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FORUM MINIREVIEW

The Non-neuronal Cholinergic System

The Biological Role of Non-neuronal Acetylcholine in Plants and Humans

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ABSTRACT—Acetylcholine, one of the most exemplary neurotransmitters, has been detected in bacteria, algae, protozoa, tubellariae and primitive plants, suggesting an extremely early appearance in the evolutionary process and a wide expression in non-neuronal cells. In plants (*Urtica dioica*), acetylcholine is involved in the regulation of water resorption and photosynthesis. In humans, acetylcholine and/or the synthesizing enzyme, choline acetyltransferase, have been demonstrated in epithelial (airways, alimentary tract, urogenital tract, epidermis), mesothelial (pleura, pericardium), endothelial, muscle and immune cells (granulocytes, lymphocytes, macrophages, mast cells). The widespread expression of non-neuronal acetylcholine is accompanied by the ubiquitous expression of cholinesterase and acetylcholine sensitive receptors (nicotinic, muscarinic). Both receptor populations interact with more or less all cellular signalling pathways. Thus, non-neuronal acetylcholine can be involved in the regulation of basic cell functions like gene expression, proliferation, differentiation, cytoskeletal organization, cell-cell contact (tight and gap junctions, desmosomes), locomotion, migration, ciliary activity, electrical activity, secretion and absorption. Non-neuronal acetylcholine also plays a role in the control of unspecific and specific immune functions. Future experiments should be designed to analyze the cellular effects of acetylcholine in greater detail and to illuminate the involvement of the non-neuronal cholinergic system in the pathogenesis of diseases such as acute and chronic inflammation, local and systemic infection, dementia, atherosclerosis, and finally cancer.

Keywords: Non-neuronal cholinergic system, Cytomolecule acetylcholine, Plant, Human, Basic cell function

Even today the biological role of acetylcholine is focused mainly on its "neurotransmitter" function, although it has been repeatedly shown that acetylcholine is present in bacteria, algae, protozoa, fungi, plants, yeast and is also widely expressed in non-neuronal cells in animals and humans (1 – 5). For example, in a recent paper, significant effects of acetylcholine on the release of cytokines and acute systemic inflammation were described, but a possible physiological role of acetylcholine was attributed to the vagal nerve only (6). Already more than forty years ago, Whittaker stated that "acetylcholine occurs in non-nervous tissues and is so widely distributed in nature to suggest a non-nervous function of it" (7). Thus, the fact can be no longer disregarded that acetylcholine represents a phylogenetically extremely old molecule, widely distributed in pro- and eucaryotic cells.

It is being increasingly recognized that non-neuronal acetylcholine, a cytomolecule, appears to be involved in the regulation of basic cell functions like proliferation, differentiation, cell-cell contact, immune functions, secretion and absorption. A first systematic review article about the cholinergic system in non-neuronal tissue was published approximately 20 years ago (1). The expression and function of the non-neuronal cholinergic system in humans has been described in recent years (2 – 5, 8, 9). Grando and colleagues have introduced the term "universal cytotransmitter", which corresponds to the widespread expression and the involvement in the regulation of basic cell functions (2 – 4, 9). We have introduced the term non-neuronal acetyl-

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choline and non-neuronal cholinergic system to underline the presence of acetylcholine in cells definitively independent of neurons or present in organisms free of neuronal tissue very early in the evolutionary process (2, 3, 9). The present article is focused on very recent findings on the expression and function of non-neuronal acetylcholine in plants (*Urtica dioica*) and humans. It is emphasized that the article cannot give a systemtic presentation of the existence of the non-neuronal cholinergic system in animalia. A first review article about the expression of the cholinergic system in protozoa and metazoa has been published 30 years ago (10).

