British Journal of Pharmacology (2005) 145, 75–83
www.nature.com/bjp

Hyperforin activates nonselective cation channels (NSCCs)

[1]Kristina Treiber, [1]Andrea Singer, [1]Bettina Henke & *,[1]Walter E. Müller

[1]Department of Pharmacology, Biocenter, N260, University of Frankfurt, Marie-Curie-Str. 9, 60439 Frankfurt, Germany

1 A large body of evidence supports the preclinical antidepressant profile of hyperforin including inhibition of the synaptosomal uptake of several neurotransmitters by hyperforin and studies in behavioural models. In contrast to other antidepressants, hyperforin does not directly inhibit neurotransmitter transporters, but instead uptake inhibition seems to be the consequence of an elevated intracellular sodium concentration ($[Na^+]_i$).

2 The mechanism of hyperforin-induced elevation of $[Na^+]_i$ was investigated using two different cell types: human platelets and rat pheochromocytoma cells (PC12 cells). In both cell systems, hyperforin increased both $[Na^+]_i$ and free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

3 One pathway for Na^+ and Ca^{2+} entry is mediated by nonselective cation channels (NSCCs), which can be blocked by SK&F 96365 and LOE 908. LOE 908 is a blocker of both NSCC1 and NSCC2 subclasses, while SK&F 96365 blocks NSCC2 only. Both SK&F 96365 and LOE 908 completely inhibited the hyperforin-induced influx of Na^+ and Ca^{2+} into platelets and PC12 cells. This indicates that hyperforin is mainly active upon NSCC2.

4 The effect of hyperforin is inhibited by La^{3+} and Gd^{3+}, indicating that there is a potential homology with canonical transient receptor potential protein channels (TRPC channels). Moreover, La^{3+} and Gd^{3+} attenuate the effect of hyperforin on serotonin uptake in human platelets. Additionally, hyperforin induces barium influx in PC12 cells and this influx can be inhibited by SK&F 96365, LOE 908, Gd^{3+} and La^{3+}.

5 In summary, these findings suggest that hyperforin represents a new principle for preclinical antidepressant activity, modulating brain neurotransmission by inhibition of neurotransmitter uptake *via* activation of NSCCs.

British Journal of Pharmacology (2005) **145,** 75–83. doi:10.1038/sj.bjp.0706155
Published online 21 February 2005

Keywords: St John's wort; hyperforin; sodium; calcium; NSCCs; TRPC channels; SK&F 96365; LOE 908; gadolinum; lanthanum

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_o$, extracellular calcium concentration; Gd^{3+}, gadolinum; HBSS, Hank's balanced salts; HEPES, 2-(4-(2-hydroxyethyl)-1-piperazinyl]-ethansulphonacid; La^{3+}, lanthanum; $[Na^+]_i$, intracellular sodium concentration; $[Na^+]_o$, extracellular sodium concentration; NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+-exchanger; NSCCs, nonselective cation channels; PC12 cells, rat pheochromocytoma cells; SOCCs, store operated calcium channels; TMA, tetramethyl-ammoniumchloride; TRP channels, transient receptor potential protein channels; TRPC channels, canonical transient receptor potential protein channels

Introduction

Extracts of the medicinal plant *Hypericum perforatum* (St John's wort) are broadly used in many countries to treat mildly to moderately depressed patients (Di Carlo *et al*., 2001).

St John's wort extract has a large number of active constituents. Of these, the phloroglucinol derivative hyperforin, and its analogue adhyperforin, inhibit neuronal uptake of several neurotransmitters including serotonin, norepinephrine, dopamine, GABA and L-glutamate into neurons and astrocytes (Chatterjee *et al*., 1998; Jensen *et al*., 2001; Neary *et al*., 2001; Wonnemann *et al*., 2001). Consequently, hyperforin administration to rats causes elevated extracellular brain concentrations of serotonin, norepinephrine, dopamine and L-glutamate (Kaehler *et al*., 1999; Phillipu, 2001). Furthermore, hyperforin is active in several behavioural models of antidepressant activity (Misane & Ogren, 2001; Cervo *et al*., 2002).

Compared to all other known antidepressant drugs, hyperforin prevents the synaptosomal uptake of several neurotransmitters and of choline with comparable potency (Chatterjee *et al*., 1998; Jensen *et al*., 2001; Wonnemann *et al*., 2001; Buchholzer *et al*., 2002). All respective transporter proteins are driven by the sodium gradient across the neuronal membrane. Our previous findings were that hyperforin, at concentrations sufficient to inhibit serotonin uptake, causes an elevation of (intracellular sodium concentration ($[Na^+]_i$)) in human platelets. This provides a convincing explanation for the effect of hyperforin on the neuronal reuptake of several neurotransmitters, including choline (Singer *et al*., 1999). That sodium conductive pathways have a role in the mechanism of action of hyperforin is further supported by the findings that amiloride, and some of its derivatives, at high concentrations blocked the effect of hyperforin on neurotransmitter reuptake.

*Author for correspondence;
E-mail: PharmacolNat@em.uni-frankfurt.de
Published online 21 February 2005

This lead us to the hypothesis that hyperforin might interfere with the Na^+/H^+-exchanger (NHE) (Wonnemann *et al*., 2000). However, the present study shows that the effect of hyperforin on $[Na^+]_i$ is independent of the NHE but can instead be explained by the activation of nonselective cation channels (NSCCs).

Methods

^{3}H-serotonin uptake in human platelets

Blood (10 ml) from healthy unmedicated volunteers was anticoagulated in S-Monovettes with 1.0 ml citrate solution and centrifuged for 15 min at $250 \times g$ at room temperature (RT). The upper two-thirds of the platelet-rich plasma were collected in a Falcon tube and centrifuged at $800 \times g$ for 30 min at RT. The resulting pellet was resuspended in a four-fold amount of Krebs–Henseleit phosphate buffer (NaCl 118 mM, KCl 4.6 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, glucose 10 mM, ascorbic acid 0.1% l^{-1}; pH 7.4 at 37°C). In total, $1–2 \times 10^7$ platelets were used for each experiment. The platelets were preincubated in a shaking water bath for 15 min with various agents before ^{3}H-serotonin (2.9 nM) was added and then incubation was continued for a further 2 min. Uptake was terminated by diluting the samples with ice-cold buffer and rapid filtration through Whatman GF/C glass fibre filters. Microtitre plates and filters were washed three times using a Brandel cell harvester. The filters were dried and radioactivity was determined by liquid scintillation counting. Each point was determined as a triplicate. Nonspecific uptake was determined in parallel experiments containing 1 mM unlabelled serotonin. The same procedure was performed using several antidepressants, for example 1 μM citalopram (Singer *et al*., 1999).

Fluorescence measurements

Loading and fluorescence measurements using different cell types and different dyes Platelets were loaded in Na-HEPES incubation buffer (145 mM NaCl, 10 mM HEPES, 1 mM $MgSO_4$, 5 mM KH_2PO_4, 5 mM KCl and 10 mM glucose, pH adjusted to 6.5 with NH_3).

Rat pheochromocytoma cells (PC12 cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum, 5% heat-inactivated horse serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin and cultured for approximately 6 weeks. Before loading, PC12 cells were plated at a density of 2.5×10^6 cells $plate^{-1}$. Dulbecco's modified Eagle's medium was replaced with Hank's balanced salts (HBSS) before loading with the fluorescence dyes fura-2/AM and fluo-3/AM. For the loading with the fluorescence dye SBFI/AM, '$3K^+$–Ca^{2+} without Na^+ Medium' (3 mM KCl, 2 mM MgCl, 5 mM TRIS, 10 mM glucose; the sodium replaced by an equimolar amount of sucrose; pH adjusted with HCl to 7.4) was used instead of Dulbecco's modified Eagle's medium.

