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Stereospecific modulation of $GABA_A$ receptor function by urocanic acid isomers

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Abstract

A deamination product of histidine, urocanic acid, accumulates in the skin of mammals as *trans*-urocanic acid. Ultraviolet (UV) irradition converts it to the *cis*-isomer that is an important mediator in UV-induced immunosuppression. We have recently shown that urocanic acid interferes with the agonist binding to $GABA_A$ receptors. We now report that the effects of urocanic acid on binding of a convulsant ligand (*t*-butylbicyclo[^{35}S]phosphorothionate) to $GABA_A$ receptors in brain membrane homogenates are dependent on pH of the incubation medium, the agonistic actions being enhanced at the normal pH of the skin (5.5). Using *Xenopus laevis* oocytes expressing recombinant rat $\alpha1\beta1\gamma2S$ $GABA_A$ receptors, the low pH potentiated the direct agonistic action of *trans*-urocanic acid under two-electrode voltage-clamp, whereas *cis*-urocanic acid retained its low efficacy both at pH 5.5 and 7.4. The results thus indicate clear differences between urocanic acid isomers in functional activity at one putative receptor site of immunosuppression, the $GABA_A$ receptor, the presence of which in the skin remains to be demonstrated.

Keywords: Urocanic acid; $GABA_A$ receptor; UV-induced immunosuppression

1. Introduction

Urocanic acid [3-(1*H*-imidazol-4-yl)-propenoic acid, UCA] is a component of the epidermis and major chromophore for ultraviolet (UV) light (for review, see Mohammad et al., 1999). Located superficially in the stratum corneum of the skin, urocanic acid efficiently absorbs UV radiation and undergoes photoisomerization from *trans*-urocanic acid to *cis*-urocanic acid (Fig. 1; Anglin et al., 1961; Everett et al., 1961). Various studies have demonstrated that *cis*-urocanic acid contributes to UV-induced immunosuppression, although the mechanisms have remained uncertain (De Fabo and Noonan, 1983; Moodycliffe et al., 1996). Histamine H_1 and H_2 receptor antagonists partially block *cis*-urocanic acid induced immunosuppression (Gilmour et al., 1992–1993; Hart et al., 1997), although *cis*-urocanic acid does not bind to histamine receptors (Mitra et al., 1993; Jaksic et al., 1995; Hart et al., 1997; Laihia et al., 1998).

The isomers of urocanic acid have been shown to interact with $GABA_A$ receptors. *Trans*-urocanic acid displaces [^{3}H]GABA binding (IC_{50} = 0.3 mM) and stimulates [^{3}H]flunitrazepam binding to brain membranes (Tunnicliff et al., 1985; Matheson et al., 1987). More recently, *cis*-urocanic acid was shown to be a more potent displacer of [^{3}H]GABA than the *trans* isomer (Laihia et al., 1998), suggesting that $GABA_A$ receptors might be involved in immunosuppressive mechanisms of *cis*-urocanic acid.

In the present study, we have further characterized the interactions of urocanic acid isomers with native whole brain and recombinant $GABA_A$ receptors. The experiments were carried out at the pH's of 5.5 and 7.4 to reveal

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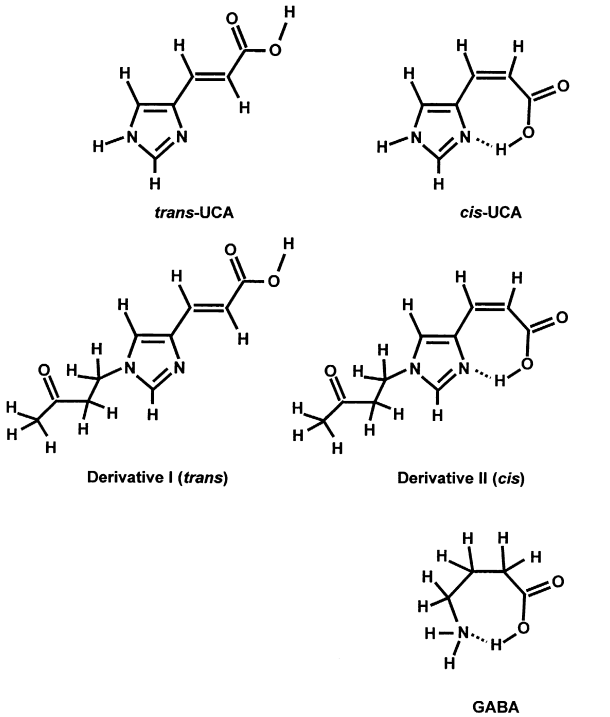

