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Effects of *Valeriana Officinalis* Extracts on [^{3}H]Flunitrazepam Binding, Synaptosomal [^{3}H]GABA Uptake, and Hippocampal [^{3}H]GABA Release*

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Extracts of Valeriana officinalis have been used in folkloric medicine for its sedative, hypnotic, tranquilizer and anticonvulsant effects, and may interact with γ-aminobutyric acid (GABA) and/or benzodiazepine sites. At low concentrations, valerian extracts enhance [^{3}H]flunitrazepam binding (EC_{50} 4.13×10^{-10} mg/ml). However, this increased [^{3}H]flunitrazepam binding is replaced by an inhibition at higher concentrations (IC_{50} of 4.82×10^{-1} mg/ml). These results are consistent with the presence of at least two different biological activities interacting with [^{3}H]flunitrazepam binding sites. Valerian extracts also potentiate K^+ or veratridine-stimulated release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA. Finally, inhibition of synaptosomal [^{3}H]GABA uptake by valerian extracts also displays a biphasic interaction with guvacine. The results confirm that valerian extracts have effects on $GABA_A$ receptors, but can also interact at other presynaptic components of GABAergic neurons.

KEY WORDS: *Valeriana;* [^{3}H]flunitrazepam; [^{3}H]GABA uptake; [^{3}H]GABA release.

INTRODUCTION

Extracts of *Valeriana officinalis* are used in folkloric medicine for its sedative, hypnotic, tranquilizer, and anticonvulsant effects (1,2). The major compounds present are the essential oils, classified as valepotriates and sesquiterpenes (3). The valepotriates, didrovaltrate, valtrate, acevaltrate, and isovaltrate, are the most important chemical group, although they are chemically unstable (4). Valepotriates potentiate hexobarbital anesthesia, suppress aggressivity, have anticonvulsant effects against pentylenetetrazol and strychnine seizures, increase thiopental sleeping time, reduce motility and have dose-dependent sedative effects (5–8). It has also been shown that a mixture of valepotriates is effective in attenuating benzodiazepine withdrawal symptoms in rats (8) suggesting that valerian extracts have some of the therapeutic effects of benzodiazepines.

The sesquiterpenes reduce locomotion, and increase pentobarbital and hexobarbital sleeping time of mice (9). Some sesquiterpenes, specially valerenic acid, influence serotonin and noradrenaline levels (9). Valerenic acid plays an important role in the depressive action and calming effects of valerian extract preparations

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suggesting that it has specific properties as a central nervous depressant (9). Moreover, studies with humans reveal that valerian extracts reduced latency to fall asleep as effectively as small doses of benzodiazepines (10).

These biological activities are consistent with a possible potentiation of GABAergic transmission. Santos et al., (11–14) report that aqueous valerian extracts inhibit the uptake and induce the release of [^{3}H]GABA. Similarly, valerian extract displaces [^{3}H]muscimol binding (14). Santos and co-workers (13,14) attribute the effects of valerian extracts to its endogenous GABA concentrations as determined by HPLC.

It is, however, doubtful that significant amounts of GABA from the extract cross the Blood Brain Barrier (BBB, 15) as to account for the in vivo effects of valerian extracts. Our working hypothesis is that there exists at least one component of the valerian extract with benzodiazepine-like effects that may account for its in vivo effects.

EXPERIMENTAL PROCEDURE

Chemicals. [2, 3-^{3}H]γ-aminobutyric acid (30 mCi/mmol) was obtained from American Radiolabelled Chemicals Inc. (St. Louis, MO). [Methyl-^{3}H]flunitrazepam (85 Ci/mmol) was obtained from DuPont NEN (Boston, MA or American Radiolabelled Chemicals Inc., St Louis, MO). Clonazepam was obtained from Roche Laboratories (Nutley, NJ). Guvacine was obtained from Research Biochemical International (Natick, MA). EcoLume was obtained from ICN Biomedicals Inc. (Irvine, CA). All other reagents were obtained from Sigma Chemical Company (St. Louis, MO). The AP 40 filters were purchased from Millipore Co. (Cidra, Puerto Rico). Rat cortical membranes were purchased from Analytical Biological Services, Inc. (Wilmington, DL).