The expression and function of acetylcholine in *Urtica dioica*

During the half of the palaeozoicum period (about 600 million years ago) plants are assumed to have developed outside the ocean and spread over the continents. Acetylcholine has been detected in moss and in *Equisetum robustum* (2, 3, 11); i.e., in primitive plants that developed very early in the evolutionary time scale. For example, *Equisetum robustum* (sphenophyta family) has been established in the Devonian period, 360 – 400 million years ago. Acetylcholine, measured by gas-liquid chromatography or by high pressure liquid scintillation spectometry followed by electrochemical detection (HPLC-EC), has been found in the following primitive and higher plants: *Amarathus*

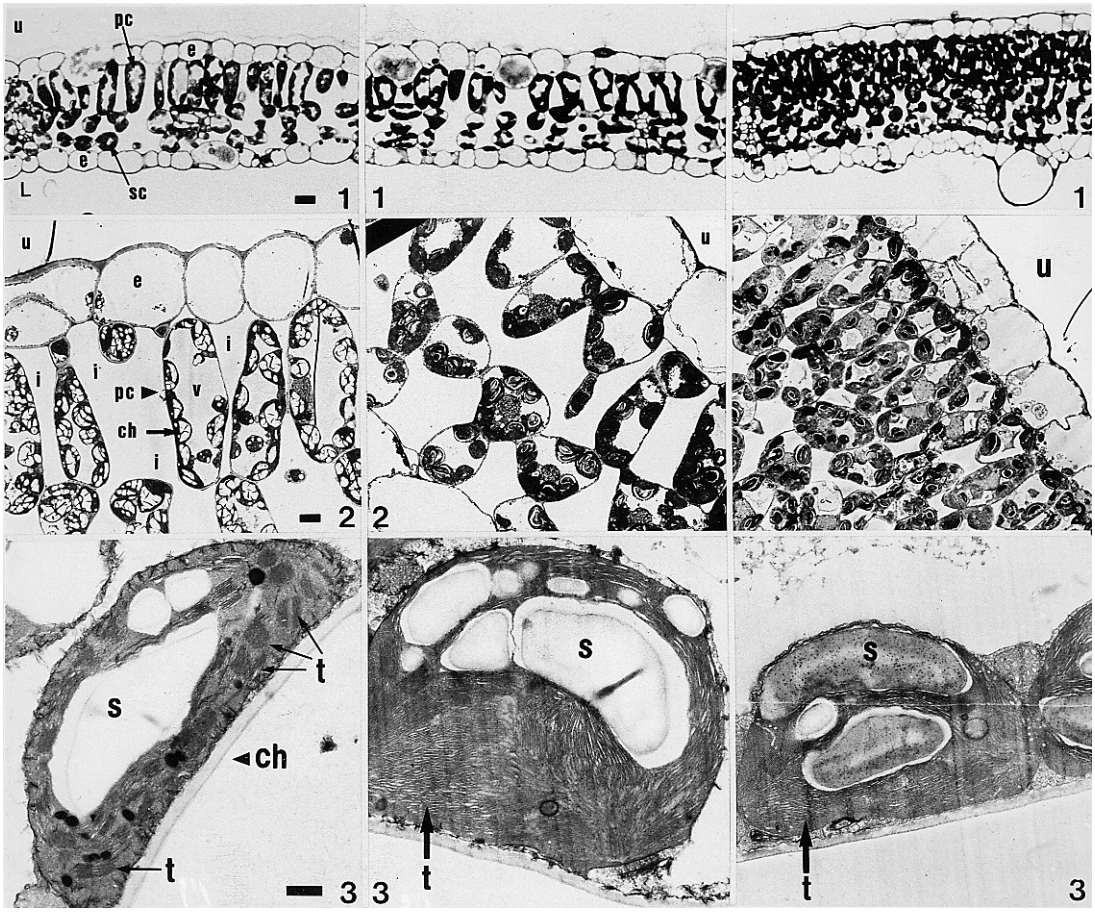

Fig. 1. Effect of tubocurarine and atropine on cell morphology of leaves of *Urtica dioica*. *Urtica dioica* were incubated (48 h) with the stems in water (control, left hand row) or in water containing 30 μM tubocurarine (middle row) or 1 μM atropine (right hand row). At the end of the incubation, leaves were fixed and prepared for light (panels 1: bar represents 14.5 μm, magnification 400 ×)- and electron (panels 2: bar represents 4 μm, magnification 1300 ×; panels 3: bar represents 0.5 μm, magnification 17000 ×)-microscopy. Shown are representative leaves. Left hand row (control): panel 1 shows the regular morphology with epidermal (e) cells at the upper (u) and lower (L) side and parenchymal cells (pc: palisade cells, sc: spongy cells); panel 2 shows the upper epidermal cells and palisade cells with chloroplasts (ch) and cell vacuole (v) and intercellular space (i); panel 3 shows an individual chloroplast containing the stratified thycaloid membranes (t) and starch (s). Middle row (effect of tubocurarine): in panels 1, 2 and 3, the reduction of palisade cell size and the proliferation of the thylacoid membrane is shown. Right hand row (effect of atropine): in panels 1, 2 and 3, the disturbed cell organization, the reduction of intercellular space, cell vacuole and cell size and the increase of the thylacoide membrane is shown.

caudatus, Arum specificum, Arum maculatum, blue green algae, *Brachytecium, Capsella bursa-pastoris, Citrus auranticus, Cucurbita pepo, Equisetum robustum, Fragaria vesca, Helianthus annuus,* moss callus, *Phaseolus vulgaris, Pisum sativum*, radish, rye ergot, *Senecio vulgaris, Sianpis alba, Spinacea oleracea* and *Urtica dioica* (2, 3, 7, 11, 12; I. Wessler et al., unpublished observation). In plants the highest amount of acetylcholine, about 0.5 μmol/g dry weight, we detected in the roots and stems of *Urtica dioica*. We also found acetylcholine in fungi like *Agaricus bisporus* and *Cantharellus cibarius*. Without any doubt a systematic analysis of the presence of acetylcholine in the plant kingdom will result to an increase of the list given above.