Human platelets and PC12 cells were loaded with 10 μM SBFI/AM, 5 μM fura-2 or 10 μM fluo-3 for 60 min (SBFI) or for 45 min (fura-2 and fluo-3).

After washing out the fluorescence dyes, aliquots of human platelets and PC12 cells were resuspended in the different incubation buffers. Aliquots of human platelets (4 μl) were added to 950 μl incubation buffer (pH 7.4, adjusted with NaOH). For measurements in calcium-rich medium, 1 mM $CaCl_2$ was added to the Na^+-HEPES buffer. When a sodium-free buffer was used, NaCl was replaced by equimolar concentrations of tetramethylammoniumchloride (TMA). PC12 cells were resuspended in HBSS for the measurement of $[Ca^{2+}]_i$ and in '$3K^+$–Ca^{2+} with 140 mM Na^+' containing 3 mM KCl, 2 mM MgCl, 5 mM TRIS, 10 mM glucose (pH = 7.4 adjusted with HCl) for measurement of $[Na^+]_i$. To measure Ba^{2+} influx, cells were washed three times following incubation with Ca^{2+}-free HBSS. The influx of Ba^{2+} in PC12 cells was evaluated in fura-2-loaded cells by measuring the fluorescence of Ba^{2+} fura-complexes. Fluorescence was measured in quartz cuvettes using an SLM-Aminco luminescence spectrometer (Luminescence Spectrometer Series 2, SLM-Aminco; Spectronic Instruments, Rochester, NY, U.S.A.) before and after addition of the tested drugs. All measurements were performed at 37°C.

Calibration of fluorescence signals

Calibration of SBFI/AM fluorescence for $[Na^+]_i$ measurement was performed according to the method of Harootunian *et al*. (1989). Human platelets or PC12 cells were added to solutions of known sodium concentration (0–150 mM) and the 340/380 nm intensity ratio was determined before and 5 min after addition of the ionophore gramicidin D (1 μM final concentration). Fura-2 signals were calibrated according to the method of Grynkiewicz *et al*. (1985) using a K_d-value of 224 nM. R_{max} was determined after addition of 10% Triton-X (human platelets) or 0.2% SDS (PC12 cells) and R_{min} was determined after injection of EGTA:

$$[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R) \times Sf2/Sb2]$$

where Sf2/Sb2 is the ratio of the signal of the calcium-free dye to the signal of the dye saturated with calcium, at a wavelength of 380 nm.

Ba^{2+} influx was evaluated in fura-2-loaded cells by measuring the fluorescence of Ba^{2+}–fura-complexes. The excitation and emission wavelengths were 340 and 509 as described for $[Ca^{2+}]_i$ measurement, except that $[Ba^{2+}]_i$ was expressed as ratio values according to the formula $= (F - F_{min})/(F_{max} - F)$.

Statistics

In addition to Microsoft Office Excel, GraphPad PRISM™ (Version 3.0) was used for statistical analyses and to create the graphs. For statistical analyses, an unpaired Student's *t*-test was used. Unless otherwise indicated, data are given as mean ± s.d.

Chemicals

Standard laboratory chemicals were obtained from Sigma-Aldrich Chemical (Deisenhofen, Germany) and VWR (Darmstadt, Germany). The fluorescence dyes (SBFI/AM, fura-2/AM, fluo-3/AM) and gramicidin were purchased from Molecular Probes (Eugene, OR, U.S.A.). ^{3}H-serotonin (specific activity 30 Ci $mmol^{-1}$) was obtained from NEN Life Science Products (Boston, MA, U.S.A.). Pluronic F-127 and SK&F 96365 were from Calbiochem (Frankfurt, Germany).

Cell culture media were obtained from GIBCO (GIBCO BRL, Carlsbad, CA, U.S.A.). Hyperforin sodium salt was generously supplied by Schwabe (Karlsruhe, Germany). LOE 908 was kindly provided by Boehringer Ingelheim (Ingelheim, Germany). Cariporid (HOE 642) was a kind gift from Aventis (Frankfurt, Germany).

Results

Serotonin uptake inhibition is associated with an increase in $[Na^+]_i$

Hyperforin (10 μM), which inhibits the uptake of serotonin into human platelets, also elevates $[Na^+]_i$ (Figure 1a), confirming our hypothesis that serotonin uptake inhibition by hyperforin might be the consequence of $[Na^+]_i$ elevation (Singer *et al*., 1999). The present data confirm this further, with the initial time course indicating that both serotonin uptake inhibition and the increase in $[Na^+]_i$ are observed shortly after the addition of hyperforin (Figure 1a). Maximal inhibition of serotonin uptake is reached much faster than maximum $[Na^+]_i$ elevation. A relatively small elevation of $[Na^+]_i$ (which is, however, nearly 100% over basal level at this point already) is enough to produce nearly maximal inhibition of serotonin uptake after an incubation time of 60 s (Figure 1a). This indicates that the IC_{50} value for uptake inhibition is much less than the concentration required to induce half-maximal increase in $[Na^+]_i$.

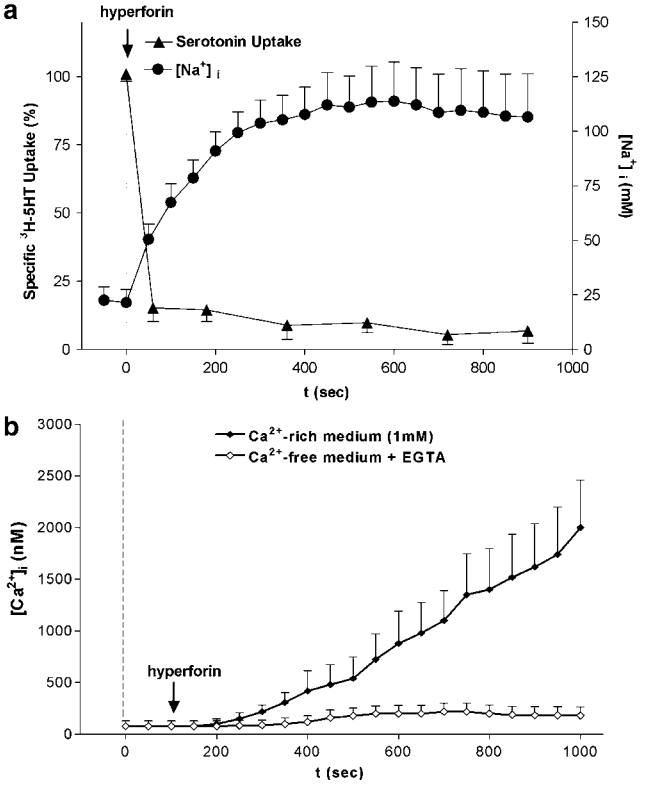

Figure 1 (a) Effect of hyperforin (10 μM) on $[Na^+]_i$ and serotonin uptake in human platelets over time (a). Specific serotonin uptake (filled triangle) was measured after the first minute and then every 30 s for 15 min. $[Na^+]_i$ (filled circle) was analysed for 15 min and was investigated as described in the Methods. (b) Additionally, the effect of hyperforin (10 μM) on $[Ca^{2+}]_i$ in human platelets was investigated according to stated methods. The experiments were conducted in calcium-free buffer with EGTA (blank diamond) and calcium-rich buffer (1 mM) (filled diamond) and were continued for 15 min. Data are mean $\pm$ s.d. of six different experiments.

