Regulatory Effects of Polyamines on Membrane-Bound Acetylcholinesterase

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The effects of putrescine, spermidine and spermine on membrane-bound acetylcholinesterase from human erythrocyte 'ghosts' and the solubilized enzyme of the electric organ of the electric eel were studied by kinetic methods. Measurements were made by using a photometric method which made it possible to record the enzyme reaction in the steady-state phase. Substrate-concentration-dependent activation and inhibition of acetylcholinesterase by polyamines is similar to that by Na^+ , K^+ , Ca^{2+} , Mg^{2+} and certain quaternary and bisquaternary amines. The kinetics suggest an allosteric reaction mechanism. On the basis of the kinetic results a role for the polyamines as modulators of synaptic acetylcholinesterase is proposed.

The polyamines spermidine and spermine and their physiological precursor putrescine are polycations of ubiquitous occurrence (Cohen, 1971). Although they are probably mainly involved in cell growth and cell proliferation (Tabor & Tabor, 1964, 1972; Bachrach, 1973, 1970; Herbst & Bachrach, 1970*a,b*; Cohen, 1971; Russell, 1973) it is possible that they also have functional roles besides those related to protein and nucleic acid metabolism. Therefore we were looking for possible interactions between these naturally occurring polycations and enzymes that are known to be modulated by cationic compounds. We have described inhibitory effects of spermidine and spermine on two bivalent cation-activated ATPases‡ (Peter *et al.*, 1973). A stabilizing effect of spermine on ($\text{Na}^+ + \text{K}^+ + \text{Mg}^{2+}$)-stimulated ATPase of microsomal and synaptosomal systems has also been reported (Nag & Ghosh, 1973).

Acetylcholinesterase (EC 3.1.1.7) is known to be modified in its activity by a large number of cationic compounds (Wolf, 1964, 1965; Changeux, 1966; Roufogalis & Thomas, 1968, 1969; Changeux *et al.*, 1968; Wins *et al.*, 1970; Belleau *et al.*, 1970; Kitz *et al.*, 1970; Wombacher & Wolf, 1971; Roufogalis & Quist, 1972; Kato *et al.*, 1972). In particular its interaction with some well known ganglion-blocking agents (tetramethylammonium iodide, pentamethonium chloride) revealed its sensitivity to changes caused by allosteric mechanisms that depend on substrate concentration. At low acetylcholine concentrations acetylcholinesterase activity was inhibited, whereas it was activated at high substrate concentration in the presence of the same concentrations of the drug that were inhibitory at low sub-

strate concentration (Wombacher & Wolf, 1971). In preliminary experiments we found some interesting effects of polyamines on the activity of membrane-bound erythrocyte acetylcholinesterase and therefore we decided to carry out kinetic analyses of the influence of putrescine, spermidine and spermine on the activity of this enzyme.

As far as we know there is no reason to assume that the membrane-bound acetylcholinesterase from nervous tissue and from erythrocyte 'ghosts' have different kinetic properties. Even the solubilized enzyme from the electric organ of the electric eel showed changes in activity similar to those shown by the erythrocyte enzyme in the presence of polyamines. On the basis of the enzyme kinetic results we therefore propose a possible role for the polyamines as modulators of synaptic acetylcholinesterase.

Our results show that polyamines have effects on acetylcholinesterase which are analogous to those produced by Na^+ , K^+ , bivalent cations (Mg^{2+} , Ca^{2+}) and certain quaternary and bisquaternary bases.

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Materials and Methods

Materials

Enzyme. Human erythrocyte 'ghosts' were prepared by the method of Wolf (1970, 1972). The specific activity of the acetylcholinesterase in this preparation was 19.6 units/mg of protein.

Soluble acetylcholinesterase from *Electrophorus electricus* was obtained from Boehringer Corp., Mannheim, Germany.

‡ Abbreviations: AH, Augustinsson-Hofstee; ATPase, adenosine triphosphatase.

Chemicals. Phenol Red and Tris base were from E. Merck, Darmstadt, Germany; acetylcholine iodide was from Fluka Corp., Buchs, Switzerland, spermidine hydrochloride and spermine hydrochloride were from Serva Corp., Heidelberg, Germany, and putrescine hydrochloride was from Schuchardt Corp., München, Germany. Water was double-distilled in quartz apparatus and used for preparation of all solutions.

