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THE INTERACTION OF THE BETA-HALOETHYL BENZYLAMINES, XYLAMINE, AND DSP-4 WITH CATECHOLAMINERGIC NEURONS

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INTRODUCTION

Xylamine and DSP-4 (Scheme 1) are members of a group of beta-haloethylamine derivatives of benzylamine that interact with components of the adrenergic synapse. Other members of this group include dibenamine and phenoxybenzamine. The compounds exhibit different selectivities for presynaptic (xylamine and DSP-4) and postsynaptic (dibenamine and phenoxybenzamine) elements. Beta-haloethylamines, or one-arm mustards, are alkylating agents that form covalent bonds with electrophilic centers on or near their site of action and exert irreversible effects. The chemistry of this process, shown in Scheme 1, involves an initial ring closure of the neutral amine to an aziridinium ion which is highly reactive toward nucleophilic functions, such as thiols and amines. When the aziridinium ion binds to a site, a neighboring nucleophilic function can form a covalent bond in the manner shown and irreversibly disrupt the function of the binding site. Xylamine was

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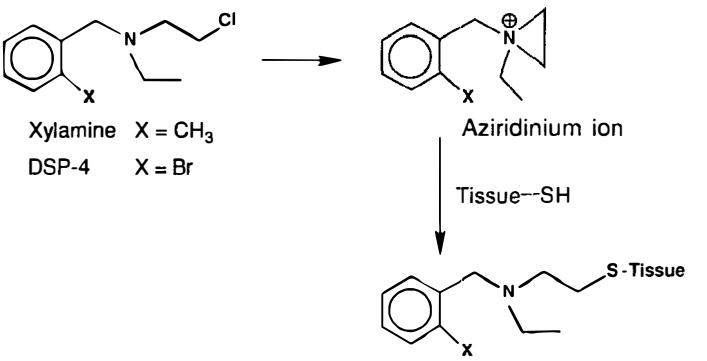
originally described by Kreuger & Cook (1) as a very weak adrenergic neuron blocking agent. DSP-4 was originally described by Ross & Renyi (2) who reported its long-term effects on peripheral and central norepinephrine (NE) levels and on neuronal uptake. When converted to their aziridinium ion form, both DSP-4 and xylamine are very similar in structure to the quaternary adrenergic neuron blocker, bretylium. The ability of these compounds to affect a long-term depletion of NE stores and to irreversibly inhibit neuronal uptake has been used in pharmacological investigations. The depleting actions have been used to study behavior and synaptic biochemistry under conditions of reduced NE nerve function. Xylamine has been used as an irreversible ligand to identify components of catecholaminergic nerve terminals. This chapter reviews studies investigating the mechanism of action of these compounds and their use in the characterization and isolation of the catecholamine transporter and in modifying catecholamine systems to assess their role in drug action.

MECHANISM OF ACTION

Xylamine was studied initially to assess its potential as a marker for the catecholamine transporter. It was selected over DSP-4 because it could be so easily radiolabeled (3). The sites of action of this compound and details of its interaction with elements of the adrenergic synapse were determined by in vitro and in vivo studies. Like DSP-4, xylamine exhibited a specificity for central noradrenergic neurons when administered systemically to rats (4). When synaptosomes were prepared from the cortices and striata of rats pretreated with xylamine, the NE content and uptake activity in cortical synaptosomes were substantially reduced whereas the dopamine (DA) content and uptake activity of striatal synaptosomes were unchanged. DSP-4 exhibited similar selectivities that could be blocked by coadministration of uptake inhibitors, such as desipramine (DMI).



Analogous observations were noted in vitro when xylamine was exposed to minces of rat brain (5), but not to synaptosomes prepared from brains of untreated animals. The selectivity was reversed in synaptosomal preparations and the IC_{50} for DA uptake by striatal synaptosomes was much lower than that for NE uptake by cortical synaptosomes. The basis for this change in selectivity with tissue organization is not known. Experiments with peripheral tissue confirmed the actions of xylamine on uptake and provided additional insight into its mechanism of action. Studies with rabbit aortic rings showed that NE uptake by this tissue was irreversibly inhibited and that the inhibitory action of xylamine required a functional transporter. Thus, changes in Na^+ concentration of the exposure medium, addition of alternate uptake substrates, such as NE and amphetamine, protected the tissue from the irreversible effects (6). The nature of the irreversible blockade of uptake by xylamine was demonstrated in experiments with cultures of rat superior cervical ganglia (7). This preparation can survive in culture for several days, and over a period of two days after collection increases its uptake activity by sixfold. NE uptake by this preparation was inhibited by xylamine but uptake activity was restored after an additional two days in culture. This preparation could also be preloaded with radiolabeled NE, which is releasable by tyramine. When preloaded tissue was exposed to xylamine, however, the actions of tyramine were blocked. Recovery from the irreversible effects of xylamine involved the synthesis of new transporter protein since recovery was inhibited by cycloheximide.