Fig. 1. Structures of *cis*- and *trans*-urocanic acids (UCA), urocanic acid derivatives, and GABA.

the effects of urocanic acid isomers at the skin and blood plasma pH's, respectively.

2. Materials and methods

2.1. Materials

t-Butylbicyclo[^{35}S]phosphorothionate ([^{35}S]TBPS) was purchased from DuPont de Nemours, NEN Division (Dreieich, Germany). RiboMax RNA synthesis kit was from Promega (Madison, WI, USA). Collagenase type IA, *trans*-urocanic acid, GABA and picrotoxinin were purchased from Sigma (St. Louis, MO, USA). *Cis*-urocanic acid was prepared from *trans*-urocanic acid with UV photoisomerization (Laihia et al., 1996). The purity of the isomers was at least 98% by high-pressure liquid chromatography.

To study possible relationships of chemical structure and biological activity, it was of interest to prepare the urocanic acid derivatives I and II in which there are side chains at the $N-1$ position of the imidazole nucleus (Fig. 1). Derivative I [*E*-3-(4-(1-(3-oxobutyl))imidazolyl)propenoic acid] was prepared by modifying the procedure of Ienaga et al. (1988), starting from urocanic acid and 3-buten-2-one in dimethyl formamide as solvent and *p*-toluenesulfonic acid as a catalyst. Derivative II [*Z*-3-(4-(1-(3-oxobutyl))imidazolyl)propenoic acid] was prepared by photochemical treatment of derivative I in water solution (Anglin and Batten, 1968) followed by separation through ion exchange chromatography (Anglin and Batten, 1968; Morrison et al., 1980b). The preparative yields for the derivatives I and II were 52% and 60%, respectively. The chemical analyses of I and II by ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR, and mass spectrometric spectra were in accordance with the expected structure (data not shown).

2.2. Preparation of cerebral cortical membranes

Adult male Wistar rats (Department of Laboratory Animals, University of Helsinki, Finland) were decapitated and the cerebral cortices were dissected and frozen. Cerebral cortical membranes were prepared essentially as described earlier (Uusi-Oukari and Korpi, 1990). In brief, cerebral cortices were homogenized with a Polytron homogenizer in 50 volumes of ice-cold 50 mM Tris–citrate buffer, pH 7.4, containing 1 mM EDTA. The homogenates were centrifuged at $20{,}000 \times g$ for 20 min. Pellets were resuspended in the same buffer and recentrifuged five times. The final suspension was prepared in 50 mM Tris–citrate buffer, pH 7.4, divided into aliquots, and stored frozen at -80°C.

