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IRREVERSIBLE BLOCKADE OF HIGH-AFFINITY CHOLINE UPTAKE IN RAT BRAIN BY *N*-ETHOXYCARBONYL-2-ETHOXY-1,2-DIHYDROQUINOLINE (EEDQ)

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Abstract—*N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), an agent that causes irreversible covalent modification of protein carboxyl residues, has been used previously to produce irreversible occlusion of neurotransmitter receptors as well as other cellular proteins. The present investigation was undertaken to ascertain the mechanism by which EEDQ inhibits stimulus-dependent acetylcholine (ACh) release from rat brain hippocampal synaptosomes. Brief pretreatment with EEDQ (up to 100 μM) eliminated completely calcium-evoked [^{3}H]acetylcholine ([^{3}H]ACh) release and reduced *de novo* synthesis of transmitter by greater than 90%. Studies revealed that pretreatment with EEDQ *in vitro* caused a time- and concentration-dependent inhibition of high-affinity [^{3}H]choline uptake (HACU) by synaptosomes. EEDQ-induced inhibition of HACU was not reversed by repeated tissue washing; however, co-incubation with hemicholinium-3, a highly specific and reversible inhibitor of HACU, protected against EEDQ-induced inhibition of HACU, as well as the loss of stimulus-dependent [^{3}H]-ACh release. *In vivo* administration of EEDQ (20 mg/kg, s.c.) to rats caused marked reductions (46–65%) in synaptosomal HACU as well as the number of membrane binding sites for the muscarinic cholinergic antagonist L-[benzilic-4,4′-^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) in the hippocampus and striatum. Treatment with atropine (100 mg/kg) prevented the reduction in [^{3}H]QNB binding but did not influence EEDQ-induced inhibition of HACU. Taken together, these results indicate that EEDQ causes a direct and irreversible inhibition of high-affinity choline transporters on CNS cholinergic nerve terminals and, therefore, may be a useful investigational tool for characterization of the turnover and regulation of this transporter protein *in vivo*.

Key words: acetylcholine; brain; choline uptake; *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ); hemicholinium-3; synaptosomes

Agents that promote irreversible chemical modification of proteins often provide a useful means to investigate the turnover and functional role(s) of catalytic enzymes, membrane-bound receptors, and other cellular proteins. One such agent, EEDQ†, has been reported to inactivate proteins through direct modification of carboxyl residues that are essential for protein function [1, 2]. In view of this rather "non-specific" mechanism for protein inactivation, it is anticipated that EEDQ could influence a myriad of diverse proteins, including those that play a role in chemically mediated neurotransmission. Indeed, within the CNS, it has been reported that EEDQ inactivates a variety of neurotransmitter receptors, including α-adrenergic [2–4], dopaminergic [5], serotonergic [6] and muscarinic cholinergic [7–10] receptors. With regard to studies *in vivo*, EEDQ appears to be particularly well-suited in view of the apparent efficiency with which this agent penetrates the blood–brain barrier following systemic administration.

Despite the predominant use of EEDQ for investigations related to the functional roles and reserve populations of neurotransmitter receptor families, it seems plausible that EEDQ could also be used to alter a variety of non-receptor proteins within the plasmalemmal membrane of neurons. Indeed, the actions of EEDQ on CNS cholinergic neurons appear to substantiate this view. Previous reports have revealed that EEDQ influences ion channels that are gated by nicotinic cholinergic receptors ([^{3}H]perhydrohistrionicotoxin binding sites) [11] and inactivates the cholinergic degradative enzyme acetylcholinesterase [1] in addition to the aforementioned actions on mAChR. With regard to mAChR, EEDQ has been reported to interact preferentially with those receptor subtypes that bind the antagonist pirenzepine with high affinity [9, 10]. In view of this purported selectivity, we recently attempted to irreversibly occlude presynaptic muscarinic autoreceptors in rat hippocampus with EEDQ in order to assess the reserve capacity within this presynaptic receptor population [12]. Surprisingly, EEDQ caused a near-complete attenuation of

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† Abbreviations: ACh, acetylcholine; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; HACU, high-affinity choline uptake; mAChR, muscarinic cholinergic receptors; [^{3}H]QNB, L-[benzilic-4,4′-^{3}H]quinuclidinyl benzilate.