These experiments showed that the actions of xylamine and DSP-4 on uptake were irreversible and required a functional transporter for the inactivation process. Studies of the disposition of xylamine were conducted with tritium-labeled xylamine, prepared by reductive dehalogenation of the bromine-substituted analog (3). Tritium-labeled xylamine was accumulated in cultured superior cervical ganglia by the catecholamine transporter (8). About 30% of the accumulated radioactivity was bound to protein and the remainder was removed by sequential washing. Similar observations were made with rabbit thoracic aorta and rat vas deferans. The accumulation by rat vas deferans was prevented by destruction of NE neurons with pretreatment of the animals with 6-hydroxydopamine (6OHDA). Xylamine was accumulated in this tissue and in the heart after systemic administration by a DMI-sensitive process (8). Synaptosomal preparations from rat brain (9) and PC12 cells (10) also accumulate and bind xylamine. The binding to homogenates of these latter preparations was mostly to thiol functions, as prior exposure to N-ethylmaleimide (NEM) almost completely blocked the labeling of proteins by [^{3}H]xylamine. Hydrolysis of xylamine or coincubation with thiosulfate, a potent nucleophile, prevented the accumulation of xylamine and rendered it inactive. These observations indicated that the accumulation and inhibitory

actions of xylamine, and presumably DSP-4, required the beta-haloamine or its ring closed, reactive electrophile, the aziridinium ion.

To determine the role of the aziridinium ion, it and other possible reaction products of xylamine such as its hydrolysis product and its dimer were prepared and their activities assessed in experiments in vitro (11). The results of these studies showed that the aziridinium ion was the only active product. The aziridinium ion was rapidly formed at pH 7 and it was moderately stable so that solutions of the haloamine could be converted to the aziridinium ion before being used in in vitro experiments. All of the in vitro activity of xylamine could be accounted for by the aziridinium ion. When used in intact animals, however, the tertiary amine must be administered to penetrate the blood-brain barrier.

Studies with brain preparations provided further evidence for the nature of xylamine action. As stated above, when xylamine is administered systemically, the site of action on the central nervous system appears to be on noradrenergic (4) and, to a limited extent, on serotonergic (12) systems. However, when exposed to synaptosomal preparations, xylamine exhibited a selectivity toward the DA terminals of the striatum (13). The irreversible binding of xylamine to components of nerve endings was demonstrated in rats with unilateral lesions in the median forebrain bundle which destroyed the DA terminals in the striatum. When synaptosomes from lesioned and control striata were exposed to [^{3}H]xylamine and the labeled peptides examined by SDS-PAGE procedures, essentially no peptides were labeled in the synaptosomes from the lesioned striata. Thus, in order to bind to tissue in significant quantities, xylamine appears to require interaction with the catecholamine transporter, to accumulate in the terminal, then form covalent bonds with thiol groups on proteins. Fractionation experiments of xylamine-labeled synaptosomes showed that mitochondrial proteins were heavily labeled and examination of mitochondrial function revealed that it was compromised. Xylamine inhibited ATP synthesis at the NADPH and succinate dehydrogenase level (9). This observation raised the possibility that xylamine could be inhibiting catecholamine uptake indirectly by inhibiting ATP synthesis. To eliminate this possibility, plasma membrane vesicles were prepared from bovine caudate. These vesicles, although they lack mitochondria, accumulate DA by a sodium-dependent process and prior exposure to xylamine blocked this activity (14). These experiments showed that xylamine inhibited catecholamine transport directly by interaction with a component of the nerve terminal. The reduction in mitochondrial activity by xylamine, however, may affect the storage capacity of the nerve terminal and thereby reduce levels of neurotransmitter in the terminals that accumulate this drug.