Valerian Extract. Commercially available dried roots of *Valeriana officinalis* were obtained from the Arecibo (PuertoRico) marketplace from local growers. The roots were ground in a blender at low speed for five minutes with 95% ethanol (1:40 w/v). The mix was left to stand for 48 hours. The mixture was filtered through filter paper by gravity and dried using a rotary evaporator. The dried mixture of the crude ethanol valerian extract was reconstituted in 95% ethanol. Aliquots of the extract were stored at 0°C.

Animals. Swiss Webster mice (female, 6–8 weeks old) and Sprague Dawley rats (female, 2–4 months old) were obtained from the University of Puerto Rico, Río Piedras. The animals had free access to food and water and NIH guidelines were used always.

[^{3}H]Flunitrazepam Binding. Assays were done with rat cortical membranes obtained from Analytical Biological Services, Inc. The reaction was initiated by the addition of tissue (20–24 μg protein) to tubes containing 2 nM [^{3}H]flunitrazepam in a final volume of 400 μL of 50 mM Tris-HCl buffer, pH 7.4 (16). Non-specific binding was determined in the presence of 10^{-4} M clonazepam (17). Different concentrations of valerian extracts were added to tubes to do competition studies. All samples were incubated at 25°C for 40 minutes. The assay was stopped by rapid filtration of 100 μl of each sample in Millipore AP 40 prefilters in a Millipore manifold followed by two-2.5 ml cold buffer washes. Radioactivity of the dried filters was quantified in a Beckman LS 1800 counter with 5 ml of EcoLume scintillation cocktail. Results are shown as percentage of total binding.

[^{3}H]GABA Uptake Assays. Crude synaptosomes were prepared from Swiss Webster mice as described by Cordero et al., (18). The uptake was initiated by the addition of whole brain synaptosomes (30–80 μg protein in a final volume of 150 μL) to samples containing 100 μM aminooxyacetic acid (AOAA, an inhibitor of GABA degradation, in 150 μl of Earle's Balanced Salt solution (EBSS) and preincubated for 2 min at 37°C. After this, [^{3}H]GABA, (final concentration of 1 μM), was added to the reaction and incubated at 37°C for 10 min. Controls were incubated at 0–4°C. Parallel assays were done in the presence of different concentrations of valerian extract, nipecotic acid or guvacine. The reaction was stopped by rapid filtration of 100 μl of each samples through Millipore AP 40 prefilters in a Schleicher and Schuell Minifold I, followed by two-100 μl washes with cold buffer (18). Filters were dried and radioactivity quantified in a Beckman LS 1800 counter.

[^{3}H]GABA Release from Hippocampal Slices. Sprague Dawley rats (female, 2–4 months old) were lightly anesthetized with ether and sacrificed by decapitation. The brains were promptly removed and the hippocampi rapidly dissected out. Hippocampal slices (400 μm) were obtained using a Stoelting slicer. The slices were equilibrated for one hour at 37°C in the presence of 100 μM AOAA in a humidified atmosphere saturated with 95% O_2/5% CO_2. Controls were incubated at 60°C. After the equilibration period, the hippocampal slices were incubated with one μM [^{3}H]GABA at 37°C for 20 min. After this, two hippocampal slices were placed between filters and transferred to a Branded superfusion chamber. The tissue was superfused with oxygenated (95% O_2/5% CO_2.) EBSS at 37°C at a flow rate of 0.3 mL/min. After a 5 min washout period, the effluent was collected in 3 min fractions. The hippocampal slices were stimulated with high potassium for three minutes (K^+, 50mM) at 12 min, followed by oxygenated EBSS. The slices were stimulated again at 36th min with 50 mM K^+, veratridine (100 μM) or valerian extracts or combination thereof. The duration of the pulses was 3 min. The same procedure was done in EBSS without Ca^{+2}, 1 mM Ethylenediaminetetreaacetic acid (EDTA), a Ca^{+2} chelator. Radioactivity of each fraction was quantified with 3 mL of EcoLume scintillation cocktail, in a Beckman LS 1800 counter. All values were normalized to the value obtained from the baseline at 14 min (17).

