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SHORT COMMUNICATIONS

Inhibition of acetylcholinesterase by neurotoxic aminonitriles

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Aliphatic aminonitriles have been recognized as possessing neurotoxic properties [1–3]. β-Aminopropionitrile has been associated with osteolathyrism, while certain amino acid-like compounds such as β-cyanoalanine and γ-cyano-γ-aminobutyric acid produce severe convulsions in experimental animals as well as being effective inhibitors of biochemical processes [2, 3]. An aminonitrile formerly used in industry which has distinctive neurotoxic effects is dimethylaminopropionitrile (DMAPN) [4–6]. In two epidemic outbreaks in 1978, DMAPN was found to cause symptoms of bladder neuropathy and sexual dysfunction. Many workers had to strain to urinate, had incomplete bladder emptying, and a reduced frequency of urination. Follow-up studies have demonstrated a persistence of urinary and sexual symptoms in DMAPN workers even after 2 years [7]. Neurological examination revealed sensory and sensorimotor neuropathies. Gad *et al.* [8] and Jaeger *et al.* [9] have reported that DMAPN produces bladder toxicity and loss of micturition in experimental investigations. DMAPN has also been shown to inhibit the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphofructokinase (PFK) with likely involvement of the nitrile group.* Our research has sought to elucidate the biochemical mechanism of the neurotoxicity of DMAPN and other aminonitriles. This study reports the results of kinetic investigations on the inhibition of acetylcholinesterase (AChE) by DMAPN and a series of related aminopropio- and butyronitriles in order to determine the possible role of the nitrile group in the inhibition of AChE.

Materials and methods

Dimethylaminopropionitrile (DMAPN) and *N*-methyl-β-alaninenitrile (monomethylaminopropionitrile, MMAPN) were obtained from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Dimethylaminobutyronitrile (DMABN) was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY, U.S.A.). *N,N,N*-Trimethyl-2-cyanoethylammonium iodide (trimethylaminopropionitrile, TMAPN) was synthesized as described by Simeral and Maciel [10]. *N,N,N*-Trimethyl-3-cyanopropylammonium iodide (trimethylaminobutyronitrile, TMABN) was synthesized similarly to TMAPN. DMABN was dissolved in chloroform and cooled in ice. Cold methyl iodide was added dropwise. The resulting crystalline product was filtered, washed with ice-cold chloroform, and recrystallized from methanol. β-Aminopropionitrile fumerate (βAPN), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine (ATCh), acetylcholinesterase (AChE), electric eel, Type V-S (EC 3.1.1.7), *S*-butyrylthiocholine (BuTCh), and butyrylcholinesterase (BuChE), horse serum, Type XI (EC 3.1.1.8), were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzymes were reconstituted with 0.1 M sodium phosphate buffer (pH 7.0) to a stock concentration of 4 units/ml. ATCh was prepared fresh daily in 0.1 M sodium phosphate buffer (pH 7.0). All other chemicals were reagent grade.

Acetylcholinesterase and butyrylcholinesterase activities were determined by the method of Ellman *et al.* [11] using a Varian DMS 100 recording UV-Vis spectrophotometer (Varian Associates, Palo Alto, CA, U.S.A.). Measurements were made at 25° by observing ATCh or BuTCh hydrolysis at 412 nm using DTNB color reagent. A typical reaction mixture contained 3.0 ml of 0.1 M phosphate buffer (pH 7.0), 100 μl DTNB (0.01 M), 50 μl enzyme (4 units/ml), 20 μl ATCh or BuTCh and various amounts of inhibitor. Inhibition of AChE and BuChE activity was plotted as Lineweaver–Burk plots with lines calculated by the least squares method of linear regression analysis.

Results and discussion

Electric eel AChE was inhibited by all the aminonitriles tested in a concentration-dependent manner. The ATCh concentrations ranged from 2×10^{-5} to 2×10^{-4} M and the concentration of inhibitor ranged from 8.3×10^{-4} to 5.3×10^{-2} M. A minimum of three determinations was made for each aminonitrile and substrates were run in triplicate. At least three inhibitor concentrations were used for each compound.