Although acetylcholine is widely expressed in the plant kingdom, our knowledge about the organization and biological role of the cholinergic system in this kingdom is very scanty. Nothing is known about the synthesizing enzyme(s), receptors and the respective biological roles. To obtain some first experimental evidence about the functional role of acetylcholine, we have studied the effects of the classical receptor antagonists. *Urtica dioica* were incubated with their stems in water (control) or in water containing the muscarinic receptor antagonist atropine (1 μM) or the nicotinic receptor antagonist tubocurarine (30 μM). Thereafter, leaves were fixed and prepared for microscopy (Fig. 1). Exposure of *Urtica dioica* to the receptor antagonists was followed by substantial morphological changes. Atropine disturbed the regular organization of the parenchymal palisade and spongy cells. Atropine caused a reduction of the intercellular space, the cell vacuole and the size of the parenchymal cells (Fig. 1). At the highest magnification, it was found that atropine reduced the size of the individual chloroplasts but that the stratified photopigment-containing thylacoid membrane increased in height (Fig. 1). Likewise, tubocurarine increased the height of the thylacoid membrane; i.e., the chloroplast was more or less completely filled up with it (Fig. 1). We observed similar effects with classical skeletal muscle relaxants such as atracurium and pancuronium. These observations can be interpreted as functional adaptation to xeromorphism and shade (13, 14). Macroscopic inspection of *Urtica dioica* exposed to the receptor antagonists atropine and tubocurarine confirmed the impaired water balance and its antagonism by applied exogenous acetylcholine (1 μM, Fig. 2). Interestingly, a dependency of the acetylcholine content on the light-dark period has already been found in higher plants (11).

These experiments provide first experimental evidence that, at least in *Urtica dioica*, the cholinergic system is involved in the regulation of water homeostasis and photosynthesis. In addition, it becomes obvious that the acetylcholine-sensitive receptors expressed in plants are comparable to animal tissue, because the classical antagonists atropine and tubocurarine, both alkaloids, were effective. In this context, we suggest that in the respective plants, acetylcholine as a receptor agonist and the receptor antagonists (atropine, tubocurarine) are expressed simultaneously to regulate plant phenotype functions intrinsically. It is emphasized that more effort should be made to investigate the role of the cholinergic system in the plant kingdom in more detail. Possibly, one may develop conditions for optimizing plant culturing and by comparison can learn more about the role of the non-neuronal cholinergic system in mammals.

