Biochemical Pharmacology, Vol. 38, No. 3, pp. 549–551, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

Irreversible inhibition of rat striatal dopamine uptake induced by *in vitro* exposure to DSP4

(*Received* 15 *January* 1988; *accepted* 10 *August* 1988)

When alkylating 2-chloroethylamine neurotoxins such as *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP4) and xylamine [1] are exposed *in vitro* to rodent brain slices or synaptosomes, [^{3}H]norepinephrine ([^{3}H]NE) uptake is inhibited markedly [2–4]. The inhibition of NE uptake is irreversible, since it persists when the tissues are washed repeatedly after neurotoxin incubation and before the addition of [^{3}H]NE. After a single systemic administration, these compounds (25–100 mg/kg) irreversibly inhibit NE uptake and produce a corresponding marked depletion of NE levels in areas (cerebral cortex, spinal cord, cerebellum and hippocampus) of the CNS receiving terminal projections from noradrenergic perikarya in the locus coeruleus [1, 2, 5–11]. Reductions in NE uptake and NE tissue levels are apparent 1 hr after administration and persist for 6–8 months [1, 2, 5–11]. Using *in vivo* voltammetry, a reduction in the extracellular concentration of NE in cerebral cortex of anesthetized rats was observed within 15 min after systemic administration of xylamine [12]. The depletion of releaseable NE following *in vivo* DSP4 treatment was confirmed in slices of hippocampus by the loss of electrophysiological responsiveness to amphetamine *in vitro* [13]. However, NE turnover is increased in terminals spared by DSP4 [10, 11].

The effects of these neurotoxins are prevented by co-incubation *in vitro* or by prior *in vivo* administration of reversible inhibitors of uptake, such as amphetamine [5], cocaine [3] or desipramine [2, 4, 12]. The protection provided by the reversible uptake blockers may be due to a prevention of the intraneuronal accumulation of DSP4, which subsequently alkylates a site within the terminal regulating uptake. Alternatively, the protection may be due to a prevention of the DSP4-induced alkylation of the uptake site on the external surface of the terminal. The direct interaction of DSP4 with the NE uptake site is further suggested by the finding that DSP4 (either *in vivo* or *in vitro* exposure) irreversibly inhibits the high-affinity binding of [^{3}H]desipramine to cortical membrane [14]. Furthermore, it is believed that the prolonged decrease in NE tissue content and degeneration of the noradrenergic nerve terminal is a secondary event triggered by the primary alkylation of a site closely associated with NE uptake [2, 4, 7, 14].

After DSP4 administration *in vivo*, a striking selectivity of the effect of DSP4 is observed, in that the same variables (uptake, levels and efflux) in dopaminergic systems are not altered [2, 5, 8–12]. In contrast, when tissues are exposed to DSP4 *in vitro*, the effect of DSP4 is no longer selective since uptake of dopamine (DA) in striatal homogenates is also inhibited [2]. In the present study, the selectivity of the irreversible blockade of uptake induced by DSP4 was examined further in rat hippocampal and striatal slices after exposure to DSP4 *in vitro*. Additionally, the effect of DSP4 on striatal slices was examined using [^{3}H]mazindol which labels recognition binding sites associated with the DA uptake pump [15].