Protein Determination. The protein concentration was measured as described by Bradford (19). Bovine Serum Albumin (BSA) was used as standard.

Statistical and Kinetics Analysis. The statistical significance of the data was examined using Student's *t*-test using INSTAT (v. 2.02, GraphPad). Effective concentration that enhances binding by 50%, (EC_{50}), the IC_{50} values and inhibition curves were obtained by nonlinear analysis using Inplot (v. 4.03 GraphPad).

RESULTS

Fig. 1 shows that very low valerian extract concentrations (10^{-14} – 10^{-11} mg/ml) do not affect [^{3}H]flunitrazepam binding to rat cortical membranes. On the other hand, 10^{-10} – 10^{-8} mg/ml valerian increases [^{3}H]flunitrazepam binding with an apparent EC_{50} of 4.13×10^{-10} mg/ml. The enhancement of [^{3}H]-flunitrazepam binding by valerian extracts starts to dis-

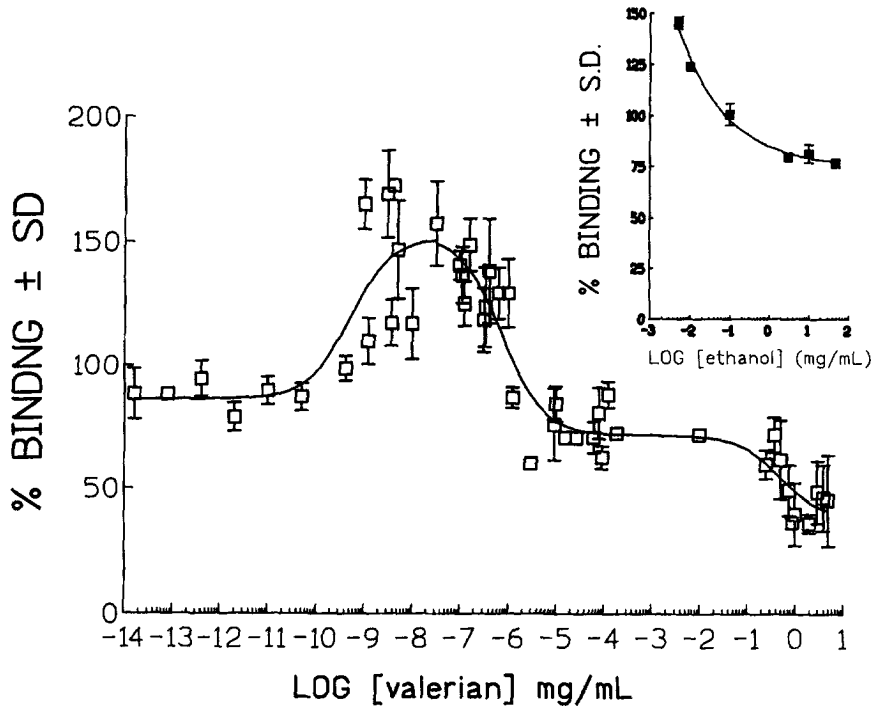

Fig. 1. Effect of crude valerian extract on [^{3}H]flunitrazepam (**Flu**) binding to rat cortical synaptic membranes. Valerian (10^{-10} – 10^{-8} mg/ml) potentiates Flu binding with an EC_{50} of 4.13×10^{-10} mg/ml. This effect is progressively lost starting at 10^{-7} mg/ml. Valerian, 10^{-6} to 10^{-2} mg/mL, reduces Flu binding to 70 %. Higher valerian concentrations, 0.5– 7.0 mg/ml cause a marked decrease in Flu binding with an apparent IC_{50} of 4.82×10^{-1} mg/ml. These effects are not due to the presence of ethanol in the extract as ethanol potentiates Flu binding (insert) at concentrations in which valerian inhibits it.

