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The Effect of Etidocaine on Spontaneous and Evoked Release of Norepinephrine from Rat Brain Synaptosomes[1] (42174)

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Abstract. Local anesthetics have been shown to have an effect on neurotransmission. In this study we examined the effect of a local anesthetic, etidocaine, on the uptake, efflux, and release of norepinephrine (NE) from central nerve terminals. The studies were performed on synaptosomes and vesicles prepared from rat brains. Etidocaine 10^{-4} *M* inhibited synaptosomal accumulation of [^{3}H]NE and did not significantly effect vesicular accumulation of this neurotransmitter. This concentration of etidocaine also augmented efflux of norepinephrine from synaptosomal preparations. This augmented efflux was primarily due to an increase in the deaminated metabolite 3,4-dihydroxyphenylglycol (DOPEG). The presence of etidocaine did not significantly alter the release of NE from synaptosomes superfused with high potassium (40 m*M*), a calcium-dependent exocytotic release process. These results indicate that in the central nervous system, as previously demonstrated in the peripheral nervous system, high concentrations of etidocaine alter vesicular storage of NE, resulting in more NE leaking into the cytoplasm where it is metabolized to an inactive metabolite.

In peripheral vascular tissue, etidocaine, one of the amide-linked, local anesthetics, causes a dose-dependent inhibition of norepinephrine (NE) release following sympathetic nerve stimulation (1). In addition etidocaine has been shown to increase the efflux of [^{3}H]norepinephrine and the deaminated metabolites from peripheral vascular tissue. Thus local anesthetics are postulated to cause vesicles to become leaky and thereby making catecholamines available for intraneuronal deamination by the catabolic enzyme monoamine oxidase (MAO).

Enhanced neurosecretion has been reported previously with other types of anesthetics. Quastel *et al.* [reviewed in (2)] reported that certain types of anesthetics and alcohols increase the frequency of miniature endplate potentials at the neuromuscular junction, and that this increase in neurosecretion did not require extracellular Ca^{2+}.

Since local anesthetics are used for both peripheral and central nervous system blockade we wanted to determine if amide-linked local anesthetics affect catecholamine storage in central neurons similar to that observed in peripheral tissues. To accomplish this objective we examined the effect of etidocaine on rat synaptosomes. Etidocaine was selected as a representative of this class of anesthetics because of our previous work in peripheral neurons.

Materials and Methods. *Synaptosome preparation for [^{3}H]NE accumulation studies.* Synaptosomes were prepared by differential centrifugation from whole brain preparations obtained from decapitated mature male Sprague–Dawley rats by the method of Gray and Whittaker (3). After differential and discontinuous gradient centrifugation the purified synaptosomes were obtained at the interface between the 0.8 and the 1.2 *M* sucrose gradient phases. The synaptosomes were then pelleted by ultracentrifugation and resuspended in 0.32 *M* sucrose. The synaptosome preparation was diluted with 4 vol of Krebs

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bicarbonate solution of the following composition (in m*M*) NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 5.6, $Na_2S_2O_5$ 0.053, and disodium EDTA 0.013. The final concentration was 2.0 ml of solution per gram of original brain tissue (wet weight).

The synaptosome suspensions (50 μl) were warmed to 37°C for 3 min in a shaking water bath underneath a stream of 95% O_2/5% CO_2 gas mixture. The incubations were started by the addition of 60 μl of [^{3}H]NE (final NE concentration, 2×10^{-7} *M*. The incubations were for varying periods of time (up to 6.0 min) and were terminated by diluting the incubate with Krebs bicarbonate and then filtering it through 0.45-μm nitrocellulose filters according to the procedure of Silbergeld (4). Filters containing synaptosomes were then solubilized and counted by a liquid scintillation counter (Packard Model 300C).

Synaptosome preparation for [^{3}H]NE efflux studies. In order to study NE efflux and release synaptosomes were preloaded by incubating for 10 min in 7×10^{-7} *M* [^{3}H]NE. Synaptosomes were pelleted by centrifugation, washed with Krebs bicarbonate solution, and then poured into a glass chamber with a 0.8-μm nitrocellulose filter supported on a fritted glass support.