calcium-stimulated [^{3}H]ACh release, which rendered it unsuitable for that particular application. Nevertheless, since no such cholinolytic action has been reported previously for EEDQ, the present investigation was undertaken in order to ascertain the mechanism through which EEDQ obliterates exocytotic release of ACh from CNS cholinergic nerve terminals. As outlined in this report, our results indicate that EEDQ causes direct irreversible inactivation of high-affinity choline transporters in CNS cholinergic nerve terminals following *in vitro* or *in vivo* treatment and that protection against this action alleviates the marked attenuation of ACh synthesis and release that is caused by EEDQ.

MATERIALS AND METHODS

Materials. [Methyl-^{3}H]choline chloride (sp. act. 87.6 Ci/mmol) and [^{3}H]QNB (sp. act. 36.4 Ci/mmol) were purchased from DuPont New England Nuclear, (Boston, MA). Atropine sulfate, choline oxidase, hemicholinium-3 and physostigmine hemisulfate were purchased from the Sigma Chemical Co. (St Louis, MO). EEDQ, which was purchased from Research Biochemicals, Inc. (Natick, MA), was routinely prepared fresh daily in DMSO as 1000-fold stock solutions and diluted to the desired final concentrations in assay buffer. Unless otherwise indicated, all other drugs were dissolved in assay buffer.

Tissue preparation and EEDQ treatments. Male albino Sprague–Dawley rats (150–300 g from Harlan Laboratories) were housed in environmentally controlled rooms with unrestricted access to food and water. On the morning of each experiment, rats were transferred to the laboratory and injected with drug(s) or used immediately. Animals were decapitated and brains were quickly removed and placed upon an ice-cold surface for dissection. Forebrain tissues were dissected and transferred into approximately 100 vol. of an ice-cold aqueous sucrose solution (0.32 M). Shortly thereafter, tissues were homogenized with a Potter–Elvehjem tissue grinder (approx. 30 rpm), and a crude mitochondrial fraction was isolated by differential centrifugation (1100 *g*, 10 min; S_1 removed and centrifuged at 17,400 *g*, 15 min) at 4°. For EEDQ treatments *in vitro*, the synaptosomal fraction (P_2) was resuspended at a concentration of approximately 22 mg/mL (original tissue wet weight) by gentle trituration in a HEPES-buffered salt solution (see below) prior to EEDQ treatment.

Drug treatments *in vivo* were administered via subcutaneous injections in a vol. of 0.1 mL/100 g body weight. For studies in which animals were treated with atropine and EEDQ, the former drug was injected 15 min prior to the latter agent. Following a specified period of time, animals were decapitated and tissues were prepared as described above. For tissue treatments with EEDQ *in vitro*, tissue (final concentration of 4.4 mg/mL based on original wet weight) was mixed with EEDQ (1000-fold stock in DMSO) or DMSO [final concentration of DMSO was 0.1% (v/v) in all samples] and incubated in a HEPES-buffered salt solution at 37° under an O_2 atmosphere. At the specified time, the reaction was terminated by adding a 4-fold excess of cold buffer, and synaptosomes were collected by centrifugation (17,400 *g*, 15 min). The pellet was washed (up to three times) with a 200-fold excess of cold buffer in order to remove any residual unbound drug. Upon final resuspension in buffer, tissue homogenates were assayed for high-affinity choline uptake and the remainder was frozen for subsequent radioligand binding analysis with [^{3}H]QNB.