Labeling experiments have also been performed with rat striatal synaptosomes (9). After exposure to [^{3}H]xylamine, a large number of syn-

aptosomal peptides were labeled. Fractionation of synaptosomes into mitochondria and plasma membrane fractions reduced the number of labeled peptides to four. The peptide associated with xylamine action was tentatively identified on the basis of changes in labeling patterns with NEM, a permeable thiol reagent, and mersalyl, an impermeable one. Exposure to NEM at 0° reduces overall binding but does not inhibit DA uptake. Using low-temperature exposure to NEM and two-dimensional electrophoretic separation of plasma membrane peptides, a peptide of 55 Kd has been tentatively identified as the site of xylamine binding that is associated with inhibition of uptake (9).

The studies described above indicate that xylamine and DSP-4 irreversibly reduced the levels of NE in nerve terminals and inhibit uptake by a process that requires a functional transporter. Depending on the exposure conditions, the actions are fairly specific and this feature has been used in studies of ncuronal function and of the catecholamine transporter. Experiments with brain fractions have also resulted in the specific labeling of a peptide that is associated with xylamine action on the catecholamine transporter. This 55 Kd peptide is very similar in molecular weight to a 54 Kd peptide identified as the site of xylamine action on a cultured cell line transporter (see below). Additional studies of the transporter have utilized cell culture experiments, for the most part utilizing the PC12 clonal cell line.

Cell Culture Studies

Two cultured cell lines, N-2a, a mouse neuroblastoma-derived cell line (15) and PC12 cell line have been examined for their interaction with xylamine. The N-2a cell line contains elements of catecholaminergic neurons, including biosynthetic enzymes and a high-affinity uptake system for catecholamines. Xylamine inhibits this uptake with an IC_{50} of about 1 μM whereas concentrations in excess of 200 μM were required for cytotoxicity (16). Studies with the PC12 cell line have been much more extensive, focusing on studies characterizing the carrier. PC12 is a clonal line of pheochromocytoma cells from the adrenal medulla of rats (17, 18). The cells synthesize NE, DA, and acetylcholine (ACh), store each in secretory vesicles, and exhibit depolarization-evoked, Ca^{++}-dependent release of the catecholamines and ACh (18). PC12 cells take up catecholamines from the extracellular space by a transporter-mediated process that has many of the characteristics of neuronal catecholamine transport (10, 19).

PC12 Cells

Under standard conditions of culture, the cells resemble endocrine cells more than neurons but they can be induced to differentiate into neuron-like cells by treatment with nerve growth factor (NGF). Thus, after a brief (a few days)

exposure to NGF, the cells essentially cease multiplication, extend neuronal processes, and acquire the appearance of neurons (18) that also store and secrete catecholamines and ACh. They also have a transporter-mediated uptake system for catecholamines; there are no known differences between this sytem in NGF-induced cells and those of undifferentiated cells (10, 19).

The uptake of both DA and NE is a saturable Na^+-dependent process. The apparent Km value for DA is 0.2 μM and the V_{max} is 3.8 pmol/min/mg protein and for NE the values are 0.5 μM and 2.7 pmol/min/mg protein, respectively (10). Both DA and NE uptake by PC12 are inhibited by DMI and benztropine, with DMI the more potent inhibitor in both cases (10). Since benztropine is more selective for the DA transporter (20), the PC12 uptake system for DA resembles NE uptake in rat brain. NE uptake by the cells is also inhibited by cocaine (IC_{50} = 0.3 μM), bretylium (IC_{50} = 7 μM), and xylamine (IC_{50} = 15μM). DA uptake into PC12 cells is inhibited by cocaine (IC50 = 0.2 μM) and is inhibited 40% by 10 μM xylamine. These results suggest that the same system likely functions in the transport of both compounds.

Studies of the binding of [^{3}H]DMI (21) and [^{3}H]mazindol (B. D. Howard, A. K. Cho, M.-b. Zhang, M. Koide & S. Lin, submitted) have estimated between 55,000 to 70,000 transporters per PC12 cell. Xylamine inhibited the binding of DMI with an IC_{50} of 2.5 μM (21).

Effect of Xylamine on PC12 Cells

Xylamine inhibits the Na^+-dependent uptake of both NE and DA (10, 21) but its effects on NE uptake have been better characterized. The inhibition was irreversible and depended on the length of xylamine exposure time, with maximum inhibition occurring after 30 min. The actions of 10 μM xylamine were blocked by 10 μM cocaine, 100 μM NE, or the absence of Na^+ in the exposure medium (10), indicating that xylamine must interact with the NE transporter to inhibit uptake. PC12 cells also take up choline, 2-deoxyglucose and alpha-aminoisobutyric acid by transporter-mediated processes that, in the latter case, is also Na^+-dependent. Since xylamine (10 μM) did not affect uptake of any of these compounds, its action on NE uptake must not be due to a nonspecific perturbation of the PC12 plasma membrane. Furthermore, xylamine is not toxic as evidenced by the absence of changes in morphology or viability of cells cultured in medium containing it (M. Koide, unpublished results).

