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Structure–activity relationships for various *N*-alkylcarbamyl esters of choline with selective nicotinic cholinergic properties

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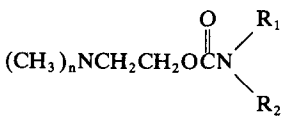
In the course of investigating the structure–activity relationships of various carbamate esters of alkylamino alcohols, it was observed that the addition of a methyl substituent to the carbamyl N of carbamylcholine increases the nicotinic cholinergic properties of carbamylcholine, while virtually abolishing its muscarinic properties [1]. This observation has led to the development of [^{3}H]methylcarbamylcholine ([^{3}H]MCC) as a prototypic ligand for investigating the nicotinic recognition site in brain tissue [1–4]. Since the addition of an aromatic or cycloalkyl substituent on the carbamyl N imparted antagonistic properties to carbamylcholine, a study was undertaken to determine the effect of varying alkyl chain lengths on the nicotinic properties of newly synthesized N-substituted carbamate esters of choline. The compounds were evaluated for their ability to compete for [^{3}H]MCC, [^{3}H]nicotine, and [^{3}H]quinuclidinylbenzilate ([^{3}H]QNB) binding and for their ability to produce prostration in rodents.

Methods

Synthesis of dimethylaminoethyl (DMAE) dimethylcarbamate and dimethylcarbamylcholine (DMCC). The compounds synthesized and evaluated in the present study had the general chemical structure:



where R_1 = H (except for one case where it was methyl); R_2 = methyl, ethyl, propyl, butyl, or phenyl; and n = 2 or 3.

To 0.06 mol of dimethylaminoethanol in 50 mL of dry toluene was added 0.03 mol of dimethylcarbamyl chloride. The solution was refluxed gently for 3 hr during which time a light brown oil formed. After cooling overnight at room temperature, the oil (bottom layer) solidified. The product (DMAE dimethylcarbamate) was recovered from the liquid phase by first evaporating half of the solvent and removing by filtration the residual dimethylaminoethanol hydrochloride. A pale yellow oil (85% yield) was obtained when the filtrate was evaporated to dryness *in vacuo*.

An analysis of DMAE dimethylcarbamate by infrared and NMR spectroscopy yielded the following:

i.r. bands 1710, 1200, 1400, 1460, 1385, 1500, (1355, 1275, 1200), 1050, 790
NMR ($CDCl_3$, TMS = 0.00 ppm) 2.3s 6H, 2.6t 2H, 2.9s 6H, 4.2t 2H.

Capillary gas chromatography–mass spectroscopy revealed a sample composition of approximately 95% purity that yielded a base peak of m/z 58, M + 1 fragment of m/z 161, and M − 1 fragment of m/z 159.

DMCC was prepared by adding 0.009 mol methyl iodide dropwise to a solution of 0.006 mol DMAE dimethylcarbamate in 50 mL of dry acetone. The slurry that formed was stirred for an additional 1.5 hr, then filtered and washed with 30 vol. of acetone followed by 30 vol. of diethyl ether, and dried. The yield was approximately 89%. The fluffy, white, crystalline material was soluble in ethanol, dimethyl sulfoxide (DMSO), and H_2O, and insoluble in acetone, diethyl ether and CH_2Cl_2. The product was used without further purification and stored in a dessicator at room temperature. Analytic: ($C_8H_{19}N_2O_2$)C,H,N,O.

Synthesis of DMAE carbamates. Various DMAE carbamates were prepared as described elsewhere [2]. Briefly, methyl, ethyl, propyl, butyl and phenyl isocyanates were refluxed for 6 hr in dry toluene with dimethylaminoethanol. The solvent and residual isocyanate were removed by evaporation *in vacuo*. The quaternary salts were prepared by reaction with methyl iodide.

Measurement of [^{3}H]nicotine, [^{3}H]methylcarbamylcholine ([^{3}H]MCC) and [^{3}H]3-quinuclidinyl benzilate ([^{3}H]QNB) binding. The procedure for the preparation and measurement of specific [^{3}H]nicotine, [^{3}H]MCC, and [^{3}H]QNB binding has been described [5]. Membranes are obtained from whole rat brain after homogenization in 0.05 M $NaPO_4$, pH 7.0, and centrifugation at 50,000 *g* for 30 min. The pellet is resuspended in phosphate buffer and stored

on ice for up to 3 days. To a 2-mL polypropylene tube was added 0.8 mL of 1.25 mg/mL membrane protein along with 0.1 mL of 1×10^{-8} M [^{3}H]nicotine (sp. act. 75 Ci/mmol), [^{3}H]MCC (80 Ci/mmol), or [^{3}H]QNB (80 Ci/mmol) with or without various concentrations of unlabeled compound or carbamate esters, in a final volume of 1.0 mL. After incubation of the tubes in an ice bath for 30 min, the tubes were centrifuged in a table top centrifuge for 5 min at 4°. The pellets were washed with 2 mL of ice-cold 0.05 M phosphate, and the bottom of the tubes was cut off with an animal nail clipper and counted for ^{3}H-disintegrations by liquid scintillation. All assays were performed in triplicate, and the experiments were repeated at least twice. Data are expressed as specific binding, i.e. total − non-specific.

