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Evaluation of the Protective Effects of Alkaloids Isolated from the Hooks and Stems of *Uncaria sinensis* on Glutamate-induced Neuronal Death in Cultured Cerebellar Granule Cells from Rats

YUTAKA SHIMADA, HIROZO GOTO, TAKASHI ITOH, IWAO SAKAKIBARA*, MASAYOSHI KUBO*, HIROSHI SASAKI* AND KATSUTOSHI TERASAWA

*Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, and *Tsumura Central Research Laboratories, 3586 Yoshiwara, Amimachi, Ibaraki 300-1192, Japan*

Abstract

We have previously shown that an aqueous extract of the hooks and stems of *Uncaria sinensis* (Oliv.) Havil., Uncariae Uncus Cum Ramulusis, protects against glutamate-induced neuronal death in cultured cerebellar granule cells by inhibition of Ca^{2+} influx. Because it is not known which components of *Uncaria sinensis* are active, in this study we have evaluated, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) staining, the neuroprotective effects of the oxyindole alkaloids corynoxeine, rhynchophylline, isorhynchophylline and isocorynoxeine, and the indole alkaloids geissoschizine methyl ether, hirsuteine and hirsutine, isolated from the hooks and stems of *Uncaria sinensis*, on glutamate-induced cell death. We also investigated the inhibitory effects of the compounds on $^{45}Ca^{2+}$ influx in cultured rat cerebellar granule cells.

Cell viability evaluated by the MTT assay was significantly increased by application of rhynchophylline (10^{-3} M), isorhynchophylline (10^{-4}–10^{-3} M), isocorynoxeine (10^{-4}–10^{-3} M), hirsuteine (10^{-4}–3×10^{-4} M) or hirsutine (10^{-4}–3×10^{-4} M) compared with exposure to glutamate only, with the effect of isorhynchophylline being the strongest. The increased $^{45}Ca^{2+}$ influx into cells induced by glutamate was significantly inhibited by administration of rhynchophylline (10^{-3} M), isorhynchophylline (3×10^{-4}–10^{-3} M), isocorynoxeine (3×10^{-4}–10^{-3} M), geissoschizine methyl ether (10^{-3} M), hirsuteine (3×10^{-4}–10^{-3} M) or hirsutine (3×10^{-4}–10^{-3} M).

These results suggest that oxyindole alkaloids such as isorhynchophylline, isocorynoxeine and rhynchophylline and indole alkaloids such as hirsuteine and hirsutine are the active components of the hooks and stems of *Uncaria sinensis* which protect against glutamate-induced neuronal death in cultured cerebellar granule cells by inhibition of Ca^{2+} influx.

Glutamate mediates the death of central neurons in several pathological conditions, for example ischaemic-hypoxic injury, epilepsy and neurodegenerative diseases (Choi 1988, 1990; Meldrum & Garthwaite 1990). The extracellular concentration of glutamate in the brain increases during brain ischaemia (Benveniste et al 1984) and overstimulation of glutamate receptors of neuronal cells, as a result of excessive release of glutamate, has been found to be toxic to the cells (Rothman & Olney 1986). Glutamate receptors can be classified as metabotropic and ionotropic; ionotropic receptors include the *N*-methyl-D-aspartate (NMDA) receptor and non-NMDA receptors (Nakanishi 1992). Overstimulation of ionotropic glutamate receptors has been found to be toxic to neuronal cells as a result of Ca^{2+} influx (Rothman & Olney 1986; Choi 1988; Kristián & Siesjö 1998).

