Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 190-196

ELECTRON SPIN RESONANCE STUDIES OF THE EFFECTS OF NATURALLY-OCCURRING EXCITOTOXIC AMINO ACID ANALOGUES ON THE PHYSICAL STATE OF MEMBRANE PROTEINS IN HUMAN ERYTHROCYTES

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Received July 27,1981

SUMMARY: The interaction of the neurotoxic natural products, kainic and ibotenic acids, both of which are also excitatory neurotransmitters and amino acid analogues of glutamic acid, along with the latter compound, with human erythrocyte membranes has been investigated by electron spin resonance methods. Only ibotenic acid caused a statistically significant alteration in the physical state of membrane proteins ($P = 0.01$) while none of these excitotoxins measurably affected motion of membrane lipids. In order to further investigate some of the molecular characteristics of ibotenic acid that may have contributed to its effect on the conformation of membrane proteins, similar spin labeling studies were performed employing the decarboxylation product and parent ring compound of this excitotoxin, muscimol and isoxazole, respectively. No effect of either of these latter compounds was observed suggesting that the carboxylic acid group of ibotenic acid is essential for its interaction with membrane proteins. These results are discussed in relation to the known different neurotoxic and physiological effects of kainic and ibotenic acids and muscimol.

The excitotoxic amino acids, kainic acid (KA) (1), and ibotenic acid (Ibo) (2) (Figure 1), which are isolated as natural products from the seaweed Digenia simplex and the mushroom Amanita muscaria, respectively, have been shown to be excitatory neurotransmitters [1-4] reminscent of L-glutamic acid (3) [5]. These natural products are analogues of glutamic acid. In contrast to the excitatory properties of KA and Ibo, muscimol (4), the decarboxylation product of Ibo, along with gamma-aminobutyric acid (5), the decarboxylation product of glutamic acid, are powerful inhibitory neurotransmitters [6]. Glutamic, kainic, and ibotenic acids all exhibit neurotoxic properties when injected into mammalian brain [7], although the action of ibotenic acid is thought to be different than the former two excitotoxic agents since an intact glutamate innervation is not required [7]. Further, in contrast to KA,

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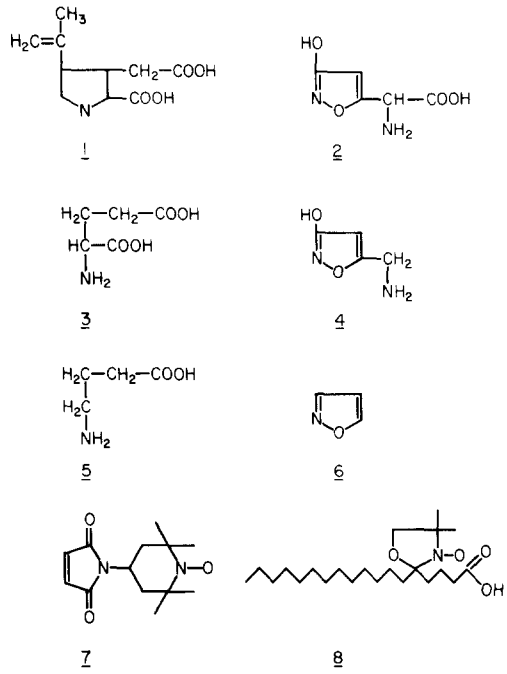

Figure 1. Molecular formulae for the compounds used or discussed in this study: (1) kainic acid; (2) ibotenic acid; (3) glutamic acid; (4) muscimol; (5) gamma-aminobutyric acid; (6) isoxazole; (7) 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl; (8) 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl

neuronal death caused by Ibo is highly specific for the injection site [7] and both result in a neuropathology similar to that seen in persons suffering from Huntington's disease [8]. Another difference between Ibo and KA is that the decarboxylation product of KA is essentially devoid of inhibitory neurotransmitter properties unlike muscimol [7]. These different characteristics of KA and Ibo may suggest different molecular bases for their effects on neurons. In order to study this possibility on cell membranes, spin labeling studies of the effects of glutamic, kainic, and ibotenic acids on the physical state of membrane proteins in human erythrocytes have been performed. Only ibotenic acid caused a statistically significant alteration in the conformation of memebrane proteins. Further experiments to relate these findings to particular structural aspects of Ibo and to assess the role

of membrane lipids in these results were also undertaken with the result that the carboxylic acid group of Ibo was found to be essential for its action with membrane proteins.

