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Differential effect of the benzophenanthridine alkaloids sanguinarine and chelerythrine on glycine transporters

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ABSTRACT

Glycine transporter inhibitors modulate the transmission of pain signals. Since it is well known that extracts from medicinal plants such as *Chelidonium majus* exhibit analgesic properties, we investigated the effects of alkaloids typically present in this plant on glycine transporters. We found that chelerythrine and sanguinarine selectively inhibit the glycine transporter GlyT1 with comparable potency in the low micromolar range while berberine shows no inhibition at all. At this concentration both alkaloids only minimally affected transport of the closely related glycine transporter GlyT2, suggesting that the effect is not mediated by the inhibitory activity of these alkaloids on the Na^+/K^+ ATPase. GlyT1 inhibition was time-dependent, noncompetitive and increased with glycine concentration. While chelerythrine inhibition was reversible, the effect of sanguinarine was resistant to wash out. These results suggest that benzophenanthridine alkaloids interact with glycine transporters and at low micromolar range selectively target glycine transporter GlyT1.

1. Introduction

Neurotransmitter transporters are a family of membrane proteins, which regulate the availability of several neurotransmitters including glycine (Nelson, 1998; Masson et al., 1999; Aragon and Lopez-Corcuera, 2003; Eulenburg et al., 2005; Gether et al., 2006). Glycine is predominantly involved in the inhibitory neurotransmission of the mammalian hindbrain (Betz, 1992). However it also modulates neurotransmission in the forebrain, where its major effect is believed to be caused by acting on the coagonist site of the NMDA receptor (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Both glycine and D-serine can potentiate the NMDA receptor, but the individual contribution of these two agonists is still not clearly defined, mainly because they share a common binding site on the NMDA receptor (Mothet et al., 2000; Panatier et al., 2006; Wolosker, 2007; Shimazaki et al., 2010). The recently developed specific glycine transporter inhibitors are able to balance the hypofunction of the NMDA receptor. This hypofunction is presumably associated with schizophrenia and inhibitors of GlyT1 represent promising drugs for the treatment of this serious psychiatric disease (Sur and Kinney, 2007; Javitt, 2009).

Accumulating evidence indicates that glycinergic and GABAergic transmission has a very specific role in the development of neuropathic pain. In some pathological states, the inflammatory prostaglandin PGE2 activates the EP2 subtype of the prostaglandin E receptor, which leads to protein kinase A dependent inhibition α3 containing glycine receptors in the superficial dorsal horn in the vicinity of nociceptive afferents terminals. The disinhibition of α3 containing glycine receptors increases the firing of superficial dorsal horn neurons and elevates the transmission of nociceptive signals to higher brain pain centers (Ahmadi et al., 2002; Harvey et al., 2004; Zeilhofer and Zeilhofer, 2008; Dohi et al., 2009). According to another hypothesis, neuropathic pain could originate from changes in the chloride gradient, resulting in insufficient glycinergic and GABAergic inhibitory input (Coull et al., 2003, 2005; Prescott et al., 2006). Additionally, glycine can also potentiate the NMDA receptor and influence the periaqueductal

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gray opioid-based antinociception (Wink et al., 1998; Maione et al., 2000; Martins et al., 2008).

Glycine transporter inhibitors ameliorate the symptoms of neuropathic pain most likely via an increase in the local glycine concentration (Dohi et al., 2009; Morita et al., 2008; Tanabe et al., 2008). Even though the exact mechanism still needs to be elucidated, pain relief represents a second important line of potential pharmaceutical use for glycine transporter inhibitors.

Several pharmacophores acting on glycine transporters were described recently (for review see Bridges et al., 2008; Gilfillan et al., 2009). Since each substance likely has its own specific mode of action and potential pharmacological side effects, there is a constant need for new pharmacophores, which could be adapted to make improved drugs.

Alkaloids are a group of biological substances, which exhibit a wide spectrum of anti-inflammatory, antimicrobial, antitumor, analgesic and spasmolytic effects (Wink et al., 1998; Verpoorte, 1998; Simanek et al., 2003). The alkaloids investigated here, chelerythrine and sanguinarine, have many cellular targets, which is probably caused by their quaternary nitrogen, polycyclic and planar structure interacting with the nucleophilic and anionic moieties of amino acids in biomacromolecules (Schmeller et al., 1997).

