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Different Effects on [^{3}H]Noradrenaline Uptake of the *Aconitum* Alkaloids Aconitine, 3-Acetylaconitine, Lappaconitine, and *N*-Desacetyllappaconitine in Rat Hippocampus

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ABSTRACT. The effect of the *Aconitum* alkaloids aconitine, 3-acetylaconitine, lappaconitine, and *N*-desacetyllappaconitine to inhibit [^{3}H]noradrenaline uptake was investigated in rat hippocampal synaptosomes. Aconitine and 3-acetylaconitine, which are known to activate sodium channels, had comparable inhibitory potencies and yielded K_i (inhibitor constant) values of 230 ± 66 nM and 316 ± 96 nM, respectively. In contrast, lappaconitine and *N*-desacetyllappaconitine failed to inhibit [^{3}H]noradrenaline uptake. When either lappaconitine or *N*-desacetyllappaconitine was applied in combination with aconitine, [^{3}H]noradrenaline uptake was not affected. The sodium channel blocker tetrodotoxin enhanced [^{3}H]noradrenaline uptake, whereas uptake was completely blocked in sodium-free incubation medium. The inhibitory action of aconitine and 3-acetylaconitine on [^{3}H]noradrenaline uptake was blocked by addition of tetrodotoxin. Patch clamp studies performed on cultured rat hippocampal neurons revealed an inhibitory action of lappaconitine and *N*-desacetyllappaconitine on whole cell sodium currents. It is concluded that the blockade of [^{3}H]noradrenaline uptake evoked by aconitine and 3-acetylaconitine is mediated indirectly by an increased sodium concentration in the synaptosomes. BIOCHEM PHARMACOL **55**;6:883–888, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Aconitum* alkaloids; [^{3}H]noradrenaline uptake; synaptosomes; hippocampus; sodium current; electrophysiology

Plant extracts of different *Aconitum* species are employed in traditional Chinese and Japanese medicine as analgesics, antirheumatics and treatment of neurological disorders. The pharmacological effects are reported to be due to diterpenoid alkaloids [1, 2]. Among these, the alkaloids aconitine, 3-acetylaconitine, lappaconitine, and *N*-desacetyllappaconitine (Fig. 1) have been reported to have analgesic properties *in vivo* [1, 3–5] and inhibitory properties on neuronal activity in rat hippocampal slices *in vitro* [6–8]. 3-Acetylaconitine and lappaconitine have been proved to be centrally acting analgesic drugs [9] without affinity for opiod receptors [5, 10]. Actions other than opioid mechanisms may therefore contribute to their analgesic efficacy. This assumption is supported by the observation that in some experiments the antinociceptive action of lappaconitine administered intracerebroventricularly to mice was reduced by pretreatment with antagonists of noradrenergic receptors, indicating an interaction of the alkaloids with the endogeneous central noradrenergic pathways [11, 12].

Since aconitine and 3-acetylaconitine are known to activate voltage-dependent sodium channels [6, 13], the present study was designed to investigate whether the action of the alkaloids on the sodium channel is associated with a modulation of the noradrenergic system. To this end, we have studied the effects of aconitine, 3-acetylaconitine, lappaconitine, and *N*-desacetyllappaconitine on [^{3}H]noradrenaline uptake into rat hippocampal synaptosomes as well as on the voltage-dependent sodium current in rat hippocampal neurons. The hippocampus is known to receive noradrenergic projections from the locus coeruleus. Furthermore, previous electrophysiological studies with these alkaloids were carried out with hippocampal slices [6–8]. Hence, we investigated the action of the alkaloids by biochemical and electrophysiological methods in the same tissue.

MATERIALS AND METHODS

[^{3}H]Noradrenaline Uptake Studies

Male Wistar rats (150–200 g) were decapitated and the hippocampus was dissected. A crude synaptosomal (P_2) fraction was prepared as described by others [14]. Briefly,

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† *Abbreviations:* TTX, tetrodotoxin; K_i, inhibitor constants.

