A Comparison of Some Stereochemical Requirements of the Acetylcholinesterase and Muscarinic Receptor Areas

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Received July 9, 1969

The muscarinic receptor and acetylcholinesterase (AChE) demonstrate stereoselectivity in their interactions with acetyl-α-methylcholine, acetyl-β-methylcholine,[2] muscarines,[3] dioxolanes,[4] 3-trimethylammonium-2-acetoxy-*trans*-decalins,[5] and 2-acetoxycyclopropyltrimethylammonium[6] compounds. While acetylcholine (ACh) is regarded as a common substrate for tissue and enzyme, the lack of parallel activity to these receptors by many optical isomers occurs.[2,3] These events indicate that each receptor area may be presented with a different aspect of ACh and that they are different. We have sought to compare the effect of the enantiomeric forms of acetyl-α-methylcholine and acetyl-β-methylcholine together with acetyl-α,α-dimethylcholine and acetyl-β,β-dimethylcholine to demonstrate how the respective receptors may interact with the choline fragment of ACh.

From the data in Table I *R*(+)-acetyl-α-methylcholine and *S*(+)-acetyl-β-methylcholine show five and nine times, respectively, more muscarinic potency than their corresponding enantiomers. Clearly, a methyl group in the choline moiety does not appear to contribute in any way to activity since dimethylation of either the α or β carbons further reduces the potency of the chemical. Therefore, for optimal muscarinic activity the methyl group should not be in juxtaposition between the receptor area and the bulk of the molecule. The facet of the choline fragment which faces the receptor is the side which resembles acetylcholine. It appears also that the steric requirements in the vicinity of the anionic site of the muscarinic receptor on guinea pig ileum are more stringent but less stereospecific than those of the esteratic site. Substitution at the α carbon reduces ACh-like activity almost 40–200-fold while substitution at the β carbon shows little significant reduction in the *S*(+) isomer, III, but a tenfold reduction with the *R*(−) isomer, V.

TABLE I

MUSCARINIC ACTIVITIES OF ACETYLCHOLINE IODIDES ON GUINEA PIG ILEUM

Iodides	Potency of ACh[a]	95% confidence limits
Acetylcholine	1.00	
R(+)-Acetyl-α-methylcholine	0.0240	0.0150–0.0370
RS-Acetyl-α-methylcholine	0.0339	0.0263–0.0437
S(−)-Acetyl-α-methylcholine	0.0045	0.0040–0.0031
Acetyl-α,α-dimethylcholine	0.0025	0.0021–0.0031
S(+)-Acetyl-β-methylcholine	0.802	0.661–0.973
RS-Acetyl-β-methylcholine	0.622	0.456–0.848
R(−)-Acetyl-β-methylcholine	0.090	0.063–0.129
Acetyl-β,β-dimethylcholine	0.001	0.0007–0.0015

[a] The λ mean value for these assays is 0.123.

The enzyme activity data in Table II show *S*(−)-acetyl-α-methylcholine to have the highest rate of hydrolysis by AChE. This compound has a greater affinity for the enzyme receptor than its enantiomer. The *R*(−)-acetyl-β-methylcholine, although poorly hydrolyzed by AChE, demonstrates competitive inhibitor activity. The competitive nature can be interpreted as receptor affinity by the molecule. The Michaelis constant, K_s (dissociation constant), for AcCh from bovine erythrocytes AChE at pH 7.5 and 25° is 9.21×10^{-4}.[7] A K_i of 9.77×10^{-4} representing also the dissociation constant of II from the enzyme indicates a favorable comparison of affinity of *R*(−)-acetyl-β-methylcholine with acetylcholine for the active site. In contrast, the *S*(+)-acetyl-β-methylcholine has a low per cent hydrolysis and no inhibitor activity while *R*(+)-acetyl-α-methylcholine is also a poor substrate compared to either its enantiomer or the racemate. The compounds that have the greatest affinity for the receptor area have the same configuration in that the side-chain Me is deployed essentially in a similar manner. Again, Me cannot be construed as contributing activity since the dimethylcholine analogs are essentially devoid of activity. Me substitution in the vicinity of the anionic site does not as profoundly affect the ability of the compound to be hydrolyzed as does substitution adjacent to the esteratic site. Hydrolysis rates of the β-methylcholine analogs are lower than those of the α-methylcholine analogs.