Estimation of [^{3}H]*acetylcholine release.* Hippocampal synaptosomes were isolated as described above and incubated for 10 min (37°) in a calcium-free bicarbonate-buffered salt solution containing (in mM) NaCl (137), KCl (4.7), $MgCl_2$ (1.0), EGTA (0.01), glucose (10), NaH_2PO_4 (1.25), $NaHCO_3$ (25) and physostigmine hemisulfate (0.1) that was purged continuously with a 95 : 5 mixture of O_2/CO_2 (pH 7.4 at 37°). Immediately thereafter, [*methyl*-^{3}H]choline chloride (50 nM final concentration) was added and synaptosomes were incubated for an additional 15 min. Aliquots of [^{3}H]choline-labeled tissue (approximately 10–15 mg wet weight) were subsequently transferred onto glass fiber filters (Whatman GF/B) within a temperature-controlled Plexiglas block and superfused (1 mL/min) with oxygenated buffer (37°) to achieve a stable rate of [^{3}H]ACh efflux. Following the washout period, superfusates from each chamber (4-min fractions) were collected and saved for [^{3}H]ACh analysis. The superfusion protocol (see Fig. 1) consisted of the following sequential buffer infusions: (1) EGTA-containing buffer from 0 to 29 min; (2) EGTA-free buffer with 1 mM $CaCl_2$ from 29 to 39 min (stimulus period); and (3) return to EGTA-containing buffer from 39 to 48 min. At the end of superfusion, filters containing synaptosomes were extracted by brief ultrasonic disruption in 1 mL of 0.1 M HCl and then neutralized with 50 mM HEPES (pH 7.4) and 0.1 N NaOH. [^{3}H]ACh in superfusate fractions and tissue extracts was isolated and measured by a modification of the choline oxidase-ion pair extraction method [13] as recently described [14]. Fractional release of transmitter was expressed as the ratio (multiplied by 100%) of [^{3}H]ACh in each fraction to the total synaptosomal content of [^{3}H]ACh at the time each fraction was collected. Basal (non-stimulated) [^{3}H]-ACh efflux was averaged from fractional release values for the 8 min-period preceding infusion of calcium-containing buffer. Net calcium-evoked [^{3}H]-ACh release was calculated from fractional release values by estimating area under the curve (above baseline) as described previously [15].

[^{3}H]*Choline uptake assay.* Tissue aliquots (100 μL) were added to triplicate tubes containing 0.3 mL of HEPES-buffered salt solution (same as above except that sodium bicarbonate was replaced with 25 mM HEPES) without or with (blank) 10 μM hemicholinium-3. The mixture was warmed to 30° (1 min), and the reaction was initiated by adding 100 μL of [^{3}H]choline (see figure legends for final concentration). After 3 min, the reaction was stopped by adding 1 mL of cold buffer containing 10 μM hemicholinium-3, filtering the reaction mixture through Whatman GF/B filter paper under negative pressure, and rinsing filters three times with cold buffer (2 mL). To determine [^{3}H]choline uptake, filters

were extracted overnight in 0.2 N NaOH, neutralized with HCl and counted in Ecolume, (ICN Biomedicals, Inc.). To estimate [^{3}H]ACh formation, filters were sonicated in 0.1 M HCl and carried through the choline oxidase-ion pair extraction method as described above. Net uptake or synthesis was defined as the difference between total and blank (hemicholinium-3) samples (blanks were less than 30% of total at all [^{3}H]choline concentrations for both assays).

[^{3}H]*QNB binding assay*. Frozen aliquots of synaptosomal homogenates were thawed, and tissue was collected by centrifugation (20,000 *g*, 10 min). Tissue pellets were resuspended in 50 mM NaH_2PO_4 with a Brinkmann polytron (10 sec at setting 5), and membranes were re-collected by centrifugation. Final pellets were resuspended in phosphate buffer at concentrations between 7.5 and 30 mg/mL depending upon the anticipated loss of binding sites associated with EEDQ treatment. Aliquots of tissue and drugs were diluted in 0.9 mL of phosphate buffer, and the binding reaction was initiated by adding [^{3}H]QNB (0.01 to 1.0 nM final concentration). Following a 1-hr incubation at 37°, tissue-bound radioactivity was separated from unbound ligand by vacuum filtration through Whatman GF/B filters using a Brandel cell harvester and washed three times with 3 mL of ice-cold phosphate buffer. Filter-bound tritium was extracted with NaOH, acidified with an excess of HCl and counted in 5 mL of Ecolume. Nonspecific ligand binding (in the presence of 3 μM atropine) comprised approximately 8% of total bound radioactivity under these conditions.