Hyperforin does not affect the Na^+/Ca^{2+} exchanger or the NHE

A possible pathway involved in the elevation of $[Na^+]_i$ was the Na^+/Ca^{2+} exchanger (NCX), which removes Ca^{2+} from the cell using the sodium gradient as a driving force. However, in human platelets under our standard conditions, hyperforin (10 μM) also causes an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Following addition of 10 μM hyperforin, in calcium-rich buffer, $[Ca^{2+}]_i$ increased from a basal level of 75 nM (1 mM $[Ca^{2+}]_o$) to about 1700 nM and in calcium-free buffer to about 200 nM (EDTA-buffered extracellular medium) (Figure 1b). This implies that, in addition to a large influx of Ca^{2+} into the cell, there might also be a minor elevation of $[Ca^{2+}]_i$ associated with release of calcium from intracellular calcium stores. However, the time course of the increase of $[Ca^{2+}]_i$ is considerably different from that of serotonin uptake inhibition.

As human platelets possess no intracellular stores for Na^+, it can be assumed that the elevation of $[Na^+]_i$ has a direct correlation with the extracellular sodium concentration ($[Na^+]_o$). Accordingly, experiments in sodium-free TMA-buffer revealed no increase in $[Na^+]_i$ following addition of hyperforin (10 μM) (data not shown). The same experiment performed in calcium-free buffer showed that the hyperforin-induced $[Na^+]_i$ elevation is independent of the extracellular calcium concentration (Figure 2a). In addition, it has been observed that the hyperforin-induced (10 μM) $[Ca^{2+}]_i$ elevation is independent from the $[Na^+]_o$. Measurements in Na^+-rich buffer (145 mM) and Na^+-free TMA-buffer revealed no significant differences in the elevation of $[Ca^{2+}]_i$ (Figure 2b). These results suggest that hyperforin does not activate Na^+/Ca^{2+} antiport mechanisms. Consequently, the NCX is probably not affected by hyperforin.

Previous observations (Wonnemann *et al*., 2000) suggested that the NHE possibly has a role in transmitter uptake inhibition by hyperforin. This was not confirmed by the present data, since cariporid (HOE 642), a potent NHE inhibitor in human platelets (Scholz *et al*., 1995), did not affect the hyperforin-induced increase in $[Na^+]_i$ (Figure 3).

Hyperforin activates nonselective cation channels

Both monovalent (Na^+) and divalent cations (Ca^{2+}) cross the platelet membrane through NSCCs (Barritt, 1999), which play an important role in many tissues and nonexcitable cells. As most alternative sodium conductive pathways could be excluded, we examined whether NSCCs play a role in the observed hyperforin-induced elevation of $[Na^+]_i$ and $[Ca^{2+}]_i$. The NSCC inhibitor SK&F 96365 (Merritt *et al*., 1990) blocked both the increase in $[Na^+]_i$ (Figure 4a) and $[Ca^{2+}]_i$ (Figure 4b) caused by hyperforin (10 μM) into human platelets, in a concentration-dependent manner. SK&F 96365 treatment alone had no effect on basal Na^+ or Ca^{2+} levels (Figure 4b). SK&F 96365 was initially introduced as a blocker of receptor-mediated Ca^{2+} influx in broad sense and its pharmacological

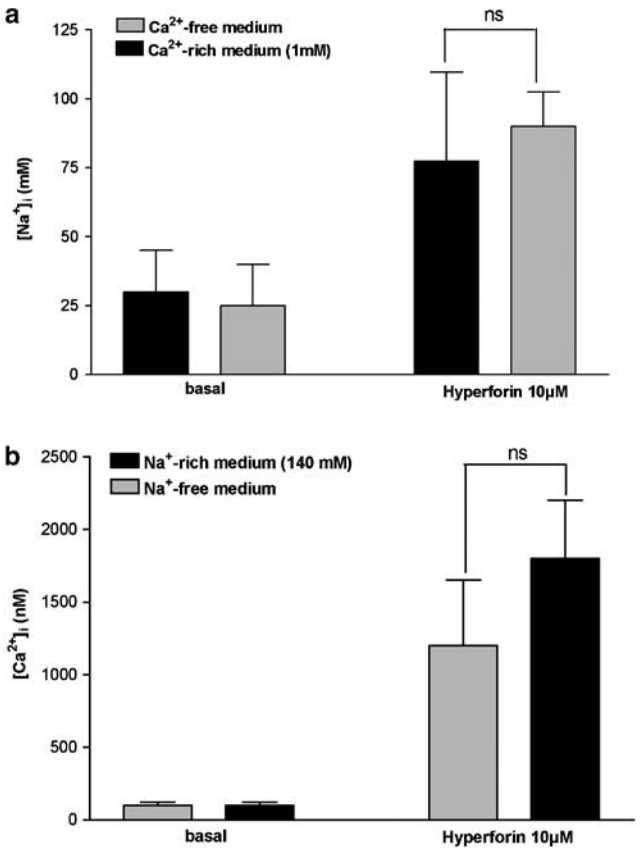

Figure 2 To investigate the possible involvement of NCXs, the effect of hyperforin on $[Na^+]_i$ or $[Ca^{2+}]_i$ in human platelets was analysed in calcium-free medium with EGTA (b) or TMA-buffered sodium-free medium (a). Platelets were incubated with fura-2 AM or SBFI AM as described in Methods. Afterwards, platelets were washed three times with calcium-free medium containing EGTA or with TMA-buffered sodium-free medium. Fluorescence was measured as stated in Methods and platelets were stimulated with hyperforin (10 μM). Data are mean $\pm$ s.d. of six different experiments (b), ns = not significant.

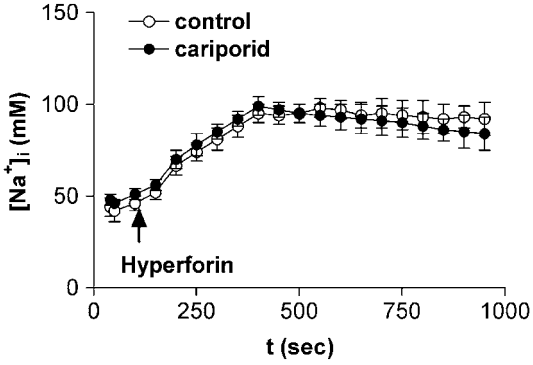

Figure 3 Time course of the effect of NHE-antagonist cariporid (HOE 642) (1 μM) treatment on the hyperforin-induced elevation of $[Na^+]_i$ in human platelets. Cariporid (HOE 642) was preincubated for 1 min followed by stimulation with hyperforin (10 μM) (filled circle). Data are mean $\pm$ s.d. of six different experiments.