2.3. [^{35}S]TBPS binding

Frozen membranes were thawed, resuspended, and recentrifuged once before final resuspension in 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 5.5, or 50 mM Tris–citrate, pH 7.4, both containing 0.2 M NaCl in a total volume of 0.5 ml/assay tube. In experiments performed at pH 7.4, membranes corresponding to 3 mg original wet weight/tube were used, while 6 mg/tube was used at pH 5.5 due to lower specific binding of [^{35}S]TBPS. The binding assays were performed essentially as described by Mäkelä et al. (1995). Briefly, membranes were incubated at room temperature for 90 min with 2 nM [^{35}S]TBPS in the absence and presence of various concentrations of *cis*-urocanic acid, *trans*-urocanic acid and urocanic acid derivatives with or without GABA. *Trans*-urocanic acid was solubilised in dimethyl sulfoxide (DMSO) and diluted in assay buffer before use. Due to its limited solubility, *trans*-urocanic acid was not used at concentrations higher than 1 mM. The final concentration of DMSO in *trans*-urocanic acid experiments was 1% in all control and test incubations. Other compounds were dissolved directly into assay buffers. Non-specific binding was determined in the presence of 100 μM picrotoxinin. After incubation, the samples were filtered onto Whatman GF/B glass fiber filters, rinsed twice with 5 ml of ice-cold 10 mM MES buffer, pH 5.5, or 10 mM Tris–HCl, pH 7.4, depending on the pH of the incubation buffer. Air-dried filters were immersed in 4 ml of scintillation fluid (Wallac Optiphase Hisafe 2) and radioactivity was determined in a Wallac 1410 liquid scintillation counter.

2.4. Preparation of cRNA and oocytes

Capped cRNAs coding for rat $GABA_A$ receptor subunits $\alpha 1$, $\beta 1$ and $\gamma 2S$ (Lüddens et al., 1990; Shivers et al., 1989; Ymer et al., 1989) were transcribed in vitro from pRK5 plasmids using RiboMAX kit (Promega) according to manufacturer's instructions. Oocytes were isolated from adult *Xenopus laevis* females (Horst Kähler, Hamburg, Germany) (Kuner et al., 1993). The frogs were anaesthetized with 0.2% tricaine, oocytes were isolated and stored in Ca^{2+}-free OR2 medium, which contained in mM: 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 Na_2HPO_4 and 5 HEPES; pH 7.5. Oocytes were then defolliculated manually and injected in 46 nl of a solution containing (1:1:1) mixtures of subunit cRNAs (1–2 mg/ml) with Drummond Nanoject injector (Drummond Scientific, Broomall, PA, USA) via a glass micropipette ∅ 20–40 μm (Drummond). Injected oocytes were digested for 30 min in OR2 medium containing 0.5 U/ml collagenase type IA (Sigma). The oocytes were stored at 19°C for up to 5 days in normal frog Ringer (NFR) solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES, pH 7.5, and supplemented with 5 mM sodium pyruvate, penicillin 100 U/ml and streptomycin 100 μg/ml. The incubation medium was changed daily.

2.5. Electrophysiological methods

Oocytes were perfused with NFR ± drugs at a flow rate of 2 ml/min at room temperature (22°C) using Ismatec pump and eight channel perfusion system with pinch valves. At pH 5.5, 10 mM MES replaced HEPES as a buffering agent. Oocytes were impaled with two microelectrodes (0.5–2.5 MΩ) filled with 3 M KCl and 10 mM EGTA and clamped at −50 mV with Turbo TEC-05 two electrode voltage-clamp amplifier (npi electronic, Tamm, Germany). Experiments were controlled by EggWorks experimental control and data acquisition software program (npi). GABA was dissolved in water, *cis*-urocanic acid in water or DMSO, and *trans*-urocanic acid was dissolved in DMSO, which was also tested as a vehicle control at 1% concentration. Drugs were applied for 30 s and 2–10 min washout period was used depending on the concentration, which protocol gave stabile and reproducible responses on repeated GABA applications (not shown).

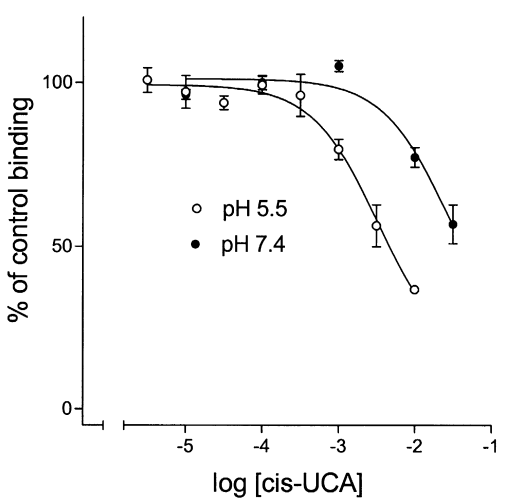

Fig. 2. Displacement of [^{35}S]TBPS by *cis*-urocanic acid at pH 5.5 and 7.4. Cerebral cortical membranes were incubated with 2 nM [^{35}S]TBPS in the absence and presence of various concentrations of *cis*-urocanic acid (UCA). The binding assay was performed as described in Materials and methods.