Expression of ChAT and presence of acetylcholine in non-neuronal cells in humans

The enzymatic protein (ChAT) mediating the intracellular synthesis of acetylcholine within the cytosol of neurons was first described by Nachmansohn and Machado, more than 50 years ago (15). ChAT protein appears to be expressed in the vast majority of human cells, i.e., represents a basic cellular component. In agreement with the widespread expression of ChAT-protein, one promotor region of the human ChAT gene does not contain the classical TATA box but a rather GC-rich region, typical of many housekeeping genes (16).

Figure 3 gives an example of positive ChAT immunoreactivity in human epithelial cells: human airway surface epithelium (basal, ciliated and goblet cells), submucosal glandular cells, small and large intestine, secretory cells of the femal breast, epidermal cells of the human skin, sebaceous and eccrine gland and kidney tubuli cells. Positive ChAT immunoreactivity and/or acetylcholine has also been detected in the epithelial cell layer of the buccal mucosa, esophagus, stomach (except the pyloric part), sigmoid, gallbladder, kidney plevis, urinary bladder, vaginal mucosa, cornea, amnion and placenta (1, 3, 4). In addition ChAT immunoreactivity and/or acetylcholine have been found in human mesothelial cells (pleura, pericardium), endothelial cells, circulating cells (leukocytes, platelets), astrocytes, sperm, hair, nail, fat tissue and muscle cells (2, 3, 5). Finally, ChAT and acetylcholine were demonstrated in more or less all immune cells. Figure 4 gives an example for positive ChAT-immunoreactivity in human leukocytes, eosinophilic granulocytes, lymphocytes, alveolar macrophages, skin mast cells and rat microglia (3 – 5, 17, 18).

Some reservation has arisen against the widespread expression of ChAT and acetylcholine in non-neuronal cells. It has been questioned whether acetylcholine is synthesized by neurons and then taken up by non-neuronal cells, giving the impression of a non-neuronal localization. This hypothesis can be definitely ruled out for two reasons. Firstly, ChAT immunoreactivity as well as ChAT enzyme activity have been identified directly in non-neuronal cells; i.e., these cells can synthesize acetylcholine by themselves. Secondly, acetylcholine has been extracted from freshly

Fig. 2. Impaired water uptake by atropine and tubocurine and its antagonism by applied acetylcholine in *Urtica dioica*. *Urtica dioica* were placed in water (CON: control) or in water containing 1 μM atropine and 30 μM tubocurarine (ATR/TC) or both antagonists together with 1 μM acetylcholine (ATR/TC + ACh), and photographs were made 48 h later. The leaves of *Urtica dioica* exposed to both antagonists became dry despite the stems standing in water. This effect was antagonized by applied acetylcholine.