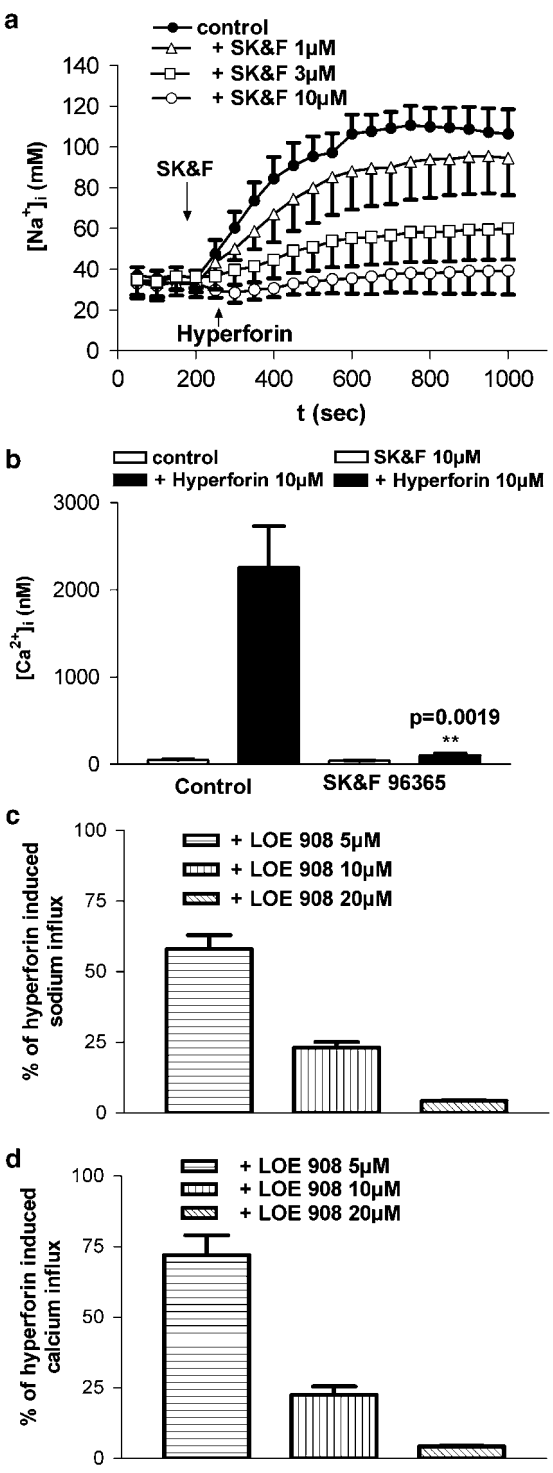

Figure 4 Effect of preincubation (1 min) with the NSCC inhibitors SK&F 96365 and LOE 908 (10 μM) on the hyperforin-induced elevation of $[Na^+]_i$ and $[Ca^{2+}]_i$ in human platelets. $[Na^+]_i$ and $[Ca^{2+}]_i$ in human platelets were measured according to stated Methods. SK&F 96365 and LOE908 were preincubated for 1 min at various concentrations followed by stimulation with hyperforin (a,b). Results are presented as a percentage of hyperforin-induced Ca^{2+} or Na^+ influx. Data are mean $\pm$ s.d., $n = 6$, $^{**}P < 0.01$ when compared with the effect of hyperforin alone.

actions are well characterised (Merritt *et al*., 1990). However, it is known that the actions of SK&F 96365 are nonspecific as it suppresses both NSCCs and store operated calcium channels (SOCCs) (Chung *et al*., 1994; Franzius *et al*., 1994; Koch *et al*., 1994). To further confirm that hyperforin activates NSCCs, we also investigated the compound LOE 908, which inhibits two classes of NSCCs (NSCC1 and NSCC2) but has no effect on

SOCCs (Krautwurst *et al*., 1992; Miwa *et al*., 2000; Kawanabe *et al*, 2002). As indicated in Figure 4c and d, LOE 908 almost completely blocked the hyperforin-induced (10 μM) elevation of $[Na^+]_i$ and $[Ca^{2+}]_i$ in human platelets with IC_{50} values around 5 μM. Neither SK&F 96365 nor LOE 908 alone had any effect on basal Na^+ or Ca^{2+} levels (data not shown), which suggest little or no constitutive activity of the hyperforin-activated conductive pathway.

As NSCCs are present in many different cell types, including neurons (Yang *et al*., 2003), the possible role of NSCCs in the hyperforin-induced elevation of $[Na^+]_i$ and $[Ca^{2+}]_i$ in PC12 cells was also investigated. Again, hyperforin concentration dependently increased $[Na^+]_i$ with an EC_{50} concentration of 0.72 μM (Figure 5a). This effect was blocked by SK&F 96365 (10 μM) (Figure 5b). Moreover, the Na^+ influx induced by hyperforin (10 μM) could also be inhibited by LOE 908 (10 μM) (Figure 5d) and by flufenamic acid (Figure 5c), another blocker of NSCCs (Goegelein *et al*., 1990). Hyperforin not only elevated $[Na^+]_i$ but also $[Ca^{2+}]_i$ with an EC_{50} concentration of 1.16 μM (Figure 6a). SK&F 96395, LOE 908 and flufenamic acid also inhibited the hyperforin-induced elevation of $[Ca^{2+}]_i$ in PC12 cells (Figure 6b–d).

Until now, NSCCs have been mainly characterised as ionic conductive pathways, with no clear concept of their molecular structure. The NSCCs may belong to a group of transient receptor potential protein channels (TRP channels), for which many different genes have been identified (Clapham, 2003). A possible overlap between NSCCs and several TRPC channels has been proposed on the basis of some common pharmacological properties (Inoue *et al*., 2001). While the pharmacological characterisation of TRP channels is ongoing, the high sensitivity of some TRP channels for the lanthanides Gd^{3+} and La^{3+} at micromolar concentrations seems to be a rather specific property (Jung *et al*., 2003). Moreover, Jung *et al*. (2003) reported in whole cell patch clamp recordings that TRPC5 was bimodally modulated by lanthanides, with potentiation at micromolar concentrations but inhibition at millimolar concentrations. In contrast, TRPC6 was inhibited by micromolar concentrations and showed no potentiation.

In our study, both lanthanides inhibited the hyperforin-induced $[Ca^{2+}]_i$ and $[Na^+]_i$ increase in PC12 cells (Figure 7a and b). Again, basal Ca^{2+} and Na^+ levels were not affected by either lanthanide (data not shown). A biphasic effect was not observed over the investigated concentration range. To investigate the selectivity of hyperforin-activated cation channels, Ba^{2+} was used as a surrogate cation. Hyperforin (10 μM) induced a Ba^{2+} influx (200 μM) in PC12 cells (Figure 7c) and this influx was blocked by SK&F 96365 (10 μM), LOE 908 (10 μM), Gd^{3+} (10 μM) and La^{3+}(10 μM) (Figure 7c). Zhang & Saffen (2001) earlier showed that endogenously expressed SOCCs in PC12D cells, a subline of PC12 cells, are not responsible for the Ba^{2+} influx in these cells thus supporting our hypothesis that hyperforin-induced Ba^{2+} influx is due to an activation of NSCCs.

Furthermore, both lanthanides attenuated the hyperforin-induced inhibition of specific serotonin uptake into human platelets (Figure 7d), while La^{3+} and Gd^{3+} alone did not affect specific ^{3}H-serotonin uptake.

