysis of three independent experiments (right panel). Results are expressed as mean ± s.e.m. (n=3). CTF formation was significantly increased after melittin treatment (*P<0.05). (B) HUVECs were stimulated with melittin in the presence or absence of broadspectrum metalloprotease inhibitor marimastat (MM, 10 µM). VE-cadherin cleavage was analyzed with a C-terminal VE-cadherin antibody by immunoblot. (D) Densitometric analysis of three independent experiments is shown in the right panel (*P<0.05). (C) Human neutrophil granulocytes were stimulated with 0.5 µM melittin for 15 min in the presence or absence of marimastat (MM, 10 µM). Cell surface expression of CD62L was determined by FACS analysis.

**FIGURE 3. Melittin augments ADAM-dependent E-cadherin shedding and EGFR activation in HaCaT keratinocytes.** (A) After incubation with different amounts of melittin, HaCaT keratinocyte pellets were subjected to immunoblot analyses using a C-terminal E-cadherin antibody. E-cadherin CTF generation was calculated by densitometric analysis of three independent experiments (right panel). Results are expressed as mean ± s.e.m. (n=3). (B) Cells were treated with the ADAM10 inhibitor GI254023X (GI, 3 µM), the ADAM17/ADAM10 inhibitor GW280264X (GW, 3 µM), the broad-spectrum metalloprotease inhibitor marimastat (MM, 10 µM), or DMSO (D) as a control for 10 min before stimulation. E-cadherin CTF generation was calculated by densitometric analysis of three
independent experiments (right panel). Melittin significantly increased E-cadherin proteolysis (*P<0.05, n=3). Marimastat, GI and GW significantly decreased this melittin effect (#P<0.05, n=3).

(C) Release of soluble TGF-alpha (sTGF-alpha) from HaCaT keratinocytes was analyzed by ELISA and compared to mock treated cells. Cells were stimulated with 0.5 µM melittin for 30 min in the presence or absence of the ADAM10 inhibitor GI254023X (GI) or the ADAM17/ADAM10 inhibitor GW280264X (GW). Data represent mean values of 5 independent experiments with s.e.m. Melittin significantly decreased sTGF-alpha release (**P<0.01, n=5). Marimastat and GW significantly decreased melittin-induced TGF-alpha shedding (#P<0.05, ###P<0.001, n=5).

(D) HaCaT keratinocytes were treated with melittin for 30 min in the presence or absence of marimastat (MM, 10 µM). Representative western blot analyses of EGFR phosphorylation and ERK1/2 phosphorylation are shown with an immunoblot of total EGFR and total ERK1/2 included as loading control.

FIGURE 4. Melittin augments metalloprotease-dependent proliferation and migration of epithelial cells. (A) Cell cycle analysis was determined by propidium iodide (PI) staining and flow cytometry. HaCaT keratinocytes were seeded at 20,000 cells/well in microtiter plates and stimulated with EGF (4 nM) as positive control and with melittin (0.5 µM). After 24 h, increased percentages of EGF and melittin treated cells are in S+G2/M phase. TAPI (10 µM) abrogated the melittin-induced effect. Analyses are representative of two independent experiments. (B; C) Melittin stimulates epithelial cell migration. HaCaT keratinocytes were grown to confluence and a cell-free area was introduced by scratching with a pipette tip. After washing, cells were mock treated or incubated with the indicated amounts of melittin (B) in the presence of the broad-spectrum metalloprotease inhibitor marimastat (MM, 10 µM) or (C) with the EGFR blocking antibody Cetuximab (Cetux, 10 µg/ml). Migration was evaluated after 24 h. Micrographs of one representative of 3 independent experiments are shown, respectively. Bar: 100 µm. Migration of three independent assays was quantified in the right panel. Asterisk (*) indicates a significant increase compared with mock-treated samples (*P<0.05, n=3). MM and Cetux significantly inhibited melittin induced migration (#P<0.05, n=3).