METHODS

Glutamic and kainic acids (>99% pure) were obtained from Sigma while ibotenic acid and muscimol (>99% pure) were obtained from Chemical Dynamics Corporation. Isoxazole (6) was purchased from Aldrich. All other chemicals used were of the highest purity available.

Human erythrocytes were obtained by venipuncture into heparinized tubes and cell membranes (ghosts) were prepared as described previously [9,10]. The spin labels used, 2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl (7) and 2-(3-Carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (8), were purchased from Syva. UV and melting point analyses [11] showed no isomaleimide spin label present.

Erythrocyte ghost membranes were spin labeled with the protein-specific piperidine maleimide spin label as previously described [9,10] and washed five times at 4°C with 5 mM sodium phosphate buffer, pH 8.0, to remove excess spin label as also previously described [9,10]. To three of four separate 0.24 ml aliquots of spin labeled, washed ghosts from the same donor were added 0.06 ml of Ibo, KA, or glutamic acid, respectively, in 5 mM sodium phosphate buffer, pH 8.0 to give a final concentration of each excitotoxic compound of 5×10^{-6} M. To the fourth aliquot of 0.24 ml of spin labeled ghosts were added 0.06 ml of the phosphate buffer as a control. Each sample was shaken gently for 15 minutes at room temperature and directly used for ESR studies. Studies with muscimol and isoxazole were performed in the same manner.

RESULTS

A typical ESR spectrum of the protein-specific spin label (7) covalently bound to cysteine and lysine residues of membrane proteins in human erythrocytes is shown in Figure 2. This type of spectrum discussed extensively elsehwere [12-17] demonstrates the existence of at least two different types of spin label binding sites: one strongly immobilized (S) and one weakly immobilized (W). As discussed by many authors [12-22], the W/S ratio is a sensitive and convenient monitor of the physical state of membrane proteins and has been used in the study of the relationships between structure and function of cell membranes [12-17] and alterations of these relationships in disease processes [18-22]. Typically, unless the membrane is completely disrupted or proteins solubilized from the membrane, the W/S ratio of this

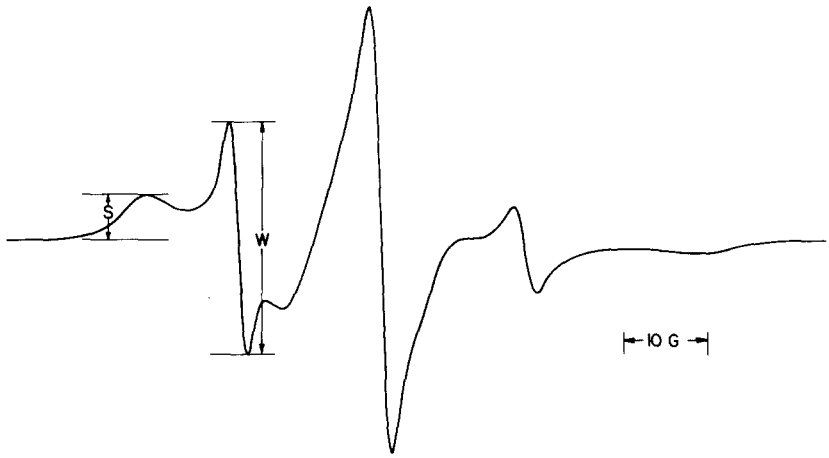

Figure 2. Typical ESR spectrum of the protein-specific spin label (7) covalently bound to membrane proteins in human erythrocyte ghosts. The ESR spectral amplitudes of the M_I = +1 lines of the spin label bound to strongly- and weakly immobilized binding sites are indicated by S and W, respectively. The spectra of this study were recorded on a Varian E-109 Century Series ESR spectrometer employing E-238 quartz aqueous sample cell and E-238 resonant cavity. Spectrometer conditions: 100 G scan range, 30 min scan time, 0.25 sec time constant, 0.32 G modulation amplitude, and 14 mw microwave power incident on the resonant cavity.