To exclude the possibility that voltage-dependent Na^+ and Ca^{2+} channels (L-type and N-type) play a role, the effects of Tetrodotoxin (TTX) (1 μM), Verapamil and ϖ-conotoxin MVIIA (1 μM) on hyperforin-induced $[Na^+]_i$ and $[Ca^{2+}]_i$ increase were investigated (data not shown). Neither TTX nor ϖ-conotoxin (MVIIA) affected hyperforin-induced $[Na^+]_i$ or $[Ca^{2+}]_i$ influx. Verapamil had no effect on

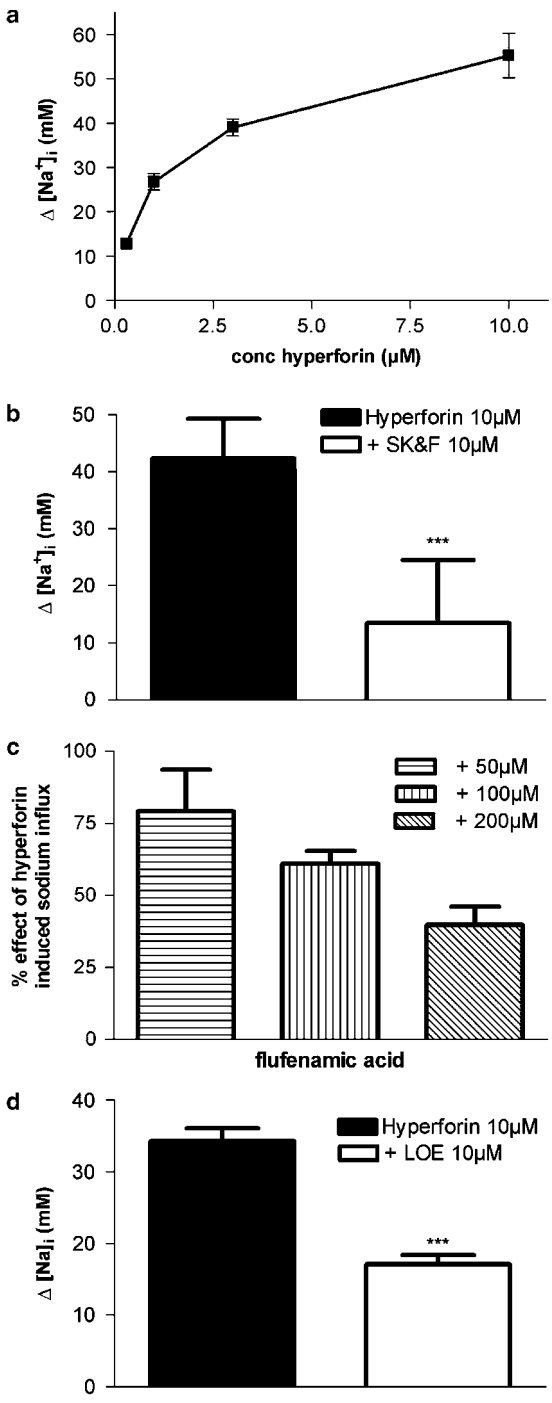

Figure 5 The effect of hyperforin on $[Na^+]_i$ in PC12 cells was investigated (a). $[Na^+]_i$ in PC12 cells was investigated as described in Methods. PC12 cells were stimulated with various concentrations of hyperforin. The NSCC inhibitors SK&F 96365 (10 μM) (b) and LOE 908 (10 μM) (d) were tested to measure their effect on hyperforin-induced $[Na^+]_i$ increase in PC12 cells. PC12 cells were preincubated for 1 min with the blockers, followed by stimulation with hyperforin (10 μM). An additional blocker of NSCCs, flufenamic acid, was also investigated. PC12 cells were preincubated with various concentrations of flufenamic acid for 1 min and afterwards stimulated with hyperforin (10 μM) (c). Data are mean $\pm$ s.e.m., $n=6$, $^{***}P<0.001$ when compared with the effect of hyperforin alone.

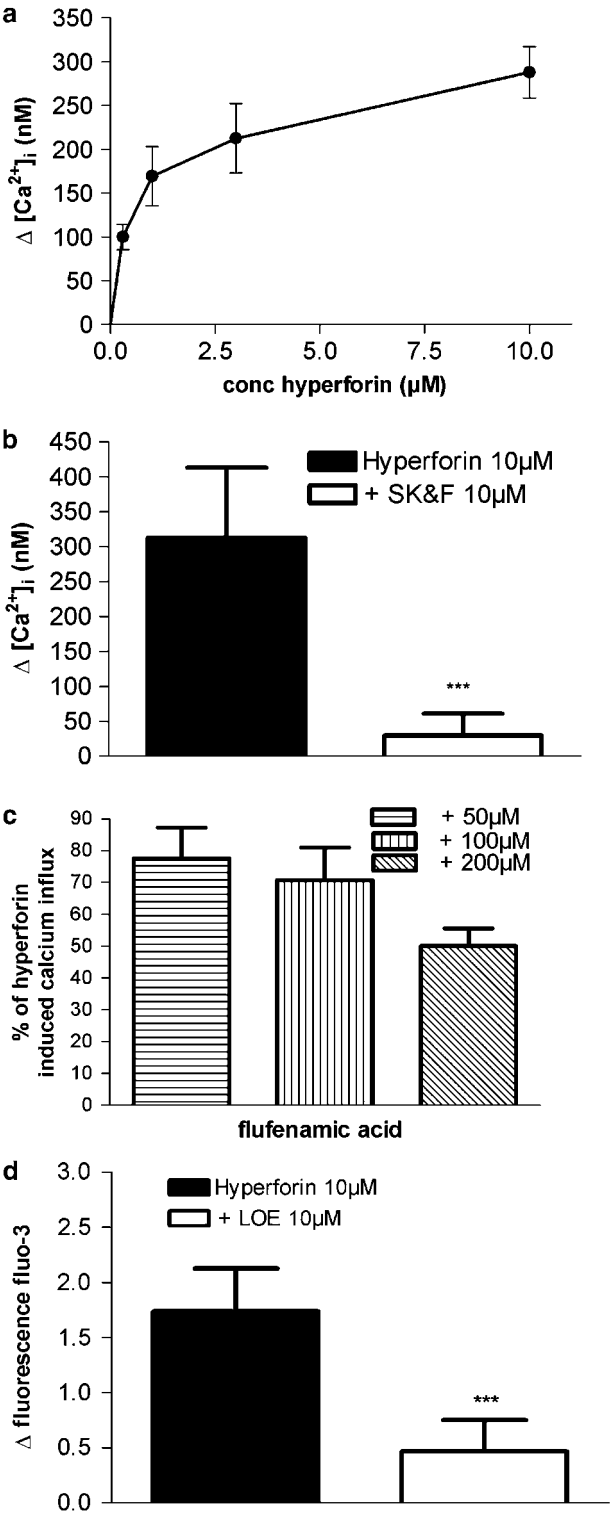

Figure 6 The effect of hyperforin on $[Ca^{2+}]_i$ in PC12 cells was also measured (a). $[Ca^{2+}]_i$ in PC12 cells was determined according to the stated methods. Various concentrations of hyperforin were used to stimulate PC12 cells. Again, the effects of SK&F 96365 (10 μM) and LOE 908 (10 μM) on hyperforin-induced $[Ca^{2+}]_i$ influx were tested. PC12 cells were preincubated for 1 min with the blockers followed by hyperforin (10 μM) stimulation (b and d). Also, the inhibiting effect of 1 min preincubation with various concentrations of flufenamic acid on hyperforin-induced $[Ca^{2+}]_i$ influx was investigated (c). Data are mean $\pm$ s.e.m., $n=6$, $^{***}P<0.001$ when compared with the effect of hyperforin alone.

hyperforin-induced $[Ca^{2+}]_i$ elevation up to 3 μM but inhibited $[Ca^{2+}]_i$ elevation at higher concentrations. Parallel findings by Zhu *et al*. (1998) that similarly high verapamil concentrations prevented Ca^{2+} entry in TRP3-stably transfected HEK cells *via* the TRP3-mediated pathway could explain our findings.