The synaptosomes and filters were then washed with 10 ml of Krebs bicarbonate solution and covered with a layer of Krebs (total volume 1.0 ml). The chamber was then closed and connected to a peristaltic pump and the preparation was superfused at the rate of 1.0 ml/min with Krebs bicarbonate solution aerated with 95% O_2 and 5% CO_2, at 37°C.

Following a 10-min superfusion period the superfusate was collected with a fraction collector at 1- or 2-min intervals. In some experiments the potassium concentration of the Krebs solution was increased to 40 m*M* by substituting KCl for NaCl. At the termination of the superfusion procedure the filter chambers were pumped dry and the filters were counted using a liquid scintillation counter.

Vesicle preparations. Synaptic vesicles were prepared from these synaptosome preparations following osmotic rupture and discontinuous sucrose gradient centrifugation according to the procedure of Whittaker *et al.* (5). The vesicles were obtained from the 0.4 *M* (gradient layer), pelleted, and then resuspended in 130 m*M* phosphate buffer at pH 7.4.

Vesicles from each rat brain were suspended in 1.5 ml of phosphate buffer. Aliquots of this preparation (300 μl, 44 to 121 μg of protein) were incubated in the presence of [^{3}H]NE, 7×10^{-7} *M*, 1 m*M* ATP and 1 m*M* $MgCl_2$ (final concentration). The final incubation volume was 930 μl. The incubation was terminated by dilution and the vesicles were filtered in a manner similar to that previously described for synaptosomes.

Chromatographic analysis of NE metabolites. In some experiments unmetabolized [^{3}H]NE was separated from its tritiated metabolites. Synaptosomes were extracted with 3.0 ml of 1 *N* acetic acid solution containing 3.0 m*M* EDTA and 1.1 m*M* ascorbic acid (6). Cold carrier metabolites were added to both the extract and the superfusate samples (7.0 ml). The pH was adjusted to 8.4 with Na_2CO_3 and the samples were passed over a cascade of alumina and Dowex columns according to the procedure of Graefe *et al.*, 1973 (7).

Aliquots of the column eluates (1.0 ml) were taken to determine the quantity of tritium in the *O*-methylated fraction, the 3,4-dihydroxyphenylglycol (DOPEG) fraction and the unmetabolized [^{3}H]NE fraction. Internal standards containing [^{3}H]NE not exposed to synaptosomes were used to monitor for nonenzymatic degradation of NE during the experimental procedure. Validation of crossover and percentage recovery of tritiated metabolites using this procedure has previously been published (8).

Drugs and statistical analysis. The following drugs were used in these experiments: cocaine hydrochloride (Mallinckrodt), reserpine (Sigma), and etidocaine HCl (Astra Pharmaceutical). For each synaptosome and vesicle preparation utilized in a [^{3}H]NE uptake study all experimental treatment procedures were tested at least three times. The average of these values obtained from each preparation was then considered as an experimental replication and the number of these experimental replications is designated by the value of *n* for each experiment. Statistical analysis was performed using multivariate analysis of variance. A probability level of less than 0.05 was considered significant. The protein content of syn-

aptosomal and vesicle samples was determined by the method of Lowry (9) after solubilization in 2 N NaOH. Bovine serum albumin was used as a standard. An Eadie–Scatchard plot was used in determination of K_m.

Results. The effect of etidocaine on the synaptosomal accumulation of [^{3}H]NE was determined in four preparations. Etidocaine (1×10^{-4} M) significantly inhibited [^{3}H]NE accumulation from 4.5 ± 0.3 to 3.2 ± 0.5 pg/mg protein.

In an effort to determine if this inhibitory activity was at the neurolemma or at the vesicular level we further purified synaptic vesicles from these whole brain synaptosomes and examined the effect of different concentrations of etidocaine (10^{-7} to 10^{-4} M) on vesicular accumulation of NE. These vesicles were first characterized for their ability to accumulate norepinephrine. Incubation of the vesicles in the presence of [^{3}H]NE over a concentration range of 1×10^{-8} to 1×10^{-5} M resulted in a K_m of 3×10^{-6} M. Accumulation of [^{3}H]NE was time-dependent with the greatest rate of uptake occurring within the first 2 min of incubation. The uptake was dependent on 1 mM Mg^{2+} and ATP, in the absence of ATP and magnesium the uptake activity was decreased by $62.4 \pm 12\%$. Average vesicular uptake under our standard incubation conditions was 18.3 ± 3.4 pmole NE/mg protein.

