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Acetylcholine and ethylene: do they share similar receptors and biological action?

B.A. Kurchii*

Institute of Plant Physiology and Genetics, NAS of Ukraine
31/17 Vasylkivska Str., Kyiv, 03022, Ukraine

Summary. From chemical point of view acetylcholine (ACh) is a quaternary ammonium salt whose biological importance is connected with its role as a neurotransmitter between neurones and other neurocellular junctions. Earlier we have postulated (Kurchii, 1998) and then confirmed (Kurchii, Kurchii, 2000) that ACh under influence of physiological solution and alkaline mixture was decomposed with releasing of ethylene. Unfortunately using gas chromatograph we could not detected another two peaks on the chromatogram (Kurchii, Kurchii, 2000). Here we identified one of two unknown peaks: it is ethylene oxide. We have revealed that ethylene oxide was released in the quantity much more than ethylene during 10-20 min of decomposition in air or in drop of physiological solution. We have concluded that biological effects of ACh can be caused by action of ethylene oxide that is a very reactive agent and this is a prompt effect for short distance. Ethylene can migrate on the long distance and cause slow effects, but it should be preliminary activated because under normal conditions (temperature and pressure) it is a very inert chemical and cannot react with any substance. Nevertheless, it can be *in vivo* activated in the free radical addition reactions. Also as follows from our results the question about specific receptors for ACh is questionable.

Keywords: acetylcholine, ethylene, ethylene oxide, free radicals, receptors.

Introduction. Biogenic amine acetylcholine is a rather simple molecule of formula $(CH_3COOCH_2CH_2N^+(CH_3)_3$ [10]. Recent investigations suggest that ACh is not present only in neurons and glial cells of the nervous system but also is synthesized in epithelial, mesothelial, endothelial cells, alveolar macrophage, and white blood cells [38, 44, 76].

ACh induces conformational changes and then concomitant cellular changes through their interactions with membrane-bound proteins. Its neurophysiological action is mediated by either nicoticic (nAChR) or muscarinic (mAChR) receptors. The released ACh by nerve impulse at the terminal region diffuses across the synaptic cleft and by binding to protein receptors in the postsynaptic membrane acts to produce a conformational change in the postjunctional membrane of the motor end-plate, providing for an increase in permeability to cations, especially Na^+ and K^+ and this results in a muscle contraction caused by depolarization of the membrane of the muscle cell. ACh is enzymatically hydrolyzed by acetylcholinesterase in the synaptic cleft into choline and acetate. Choline is then transported back into the presynaptic cell and through the action of acetyltransferase is transformed into ACh [10, 32, 33, 42, 70].

Thus, nAChR converters exstracellular signals into intracellular effects. In the most cases the receptors are composed of three functional parts: a signal receiving (ligand binding) part "R", effector part "E" that converts signal on to the inner cell, and part "T", the so-called transducer that connects "R" and "E" parts (Fig. 1) [38].

*** Corresponding author.**
Tel.: +38044-2575160
E-mail address: kurchii@mail.ru

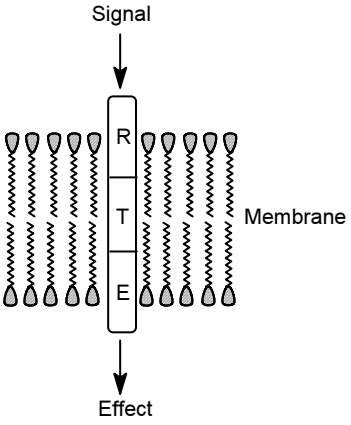


Fig. 1. The triune receptor concept (adapted from 38).

