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Enhancement of proteolytic processing of the β-amyloid precursor protein by hyperforin

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Abstract

We studied the effect of hyperforin, a component of St. John's wort (*Hypericum perforatum*) extracts, on the processing of the amyloid precursor protein (APP) in rat pheochromocytoma PC12 cells, stably transfected with human wildtype APP. We observed transiently increased release of secretory APP fragments upon hyperforin treatment. Unique features, like a strong reduction of intracellular APP and the time course of soluble APP release, distinguished the effects of hyperforin from those of alkalizing agents and phorbol esters, well known activators of secretory processing of APP. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a protonophore, induced an almost identical decrease in intracellular pH in PC12 cells as does hyperforin. Despite this, FCCP induced a less pronounced release of soluble APP fragments and only slightly reduced intracellular APP levels. These results suggest that hyperforin is an activator of secretory processing of APP with a novel mechanism of action not solely dependent on its effects on intracellular pH.

Keywords: Hyperforin; PC12 cells; APP processing; Secretase; Intracellular pH

1. Introduction

The processing pathways of the APP represent an increasingly important target for the development of treatment strategies for AD [1]. It is established by now that massive accumulation and aggregation of extracellular Aβ, a proteolytic product of APP, into amyloid plaques in the brain of AD patients is at least in part a consequence of altered proteolytic processing of the precursor protein. APP is cleaved by three different proteolytic activities, the so-called secretases, of which only one, the β-secretase, has been identified unambiguously to date [2]. Several candidate enzymes, especially of the disintegrin-metalloprotease family, have shown α-secretase-like activity so far. For γ-secretase activity, the presenilins, proteins mutated in a large number of early-onset familial AD cases, are strong candidates. But it is still a matter of ongoing discussion, if the presenilins are indeed the catalytic component of this apparently large protease complex (reviewed in [3]).

Hypericum perforatum (St. John's wort) extracts have been used since long to treat mild depressive episodes. Many, but not all clinical trials have confirmed its antidepressant properties [4–7]. The extract contains several components, one of which is the phloroglucinol-derivative hyperforin. A number of actions have been attributed to hyperforin. These are: broad reuptake inhibition for a large number of neurotransmitters [8], effects on intracellular sodium and calcium homeostasis [8,9] and on ion channels [10], antioxidative properties [11], effects on cytochrome P450 drug-metabolizing enzymes [12,13], inhibition of cyclooxygenase-1 and 5-lipoxygenase [14] and effects on membrane fluidity [15]. In addition, at high concentration, hyperforin has shown growth-inhibitory and apoptosis-inducing effects in cultured cells [16–18]. Hyperforin also affects pH regulation [8,19,20], which prompted us to investigate the effect of hyperforin on the processing of the APP, since a number of compounds affecting intracellular pH (such as ammonium chloride, chloroquine, monensin,

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; sAPP, soluble fragment of APP; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; BCECF/AM, 2′,7′-bis(2-carboxyethyl)-5(6′)-carboxyfluorescein, acetoxymethyl ester; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMA, phorbol-12-myristate-13-acetate.

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bafilomycin A1 and FCCP) have previously been shown to alter APP processing in a variety of cells [21–31]. We compared the effects of hyperforin with two of these compounds, bafilomycin A1 and FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazon). Bafilomycin A1 is a specific inhibitor of vacuolar ATPases at low concentrations and thus inhibits acidification of a number of intracellular organelles [32]. FCCP is a protonophore and an uncoupler of mitochondrial oxidative phosphorylation and thus primarily dissipates the pH gradient over the mitochondrial inner membrane, but has also shown effects at the level of the plasma membrane [33–35]. We analyzed the influence of these drugs on cytosolic pH using fluorescence imaging with the fluorescent dye BCECF.

2. Materials and methods

2.1. Cell culture

All cell culture media and media supplements were from Invitrogen. Rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 5% heat-inactivated horse serum, 50 units/mL penicillin and 50 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°. PC12 cells stably transfected with human wildtype APP (wt APP) were cultured in media supplemented with 400 μg/mL G418 (PAA Laboratories) and have been described previously [36].