protein specific spin label is altered by 5-15% in erythrocyte ghosts over control values by various procedures and agents or in pathological states [12-22]. The effects of addition of glutamic, kainic, or ibotenic acids to spin labeled erythrocyte ghosts are shown in Table I. Only ibotenic acid caused a statistically significant alteration in the W/S ratio of the piperidine maleimide spin label ($P = 0.01$) the mean value of which was reduced upon Ibo treatment compared to the control value. This result suggests that only this excitotoxic neurotransmitter caused an alteration in the conformation and/or organization of membrane proteins.

Examination of the lipid phase of the membrane upon addition of Ibo revealed no changes in ESR spectra of 5-doxylstearic acid (8) relative to control samples suggesting no alteration in lipid fluidity is caused by this excitotoxic substance. This result may imply that Ibo interacts with cell surface protein components and not within the lipid bilayer. Consistent with this suggestion, in preliminary experiments Ibo appeared to attenuate the

TABLE I

EFFECTS OF GLUTAMIC (GLU), KAINIC (KA) AND IBOTENIC (IBO) ACIDS ON THE W/S RATIO OF THE PROTEIN-SPECIFIC SPIN LABEL (7) ATTACHED TO MEMBRANE PROTEINS IN HUMAN ERYTHROCYTE GHOSTS.

	Control	+ Glu	+ KA	+ Ibo
MEAN ± S.D.[a]	5.17 ±0.17	5.52 ± 0.34	5.48 ± 0.45	4.72 ± 0.21
P[b]	—	<0.1, NS[c]	<0.5, NS[c]	0.01

a. N = 5 separate experiments were performed.
b. P-value calculated from a two-tailed Student's t-test.
c. NS = not significantly different than the control value.

decrease in chloride efflux rate constant with time previously observed [23,24]. Chloride transport across intact erythrocytes is controlled at the external surface of the major transmembrane protein of the red cell [25].

In order to further study the chemical basis of the action of Ibo on membrane proteins, muscimol, the decarboxylation product of Ibo, and isoxazole the parent ring compound of Ibo, were similarly examined. No effect of either agent on three different membrane preparations could be demonstrated by the piperidine maleimide spin label (7) [percent change in the W/S ratio relative to control treated with 5 mM phosphate buffer: muscimol: -1.8 ± 2.1%; isoxazole: 0.1 ± 1.7%] suggesting that these agents do not affect the physical state of membrane proteins.

DISCUSSION

The present results of an alteration of the physical state of membrane proteins in human erythrocytes by the neurotoxin, Ibo (3), and the absence of effects of muscimol (4) and isoxazole (6) suggest that the carboxyl group of this compound is critical in the observed effects. The fact that neither muscimol or KA had a statistically significant effect on membrane proteins also suggests that the location of the alpha-amino group in the ring (KA) or external to it (muscimol) is not important in the results observed with Ibo. Similarly, the absence of effects with isoxazole suggests that neither the

ring itself or the 3-hydroxyl group may play a crucial role in the interaction of Ibo with proteins of erythrocyte membranes. In contrast to these results with Ibo, whose neurotoxic effects are different than those of kainic acid [7], the isopropylene group of KA was shown to be essential for the neurotoxic properties of this compound [20]. The results with muscimol are in contrast to the reported effects of GABA (5), the decarboxylation product of glutamic acid, which caused a highly significant increase in the W/S ratio of this spin label in erythrocyte ghosts [24].

The different results obtained with KA and Ibo in this study may suggest that the differential neuropathological and physiological characteristics of these excitotoxins may be related to their differential interactions with membrane proteins. These studies are being extended to examine the nature of the membrane protein interaction site(s) of ibotenic acid, possible biochemical consequences of this interaction (e.g. altered enzymatic activities), and whether erythrocyte membranes in pathologic states respond differently to this agent.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (NS-13791) and the Muscular Dystrophy Association of America.

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