appear at 10^{-7} mg/ml and binding returns near to control levels by 10^{-6} mg/ml. Inhibition of [^{3}H]flunitrazepam binding is observed at higher extract concentrations (0.5–7.0 mg/mL), with an apparent IC_{50} of 4.82×10^{-1} mg/mL. These effects are not likely to be the result of the ethanol in the extract as ethanol potentiates [^{3}H]flunitrazepam binding at the same concentration (0.01 mg/ml) that valerian extract inhibits the binding (insert).

The inhibitory effects of two separate valerian extracts on synaptosomal [^{3}H]GABA uptake is shown in Fig. 2A. The insert shows that ethanol does not affect GABA uptake. Fig. 2B shows that nipecotic acid inhibits [^{3}H]GABA uptake at low concentrations. However, when mixed with varying concentrations of valerian extract, nipecotic acid does not affect the inhibition caused by the valerian extract on [^{3}H]GABA uptake. On the other hand, figure 2C shows the inhibition curves of [^{3}H]GABA uptake by guvacine, valerian and its mixture. Low valerian extract concentrations (2×10^{-4} – 1×10^{-2} mg/ml) combined with guvacine potentiate the inhibitory effects of guvacine alone. At higher valerian extract concentrations (0.1–3 mg/ml), the guvacine-valerian mixture inhibits 63% of the uptake.

Fig. 3A shows the effects of valerian extract on the release of [^{3}H]GABA from hippocampal slices by 50 mM K^+- or 100 μM veratridine-induced depolarization. Valerian extracts increase both K^+- and veratridine-stimulated [^{3}H]GABA release. Low amounts of valerian extract by itself, figure 3B, do not induce [^{3}H] GABA release. As the concentration increases, [^{3}H] GABA release increases suggesting a dose-dependent effect. This effect is significantly increased in the presence of depolarization.

We examined the possible effects of ethanol on K^+-stimulated [^{3}H]GABA release to rule out the possibility that the effects of valerian extract could be due to the ethanol content in the extract. Fig. 4 shows that ethanol has no effects on K^+-stimulated [^{3}H]GABA release. The insert shows that by itself, ethanol inhibits the release of radioactivity. These results suggest that the effects of valerian extract on K^+-stimulated release are not due to the presence of ethanol in the extract.

Valerian extracts may contain GABA and may induce its own release by a homoexchange mechanism