Xylamine can be accumulated by PC12 cells (10) by a saturable and Na^+-dependent process. The Km for xylamine is 13 μM and the Vmax is 36.8 pmol/min/mg protein. The Vmax is 10 times greater than that for DA or NE uptake, but the efficiency of uptake (Vmax/Km) is lower. As in rat tissue, xylamine uptake is inhibited by DMI (IC_{50} <1 μM), cocaine (IC_{50} = 0.6

μM), Ne (IC_50) = 1 μM), and bretylium (IC_{50} = 2.5 μM). Gramicidin D, which dissipates Na^+ gradients by forming Na^+ channels across membranes, blocks the Na^+-dependent uptake of xylamine into PC12 as effectively as it does the uptake of these compounds. Similar observations have been made with synaptic plasma membrane vesicles (14).

[^{3}H]Xylamine Labeled Proteins of PC12

When intact PC12 cells were incubated with [^{3}H]xylamine, several proteins were radioactively labeled (10). The most prominently labeled proteins had molecular weights of 17, 24, 31, 33, 41, 43, 52, 54, and 80 Kd, respectively. The labeling of all these proteins was decreased when the exposure to xylamine occurred in Na^+-free buffer or in standard buffer containing gramicidin D, cocaine (10 μM), or DMI (10 μM), indicating that xylamine must enter the cells for covalcnt binding to cellular proteins to occur. Koide et al (10) interpreted these results to mean that xylamine enters the cells via the catecholamine transporter and then covalently reacts with several proteins. They suggested that at least one of these proteins is the catecholamine transporter or one of its subunits because of the evidence that xylamine irreversibly inhibits the catecholamine transporter by covalently binding to it. The fact that no PC12 protein is prominently labeled by xylamine when the cells are treated with gramicidin D indicates that xylamine does not bind covalently to a portion of the transporter exposed to the cell exterior, at least not in the absence of a transmembrane Na^+ gradient, but rather binds reversibly to the exterior of the transporter and binds covalently to a site on the interior only upon transport across the membrane. The other proteins labeled by xylamine reflect its ability to react with nucleophilic centers in a nonspecific manner.

Nerve growth factor treated cells also take up catecholamines and this uptake is inhibited by xylamine with an IC_{50} of 10.0 μM. The proteins labeled by [^{3}H]xylamine in cells treated with nerve growth factor are similar to those labeled in cells not treated with nerve growth factor (10).

When homogenates of PC12 cells are incubated with [^{3}H]xylamine, all of the proteins mentioned above except for the 41 Kd and 54 Kd, become covalently labeled. The proteins labeled in homogenates can be eliminated as candidates for the transporter because their labeling in homogcnates cannot be inhibited by competitive blockers of the uptake of xylamine and catecholamines and most are also found in cells such as fibroblasts that do not have the catecholamine transporter (10). These observations and others based on protection experiments with unlabeled xylamine suggest that the 54 Kd protein is the catecholamine transporter or a subunit of it (B. D. Howard, A. K. Cho, M.-b. Zhang, M. Koide & S. Lin, submitted). Howard et al have proposed that since xylamine can alkylate the transporter only when it passes through

the transporter, the covalent binding site must be distinct from the extracellular site to which xylamine, catecholamines, cocaine, etc, bind reversibly. They suggested that upon homogenization, the transporter undergoes a conformational change such that xylamine can no longer interact with its covalent binding site. Therefore, none of the proteins that are labeled by xylamine in homogenates can be the transporter. This interaction would account for the finding that, even at concentrations up to 1 mM, neither catecholamines nor various specific blockers of catecholamine transport (e.g. cocaine, desipramine) decrease xylamine labeling of these proteins in homogenates.

There is additional evidence that the 54 Kd protein is the transporter. It is the most strongly labeled by [^{3}H]xylamine when intact cells are previously treated with unlabeled xylamine (10 μM) in the presence of cocaine. At high concentrations xylamine can enter the cell by a nontransporter-mediated route (10). The transporter is protected from xylamine by cocaine so that when the unlabeled xylamine and cocaine are removed by washing, the transporter is available for reaction with [^{3}H]xylamine. The other proteins, however, are not labeled because their binding sites have been alkylated by the unlabeled xylamine.