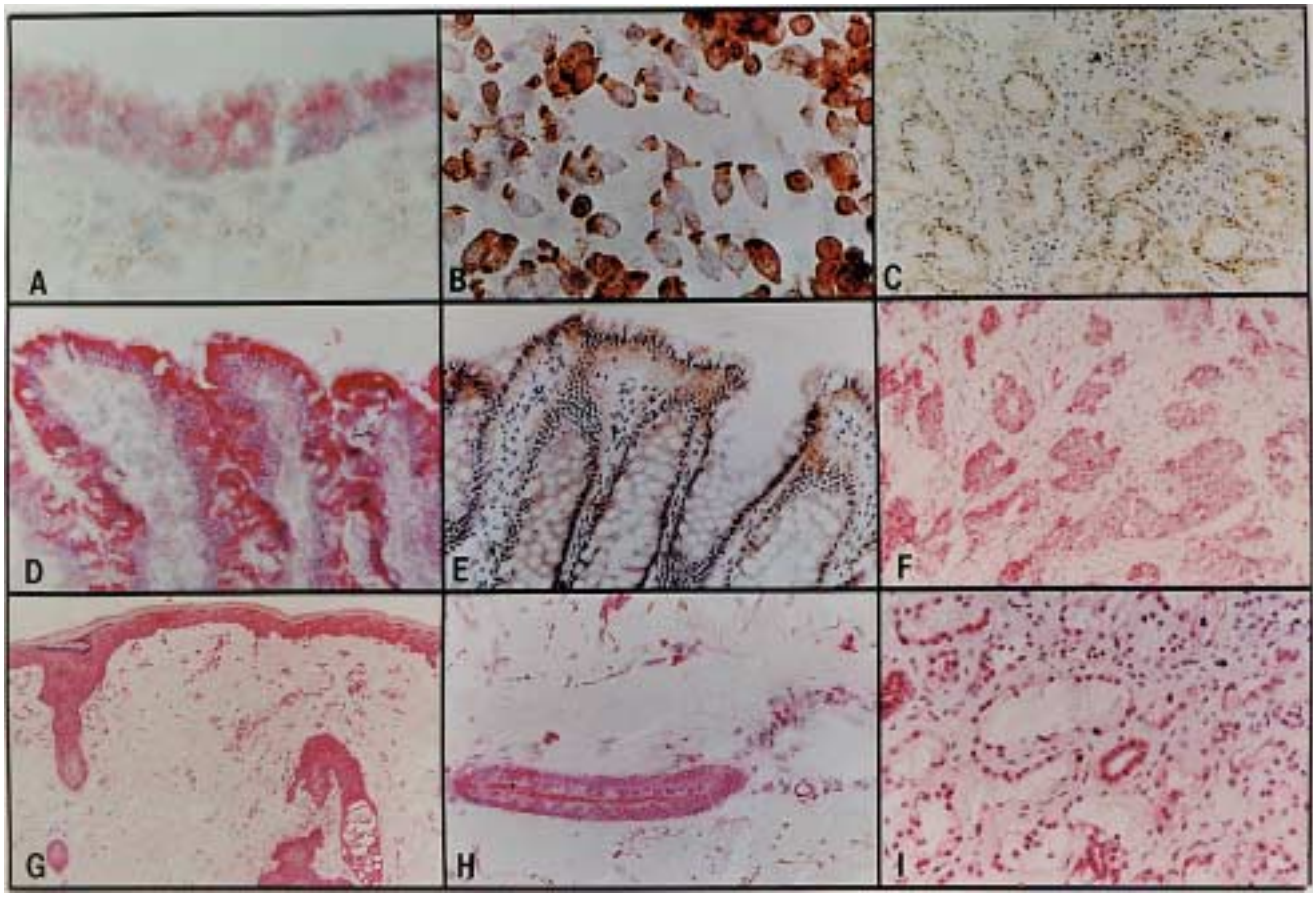

Fig. 3. Positive ChAT immunoreactivity in human epithelial cells. Human tissue was snap frozen immediately after surgical dissection and prepared for immunocytochemistry. The primary antibody was either a polyclonal rabbit or a monoclonal IgG1 mouse anti-choline acetyltransferase antibody; secondary antibody was either a goat anti-rabbit IgG antibody-alkaline phosphatase conjugate (alkaline phosphastase system, pink staining) or an anti-mouse IgG biotinylated antibody (peroxidase system; brown staining). Positive choline acetyltransferase immunoreactivity was found in A: surface epithelium of human bronchi, B: ciliated ariway cells, C: submucosal glandular airway cells, D: epithelium of the small intestine, E: epithelium of the colon, F: glandular cells of the female breast, G: skin epidermal cells and sebaceous gland, H: eccrine gland, I: tubulus cells of the kidney.