2.2. Experimental treatment of PC12 cells

Cells were plated at 4×10^6 cells per dish in poly-L-lysine-coated 60 mm culture dishes 20 hr before drug exposure. Cells were washed twice with phosphate-buffered saline (PBS) and media changed to Opti-MEM supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin and 400 μg/mL G418 (serum free). Cultures were then exposed to vehicle (DMSO 1%) or varying concentrations of the sodium salt of hyperforin (50 nM–10 μM, freshly prepared solutions; kind gift from Schwabe), bafilomycin A1 (100 nM, Alexis), FCCP (5 μM, Fluka) and PMA (100 nM, Alexis) for the indicated periods of time. All drugs were dissolved in DMSO, the final concentration of DMSO in media did not exceed 1%.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting analysis

Conditioned media were analyzed for secreted N-terminal APP fragments and cell lysates were analyzed for full-length intracellular APP by SDS–PAGE and western blotting. Proteins from conditioned media were precipitated with trichloroacetic acid and resuspended in 10 mM Tris base. Cells were washed with cold PBS and lysed by 20 min incubation in lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 2 mM each of EDTA and DTT, 1% NP-40, protease inhibitor cocktail) (Complete®, Roche Diagnostics). The lysates were centrifuged at 20,000 *g* and the supernatant used for gel electrophoresis. Equal amounts of protein were resolved by 8% SDS–PAGE, followed by electroblotting onto nitrocellulose membrane (Whatman Biometra). Membranes were blocked for 2 hr in 5% nonfat dry milk in TBST (Tris-buffered saline with 0.1% Tween 20), and incubated overnight at 4° with antiserum 5313 (1:1400 in 50% horse serum in TBST). 5313 is a human specific antiserum raised against an epitope located just N-terminal of the Aβ region (amino acids 444–592, human APP695 numbering) [37] and thus does not detect the endogenous rat APP in PC12 cells. An antibody against actin (goat polyclonal antibody, against amino acids 357–375 of human actin, Santa Cruz) was used as a control. After washing, the membranes were incubated with secondary goat–anti-rabbit horseradish peroxidase-conjugated antibody (1:10,000) for 1 hr at room temperature. Detection of immunoreactive proteins was performed with the enhanced chemiluminescence (ECL; Amersham) system.

2.4. Densitometry and data analysis

Densitometry of western blot data was done with Kodak Digital Science™ 1D Image Analysis Software, statistical analysis was performed using unpaired *t*-test or one-way ANOVA, followed by Tukey-test.

2.5. Intracellular pH measurements

Intracellular pH ($[pH]_i$) in cells was measured by fluorescence imaging of the H^+-sensitive fluorescent dye BCECF. Cells were seeded at 9×10^5 cells per well onto poly-L-lysine-coated cover slips in 35 mm culture dishes 20 hr before loading with BCECF-AM (Molecular Probes). Cells were washed once and media were changed to loading buffer (HBSS, pH 7.4; 37°). Cells were loaded with a final concentration of 5 μM BCECF-AM at 37° in the dark for 30 min. BCECF-loaded cells were stored in the dark at 37° for up to 3 hr. Immediately before measurement of intracellular pH, cells were washed with HBSS. The fluorescence was measured at 488/460 nm (excitation) with emission at 520 nm on a Zeiss Axiovert inverted microscope (40× oil immersion objective) coupled to an Attofluor imaging system. pH calibration was done according to James-Kracke [38]. Alternatively, cells were seeded at 2.5×10^6 cells/well in 6 cm uncoated culture dishes and were loaded with BCECF as before, washed three times in HBSS to remove external dye and analyzed in suspension using the SLM-Aminco Luminescence Spectrometer at 490 nm (excitation) with emission at 535 nm. pH calibration was performed using BCECF-loaded cells equilibrated in HBSS of different pH and measuring fluorescence

after lysis with 10% Triton X-100. Results were essentially the same with both methods and have been combined for Figs. 3B, 4A and 5C.

3. Results

We used PC12 cells that were stably transfected with human wt APP [36]. Human intracellular APP from cell lysates and N-terminal secretory APP fragments from conditioned media were selectively detected with a human-specific antiserum, 5313 [37], raised against an epitope located just N-terminal of the Aβ region (amino acids 444–495, human APP695 numbering). PC12 cells are derived from a rat pheochromocytoma and are widely used as a model system for many different aspects of neuronal function [39,40]. Hyperforin has been shown to induce apoptosis in several tumor cell lines at high concentrations [17]. However, under our conditions (incubation up to 2 hr, hyperforin up to 10 μM) no effects on PC12 cell viability (MTT- and LDH-assay) could be detected (data not shown).