Methods

Assay method. The reaction-rate measurements were done by using the photometric method of Gregoire *et al.* (1955), which was modified so that the reaction rate in the steady-state phase could be recorded (Wolf, 1964; Wombacher & Wolf, 1971). The test solution contained in a final volume of 10.0ml: 1.0mM-Tris-HCl buffer, pH8.0; 80 μ g of Phenol Red; 16–26 μ g of 'ghost' preparation (equivalent to 8–13 μ g of protein), or 1 μ g of soluble acetylcholinesterase. Acetylcholine concentrations were varied from 0.02 to 10mM in the experiments with spermine and spermidine, and from 0.02 to 4mM with putrescine. The concentrations of the amines are indicated in the legends of the Figures. Their buffering capacity was considered by appropriate corrections. The reaction was started by addi-

tion of substrate and the rate was recorded at 546nm, within the initial phase of linear reaction, with an Eppendorf photometer combined with an Eppendorf recorder. The temperature of the cuvette holder was maintained at $30 \pm 0.1^\circ\text{C}$.

Data are mean values of at least four determinations. For substrate concentrations of 0.1mM and higher, individual measurements used for AH plots were found to vary by 3% at the most from the mean. For substrate concentrations of less than 0.1mM, the variation between individual measurements was more than 10%, although in the presence of putrescine, the accuracy was only slightly less than for high substrate concentrations. The rate was proportional to the amount of 'ghost' preparation. Phenol Red at the concentration used did not affect the reaction rate, this was checked by the Warburg method (H. U. Wolf, unpublished work). This assay method is not suitable for measurements with substrate concentrations of less than 0.02mM, since it is not possible to establish a steady-state phase under these conditions.

Results

Figs. 1–3 show the AH plots (Augustinsson, 1948; Hofstee, 1956) for putrescine, spermidine and spermine respectively in which only the reaction-rate

Fig. 1. AH plots for various putrescine concentrations

The test solution contained 16 μ g of 'ghost' preparation. For further details see the Materials and Methods section. The inset shows reaction rate versus logarithm of [S] for various putrescine concentrations. The following putrescine concentrations were used: $\blacktriangle$, 2mM; $\blacksquare$, 0.5mM; $\square$, 0.2mM; $\bullet$, 0.1mM; $\circ$, 0.05mM; $\blacklozenge$, 0.01mM; $\diamond$, control.

Fig. 2. AH plots for various spermidine concentrations

The test solution contained 13 g of 'ghost' preparation. For further details see the Materials and Methods section. The inset shows reaction rate against logarithm of [S] for various spermidine concentrations. The following spermidine concentrations were used: ■, 1 mM; ◇, 0.2 mM; ●, 0.1 mM; □, 0.04 mM; ▲, 0.02 mM; ○, 0.01 mM; ▽, control.

Fig. 3. AH plots for various spermine concentrations

The test solution contained 20 μg of 'ghost' preparation. For further details see the Materials and Methods section. The inset shows reaction rate against logarithm of [S] for various spermine concentrations. The following spermine concentrations were used: ■, 0.5 mM; ◇, 0.2 mM; ●, 0.1 mM; □, 0.04 mM; ▲, 0.02 mM; ○, 0.01 mM; ◆, 0.005 mM; △, 0.001 mM; ▽, control.

values obtained for the ascending parts of the respective reaction-rate versus logarithm of substrate concentration curves are shown. The AH plots appear to be linear for putrescine within the limits of experimental error, but for higher spermidine and spermine concentrations the plots show concave-upward curves, which are more pronounced the higher the concentration. In the inset of Fig. 1 the reaction rate for various putrescine concentrations is plotted against logarithm of substrate concentration. The reaction rate observed at the optimum concentration increases with increasing putrescine concentration, whereas at low substrate concentrations weak inhibition occurs, which is more pronounced at higher putrescine concentrations.

The inset of Fig. 2 shows the analogous plot for spermidine. At low substrate concentrations the reaction rate markedly decreases with increasing spermidine concentrations. On the other hand, activation occurs at optimum substrate concentrations (with [spermidine] = 0.01, 0.02, 0.04 and 0.1 mM) with the reaction rate again decreasing with increasing spermidine concentrations.

The corresponding plot for spermine is shown in the inset of Fig. 3. The reaction rate at optimum substrate concentrations increases with increasing spermine concentrations ([spermine] = 0.001–0.005 mM). When spermine is 0.01–0.04 mM a decrease in reaction rate is observed. A slight increase is observed at a spermine concentration of 0.1 mM, whereas the reaction rate at optimum substrate concentrations decreases with further increase in spermine concentration. At low substrate concentrations inhibition by spermine at concentrations higher than 0.001 mM is as great as for spermidine.

As well as investigations on erythrocyte acetylcholinesterase in the membrane-bound state, experiments have also been carried out with soluble acetylcholinesterase from the electric-organ of the electric eel. Although these have not been done in the same detail, the rates calculated from these measurements showed similar dependences on substrate and effector concentrations.

Discussion

General considerations

The features of the different plots indicate a rather complicated allosteric reaction mechanism. An attempt to unequivocally elucidate this mechanism merely on the grounds of the kinetic data would certainly be a daring approach. We feel that it is necessary to consider at least briefly the most likely possibilities that would produce the observed effects. However, since we are dealing with a membrane-bound enzyme, special care has to be exercised in interpretation of the results.