Data analysis and statistical comparisons. Values for specific [^{3}H]QNB binding and [^{3}H]choline uptake were normalized with total tissue protein as determined by the method of Lowry *et al.* [16], using bovine serum albumin standards. Data from saturation binding experiments were analysed by nonlinear regression analysis of unweighted data using INPLOT (GraphPad Software) according to the method described previously [17]. For statistical analysis of possible differences between experimental and control groups, a one-way analysis of variance (ANOVA) followed by Dunnett's test to isolate group differences was carried out. Differences with a P value of less than 0.05 were accepted as a minimum level for statistical significance.

RESULTS

The effect of EEDQ on stimulus-dependent [^{3}H]-ACh release was studied *in vitro* using superfused synaptosomes from rat hippocampus. In this model system, isolated nerve endings are deprived of extracellular calcium (zero $CaCl_2$/10 μM EGTA buffer), and exocytotic release of transmitter is evoked by brief re-introduction of 1 mM $CaCl_2$ into the superfusion medium. As shown in Fig. 1A, calcium infusion caused a robust and reversible increase in the rate of [^{3}H]ACh release from calcium-naive synaptosomes. However, following pretreatment with EEDQ (30 μM), calcium failed to elicit any measurable increase in synaptosomal transmitter output (Fig. 1B). In contrast with stimulus-dependent [^{3}H]ACh release, the fractional

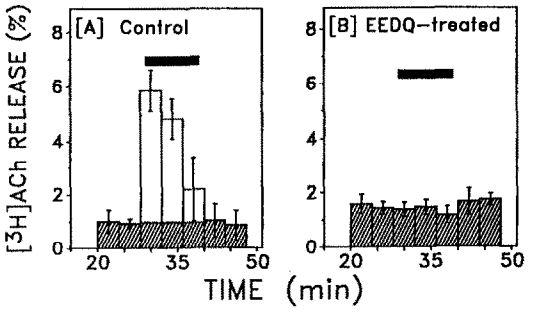

Fig. 1. Attenuation of calcium-evoked [^{3}H]acetylcholine release from hippocampal synaptosomes following EEDQ (30 μM) pretreatment *in vitro*. Calcium-naive synaptosomes were incubated with 0.1% DMSO (panel A) or 30 μM EEDQ in 0.1% DMSO (panel B) for 30 min and then were washed twice prior to labeling with [^{3}H]choline. Bar heights represent percent fractional [^{3}H]ACh efflux (mean ± SEM of four chambers) under basal (calcium-free) conditions as well as stimulated transmitter release during the infusion of calcium-enriched buffer (horizontal bar). Open bars represent net stimulated (calcium-dependent) [^{3}H]ACh release above basal efflux. The total amount of [^{3}H]ACh recovered from superfused tissues (sum of all superfusate fractions plus residual tissue content) was 318,200 ± 17,900 dpm (DMSO-treated) and 27,800 ± 9,900 dpm (EEDQ-treated).

rate of basal (non-stimulated) transmitter efflux appeared to be unchanged or modestly increased following EEDQ treatment (0.44 ± 0.07 vs 0.25 ± 0.06%/min for EEDQ-treated and control tissues, respectively); however, since fractional efflux rate is based upon total tissue [^{3}H]ACh content, it should be noted that the actual rate of transmitter efflux under calcium-free conditions (i.e. dpm [^{3}H]-ACh released/min) is substantially lower in EEDQ-treated synaptosomal preparations. This difference between fractional [^{3}H]ACh release and the amount released is readily appreciated if one considers that total tissue [^{3}H]ACh content was reduced by greater than 90% in EEDQ-treated synaptosomes (see legend of Fig. 1).