We found that hyperforin treatment of wt APP-transfected PC12 cells activated secretion of sAPP into the medium with a highly significant concentration effect as indicated by one-way ANOVA (Fig. 1). Analysis of the secreted APP fragments in the media with antibody 6E10 [41], which was raised against an epitope in the N-terminal Aβ-region, and thus detects only α-secretase-derived sAPP species, revealed a very similar pattern to that of the antiserum 5313, which recognizes α-secretase as well as β-secretase cleaved N-terminal APP fragments (Fig. 2). This indicates, that the majority of sAPP fragments of PC12 cells are α-secretase-derived, as has also been shown for other cell types [42].

Hyperforin induces a decrease in intracellular pH, a representative time course is shown in Fig. 3A. In the concentration range tested (100 nM–10 μM), hyperforin

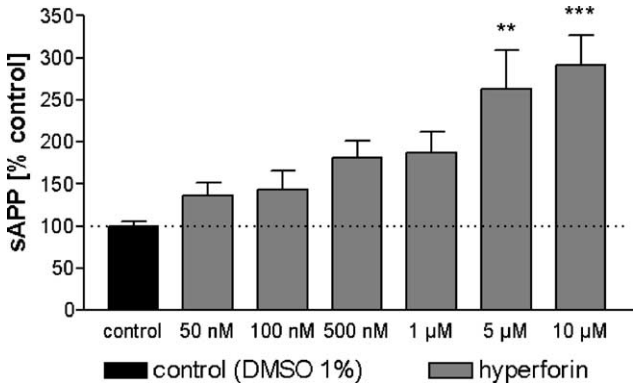

Fig. 1. Hyperforin increases secretion of sAPP. wt APP-transfected PC12 cells were treated with 1% DMSO (control) or the indicated concentrations of hyperforin for 2 hr. sAPP fragments were quantified from media by western blot analysis using antiserum 5313. Results are expressed as percentage of the vehicle-treated control group. Means and SEM are shown for 6–8 separate experiments. One-way ANOVA: $P < 0.0001$; Tukey's post-hoc analysis: $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, hyperforin vs. vehicle-treated control.

induced a decrease in cytosolic pH of 0.2–0.6 pH units, with significant effects at rather low concentrations (Fig. 3B). When compared, 5 and 10 μM FCCP and hyperforin show a quite similar pattern. Both significantly reduced intracellular pH by about 0.6 pH units (Fig. 4A). sAPP release from PC12 cells is significantly, although only transiently elevated by hyperforin under these conditions (maximal effect at 30 min, back to baseline level after 4 hr, data not shown). FCCP shows a less pronounced, but similar pattern, also with a slightly stronger effect at 30 min than at 2 hr of treatment (Fig. 4B). Intracellular APP is significantly reduced by both compounds, but the effect of hyperforin, especially at 2 hr of treatment, is much stronger (Fig. 4C). These data are in contrast to Connop *et al.* [31], who found strongly reduced sAPP levels and no effect on intracellular APP levels upon FCCP treatment. However, a different cell line and different incubation conditions were used. When tested under the same conditions,

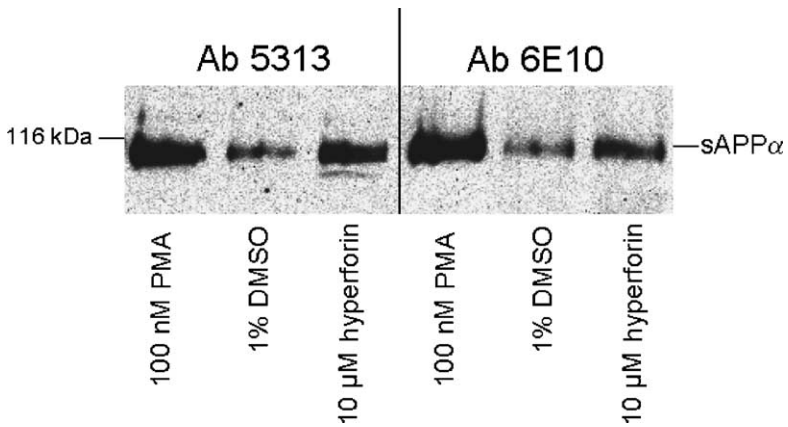

Fig. 2. Hyperforin increases α-secretase-generated sAPP fragments. wt APP-transfected PC12 cells were treated with 100 nM PMA (positive control), 1% DMSO (vehicle control) and 10 μM hyperforin for 2 hr. sAPP fragments were analyzed by western blot with antiserum 5313 or with monoclonal antibody 6E10. 6E10 only recognizes α-secretase-generated sAPP fragments, whereas 5313 detects α- as well as β-secretase-generated sAPP fragments. Note the very similar pattern for both antibodies.