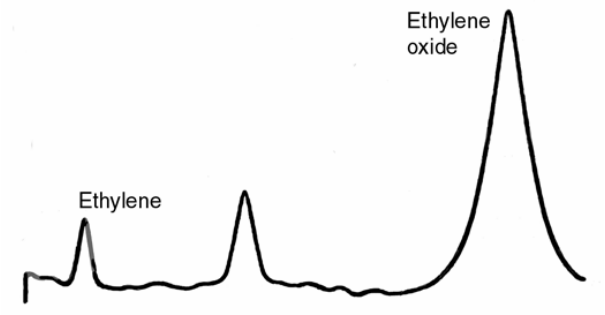
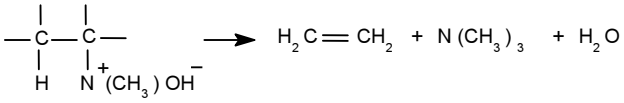


Fig. 2. Chromatogram from gas chromatography analysis of products formed during ACh decomposition. The retention times of the compounds in the chromatogram are: 39 sec, ethylene; 315 sec, ethylene oxide.

Surprisingly ACh is not only distributed in vertebrates and invertebrates but also in a plant kingdom [30, 35, 72]. As in the animals ACh is synthesized in plants via acetyltransferase (EC 2.3.1.6) [69] and decomposed by acetylcholinesterase (EC 3.1.1.7) [55, 56]. Little is known about the role of ACh in the plants. It is proposed that ACh can act in the plants as a local hormone in phytochrome-mediated responses [40] and produces alteration of ion channel activity [33, 35, 72]. Hartmann and Gupta [35] reported data confirming the existence of 'ACh-binding sites' in bean seedlings. However, the biochemical characteristics of ACh receptors remain unclear in plants.

It is interestingly to note that from chemical viewpoint ACh is a quaternary ammonium salt that theoretically in appropriate conditions can be decomposed in accordance to Hofmann's rule with olefin formation [22, 28, 29]:



If ACh may be decomposed *in vivo* with ethylene releasing, hence the question on the ACh receptor still seems to be open. In order to understand the molecular mechanisms that are responsible for the biological action of ACh we have examined the hypothesis [45] that physiological effects of ACh might be attributable to its decomposition with ethylene formation. Herein we report an extension of this approach toward the generation of ethylene and ethylene oxide from ACh.

Material and methods. The chemicals ACh·HCl and cholin·HCl used in this work were from «Mosmedpreparaty». Ethylene oxide was purchased from «Sigma». Chemicals were decomposed in 10 cm^3 flasks with rubber caps at room temperature and 1 cm^3 of head space gas was analyzed for ethylene. Ethylene was determined by a gas chromatograph («Selmihrom», Sumy, Ukraine) filled with a flame-ionization detector and a 2 m x 3.2 mm stainless-steel column packed with Poropack-Q (80-100 mesh). The oven temperature was 100 °C and the N_2, H_2 and O_2 (air) flow rates were 40, 40 and 350 ml min^{-1}, respectively. Ethylene identification was based on the retention time compared with the C_2H_4 standard. A computer program was used to calculate the quantities of ethylene in the samples.

Experimental data. Earlier we reported that ACh·HCl was decomposed to form ethylene [49]. Unfortunately we could not detect two another peaks. In this work we have identified that one from the unknown peaks is ethylene oxide (Fig. 2). Also ethylene oxide was found in the flasks where to ACh we added several drops of 0,9 NaCl. Ethylene and ethylene oxide were already found in the flasks after 10 min of exposition. Thus, ACh was decomposed with formation of ethylene and ethylene oxide. In our experiments the quantity of formed ethylene oxide was markedly higher than such of ethylene. Unfortunately we could not detect one peak in the chromatogram.