FIGURE 5. P2 receptor signaling is involved in activation of melittin effects in HaCaT keratinocytes. (A) HaCaT keratinocytes were stimulated with melittin (0.5 µM) for 30 min in the presence of increasing amounts of PPADS. Representative western blot analyses of ERK1/2 phosphorylation with an immunoblot of total ERK1/2 included as loading control is shown in the upper panel. PPADS (PP, 100 µM) inhibition of melittin-induced E-cadherin proteolysis was nearly as effective as marimastat (MM, 10 µM), lower panel. (B) P2 receptor antagonist suramin (Sur) led to decrease of ERK1/2 phosphorylation and E-cadherin CTF generation at concentrations of 100 and 200 µM. (C) The P2 receptor antagonist Evans blue (EB) was found to inhibit melittin effects effectively at a concentration of 1 µM. (D) ATPase hexokinase dose-dependently decreased ERK1/2 phosphorylation and E-cadherin shedding. (E) HEK293T cells were mock-transfected or transfected with P2X7. 48 h afterwards, cells were stimulated with melittin (0.5 µM) in the presence of marimastat (MM, 10 µM) or ATPase apyrase (Ap, 2 U/ml). Stimulation with bzATP (300 µM) was used as positive control. One representative immunoblot of three independent experiments is shown for all analyses.

FIGURE 6. Schematic representation of melittin stimulated effects. Melittin-induced ATP release leads to activation of P2X and P2Y receptors, which trigger several signaling pathways. Whether and how these signaling cascades activate ADAM10 and ADAM17 remains nebulous. ADAM10 and ADAM17 activation results in shedding of E-cadherin (E-cad) and in the release of EGFR ligands. E-cadherin shedding decreases cell-cell adhesion and induces cell migration. EGFR phosphorylation triggers downstream signaling cascades leading to increased HaCaT keratinocyte migration and proliferation.
Figure 2

A

Wt MEFs

ADAM10−/− MEFs

kDa 130

N-cad/ FL

CTF

B

HUVEC

kDa 130

VE-cad/ FL

CTF

C

granulocytes

Cell Counts

Mel 0.5 µM + MM

control

Mel 0.5 µM

L-selectin

10^0 10^1 10^2 10^3
Figure 3

A

B

C

D

E-cad/FL

120 kDa

CTF1

HaCaT

Mel 0.5 µM

sTGF-alpha

10

5

0

co

co

MM

GI

GW

Mel 0.5 µM

X-fold increase

(compared to Ctrl)

DMSO

MM

Mel 0.5 µM

pEGFR

EGFR

pERK

ERK

HaCaT

**

###

#
Figure 4

A

| Condition          | S+G2/M: |
|--------------------|---------|
| Control            | 17.2%   |
| EGF                | 56.2%   |
| Mel 0.5 µM         | 61.4%   |
| Mel 0.5 µM + TAPI  | 35.2%   |

B

| Condition          | 0 h | 24 h |
|--------------------|-----|------|
| control DMSO      |     |      |
| Mel 0.5 µM DMSO   |     |      |
| Mel 0.5 µM MM     |     |      |

C

| Condition          | 0 h | 24 h |
|--------------------|-----|------|
| control            |     |      |
| Mel 0.5 µM         |     |      |
| Mel 0.5 µM + Cetux |     |      |

HaCaT
Figure 5

**A**

Melittin 0.5 µM

| µg/ml PPADS | pERK | ERK |
|-------------|------|-----|
| -           | -    | -   |
| 1           |      |     |
| 10          |      |     |
| 50          |      |     |
| 100         |      |     |
| 100         |      |     |

Melittin 0.5 µM

| µg/ml Sur | pERK | ERK |
|-----------|------|-----|
| -         | -    | -   |
| 1         |      |     |
| 10        |      |     |
| 50        |      |     |
| 100       |      |     |
| 200       |      |     |

**B**

Melittin 0.5 µM

| µg/ml Melittin | pERK | ERK |
|----------------|------|-----|
| -              | -    | -   |
| 0.5            |      |     |

**C**

Mel 0.5 µM

| µg/ml Melittin | pERK | ERK |
|----------------|------|-----|
| -              | -    | -   |
| 0.5            |      |     |

**D**

HEK293T

| Hexokinase | pERK | ERK |
|------------|------|-----|
| -          | -    | -   |
| 0.25       |      |     |
| 0.5        |      |     |
| 1          |      |     |

**E**

HEK293T

| P2X7 transfection | pERK | ERK |
|--------------------|------|-----|
| -                  | -    | -   |
| +                  |      |     |
| -                  | -    | -   |
| +                  |      |     |
Figure 6

Deadhesion Migration

ATP
Melittin
Ca²⁺
P2X
P2Y
EGFR Ligand
Ca²⁺
Gq,i
PLC
DAG
PKC
CaMK
MAPK
pERK
Proliferation Migration

E-cad A10 A17

EGFR

CPLA₂, PLD

?
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