The inhibitory effect of chelerythrine and sanguinarine on several protein kinases and Na^+/K^+ ATPase activity is well known (Herbert et al., 1990; Wang et al., 1997). The potency of these effects can vary significantly, however. Chelerythrine blocks the ATP-induced cation fluxes mediated by the P2X7 receptor (Shemon et al., 2004), while sanguinarine binds to the angiotensine AT1 receptor (Caballero-George et al., 2003) and inhibits phosphatase PP2C (Aburai et al., 2010), aminopeptidase N and dipeptidyl peptidase IV (Sedo et al., 2002).

Two types of interactions between benzophenanthridine alkaloids and DNA have been described. Sanguinarine intercalates into DNA in a similar way as ethidium bromide (Maiti et al., 1982). Sanguinarine and chelerythrine are both metabolized by hepatic microsomes to species, which form covalently bound DNA, adducts under certain conditions (Stiborova et al., 2002).

Benzophenanthridine alkaloids affect apoptosis and despite their structural similarities, chelerythrine and sanguinarine target different binding sites on the pro-survival Bcl-X_L protein (Zhang et al., 2006).

Concerning the effects on the glycine neurotransmitter system, Chelidonium herba, having a high content of benzophenanthridine alkaloids, inhibits glycine-activated currents and potentiates glutamate-activated ion currents in rat periaqueductal gray neurons (PAG) (Shin et al., 2003). Additionally, interaction with several other receptors has been described previously (Wink et al., 1998).

In this work we report that the benzophenanthridine alkaloids chelerythrine and saguinarine inhibit glycine transporters and in low micromolar concentration preferentially target the glycine transporter GlyT1.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagles medium (DMEM), fetal calf serum, streptomycin, penicillin, L-glutamine, Trypsine-EDTA solution, sanguinarine in 98.1% purity, M.P. (279–282 °C) and chelerythrine in 95% purity, M.P. (200–204 °C), berberine, ouabain octahydrate (MP. 182–205 °C), luminol, PCA, hydrogen peroxide, 2-mercaptoethanol, PEI 80 kDa were purchased from Sigma Chemicals (St. Louis, MO, USA). Affinity purified primary antibodies (epitope: 554–625) against mouse GlyT1C terminus were used as previously described (Baliova and Jursky, 2010). Secondary horseradish peroxidase conjugated antibodies were purchased from Millipore (Temecula, CA, USA). [^{3}H]Glycine (2.08 TBq/mmol) was supplied by ICN (Irvine, CA, USA). Tris(Hydroxymethyl)aminomethane (Tris) free base, HEPES free acid, sodium chloride (NaCl), lithium chloride (LiCl), all molecular biology grade were from Merck Chemicals, Slovakia (Bratislava). Stock solutions of alkaloids used for subsequent dilutions were prepared as follows: 3 mg of berberine (MW 371.81) was dissolved in 1 ml of methanol to a final concentration 8 mM; 1 mg of chelerythrine (MW 383.8) was dissolved in 0.3 ml of redistilled water to make 8.68 mM; 5 mg of sanguinarine (MW 367.78) was dissolved in 1.550 ml of methanol to make 8.77 mM. Stock solutions 200× diluted in redestilled water had the following absorbances at 328 nm. Berberine 0.532, chelerythrine 0.552, sanguinarine 0.441.

2.2. Glycine transporter genes and cell lines

Rat GlyT2a (Liu et al., 1993), human GlyT1c (gift from Prof. Vandenberg, Department of Pharmacology, Institute for Biomedical Research, University of Sydney, Australia) and human GlyT2a (gift from Prof. Robert Harvey, Department of Pharmacology, School of Pharmacy, London, UK) were transferred to a pEDFPN1Δ plasmid containing a G418 resistance gene. This plasmid was obtained from pEGFPN1 (Clontec, Palo Alto, CA, USA) in the following way: the GFP gene was excised with ApaI/XbaI, and then the DNA ends were filled with the Klenow fragment and ligated. Glycine transporter genes were inserted downstream of the CMV promoter. HEK293T-hGlyT1c and HEK293T-rGlyT2a stable cell lines were prepared via the integration of ApaLI linearized pEDFPN1Δ plasmids bearing glycine transporter genes into the HEK293T cell line (ATCC CRL-1573 293) using PEI transfection (Boussif et al., 1995). Single colonies were isolated after selection with G418 at a concentration of 0.5 mg/ml. Cell lines were further propagated in complete DMEM 0.25 mg/ml G418.