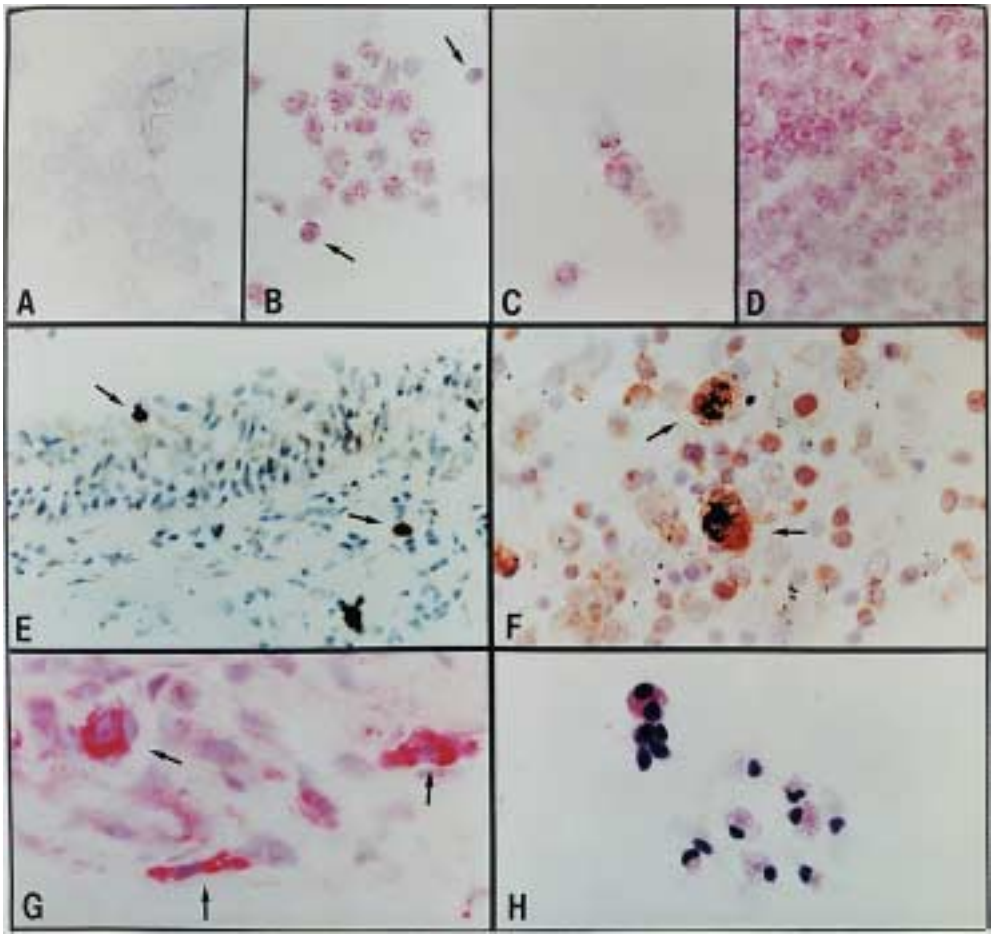

Fig. 4. Positive ChAT immunoreactivity in human immune cells. For experimental details, see legend to Fig. 3. A: human leukocytes (polymorphnuclear cells), negative control using an unspecific rabbit serum: Positive ChAT immunoreactivity in B: human polymorphnuclear cells and lymphocytes (arrows), C: eosinophilic granulocytes, D: mononuclear cells, E: alveolar macrophages in situ (arrows), F: isolated alveolar macrophages (arrows), G: skin mast cells (arrows), H: primary culture of rat microglia.

isolated or cultured cells in the absence of neuronal tissue (absence of neuronal markers like S100 or neurofilaments, 19).

ChAT protein cannot be isolated from homogenized tissue on the basis of a lectin-loaded column. This suggests that ChAT does not represent a glycoprotein, which makes it unlikely that ChAT is bound to the outer cell membrane (20). The characterization of the physical properties of ChAT reveals additional difficulties because ChAT shows a tendency to interact with various proteins and membrane structures (20). The exact number of ChAT isoenzymes (or subunits) is, as yet, unknown. Western blot analysis of human airway epithelial cell extracts has visualized ChAT-like proteins with a molecular mass of 41 and 54 kd (9). The neuronal ChAT is a 69-kd protein (21, 22). A 51-kd subunit of the neuronal ChAT protein has also been found and a 84-kd ChAT protein appears to be composed of 6 identical 14-kd subunits (20). In *Drosophila,* ChAT proteins of 67, 54 and 13 kd have been described, thus showing a remarkable homology to the mammalian ChAT protein (23).