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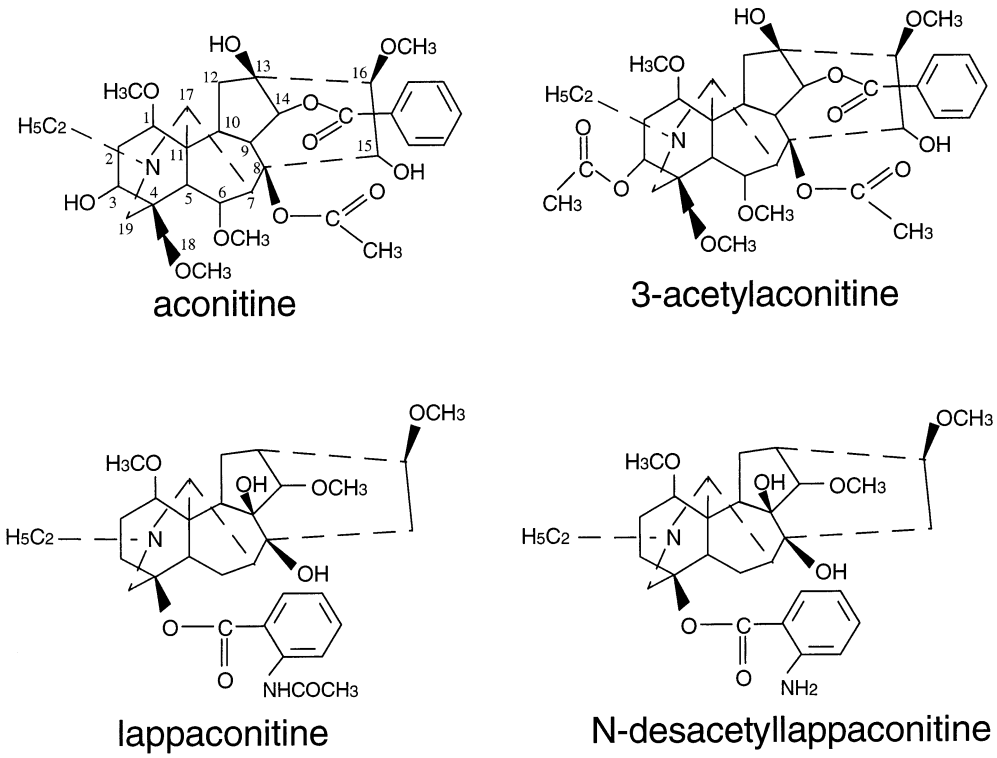

FIG. 1. Chemical structure of the *Aconitum* alkaloids aconitine, 3-acetylaconitine, lappaconitine and N-desacetyllappaconitine.

the tissue was homogenized in 15 mL of ice-cold solution containing: sucrose (320 mM), EDTA (0.5 mM), bovine serum albumin (1 mg/mL), *N*-Tris(hydroxymethyl)-methyl-2-aminoethan sulfonic acid (5 mM), pH 7.4, in a glass potter with a teflon pestle (7 strokes, 800 rpm). The homogenate was centrifuged at 4° for 10 min at 1,000 *g*. The resulting supernatant was then centrifuged at 28,000 *g* for 20 min and the supernatant was discarded. The pellet (P_2) was gently resuspended in incubation buffer containing (in mM): glucose 10, HEPES 10, NaCl 150, KCl 6.2, $MgSO_4$ 1.2, NaH_2PO_4 1.2, pargyline 0.001, and ascorbic acid 0.5, pH 7.4. In some experiments, a modified incubation buffer was used which was nominally Na^+-free. In this buffer, NaCl was replaced by choline chloride and 100 μM ouabain was added to block the Na^+/K^+-ATPase. Protein content was determined by the method of Bradford [15] using bovine serum albumin as a standard.

Monoamine uptake assays were performed according to the method of Bolden-Watson and Richelson, with modifications [16]. Briefly, the synaptosomal suspension (1.0 mg protein/mL) was incubated at 37° in a total volume of 0.5 mL containing varying concentrations of desipramine as reference drug or the four alkaloids. After a 10 min preincubation, the uptake was initiated by the addition of 30 nM [^{3}H]noradrenaline and was terminated by a rapid cooling of samples on ice for 5 min. Afterwards, the synaptosomes were collected on a glass-fiber filter (Whatman GF/C), washed four times with 2.5 mL incubation medium and counted by liquid scintillation spectroscopy (LS6000 TA, Beckmann Instruments). To determine the passive diffusion of [^{3}H]noradrenaline, we subtracted the uptake on ice from that at 37°.