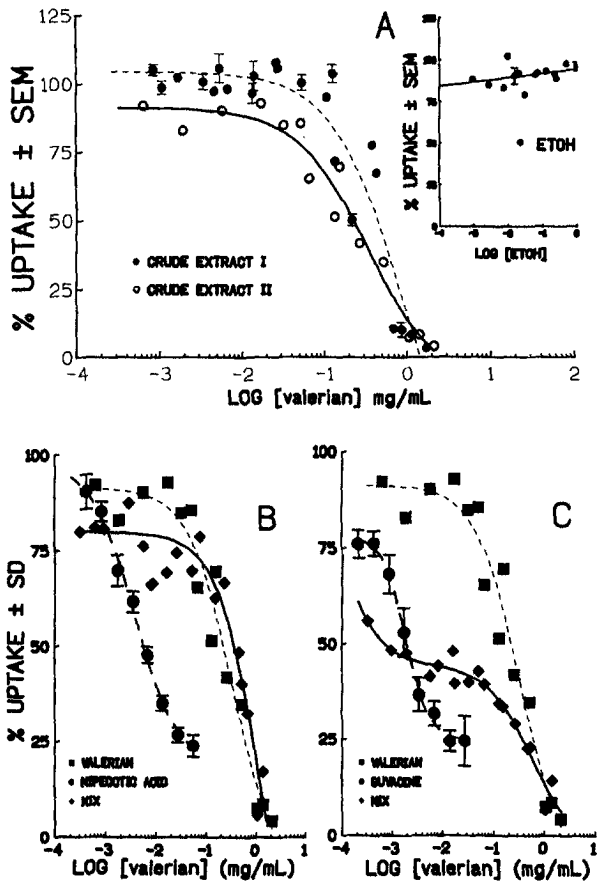

Fig. 2. (**A**) Two different valerian extracts inhibit synaptosomal [^{3}H]GABA uptake with an apparent IC_{50} of $9.75 \times 10^{-1} \pm 0.21$ mg/ml for extract I and $3.31 \times 10^{-1} \pm 0.13$ mg/mL for extract II. The insert shows that ethanol has no noticeable effects on [^{3}H]GABA uptake; (**B**) Nipecotic acid inhibits [^{3}H]GABA uptake with an apparent IC_{50} of $3.7 \times 10^{-3} \pm 0.06$ mg/ml. A mixture of different valerian concentrations with 6.5×10^{-4} nipecotic acid results in an inhibition curve indistinguishable from that of valerian alone; (**C**) Increasing amounts of valerian in the presence of 2.94×10^{-3} mg/ml of guvacine results in a biphasic inhibition curve with IC_{50}' of $5.4 \times 10^{-3} \pm 2.3$ mg/mL for the first site and $5.6 \times 10^{-1} \pm 0.11$ mg/ml for the second site.

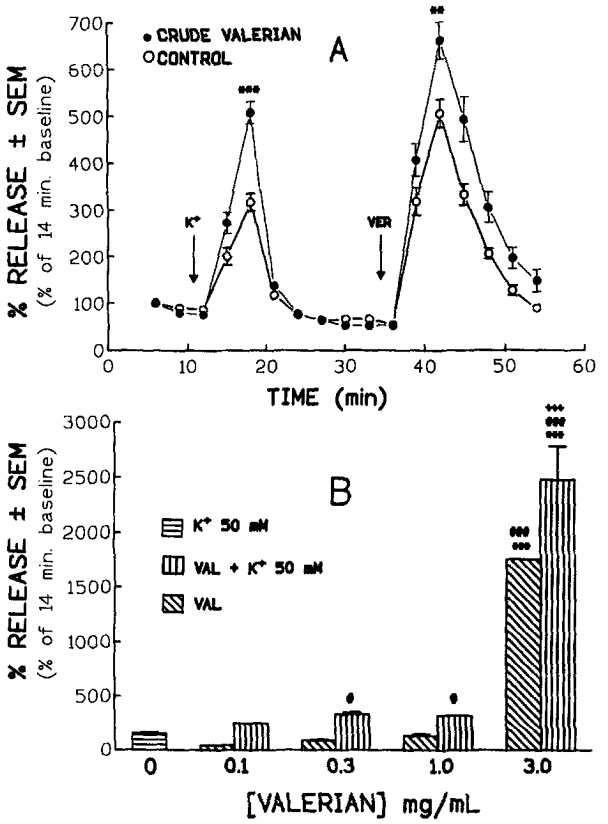

Fig. 3. (**A**) Valerian potentiates 50 mM K^+ (first arrow) or 100 μM veratridine (second arrow)-evoked release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA. All values are normalized to the 14 min baseline; ***$p < 0.001$, **$p < 0.01$ vs. control (**B**) The potentiation of K^+-evoked release of radioactivity by valerian increases with increases in the valerian concentration. ***$p < 0.001$ vs valerian extract alone.

(12–14). We were unable to detect any endogenous GABA in our valerian extracts using HPLC. (Data not shown). Fig. 5A shows that 100 μM GABA, (second pulse) induces the release of radioactivity in the absence of depolarization. On the other hand, Fig. 5B shows the effects of reducing extracellular Ca^{+2} (1 mM EDTA) on the release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA. High K^+ (50 mM) stimulates Ca^{+2}-dependent release from preloaded hippocampal slices, while having essentially no effect in the absence of external Ca^{+2}. Valerian markedly potentiates the K^+-stimulated release of [^{3}H]GABA in the presence of external Ca^{+2}. In reduced Ca^{+2}, the potentiation by valerian of K^+-stimulated [^{3}H]GABA release is not observed. Thus, the effect of valerian extract on K^+-stimulated [^{3}H]GABA release is dependent on external Ca^{+2}.