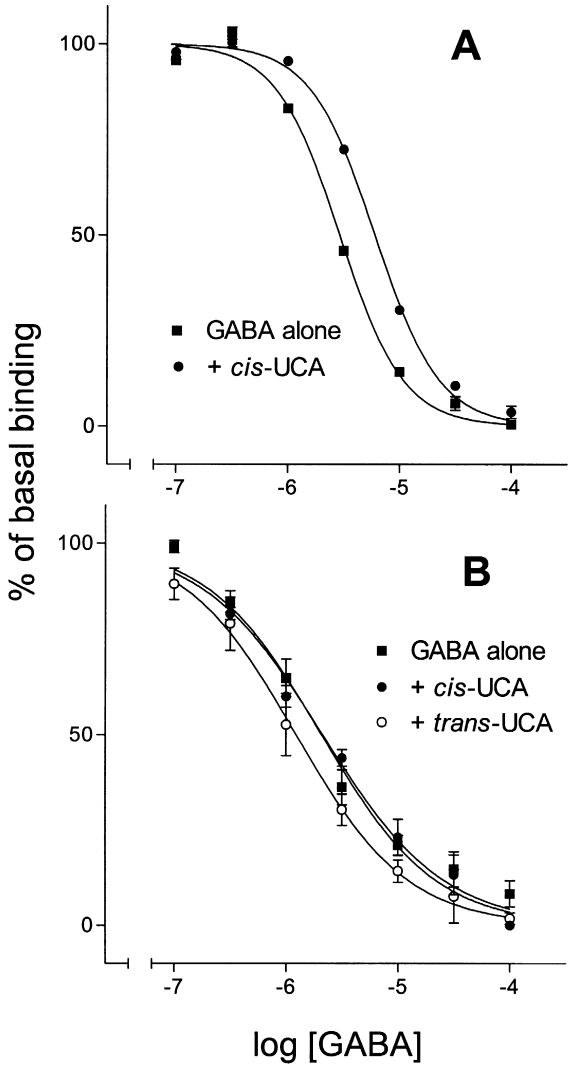

Fig. 3. Effect of *cis*-urocanic acid (UCA) (A; pH 7.4) or *cis*- and *trans*-urocanic acids (B; pH 5.5) on GABA-induced displacement of [^{35}S]TBPS. Cerebral cortical membranes were incubated at pH 7.4 with 2 nM [^{35}S]TBPS with or without *cis*- or *trans*-urocanic acid [1.0 mM (A); 0.3 mM (B)] in the absence and presence of various concentrations of GABA. The binding assay was performed as described in Materials and methods.

2.6. Data analysis

Prism 2.0 program (GraphPad Software, San Diego, CA, USA) was used to calculate the IC_{50} values for

cis-urocanic acid and GABA displacement of [^{35}S]TBPS binding. Origin 5.0 (Microcal Software, Northampton, MA, USA) was used to calculate EC_{50} values for GABA responses. Statistical significance of the differences were analysed using paired *t*-test.

3. Results

3.1. [^{35}S]TBPS binding

A preliminary experiment was performed to assess the effect of *cis*- and *trans*-urocanic acid on [^{35}S]TBPS binding and on GABA-induced inhibition of [^{35}S]TBPS binding. At pH 7.4, both isomers of urocanic acid were unable to inhibit [^{35}S]TBPS binding at the highest concentration tested (1 mM). *Cis*-urocanic acid (1 mM) partially reversed the 3-μM GABA-induced inhibition of the binding, while *trans*-urocanic acid had no effect on it. At pH 5.5, both isomers (1 mM) inhibited [^{35}S]TBPS binding. A low, alone ineffective concentration (0.1 mM) of *cis*-urocanic acid partially reversed GABA-induced inhibition, while *trans*-urocanic acid potentiated the effect of GABA. The displacing potency of urocanic acid derivatives I and II was not improved as compared to urocanic acid (data not shown).