Apart from the fact that reaction rates might be influenced by diffusion-layer problems and steric hindrance, the following cases have to be considered: (a) direct interaction of the polyamines with acetylcholinesterase evoked by binding to allosteric sites; (b) interaction of the polyamines with another protein, or with other proteins, or even with lipids, within the membrane, leading to conformational changes that are transferred to acetylcholinesterase, thereby influencing its activity; (c) a mixture of these interactions.

Measurements in the presence of polyamines on solubilized electric-eel acetylcholinesterase showed a substrate concentration-dependent shift from inhibition to activation as was observed for the membrane-bound enzyme. These measurements were mainly done with the aim of discovering whether polyamines interact at all with acetylcholinesterase itself, although in view of the known tendency of cationic compounds to interact with this enzyme, non-interaction would have been surprising. These measurements were not carried out in detail because the membrane-bound enzyme bears, in our opinion, more physiological relevance than the solubilized enzyme. As the findings with the membrane-bound and soluble enzyme are similar it seems reasonable to assume that the effects described here are at least partly due to the direct interaction of the polyamines with the membrane-bound acetylcholinesterase. This is further supported by the fact that the localization of acetylcholinesterase at or near the outer surface of the human erythrocyte has been established (Herz & Kaplan, 1973).

Our data can formally be treated on the basis of the single substrate–single modifier model of Frieden (1964), by regarding those of the possibilities of interactions mentioned above that are relevant. By binding to the enzyme the effector lowers the affinity for the substrate, however, the enzyme–effector–substrate complex breaks down at a higher product yielding rate than the enzyme–substrate complex. Therefore whether inhibition or activation is observed in the presence of the effector depends on the substrate concentration. This situation is described in Frieden's (1964) model for $K_3/K_2 > k_6/k_5 > 1$ (see Fig. 2E in Frieden's paper; note, however, that $1/v$ versus $1/[S]$ plots are used there instead of the AH plots which we use in the present paper). With respect to substrate binding and substrate transformation to products, this mechanism is analogous to a partially competitive partially non-competitive inhibition, in which, however, $k_6 > k_5$.

By extending this case to an enzyme that possesses two modifier binding sites, AH plots of the kind shown in Fig. 1 for putrescine will result. Scheme 1 shows the reaction scheme for this case. The ratios of the respective dissociation constants given in the legend to the Scheme must apply in order to obtain AH

Scheme 1. Reaction scheme for an enzyme with one catalytically active site and two modifier binding sites

For AH plots of the kind obtained for putrescine, the following ratios of the dissociation constants must be valid: $K_4/K_3 > \beta > 1$; $\gamma \approx \beta$; $K_6 > K_3$ (at least by one order of magnitude); $K_7 > K_4$ (by at least one order of magnitude); $K_{13}/K_{12} > \delta > 1$; $\delta > \beta$. [K_1 , K_3 , K_4 , k_2 and βk_2 correspond to K_1 , K_2 , K_3 and k_6 respectively of the case treated by Frieden (1964).]

plots of the observed characteristics (i.e. two points of intersection in the first quadrant). Of course, no statement is possible about the position of these modifier sites [see cases (a) and (b) above], although the results obtained with electric-eel acetylcholinesterase seem to show that at least one site is located on the acetylcholinesterase molecule.

The AH plots obtained for spermidine and spermine suggest the existence of one and two modifier sites respectively on the acetylcholinesterase molecule or within the membrane. These two sites follow from the points of intersection obtained in the second quadrant on extrapolation of the linear portion of the plots and from the point of intersection occurring between the AH plots for spermine within the first quadrant respectively. In addition at least one more binding site has to be assumed for these amines. These sites represent high-affinity sites and should be indicated by a common point of intersection at high $v/[S]$ values between the control AH plots and those obtained for lower amine concentrations than were used in the present work. Owing to the limited accuracy of the assay method it was not possible to establish these sites. However, the circular-dichroism measurements on erythrocyte 'ghosts' in the presence of these amines furnished evidence for conformational changes which parallel the results from the kinetic measurements and also reveal the existence of the postulated high-affinity sites (A. Kossorotow & H. U. Wolf, unpublished work).