Studies were undertaken to ascertain the basis for EEDQ-induced depletion of synaptosomal [^{3}H]ACh stores. As shown in Fig. 2, pretreatment with EEDQ or hemicholinium-3 caused marked reductions in high-affinity synaptosomal [^{3}H]choline uptake. However, repeated attempts to reverse the inhibition by these agents (i.e. washing treated tissues with large volumes of drug-free buffer) revealed a significant difference in the nature of the inhibitory actions exerted by these agents. Inhibition of high-affinity [^{3}H]choline uptake following hemicholinium-3 pretreatment was readily reversible and even exhibited a modest but non-significant increase over control values following three washes. By comparison, EEDQ inhibition of high-affinity [^{3}H]-choline uptake was not reversed by an identical washing procedure (Fig. 2). Additional features of EEDQ actions are shown in Figs. 3 and 4. First, it is readily apparent that the magnitude of [^{3}H]choline uptake inhibition was highly-dependent upon the

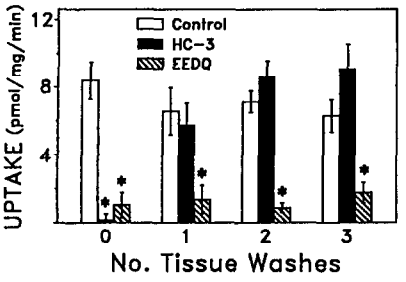

Fig. 2. Differential recovery of [^{3}H]choline uptake by repeated tissue washing following treatment with hemicholinium-3 or EEDQ. Hippocampal synaptosomes were pretreated for 20 min with vehicle (open bars), 10 μM hemicholinium-3 (solid bars) or 10 μM EEDQ (striped bars) and then were washed (up to three times) with a 200-fold excess of fresh buffer. Each bar represents the average (mean ± SEM) of triplicate determinations from three separate experiments. Uptake was determined in the presence of 1 μM [^{3}H]choline as described under Materials and Methods. Asterisks (*) indicate a significant difference ($P < 0.05$) from corresponding control groups.

concentration of EEDQ, as well as the duration of the pretreatment period. When the pretreatment period was limited to 20 min, EEDQ (0.3 to 100 μM) caused a graded inhibition of high-affinity [^{3}H]-choline uptake with an apparent IC_{50} of 9.8 μM (Fig. 3A). This reduction in uptake was accompanied by a substantial decrease in the maximal rate of [^{3}H]-choline transport (estimated values for V_{max} of 24.5 ± 5.0 and 13.1 ± 6.3 pmol/mg/min for control and EEDQ-treated tissues, respectively) with no concomitant change in substrate affinity (Fig. 3B).

Under identical assay conditions, EEDQ exhibited very similar potencies for inhibition of [^{3}H]ACh synthesis (apparent IC_{50} = 6.5 μM) and [^{3}H]choline uptake (Fig. 3A) with a similar profile obtained in rat striatal synaptosomes (data not shown). However, the potency of EEDQ as an inhibitor of high-affinity synaptosomal [^{3}H]choline uptake was affected markedly by the duration of tissue exposure to drug. As shown in Fig. 3C, there was a linear relationship between the reduction in high-affinity [^{3}H]choline uptake and the time over which tissue was exposed to EEDQ. In view of these observations, additional experiments were undertaken to ascertain whether EEDQ causes a disruption of [^{3}H]choline uptake (and [^{3}H]ACh synthesis) through an interaction with a hemicholinium-3-sensitive site. As shown in Fig. 4A, pretreatment of synaptosomes with hemicholinium-3 afforded complete protection of high-affinity [^{3}H]choline uptake against irreversible blockade by EEDQ. Using an identical pretreatment protocol, it was found that hemicholinium-3 also protected against the marked decrease in [^{3}H]ACh formation and calcium-stimulated [^{3}H]ACh release that was produced by EEDQ treatment alone (Fig. 4B).

Finally, experiments were carried out to determine whether EEDQ administration *in vivo* could also attenuate high-affinity [^{3}H]choline uptake. EEDQ (20 mg/kg) was injected and animals were killed 3 hr later in order to measure high-affinity synaptosomal [^{3}H]choline uptake and membrane binding sites for the mAChR radioligand [^{3}H]QNB. As shown in Fig. 5, EEDQ treatment reduced [^{3}H]choline uptake by 48% in hippocampus and 46% in striatum. By comparison, [^{3}H]QNB binding was reduced by 50% (hippocampus) and 65% (striatum) in the same EEDQ-treated animals. Additional experiments