DISCUSSION

$GABA_A$ receptors are involved in the action of many sedatives-hypnotic and anticonvulsants. Many in vivo effects of valerian are consistent with activation of $GABA_A$ receptors. Cavadas and collaborators (14) proposed interactions of valerian extract with $GABA_A$ receptors based on displacement of [^{3}H]muscimol binding. These authors attribute the valerian effects to the endogenous GABA content of the extract. However, GABA could not be detected in our extracts and it is unlikely that the in vivo effects of valerian are due to the endogenous GABA as it does not readily cross the blood brain barrier (15).

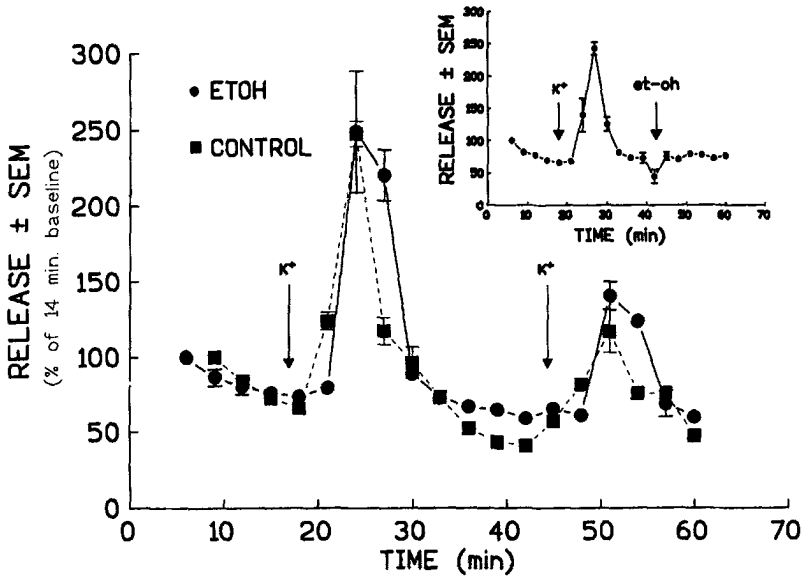

Fig. 4. Ethanol does not alter the 50 mM K^+-evoked release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA. The insert shows that ethanol by itself, is not capable of releasing [^{3}H]GABA.

The results of our [^{3}H]flunitrazepam binding experiments confirm the hypothesis of an interaction with the $GABA_A$ receptor complex. Our binding studies show that low concentrations of crude valerian extract potentiate benzodiazepine binding, but inhibit it at high concentrations. These biological activities are consistent with the presence of at least two active compounds, of different potencies that can differentially modify [^{3}H] flunitrazepam binding. At low valerian concentrations, the amounts of the inhibitory compound would be so low as to be without any noticeable effect allowing the activating compound to potentiate [^{3}H]flunitrazepam binding. Attempts to purify [^{3}H]flunitrazepam binding activities have not been successful as the activity is labile, (unpublished results). On the other hand, the inhibitory activity is stable and has been previously observed by Medina and colleagues (Medina, J., personal communication, 1997). Furthermore, there is considerable variability among different commercial valerian preparations (unpublished results). Nonetheless, potentiation of benzodiazepine binding is clearly consistent with the reported in vivo effects (7–10).

Aqueous valerian extract inhibits the transport of GABA (11). High valerian concentrations inhibit synaptosomal [^{3}H]GABA uptake in our experiments. When combined with nipecotic acid, valerian could mask the effects of nipecotic acid. This observation suggests that at, least a component of the valerian extract, competes with nipecotic acid. On the other hand, a mixture of low valerian extract concentrations and guvacine is more effective than either of the two compounds alone. At higher valerian concentrations, the effects of guvacine are reduced. Saturation kinetic analysis reveals a complex interaction when either nipecotic acid or guvacine is co-incubated in the presence of valerian. Potentiation of the effects of guvacine deserves further study as a possible adjuvant in the treatment of conditions in which GABAergic transmission should be enhanced.