Pharmacologic measurements. The various carbamate esters were tested for their ability to produce prostration in rats. The procedure for determining prostration in rats following administration of 10 μL of test agent intraventricularly through chronically implanted cannulae is described in detail elsewhere [5]. Briefly, 10 μL of various concentrations of agent was administered into the lateral ventricles of rats through chronically implanted cannulae. Prostration involving all four limbs was considered as a full response, while prostration of the hind limbs only was taken as the ED_{50}. Five rats were used for each agent, the results being expressed as ED_{50}.

Results and Discussion

The various carbamate esters of dimethylaminoethanol and trimethylaminoethanol (choline) were tested for their ability to compete for [^{3}H]nicotine and [^{3}H]MCC binding to rat brain membranes. In addition, they were evaluated for relative potency in producing prostration in rats following administration into the lateral ventricles (Table 1). The highest affinity for the nicotinic receptor was observed with methylcarbamylcholine and dimethylcarbamycholine, with K_i values of about 5×10^{-9} M using either [^{3}H]nicotine or [^{3}H]MCC as radioligands (Table 1). Replacement of the methyl group of MCC by ethyl or propyl reduced the binding affinity about 3- and 6-fold respectively, while the relative potency for producing prostration decreased 2- and 3.5-fold respectively. Replacement with a butyl group reduced binding affinity by over two orders of magnitude, as compared to MCC, and psychotropic potency over 20-fold. The K_i values for the choline derivatives were generally two orders of magnitude lower than those for the corresponding dimethylaminoethyl carbamate esters, and the psychotropic potency about 20-fold lower. Replacement of methyl by a phenyl group reduced the affinity of MCC by three orders of magnitude, while imparting antagonist properties to the carbamate [2]. It can be concluded that substitution of an alkyl group on the carbamyl N diminishes affinity for the muscarinic receptor while greatly enhancing affinity for the nicotinic receptor of rat brain.

The present study presents additional data on the structure–activity relationships of the nicotinic cholinergic properties of carbamate esters of choline with various alkyl substituents on the carbamyl N. A previous study had shown that a methyl substituent on the carbamyl N of carbamylcholine increases the nicotinic properties of carbamylcholine by two orders of magnitude while almost abolishing the muscarinic properties [1]. The selective nicotinic cholinergic activity of MCC has been determined recently by employing a battery of pharmacologic tests characteristic of the nicotinic action and demonstrating their reversal by the nicotinic antagonists hexamethonium and mecamylamine [6]. Results from the present study demonstrate that the addition of a second methyl substituent slightly enhanced the nicotinic properties of MCC, while replacement of the methyl group of MCC by increasing alkyl chain-length resulted in a progressive loss of binding affinity and psychotropic potency. Replacement with a butyl substituent resulted in a decrease of three orders of magnitude in binding affinity and a marked loss in psychotropic efficacy. Replacement of methyl by phenyl resulted in a further reduction in binding affinity and

Table 1. Inhibition of [^{3}H]nicotine, [^{3}H]QNB and [^{3}H]MCC binding by various carbamates and their ability to induce prostration

Compound	Chemistry* R_1	R_2	n	[^{3}H]Nicotine binding K_i (M)	[^{3}H]MCC binding K_i (M)	Prostration ED_{50} (nmol)	[^{3}H]QNB K_i (M)
DMAE methylcarbamate	H	CH_3	2	5×10^{-7}	8×10^{-7}	300	4×10^{-4}
Methylcarbamylcholine	H	CH_3	3	8×10^{-9}	6×10^{-9}	10	1×10^{-4}
DMAE ethylcarbamate	H	C_2H_5	2	8×10^{-7}	1×10^{-6}	400	ND†
Ethylcarbamylcholine	H	C_2H_5	3	1×10^{-8}	1×10^{-8}	40	2×10^{-4}
DMAE propylcarbamate	H	C_3H_7	2	4×10^{-6}	8×10^{-6}	600	5×10^{-3}
Propylcarbamylcholine	H	C_3H_7	3	6×10^{-8}	4×10^{-8}	35	2×10^{-4}
DMAE dimethylcarbamate	CH_3	CH_3	2	1×10^{-5}	1×10^{-6}	300	5×10^{-3}
Dimethylcarbamylcholine	CH_3	CH_3	3	5×10^{-9}	5×10^{-9}	8	1×10^{-4}
Butylcarbamylcholine	H	C_4H_9	3	4×10^{-7}	5×10^{-7}	>200	8×10^{-5}
Phenylcarbamylcholine	H	phe	3	6×10^{-6}	8×10^{-7}	Antagonist‡	2×10^{-5}
Carbamylcholine	H	H	3	4×10^{-7}	1×10^{-6}	100	5×10^{-7}