Control experiments with tetrodotoxin (TTX, 0.5 μM) were performed in the same manner to investigate a dependence of [^{3}H]noradrenaline uptake on sodium channel activity.

Patch-Clamp Experiments

For patch-clamp recordings of whole-cell sodium currents, rat hippocampal pyramidal cells which had been held in culture for 10–14 days (obtained from S. Himmelseher, Experimental Anesthesiology, University of Ulm) were employed. The external solution consisted of (in mM): NaCl 150, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, HEPES-Na 5, HEPES 5, and glucose 10, pH 7.4. Experiments were performed using an Axopatch 200 A-amplifier (Axon Instruments) and an ITM-2 phase-contrast microscope (Olympus). Patch pipette electrodes (2 −4 MΩ) were filled with (in mM) artificial cerebro-spinal fluid (ACSF) 125, Tris-Cl 10, HEPES 10, and EGTA 10, pH 7.2. Internal Cs^+ (109.4 mM) was necessary for suppression of voltage-dependent potassium currents in order to allow stable recordings of sodium current. To avoid space clamp, only medium-sized cells with processes shorter than the diameter of the cell body were employed for patch-clamp recordings, because they are particularly advantageous for space-clamp conditions [17, 18]. Voltage commands and data acquisition were performed with a TIDA system (HEKA Electronic). Whole-cell currents were recorded at room temperature

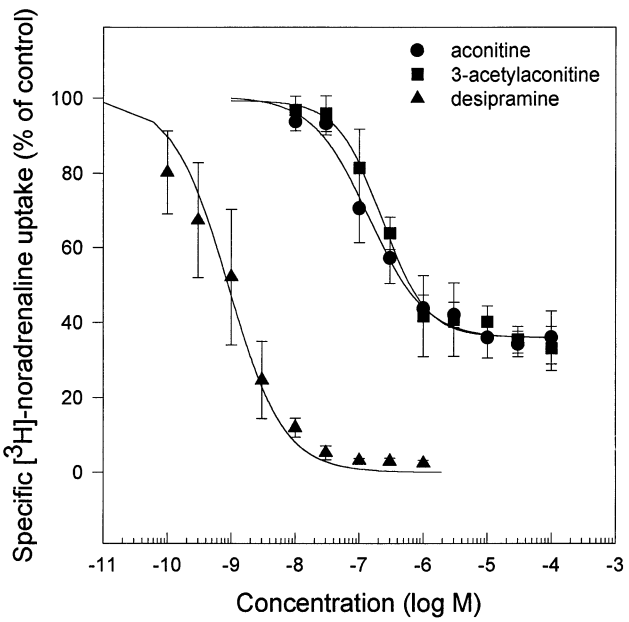

FIG. 2. Effect of increasing concentrations of the alkaloids aconitine and 3-acetylaconitine in comparison with desipramine on [^{3}H]noradrenaline uptake into rat hippocampus synaptosomes. Data points represent mean values ± SD of four independent experiments each performed in triplicate. Inhibition of [^{3}H]noradrenaline uptake was significant for a concentration of each alkaloid as low as 100 nM and desipramine as low as 0.1 nM ($p < 0.05$).

(20–22°), filtered at 5 kHz with a four-pole Bessel filter, and sampled at 10 kHz. The seal resistances ranged from 10 to 20 GΩ. The series resistance of the whole-cell patches ranged between 5 and 15 MΩ and 0.3 and 0.8 MΩ when compensated by 75–85%. The voltage drop caused by the compensated series resistance never exceeded 4 mV in these experiments. Drugs were added to the external solution. The flow rate of the perfusion medium was adjusted to 1.5 mL/min.

Drugs

Aconitine (Sigma), 3-acetylaconitine (Shanghai Institute of Materia Medica), lappaconitine (Latoxan), and *N*-desacetyllappaconitine (Latoxan, France) were dissolved in DMSO to give stock solutions of 100 mM. The final concentration of DMSO in the assays amounted to 0.1% and did not affect any of the measured parameters. Desipramine HCl, TTX and ouabain (Sigma) were dissolved in incubation buffer. L-[7,8-^{3}H]noradrenaline (37.0 Ci/mmol) was obtained from Amersham Life Science.