Cis-urocanic acid inhibited [^{35}S]TBPS binding with IC_{50} values of 39 mM and 4.7 mM for pH 7.4 and pH 5.5, respectively (Fig. 2). *Trans*-urocanic acid up to 1 mM concentration (highest concentration that could be solubilized without greatly affecting the binding by the vehicle, DMSO) had no effect on the binding at pH 7.4, while at pH 5.5, 1 mM *trans*-urocanic acid inhibited the binding by 26.2 ± 4.4% (mean ± S.E.M., $n = 3$).

The highest concentrations of *cis*- and *trans*-urocanic acid that had no effect on [^{35}S]TBPS binding (0.3 mM for both ligands at pH 5.5, 1 mM for *cis*-urocanic acid at pH 7.4) were used to evaluate their effects on the GABA inhibition of [^{35}S]TBPS binding. At pH 7.4, GABA inhibited [^{35}S]TBPS binding with an IC_{50} value of 2.9 μM (Fig. 3A). In the presence of 1 mM *cis*-urocanic acid, the potency of GABA was reduced ($IC_{50} = 6.0$ μM) (Fig. 3A). At pH 5.5, GABA inhibited the binding with an IC_{50} value of 2.1 μM, that was not significantly affected by 0.3 mM *cis*-urocanic acid (Fig. 3B, $IC_{50} = 2.1$ μM), whereas *trans*-urocanic acid (0.3 mM) increased the potency of GABA to displace [^{35}S]TBPS binding (Fig. 3B, $IC_{50} = 1.2$ μM).