TABLE II

AChE BOVINE ERYTHROCYTE[a] SUBSTRATE AND INHIBITOR ACTIVITIES OF ACETYLCHOLINE IODIDES

Iodides	Substrate activity: K_m 10^{-4}	Substrate activity: % hydrolysis
R(+)-Acetyl-α-methylcholine	2.00	61.3
RS-Acetyl-α-methylcholine	2.37	76.9
S(−)-Acetyl-α-methylcholine	1.79	87.7
Acetyl-α,α-dimethylcholine	2.20	36.7
Acetylcholine	2.60	100.0
S(+)-Acetyl-β-methylcholine		45.7
RS-Acetyl-β-methylcholine		27.2
R(−)-Acetyl-β-methylcholine		15.9[b,c]
Acetyl-β,β-dimethylcholine		22.0
Acetylcholine		100.0

[a] Nutritional Biochemicals Corp., Cleveland, Ohio. [b] $K_i = 9.77 \times 10^{-4}$. [c] Physostigmine sulfate under similar conditions gave a K_i value of 4.25×10^{-8}. It was a competitive inhibitor.

A comparison of the enzymatic and muscarinic activity of the α-Me isomers reveals that the isomer with

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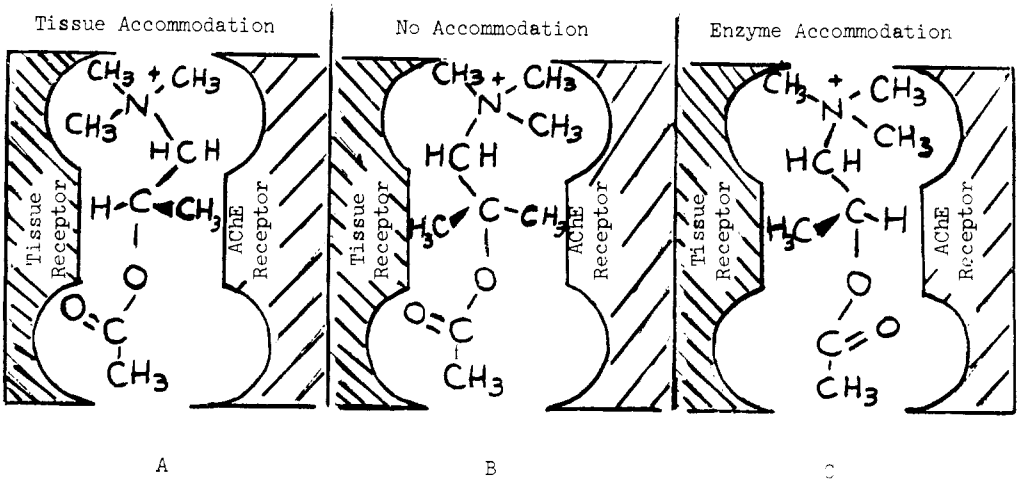

Figure 1.

poor muscarinic activity has good enzymatic hydrolytic activity and *vice versa*. In the β-Me series, the *S*(+) isomer with good muscarinic activity resists enzymatic hydrolysis, and the *R*(−) isomer which has poor muscarinic activity is a competitive inhibitor of AChE. The dimethyl compounds show poor affinity for both receptors.

The muscarinic receptor of guinea pig ileum and the enzymatic receptor area of bovine erythrocyte AChE, therefore, each best accommodate one isomer of an enantiomeric pair. The area between the esteratic and anionic sites of the two receptors differs such that the muscarinic site will accommodate the *R* configuration at the α carbon and *S* configuration at the β carbon of the choline moiety, while the enzyme site will react more favorably to the *S* and *R* configurations, respectively.

A schematic comparison of the two receptor areas consistent with the data is shown in Figure 1 and demonstrates the effect of the deployment of the methyl group of the β-methylcholines. In *A*, the β-methyl group of the *S*(+) isomer reduces accommodation to the enzyme site while tissue accommodation does occur in a manner similar to acetylcholine. In *B*, the dimethylcholine causes steric obstruction to both sites and accounts for the low activity in both tissue and enzyme. In *C*, the (*R*−) isomer is capable of accommodation to the enzyme site again in a manner similar to acetylcholine. However, accommodation to the tissue site is impeded by the Me group.

It may now be possible to view the sequence of events at the muscarinic receptor during nerve impulse transmission in light of the above observations. AcCh is released and interacts with the muscarinic tissue receptor to form a reactive complex. The complex now dissociates releasing ACh. Termination of ACh activity by AChE occurs so rapidly that a possible transfer stage may exist where AChE approaches the ACh-tissue complex as close as van der Waals radii will permit and reacts with ACh on its "backside" while it is still in the conformation needed to satisfy the stereochemistry of the muscarinic receptor. While the stereochemistry of the enantiomers of α- and β-substituted cholines may prevent this sequence because of steric interference from Me, the symmetry of ACh does not.

Experimental Section

Melting points were taken on a Mel-Temp apparatus and are corrected. Specific rotations were measured on a Cary-60 spectropolarimeter at 30° and 589.3 mμ. Microanalyses were performed by Alfred Bernhardt Microanalytical Laboratories, Mülheim (Ruhr), Germany. The analyses reported and indicated only by symbols are within ±0.4% of the theoretical values. Ir spectra were taken on a Model 137 Perkin-Elmer spectrophotometer.