Discussion. As mentioned above biological action of ACh is explained in terms of an interaction with receptors for this chemical. This interaction is believed to be a physicochemical reaction that depends on the molecule of ACh

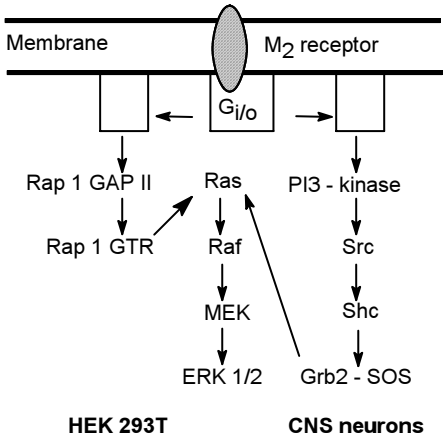


Fig. 3. Schematic representation of some pathways described for the M2 mAChR in various cell lines or in CNS neurons (adopted from 50).

being attracted to a corresponding or a complementary molecular structure of the receptor. Two types of ACh receptors are known as associated with animal cell membranes mediating physiological effects of ACh: nicotinic and muscarinic, being selectively activated by the agonists nicotine and muscarine, respectively [4, 17, 23]. There are also numerous subtypes of these receptors.

If hormone perception is necessary for the coordinated growth and development of multicellular eukaryotes one of the most intriguing questions is how do the receptors transmit the signal? In animals it is involved in two distinct pathways: the nicotinic receptor, in which the channel is activated directly by acetylcholine, and the muscarinic receptor, which requires an indirect G protein-based pathway [25, 27, 65].

Nicotinic Receptors. These receptors are found in both the central nervous system (CNS) and the peripheral nervous system (PNS) [52]. Nicotinic receptors are divided into the subtypes nicotinic-N receptors (at ganglionic autonomic synapses) and nicotinic-M receptors (at neuromuscular synapses). Nicotinic-M receptors cause end-plate depolarization and muscle contraction, whereas nicotinic-N receptors are involved in ganglionic transmission [9, 34, 37, 43].

Muscarinic Receptors. Five subtypes of muscarinic receptors have been identified: M1, M2, M3, M4, and M5 [26, 39, 50, 53]. M1 receptors are widespread in the brain, whereas M2 receptors primarily cluster in the cardiovascular system. M3 receptors are found mainly in smooth muscles and secretory glands. Much research has been done to distinguish these sub-types, but much is stil unfolding. Functions of M4 and M5 are speculative at present. Muscarinic receptors play a major role in the functioning of the pPNS and CNS.

It is believed interaction of acetylcholine with members of the muscarinic receptor family (M1-M5), which couple to two G proteins: G_i and G_q. When the G_q-coupled receptors, M1, M3, and M5, are stimulated, the β_γ subunit of G_q dissociates from the subunits and stimulates phospholipase C-β. Phospholipase C-β in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and di-acylglycerol, both of which activate protein kinase C (PKC). PKC then activates MAPK by unknown mechanisms. Also mAChRs activates MAPK through the G_i-coupled receptors [24].

It is suggested that mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that play a crucial role in transmitting signals from the cell surface to regulate various cellular functions. MAPK is activated when phosphorylated on both a threonine and a tyrosine residue [67]; thus, changes in the phosphorylation state reflect changes in activity [36, 66]. The mode of signaling that uses this type of phosphorylation has been referred to as the two-component system.

Ethylene receptors. The plant hormone ethylene is a simple two-carbon (CH_2=CH_2) gaseous plant growth regulator that has profound effects on plant growth and development. Ethylene is formed from methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino acid 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet by the action of ACC synthase (ACS) and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO) [1, 44].

This plant growth regulator plays essential roles in integrating numerous responses to ethylene throughout life of the plant, including promotion of seed germination, induction of ripening in climacteric fruits, promotion or inhibition of flowering, leaf and petal abscission, senescence, and plant responses to stresses such as those induced by pathogens, flooding or drought [1, 58, 59]. Regulation of ethylene controlled

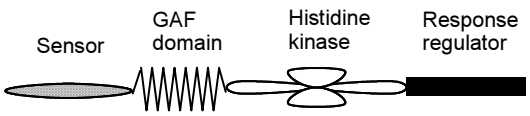


Fig. 4. Schematic representation of the arabidopsis ethylene receptor family (adopted from 6, 20). The four main domains (sensor, GAF domain, histidine kinase and response regulator) of the proteins are indicated.

events can occur at the level of biosynthesis, catabolism or perception [1, 75]. Although over the past decade great progress has been made in the study of biochemical events involved in ethylene:receptor interaction, it is still unclear how this small organic molecule can influence so many aspects of plant growth and development.