Methods

Transmitter uptake experiments. The striata and hippocampi from male Sprague–Dawley rats (200 g) were rapidly dissected and sliced (500 μm) using a McIlwain tissue chopper. Slices were placed in ice-cold aerated artificial CSF (in mM; 124 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 2.4 $MgSO_4$, 2.5 CaCl, 25.7 $NaHCO_3$, 10.0 glucose, additionally containing 1.1 L-ascorbate and 0.1 pargyline; pH 7.4) and were incubated in a metabolic shaker for 60 min at 34° in order to allow replenishment of energy stores within the slice [16]. Duplicate slices were transferred to 2 ml of ice-cold artificial CSF to which 20 μl of DSP4 (100 pM to 1 mM final concentration) or vehicle (1 mM HCl) was added, and the slices were incubated for 30 min in the metabolic shaker. Slices were rinsed in 50 ml of ice-cold artificial CSF, transferred to 2 ml of artificial CSF to which 20 μl of [^{3}H]DA (28.0 Ci/mmol, New England Nuclear, final concentration 10 nM) or [^{3}H]NE (44.4 Ci/mmol, New England Nuclear, final concentration 2.5 nM) was added, and incubated for 10 min at 34°. Slices were transferred to glass fiber filters, washed twice with 10 ml of ice-cold artificial CSF containing pyrocatechol (1 mM), weighed and solubilized. Nonspecific uptake was determined by the amount of [^{3}H]DA or [^{3}H]NE accumulation in the absence of sodium ions which were substituted with molal equivalents of choline. Scintillation fluid was added to triplicate 50-μl aliquots of incubation medium and to solubilized tissue slices. Radioactivity was determined by liquid scintillation spectrometry.

[^{3}H]*Mazindol binding experiments.* After the initial 60-min incubation period, duplicate striatal slices were transferred to 2 ml of ice-cold artificial CSF to which 50 μl of vehicle or DSP4 (10 μM final concentration) was added, and the slices were incubated for 30 min at 34°. Slices were rinsed in 50 ml of ice-cold artificial CSF and transferred to, and homogenized in, 15 ml of ice-cold buffer [in mM: 50 Tris, 150 NaCl, 1 ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), 5 KCl; pH 7.4]. The homogenates were centrifuged at 30,000 *g* for 15 min at 4°. The pellet was washed twice by this procedure. The membranes were resuspended at a concentration of 175 μg protein/ml buffer. Assays contained final concentrations of 2.5 nM [^{3}H]mazindol (15.0 Ci/mmol, New England Nuclear), 10 nM to 1 μM mazindol and tissue in a volume of 250 μl and were incubated for 25 min at 0°. Nonspecific binding was defined in the presence of 100 μM benzotropine. Reactions were terminated by addition of 5 ml of ice-cold buffer and rapid filtration (three times). Radioactivity was determined by liquid scintillation spectrometry. Competition curves were analyzed by the curve-fitting program LIGAND [17]. Protein concentrations were determined [18] using bovine serum albumin as the standard.

Results and discussion

Results from *in vivo* studies indicate that DSP4 selectively and irreversibly inhibits NE uptake into noradrenergic nerve terminals (see Introduction). In the present study, *in vitro* exposure to DSP4 (100 pM to 1 mM) for 30 min irreversibly inhibited not only [^{3}H]NE accumulation in hippocampal slices but also [^{3}H]DA accumulation in striatal slices with IC_{50} values of 1 and 30 μM respectively (Fig. 1). The IC_{50} value for the inhibition of NE uptake is in good agreement with the results of previous studies [2] in which the effect of *in vitro* DSP4 was examined in cortical synaptosomes. Ross [2], however, showed only partial inhibition (40%) of DA uptake after exposing striatal synaptosomes to 30 μM DSP4 *in vitro*. In contrast, in the present study, complete inhibition of DA uptake into striatal slices

