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THE MECHANISM OF IRREVERSIBLE ADRENERGIC BLOCKADE BY *N*-CARBETHOXYDIHYDROQUINOLINES—MODEL STUDIES WITH TYPICAL SERINE HYDROLASES*

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Abstract—Carbamates I, II and III of the 1,2-dihydroquinoline series have been recently shown to induce selective irreversible inhibition of the catecholamine α-receptors. These compounds bear no discernible analogy to known adrenergic blockers. Only the pseudobase III possesses the required chemical reactivity for covalent bond induction at the receptor level. On the other hand, I and II react spontaneously with molecular oxygen to give the peroxide IV, which possesses chemical reactivity similar to that of III. The hypothesis offers itself that I and II undergo oxidation *in vivo* in position 2 prior to inhibiting the α-receptor. Pseudobase III has been shown to act as an excellent peptide bond-forming reagent by way of a selective activation of carboxyl functions. This property suggested that III may act as a selective modifier of those esterases which include a carboxyl function as part of their active centre. With acetylcholinesterase (AChE), enzymatic activity toward acetylcholine is abolished by III by way of a mechanism implicating the obliteration of the anionic binding site. The thus modified enzyme retains its esteratic activity toward indophenyl acetate. Chymotrypsin is also inhibited by III, but spontaneous reactivation was observed. However, subtilisin, which does not carry a carboxyl function on its active sequence suffers only competitive inhibition by III. Carbamates I and II react at a negligible rate with AChE and chymotrypsin, whereas peroxide IV reacts like III with these enzymes. These observations support the hypothesis that the adrenergic α-receptor may bear analogy to the "carboxyl" serine hydrolases.

UNTIL recently,[1] irreversible inhibition of the catecholamine α-receptor was exclusively associated with alkylating agents of the dibenamine family of drugs.[2,3] Our discovery[1] of the specific and profound irreversible inhibition of this receptor by carbamates of the 1,2- and 1,4-dihydroquinoline series (I, II, III) poses a problem of considerable interest from the mechanism point of view, because these agents cannot be fitted into any of the classical structural patterns[4] that are known to favour specific interaction with the catecholamine α-receptor. In fact, these structures (Fig. 1) would appear at first sight to be better suited for reaction with the cholinesterases, in view of the strong affinity of the latter for several types of carbamates.[5]

Since carbamates I and II are neutral and stable at acid or alkaline pH, their peculiar effects at the α-receptor level cannot be as readily rationalized as in the case of the alkylating agents.[3] However, the carbamate III, a pseudobase, is chemically highly

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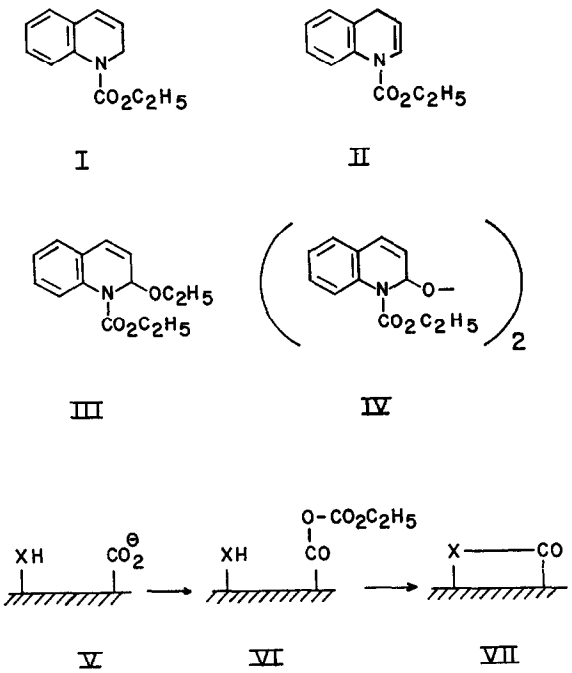

FIG. 1. Structural patterns of *N*-carbethoxydihydroquinolines and their possible mechanism of carboxyl group transformation.

reactive[1] and would thus appear suitable for the rapid induction of some kind of covalent linkage at the receptor level.