***S*(+)-, *R*(−)-, and *RS*-Acetyl-β-methylcholine Iodide (III and V).**—Resolution of 1-dimethylamino-2-propanol was accomplished according to the procedure of Majors and Bonnett.[8] One gram of each free base was mixed with 10 ml of *i*-PrOH and 10 ml of Ac_2O and allowed to sit overnight at room temperature. Then, 10 ml of MeI was added and the mixture allowed to remain at room temperature for 4 hr. Addition of a small amount of dry EtOEt caused slow precipitation of III. The product was recrystallized from *i*-PrOH: mp 176–177° (lit.[9] 177.5–178.5°); $[\alpha]^{30}_{589.3\ m\mu}$ +27.0 ± 0.5° (*c* 1.00, H_2O). *Anal.* ($C_7H_{18}NO_2I$) C, H, N. The *R*(−) isomer, V, melted at 175–176° (176–178° lit.[9]); $[\alpha]^{30}_{589.3\ m\mu}$ −26.8 ± 0.5°, (*c* 1.00, H_2O). *Anal.* ($C_7H_{18}NO_2I$) C, H, N. Both enantiomers had a ν^{KBr}_{max} 1730 cm^{-1} for the C=O group.

RS-Acetyl-β-methylcholine was prepared by acetylation and quaternization of racemic 1-dimethylamino-2-propanol as described above, mp 150–151° (lit.[9] 151–152°). *Anal.* ($C_7H_{18}INO_2$) C, H, N.

***R*(+)-, *S*(−)-, and *RS*-Acetyl-α-methylcholine Iodide (IV and VI).**—Each isomer was prepared from L- and D-alanine, respectively, according to the method of Bowman and Stroud.[10] The *R*(+) isomer melted at 106–107° (lit.[9] 107–108°); $[\alpha]^{30}_{589.3\ m\mu}$ 6.55 ± 0.5° (*c* 1.00, H_2O). *Anal.* ($C_7H_{18}NO_2I$) C, H, N. The *S*(−) isomer melted at 107.5–108° (lit.[9] 107–180°); $[\alpha]^{30}_{589.3\ m\mu}$ −6.30 ± 0.5° (*c* 1.00 H_2O). *Anal.* ($C_7H_{18}NO_2I$) C, H, N. Both enantiomers have a ν^{KBr}_{max} 1730 cm^{-1} for the C=O group.

RS-Acetyl-α-methylcholine was prepared from DL-alanine in the same manner as the enantiomers. The product was recrystallized from *i*-PrOH, mp 137–138° (137–138° lit.[9]). *Anal.* ($C_7H_{18}INO_2$) C, H, N.

Acetyl-β,β-dimethylcholine Iodide.—2-Methyl-2-hydroxy-1-dimethylaminopropanol[11] was acetylated and treated with MeI in the same manner as described above. The product was recrystallized from *i*-PrOH, mp 185–186°. *Anal.* ($C_8H_{20}INO_2$) C, H, N.

Acetyl-α,α-dimethylcholine Iodide.—2-Dimethylamino-2-methyl-1-propanol was treated as described above. The product was recrystallized from *i*-PrOH, mp 193–194° (194–195° lit.[9]). *Anal.* ($C_8H_{20}NO_2I$) C, H, N.

Pharmacology.—The muscarinic potency in relation to ACh of each compound was determined by a 3 × 3 bioassay on the iso-

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lated guinea pig ileum. The ileum was suspended in Krebs–Henseleit solution, aerated with 95% O_2 and 5% CO_2, and maintained at 37°. AcCh and unknown drug were tested at minimal, medium, and submaximal concentrations as determined prior to the actual assay. The compounds were run on separate days using different guinea pigs for each experiment. The data were subjected to variance analysis which showed that none of the compounds altered the sensitivity of the tissue to AcCh.

Enzymology.—Enzyme-catalyzed hydrolysis of the compounds and inhibition of ACh hydrolysis were determined at pH 7.5 by titration of the liberated AcOH with NaOH solution (0.0065 N) using a Sargent pH-Stat. Concentrations of substrate varying from 1.0 to 5.0 $\times 10^{-4}$ M were used in a medium consisting of 0.01 M $MgCl_2$, 0.1 M Na Cl, and 0.01 mg/ml of AChE for the K_m determinations. Inhibitor concentrations were 3.0 $\times 10^{-4}$ M. The reaction rates were measured at 25° and were linear. A graphic plot of S/V *vs.* S provided K_m and K_i values. Comparisons of hydrolysis rates were carried out at 5.0 $\times 10^{-4}$ M substrate concentration.