As expected for the catecholamine transporter, the 54 Kd protein is an integral plasma membrane protein (10). It is a glycoprotein that binds to and can be eluted from a wheat germ agglutinin column (B. D. Howard, A. K. Cho, M.-b. Zhang, M. Koide & S. Lin, submitted). In a relevant study, Sallee et al (23), report that a binding site for GBR-12935, a specific inhibitor of DA transport, on canine brain membranes can be solubilized and also fractionated on a wheat germ agglutinin column.

Other evidence for the 54 Kd protein as the transporter comes from studies of PC12 variants. The catecholamine-uptake system in PC12 appears to be very susceptible to genetic variation and it has been easy to isolate PC12 cell variants markedly deficient in transporter-mediated uptake of DA (24). The variants have been obtained nonselectively, or selected by their resistance to certain drugs. Two such variants are called B9 and MPT1. B9 was obtained nonselectively as a subclone of wild-type PC12; MPT1 was selected by its resistance to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). There is evidence that the MPT1 variant has decreased expression of multiple genes. Many of these genes appear to code for proteins involved in neurotransmitter-related activities (e.g. transport, release) in wild-type cells.

When intact MPT1 cells were exposed briefly to [^{3}H]xylamine, proteins were labeled to a much lesser extent than with wild-type cells (24). The same observations were made with other transport-deficient variants. The reduced labeling of proteins in intact variant cells can be attributed to their defective catecholamine transport system; furthermore, it supports the contention that

xylamine binds covalently to PC12 proteins only after entering the cell via the catecholamine transporter under normal circumstances. However, if the variant cells are exposed to [^{3}H]xylamine for a prolonged period of time (30 min), sufficient xylamine enters the cells without benefit of the transporter to result in protein labeling, albeit at reduced levels (B. D. Howard, A. K. Cho, M.-b. Zhang, M. Koide & S. Lin, submitted). In the case of the MPT1 variant, the 54 Kd protein is not labeled and probably not present. This finding is consistent with the fact that many genes involved in specific neurotransmitter-related activities (e.g. tyrosine hydroxylase) are not expressed in MPT1 cells. In the case of the B9 variant, with the exception of the 33 Kd protein, the most prominently labeled protein is the 54 Kd protein. The 33 Kd protein is always the most readily labeled protein; it is not the transporter because it is found in the cells that do not exhibit catecholamine transport. In wild-type PC12 cells the 54 Kd protein is not very prominently labeled. It appears that in B9 the transporter malfunctions so that the DA and xylamine are either transported only part way through the transporter or transported all the way but at a reduced rate. The resulting longer exposure time allows for greater covalent binding of [^{3}H]xylamine to the transporter.

Experiments with whole animals have addressed the nature of the depleting actions of DSP-4 and xylamine, an evaluation of their neurotoxicity, and their effects on pre- and postsynaptic components.

Neuronal Integrity Following NE Depletion

The neurotoxicity of xylamine and DSP-4 have been extensively studied and differences in long-term effects between these compounds and 6OHDA are marked. Catecholamine histofluoresence studies have shown that while xylamine produces a dose-dependent decrease in fluorescence in the cortex, the number of catecholamine-containing processes and/or neuronal terminals was not severely diminished. Although the catecholamine content was substantially decreased, several neuronal elements continued to fluoresce. Furthermore, there was no gliosis or damage to the blood-brain barrier, as is seen after cell damage by 6OHDA (4). Similarly, no gross nonspecific cytotoxic effects were noted in cresyl violet stained sections of cerebellar tissue of rats examined 14 days after a single dose of DSP-4 (50 mg/kg, i.p.). This dose had no effect on the rate or pattern of cerebellar Purkinje cell spontaneous discharge, whereas 6OHDA increased discharge rate (25). NE-uptake activity, used as a marker of presynaptic function, is substantially reduced after 6OHDA. This reduction, measured by NE uptake (26) and by [^{3}H]DMI binding, remained 30 days after 6OHDA dosage (27). Similar losses in NE uptake and DMI binding were noted as soon as 4 hr after xylamine (20 mg/kg) (28) or DSP-4 (50 mg/kg and higher) (29) administration. However, 35 days after the low dose of xylamine, both NE uptake and [^{3}H]DMI binding

returned to control levels (28). The effects of the higher doses of DSP-4 were longer lasting and persisted beyond 35 days (2). Since these actions are presumably due to alkylation of elements of the nerve terminal, recovery would depend on the continued synthesis and transport of protein from the cell body. The difference between the observations with xylamine and DSP-4 may reflect dose dependency, such that recovery from the effects of the 50 to 100 mg/kg dose of DSP-4 takes longer.