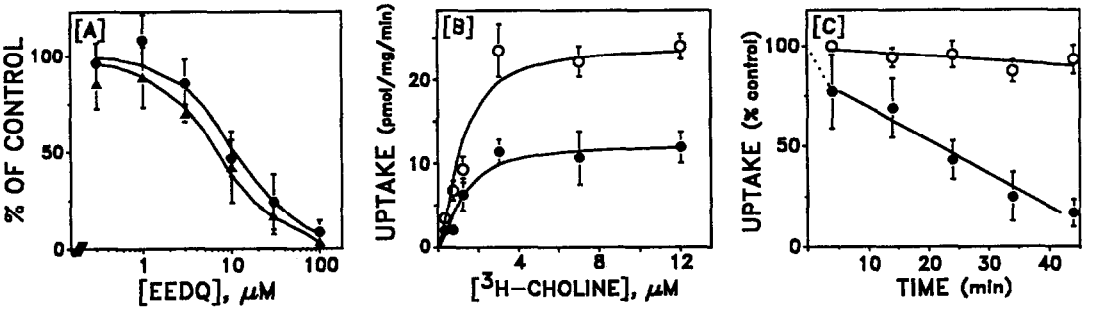

Fig. 3. Potency and temporal-dependence for EEDQ inhibition of [^{3}H]choline uptake by hippocampal synaptosomes. All data are the means ± SEM from three to five separate measurements in duplicate. Final [^{3}H]choline concentration was 1 μM except in panel B. (Panel A): Concentration dependence for EEDQ-induced inhibition of [^{3}H]choline uptake (circles; control uptake = 5.4 ± 1.0 pmol/mg/min) and [^{3}H]acetylcholine formation (triangles; control [^{3}H]ACh formation = 192,600 ± 15,900 dpm/mg). Tissues were incubated with the indicated concentration of EEDQ for 20 min prior to measurement of uptake. (Panel B): Saturation kinetics for [^{3}H]choline uptake by synaptosomes following a 20-min pretreatment with vehicle (open circles) or 10 μM EEDQ (filled circles). Solid lines represent fitted curves from best-fit models. (Panel C): Time course for EEDQ inhibition of [^{3}H]choline uptake. Tissue was pretreated with vehicle (open circles) or 10 μM EEDQ (filled circles) for the indicated time periods and then was washed twice and assayed for uptake. Data are expressed as a percentage of specific [^{3}H]-choline uptake (6.2 ± 0.5 pmol/mg/min) by synaptosomes that underwent no pretreatment period.

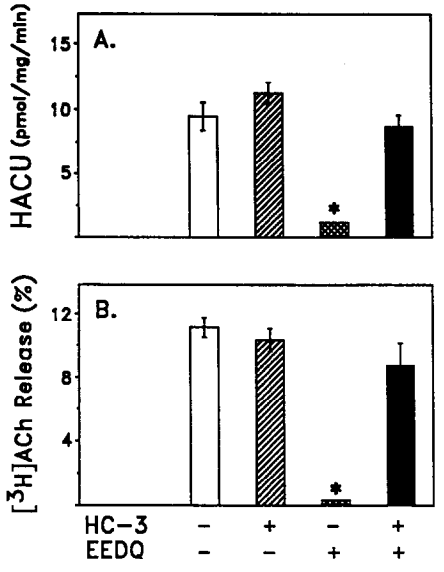

Fig. 4. Protection by hemicholinium-3 against EEDQ-attenuated [^{3}H]choline uptake and calcium-stimulated [^{3}H]ACh release. Hippocampal synaptosomes were pretreated with hemicholinium-3 (10 μM) and/or EEDQ (10 μM) for 20 min and then were washed twice with drug-free buffer prior to measurement of [^{3}H]choline (1 μM) uptake (panel A) or net calcium-evoked [^{3}H]ACh release (panel B). All data are expressed as means ± SEM from three to five separate experiments. Asterisks (*) indicates a significant difference ($P < 0.02$) from vehicle-treated and hemicholinium-3 plus EEDQ-treated tissues.