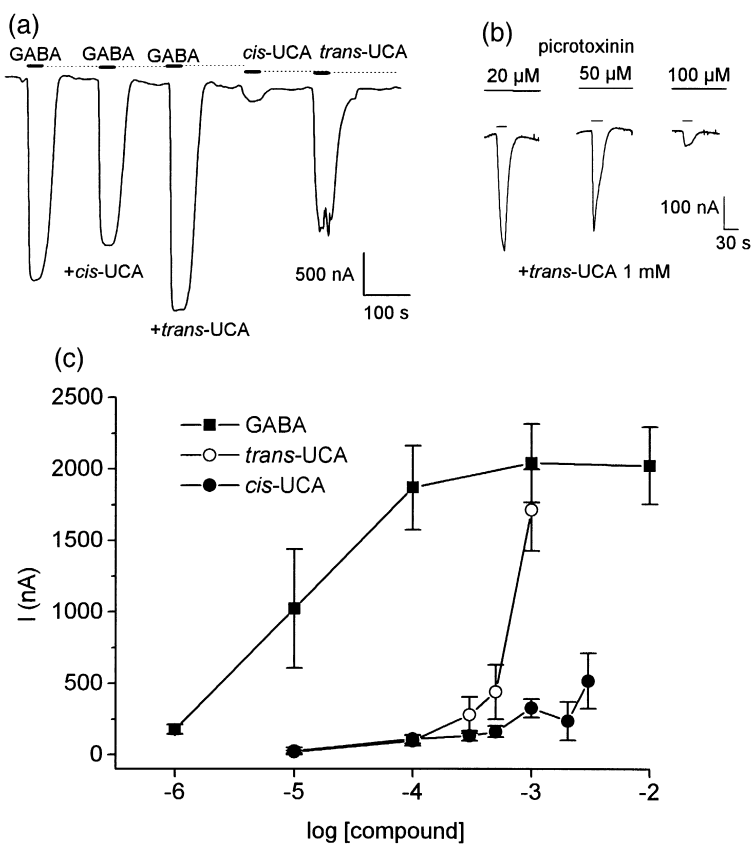

Fig. 4. Representative current trace (a) at pH 5.5 showing (from left to right) the effects of GABA alone (30 μM), GABA + *cis*-urocanic acid (UCA) (1 mM), GABA + *trans*-urocanic acid (1 mM), *cis*-urocanic acid alone (1 mM) and *trans*-urocanic acid alone (1 mM) in *X. laevis* oocytes expressing α1β1γ2S $GABA_A$ receptor. The horizontal bars depict the times of drug application. Representative current traces (b) of picrotoxinin blockade of *trans*-urocanic acid-induced inward currents at pH 5.5 in an oocyte expressing the α1β1γ2S $GABA_A$ receptor. Concentration–response curves (c) for GABA, *trans*- and *cis*-urocanic acids in α1β1γ2S receptor-expressing oocytes at pH 5.5. Points are means ± S.E.M. ($n = 4$).

3.2. Electrophysiology

Concentration–response curves for GABA were obtained for $\alpha1\beta1\gamma2S$ subunit containing $GABA_A$ receptors. At pH 5.5, there was a decrease in the efficiency of the GABA response without a change in GABA potency as compared to pH 7.4. The EC_{50} values for GABA responses were 5.8 ± 0.4 μM at pH 7.4 (mean ± S.E.M., $n = 5$) and 5.0 ± 0.3 μM at pH 5.5 ($n = 4$). Maximal currents of these responses were 4.3 ± 0.6 and 2.0 ± 0.3 μA at the pH 7.4 and 5.5, respectively. Representative effects of *cis*-urocanic acid and *trans*-urocanic acid at pH 5.5 are shown in Fig. 4. *Cis*-urocanic acid (1 mM) significantly inhibited the 30-μM GABA-induced chloride currents at both pH 5.5 and pH 7.4, while *trans*-urocanic acid (1 mM) slightly increased the GABA responses (Fig. 5). No responses to GABA were detected in control, non-injected oocytes when applied alone or with *cis*- or *trans*-urocanic acid.