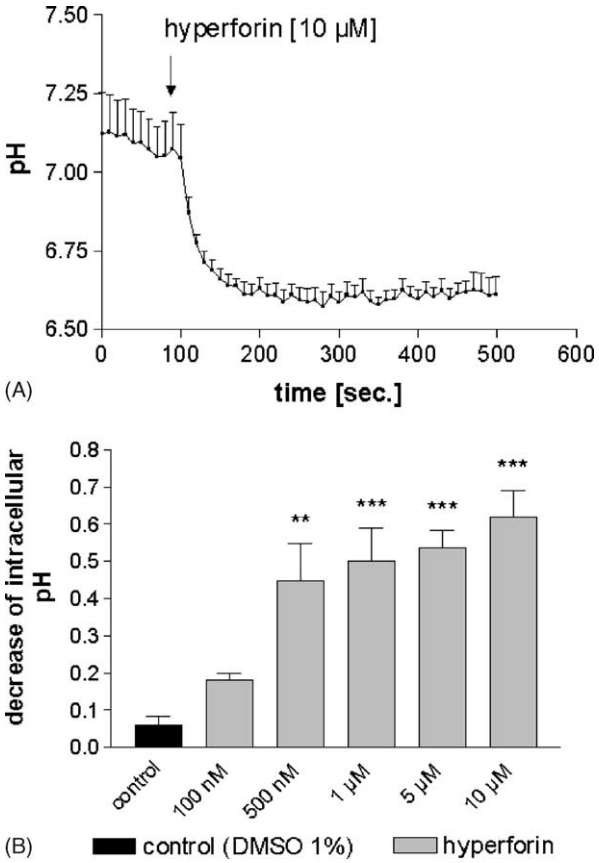

Fig. 3. Hyperforin decreases cytosolic pH in PC12 cells. Intracellular pH of wt APP-transfected PC12 cells was measured by fluorescence imaging of the pH-sensitive dye BCECF. Time course of the change in intracellular pH, measured in suspension, after the addition of 10 µM hyperforin at 100 s (A). Means and SEM are shown for 6 separate experiments. Decrease of intracellular pH after treatment with 1% DMSO (vehicle control) and the indicated concentrations of hyperforin (B). Means and SEM are shown for 7–9 separate experiments. One-way ANOVA: $P < 0.0001$; Tukey's post-hoc analysis: $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, hyperforin vs. vehicle-treated control.