Tyrosine hydroxylase (TyrOH) and dopamine-beta-hydroxylase (DBH) have also been used as markers of presynaptic integrity. Five and twenty-one days after 6OHDA treatment (30), DBH activity in the nerve terminals of the tractus intermediolateralis is reduced by 68% and by 85%, respectively. However, even with high dose DSP-4 (50 mg/kg) treatment, enzyme levels in the tractus intermediolateralis recover from a 56% loss on day 5 to only a 28% loss by day 21 (31). Thirty-five days after low-dose xylamine treatment, DBH levels in rat cortex had returned to control values (28).

Booze et al (32), using light or electron microscopy, found no evidence of neuronal fiber degeneration in the brain following high-dose DSP-4 treatment when examined 2 or 5 weeks after treatment. They did report accumulation of TyrOH immunoreactivity in neuronal fibers and suggested that although DSP-4 may not destroy neuronal terminals, it may produce an intraneuronal lesion leading to the accumulation of TyrOH and the depletion of NE in the terminal fields. Fety et al (31) have shown that after a 50 mg/kg dose of DSP-4, TyrOH activity did decrease in the NE terminals of the tractus intermediolateralis, but returned to control levels by 21 days after treatment. Electrophysiological studies on the effects of DSP-4 on rat locus coeruleus have shown that 10 days after DSP-4 administration, mean neuronal firing rates were 50% of control animals. Firing rates recovered to near normal levels by 50 days. In addition, histological examinations revealed no morphological changes in locus coeruleus cell bodies at either the 10 or 50 day time points (33). Thus, in contrast to 6OHDA, the neurotoxicity of xylamine and DSP-4 appear to be less damaging and the terminals are capable of restoring their lost or reduced functions by turnover.

Depletion

A single dose of xylamine (4) or DSP-4 (34, 35) produced a dose-dependent decrease in NE content in rat brain that persisted for as long as eight months (34). The cortex and cerebellum are the most sensitive and the hypothalamus less so (4). When DSP-4 was given to newborn rats, NE levels were reduced in the cortex but increased in the brain stem and cerebellum (35). When given a few days after birth, however, NE levels were reduced throughout the brain. Because these compounds cross the placental barrier, they also may be administered during prenatal development. DSP-4 effects at that time are

similar to those resulting from dosage immediately after birth. These actions are selective for NE as neither DSP-4 nor xylamine deplete DA from rat striata when administered systemically (4, 35). However, xylamine does deplete 5-hydroxytryptamine (5-HT) in the cortex by a mechanism involving the 5-HT uptake system, as evidenced by the protective ability of fluoxetine (12, 28).

NE levels in some peripheral nerves are also reduced after these compounds, but the actions vary with the tissue. In vivo, DSP-4 depleted NE from nerve terminals in the heart (34, 35) but the effects are reversed after two weeks. In vitro, when rabbit aortic rings are exposed to xylamine (1.0 IM) for 60 min, the NE content is reduced by 90% (36). However, in studies with cultured rat superior cervical ganglia, Fischer & Cho (37) found very little depletion in this tissue under conditions in which uptake was substantially inhibited. Vasa deferentia of rats were also somewhat resistant to the effects of these compounds as systemic doses of 50 mg/kg of xylamine reduced NE levels in this tissue by less than 10% whereas uptake activity was reduced by almost 90% (38).