Discussion

It has previously been demonstrated that the mechanism by which hyperforin inhibits serotonin uptake in human platelets is associated with an elevation of $[Na^+]_i$ (Singer *et al*., 1999). Previous reports from our laboratory, however, revealed that hyperforin affects neither the Na^+/K^+-ATPase nor voltage-gated sodium channels (Wonnemann *et al*., 2000). This latter observation was confirmed in the present study. The present investigation therefore focused on the effect of hyperforin on other sodium conductive pathways in human platelets and in PC12 cells, in particular the NCX, the NHE and NSCCs.

One possible pathway that could be responsible for the effect of hyperforin on $[Na^+]_i$ could be through activation of the NCX, since hyperforin also modulates $[Ca^{2+}]_i$. In nearly all cells, including neurons, NCX exchanges three extracellular Na^+ to one intracellular Ca^{2+}. The transport of calcium ions into cells is elevated when $[Na^+]_i$ increases or $[Na^+]_o$ decreases (Fontana *et al*., 1995). Therefore, we investigated whether the hyperforin-induced alterations in $[Na^+]_i$ and $[Ca^{2+}]_i$ are linked together. However, experiments in sodium- or calcium-free incubation buffers indicated that the hyperforin-induced increase in $[Ca^{2+}]_i$ or $[Na^+]_i$ was not affected by the extracellular concentration of the counter-transported ion (Figure 2a and b). These findings indicate that the NCX is not involved in the elevation of $[Na^+]_i$ and $[Ca^{2+}]_i$ induced by hyperforin.

Similarly, based on the inability of the potent NHE inhibitor, cariporid (HOE 642), to interfere with the hyperforin-induced increase in $[Na^+]_i$, a possible role of the NHE could be excluded (Figure 3). This is also supported by the finding that under conditions of elevated $[Na^+]_i$, hyperforin decreases rather than increases intracellular pH (Froestl *et al*., 2003; Roz & Rehavi, 2003). Furthermore, several blockers of voltage-gated Na^+ and Ca^{2+} channels (TTX, Verapamil and ϖ-conotoxin MVIIA) did not inhibit the effects of hyperforin on $[Ca^{2+}]_i$ or $[Na^+]_i$ in relevant concentrations (data not shown).

NSCCs are permeable for mono- and divalent cations such as Na^+ and Ca^{2+} ions and represent a rather heterogeneous group of cation channels (Barritt, 1999). Currently, three different kinds of NSCCs have been proposed in excitable and nonexcitable tissues (Suzuki *et al*., 1998). They can be activated by intracellular calcium ions, hydrostatic pressure or stretching, G-protein-coupled receptors, ATP and further unknown parameters (Suzuki *et al*., 1998; Liu *et al*., 2002). NSCCs were identified in platelets (Stamouli *et al*., 1993), synaptosomes (Reichardt & Kelly, 1983) and PC12 cells (Tesfai *et al*., 2001).

In platelets, the main sodium conducting pathway seems to be influx through NSCCs and to a lesser extent *via* NHEs (Sage *et al*., 1991; Stamouli *et al*., 1993). The lipophilic imidazole derivative SK&F 96365 has been identified as an inhibitor of NSCCs and prevents the influx of Na^+ and Ca^{2+} *via* NSCCs in HL-60 cells (Krautwurst *et al*., 1992). Moreover, SK&F 96365 also inhibits NSCCs in platelets (Sage *et al*., 1991) and many other cells (Merritt *et al*., 1990). All these

reports support the assumption that hyperforin-induced elevations of $[Na^+]_i$ as well as $[Ca^{2+}]_i$ are mediated by activation of NSCCs, since SK&F 96365 blocked the effect in human platelets as well as in PC12 cells at low micromolar concentrations (Figures 4a, b, 5b; 6b).

However, the subtypes, structures and mechanisms of activation of NSCCs are not well understood. Pharmacologically, two subclasses have been identified by the parallel use of SK&F 96365 and LOE 908. SK&F 96365 only blocks NSCC2 channels but also to some extent SOCCs, while LOE 908 is specific for NSCCs but does not differentiate between the presumed subclasses NSCC1 and NSCC2 (Krautwurst *et al*., 1992; Miwa *et al*., 2000). Our findings with SK&F 96365 and LOE 908 (Figures 4; 5b,d; 6b,d) for both cell types suggest that the activation of NSCCs by hyperforin is possibly restricted to the NSCC2 subtype. The nonselectivity of the hyperforin-activated cation channels is supported by the findings that hyperforin also induced Ba^{2+} influx in PC12 cells (Figure 7c). Again, this influx was inhibited by SK&F 96365 and LOE 908. Very little is known about the structure and physiological function of NSCCs. Since structure and activation mechanism of NSCCs are not clear and up to now only the NSCC1 subtype has been cloned (Suzuki *et al*., 1998), the mechanism by which hyperforin activates NSCC2 is not known but is currently under investigation. A possible overlap between SK&F 96365-sensitive α_1 adrenoceptor-activated calcium channels (NSCCs) with TRPC6 channels has been proposed (Inoue *et al*., 2001). TRPC6 channels have been identified in PC12 cells and human platelets (Tesfai *et al*., 2001; Hassock *et al*., 2002). Since the rather specific inhibition of TRPC6 channels by the two lanthanides Gd^{3+} and La^{3+} (Jung *et al*., 2003) could also be demonstrated for the hyperforin-induced sodium, calcium, and barium influx, we speculate that hyperforin is an activator of a TRP channel (Figure 7a–c). Our observation that the inhibition of serotonin uptake by hyperforin can be attenuated by both lanthanides further confirms a functional role for these channels in the mechanism of action of hyperforin (Figure 7d).

In conclusion, data obtained from this study combined with data from previous reports suggests a possible mechanism explaining the preclinical antidepressant properties of hyperforin. Hyperforin causes an increase in $[Na^+]_i$ and $[Ca^{2+}]_i$ due to activation of NSCCs, leading to a reduced presynaptic uptake and possibly to an increased release of several neurotransmitters. Subsequently, this leads to changes in extracellular and intracellular neurotransmitter concentrations, which are a parameter for antidepressant activity. Finally, these findings could explain the activity of hyperforin in behavioural tests indicative for antidepressant activity, for example, the forced swimming test or the tail suspension test.

While classical antidepressants directly interact with neurotransmitter transporters, hyperforin instead elevates $[Na^+]_i$