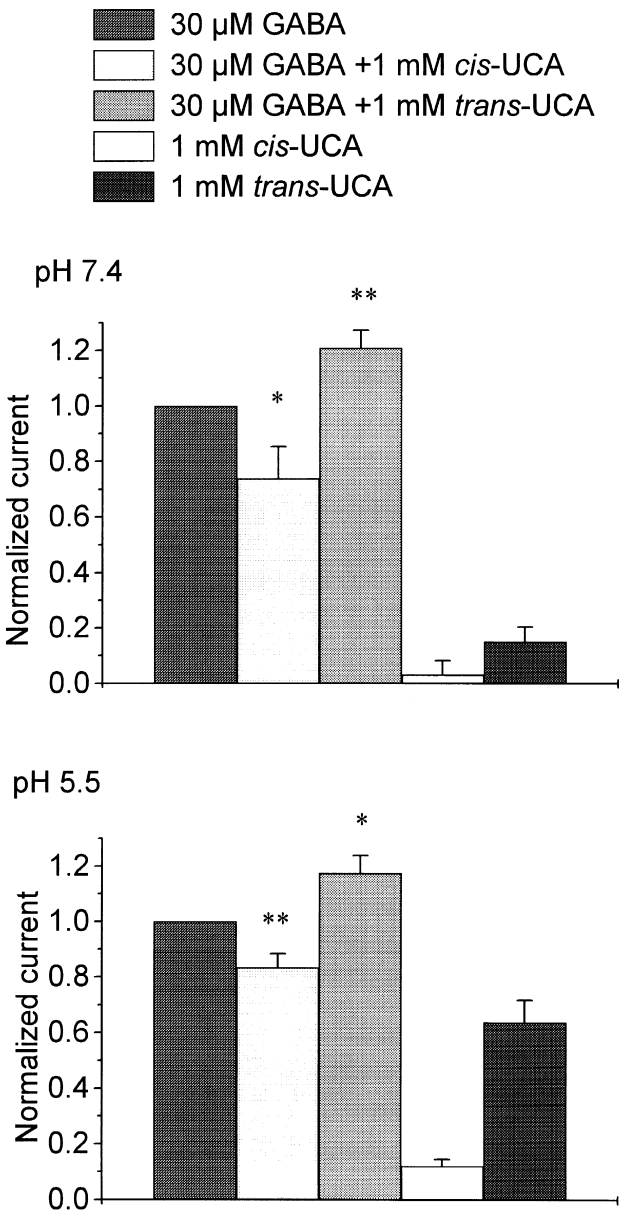

Fig. 5. Effects of *cis*- and *trans*-urocanic acids (UCA) on 30-μM GABA-activated currents and the effects of *cis*-urocanic acid and *trans*-urocanic acid alone in *X. laevis* oocytes expressing $\alpha1\beta1\gamma2S$ $GABA_A$ receptor. Data is normalized response ± S.E.M. of the control GABA response [3.4 ± 0.4 vs. 3.1 ± 03 μA (mean ± S.E.M.) for pH 7.4 ($n = 10$) and pH 5.5 ($n = 8$), respectively]. Statistical significance of the difference from GABA alone: $^{*}P < 0.05$, $^{**}P < 0.01$ (paired *t*-test).

Especially *trans*-urocanic acid alone was able to induce picrotoxin-sensitive currents (Figs. 4 and 5). Further characterization of currents elicited by *cis*-urocanic acid was rendered unfeasible by its low efficacy. At 1-mM concentration the response of *cis*-urocanic acid alone was about 10% of that elicited by 30 μM GABA (Figs. 4 and 5). At pH 5.5, *trans*-urocanic acid alone from 10-μM concentration on elicited currents concentration-dependently, but starting from the concentration of 1 mM the effects became often oscillatory and non-reversible on washout (Fig. 4). *Trans*-urocanic acid did not induce any currents in non-injected oocytes (data not shown). DMSO (1%) alone did not affect membrane currents of injected oocytes.

4. Discussion

We studied the effects of urocanic acid isomers on $GABA_A$ receptor function using [^{35}S]TBPS binding and electrophysiology. The two methods gave mostly similar results, making it possible to draw the following basic conclusions: (1) urocanic acid isomers are more potent at pH 5.5 than at pH 7.4; (2) the direct agonist effect of *trans*-urocanic acid is stronger than that of *cis*-urocanic acid; (3) *trans*-urocanic acid slightly potentiates GABA responses, while *cis*-urocanic acid inhibits them.

The protonation states of both $GABA_A$ receptors and urocanic acid isomers may play a role in the higher potency of urocanic acid at pH 5.5 than at pH 7.4. Urocanic acid contains three protonation sites, a carboxy group and secondary and tertiary nitrogens. At pH 5.5, urocanic acid isomers exist predominantly as zwitterions, both nitrogens are protonated, while the carboxy group is negatively charged (Mehler and Tabor, 1953; Morrison et al., 1980a; Roberts et al., 1982; Öhman and Vahlquist, 1994). When pH is shifted to neutral (pH 7.4), the ionic state of urocanic acid is predominantly carboxylate ion (Morrison et al., 1980a), suggesting that the higher potencies of urocanic acid isomers at acidic pH are based on zwitterionic form of the molecules. There is, however, indications that pH changes also affect $GABA_A$ receptor responses (for review, see Kaila, 1994). The pH effects depend on subunit combination, the β variant being responsible for the pH sensitivity/insensitivity in recombinant receptors (Krishek et al., 1996). $\alpha1\beta1\gamma2S$ combination is not affected by external pH, while β2 in place of

β1 confers sensitivity to pH. Hence, we used α1β1γ2S combination to eliminate the effect of pH on receptor function. Although, in the present study, the efficiency of GABA was affected by external pH despite the use of α1β1γ2S combination, the potency of GABA remained unaltered. Therefore, the different effects of urocanic acid isomers on GABA responses at various pH's are likely due to their molecular features rather than altered receptor structures.