Calculations and Data Analysis

Specific uptake was calculated by subtracting the uptake at 0° from that at 37°. K_1 values were calculated according to Cheng and Prussoff [19]. Results represent mean values ± SD of at least 3–6 independent experiments, each performed in triplicate. Statistical differences between control values and those obtained in presence of the test drug were determined by Student's *t* test. Significance was set at $p < 0.05$.

RESULTS

Preliminary uptake experiments with incubation times up to 15 min showed linear uptake of [^{3}H]noradrenaline in the crude synaptosomal preparation for up to 15 min (not shown). Therefore, the chosen incubation period of 10 min was within the linear range and in accordance with Petterson [14]. Synaptosomes were incubated with [^{3}H]noradrenaline either in the presence or absence of desipramine, aconitine, 3-acetylaconitine, lappaconitine, or *N*-desacetyllappaconitine. [^{3}H]Noradrenaline uptake was inhibited by desipramine (10^{-10} to 10^{-7} M) as well as by aconitine and 3-acetylaconitine (10^{-8} to 10^{-4} M) in a concentration-dependent manner (Fig. 2). In contrast, lappaconitine and *N*-desacetyllappaconitine (10^{-8} to 10^{-4} M) failed to inhibit [^{3}H]noradrenaline uptake (Table 1). Desipramine, a specific noradrenaline reuptake blocker, inhibited [^{3}H]noradrenaline uptake with a K_i of 0.78 nM, which is in line with previous findings [20]. The K_i values of aconitine and 3-acetylaconitine were not significantly different, amounting to 230 nM and 316 nM, respectively (Table 1). At maximal concentrations of these two alkaloids (30 μM), [^{3}H]noradrenaline uptake was inhibited by up to 56–66% of control (Table 1).

To investigate whether the blocking effect of aconitine on the [^{3}H]noradrenaline uptake is antagonized by lappaconitine and *N*-desacetyllappaconitine, experiments were performed with a combination of 300 nM aconitine and 10 *N*-desacetyllappaconitine. When aconitine was applied alone to the synaptosomes, it reduced [^{3}H]noradrenaline uptake by 57.2 ± 6.8% ($n = 4$, $p < 0.001$). However, when it was applied together with either lappaconitine (n ± 9.7%) or *N*-desacetyllappaconitine (n =

TABLE 1. Inhibitor constants (K_i) and maximal inhibition in % of control for specific [^{3}H]noradrenaline uptake into rat hippocampal synaptosomes

	Desipramine	Aconitrine	3-Acetylaconitrine	Lappaconitrine	N-Desacetyllapperconitrine
K_i(nM)	0.78 ± 0.15	230 ± 66	316 ± 96	n.d.	n.d.
Maximal	98.8 ± 0.7**	65.7 ± 3.5*	56.5 ± 7.8*	4.1 ± 6.4	4.3 ± 4.9

K_i values were calculated with the Cheng-Prusoff equation. Maximal inhibition was determined at a concentration of 30 μM for each alkaloid and at a concentration of 100 nM for desipramine. Data are mean values ± SD of at least three to six experiments (n.d, not determined; * $p < 0.01$ and ** $p < 0.001$ as compared to respective control uptake).

