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Influence of Two Naturally Occurring Abietane Monocarboxylic Acids (Resin Acids) and a Chlorinated Derivative on Release of the Inhibitory Neurotransmitter γ-Aminobutyric Acid from Trout Brain Synaptosomes

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Naturally occuring abietane monocarboxylic acids, and certain of their chlorinated derivatives produced by the use of chlorine as a pulp bleaching agent, are commonly found in pulp and paper mill effluents (Leach and Thakore 1973 and 1975). A number of these aquatic pollutants are known to be highly toxic to fish. For example, in rainbow trout (*Salmo gairdneri*) the acute 96-hr LC_{50}s of abietic acid and dehydroabietic acid range from 0.7-1.5 mg/L (Leach and Thakore 1976; Chung et al. 1979) and 0.8-1.74 mg/L (Leach and Thakore 1976; Davis and Hoos 1975; Chung et al. 1979) respectively under static conditions. The halogenated derivative, 12,14-dichlorodehydroabietic acid, is slightly more toxic than its non-chlorinated counterpart with LC_{50}s ranging from 0.6-1.2 mg/L (Leach and Thakore 1975; Chung et al. 1979). After acute exposure of rainbow trout to dehydroabietic acid, this compound is efficiently absorbed and readily distributed to most organs including the brain (Oikari 1982). The brain is also known to be a prominent site of accumulation of dehydroabietic acid in sockeye salmon (Kruzynski 1979). Dehydroabietic acid effectively reduces the hepatic clearance of bilirubin from the blood, producing jaundice-like symptoms which are associated with reduced UDP-glucuronyl transferase activity and inhibition of bile acid transport in trout hepatocytes (Matsoff and Oikari 1987; Rabergh et al. 1992). Exposure to dehydroabietic acid is also accompanied by a reduction in the activity of UDP-glucuronyl transferase in kidney, and elevations in lactate dehydrogenase and aspartate aminotransferase have been reported in heart muscle (Oikari et al. 1983).

Poisoning signs consistently observed in trout exposed to abietane monocarboxylic acids include both disorientation and abnormal responses to external stimuli which are indicative of nervous system dysfunction (Oikari et al. 1982). Paralysis involving the hind limbs and co-ordination

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problems have been documented in rodents following oral dosing with dehydroabietic acid (Villeneuve et al. 1977). Abietane monocarboxylic acids have also been implicated in a neurotoxic syndrome observed in cattle when rosin gum (a concentrated form of these and related compounds extracted from conifers) was administered by gavage (Gardner et al. 1994). The present research stems primarily from our recent study, which clearly demonstrated that dehydroabietic acid has a potent neurotransmitter releasing effect on synaptosomes (pinched-off nerve endings) prepared from mammalian brain (Nicholson 1994). The objective of this investigation was to establish whether this excitatory action occurs in the organism of immediate ecotoxicological concern (i.e. the fish), and to characterize the presynaptic actions of a chlorinated and two non-chlorinated pollutants within the abietane subgroup of resin acids.

MATERIALS AND METHODS

Dehydroabietic acid and 12,14-dichlorodehydroabietic acid were obtained from Helix Biotech Corporation (Vancouver, BC, Canada). Abietic acid was provided by Anachemia Canada Inc. (Toronto, ON, Canada). [3H] γ-aminobutyric acid was purchased from NEN Products (Boston, MA, U.S.A.) and remaining compounds were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Rainbow trout *(Onchorynchus mykiss*) approximately 250 g were provided by West Creek Trout Farms (Aldergrove, BC, Canada). Fish were maintained on a 12 hr (light) 12 hr (dark) cycle and reared on sterling silver cup fish feed obtained from Nelson's (Murray, Utah, U.S.A.). The preparation of trout brain synaptosomes was based on the method of Whittaker and Greengard (1971) with modifications. Briefly, a trout was decapitated, the brain rapidly removed and homogenized in 20 mL of ice-cold sucrose (0.32 M, pH 7.4). The homogenate was centrifuged at 1000 g for 10 min and the resulting supernatant was centrifuged at 31,400 g for 30 min to obtain the crude synaptosomal pellet which was resuspended in standard saline (128 mM NaCI, 5 mM KCl, 2.7 mM $CaCl_2$ 1.2 mM $MgSO_4$ 1 mM Na_2HPO_4 16 mM glucose and 20 mM Hepes adjusted to pH 7.4 with Trizma base. All fractionation procedures were carried out at 4 °C. Synaptosomes were incubated with [^{3}H] γ-aminobutyric acid to load releasable pools with neurotransmitter as described by Nicholson and Merletti (1990) except that incubation time was increased to 25 min and the temperature was 25 °C. After loading, 20 mL of ice-cold standard saline was added and the suspension centrifuged at 11,300 g for 10 min. Loaded synaptosomes were gently resuspended in standard saline (1 mL) and 90 µL transferred to each of ten super-fusion units containing GF/B filters. [^{3}H] γ-aminobutyric acid-loaded synaptosomes in individual super-fusion units were super-fused