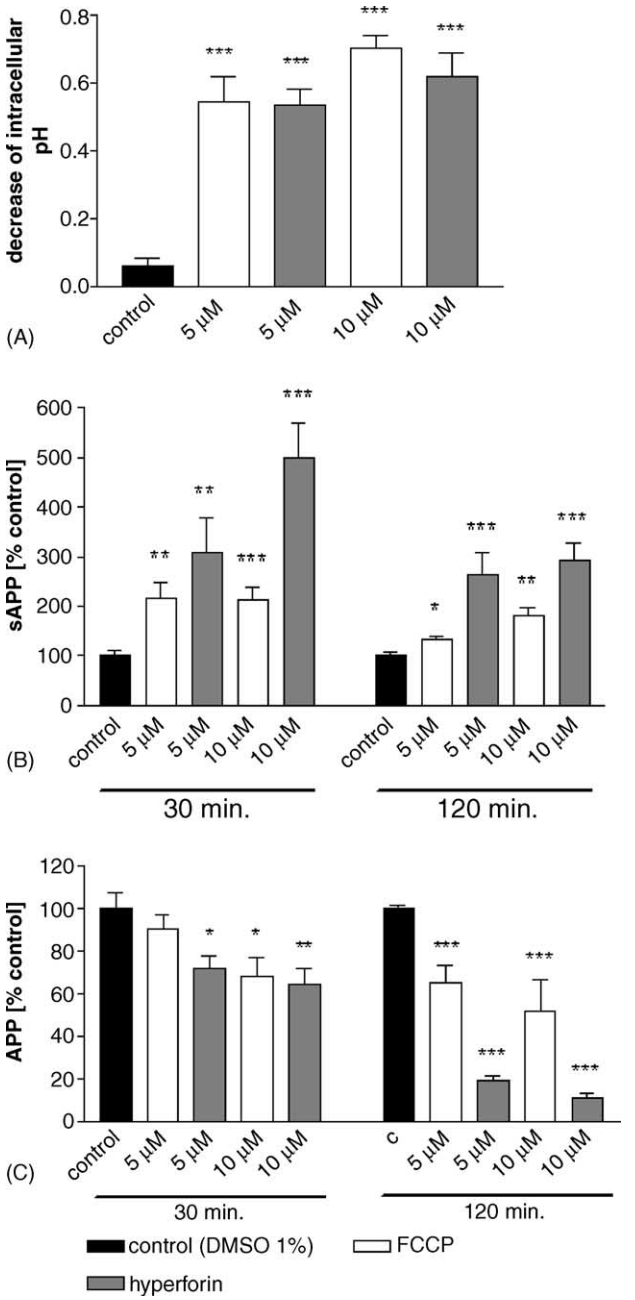

Fig. 4. Comparison between hyperforin and FCCP. Effects on intracellular pH (A). Means and SEM are shown for 6–9 separate experiments. Unpaired *t*-test: $^{***}P \leq 0.001$, hyperforin and FCCP vs. vehicle-treated control. Effects on sAPP release (B) and intracellular APP (C) were analyzed as described above. Results in 'B' and 'C' are expressed as percentage of vehicle-treated control for each individual time point. Means and SEM are shown for 6–8 separate experiments. Unpaired *t*-test: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, hyperforin or FCCP vs. control for each individual time point.

100 nM PMA, a phorbol ester and known α-secretase activator [43], increases sAPP production continuously and does not reduce intracellular APP production within the time frame analyzed (data not shown), which confirms previous data, also obtained with PC12 cells [44,45]. The effect of hyperforin was not just the consequence of a nonspecific loss of protein, as our data are normalized for total protein. Moreover, actin concentrations measured in the lysate as a control were not significantly altered by 10 µM hyperforin and incubation times of 30 and 120 min (data not shown).

Bafilomycin A1, induced an increase in the release of sAPP fragments already at 100 nM, which is in agreement with earlier reports [27,28,30,31]. However, the effect of bafilomycin A1, especially at 30 min of treatment, is only modest compared to hyperforin (Fig. 5A). While both drugs increase the secretion of sAPP fragments, the effects on intracellular APP are quite different. Hyperforin shows a rapid and strong reduction in intracellular APP levels, whereas a significant increase is observed upon bafilomycin A1 treatment (Fig. 5B), which is in line with previous findings [27,28,30,31]. Regarding the effects on intracellular pH, hyperforin shows a much stronger effect, reducing cytosolic pH by about 0.6 pH units, while bafilomycin A1 only shows a small effect (Fig. 5C). Apparently, alkalinization of the endosomal compartment by bafilomycin