It is commonly recognized that ethylene may function as signal molecules that trigger the signal transduction pathways in cells. It is now known that eubacteria, archaea, fungi, and plants also have the two-component system [15].

Ethylene perception is the most well studied in *Arabidopsis* and is mediated by a family of five receptors: ETR1, ERS1, ETR2, ERS2, and EIN4 that have similarity to two-component regulators from bacteria [6, 7, 16, 20]. Each receptor is mostly composed from the four main domains: sensor, GAF domain (found in cGMP phosphodiesterases, adenylate cyclases and Fh1a transcription factors), histidine kinase and response regulator (Fig. 4). The GAF domain is a sequence motif that has been associated with cyclic nucleotide binding sites in a variety of proteins [3].

Ethylene signalling pathway contains both positive and negative regulators. According to the described models [6, 18] the ethylene receptors activate the kinase activity of CTR1 in the air (absence of ethylene). CTR1 then actively suppresses the downstream responses, such that EIN2 and the EIN3/EIL transcription factors remain inactive. The binding of ethylene to the receptor is mediated by a copper cofactor [63]. With ethylene binding mediated by a single copper ion (Cu) the receptors no longer activate CTR1, and thus CTR1 no longer suppresses the pathway. This leads to the activation of EIN2, induction of the transcriptional cascade, and the establishment of ethylene responses (Fig. 5). CTR1 is a Raf-like ser/thr kinase with similarity to a mitogen-activated protein kinase kinase

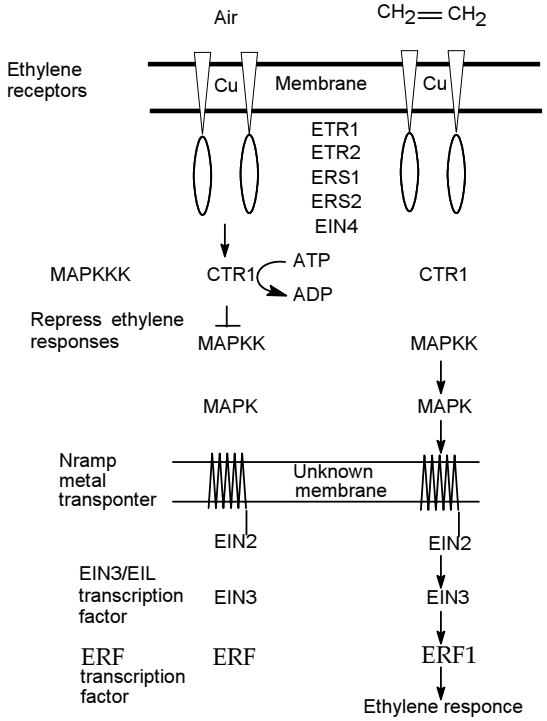


Fig. 5. Model for ethylene signal transduction (adapted from 18). In air, ethylene receptors maintain CTR1 (it is a Raf-like ser/thr kinase with similarity to a mitogen-activated protein kinase kinase kinase, MAPKKK) in an active state that serves to repress ethylene responses. Inactivating of CTR1 occurs when ethylene is biding to the receptors. This leads to activation of EIN2 and initiation of transcriptional cascade.

kinase (MAPKKK) (mitogen-activated protein kinase kinase kinase) [2, 14].