We have previously shown that an aqueous extract of the hooks and stems of *Uncaria sinensis* (Oliv.) Havil., Uncariae Uncus Cum Ramulusís, protects against glutamate-induced neuronal death

Correspondence: Y. Shimada, Department of Japanese Oriental Medicine, Faculty of Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan.

in cultured cerebellar granule cells by inhibition of Ca^{2+} influx (Shimada et al 1998). The active components of *Uncaria sinensis* have not been identified. The *Uncaria* genus produces many types of alkaloid, including oxyindole alkaloids such as corynoxeine, rhynchophylline, isorhynchophylline and isocorynoxeine, and indole alkaloids such as geissoschizine methyl ether, hirsuteine and hirsutine (Yamanaka et al 1983). It is known that these alkaloids have many activities, including an anticonvulsive effect on glutamate-induced convulsion in mice (Mimaki et al 1997) and vasodilative and Ca^{2+}-channel-blocking activity in isolated rat thoracic aorta (Yamahara et al 1987; Yano et al 1991; Horie et al 1992).

In this study, to identify the active components, we evaluated the protective effects of alkaloids isolated from the hooks and stems of *Uncaria sinensis* against glutamate-induced neuronal death and their inhibitory effects on glutamate-induced Ca^{2+} influx in cultured rat cerebellar granule cells.

Materials and Methods

Materials

The oxyindole alkaloids corynoxeine, rhynchophylline, isorhynchophylline and isocorynoxeine, and the indole alkaloids geissoschizine methyl ether, hirsuteine and hirsutine (Figure 1) were isolated from the hooks and stems of *Uncaria sinensis* (Oliv.) Havil. at Tsumura Central Research Laboratories, as described elsewhere (Sakakibara et al 1997). Because these alkaloids are insoluble in water they were dissolved in dimethylsulphoxide (DMSO; Wako Pure Chemicals, Tokyo, Japan) at a concentration of 10^{-1} M.

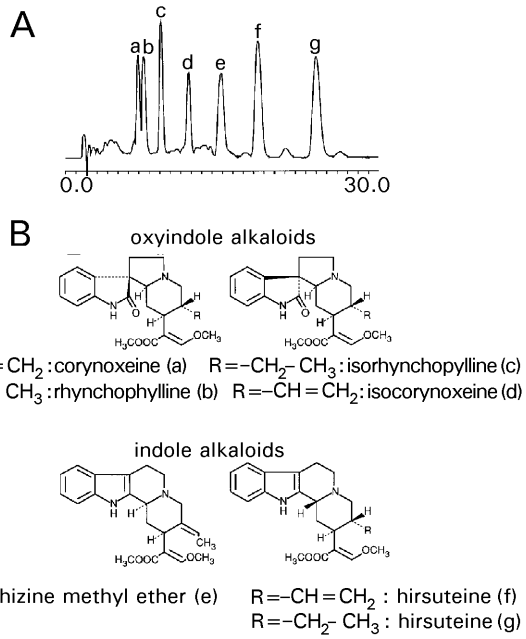

Figure 1. HPLC (A) and chemical structures (B) of oxyindole and indole alkaloids isolated from the hooks and stems of *Uncaria sinensis*.

Cell culture

Cerebellar granule cells were cultured as described elsewhere (Gallo et al 1982; Shimada et al 1998). In brief, ten (approx.) cerebella were dissected from the brains of 7–8-day-old Wistar rats, chopped into small pieces and placed in Krebs–Ringer buffer solution (KRB; 25 mL). This was centrifuged at 150 *g* for 30 s, the pellet was resuspended in KRB (25 mL) containing 0·025% trypsin (Sigma, St Louis, MO), and incubated at 37°C for 13 min. The trypsinization was stopped by addition of 0·005% trypsin inhibitor (Sigma) with 0·01% deoxyribonuclease (Sigma). The resulting pellet was centrifuged, dissociated into cells, and suspended in basal Eagle medium (Sigma) containing 10% foetal bovine serum (Sigma), 2 mM glutamine, 20 μg mL^{-1} gentamicin (Sigma) and 25 mM KCl. The cells were seeded at a density of 10^6 cells mL^{-1} in poly-L-lysine (Sigma) -coated 96-well culture plates. The cultures were maintained at 37°C with 5% CO_2 in a humidified incubator. Cytosine arabinoside (Sigma; 10 μM) was added 18 h after plating to prevent the proliferation of glial cells. The culture medium was not changed thereafter. The cultured cells were used at 7–8 days in-vitro for the experiments.

Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described elsewhere (Mosmann 1983; Shimada et al 1998). MTT is converted to an insoluble blue formazan product by dehydrogenases within mitochondria in living cells but not in dying cells (Mosmann 1983). Cultured cells were washed with Mg^{2+}-free Locke's solution (mM: NaCl 154, KCl 5·6, $NaHCO_3$ 3·6, HEPES 5·0, $CaCl_2$ 2·3, glucose 5·6, pH 7·4), then incubated with Mg^{2+}-free Locke's solution with (control) or without (vehicle) 100 μM L-glutamic acid (as glutamate; Sigma). In accordance with previous studies (Wagelius & Korpi 1995; Taniwaki et al 1997; Shimada et al 1998) Mg^{2+} was omitted from the incubation solution to prevent Mg^{2+} blockade of the NMDA receptor channel. Different concentrations (10^{-5}–10^{-3} M) of 2-amino-5-phosphonovaleric acid (AP5; RBI, Natick, MA, USA), a specific NMDA receptor antagonist, or alkaloids isolated

from the hooks and stems of *Uncaria sinensis*, were also dissolved in Mg^{2+}-free Locke's solution, with or without glutamate (100 μM), and then applied to the cells. All these incubation solutions were adjusted to contain 1% DMSO; we had previously confirmed that this concentration of DMSO had almost no influence on glutamate-induced cell death. After 1 h incubation at 37°C, MTT (Sigma; 500 $\mu g\,mL^{-1}$) was added and incubation was continued for 30 min at 37°C. Cells were then washed and lysed in isopropanol with 0·04 M HCl to dissolve the blue formazan products. Optical density was read at 570 nm with a spectrophotometer and expressed as percentage of the vehicle.

$^{45}Ca^{2+}$ *influx*

Influx of $^{45}Ca^{2+}$ was measured as described elsewhere (Lazarewicz et al 1990; Shimada et al 1998). Granule cells were incubated for 1 h in Mg^{2+}-free Locke's solution containing different drugs, $^{45}CaCl_2$ (Amersham, Little Chalfont, Buckinghamshire, UK; 1 $\mu Ci\,mL^{-1}$) and 1% DMSO at 37°C, and washed 3 times with Ca^{2+}- and glucose-free Locke's solution containing 2 mM EGTA. The incubation solution was discarded and cells were solubilized with 1 M NaOH. The amount of radioactivity incorporated by the cells was measured by liquid scintillation counting and expressed as an increase compared with vehicle.

Statistics

Six wells of culture plates were used for each group and their mean was calculated in each experiment. Values are means ± s.d. from four separate experiments. The data were analysed by the Kruskal–Wallis test then the Bonferroni *t*-test. $P < 0{\cdot}05$ was regarded as indicative of statistical significance.

Results

We evaluated the protective effect of each alkaloid isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death by MTT assay, and calculated cell viability. Exposure to glutamate only reduced cell viability to 41% (approx.). This neuronal death was dose-dependently prevented by AP5, a specific NMDA receptor antagonist (10^{-5} M, 44·7%; 3×10^{-5} M, 52·7%; 10^{-4} M, 84·1%; 3×10^{-4} M, 96·4%; 10^{-3} M, 97·0%). Corynoxeine had no significant protective effect at any concentration (Figure 2A). Rhynchophylline significantly inhibited glutamate-induced cell death at 10^{-3} M only (85·2%; Figure 2B). Isorhynchophylline had a distinct dose-dependent protective effect against glutamate-induced cell death; concentrations of 10^{-4} to 10^{-3} M afforded significant protection compared with exposure to glutamate only (10^{-4} M, 55·7%; 3×10^{-4} M, 88·0%; 10^{-3} M, 97·0%; Figure 2C). Isocorynoxeine at concentrations of 10^{-4} to 10^{-3} M also significantly inhibited glutamate-induced cell death, but the most effective dose was 3×10^{-4} M (10^{-4} M, 56·8%; 3×10^{-4} M, 84·3%; 10^{-3} M, 74·3%; Figure 2D). Geissoschizine methyl ether had no significant protective effect at any concentration (Figure 2E). Hirsuteine and hirsutine at 10^{-4}–3×10^{-4} M significantly inhibited glutamate-induced cell death (hirsuteine 10^{-4} M, 53·4%; 3×10^{-4}, 63·7%; hirsutine 10^{-4} M, 49·9%; 3×10^{-4} M, 61·0%), but their effects were weaker than those of isorhynchophylline and isocorynoxeine (Figures 2F, G).