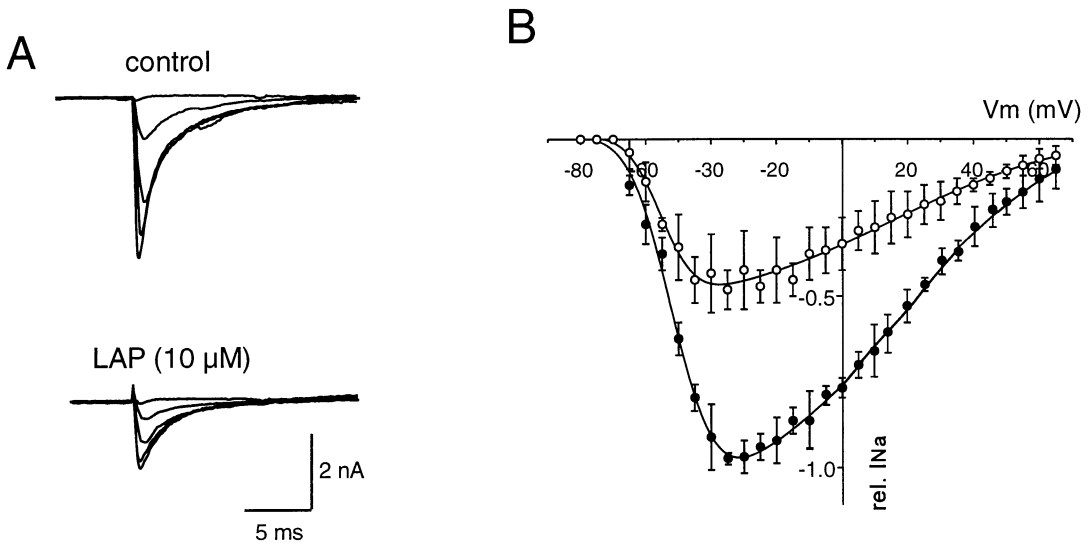

FIG. 3. Effect of lappaconitine (LAP, 10 µM) on voltage-dependent sodium currents in hippocampal neurons. (A) Original traces of currents evoked by voltage steps from −80 mV at control and in presence of lappaconitine. (B) Current-voltage relationship of peak sodium currents in the absence (●) and presence (○) of lappaconitine (10 µM). Peak sodium currents are plotted as a function of membrane potential (V_m). Data points represent the means ± SD of five experiments. Cells were maintained at a holding potential of −90 mV.

6, 97.2 ± 7.2%), [^{3}H]noradrenaline uptake was not significantly altered.

Both aconitine and 3-acetylaconitine have been reported to interact with the voltage-dependent sodium channel and to shift the current-voltage relationship in the hyperpolarized direction such that sodium currents were already activated at the resting potential [6, 13]. To examine if the [^{3}H]noradrenaline uptake is influenced by sodium channel activity, a further series of experiments was carried out with the sodium channel blocker TTX (0.5 µM). When TTX was added to the synaptosomes, [^{3}H]noradrenaline uptake was enhanced to 168.0 ± 14.1% of control ($n = 6$, $p < 0.001$). There was no significant difference when TTX was applied in combination with either 1 µM aconitine (166.7 ± 8.2%, n = 6) or 1 µM 3-acetylaconitine (164.6 ± 16.6%, $n = 6$).

A further set of experiments ($n = 24$) was performed to investigate a dependence of [^{3}H]noradrenaline uptake on the ionic environment. In these experiments, synaptosomes were incubated in Na^+-free buffer containing 100 µM ouabain. In absence of extracellular Na^+, [^{3}H]noradrenaline uptake was reduced to 5.87 ± 3.2% ($n = 7$) and was not significantly changed when aconitine (1 µM) was added (8.49 ± 2.1%, $n = 6$).

To investigate whether the structurally related alkaloids lappaconitine and *N*-desacetyllappaconitine also interact with the sodium channel, patch-clamp recordings in whole-cell configuration were performed at cultured hippocampal pyramidal cells. Inward currents were elicited by depolarizing voltage steps from a holding potential of −90 mV to potentials between −80 and +55 mV. They had a peak amplitude of 3.24 ± 0.7 nA ($n = 5$) and were blocked by 0.5 µM tetrodotoxin. Both lappaconitine and *N*-desacetyllappaconitine failed to affect the sodium current at concentrations of 1 µM. After addition of lappaconitine (10 µM) to the perfusion medium, peak amplitude of the sodium currents was reduced (Fig. 3). There was no difference in the action of lappaconitine and *N*-desacetyllappaconitine (10 µM; n = 3). Due to solubility problems, higher concentrations of these alkaloids could not be tested.