Ethylene oxide. Ethylene oxide (EO) is an agent that reacts easily with cellular substances such as, for example, deoxyribonucleic acid (DNA) and proteins, without metabolic activation [68]. Among the major reaction product in DNA is *N*-7(2-hydroxyethyl)guanine, and major reactive sites in hemoglobin are cysteine, histidine, and in particular *N*-terminal valine [12].

EO gas is widely used in the sterilization of medical equipment and materials [13, 73]. EO is an excellent gas for sterilization, but it also affects central and peripheral nervous systems [60-62].

General model for ACh and ethylene action in biological systems. Aaccording to the classical viewpoint of fast «wiring» transmission, the neurotransmitter is released in the synaptic cleft at a high concentration (up to 0.3 mM) and brief pulse (approximately 1 ms), whereas in the «vol-

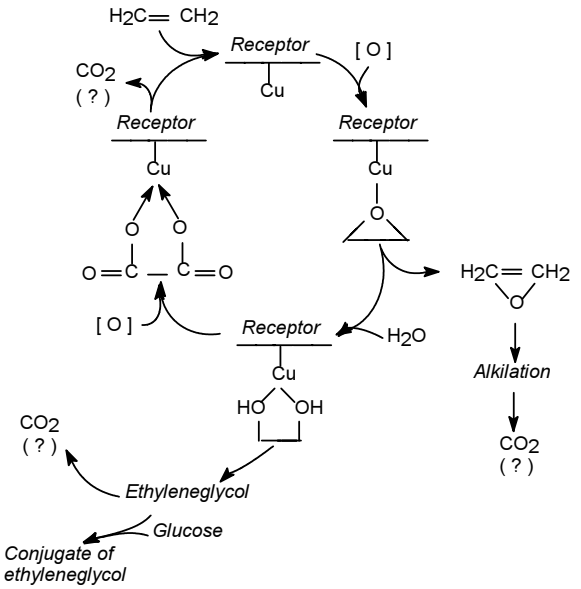


Fig. 6. Hypothetical scheme of initial ways for metabolism and action of ethylene (adapted from 5).

ume transmission», lower concentrations of the neurotransmitter may more slowly reach a distant target through intercellular space [23]. We believe that ethylene and ethylene oxide formed from ACh share different biological effects: 1) a prompt effect cased by ethylene oxide, and 2) a longer-lasting effect caused by ethylene.

Experimental data suggest that exogenously applied labelled ethylene was transformed in plants to ethylene oxide. Thus, Jerie and Hall [41] found that ethylene at physiological concentration was metabolized very rapidly to ethylene oxide. Subsequently Blomstrom and Beyer [8] demonstrated that in *Pisum* the earliest detectable metabolites were ethylene oxide and its glucose conjugate. The initial ways for metabolism and action of ethylene were described by Beyer [5] in the model (Fig. 6), where the first step is ethylene epoxidation.

Unfortunately as you can see from Fig. 5 and 6 there is some transformation of ideas during last decades: researchers omitted earlier experimental data concerning ethylene oxide formation. Also we believe that transition metals including copper in the reactive mixture is needed to form free radicals which then is bound to ethylene. Transformation of ethylene into reactive substances *in vivo* is presented in Fig. 7 [45-48].

There is a little scene of confusion in the biology of ethylene: ethylene is not an active substance under normal temperature and pressure,

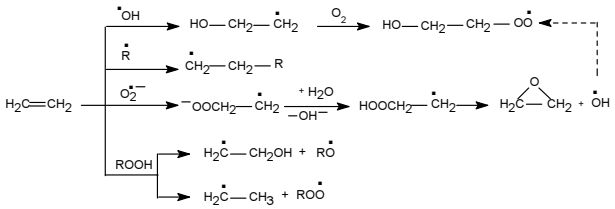
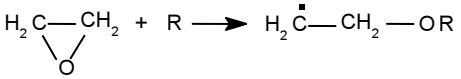


Fig. 7. Possible mechanisms of in vivo ethylene activation.

and hence, it cannot react with cellular protein-like substances. Nevertheless there is only one type of chemical reactions with ethylene: it can react only with free radicals and thus to form active structures [28, 57]. Some these reactions are illustrated in Fig. 7 [45-48].