By using $^{45}Ca^{2+}$ as a radioactive tracer we next evaluated the inhibitory effect of different concentrations (10^{-5}–10^{-3} M) of each alkaloid isolated from the hooks and stems of *Uncaria sinensis* against glutamate-induced Ca^{2+} influx, and expressed it as an increase compared with treatment with vehicle only. Glutamate-only exposure increased $^{45}Ca^{2+}$ influx 7·6-fold (approx.). This glutamate-induced $^{45}Ca^{2+}$ influx was dose-dependently prevented by AP5 (10^{-5} M, 6·7-fold; 3×10^{-5} M, 5·9-fold; 10^{-4} M, 3·5-fold; 3×10^{-4} M, 1·9-fold; 10^{-3} M, 1·5-fold). Corynoxeine had no effect on glutamate-induced $^{45}Ca^{2+}$ influx into cerebellar granule cells (Figure 3A). Rhynchophylline and geissoschizine methyl ether significantly inhibited glutamate-induced $^{45}Ca^{2+}$ influx only at 10^{-3} M (rhynchophylline, 4·9-fold; geissoschizine methyl ether, 2·4-fold; Figures 3B, E). Isorhynchophylline, isocorynoxeine, hirsuteine and hirsutine clearly and dose-dependently inhibited glutamate-induced $^{45}Ca^{2+}$ influx, and for concentrations of 3×10^{-4} and 10^{-3} M inhibition was significant compared with exposure to glutamate alone (isorhynchophylline 3×10^{-4} M, 4·7-fold; 10^{-3} M, 1·9-fold; isocorynoxeine 3×10^{-4} M, 3·4-fold; 10^{-3} M, 1·6-fold; hirsuteine 3×10^{-4} M, 3·8-fold; 10^{-3} M, 1·7-fold; hirsutine 3×10^{-4} M, 4·4-fold; 10^{-3} M, 1·5-fold; Figures 3C, D, F, G).

Finally, to examine whether these alkaloids were toxic to cultured cerebellar granule cells we evaluated the effect of each alkaloid on cell viability in the absence of glutamate (Table 1). Corynoxeine, rhynchophylline and isorhynchophylline did not significantly reduce cell viability at any concentration. Isocorynoxeine and geissoschizine methyl ether significantly reduced cell viability only at 10^{-3} M. Hirsuteine and hirsutine sig-