For uptake purposes HEK293T-hGlyT1c cells and HEK293T-hGlyT2a cells were seeded on 24-well plates. Following transfer of the cells to atmospheric CO_2 concentration, the original medium (0.5 ml) was replaced with the same volume of DMEM medium buffered with 20 mM HEPES-NaOH pH 7.4.

2.3. Dose–response of hGlyT1 and hGlyT2 glycine uptake to chelerythrine and sanguinarine

Before the assay, HEK293T-hGlyT1c and HEK293T-hGlyT2a expressing cells seeded on 24-well plates were washed once with 0.5 ml of uptake buffer (25 mM Tris–HCl, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 5 mM alanine, pH 7.4) (Alanine was added to inhibit the endogenous general amino-acid transport system in HEK293T cells). Uptake buffer (0.2 ml) containing various amounts of chelerythrine or sanguinarine (Fig. 2A and B) was then added. Cells were preincubated with alkaloids for 3 min at 23 °C, then the solution was aspirated and 0.2 ml of uptake buffer containing 10 μM [^{3}H] glycine with an alkaloid concentration equal to that in the preincubation solution was added to assay glycine uptake for 1 min (GlyT1) or 5 min (GlyT2) at 23 °C. Uptake was stopped by two 0.5 ml washes of uptake buffer containing lithium instead sodium (25 mM Tris–HCl, 150 mM LiCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 5 mM alanine). Cells were dissolved in Triton buffer (0.1 M Tris–HCl pH 8.0, 0.5% TritonX100), transferred into scintillation liquid and the samples were measured with a scintillation counter.

2.4. Time dependence of chelerythrine and sanguinarine inhibition

Cells HEK293T-hGlyT1c seeded on 24-well plates were washed once with 0.5 ml of uptake buffer. Uptake buffer (0.2 ml) containing 10 μM chelerythrine, 10 μM sanguinarine or 1 mM ouabain was then added. Cells were preincubated with alkaloids for distinct time intervals (see Fig. 2C) at 23 °C, then the solution was aspirated and 0.2 ml of uptake buffer containing 10 μM [^{3}H] glycine with 10 μM alkaloid or 1 mM ouabain was added to assay the glycine uptake for 1 min. In control samples the uptake assay was preformed in the same way, except that alkaloids were omitted during the pre-incubation and uptake assay. Uptake was stopped by two 0.5 ml washes of lithium containing uptake buffer. Cells were dissolved in Triton buffer, transferred into scintillation liquid and samples were measured with a scintillation counter.

2.5. Reversibility of chelerythrine and sanguinarine inhibition

HEK293T-hGlyT1c cells seeded on 24-well plates were washed once with 0.5 ml of uptake buffer. Uptake buffer (0.2 ml) containing 10 μM chelerythrine or 5 μM sanguinarine was then added and preincubated for 3 min. Following aspiration of the liquid, the cells were washed 2× with 0.5 ml of uptake buffer and covered with 0.7 ml of uptake buffer containing 10 mM glucose. Cells were then placed on a gentle shaker. At various time intervals, the solution was aspirated and cells were additionally washed once with 0.5 ml uptake buffer. Glycine uptake was then assayed by adding 0.2 ml of uptake solution containing 10 μM [^{3}H] labeled glycine for 1 min at 23 °C. Uptake was stopped by two 0.5 ml washes of lithium-containing uptake buffer. Cells were dissolved in Triton buffer, transferred into scintillation liquid and samples were measured on scintillation counter.

2.6. Determination of inhibition type

HEK293T-hGlyT1c cells plated on 24-well plates were washed with 0.5 ml of uptake buffer and preincubated 3 min in the same buffer with and without 2.5 μM