DISCUSSION

The major finding of the present study is that the tested alkaloids interact in a different manner in both [^{3}H]noradrenaline uptake and the voltage-dependent sodium channel. Our results demonstrate an inhibitory effect of aconitine and 3-acetylaconitine on [^{3}H]noradrenaline uptake. These two alkaloids inhibited [^{3}H]noradrenaline uptake in a concentration-dependent manner. In contrast, there was no inhibitory effect of lappaconitine and *N*-desacetyllappaconitine on [^{3}H]noradrenaline uptake. Although an inhibition of noradrenaline uptake is thought to be one mode of action of centrally acting analgesics [21], it cannot be excluded that lappaconitine and *N*-desacetyllappaconitine stimulate the noradrenergic system in another way, e.g., by an enhancement of noradrenaline release. The different modifications of the voltage-dependent sodium channel by aconitine and 3-acetylaconitine which activate Na^+ channels [6, 13] on the one hand and lappaconitine and *N*-desacetyllappaconitine, which inhibit Na^+ currents (Fig. 3) on the other, may also explain the present finding that the latter two alkaloids antagonized the ability of aconitine to inhibit noradrenaline uptake. Aconitine is known to bind at the neurotoxin binding site 2 of the voltage-dependent Na^+ channel [13]. Due to the structural rela-

tionship of the *Aconitum* alkaloids investigated in the present study, the blockage of the aconitine-induced inhibition of noradrenaline uptake by lappaconitine and *N*-desacetyllappaconitine might reflect a competitive antagonism at the same binding site at the Na^+ channel protein.

Neurotransmitter transporters are influenced not only by the intracellular concentration of neurotransmitters but also by extracellular ions, which may be in part due to the fact that the transporter proteins share structural and functional analogies with ion channels [22]. The energy for re-uptake of noradrenaline, which is often against the concentration gradient, is derived from the cotransport of Na^+ [22]. An increase in intracellular Na^+ concentration and, in consequence, a decrease in the electrochemical gradient diminishes the uptake of noradrenaline. It is known that aconitine as well as 3-acetylaconitine activate sodium channels already at resting membrane potential [6, 13]. Due to the increase in intracellular Na^+ concentration evoked by aconitine and 3-acetylaconitine, the Na^+-gradient and consequently the noradrenaline uptake is disturbed. A dependence of the noradrenaline transporter on sodium channel activity is supported by the present findings, which demonstrate an enhancing effect of the sodium channel blocker TTX on [^{3}H]noradrenaline uptake. Moreover, addition of TTX in combination with aconitine and 3-acetylaconitine blocked the inhibitory action of these alkaloids on noradrenaline uptake. This result can be explained by the fact that TTX blocks sodium channels independent of their conformational state [13]. Furthermore, the lack of [^{3}H]noradrenaline uptake in Na^+-free buffer provides strong evidence that the facilitation of the opening of sodium channels by aconitine and 3-acetylaconitine likely alters the ionic environment of the noradrenaline transporter in such a manner that its function is impaired. Further experiments are required, where the use of gradual changes of external sodium will make it possible to assess which change in the gradient may be induced by the addition of the alkaloids. However, it is intriguing that aconitine and 3-acetylaconitine are not capable of completely suppressing [^{3}H]noradrenaline uptake. This stands in contrast with the blockade observed in Na^+-free buffer as well as in presence of desipramine. This finding implies that the blockade of [^{3}H]noradrenaline uptake by aconitine and 3-acetylaconitine on the one hand and desipramine on the other involves different mechanisms. While desipramine is a direct blocker of the nonadrenaline/Na^+-cotransporter, the two aforementioned *Aconitum* alkaloids seem to act indirectly via an increase in intracellular Na^+ concentration. Due to the fact that there is still a residual uptake in the presence of aconitine and 3-acetylaconitine (Fig. 2), it is likely that the Na^+-dependent cotransporter is impaired but still functioning.

Taken together, the present results obtained with aconitine and 3-acetylaconitine imply that an increased sodium current is accompanied by a decreased uptake of noradrenaline. In contrast, lappaconitine and *N*-desacetyllappaconitine failed to affect [^{3}H]noradrenaline uptake. This is in line with the results of the electrophysiological experiments that demonstrated a blocking action of these two alkaloids on the voltage-dependent sodium channel.

3-Acetylaconitine and lappaconitine have been reported to exert analgesia by stimulating noradrenergic descending pathways [11, 12]. However, it is obvious from the present study that these two alkaloids have a different mode of action. It is intriguing that aconitine and 3-acetylaconitine, which both bear a benzoylester group at C-14 position (Fig. 1), also exert a similar effect on [^{3}H]noradrenaline uptake as well as on sodium channel. In contrast, lappaconitine and *N*-desacetyllappaconitine, which possess a benzoylester group at C-4 instead of C-14, failed to inhibit [^{3}H]noradrenaline uptake and are both blockers of the voltage-dependent sodium channel.