The curves of the AH plots might be due to the fact that erythrocyte acetylcholinesterase presumably exists within the membrane as an oligomeric protein (Bellhorn *et al.*, 1970; Wright & Plummer, 1973). In this case the possibility of negative homotropic effects evoked by acetylcholine binding to the subunits of the acetylcholinesterase oligomer has to be considered. The polyamines might amplify the negative co-operativity by exerting negative heterotropic effects on acetylcholine affinity for acetylcholinesterase by binding to acetylcholinesterase or to other membrane proteins. Similarly, various influences on the values of the catalytic rate constants have to be considered (see, e.g. Levitzki & Koshland, 1969). Therefore, considering the complex pattern of the AH plots, a treatment of the reaction mechanism on the basis of the sequential model for subunit interactions (Koshland *et al.*, 1966; Kirtley & Koshland, 1967; Conway & Koshland, 1968) appears promising, provided some binding data are collected. The term 'subunit' in this connexion could designate the individual promoters of the acetylcholinesterase oligomer as well as different molecular entities co-operatively interacting within the membrane.

A noteworthy feature shown in the insets of Figs. 1, 2 and 3 is the shift, in the presence of the polyamines, of the threshold of substrate inhibition to higher substrate concentrations. At present substrate inhibition of acetylcholinesterase is attributed

to acetylcholine binding at regulatory sites (see, e.g. Kato *et al.*, 1972). It seems likely that the polyamines, by competing with acetylcholine for these sites, or by allosteric effects, relieve substrate inhibition.

Some physiological considerations

Although it is not possible at present to describe the complete molecular mechanism of polyamines as effectors of acetylcholinesterase, they have, nevertheless, proved useful in the study of the conformational changes in this enzyme, which appear to be important in its regulation. However, more important is that the observations allow the suggestion of a probable role for polyamines in the regulation of the acetylcholine concentration at sites of physiological importance, for instance the synaptic cleft of cholinergic nerve endings. (We will exclusively consider the nerve endings, since only at this site is the physiological role of acetylcholinesterase well enough understood. It is self evident, however, that analogous considerations can be made in principle for any other physiological activity of acetylcholinesterase.)

Polyamines are normal constituents of all cell types. The concentrations of spermidine and spermine are of the order $0.1\text{--}2\text{ }\mu\text{mol/g}$ of tissue (Cohen, 1971; Bachrach, 1973) in all tissues including brain; putrescine occurs in comparable concentrations only in lower vertebrates and in invertebrates (Caldarera *et al.*, 1969; Russell *et al.*, 1969; Herbst & Dion, 1970; Cohen, 1971; Seiler & Lamberty, 1973); its concentration in adult mammalian tissue is lower by a factor of approx. 0.03 (Seiler & Askar, 1971). These concentrations (except for that of putrescine in mammalian tissues) are well within the range for the effects that we have observed on acetylcholinesterase activity. Although most observations on the subcellular localization of spermidine and spermine indicate the preferential occurrence of these amines in structures rich in RNA and DNA [i.e. endoplasmic reticulum and cell nuclei (Stevens, 1970; Cohen, 1971; Seiler, 1973)], there is some preliminary evidence (N. Seiler & K. Deckhardt, unpublished work) for the presence of the polyamines in nerve endings. The recent detection of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, enzymes involved in polyamine biosynthesis, in the synaptosomal fraction of rat brain (Schmidt & Cantoni, 1973) is further evidence for their presence in this part of nerve cells.

If we assume that either spermidine or spermine, or both, can be liberated physiologically from cholinergic nerve terminals, together with acetylcholine in sufficiently high amounts, then it is possible that these amines modulate the concentration of acetylcholine in the synaptic cleft, by their interaction with acetylcholinesterase. On the basis of our kinetic observations it would be expected that at low fre-

quency of discharge, i.e. at low physiological acetylcholine concentrations in the synaptic cleft, acetylcholine would be preserved by the inhibitory action of spermidine and spermine on acetylcholinesterase, so that the physiological effect of acetylcholine would be prolonged. On the other hand acetylcholine inactivation would be enhanced by the polyamines when it was present at high concentration. This effect would help prevent blocking of the synapse.

In principle putrescine (and probably other diamines) could exert effects similar to spermidine and spermine at cholinergic nerve endings, although its inhibitory effects on acetylcholinesterase at low substrate concentration are not as pronounced as those of the polyamines. Buffering of the acetylcholine effect at the synaptic site can be expected not only from interaction of the polyamines with acetylcholinesterase, but also from a direct interaction of the polyamines with the cholinergic receptors. It is well known (Triggle, 1971; Heilbronn, 1971) that many pharmacologically active compounds exhibit similar effects on both acetylcholinesterase and the acetylcholine receptor. Indeed both spermidine and spermine antagonize the contractions of rat uterus (Hashimoto *et al.*, 1973) and of guinea-pig ileum produced by acetylcholine, nicotine and 5-hydroxytryptamine (Onodera *et al.*, 1968; Shaw, 1972).

Although at present experimental evidence on the interaction of polyamines with acetylcholinesterase and the acetylcholine receptor under physiological conditions is lacking, it should be possible to obtain further information by microiontophoretic studies and studies on stimulated polyamine release from cholinergic preparations.

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