Crude valerian extract causes a dose-dependent release of [^{3}H]GABA in the absence of depolarization and potentiates veratridine- or K^+-stimulated [^{3}H]GABA release. However, the absence of external Ca^{+2} does not alter the K^+-stimulated release of [^{3}H]GABA but abolishes the stimulatory effect of valerian extracts. Santos et al (12,13) report that valerian extract increases GABA release in a Ca^{+2}-independent way through the reversal of the transport system (13). This effect is attributed to the content of GABA in the extract (13) and markedly disagrees with our observations. The possibility that the effects of the valerian extract are mediated by presynaptic $GABA_B$ subtypes of receptors cannot be entirely ruled out.

In summary, at very low concentrations, valerian extracts increase [^{3}H]flunitrazepam binding. Furthermore, at higher valerian concentrations, the potentiation effect is lost consistent with the presence of at least two different active components in the extracts. It is

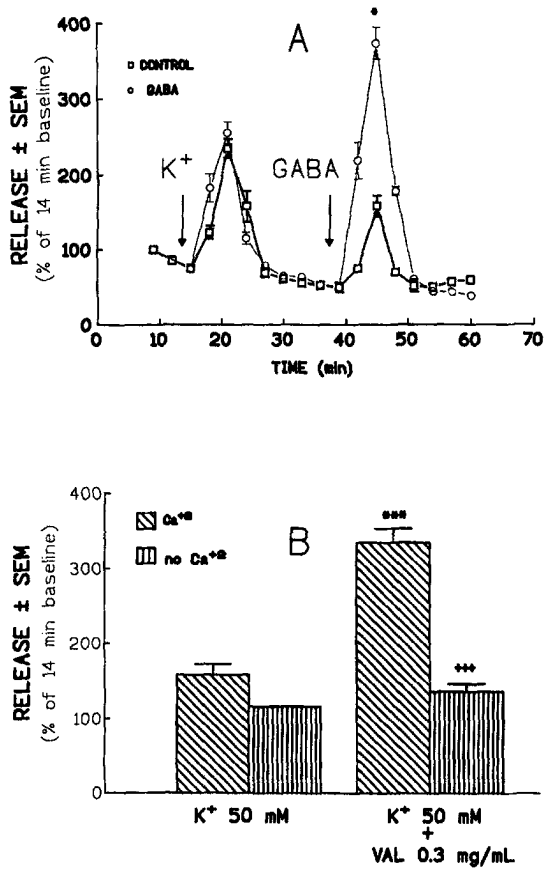

Fig. 5. (**A**) Exogenous GABA (100 μM, second arrow) is capable of stimulating the release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA; (**B**) Effects of extracellular Ca^{+2} on the release of radioactivity from hippocampal slices preloaded with [^{3}H]GABA. Reduction of extracellular Ca^{+2} prevents the release of K^+-stimulated release of radioactivity and markedly attenuates the potentiating effect of valerian on K^+-stimulated release. All values correspond to a second K^+ pulse and are normalized to the 14 min baseline. ***$p < 0.001$ vs. 50 mM K^+; $^{+++}p < 0.001$ vs. K^+ + Val (0.3 mg/ml).

likely that this combination of effects is responsible for the in vivo anxiolytic and sedative effects of valerian. In addition, valerian extracts potentiate veratridine- or K^+-stimulated, Ca^{+2}-dependent [^{3}H]GABA release and inhibit [^{3}H]GABA uptake.