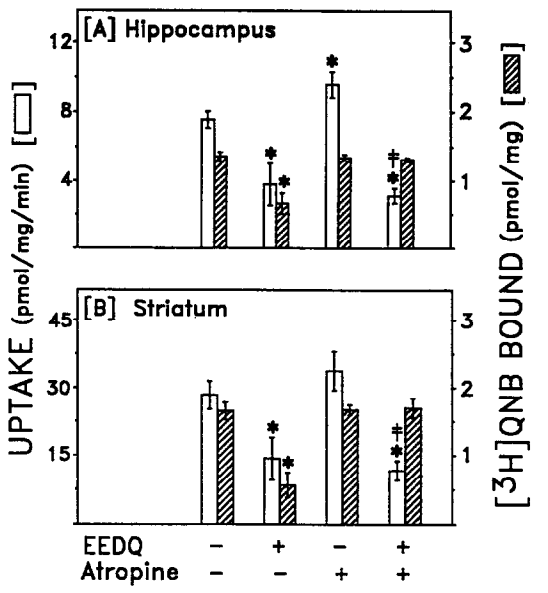

Fig. 5. Effects of *in vivo* treatments with EEDQ and atropine on [^{3}H]choline uptake and [^{3}H]QNB binding sites in forebrain tissues. Rats (six to eight animals per group) were injected with atropine (100 mg/kg, s.c.) or saline vehicle 15 min prior to the injection of EEDO (20 mg/kg, s.c.) or DMSO. Animals were killed 3 hr after the second injection, and brain synaptosomes were assayed for [^{3}H]-choline uptake (open bars). Synaptosomal membranes were stored at −20° and subsequently assayed for [^{3}H]-QNB (400 pM) binding (striped bars). All data are expressed as means ± SEM for each treatment group. Key: (*) significantly different ($P < 0.05$) from vehicle-treated rats; and (‡) significantly different ($P < 0.05$) from atropine-treated rats.

were carried out to determine whether the mAChR antagonist atropine would differentially affect the actions of EEDQ *in vivo*. As shown in Fig. 5, atropine (100 mg/kg) alone caused no change in [^{3}H]QNB binding but protected against the loss of mAChR binding sites caused by EEDQ treatment. In contrast to these results, atropine did not protect against EEDQ-induced inhibition of [^{3}H]choline uptake (68 and 65% reductions in hippocampus and striatum, respectively) despite modest increases in high-affinity [^{3}H]choline uptake by synaptosomes from animals treated with atropine alone (Fig. 5).

DISCUSSION

Data presented in this report demonstrate that EEDQ, a hydrophobic carboxyl modifying reagent, can cause long-lasting inhibition of high-affinity choline transport when administered either *in vitro* (rat brain synaptosomes) or *in vivo*. Experimental results that support this conclusion include the following: (1) a marked and essentially non-reversible inhibition of synaptosomal [^{3}H]choline uptake following EEDQ treatment *in vitro*; (2) complete ablation of stimulus-dependent [^{3}H]ACh release in EEDQ-treated synaptosomes; (3) nearly equivalent potencies (apparent IC_{50} values less than 10 μM) for EEDQ-induced inhibition of synaptosomal HACU and [^{3}H]ACh formation; (4) protection against EEDQ-induced inhibition of HACU and stimulus-dependent [^{3}H]ACh release in synaptosomes following cotreatment with hemicholinium-3, a highly selective and competitive inhibitor of HACU [18–20]; and (5) a marked decline in [^{3}H]choline uptake following systemic administration of EEDQ to rats *in vivo*.

Previous studies have provided evidence that intimates a close coupling between neurotransmitter formation and neuronal activity (firing rate) within cholinergic neurons [21, 22]. The majority of published results indicate that coupling is accomplished primarily through the rapid regulation of HACU, the rate-limiting step for neuronal ACh biosynthesis. Therefore, in view of the relationship between neuronal activity and ACh synthesis, it is likely that HACU is critical for the proper maintenance of synaptic efficacy within cholinergic pathways. Despite the potential importance of this regulatory mechanism, the molecular steps that serve to couple cell firing with choline transport *in vivo* remain largely unresolved, though studies *in vitro* indicate that activity-dependent regulation of HACU may involve carrier phosphorylation via phospholipase A_2-dependent or calcium/calmodulin-dependent mechanisms [23–26]. Nevertheless, studies of mechanisms that underlie HACU regu-