Etidocaine (10^{-9} to 10^{-4} M) did not significantly inhibit NE accumulation by the synaptic vesicle preparations (Table I). But, reserpine (1×10^{-6} M), a classical blocker of vesicular uptake, inhibited the accumulation of NE by $84.7 \pm 3.9\%$ (Table I).

To determine if etidocaine altered synaptosomal efflux we superfused synaptosomes with Krebs bicarbonate solution containing 1 $\times 10^{-5}$, 5×10^{-5}, or 1×10^{-4} M etidocaine. The highest concentration of etidocaine significantly augmented the efflux of tritum from synaptosomes preincubated in [^{3}H]NE (Fig. 1). This increased efflux was reversible. Lower concentrations of etidocaine had no significant effect on tritium efflux.

To determine if this enhanced outflow of tritium was unmetabolized NE or its tritiated metabolites, the superfusate and tissue extracts were analyzed following separation by column chromatography (Table II). The augmentation of tritium efflux could be accounted for by a significant increase in [^{3}H]DOPEG and a smaller increase in the formation of the [^{3}H]-O-methylated compounds. There was no significant effect of etidocaine on release of unmetabolized [^{3}H]NE from these synaptosomes. There was also a significant decrease in the amount of unmetabolized [^{3}H]NE and a significant increase in the amount of [^{3}H]DOPEG extracted from the tissue at the end of the superfusion.

High potassium (40 mM) increased the release of tritium from synaptosomes prelabeled with [^{3}H]NE from 0.15 ± 0.02 to 0.58 ± 0.04 pmole. The presence of etidocaine (10^{-4} M) did not significantly alter this potassium evoked release of tritium (Fig. 2).

Discussion. Using synaptosomes and synaptic vesicles obtained from rat brains we have examined the effect of etidocaine on NE uptake, storage, and release using [^{3}H]NE. We have demonstrated that 10^{-4} M etidocaine reduced NE accumulation by 30%. At this concentration of etidocaine synaptosomal uptake was inhibited but vesicular uptake was not significantly affected. These data suggest that the anesthetic at high doses blocks uptake at the neurolemma but not at the vesicular

TABLE I. THE EFFECT OF ETIDOCAINE ON THE UPTAKE OF [^{3}H]NE BY SYNAPTIC VESICLES[a] PREPARED FROM WHOLE RAT BRAINS

Drug concentration (M):	10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Etidocaine (n = 6)	nt[b]	nt	116.3 ± 8.8	114.0 ± 7.7	114.6 ± 6.9	82.1 ± 9.6
Reserpine (n = 4)	91.4 ± 4.4	46.6 ± 5.1	42.5 ± 8.1*	15.3 ± 3.9*	nt	nt

[a] Results expressed as % of control values run in the absence of drug (mean ± standard error). Control values for etidocaine and reserpine experiments were 0.45 ± 0.10 and 0.47 ± 0.19 $p$mole NE/filter respectively.

[b] nt, not tested.

* Difference from control is statistically significant $P < 0.05$.