On that basis, the strong but inferior activity of I and II was tentatively attributed to initial activation *in vivo* by oxidation to some species equivalent to III.[1] Quite revealing is our recent observation[6] that the latter behaves as an excellent peptide bond-forming reagent owing to its specific ability to convert carboxyl functions quantitatively to their corresponding highly reactive mixed carbonic anhydrides. This observation suggests that III may irreversibly inactivate the α-receptor by a mechanism of cross-link formation between the long-postulated receptor carboxyl function[3,7,8] and a sterically accessible nucleophile on the active surface (V → VI → VII).

It is known that several hydrolytic enzymes carry a carboxyl function as part of their active sequence[9] ("carboxyl" serine hydrolases), and accordingly one might expect that III could also inactivate such enzymes by some analogous mechanism.

The purpose of this communication is to describe some basic observations on: (1) the interaction of I, II and III with the two "carboxyl" serine hydrolases, acetylcholinesterase (AChE) and α-chymotrypsin;[10] (2) the interaction of III with the threonyl-serine hydrolase, subtilisin;[10] and (3) the spontaneous oxygenation of I and II to species IV, which shares with III the same type of chemical reactivity.

MATERIALS AND METHODS

Bovine erythrocyte AChE (Nutritional Biochemicals Corp.) was diluted with salt solution (0·02 M in $MgCl_2$ and 0·1 M in NaCl) so that the enzyme concentration was equivalent to 4000 units/ml of stock solution. All titrimetric assays were carried out at 25 ± 0·1° and at pH 7·4, according to the previously described techniques.[11] The

same applies to dialysis and attempted reactivation experiments. The esteratic activity of AChE was also measured spectrophotometrically by the indophenyl acetate (IPA) method described by Purdie and McIvor.[12] Typical runs were as follows.

Acetylcholinesterase

Incubation and acetylcholine assay methods. To 24·5 ml of salt solution (pH 7·4) containing 0·82 per ml of enzyme, was added, in separate experiments, I, II or III at final concentrations of 3×10^{-4} M, 3×10^{-4} M and $2{\cdot}4 \times 10^{-5}$ M respectively. The solutions were incubated at $25 \pm 0{\cdot}1°$ and 2·5-ml portions were transferred to the assay cell at suitable time intervals, followed by the addition of 15 μl acetylcholine bromide (ACh) solution, giving a final concentration of $3{\cdot}59 \times 10^{-4}$ M. The initial velocities of ACh hydrolysis were recorded titrimetrically with a pH-stat instrument.[11] When inhibition by III reached completion, the incubation mixture was dialyzed against salt solution for 24 hr at 5°. Suitable controls were run in parallel fashion and the solutions were assayed against ACh. For the purpose of comparing IV with III, the respective final concentrations in three separate incubation mixtures were 8×10^{-6} M for IV and 8 and 16×10^{-6} M for III.

Attempted reactivation and protection. A dialyzed sample was incubated with pyridine aldoxime methochloride (PAM; Aldrich Chemical Co.) at a final concentration of $4{\cdot}66 \times 10^{-4}$ M for 18 hr prior to assaying with ACh. In one set of experiments, the incubation mixture was made $1{\cdot}8 \times 10^{-3}$ M in tetramethylammonium iodide (TMA) prior to adding inhibitor III at a final concentration of $2{\cdot}4 \times 10^{-5}$ M. In a parallel control experiment, this incubation mixture was used, but III was omitted.

Indophenyl acetate assay. The dialyzed enzyme (56 units/ml), previously inhibited by III to completion, was also assayed by the IPA method[12] in phosphate buffer at pH 7·83. In this case, 0·95-ml portions of enzyme solution were transferred to a quartz cuvette containing 4 ml phosphate buffer. To this was added 50 μl of IPA solution at a final concentration of $2{\cdot}0 \times 10^{-3}$ M. After mixing, the change in absorbance at 625 mμ was recorded over a period of 3 min.

α-Chymotrypsin

Incubation and assay methods: the same titrimetric technique described above was applied to α-chymotrypsin (Sigma Chemical Co.). The incubation mixture (10 ml) consisted of 0·1 M NaCl plus 0·02 mg per ml of enzyme and inhibitor at a final concentration of $5{\cdot}4 \times 10^{-4}$ M. The pH was 7·0 and the temperature $25 \pm 0{\cdot}1°$. Aliquots (200 μl) were transferred to an assay cell containing salt solution (3·8 ml) made 1×10^{-2} M in acetyltyrosine ethyl ester (ATEE). Initial velocities of hydrolysis were recorded as for AChE.