Reactive agent ethylene oxide does not require any activation and can act as an initiator of free chain reactions of many cellular substances. Several such reactions are presented in Fig. 8 [46].

From the chemical ethylene oxide properties also the opening of the ethylene oxide ring can result in the free radical formation with cellular substances:



Based on the received experimental data we have proposed the following mechanism of ACh and ethylene action (Fig. 9).

The cellular functions of mammalian nervous systems depend on tight regulation of both extracellular and intracellular pH. It is shown that synaptic vesicles have a low pH (about 5.5) [54] and the buffering capacity of the synaptic cleft is limited during brief synaptic events [19, 71]. Coreleased with the ACh protons may tran-

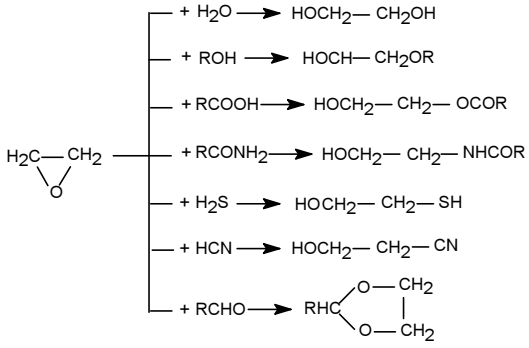


Fig. 8. Possible reactions of ethylene oxide with cellular substances in vivo [46].

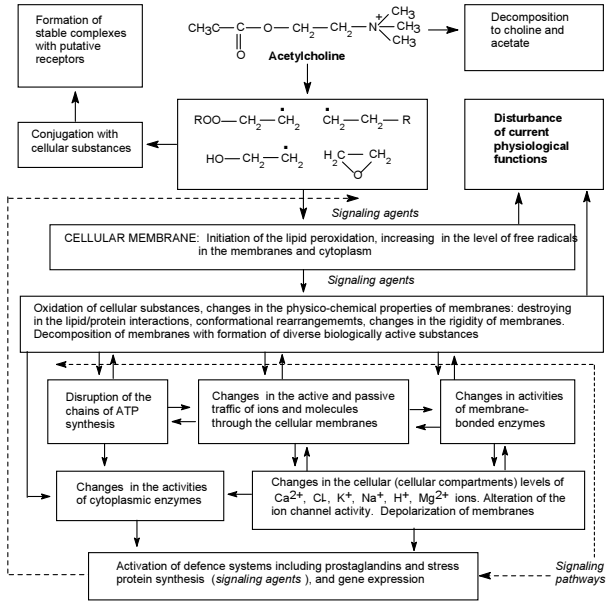


Fig. 9. Schematic mechanisms representing key steps in acetylcholine and ethylene in vivo activation and biological action.

siently change pH in the synaptic cleft. Hence, rapid and brief pH changes in the synaptic cleft could modulate synaptic transmission by direct interaction of protons, for example, with postsynaptic receptors. It is believed that approximately 1 mM ACh is rapidly released into the cleft, causing nearly synchronous activation of all of the postsynaptic nAChRs, in approximately 1 ms. [21]. Nevertheless there is alternative possibility: releasing of ACh from synaptic vesicles (pH ~5.5) to synaptic cleft (pH ~7.4) leads to ACh decomposition with ethylene oxide and ethylene formation. Unfortunately the concentrations of ACh that activate postsynaptic AChRs during synaptic events remain unclear. At the same time some part of the ACh is enzymatically hydrolyzed by acetylcholinesterase in the synaptic cleft to choline and acetate.