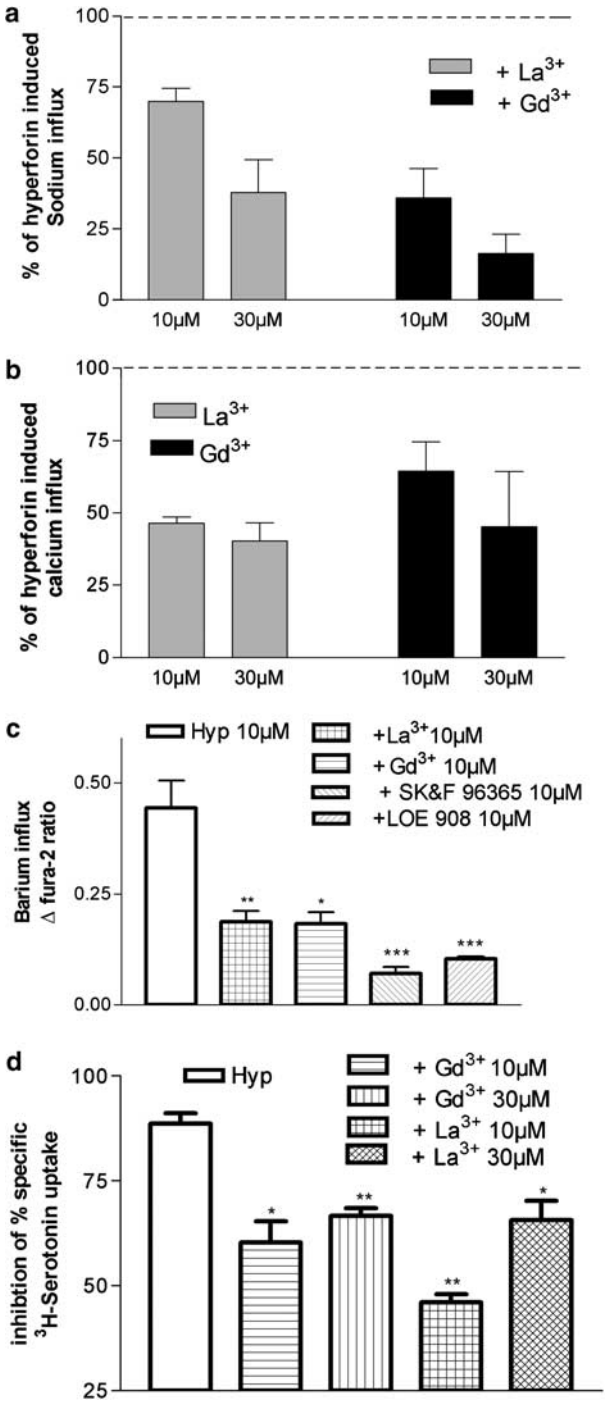

Figure 7 To investigate a possible overlap between NSCCs and TRP channels, the effects of TRP channel blockers lanthanum (La^{3+}) and gadolinum (Gd^{3+}) on hyperforin-induced $[Ca^{2+}]_i$ and $[Na^+]_i$ increase were tested. $[Ca^{2+}]_i$ and $[Na^+]_i$ levels in PC12 cells were determined as described in Methods. PC12 cells were preincubated for 1 min with the various concentrations of Gd^{3+} and La^{3+} and afterwards stimulated with hyperforin (10 μM). The results are presented as a percentage of hyperforin-induced Na^+ or Ca^{2+} influx. Additionally, Ba^{2+} influx was measured to investigate the nonselectivity of the hyperforin-induced cation influx. PC12 cells were loaded with fura-2 AM as described in Methods. In calcium-free HBSS, Ba^{2+} (200 μM) was added, then cells were stimulated with hyperforin (10 μM). Further, the effects of SK&F 96365 (10 μM), LOE 908 (10 μM), La^{3+} and Gd^{3+} on hyperforin-induced $[Ba^{2+}]_i$ influx were investigated. The blockers were preincubated for 1 min followed by stimulation with hyperforin (10 μM). To determine the effects of La^{3+} and Gd^{3+} on serotonin uptake inhibition induced by hyperforin in human platelets, platelets were incubated for 2 min with the inhibitors, then hyperforin (1 μM) was added and incubated for 2 min. Afterwards, ^{3}H-serotonin was added and uptake was investigated for a further 2 min. (d) Uptake inhibition is presented as a percentage of specific uptake of ^{3}H-serotonin in human platelets. Data are mean $\pm$ s.e.m. of four to six determinations. $^{***}P<0.001$; $^{**}P<0.01$ or $^{*}P<0.05$ when compared with the effect of hyperforin alone.

and thereby inhibits neurotransmitter uptake. This might explain why hyperforin's pharmacological profile shows important similarities to that of other antidepressants. However, if the primary mechanism of hyperforin is activation of NSCC2, its pharmacological effects may extend beyond those of typical antidepressant drugs and could explain hyperforin's beneficial effects on cognition (Klusa *et al*., 2001) and amyloid precursor protein processing (Froestl *et al*., 2003). Finally, our data suggest that the NSCC2 channels activated by hyperforin are part of the large TRP channel family, possibly within the TRPC subclass.

The regional and cellular distribution of NSCCs and TRP channels compared to that of transporter molecules provides a more plausible explanation to previously reported regional-specific effects of hypericum extract on neurotransmitter concentrations in the rat brain (Yoshitake *et al*., 2004).

This study was supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany), and Dr W. Schwabe Arzneimittel (Karlsruhe, Germany). LOE 908 was kindly provided by Boehringer Ingelheim (Ingelheim, Germany). Cariporid (HOE 642) was a kind gift from Aventis (Frankfurt, Germany).

References

BARRITT, G.J. (1999). Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem. J.,* **337,** 153–169.

BUCHHOLZER, M.L., DVORAK, C., CHATTERJEE, S.S. & KLEIN, J. (2002). Dual modulation of striatal acetylcholine release by hyperforin, a constituent of St John's wort. *J. Pharmacol. Exp. Ther.,* **301,** 714–719.

CERVO, L., ROZIO, M., EKALLE-SOPPO, C.B., GUISO, G., MORAZZONI, P. & CACCIA, S. (2002). Role of hyperforin in the antidepressant-like activity of *Hypericum perforatum* extracts. *Psychopharmacology,* **164,** 423–428.

CHATTERJEE, S.S., BHATTACHARYA, S.K., WONNEMANN, M., SINGER, A. & MÜLLER, W.E. (1998). Hyperforin as a possible antidepressant component of hypericum extracts. *Life Science,* **63,** 499–510.

CHUNG, S.C., MCDONALD, T.V. & GARDNER, P. (1994). Inhibition by SK&F 96365 of Ca^{2+} current, IL-2 production and activation in T lymphocytes. *Br. J. Pharmacol.,* **113,** 861–868.

CLAPHAM, D.E. (2003). TRP channels as cellular sensors. *Nature,* **426,** 517–524.

DI CARLO, G., BORRELLI, F., ERNST, E. & IZZO, A.A. (2001). St John's wort: prozac from the plant kingdom. *Trends Pharmacol. Sci.,* **22,** 557–559.

FONTANA, G., ROGOWSKI, R.S. & BLAUSTEIN, M.P. (1995). Kinetic properties of the sodium–calcium exchanger in rat brain synaptosomes. *J. Physiol. (London),* **485,** 349–364.

FRANZIUS, D., HOTH, M. & PENNER, R. (1994). Non-specific effects of calcium entry antagonists in mast cells. *Pfluegers Arch.,* **428,** 433–438.

FROESTL, B., STEINER, B. & MÜLLER, W.E. (2003). Enhancement of proteolytic processing of the β-amyloid precursor protein by hyperforin. *Biochem. Pharmacol.,* **66,** 2177–2184.

GOEGELEIN, H., DAHLEM, D., ENGLERT, H.C. & LANG, H.J. (1990). Flufenamic acid, mefenamic acid and niflumic acid inhibit single non-selective cation channels in the rat exocrine pancreas. *FEBS Letters,* **268,** 79–82.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HAROOTUNIAN, A.T., KAO, J.P., ECKERT, B.K. & TSIEN, R.Y. (1989). Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. Biol. Chem.,* **264,** 19458–19467.

HASSOCK, S.R., ZHU, M.X., TROST, C., FLOCKERZI, V. & AUTHI, K.S. (2002). Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. *Blood,* **100,** 2801–2811.