Although the precise mechanism by which xylamine and DSP-4 cause depletion is not established, the aziridinium ion does appear to be involved. DSP-4, when injected into the lateral ventricle, either immediately or after preincubation to form the aziridinium ion, resulted in an equal loss of NE by both preparations. On the other hand, when the preincubated solution containing the aziridinium ion was injected peripherally, there was no loss of central NE, reflecting the inability of the aziridinium ion to penetrate the blood-brain barrier (39). The possible involvement of monoamine oxidase B (MAO B) in the actions of these compounds was suggested by the studies of Gibson (40) who showed that NE depletion in the brains of mice could be blocked by pretreatment with the MAO B selective inhibitor, L-deprenyl. Furthermore, DSP-4 inhibits MAO A and B with Ki values of 6 and 80 μM, respectively (41). However, subsequent studies showed that the protective effects of L-deprenyl may be due to its metabolites, amphetamine and methamphetamine (42, 43), which inhibit neuronal uptake and release amines (44). This can be verified by either pretreatment with the selective MAO B inhibitor, MDL 72,974, which is devoid of releasing activity (45), or pretreatment with L-deprenyl 24 hr before xylamine administration. This latter protocol did not protect against its depleting actions (46). The depleting actions of the nitrogen mustards may involve mitochondria. The extensive binding of [^{3}H]xylamine to mitochondrial peptides led to examination of mitochondrial function in synaptosomes exposed to this compound (9). The results showed that the compound inhibited oxidative phosphorylation through effects on NADPH and succinate dehydrogenase. This inhibition of ATP synthesis relates to NE depletion because of the ATP-dependent storage of this amine. By reduc-

ing levels of ATP in the terminal, the storage capacity of the neuron may be reduced. Sensitivity to and recovery from such an effect would depend on the availability of additional mitochondria which could differ with nerve structure so that variability in depletion with nerve location might be expected.

Effect on Synaptic Levels of NE

Treatment with a single dose of xylamine or DSP-4 may not only reduce intraneuronal levels of NE but also the amount of NE released onto the synapse (28). The reduction of synaptic release should result in decreased postsynaptic function and in vitro studies with peripheral tissues have provided evidence for this notion. Thus, Nedegaard (personal communication) has observed a time-dependent decrease in electrical field–stimulated contraction of rabbit pulmonary artery during xylamine exposure. One interpretation of these findings is a decrease in nerve-stimulated NE release as a result of xylamine-induced depletion. In studies of the brain, thirty-five days after a single 20 mg/kg dose of xylamine, NE levels were reduced by approximately 50% (28). There was also a parallel and sustained decrease in the two principal metabolites of NE, 3,4-dihydroxyphenylethylene glycol (DHPG) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) to 61% and 75%, respectively (28). A 50 mg/kg dose of DSP-4 produced a similar reduction in NE and MHPG levels in rat cortex and hippocampus (47). An in vivo electrochemical study showed that low-dose xylamine (12.5 mg/kg) reduced the extracellular concentration of NE in the rat cortex while having no effect on the extracellular concentration of DA in the striatum (48). Xylamine and DSP-4 reduce presynaptic NE release and as a result, the pre- and postsynaptic cells are exposed to an environment of reduced NE concentration. The long-term depletion and the resulting reduction in synaptic NE concentration may affect pre- and postsynaptic receptor density and function. The changes in these components of the NE synapses in the brain caused by these compounds have been examined and are summarized below.

Receptor Response To Chronic NE Depletion After High Doses of DSP-4

Many investigations have documented the adaptive changes in rat cortical adrenoceptors or other postsynaptic neurons after chronic and substantial decreases in central NE concentrations brought about by high doses of DSP-4. These changes have been examined after extensive depletion (>90%) of NE. Ten days after a 60 mg/kg dose of DSP-4, Dooley et al (49) found cortical alpha-1 adrenoceptors unchanged and regional variations in alpha 2 adrenoceptors. In some areas the Kd for the agonist, *p*-aminoclonidine, was increased and receptor density decreased. The beta-adrenoceptor density in rat

neocortex, measured by [^{3}H]dihydroalprenolol binding, was significantly increased as was isoproterenol-sensitive adenylate cyclase. These changes in beta-adrenoceptor number and function were not reversed by subchronic DMI treatment, but were partially or completely reversed by subchronic administration of clenbuterol, a centrally acting beta-adrenoceptor agonist. Beta-adrenoceptor number and agonist-stimulated cAMP accumulation was shown to return to control levels 50 days after treatment with DSP-4 (49). Other investigators (50–52) have reported a similar up-regulation in brain beta-adrenoceptor density or function following high dose DSP-4 after 10 to 14 days. Johnson et al (53) have reported increased alpha-1 adrenoceptor density 14 days after a single dose of DSP-4, as measured by BE2254 binding, and increased responsiveness to NE-stimulated inositol phosphate accumulation in several rat brain regions.