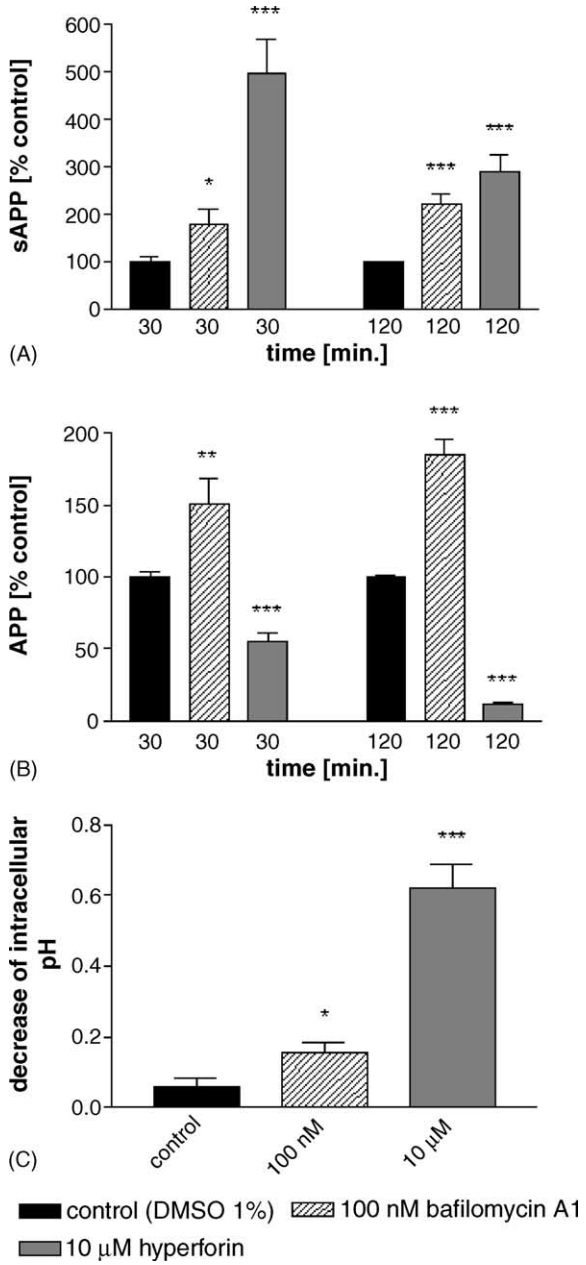

Fig. 5. Comparison between hyperforin and bafilomycin A1. Effects on sAPP release (A) and intracellular APP (B) were analyzed as described above. Results in 'A' and 'B' are expressed as percentage of vehicle-treated control for each individual time point. Means and SEM are shown for 6–8 separate experiments. Unpaired *t*-test: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, hyperforin or bafilomycin A1 vs. control for each individual time point. Effects on intracellular pH (C). Means and SEM are shown for 7–9 separate experiments. Unpaired *t*-test: $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$, hyperforin and bafilomycin A1 vs. vehicle-treated control.

A1 is not detected by BCECF, in contrast, a slight acidification of the cytosolic compartment is observed.

4. Discussion

Hyperforin has recently been shown to possess cell toxic, e.g. apoptosis-inducing and cell proliferation-inhibiting properties [16–18]. Brain cells seem to be less sensitive than tumor cells [18]. These effects were usually seen at high hyperforin concentrations (10 μM and higher) and long incubation periods (24 hr). It is therefore important to note that for the conditions used in the present paper, no cell toxic effects were observed (MTT- and LDH-assay, data not shown).

The present study analyzes the effect of hyperforin, a component of *H. perforatum* (St. John's wort) extracts, on the processing of the APP. We found that treatment with hyperforin of cultured PC12 cells, stably transfected with human wt APP, activates the release of sAPP fragments into the media (Fig. 1). This indicates that hyperforin might act as an activator of secretory processing of APP or that it might increase the availability of the substrate APP to secretory pathways. Further analysis however, revealed marked differences to the actions of phorbol esters, well-known activators of α-secretase [43], which increase α-secretase activity by activating protein kinase C pathways. In contrast to the transient effect of hyperforin we observed on sAPP release (Fig. 4B), phorbol esters lead to a continuous activation of sAPP secretion over many hours under the same conditions (data not shown and [44]). In addition, hyperforin rapidly and strongly decreases intracellular APP levels (Fig. 4C), whereas phorbol esters showed no effect on intracellular APP in the time frame analyzed (data not shown and [45]). Even if first effects of hyperforin on APP processing were already seen at concentrations around 1 μM, most of the data were obtained at maximal effective concentrations (5 and 10 μM) to ensure stable differences. However, it should be kept in mind that IC_{50} values for these effects are around 1 μM and the IC_{50} for the decrease of pH is even lower, which differentiates these effects from the cell toxic effects mentioned above [17,18]. In this concentration range (around 1 μM), hyperforin specifically affects the annular fluidity of the fluorescence probe pyrene, which probably reflects fluidity close to membrane proteins [15]. Effects of hyperforin on the flexibility of the hydrocarbon core were only seen at concentrations >10 μM [15] and might speculatively be associated with the cell toxic effects described above.

As hyperforin decreases intracellular pH [8], we compared the effect of hyperforin with two other compounds known to affect pH regulation as well as APP processing: bafilomycin A1 and FCCP. Bafilomycin A1 is a macrolide antibiotic that is an inhibitor of vacuolar ATPases. It thus inhibits acidification of intracellular compartments such as lysosomes, endosomes and the *trans*-Golgi network [32]. Bafilomycin A1 does not lead to morphological alterations of the vacuolar compartment, as it is known for weak bases, the ionophore monensin and for brefeldin A [46]. Under our experimental conditions, hyperforin decreases cytosolic pH in wt APP-transfected PC12 cells by about 0.6 pH units, whereas bafilomycin A1 only showed a very moderate effect (Fig. 5C). Similarly, the increase in sAPP secretion, at least at 30 min of treatment, is much more