Acknowledgment.—The authors wish to express appreciation to the North Carolina Pharmaceutical Research Foundation for their support of the Undergraduate Research Project during the academic year and to the National Science Foundation for their support during the summer period. We are also indebted to Dr. Jan Hermans and Mr. Dave Pruett of the Department of Biochemistry for the polarimetric determinations.

Phosphorus–Nitrogen Compounds. XI. Phosphamidase Studies. I. Unsubstituted Amides[1,2]

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Received September 3, 1969

That enzymes capable of catalyzing reactions involving P–N bonds occur in higher concentration in cancer than in normal cells has been well established.[3] These may be the same as, or closely related to, phosphamidase which has been isolated from *Escherichia coli*,[4] hog eye,[5] and beef spleen.[6] Neither a natural substrate nor a substrate-like inhibitor has been determined for this enzyme. In the design of the latter a common preliminary step is the selection of compounds with high affinity for the enzyme receptor site for alteration, if necessary, to produce increased binding capacity.

There have been only 12 different P–N compounds, excluding a few qualitative histochemicals,[3] previously used to study phosphamidase preparations, including 2 phosphorodiamides and 5 substrates with unsubstituted amido groups. This initial paper reports the examination of 25 P–N substrates of type $=P(O)NH_2$, $P(O)(OH$ or $OEt)NH_2$, and $P(O)(NH_2)_2$ with 60 to 90 SAS beef spleen fraction. Twenty-one of these have not been investigated previously with phosphamidase and 7 are new chemical entities.

The only substrates subject to P–NH_2 hydrolysis by the beef spleen preparation are certain triamides having two unsubstituted amido groups and two phosphonic diamides (XIII, XIV). As shown in Table I, the order of degree of hydrolysis is XVI > XIII > XVIII > XIX > XIV, XX > XXIII, XXV. With the triamides XVI–XXIII, where the alkyl substituents were N,N-Me_2, N,N-Et_2, N-Bu, N-hex, and N-cyclohexyl, hydrolytic activity decreased with increasing number of carbons. Loss of activity resulted when the alkyl chain was lengthened to eight carbons (XXI), when a double bond was introduced (XVII), and when the β carbon atoms of XVIII were linked to give a pyrrolidine derivative (XXIV). Hydrolysis of the P–NH_2 bond is, however, restored in the piperidyl homolog, XXV. The high activity of XIII is especially interesting since it contains a $ClCH_2P(O)$ moiety, an analog of the halomethylcarbonyl group which has been used as a covalent bonder in the design of inhibitors.[7]

Since the ultimate goal of this continuing study is the design of phosphamidase inhibitors, several substrates were screened for this property. Representative inactive compounds IV, V, X, XII, and XXIV did not affect phosphoramidate cleavage at 12 mM whereas XI and XV gave evidence of inhibition at this concentration, reducing NH_3 liberation by 50%.

Although the P–NH_2 bond in XI was shown not to be cleaved enzymatically in this study it is used as a histochemical substrate for the detection of phosphamidase.[8] This compound is probably cleaved preferentially at the aryl N–P bond to phosphoramidic acid which, in turn, is converted into H_3PO_4 and NH_3. Under conditions herein reported this acid, being formed in low concentration, is not appreciably acted upon. Each of the triamides, as well as IX and XII, also contains substituted amido groups subject to phosphamidase activity, and the short series of five N-substituted phosphoramidic acids previously studied with beef spleen preparation[6] does not permit the relating of the effect of this substituent on enzymatic activity. A future study in this series is expected to yield more definite information concerning the effect of N-substitution of phosphoramidic acid on enzyme interaction. It may then be possible to devise a substrate, *i.e.*, R(R or H)P(O)$N(CH_3)_2$ where R is an optimal substituent(s), possessing high enzyme affinity.

Experimental Section

Chemistry.—Previously unreported XIII, XVII, and XX–XXIV were prepared from the appropriate phosphorodichloridates according to the procedure of Goehring and Niedenzu.[9] Their elemental analyses (C, H, N) were obtained with Coleman analyzers with the results being within ±0.4% of the theoretical values and spectra using a Beckman IR-8 were as expected. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. NH_3 (2.0-ml sample) was determined using Conway microdiffusion dishes[10] with Obrink modification.

Enzyme Studies.—Substrates I–XXV (0.012 M), 0.1 M acetate buffer (pH 6.0), enzyme preparation 60 to 90 SAS 1.0 $(EU)^{PA}$/ml, and 0.02 M β-mercaptoethanol were incubated 10 min at 37° according to a previously described procedure.[6] Although this

(1) This investigation was supported by Grant E-297 from the Robert A. Welch Foundation, Houston, Texas, and Grant CA-08711 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.

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