INOUE, R., OKADA, T., ONOUE, Y., HARA, Y., SHIMIZU, S., NAITOH, S., ITO, Y. & MORI, Y. (2001). The transient receptor potential protein homologue TRP6 is the essential component of vascular α_1-adrenoceptor-activated Ca^{2+}-permeable cation channel. *Circ. Res.,* **88,** 325–332.

JENSEN, A.G., HANSEN, S.H. & NIELSEN, E.O. (2001). Adhyperforin as a contributor to the effects of *Hypericum perforatum*, L. in biochemical models of antidepressant activity. *Life Sci.,* **68,** 1593–1605.

JUNG, S., MÜHLE, A., SCHAEFER, M., STROTMANN, R., SCHULTZ, G. & PLANT, T.D. (2003). Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. *J. Biol. Chem.,* **278,** 3562–3571.

KAEHLER, S.T., SINNER, C., CHATTERJEE, S.S. & PHILIPPU, A. (1999). Hyperforin enhances the extracellular concentrations of catecholamines, serotonin and glutamate in the rat locus coeruleus. *Neurosci. Lett.,* **262,** 199–202.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2002). Characterisation of G proteins involved in activation of nonselective cation channels by endothelin (B) receptor. *Br. J. Pharmacol.,* **136,** 1015–1022.

KLUSA, V., GERMANE, S., NOLDNER, M. & CHATTERJEE, S.S. (2001). Hypericum extract and hyperforin: memory-enhancing properties in rodents. *Pharmacopsychiatry,* **34** (Suppl. 1), S61–S69.

KOCH, B.D., FAUROT, G.F., KOPANITSA, M.V. & SWINNEY, D.C. (1994). Pharmacology of a Ca^{2+}-influx pathway activated by emptying the intracellular Ca^{2+} stores in HL-60 cells: evidence that a cytochrome P-450 is not involved. *Biochem. J.,* **302,** 187–190.

KRAUTWURST, D., SEIFERT, R., HESCHELER, J. & SCHULTZ, G. (1992). Formyl peptides and ATP stimulate Ca^{2+} and Na^+ inward currents through non-selective cation channels *via* G-proteins in dibutyryl cyclic AMP-differentiated HL-60 cells. Involvement of Ca^{2+} and Na^+ in the activation of beta-glucuronidase release and superoxide production. *Biochem. J.,* **288** (Part 3), 1025–1035.

LIU, R.J., VAN DEN POL, A.N. & AGHAJANIAN, G.K. (2002). Hypocretins (Orexin) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions. *J. Neurosci.,* **22,** 9453–9464.

MERRITT, J.E., ARMSTRONG, W.P. & BENHAM, C.D. (1990). SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.,* **271,** 515–552.

MISANE, I. & OGREN, S.O. (2001). Effects of *Hypericum perforatum* (St John's wort) on passive avoidance in the rat: evaluation of potential neurochemical mechanisms underlying its antidepressant activity. *Pharmacopsychiatry,* **34** (Suppl. 1), S89–S97.

MIWA, S., IWAMURO, Y., ZHANG, X.F., KAWANABE, Y. & MASAKI, T. (2000). LOE 908: a specific blocker of non-selective cation channel. *Cardiovasc. Drug Rev.,* **18,** 61–72.

NEARY, J.T., NEARY, J.T., WHITTEMORE, S.R., BU, Y., MEHTA, H. & SHI, Y.F. (2001). Biochemical mechanisms of action of Hypericum LI 160 in glial and neuronal cells: inhibition of neurotransmitter uptake and stimulation of extracellular signal regulated protein kinase. *Pharmacopsychiatry,* **34,** S103–S107.

PHILLIPU, A. (2001). *In vivo* neurotransmitter release in the *Loceus coereleus* – effects of hyperforin, inescapable shock and fear. *Pharmacopsychiatry,* **34** (Suppl. 1), S111–S115.

REICHARDT, L.F. & KELLY, R.B. (1983). A molecular description of nerve terminal function. *Annu. Rev. Biochem.,* **52,** 871–926.

ROZ, N. & REHAVI, M. (2003). Hyperforin inhibits vesicular uptake of monoamines by dissipating pH gradient across synaptic vesicle membrane. *Life Sci.,* **73,** 461–470.

SAGE, S.O., RINK, T.J. & MAHAUT-SMITH, M.P. (1991). Resting and ADP-evoked changes in cytosolic free sodium concentration in human platelets loaded with the indicator SBFI. *J. Physiol. (London),* **441,** 559–573.

SCHOLZ, W., ALBUS, U., COUNILLON, L., GOGELEIN, H., LANG, H.J., LINZ, W., WEICHERT, A. & SCHOLKENS, B.A. (1995). Protective effects of HOE642, a selective sodium–hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc. Res.,* **29,** 260–268.

SINGER, A., WONNEMANN, M. & MÜLLER, W.E. (1999). Hyperforin, a major antidepressant constituent of St John's wort, inhibits serotonin uptake by elevating free intracellular Na^+. *J. Pharmacol. Exp. Ther.,* **290,** 1363–1368.

STAMOULI, V., VAKIRTZI-LEMONIAS, C. & SIFFERT, W. (1993). Thrombin and NaF, but not epinephrine, raise cytosolic free Na^+ in human platelets. *Biochim. Biophys. Acta.,* **1176,** 215–221.

SUZUKI, M., MURATA, M., IKEDA, M., MIYOSHI, T. & IMAI, M. (1998). Primary structure and functional expression of a novel non-selective cation channel. *Biochem. Biophys. Res. Commun.,* **242,** 191–196.

TESFAI, Y., BRERETON, H.M. & BARRITT, G.J. (2001). A diacylglycerol-activated Ca^{2+} channel in PC12 cells (an adrenal chromaffin cell line) correlates with the expression on TRP-6 (transient receptor potential) protein. *Biochem. J.,* **358,** 717–726.

WONNEMANN, M., SINGER, A. & MÜLLER, W.E. (2000). Inhibition of synaptosomal uptake of ^{3}H-L-glutamate and ^{3}H-GABA by Hyperforin, a major constituent of St John's wort. The role of amiloride sensitive sodium conductive pathways. *Neuropsychopharmacology,* **23,** 188–197.

WONNEMANN, M., SINGER, A., SIEBERT, B. & MULLER, W.E. (2001). Evaluation of synaptosomal uptake inhibition of the most relevant constituents of St John's Wort. *Pharmacopsychiatry,* **34** (Suppl. 1), S148–S152.

YANG, B., SAMSON, W.K. & FERGUSON, A.V. (2003). Excitatory effects of orexin-A on nucleus tractus solitarius neurons are mediated by phospholipase C and protein kinase C. *J. Neurosci.,* **23,** 6215–6222.

YOSHITAKE, T., IIZUKA, R., YOSHITAKE, S., WEIKOP, P., MÜLLER, W.E., ÖGREN, O. & KEHR, J. (2004). *Hypericum perforatum* L (St John's wort) preferentially increases extracellular dopamine levels in rat prefrontal cortex. *Br. J. Pharmacol.,* **142,** 414–418.

ZHANG, L. & SAFFEN, D. (2001). Muscarinic acetylcholine receptor regulation of TRP6 Ca^{2+} channel isoforms. Molecular structures and functional characterization. *J. Biol. Chem.,* **276,** 13331–13339.

ZHU, X., JIANG, M. & BIRNBAUMER, L. (1998). Receptor-activated Ca^{2+} influx *via* human Trp3 stably expressed in human embryonic kidney (HEK) 293 cells. *J. Biol. Chem.,* **273,** 133–142.

(Received September 29, 2004
Revised November 25, 2004
Accepted December 21, 2004)