The data indicate that the inhibition of AChE by the aminonitriles was of the linear mixed type [12]. The inhibitor affected both the apparent K_m and V_{max} of the hydrolysis of ATCh by AChE, indicating both competitive and noncompetitive inhibition. Mixed reversible inhibition of AChE by amines has been reported by numerous authors [13–19]. The noncompetitive component of the inhibition is associated with complex formation between the acylated enzyme and inhibitor. K_{comp} (K_I) and $K_{noncomp}$ (K'_I) represent the dissociation constants for the enzyme–inhibitor complex and the acylated enzyme–inhibitor complex respectively. The values of K_{comp} (K_I) and $K_{noncomp}$ (K'_I) for the aminonitriles tested are listed in Table 1. An example of a reciprocal plot for DMAPN is shown in Fig. 1.

Preincubation of the enzyme–inhibitor mixture prior to the addition of substrate for different time intervals ranging from 10 min to 12 hr and at temperatures of 25° and 37° had no effect on the inhibition of AChE. Similarly, simultaneous addition of inhibitor and substrate had no effect on the degree of inhibition.

Inhibition of AChE increased as the degree of methylation of the amine increased from $n = 0$ to $n = 3$ for all the aminonitriles studied. The quaternary ammonium compounds were more effective inhibitors than the tertiary analogues. βAPN and MMAPN were weak inhibitors of AChE. The values for βAPN and MMAPN, respectively, are reasonably consistent with values for methylamine, $K_I = 6.3 \times 10^{-2}$, $K'_I = 1.1 \times 10^{-2}$ and for dimethylamine, $K_I = 3.3 \times 10^{-2}$ and $K'_I = 2 \times 10^{-2}$ [16]. DMAPN was a poor inhibitor of BuChE. K_I and K'_I values were 1.6×10^{-2} and 8.5×10^{-2}. The butyronitrile derivatives inhibited AChE over their propionitrile analogs.

The cyano group in the aminopropionitriles may be expected to influence the inhibition of AChE by virtue of its electron withdrawing capabilities. The DMAPN pK_a of 7.5 [20] is lower because of the proximity of the cyano group to the amine. The aminonitrile would then be expected to be a somewhat poorer inhibitor of AChE than amino compounds with no electron withdrawing groups to

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Table 1. $K_{competitive}$, $K_{noncompetitive}$ for reversible inhibition of AChE by aminonitriles

	K_{comp} (K_I)	K_{noncom} (K'_I)	K_I/K'_I
β-Aminopropionitrile (βAPN)	$4.8 \pm 1.0 \times 10^{-2}$*	$7.8 \pm 1.6 \times 10^{-2}$	0.62
Monomethylaminopropionitrile (MMAPN)	$2.1 \pm 0.4 \times 10^{-2}$	$5.3 \pm 1.5 \times 10^{-2}$	0.40
Dimethylaminopropionitrile (DMAPN)	$6.7 \pm 2.2 \times 10^{-3}$	$3.1 \pm 2.5 \times 10^{-2}$	0.22
Trimethylaminopropionitrile (TMAPN)	$2.4 \pm 0.5 \times 10^{-3}$	$5.7 \pm 0.5 \times 10^{-3}$	0.42
Dimethylaminobutyronitrile (DMABN)	$5.9 \pm 2.0 \times 10^{-3}$	$8.6 \pm 0.7 \times 10^{-3}$	0.69
Trimethylaminobutyronitrile (TMABN)	$8.9 \pm 0.1 \times 10^{-4}$	$5.6 \pm 0.1 \times 10^{-3}$	0.16

* Units are M. Measurements were made at 25° and pH at 7.0. Results are given as average ± S.D.; N = 3 except for DMAPN where N = 4.

influence the basicity of the amine group. The dissociation constant, K_I, for trimethylamine is 4.8×10^{-3} and for dimethylaminoethanol, 3.7×10^{-3}, as compared to 6.7×10^{-3} for DMAPN [16].