with 25 mL of standard saline to remove extrasynaptosomal radioactivity, whereupon a further 10 mL of standard saline was added and collection of 3-mL fractions started. For studies on the effects of abietane monocarboxylic acids on release of neurotransmitter, standard saline (20 mL) containing these compounds was added to the superfusion system at fraction four (see Fig. 1). The flow rate was 1 mL/min throughout. In some experiments channel blockers were added with the study compounds. Dimethyl sulfoxide (0.1% final concentration) was used as a carrier for all test compounds except tetrodotoxin which was added in 3 µL water. Appropriate volumes of solvent were added to all control salines. All superfusion experiments were conducted at room temperature (24 °C). Initial experiments to check functional competence confirmed that trout brain synaptosomes superfused in this way release neurotransmitter in response to standard depolarizing treatments (for example the sodium channel activating alkaloid mixture veratrine and high K^+) and that tetrodotoxin blocks veratrine's action.

RESULTS AND DISCUSSION

All three abietane monocarboxylic acids examined in this study have the ability to stimulate release of radioactivity from trout brain synaptosomes preloaded with [^{3}H] γ-aminobutyric acid (Fig. 1). The release profiles confirm that the study compounds cause rapid effects, with the greatest quantity of neurotransmitter release occuring after treatment with abietic acid. In separate experiments, chromatographic analysis of superfusates verified that with each resin acid, [^{3}H] γ-aminobutyric acid was the only radiolabeled substance released from the nerve terminals.

The relationship between concentration of individual abietane mono carboxylic acids and release of neurotransmitter is shown in Fig. 2. The data clearly show that 12,14-dichlorodehydroabietic acid is the more potent of this group with threshold responses detectable at 2.5 µM. In contrast to the non-chlorinated abietane monocarboxylic acids, the response to 12,14-dichlorodehydroabietic acid is maximal at 25 µM, indicating the latter may be more selective in its action.

The extent to which tetrodotoxin, an inhibitor of voltage-sensitive sodium channels, and the calcium channel blockers nifedipine and verapamil, influence the release of γ-aminobutyric acid from synaptosomes is shown in Table 1. It is evident that opening of voltage-sensitive sodium channels can be excluded as action of resin acids on central nerve endings of fish. However, the activation of transmitter release by 12,14-dichlorodehydroabietic acid, in marked contrast to that of abietic acid and dehydroabietic acid, is substantially inhibited by nifedipine and verapamil which bind to different recognition sites on the calcium channel.

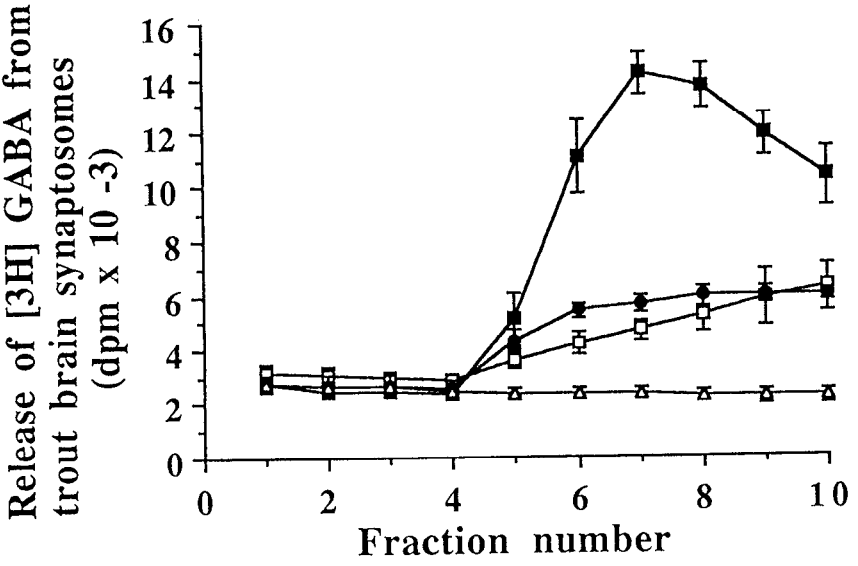

Figure 1. Stimulation of [^{3}H] GABA release from trout brain synaptosomes by abietic acid (■), dehydroabietic acid (□), 12,14-dichlorodehydroabietic acid (●). Abietane monocarboxylic acids (75 µM) added at fraction four. [Control (Δ); mean ± standard error].