References

1. Bisset NG, Arrow poisons in China. Part II. Aconitum—botany, chemistry, and pharmacology. *J Ethnopharmacol* **4:** 247–336, 1981.
2. Han GQ and Chen YY, Distribution of alkaloids in traditional Chinese medicine plants. In: *The Alkaloids* (Ed. Brossi A), pp. 241–270. Academic Press, New York, 1988.
3. Ono M and Satoh T, Pharmacological studies of lappaconitine. Analgesic activities. *Arzneim-Forsch/Drug Res* **38:** 892–895, 1988.
4. Ono M and Satoh T, Pharmacological studies of lappaconitine. Analgesia produced by intracerebroventricular, intercisternal and intrathectal injections. *J Pharmacobio-Dyn* **13:** 374–377, 1990.
5. Tang X, Liu X, Feng J, Zhu M and Li A, Analgesic action and no physical dependence of 3-acetylaconitine. *Acta Pharmacol Sin* **7:** 413–418, 1986.
6. Ameri A, Inhibition of rat hippocampal excitability by the plant alkaloid 3-acetylaconitine mediated by interaction with voltage-dependent sodium channel. *Naunyn-Schmiedebergs Arch Pharmacol* **355:** 273–280, 1997.
7. Ameri A, Metzmeier P and Peters T, Frequency-dependent inhibition of neuronal activity by lappaconitine in normal and epileptic hippocampal slices. *Br J Pharmacology* **118:** 577–584, 1996.
8. Ameri A, Shi Q, Aschoff J and Peters T, Electrophysiological effects of aconitine in rat hippocampal slices. *Neuropharmacology* **35:** 13–22, 1996.
9. Ono M and Satoh T, Pharmacological studies of lappaconitine. Supraspinal interaction in antinociception. *Arch Int Pharmacodyn* **309:** 32–41, 1991.
10. Ono M and Satoh T, Pharmacological studies of lappaconitine. Occurrence of analgesic effect without opioid receptor. *Res Common Chem Pathol Pharmacol* **12:** 13–25, 1989.
11. Lu D, Guo X and Tang X, Effects of monoamine transmitters on 3-acetylaconitine analgesia. *Acta Pharmacol Sin* **9:** 216–220, 1988.
12. Ono M and Satoh T, Pharmacological studies on lappaconitine: possible interaction with endogenous noradrenergic and serotonergic pathways to induce antinociception. *Japan J Pharmacol* **58:** 251–257, 1992.
13. Catterall WA, Neurotoxins that acts on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharm Toxicol* **20:** 15–43, 1980.
14. Petterson E, Studies of four diphenylbutylpiperazinepyridyl

derivatives on release and inhibition of reuptake of dopamine, serotonin and noradrenaline by rat brain *in vitro*. *Eur J Pharmacol* **282:** 131–135, 1995.
15. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye binding. *Anal Biochem* **72:** 248–254, 1976.
16. Bolden-Watson C and Richelson E, Blockade by newly developed antidepressants of biogenic amine uptake into rat brain synaptosomes. *Life Sci* **52:** 1023–1029, 1983.
17. Huguenard JA, Hamill OP and Prince DA, Developmental changes in Na^+ conductances in neocortical neurons: appearance of a slowly inactivating component. *J Neurophysiol* **59:** 778–779, 1988.
18. Ogata N and Tatebayashi H, Sodium current kinetics in freshly isolated neostriatal neurones of adult guinea pig. *Pflügers Arch* **416:** 594–603, 1990.
19. Cheng YC and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099–3108, 1988.
20. Stanford SC, Prozac: panacea or puzzle? *Trends Pharmacol Sci* **17:** 150–154, 1996.
21. Reimann W and Hennies HH, Inhibition of spinal noradrenaline uptake in rats by centrally acting analgesic tramadol. *Biochemical Pharmacol* **47:** 2289–2293, 1994.
22. Lester HA, Mager S, Quick MW and Corey JL, Permeation properties of neurotransmitter transporters. *Annu Rev Pharmacol Toxicol* **34:** 219–49, 1994.