Subtilisin

To subtilisin (Sigma Chemical Co.) the same procedure as for α-chymotrypsin was applied, except for the following differences in reagent concentrations: 2 mg/ml of enzyme, 1 ml of incubation mixture (consisting of 0·02 M phosphate buffer, pH 7·0, and inhibitor at a final concentration of 1×10^{-3} M). Aliquots of 50 μl were transferred to 3 ml of 0·05 M NaCl in the assay cell containing tyrosine ethyl ester hydrochloride (TEE) at final concentrations ranging from $1{\cdot}66$ to $6{\cdot}66 \times 10^{-2}$ M. The results are summarized in Fig. 2.

Air oxidation of I and II

Exposure of thin films of I or II to air for 24 hr, followed by washing with ether, gave white crystals which after recrystallization from ether–petroleum ether (b.p. 30–60°) had an m.p. of 105–106° (decomp.); infrared bands ($CHCl_3$) at 1710, 1480, 1400, 1370, 1318 and 1285 cm^{-1}. The n.m.r. spectrum was entirely consistent with structure IV.

Analysis. Calculated for $C_{24}H_{24}O_6$: C, 70·57; H, 5·92. Found: C, 70·81; H, 5·80. The compound liberated the theoretical quantity of iodine from a potassium iodide solution. Only structure IV is consistent with the data.

RESULTS AND DISCUSSION

Acetylcholinesterase

The data summarized in Fig. 2 establish that the carboxyl-group activator III[6] and the related peroxide IV are potent irreversible inhibitors of AChE. Within 80–90 min

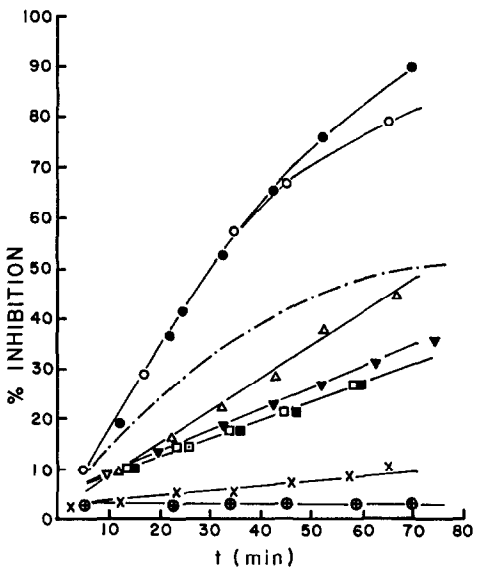

FIG. 2. Time course of the interaction of I, II, III and IV with AChE, chymotrypsin (CT) and subtilisin. The incubation mixtures consisted, respectively, of 0·82 unit/ml of AChE in 0·02 M $MgCl_2$ and 0·1 M NaCl, 0·02 mg/ml of CT in 0·1 M NaCl and 2 mg/ml of subtilisin in 0·02 M phosphate buffer, pH 7·0. The temperature was 25 ± 0·1° and, in the case of AChE, 25-ml aliquots were assayed titrimetrically toward ACh at a concentration of $3·59 \times 10^{-4}$ M or toward indophenyl acetate (IPA) in phosphate buffer, pH 7·83, at a concentration of 2×10^{-3} M by following the change in absorbance at 625 mμ for 3 min. In the case of CT, 200-μl aliquots were assayed titrimetrically toward acetyltyrosine ethyl ether (ATEE) at a concentration of 1×10^{-2} M; for subtilisin, 50-μl aliquots were assayed titrimetrically toward tyrosine ethyl ether hydrochloride (TEE) at concentrations ranging from 1·66 to $6·66 \times 10^{-2}$ M. ●—● = AChE + III, $8·1 \times 10^{-5}$ M, pH 7. Dialysis at 5° for 24 hr, pH 7, did not regenerate activity (ACh assay). ○—○ = CT + III, $5·4 \times 10^{-5}$ M, pH 7. Dialysis at 5° for 54 hr, pH 6·5, led to 74% reactivation. ⊕—⊕ = Subtilisin + III, 1×10^{-3} M, pH 7. Competitive inhibition, $K_i = 5 \times 10^{-6}$ M. —○— = AChE + III, $8·1 \times 10^{-5}$ M, pH 7 (indophenyl acetate assay). △—△ = AChE + III, $2·4 \times 10^{-5}$ M, pH 7 (ACh assay). ▼—▼ = AChE + III, $2·4 \times 10^{-5}$ M and TMA, $1·8 \times 10^{-3}$ M, pH 7, (ACh assay). Control: AChE + TMA, $1·8 \times 10^{-3}$ M. □—□ = AChE + III, 16×10^{-6} M, pH 7 (ACh assay). ■—■ = AChE + IV, 8×10^{-6} M, pH 7 (ACh assay). ×—× = AChE + I or II, 3×10^{-4} M, pH 7 (ACh assay). ×—× = CT + I or II, $5·4 \times 10^{-5}$ M, pH 7 (ATEE assay).