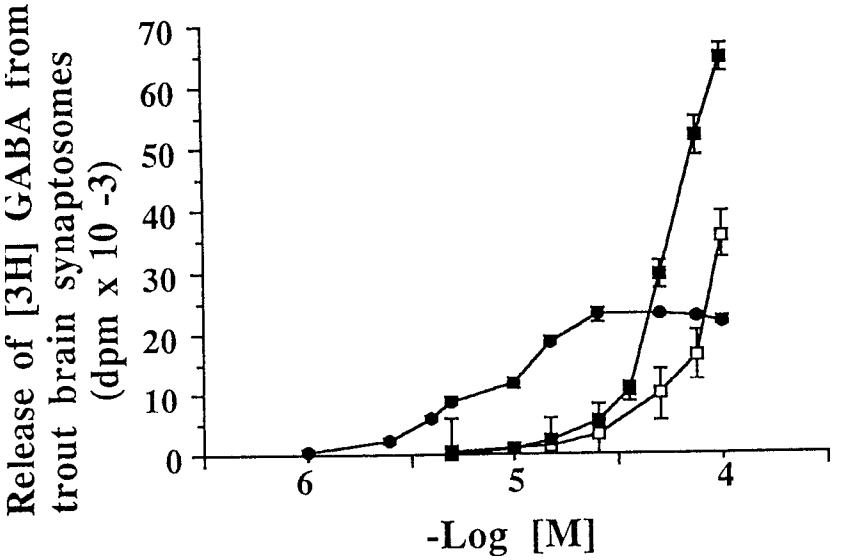

Figure 2. Relationship between the concentration of abietic acid (■), dehydroabietic acid (□), 12,14-dichlorodehydroabietic acid (●) and release of [^{3}H] GABA from trout brain synaptosomes. Values show mean ± standard error.

Table 1. The sensitivity of the transmitter releasing effects of abietic acid (AA), dehydroabietic acid (DHAA) and 12,14-dichloro-dehydroabietic acid (Cl_2-DHAA) to inhibition by tetrodotoxin (TTX), nifedipine (NP) and verapamil (VP). (Mean ± standard error; n= 3 - 7 independent experiments; C= control)

Treatment	Total release of [^{3}H] GABA (dpm x 10^{-3})
C	13.82 ± 0.21
AA (50 μM)	41.42 ± 0.54
AA (50 μM) + TTX (2 μM)	37.65 ± 0.73
AA (50 μM) + NP (50 μM)	40.18 ± 0.38
AA (50 μM) + VP (50 μM)	38.54 ± 0.32
DHAA (75 μM)	30.70 ± 0.44
DHAA (75 μM) + TTX (2 μM)	29.30 ± 0.68
DHAA (75 μM) + NP (50 μM)	33.49 ± 0.31
DHAA (75 μM) + VP (50 μM)	30.69 ± 0.26
Cl2-DHAA (15 μM)	25.98 ± 0.27
Cl2-DHAA (15 μM) + TTX (2 μM)	24.89 ± 0.73
Cl2-DHAA (15 μM) + NP (50 μM)	18.35 ± 0.34
Cl2-DHAA (15 μM) + VP (50 μM)	16.86 ± 0.23
TTX (2 μM)	12.76 ± 0.29
NF (50 μM)	13.13 ± 0.10
VP (50 μM)	12.89 ± 0.11

In summary, our results demonstrate that abietane monocarboxylic acids are strong activators of the release of the neurotransmitter γ-aminobutyric acid from nerve endings isolated from trout brain. In addition our data support the contention that compared to the non-chlorinated analogues, 12,14-dichlorodehydroabietic acid is both more potent and more specific in its action, with calcium channel activation a possible feature.

The ecotoxicological relevance of presynaptic impairment in fish is indicated by the fact that the concentrations required to precipitate transmitter release in the present experiments are generally lower than those concentrations known to be present in the brains of trout after exposure to resin acids (Oikari et al. 1982).

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