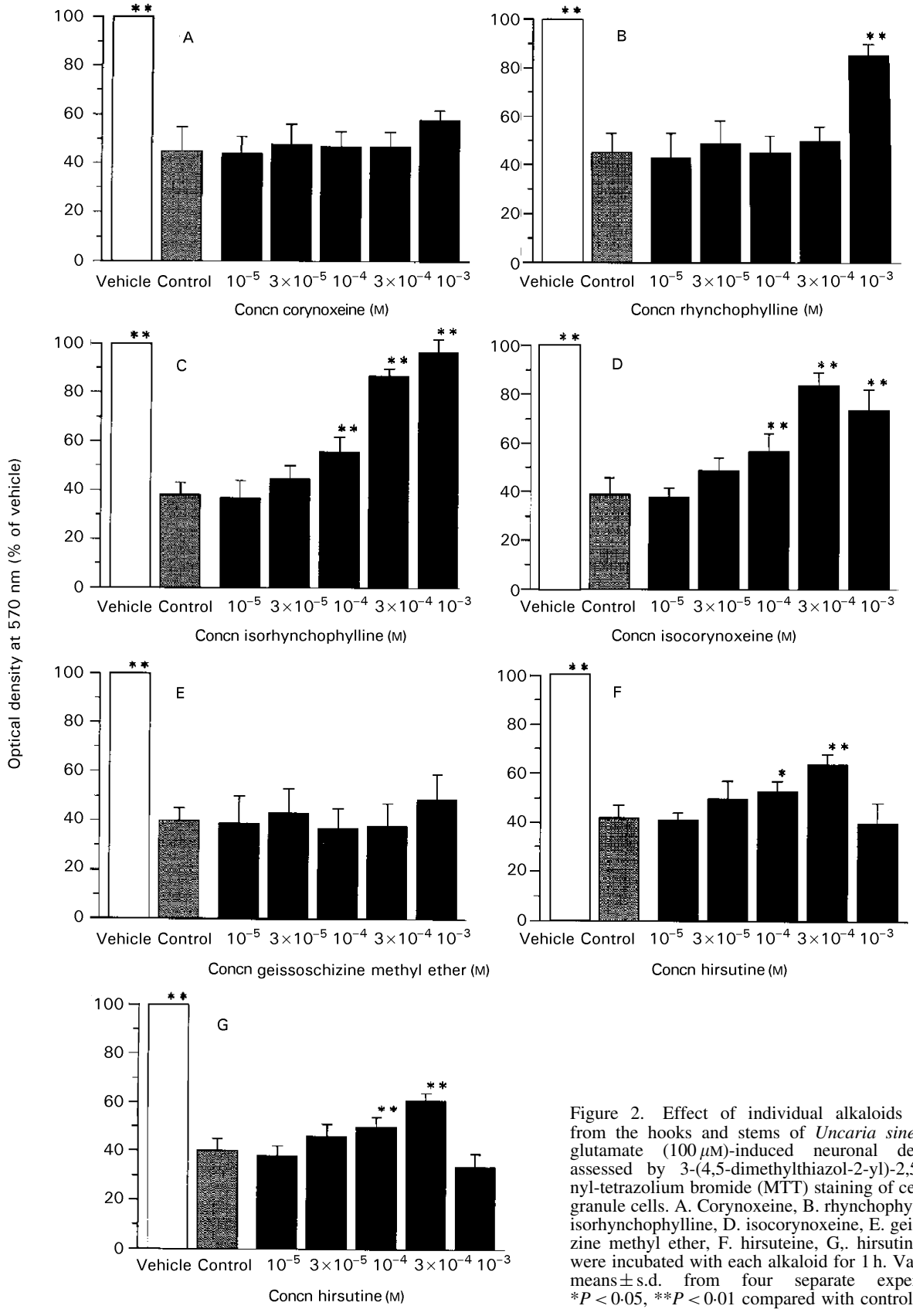

Figure 2. Effect of individual alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate (100 μM)-induced neuronal death as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) staining of cerebellar granule cells. A. Corynoxeine, B. rhynchophylline, C. isorhynchophylline, D. isocorynoxeine, E. geissoschizine methyl ether, F. hirsuteine, G,. hirsutine. Cells were incubated with each alkaloid for 1 h. Values are means ± s.d. from four separate experiments. $*P < 0.05$, $**P < 0.01$ compared with control.