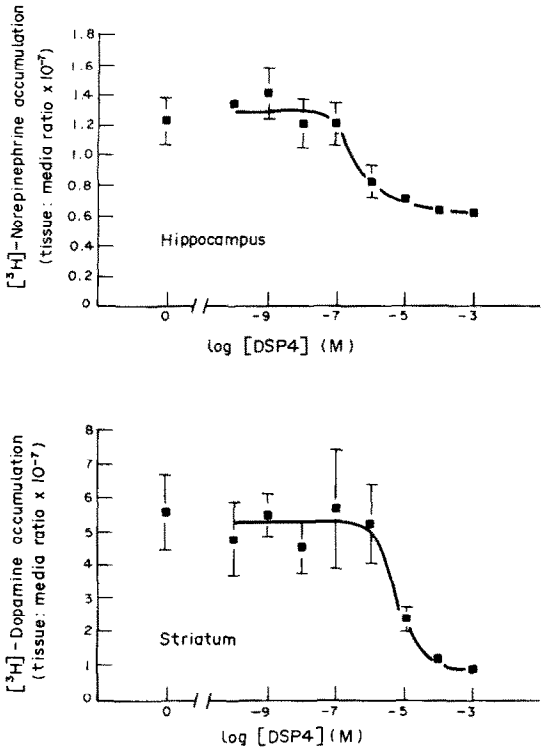

Fig. 1. Dose–response curves for the inhibition of uptake of [³H]NE into hippocampal slices (top) and [³H]DA into striatal slices (bottom). Uptake was measured after a 30-min pretreatment with DSP4 and subsequent washing. Under conditions of maximal inhibition, accumulation of transmitter was similar to the nonspecific uptake measured in the sodium-free buffer. Mean values ± SEM are shown for N = 3.

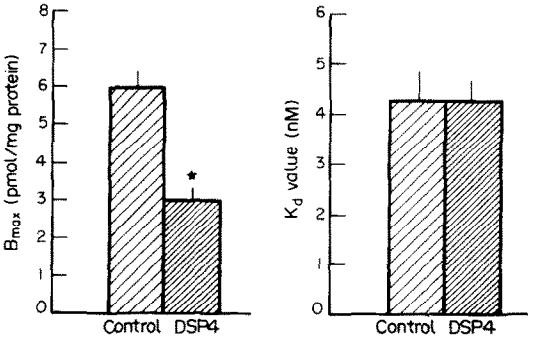

Fig. 2. Effect of *in vitro* exposure of striatal slices to DSP4 on the density and the affinity of [³H]mazindol binding sites in membranes. Slices were exposed to 10 μM DSP4 or vehicle and subsequent washing prior to the saturation binding assay. Competition curves were analyzed by the curve-fitting program LIGAND. Mean values ± SEM are shown for N = 3. The asterisk (*) equals $P < 0.05$, using Student's *t*-test.

was found upon examination of the full dose–response curve. Therefore, our results indicate that the selectivity for noradrenergic systems is lost when tissues are exposed to DSP4 *in vitro*.

Furthermore, in the present study pretreatment of striatal slices *in vitro* with DSP4 (10 μM) resulted in a 50% decrease in the density, with no change in the affinity, of [³H]mazindol binding sites in striatal membranes (Fig. 2). There is good agreement between the concentrations of DSP4 which half-maximally inhibited [³H]DA accumulation and decreased the density of [³H]mazindol binding sites. Therefore, the present study extends the previous work [2] by providing evidence for a direct interaction of DSP4 with DA uptake sites on the external surface of the dopaminergic neuronal membrane. The irreversible loss of DA uptake sites is suggested to result from DSP4-induced alkylation of these sites which cannot be reversed by repeated washing of the membranes. The loss of DA uptake sites, thereby, accounts for the DSP4-induced decrease in [³H]DA accumulation.

In summary, *in vitro* exposure to DSP4 resulted in an irreversible alkylation or loss of DA uptake sites in rat striatal slices, which was manifested by an irreversible inhibition of DA uptake. Therefore, despite the selectivity of DSP4 for noradrenergic neurons *in vivo*, *in vitro* exposure to DSP4 produced an irreversible inhibition of both NE and DA uptake in rat brain slices, although NE uptake was affected more potently. It remains to be determined why DSP4 selectively alters the noradrenergic system when administered *in vivo*, but also affects the dopaminergic system upon *in vitro* exposure. The effects produced by DSP4 may simply depend on its concentration at its site of action which, in turn, depends upon whether the tissue is exposed to DSP4 *in vivo* or *in vitro*. Furthermore, studies examining the effect of *in vitro* exposure to DSP4 may provide information concerning its mechanism of action and may prove to be a useful tool to examine the relationship between DA uptake and DA release from presynaptic terminals.

Acknowledgements—DSP4 was a gift from Dr. G. Jonsson, Karolinska Institute, Stockholm, Sweden. This work was supported by USPHS Grant NS09199 (N.R.Z.) and MH42934 (L.P.D.).

Department of Pharmacology
University of Colorado Health Sciences Center
Denver, CO 80262, U.S.A.