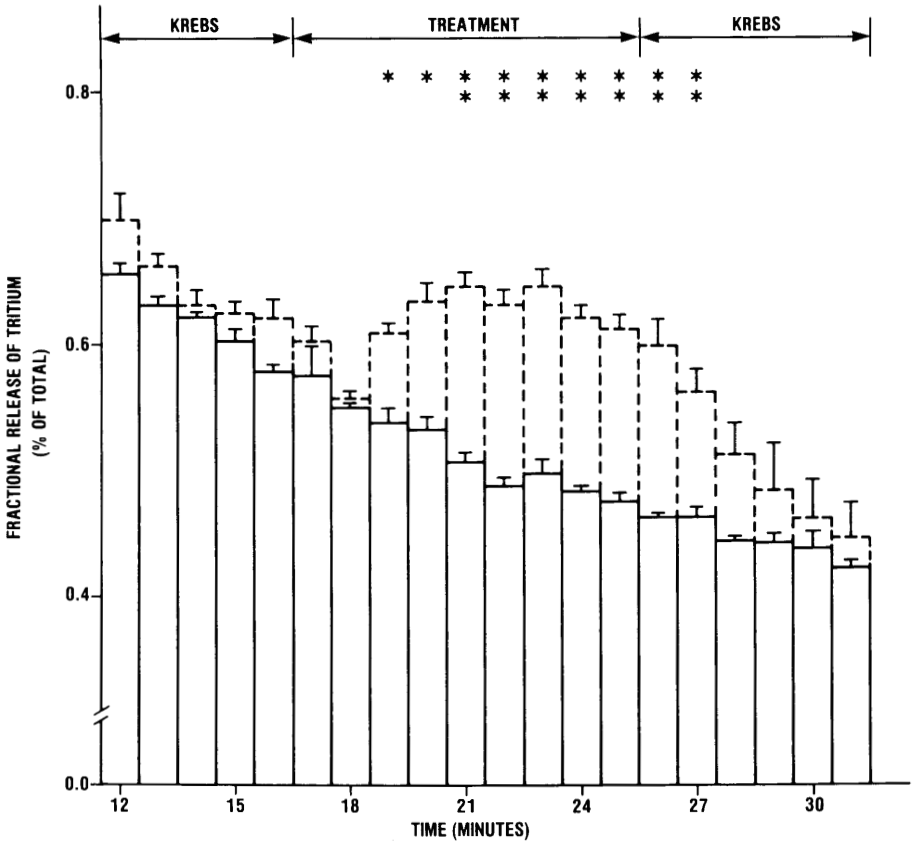

FIG. 1. The effect of 1×10^{-4} *M* etidocaine on tritium release from synaptosomes. Synaptosomes were superfused with Krebs bicarbonate solution and 1-ml fractions were collected each minute. Arrows represent treatment application and termination. The experimental set up required a period of 45 sec for the treatment solution to reach the synaptosome chamber.

membrane. To support this observation the action of etidocaine was compared with that of reserpine, a classical blocker of NE accumulation by synaptic vesicles. Although reserpine had a potent inhibitory action on vesicular accumulation of NE, etidocaine was without effect in this preparation. The observed effect of etidocaine on decreased synaptosomal accumulation of NE may be due in part to a decrease in the synaptosomes ability to store NE since we also demonstrated that in the presence of etidocaine there was an increase in spontaneous release of tritium.

The observed spontaneous increase in release of tritium was primarily due to an increase in the formation of the deaminated metabolite DOPEG. Etidocaine appears to enhance vesicular release which in turn results in higher levels of cytoplasmic NE available for enzymatic degradation by monoamine oxidase. Evoked release due to 40 m*M* K^+, a calcium-dependent process, was not significantly altered in our preparation by the presence of etidocaine suggesting that etidocaine altered storage of vesicular bound NE, not exocytotic release of NE.

In this work we used vesicles prepared from whole rat brain. Slotkin *et al.* (10) have previously shown, using selective neurotoxins, that the uptake of NE by these preparations occurs in vesicles from NE and Dopamine neurons, not in vesicles from serotonin neurons. Slotkin *et al.* also reported that vesicles prepared from whole brains have similar catecholamine transport systems, compared to those isolated from splenic nerve and a wide variety of other peripheral sympathetic tissues. Therefore, our objective was to determine if