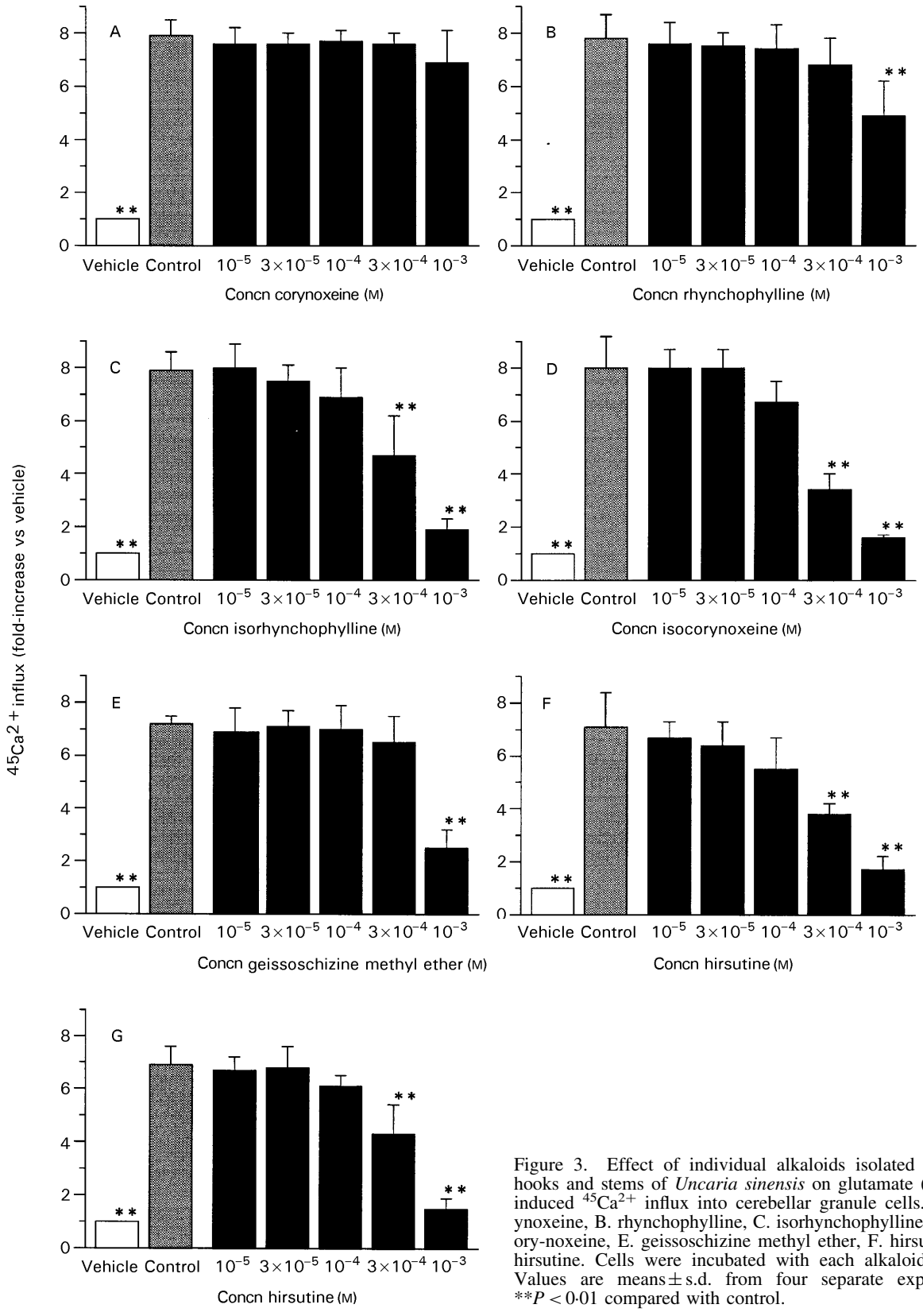

Figure 3. Effect of individual alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate (100 μM)-induced $^{45}Ca^{2+}$ influx into cerebellar granule cells. A. Corynoxeine, B. rhynchophylline, C. isorhynchophylline, D. isocory-noxeine, E. geissoschizine methyl ether, F. hirsuteine, G. hirsutine. Cells were incubated with each alkaloid for 1 h. Values are means $\pm$ s.d. from four separate experiments. $^{**}P < 0.01$ compared with control.