Since TMAPN is a permanent quaternary ammonium compound, its potential for inhibition of AChE might be expected to be similar to other quaternary compounds. TMAPN ($K_I = 2.4 \times 10^{-3}$) has the same dissociation constant within experimental error as chlorocholine chloride and tetramethyl ammonium. K_I values for chlorocholine chloride and tetramethyl ammonium are 1.85×10^{-3} and 2.8×10^{-3} respectively [16].

In contrast to the aminopropionitriles, the aminobutyronitriles will have a higher pK_a by virtue of the additional methylene group between the amine and cyano groups and would therefore have somewhat different characteristics than DMAPN. DMABN and TMABN were both stronger inhibitors of AChE than DMAPN and TMAPN, although the K'_I for TMABN was the same as for TMAPN. This is consistent with the work of Bergmann and Segal [21] who found that inhibition increases as a function of chain length for trimethylalkyl ammonium salts.

Examination of the structures of DMABN and TMABN suggests that these molecules might have enhanced inhibitory power because of the potential for interaction between the nitrile carbon and the seryl residue of the esteratic site. The carbon of the nitrile occupies a similar position to that which would be occupied by the carbonyl carbon in acetylcholine. No marked enhancement of inhibition was observed for butyronitrile derivatives. The K_I for TMABN was 8.9×10^{-4} as compared to 2.0×10^{-3} and 1.1×10^{-3} for *n*-butyltrimethylammonium and *n*-amyltrimethylammonium.

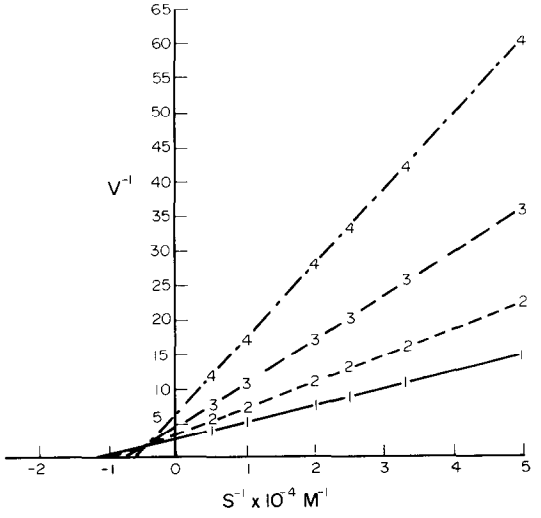

Fig. 1. Lineweaver–Burk plot demonstrating mixed inhibition of electric eel AChE (25°, pH 7.0) activity by DMAPN as analyzed by the least squares method of linear regression. Substrate (ATCh) was measured by the method of Ellman *et al.* [11]. Inhibitor concentrations were as follows: (1) 1.67×10^{-3} M; (2) 3.33×10^{-3} M; (3) 6.67×10^{-3} M; and (4) 13.33×10^{-3} M.

The focus of this study was to determine whether the nitrile group on the aminonitrile would interact with nucleophilic sites on the enzyme, resulting in an enhancement of inhibition. The results indicate that any interaction between the nitrile carbon and the esteratic site has a minimal impact on the inhibition of AChE. In general, the nitrile group in the compounds studied is unimportant in the inhibition of AChE.

The symptoms described as resulting from DMAPN exposure are not the result of the inhibition of the catalytic activity of acetylcholinesterase. Whether the DMAPN binds with cholinergic sites with resulting antimuscarinic activity is currently under investigation. Structural analogues of DMAPN, which are also weak inhibitors of AChE, have been shown to be effective inhibitors of ACh binding to receptor sites [22]. The report of Gad *et al.* [8] that carbachol or neostigmine treatment of DMAPN-exposed rats relieves the symptoms of urinary retention is relevant to this hypothesis. The aminonitriles investigated here have been studied to determine whether they produce urinary retention similar to that reported for DMAPN. Only DMAPN produces urinary retention.* Thus, the mechanism of bladder neuropathy from DMAPN exposure may be more complex than being simply of pharmacologic origin.