Experimental evidences suggest that the function of the AChR is influenced by its lipid microenvironment [3]. Ethylene oxide being very reactive agents may immediately react with constituents of the postsynaptic membranes causing structural, functional and conformational changes in them. For example, it can react with the membrane enzymes or with the membrane lipidic phase. In the last case it initiates free radical chain reactions that lead to the changes in physico-chemical properties of membranes and thus triggers numerous biochemical and physiological reactions. For example, initiation in the membranes of non-controlled by antioxidative molecules and enzymatic free radical chain reactions can lead to destroying in the lipid/protein interaction and conformational rearrangements, the opening of sodium channels, hence depolarization, then muscle contraction, etc. The disbalance of ions in the compartments of the cell as in the different cells can also take place. Finally, as you can see from the scheme the reactions of ACh with cellular substances occur after ACh activation, i.e. transformation into reactive forms. Hence, the formation of ACh/protein complexes may not be a cause but a consequence of its biological activation.

In conclusion, we would like to stress that the major finding of this report is that ACh is not stable substances and under some conditions can be decomposed *in vitro* to form ethylene and ethylene oxide. In this connection we proposed that complexes ACh (ethylene and ethylene oxide) with putative receptors are formed after their biological activation, i.e. transformation into free radical state. Chemicals in the free radical state may conjugate with any cellular substance to form complexes with putative receptors that can be reactive or not.

Nevertheless at least one question remains to be answered. How many receptors can be present at the plasmalemma? The question is not idle. The answers to these questions will provide important insight not only into the mechanism activation but also into the molecular mechanisms of action of biologically active substances. It is believed that about 20 % of all cellular proteins are presented at the membranes of living cells [51] and this can count approximately 10 000 proteins. Unfortunately the quantity of plasmalemma proteins is not yet fully known. Moreover the quantity of these proteins is in contradiction with information about known several thousands of natural biologically active agents. For instance only in the plant kingdom many tens of thousands of plant bioactive substances have been identified [11]. Thus, there are no structural grounds to think that all biological effects of growth regulating substances are mediated through protein receptors. In addition the precise molecular mechanisms underly-

ing, for example, nicotinic receptor activation and as a consequence ligandgated ion channel activation remain largely unresolved. Hence, non-receptor mechanisms activation and action of biologically active agents, including acetylcholine, can take place in living systems.

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Ацетилхолін та етилен: чи подібні їх рецептори й аналогічна біологічна дія?

Б.О. Курчій

Інститут фізіології рослин і генетики НАН України
вул. Васильківська, 31/17, Київ, 03022, Україна

Резюме. З точки зору хімії ацетилхолін (АХ) є четвертинна амонієва сполука, біологічна роль якої пов'язана з нейропередачею між нейронами та іншими нейроклітинними з'єднаннями. Раніше нами запропоновано (Kurchii, 1998), а потім і підтверджено (Kurchii, Kurchii, 2000), що АХ за дії фізіологічного розчину і лугу розкладався з утворенням етилену. На жаль, ми не змогли визначити два інших піки, отриманих на газовому хроматографі. У цій статті ми ідентифікували один із двох невідомих піків — окис етилену. Встановлено, що окис етилену виділявся в кількості значно більшій за таку ж кількість етилену в атмосфері повітря або в краплі фізіологічного розчину. З'ясовано, що біологічні ефекти ацетилхоліну можуть бути зумовлені дією окису етилену, який є хімічно активною сполукою, і це є швидка його дія на короткі відстані. Етилен же може мігрувати на далекі відстані, спричинюючи сповільнені ефекти, але тільки за умови його активації, бо за нормальних умов (температури і тиску) він є неактивною сполукою і не може вступати в реакції. Проте *in vivo* етилен може активуватися в реакціях приєднання вільних радикалів. Також, як свідчать здобуті дані, питання про специфічні рецептори АХ залишається дискусійним.

Ключові слова: ацетилхолін, етилен, окис етилену, вільні радикали, рецептори.

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