Table 1. Effect of individual alkaloids isolated from the hooks and stems of *Uncaria sinensis* on cell viability in cerebellar granule cells in the absence of glutamate as assessed by MTT assay.

Treatment	Cell viability					
	Vehicle	10^{-5} M	3×10^{-5} M	10^{-4} M	3×10^{-4} M	10^{-3} M
Corynoxeine	100·0 ± 0·0	103·5 ± 3·4	98·7 ± 5·5	99·6 ± 7·4	99·6 ± 11·4	97·6 ± 9·4
Rhynchophylline	100·0 ± 0·0	103·5 ± 5·4	103·4 ± 6·2	103·8 ± 6·7	103·7 ± 10·0	99·3 ± 8·6
Isorhynchophylline	100·0 ± 0·0	100·2 ± 2·7	104·7 ± 7·2	104·6 ± 6·1	102·5 ± 3·7	97·2 ± 4·7
Isocorynoxeine	100·0 ± 0·0	103·0 ± 3·1	99·9 ± 9·2	98·3 ± 4·2	98·7 ± 5·9	76·1 ± 9·6**
Geissoschizine methyl ether	100·0 ± 0·0	99·7 ± 4·5	101·0 ± 4·6	99·3 ± 3·6	91·0 ± 8·4	61·0 ± 8·1**
Hirsuteine	100·0 ± 0·0	102·3 ± 4·9	102·7 ± 2·9	102·5 ± 4·8	80·9 ± 4·7**	41·3 ± 3·7**
Hirsutine	100·0 ± 0·0	98·7 ± 2·0	99·5 ± 3·5	99·0 ± 1·3	78·2 ± 1·8**	35·7 ± 2·3**

Cells were incubated with each alkaloid for 1 h. Values are mean ± s.d. from four separate experiments. **$P < 0·01$ compared with vehicle.

nificantly reduced cell viability at 3×10^{-4} M and 10^{-3} M.

Discussion

Glutamate is the major excitatory neurotransmitter in the central nervous system and is important in learning and memory. Brain ischaemia leads to excessive release of glutamate, and neuronal death as a result of Ca^{2+} influx (Benveniste et al 1984; Rothman & Olney 1986). Neuronal cells have different types of ionotropic glutamate receptor, NMDA and non-NMDA receptors, which include the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor and the kainate receptor (Seeburg 1993). The NMDA receptor is characterized by high Ca^{2+} permeability; the other two receptors have very low Ca^{2+} permeability but are still involved in Ca^{2+} permeability as a result of membrane depolarization (Choi 1988). Further, neuronal cells have metabotropic glutamate receptors, voltage-dependent Ca^{2+} channels and Ca^{2+} storage (Henzi & MacDermott 1992; Seeburg 1993; Lnenicka & Hong 1997). Influx of Ca^{2+} into cells, induced by ischaemia and excessive release of glutamate, leads to cell death as a result of degradation of cytoskeletal protein, because of the enhanced activity of various Ca^{2+}-dependent enzymes, for example phospholipase C and protein kinase C (Choi 1988; Kristián & Siesjö 1998). It is also clear that there is some connection between the influx of Ca^{2+} into cells and the enhanced production of reactive oxygen species such as $O_2 \cdot^-$, H_2O_2, $\cdot OH$ and NO·. It has become equally clear that the combination of $O_2 \cdot^-$ and NO· (nitric oxide) can yield $ONOO^-$ (peroxynitrate), a metabolite with potentially devastating effects (Samdani et al 1997; Kristián & Siesjö 1998).