at $8{\cdot}1 \times 10^{-5}$ M, III causes the complete loss of activity toward ACh as the substrate. However, the resulting enzyme retains 45–50 per cent of its esteratic activity toward the uncharged substrate, IPA, thus establishing that the esteratic nucleophiles are left largely unaffected by III, in agreement with a mechanism primarily involving attack of the anionic (carboxyl?) binding site.

A qualitatively similar result was previously obtained[11,12] with the anionic site-directed alkylating agent, *N,N*-dimethyl-2-phenylaziridinium chloride (DPA), which completely inhibits AChE toward ACh as the substrate, while enhancing the esteratic activity by a factor of 2 toward IPA. It seems clear, then, that III also behaves as an anionic site-directed reagent toward AChE. In agreement with this conclusion, the tetramethylammonium ion affords protection against III, while PAM fails to reactivate the inhibited enzyme.

When the special chemical reactivity of III[6] is suppressed, as in carbamates I and II (from which peroxides have been rigorously excluded), little reaction with AChE and the two other enzymes studied is observed at 3×10^{-4} M. However, the spontaneously derived peroxide IV is as potent as III in inhibiting AChE, thus supporting the hypothesis that I and II may undergo activation *in vivo* by oxidation prior to inactivating the adrenergic α-receptor.

α-Chymotrypsin and subtilisin

α-Chymotrypsin behaves with III qualitatively like AChE, but unlike the latter, prolonged dialysis (54 hr at 5°) of the blocked enzyme causes a slow regeneration of activity (74 per cent) toward ATEE. In this case, it is not yet possible to distinguish between an initial attack by III of the carboxyl function of the active sequence and a direct interaction with the serine hydroxyl group. Experiments with labeled III are under way in order to clarify this point.

However, it should be noted that subtilisin, which lacks a carboxyl function on its active sequence,[10] does not react at all with III, in spite of its strong affinity (competitive binding, against TEE; $K_i = 5 \times 10^{-6}$ M). Since both enzymes have a strong inherent affinity for aromatic molecules, it seems to follow that the observed difference in the reactivity of α-chymotrypsin and subtilisin toward III may be relevant to the presence (in the former) and absence (in the latter) of a carboxyl function on the active sequence. This contention agrees with the demonstrated selective activity of III toward carboxyl functions.[6]

The long-standing postulate that the catecholamine α-receptor includes a carboxyl function on its binding surface,[3,7] and that it may somehow resemble the active sequence of "carboxyl" serine hydrolases[8] agrees with the demonstrated ability *in vitro* of III to inhibit irreversibly enzymes that carry a carboxyl function as part of their binding surface.*

The sensitivity of I and II to oxidation by molecular oxygen to a peroxide (IV), which shares with III similar chemical reactivity, accounts at least in part for their activity *in vivo* as irreversible blockers of the adrenergic α-receptor. It would appear that III behaves as a carboxyl-directed inhibitor of enzymes and adrenergic α-receptors.

* We have been informed by Dr S. A. Narang of the National Research Council of Canada, Ottawa, that III does not activate or react with the phosphate residues of nucleotides, an observation which is at variance with the hypothesis that adenosine triphosphate would be the site suffering attack by adrenergic blocking drugs.[13]

The interposition of transport and translocation barriers *in vivo* would seem to deny III access to other potential sites of action at doses which irreversibly modify the adrenergic α-receptors. This adequately accounts for the observed selectivity of III *in vivo*. Moreover, it is probable that some degree of complementarity between III (or an equivalent species) and some area of the receptor surface is required in order that selective attack of a carboxyl group can be achieved. Whether the carboxyl group in question acts as an anchoring site for the catecholamines themselves cannot be decided at present, although current theoretical models of the receptor surface[8] deny the carboxyl group a direct involvement in catecholamine binding.

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