lation *in vivo* have been impaired, in part, by the lack of a convenient means to directly influence this transporter process. In this regard, the actions of EEDQ reported here may provide a basis for its use as a tool to investigate the regulation of HACU *in vivo*. However, it should be noted that the ability of EEDQ to directly and irreversibly inhibit carrier-mediated, high-affinity transport of choline into CNS neurons is by no means unique. Indeed, previous reports have ascribed such an action to several agents, including choline mustard aziridinium ion and ethylcholine mustard aziridinium ion (AF64A) [27–29], as well as bromine-containing mustard derivatives of hemicholinium [30]. In view of the structural similarities between these alkylating agents and choline, it is likely that choline mustard homologs will display far greater selectivity for HACU than EEDQ does. However, it should also be noted that these agents are hydrophilic and highly reactive and, therefore, must be administered via direct intracerebral injection to exert CNS actions. In this regard, EEDQ inhibition of HACU in forebrain tissues following systemic administration (Fig. 5) represents a distinct advantage for situations wherein it is desirable to elicit widespread inhibition of forebrain HACU *in vivo*.

Although our data support the conclusion that HACU is the primary target that underlies EEDQ-induced ablation of calcium-stimulated [^{3}H]ACh release, it is conceivable that other processes that have a role in ACh release are also affected by EEDQ. Indeed, it is recognized that a significant number of proteins with widely varied functions can impact transmitter release in cholinergic neurons in some manner. Examples include the biosynthetic enzyme choline acetyltransferase, mitochondrial enzymes involved with acetyl CoA production, carriers that transport ACh into storage vesicles, proteins that mediate vesicular exocytosis, ion channels or other proteins that maintain proper transmembrane ion gradients, as well as many others. While any of these proteins could be covalently modified by EEDQ, we have concluded that HACU is the predominant site that underlies the loss of stimulus-dependent [^{3}H]ACh release in view of the protective actions afforded by hemicholinium-3 and the excellent agreement between potencies displayed by EEDQ for inhibition of HACU and ACh synthesis. Nevertheless, it is possible that hemicholinium-3 prevents uptake of EEDQ into cholinergic nerve terminals and thereby prevents it from exerting other intraneuronal actions. However, this possibility appears unlikely since EEDQ is a highly lipophilic substance that should readily diffuse through biological membranes without need of a specific carrier-mediated mechanism.

Finally, it is interesting to note that EEDQ may be useful for irreversible inactivation of other neurotransmitter transporter proteins. A published report that details the primary amino acid sequence for a cloned putative sodium-dependent choline transporter from rat CNS [31] also noted a high degree of sequence homology between this transporter and other cloned Na^+-dependent neurotransmitter transporters (notably dopamine, γ-aminobutyric acid, norepinephrine and serotonin transporters). However, more recent studies by another group [32] indicate that the clone isolated by Mayser and colleagues [31] actually codes for a protein that transports creatine rather than choline. This observation is consistent with the transport properties observed following expression of the clone in COS-7 cells [32] and explains the unexpected insensitivity to hemicholinium-3 by the putative choline transporter [31]. Nevertheless, in view of the homology among these Na^+-driven plasma membrane transporters with diverse substrate specificities, it is conceivable that EEDQ could be used to inactivate other transporters for which selective irreversible inhibitors have not yet been identified. Furthermore, since the mechanism by which EEDQ inactivates proteins involves irreversible chemical modification of free carboxyl residues [1, 2], EEDQ-sensitivity by HACU implies that a carboxyl group plays an important role in either substrate (choline) recognition and/or ion (Na^+) translocation by this neuronal transporter.

In summary, data contained in this report demonstrate that EEDQ caused direct and irreversible inhibition of choline transport in CNS cholinergic nerve terminals. As a consequence of this action, both the synthesis and stimulus-dependent release of ACh were attenuated markedly following EEDQ treatment. Since EEDQ appears to exert similar actions when administered *in vitro* or *in vivo*, this agent may prove useful for investigations concerning the regulation, turnover and functional reserve among HACU transporters in cholinergic nerve cells. However, the lack of selectivity by EEDQ requires that such studies be designed with great care and that considerable attention must be given to possible indirect effects mediated by secondary actions of EEDQ.

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