We have previously discovered, by means of a well-controlled and double-blind study, that a Japanese traditional (Kampo) medicine, Choto-san, was effective in the treatment of vascular dementia (Shimada et al 1994; Terasawa et al 1997). Choto-san is a Kampo formulation comprising 11 crude drugs (Terasawa 1993). The hooks and stems of *Uncaria sinensis*, the most important component of Choto-san, have been used in Japan for the treatment of many symptoms accompanied by hypertension and cerebrovascular disorders. This formulation has been proven to have hypotensive and vasodilative effects (Endo et al 1983; Kuramochi et al 1994), and improving effects on both scopolamine- and Δ^9-tetrahydrocannabinol-induced spatial cognitive disruption in rats (Egashira et al 1993). We recently demonstrated that an aqueous extract of the hooks and stems of *Uncaria sinensis* prevents glutamate-induced neuronal death in cultured cerebellar granule cells by inhibition of Ca^{2+} influx (Shimada et al 1998), although the active components were not identified.

It is known that the alkaloids of *Uncaria sinensis* have much pharmacological activity. Oxyindole alkaloids such as rhynchophylline, isorhynchophylline, corynoxeine and isocorynoxeine, and the indole alkaloid hirsutine have vasodilative and Ca^{2+}-channel-blocking activity in isolated rat thoracic aorta (Yamahara et al 1987; Yano et al 1991; Horie et al 1992). Hirsuteine, an indole alkaloid, non-competitively antagonizes nicotine-evoked dopamine release by blocking ion permeation through nicotinic receptor-channel complexes in rat pheochromocytoma PC12 cells (Watano et al 1993). Alkaloids obtained from *Uncaria sinensis*, for example geissoschizine methyl ether, corynantheine and dihydrocorynantheine, reduced specific [^{3}H]5-hydroxytryptamine binding to membrane preparations from rat brain (Kanatani et al 1985). Oral administration of geissoschizine methyl ether or hirsuteine dose-dependently inhibits glutamate-induced convulsion in mice (Mimaki et al 1997).

In this study, to determine the active components we evaluated the effects of alkaloids isolated from the hooks and stems of *Uncaria sinensis* on glutamate-induced neuronal death and Ca^{2+} influx in cultured cerebellar granule cells. By MTT assay we found that oxyindole alkaloids such as isorhynchophylline, isocorynoxeine, rhynchophylline, and the indole alkaloids hirsuteine and hirsutine significantly increased cell viability. Among these alkaloids, isorhynchophylline and isocorynoxeine had a stronger protective effect than the others. Rhynchophylline had a strong protective effect only at high concentrations. Hirsuteine and hirsutine were less active than isorhynchophylline and isocorynoxeine. These alkaloids have protective effects against glutamate-induced neuronal death and also inhibited glutamate-induced $^{45}Ca^{2+}$ influx into cells. Thus, the protective effects of these alkaloids against glutamate-induced neuronal death are thought to be because of their inhibition of Ca^{2+} influx. The protection against glutamate-induced cell death afforded by isocorynoxeine, hirsuteine and hirsutine followed the pattern of a bell-shaped curve, despite their dose-dependent inhibition of Ca^{2+} influx. This discrepancy is thought to arise because at high concentrations they are toxic to cultured cerebellar granule cells. The reason for the relatively weaker protective effects of hirsuteine or hirsutine against glutamate-induced neuronal death compared with isorhynchophylline and isocorynoxeine, despite their almost similar inhibitory effects on Ca^{2+} influx, is also considered to be related to the different cell toxicity of these alkaloids.

In conclusion, the results of this study suggest that the oxyindole alkaloids isorhynchophylline, isocorynoxeine and rhynchophylline and the indole alkaloids hirsuteine and hirsutine, isolated from the hooks and stems of *Uncaria sinensis*, are active components which provide protection against glutamate-induced neuronal death in cultured cerebellar granule cells by suppressing Ca^{2+} influx. Further studies will need to focus on the mechanisms of action of these alkaloids at the molecular receptor level.

Acknowledgements

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