TABLE II. EFFECT OF ETIDOCAINE ON EFFLUX OF [^{3}H]NE AND ITS METABOLITES FROM SUPERFUSED SYNAPTOSOMES

	Picograms in superfusate		% of total	
	Control	Etidocaine	Control	Etidocaine
		Superfusate (n = 6)		
Total efflux[a]	151.5 ± 5.6	195.6 ± 11.0**	100	100
O-Methylated	25.5 ± 2.3	33.1 ± 1.6**	17.15 ± 2.06	17.3 ± 1.5
DOPEG	29.5 ± 0.9	56.0 ± 3.8**	19.6 ± 0.9	28.5 ± 0.8**
NE	88.9 ± 4.1	90.5 ± 4.5	58.6 ± 1.1	43.6 ± 1.0**
		Tissue (n = 4)		
Total content[a]	3564.0 ± 180.7	3357.6 ± 172.7	100	100
O-Methylated	183.1 ± 17.3	189.8 ± 7.1	5.1 ± 0.2	5.7 ± 0.1
DOPEG	51.2 ± 10.8	71.8 ± 11.3*	1.4 ± 0.2	2.1 ± 0.3*
NE	2661.1 ± 125.7	2424.3 ± 130.4*	74.7 ± 0.7	72.2 ± 0.9*

[a] Sample total before column fractionation.
* Difference from control is statistically significant $P < 0.05$.
** Difference from control is statistically significant $P < 0.01$.

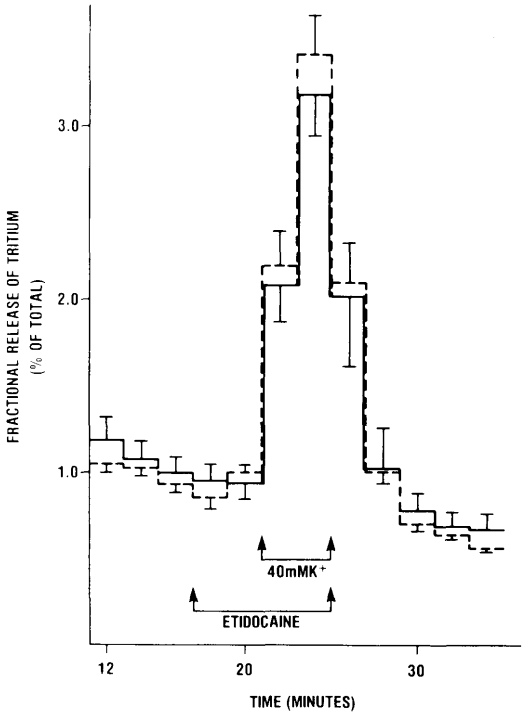

FIG. 2. Representative experiment demonstrating the influence of 1×10^{-4} M etidocaine on tritium release from prelabeled synaptosomes during 40 mM K^+ depolarization. Fractions (2.0 ml) were collected every 2 min. Arrows indicate application and termination of 1×10^{-4} M etidocaine.

etidocaine had the same effect on synaptosomes and vesicles prepared from whole brain preparations as observed in peripheral neurons.

Our results with synaptosomes under basal release conditions are similar to the previously reported studies using peripheral vascular nerves (1). In these earlier studies etidocaine at concentration of 3×10^{-5} M significantly altered efflux and metabolism of [^{3}H]NE from peripheral sympathetic nerve endings. In these experiments under basal conditions etidocaine significantly enhanced efflux of deaminated metabolites of NE.

There was a difference in the results of norepinephrine release in the presence of high potassium between our synaptosome studies, where we found no significant changes in release, and the peripheral study, where the radioactivity of the superfusate was significantly augmented in the presence of etidocaine. Chromatographic analysis showed that this augmentation was due to an increase in release of DOPEG. Also in these peripheral nerves there was no evidence that etidocaine inhibited NE uptake. The reasons for the discrepancy remain unknown, but a similar conclusion can be drawn from both studies in that the observed effect of etidocaine may be due to the result of altered storage of NE in vesicles, re-

sulting in more NE leaking into the cytoplasm for metabolism by monoamine oxidase.

Another local anesthetic, cocaine, has a much greater effect on blocking neuronal accumulation of NE both peripherally and centrally. High concentrations of cocaine also have been shown to augment leakage and cause displacement of NE from adrenergic nerve endings in blood vessel walls (11), and strips of rat vas deferens (7). It is possible that other structurally related local anesthetics may also cause enhanced efflux of NE.

In summary, our data indicate that etidocaine has effects on central nervous system stores of catecholamines which are qualitatively similar to effects observed in peripheral sympathetic nerve endings. It is postulated from these observed *in vitro* effects using synaptosome preparations that high concentrations of etidocaine may alter uptake and storage of NE in central catecholamine containing neurons following its administration to man.

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