Thus, the complete loss of NE following a high dose of DSP-4 can result in up-regulation of beta-adrenoreceptors in rat brain. This finding is similar to the beta-adrenoceptor supersensitivity which develops after chronic (18 day) reserpine treatment (54). The increase in receptor density following high dose DSP-4 was paralleled by an increased functional response to agonist activation of the receptor. DMI could not reverse the up-regulation of beta-adrenoceptors, but administration of a beta-agonist (49) or repeated electroshock (55) did. Presumably, DMI is ineffective because of the lack of releasable presynaptic NE, whereas beta-agonists work directly at the postsynaptic beta-adrenoceptor. The mechanism by which repeated electroshock reduced beta-adrenoceptor number following near total depletion of NE by DSP is unknown (55). The observation that beta-adrenoceptor density can be down-regulated in the absence of NE suggests the involvement of other neurotransmitters in controlling beta adrenoceptor density. Similar results have come from experiments with the forced swim test, an animal model of depression, where animals pretreated with a 50 mg/kg dose of DSP-4 did not antagonize the DMI-induced reduction in immobility (56). These studies suggest that NE itself is unnecessary for the antidepressant effect of DMI and questions the role of NE as the sole regulator of beta-adrenoceptor responsiveness and its role in the etiology of depression. A number of authors have suggested that depression results from a defective central noradrenergic system and that antidepressant therapy corrects this deficiency (57). More recently, it has been suggested that the reduction of noradrenergic neuron activity would lead to an adaptive response manifested as beta-adrenoceptor up-regulation or supersensitivity (58, 59). However, the reports of up-regulation of beta-adrenoceptor after NE depletion described involve conditions of total, or near total, loss of neurotransmitter, a highly unlikely situation in clinical depression. Studies of depressed patients have shown that neither urine nor cerebrospinal fluid levels of NE metabolites have varied more than 20% from normal control levels (60, 61, 62).

A more realistic model of depression might be a chonic, partial depletion of NE without loss of terminal integrity as provided by a low dose of xylamine. A single 20 mg/kg dose of xylamine can reduce cortical NE levels by approximately 50%. The depletion persisted for 35 days after treatment with a parallel decrease in synaptic levels of NE, reflected by the reduced levels of DHPG and MHPG. This long-term partial reduction of synaptic NE levels did not alter the number of alpha-1, alpha-2, beta or 5HT-1 receptors in the rat cortex, or D-2 receptors in rat striatum (28). Receptor affinities for the ligands prazosin (alpha-1), rauwolscine (alpha-2) and dihydroalprenolol (beta) were also unaffected, as was the ability of NE or isoproterenol to stimulate cAMP accumulation in cortical slices (M. W. Dudley & B. M. Baron, unpublished results). Chronic administration of DMI (10 mg/kg administered on days 21 to 34 after xylamine) resulted in down-regulation of cortical beta-adrenoceptors in both saline- and xylamine-treated animals. In addition to the down-regulation of beta-adrenoceptors, chronic DMI treatment also decreased NE and isoproterenol stimulated cAMP accumulation in the NE-depleted animals (28).

The xylamine studies show that despite a 50% decrease in available NE, both the number and function of the adrenergic receptors remained unchanged, indicating that there is a good deal of plasticity in this neuronal system. The ability of the NE-depleted system to respond to chronic DMI treatment in a manner similar to that reported in normal animals (63), would indicate that the presynaptic terminals are intact and functional. Thus, unless clinical depression involves extensive (>90%) depletion of NE, changes in beta-adreno-receptors density or function may not occur simply as a result of loss of NE.

Rats treated with DSP-4 or xylamine (64) have attenuated responses in paradigms that measure their responsiveness to threatening stimuli, such as the effect of hallucinogens to potentiate their normal tendencies to avoid novel or open areas. These observations may be of importance in understanding the role of NE in neophobia and agorophobia.

CONCLUSIONS

Xylamine and DSP-4 are site-directed alkylating agents with affinities for the neuronal uptake transporter. They are accumulated within the nerve ending and appear to affect transport of catecholamine and deplete catecholamine, possibly by reducing mitochondrial function. The specificity of these compounds for NE and 5-HT neurons in vivo is probably due to their affinity for the specific transporter which concentrates the compounds in the nerve terminal and reduces NE levels. As they enter cells the compounds can also form covalent bonds with nearby thiol functions, including one on the carrier or a

regulatory protein. This interaction is being used in studies isolating the transporter. The depleting actions of these compounds has been used to determine the role of NE in certain behavior responses and to assess the mechanism of action of antidepressants. One of the problems with these type of alkylating agents is their lack of specificity and there is recent evidence that indicates xylamine forms bonds with other elements of the adrenergic synapse such as the uptake-2 transporter and the alpha-1 receptor so that care must be taken in the interpretation of such experiments.

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