In summary, the kinetics of inhibition of AChE by a series of neurotoxic aminopropionitriles and aminobutyronitriles have been studied. AChE was inhibited by all the aminonitriles tested in a concentration-dependent manner. Kinetic data indicate that the inhibition was of the reversible linear mixed type. Inhibition increased as the degree of methylation increased from the unsubstituted primary amine to the quaternary amine and from the aminopropionitrile to the butyronitrile. Dissociation constants for competitive inhibitions (K_I) ranged from 4.8×10^{-2} to 8.9×10^{-4} for the aminonitrile and the noncompetitive constants ranged from 7.8×10^{-2} to 5.6×10^{-3} for the same compounds. There was no evidence for an interaction between the nitrile group and AChE.

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Model for action of local anaesthetics with cytochrome oxidase

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In spite of the large number of studies carried out to elucidate the effect of local anaesthesia, the precise molecular mechanism by which local anaesthetics achieve this effect is not understood. The interaction of these compounds with cellular membranes plays an important part in their pharmacological action [1–3]. The understanding of these interactions is essential in the definition of an exact molecular mechanism of action [4–10]. We therefore decided to study the interactions between a series of eight local anaesthetics having different molecular structures with a model membrane system. This allowed the research of both the drug's affinity for membrane lipids and their functional effects on membrane proteins. Cytochrome oxidase seemed to be a good model membrane system on account of its structure [11, 12] and its well-defined functional properties [13–18].

Results and discussion

The drugs investigated were: procaine, lidocaine, prilocaine, carticaine, parethoxycaine, bupivacaine, pramocaine and quinisocaine.

To begin with [19] we measured the dissociation constant pK_a, the octanol–water partition coefficient as well as the anaesthetic activity of infiltration of the various compounds.

We then studied [20] the inhibitory effects of the same compounds on cytochrome oxidase enzymatic activity at the electron transfer level from cytochrome *c* to molecular oxygen. The "local anaesthetic affinity" for the enzyme was estimated from the reciprocal of the inhibition constant K_i determined for each compound.

Finally, direct association of local anaesthetics with cytochrome oxidase was investigated [21] from a study of the binding of the anaesthetics molecules to the enzyme. Association of anaesthetics to quinacrine bound cytochrome oxidase was studied quantitatively from the rate of fluorescence quenching, K, caused by the presence of local anaesthetics. K is the quenching constant measured from the Stern Volmer plots.

In Table 1 are reported all the parameters determined from the precedent studies; the various correlations established between these parameters are illustrated by the relationships presented in Table 2. The two different ways of studying local anaesthetic interactions with cytochrome oxidase allowed us to measure two rates of interaction, K_i^{-1} and K, which correlate quantitatively, as can be seen in equation (2) (Table 2). The following comments suggest that the results are also closely related qualitatively in several ways.

The site of local anaesthetics action appears to deal with cytochrome oxidase associated phospholipids. Indeed, local anaesthetics inhibit cytochrome oxidase activity in the low affinity interaction of cytochrome *c* which is thought [22] to account for the binding of cytochrome c to the enzyme lipidic environment. The inhibition process can be explained by a competition between local anaesthetics and cytochrome *c* to bind to the enzyme associated phospholipids and, the affinity of drugs for enzyme, K_i^{-1}, could describe the amount of anaesthetic interaction with phospholipids involved in the enzymatic activity. Also, local anaesthetics decrease the fluorescence of quinacrine bound to a region of low polarity in the cytochrome oxidase complex, probably with phospholipids of the enzymatic complex, and so the quenching constant K corresponds to