pronounced for hyperforin than for bafilomycin A1. At 2 hr of treatment, the response to both drugs is quite similar. In contrast, the effects of the two compounds on intracellular APP levels were clearly very different (Fig. 5B). Hyperforin significantly reduces intracellular APP levels, while they are significantly elevated by bafilomycin A1. In human embryonic kidney (HEK293) cells, stably transfected with human wt APP, bafilomycin A1 also leads to the accumulation of mature intracellular full-length APP and increased secretion of sAPP, as well as Aβ and p3 [27,28,30,31]. Maturation, transport to the membrane and reinternalization of APP were not impaired under these conditions [28]. Bafilomycin A1 most probably stabilizes full-length APP by reducing degradation due to the alkalinization of the endosomal compartment. Only 30% of APP is diverted to secretory pathways in HEK293 cells, the rest is rapidly degraded [24]. Inhibition of this degradation pathway by bafilomycin A1 would therefore lead to increased availability of the substrate APP to secretases, which would explain the observed increase of all secretory APP fragments in the media. Since intracellular APP is reduced by hyperforin, we conclude that hyperforin does not have a bafilomycin-like effect on the endosomal compartment. Activation of secretory processing of APP by hyperforin is certainly not due to an increase in substrate availability, but might rather result from a direct or indirect activation of the secretases themselves.

FCCP is a protonophore affecting the proton gradient over the mitochondrial inner membrane [33]. However, significant effects on plasma membrane potential and intracellular sodium and proton concentrations have also been shown [34,35]. It has been reported that hyperforin has similar properties to the protonophore FCCP in synaptic vesicles [19,47] and in a smooth muscle cell line [20]. We therefore compared the effects of hyperforin and FCCP on intracellular pH and on APP processing in PC12 cells. Hyperforin and FCCP both reduced intracellular pH by about 0.6 pH units (Fig. 4A). Under conditions that did not reduce intracellular ATP levels and did not impair endosomal function or cell viability in wt APP-transfected HEK293 cells, Connop *et al.* [31] found decreased sAPP and Aβ secretion upon FCCP treatment. In addition, no change in the level of intracellular APP was observed, indicating that FCCP does not impair maturation or degradation of APP. Unlike Connop *et al.* [31] we found a small decrease in the level of intracellular APP and observed modestly elevated sAPP secretion in PC12 cells upon FCCP treatment (Fig. 4B and C) which might be due to the different cell line used. Our results demonstrate that, although hyperforin and FCCP induce a very similar decrease in cytosolic pH in PC12 cells, their effects on APP processing are different. Hyperforin reduces intracellular APP and stimulates sAPP release much more prominently under our conditions than does FCCP. This suggests that the effects of hyperforin on secretory APP processing might not solely depend on its effects on intracellular pH.

The strong reduction of intracellular APP levels that is observed upon hyperforin treatment is much more pronounced than the qualitatively similar effect of FCCP. This excludes that increased substrate availability is the reason for the increased sAPP secretion, but might explain its transient time course. We therefore propose that hyperforin is an activator of secretory processing of APP. Our results with the 6E10 antibody (Fig. 2) might suggest an effect primarily on α-secretase, but effects on β-secretase cannot be excluded. We do not assume that hyperforin is a phorbol ester-like activator of α-secretory processing, since reductions of intracellular APP levels are seen only after prolonged incubation with phorbol esters [45], whereas the effect of hyperforin is evident after only 15 min.

In conclusion our results suggest that hyperforin may present a new principle to influence APP processing. α-Secretase as well as β-secretase have been shown to be membrane-associated enzymes. It has been speculated that specific changes of the properties of neuronal membranes already induced by hyperforin concentrations of 500 nM are the basis for its effects on several ionic conductance mechanisms [15]. It is therefore possible that effects of hyperforin on membrane fluidity [15] may also be relevant for its effects on APP processing. Cholesterol, which also modulates membrane properties, has been shown to decrease α-secretase activity [48–50], whereas β- and γ-secretase activities are increased [51]. Besides affecting many ionic conductance mechanisms speculatively by changing membrane properties [8,10,52,53] hyperforin is also a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase [14]. Both mechanisms are involved in APP processing [54,55]. Further studies are needed to finally elucidate mechanisms of hyperforin effects on APP processing.

Acknowledgments

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