* General formula: $(CH_3)_nNCH_2CH_2O\overset{O}{\overset{\|}{C}}N\begin{matrix}R_1\\ \diagdown\\ \diagup\\ R_2\end{matrix}$

The prostration data are expressed as an average of five determinations agreeing within 10%. The ED_{50} and K_i values (except for [^{3}H]QNB) were obtained from a previous publication [2]. Except for DMAE dimethylcarbamate, dimethylcarbamylcholine and butylcarbamylcholine, data were obtained from Ref. 2.

† ND = not done.

‡ See Ref. 5.

imparted antagonistic properties, as determined by nicotine-induced prostration. A good correlation was observed between the nicotinic receptor binding characteristics of the *N*-alkylcarbamyl esters and their efficacy in producing prostration and seizures in rodents.

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Semicarbazide-sensitive amine oxidase activity (SSAO) of rat epididymal white adipose tissue

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In recent years the presence of amine oxidase (AO) activities, resistant to the irreversible mitochondrial monoamine oxidase inhibitors (MAO, flavin dependent amine oxygen oxidoreductase EC 1.3.4), have been described in many mammalian tissues [1]. To study this activity tissue homogenates are usually exposed to a millimolar concentration of an irreversible MAO inhibitor, such as pargyline. In these conditions, any remaining AO activity measured with benzylamine as substrate and inhibited by semicarbazide is conventionally referred to as "semicarbazide-sensitive amine oxidase activity (SSAO)".

Barrand and Callingham [2], described the SSAO of rat brown adipose tissue (BAT) and more recently the presence of a SSAO activity was also found in cultured white and brown preadipocytes transformed by lipogenic agents [3]. These findings might reinforce the hypothesis that SSAO could play a physiological role related to some BAT metabolic functions. Because BAT represents only a small fraction of the total adipose tissue disseminated in the body, our aim was to investigate on the presence of this enzyme in white adipose tissue (WAT).

Materials and Methods

WAT was obtained from epididymal fat pads of adult male Wistar (150–300 g) rats (Morini, S. Polo D'Elsa, Italy) killed by cervical dislocation. About 300 mg of minced tissue was washed in cold saline solution and then homogenized in 3 mL of ice-cold 1 mM potassium phosphate buffer containing 0.25 M sucrose (sucrose–phosphate buffer) pH 7.8. Mature adipocytes were prepared according to Rodbell [4]. The purified cells were resuspended and homogenized in ice-cold sucrose buffer, pH 7.8, to get a final protein concentration of 0.3–0.5 mg/mL [5].

SSAO activity was assayed radiochemically according to Buffoni and Ignesti [6], in the condition of MAO inhibition by 1 mM pargyline. Unless otherwise stated labelled substrates (50 μL) were reacted with the enzyme for 5 min.

When unlabelled amines were screened as possible substrates enzymatic activity was measured spectrophotometrically [6, 7] in the presence of 1 mM pargyline.

White adipocyte homogenates were then processed for subcellular fractionating. The supernatant from a first sedimentation at 600 $g \times 20$ min was again centrifuged at 12,000 $g \times 30$ min. After this the resulting supernatant was then spun at 105,000 g for 60 min. All procedures were run at 4°. Each pellet was washed in cold sucrose–phosphate buffer and resuspended in 2 mL of the same ice-cold buffer and assayed radiochemically for SSAO, for 5′-nucleotidase [8], and for cytochrome *c* oxidase [9].

Moreover, some "sandwich" immunoassays were carried out using pure pig plasma benzylamine oxidase (BAO), prepared according to Dixon and Purdom [10], and the 12,000 g pellet from white adipocytes as antigens and a rabbit serum challenged with pure BAO [11]. Assays were performed according to Ref. 12.

Antigens were coated on 96 multiwell plates and a 1:5000 dilution of the anti-BAO serum and 1:3000 dilution of a goat anti-rabbit Ig–peroxidase conjugate were used (Biorad). Extinction at 392 nm was always subtracted for the respective control value. [7-^{14}C]Benzylamine hydrochloride (57 mCi/mmol) was purchased from ICN Chemical Radioisotope Division (Irvine, CA, U.S.A.), β-[ethyl-1-^{14}C]phenylethylamine hydrochloride (50 mCi/mmol) from New England Nuclear (Boston, MA, U.S.A.). All the other reagents were analytical grade products.

Results and Discussion

The presence of an AO activity which is resistant to