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REFERENCES

1. Petkov, V., Manolov, P., Marekov, N. L., Popov, S. S., and Khandzhieva, N. B. 1974. Pharmacological studies on a mixture of valepotriates isolated from *Valeriana officinalis*. Dokl. Bolg. Akad. Nauk. 27:1007–1010.
2. Cott, J. 1995. Medicinal plants and dietary supplements: sources for innovative treatments of adjuncts. Psychopharmacol. Bull. 31:137–137
3. Granicher, F., Christen, P., and Kapetanidis, I. 1995. Essential oils from normal and hairy roots of *Valeriana officinalis* var *sambucifolia*. Phytochemistry 40:1421–1424.
4. Haas, L. F. 1996. Neurological stamp *Valeriana officinalis*. J. Neurol. Neurosurg. Psychiatry 60:255.
5. Petkov, V., and Manolov, P. 1975. To the pharmacology of iridoids. Agressologie 16:25–30.
6. Leuschner, J., Muller, J., and Rudmann, M. 1993. Characterization of the central nervous depressant activity of a commercially available valerian root extract. Arzneim. Forsch./Drug. Res. 43:638–641.
7. Andreatini, R., and Leite, J. R. 1994. Effect of valepotriates on the behavior of rats in the elevated plus-maze during diazepan withdrawal. Eur. J. Pharmacol. 260:233–235.
8. Hendriks, H., Bos, R., Allersma, D. P., Malingre, T. H. M., and Koster, A. S. J. 1981. Pharmacological screening of valerenal and some other components of essential oil of *Valeriana officinalis*. Planta Med. 42:62–68.
9. Hendriks, H., Bos, R., Woerdenbag, H. J., and A. Sj. Koster, 1985. Central nervous depressant activity of valerenic acid in the mouse. Planta Med. 1:28–31.
10. Leathwood, P. D., and Chauffard, F. 1985. Aqueous extract of valerian reduces latency to fall asleep in man. Planta Med. 2:144–148.
11. Santos, M. S., Ferreira, F., Cunha, A. P., Carvalho, A. P., and Macedo, T. 1994. Aqueous extract of valerian influences the transport of GABA in synaptosomes. Planta Med. 60:278–279.
12. Santos, M. S., Ferreira, F., Cunha, A. P., Carvalho, A. P., Ribeiro, C. F., and Macedo, T. 1994. Synaptosomal GABA release as influenced by valerian root extract -involvement of the GABA carrier. Arch. Int. Pharmacodyn. 327:220–231.
13. Santos, M. S., Ferreira, F., Faro, C., Pires, E., Carvalho, A. P., and A. P., Cunha, 1994. The amount of GABA present in aqueous extracts of valerian is sufficient to account for [^{3}H]GABA release in synaptosomes. Planta Med. 60:475–476.
14. Cavadas, C., Araujo, I., Cotrim, M. D., Amaral, T., Cunha, A. P., Macedo, T., and Ribeiro, C. F. 1995. In vitro study on the interaction of *Valeriana Officinalis* L. extracts and their amino acids on $GABA_A$ receptor in rat brain. Arzneim-Forsch / Drug Res. 45: 753–755.
15. Rosenstein, J. M. 1996. Permeability of the blood-brain barrier to protein and [^{3}H]GABA in intraparenchymal fetal CNS tissue grafts. Exp. Neurol. 142:66–79.
16. Chiu, T. M., Dryden, D. M., and H. C. Rosenberg., 1982. Kinetics of [^{3}H]flunitrazepam binding to membrane bound benzodiazepine receptors. Mol. Pharmacol. 21:57–65.
17. Ortiz, J. G., Negron, A., Thomas, A., Heimer, H., Moreira, J. A., Cordero, M. L., Aranda, J., and Bruno, M. S. 1991. GABAergic neurotransmission in the C57BL/10 sps/sps mouse mutant: a model of absence of seizures. Exp. Neurol. 113:338–343.
18. Cordero, M. L., Ortiz, J., Santiago, G., Negrón, A., and Moreira, J. A. 1994. Altered GABAergic and glutamatergic transmission in audiogenic seizure-susceptible mice. Mol. Neurobiol. 9:253–258.
19. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
20. Lindah, O., and Lindwall, L. 1989. Double blind study of a valerian preparation. Pharmacol. Biochem. Behavior 32: 1065–1066.