LINDA P. DWOSKIN*
ROBERT P. YASUDA
NANCY R. ZAHNISER

REFERENCES

1. Ross SB, Johansson JG, Lindborg B and Dahlbom R, Cyclizing compounds: Tertiary *N*-(2-bromobenzyl)-*N*-haloalkylamines with adrenergic blocking action. *Acta Pharm Suec* **10**: 29–42, 1973.
2. Ross SB, Long-term effects of *N*-2-chloroethyl-*N*-ethyl-2-bromo-benzylamine-hydrochloride on noradrenergic neurones in the rat brain and heart. *Br J Pharmacol* **58**: 521–527, 1976.
3. Kammerer RC, Amiri B and Cho AK, Inhibition of uptake of catecholamines by benzylamine derivatives. *J Med Chem* **22**: 352–355, 1979.
4. Landa ME, Rubio MC and Jaim-Etcheverry G, The neurotoxic compound *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine hydrochloride (DSP4) depletes endogenous norepinephrine and enhances release of [³H]norepinephrine from rat cortical slices. *J Pharmacol Exp Ther* **231**: 131–136, 1984.

* Author to whom correspondence should be sent. Present address: Division of Pharmacology and Toxicology, College of Pharmacy, University of Kentucky, Pharmacy Building, Rose St., Lexington KY 40536.

5. Ross SB and Renyi AL, On the long-lasting inhibitory effect of *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP4) on the active uptake of noradrenaline. *J Pharm Pharmacol* **28**: 458–459, 1976.
6. Jaim-Etcheverry G and Zieher LM, DSP-4: A novel compound with neurotoxic effects on noradrenergic neurons of adult and developing rats. *Brain Res* **188**: 513–523, 1980.
7. Jaim-Etcheverry G and Zieher LM, 2-Chloroethylamines: New chemical tools for the study of noradrenergic neurons. *Trends Pharmacol Sci* **4**: 473–475, 1983.
8. Jonsson G, Hallman H, Ponzio F and Ross S, DSP4 (*N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine)—A useful denervation tool for central and peripheral noradrenaline neurons. *Eur J Pharmacol* **72**: 173–188, 1981.
9. Dudley MW, Butcher LL, Kammerer RC and Cho AK, The actions of xylamine on central noradrenergic neurons. *J Pharmacol Exp Ther* **217**: 834–840, 1981.
10. Hallman H, Sundstrom E and Jonsson G, Effects of the noradrenaline neurotoxin DSP4 on monoamine neurons and their transmitter turnover in rat CNS. *J Neural Transm* **60**: 89–102, 1984.
11. Logue MP, Growdon JH, Coviella ILG and Wurtman RJ, Differential effects of DSP-4 administration on regional brain norepinephrine turnover in rats. *Life Sci* **37**: 403–409, 1985.
12. Howard-Butcher S, Blaha CD and Lane RF, Differential effects of xylamine on extracellular concentrations of norepinephrine and dopamine in rat central nervous system: An *in vivo* electrochemical study. *J Pharmacol Exp Ther* **233**: 58–63, 1985.
13. Dunwiddie TV, Mueller AL, Bickford PC and Zahniser NR, Electrophysiological and biochemical sequelae of the destruction of hippocampal noradrenergic afferents by DSP4. *Brain Res* **269**: 311–317, 1983.
14. Lee CM, Javitch JA and Snyder SH, Characterization of [^{3}H]desipramine binding associated with neuronal norepinephrine uptake sites in rat brain membranes. *J Neurosci* **2**: 1515–1525, 1982.
15. Javitch JA, Blaustein RO and Snyder SH, [^{3}H]Mazindol binding associated with neuronal dopamine uptake sites in corpus striatum membranes. *Eur J Pharmacol* **90**: 461–462, 1983.
16. Fredholm BB, Dunwiddie TV, Bergman B and Lindstrom K, Levels of adenosine and adenine nucleotides in slices of rat hippocampus. *Brain Res* **295**: 127–136, 1984.
17. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.