The different agonist profiles of *trans*- and *cis*-urocanic acid are apparently based on the characteristic orientation of the carboxylate group in the two stereoisomers. Although both molecules have planar geometries, the structure of *cis*-urocanic acid is stabilized by intramolecular hydrogen bonding between the imidazole and the propenoic acid moieties to form a seven-atom ring (Lahti et al., 1997) (Fig. 1). A resembling ring structure may be depicted with GABA (Fig. 1). In addition, *cis*-urocanic acid is substantially more soluble to water (Mohammad et al., 1999), and thus the isomerization of *trans*-urocanic acid to *cis*-urocanic acid by UV light in the skin could make the molecule more accessible to circulation (Kammeyer et al., 1997). *Trans*-urocanic acid produced clear agonistic effects on recombinant receptors at pH 5.5, while the responses of *cis*-urocanic acid were very slight. *Trans*-urocanic acid is localized in the epidermis where the pH is near 5.5, but because there are no reports of $GABA_A$ receptors in the skin, the significance of this agonist action remains unclear.

Both urocanic acid isomers had a relatively low potency on $GABA_A$ receptors, but due to local synthesis in the skin, the natural cutaneous concentrations of the isomers are high. The molar concentrations have been estimated to be in the range of 0.3–8.9 mM in the human epidermis (Laihia et at., 1998) and 10–20 mM in the stratum corneum (Jones et al., 1996), which are in the relevant range for the present results. Our attempt to produce more potent urocanic acid derivatives by attaching soluble side-chains to the nitrogen ring yielded only compounds that were of similar potency as *cis*-urocanic acid. This supports the fact that urocanic acid acts on $GABA_A$ receptors via GABA sites (Laihia et al., 1998).

It has been suggested that *cis*-urocanic acid has an important mediator role in the induction of immunosuppression and possibly of systemic tolerance initiated by UV irradiation (Moodycliffe et al., 1996; Mohammad et al., 1999). Histaminergic signalling takes place in the process, but histamine receptors are now considered not to be the receptors for *cis*-urocanic acid (Mitra et al., 1993; Jaksic et al., 1995; Hart et al., 1997; Laihia et al., 1998). According to current hypotheses, UV irradiation of the skin induces local bursts of neuropeptides, especially calcitonin gene related peptide (CGRP), from cutaneous sensory nerves (Niizeki et al., 1997; Garssen et al., 1998). Dermal mast cells are mediators of the *cis*-urocanic acid-induced immunosuppression (Hart et al., 1999) and these cells may be those responding to neuropeptides by degranulation followed by the release of histamine (Niizeki et al., 1997). Histamine acts on keratinocytes and/or fibroblasts to induce prostaglandin E_2 production (Jaksic et al., 1995; Hart et al., 1997). This mediator can, in turn, affect the circulating immune cell types to communicate more systemically via soluble cytokines IL-4 and IL-10 (Shreedhar et al., 1998). IL-10 is an efficient suppressor protein of antigen-presenting-cell activity in vivo and a major UV immune modulator (Shreedhar et al., 1998). The mechanism by which UV photons could initially induce skin nerves to release CGRP is unclear. Assuming that the effect of GABA is inhibitory on CGRP release, the process could involve antagonism of $GABA_A$ receptor function by the photoisomerization product *cis*-urocanic acid (Laihia et al., 1998 and the present results). Furthermore, as *cis*-urocanic acid decreases the effect of GABA on $GABA_A$ receptors, it could reverse the inhibitory effect of GABA on histamine- and/or prostaglandin E_2-producing cells. This possibility arises from recent demonstrations of $GABA_A$ receptor subunit mRNA and protein in immunocompetent cells (Bergeret et al., 1998) and of functional, proliferation-inhibiting $GABA_A$ receptors in T cells (Tian et al., 1999).

In conclusion, *cis*- and *trans*-urocanic acids act directly on $GABA_A$ receptors. The involvement of $GABA_A$ receptors on modulation of immunoresponses should be studied to reveal their possible role on *cis*-urocanic acid-induced immunosuppression.

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