The specificity of ChAT is very high for the substrate choline but not for the second substrate, the activated acetyl-donor. For example, rat brain ChAT showed the same affinity for acetyl-CoA, propionyl-CoA and butyryl-CoA (24). ChAT enzyme activity may synthesize not only acetylcholine but also other molecules, for example, propionylcholine or butyrylcholine (7). Closely related enzymes may also form acetylcholine under in vivo conditions. For example, in mammals, acetylcholine can be synthesized by carnitine acetyltransferase which is expressed in mammalian heart and skeletal muscle (25, 26). The following factors have been described to increase ChAT expression in neuronal tissue: activators of protein kinase A or cyclic AMP analogs, nerve growth factor, ciliary neurotrophic factor, leukemia inhibitory factor and retinoids; additionally, sex

hormones and glucocorticoids can regulate ChAT gene expression and/or ChAT enzyme activity (1, 3, 4, 19, 21).

Expression of acetylcholine-sensitive receptors and of cholinesterase in non-neuronal cells in humans

The presence of acetylcholine in more or less every human cell appears useless without the simultaneous expression of nicotinic and muscarinic receptors on non-neuronal cells. It has already been shown in the last decade that nicotinic and muscarinic receptors, comparable to the distribution of non-neuronal acetylcholine, are widely expressed on human non-neuronal tissue, for example epithelial cells (airways, intestine, epidermis), endothelial cells, placenta and circulating blood cells (1 – 5, 27 – 40). These findings confirm the potential role of the non-neuronal cholinergic system. A chimeric nicotinic receptor responding to both nicotinic and muscarinic agonists has been discovered in insect and bovine neurosecretory cells (41). This receptor forming the α9-subunit is also expressed in human keratinocytes (2) and appears as a likely target for non-neuronal acetylcholine acting via its nicotinic and/or muscarinic property. Thus, the classical cholinergic receptors represent the target for non-neuronal acetylcholine to mediate auto-/paracrine effects and therewith to control cell and organ homeostasis. Non-neuronal acetylcholine may also interfere with receptors inside the cells. Nicotinic and muscarinic receptors are processed along the endoplasmic reticulum; the receptors are internalized upon agonist stimulation and may be incorporated into intracellular membranes. Thus, acetylcholine may become active without leaving the cell by targeting intracellularly localized receptors.

Signal transduction via nicotinic and muscarinic receptors mediates an enormous impact on a cell. Together, both receptor types interact with more or less all ion channels and key enzymes forming second and third effectors. Subtypes of nicotinic receptors mediate a rapid and transient opening of ion channels translocating sodium, potassium or calcium ions. G-protein-coupled muscarinic receptors modulate the activity of cAMP- and cGMP-gated channels as well as potassium and calcium channels (voltage-dependent and independent). In conclusion, nicotinic and muscarinic receptor activation can trigger multiple signalling events: the activation of transmembrane ion flux (sodium, potassium, calcium); the mobilization of intracellular calcium; the increase in cGMP; the release of NO, prostanoids and other modulators; the activation of tyrosine kinases, the small G-proteins and the mitogen-activated protein kinases. Beside these receptor-mediated effects, the non-neuronal cholinergic system may also interfere with the biochemical properties of a cell. Synthesis and degradation of non-neuronal acetylcholine may affect the availability of choline and acetyl-CoA inside the cell. Both compounds are key molecules within metabolic pathways like the Krebs cycle, metabolism of fatty acids, carbohydrates, amino acids (acetyl-CoA), and the metabolism of choline-containing phospholipids, phosphatidylcholine, eicosanoids and the synthesis of glycine (choline).

Acetylcholine is broken down by specific (acetylcholinesterase; E.C. 3.1.1.7) and non-specific cholinesterase (pseudocholinesterase, butyrylcholinesterase, plasmacholinesterase or non-specific cholinesterase; E.C. 3.1.1.8) to choline and acetate. Cholinesterase activity is ubiquitously expressed (intra- and extracellularly), for example, in circulating cells, epithelial cells (airways, intestine, skin), endothelial cells, thymus cells and in the liver (42 – 48). Erythrocytes are heavily packed with the specific cholinesterase (42). Thus, erythrocytes together with plasma cholinesterase represent effective humoral principles available to destroy non-neuronal acetylcholine that has escaped into the circulation. Non-specific cholinesterase is found in the liver, lung and the circulation (42, 46).

The wide expression of cholinesterase acivity independent of neurons has been known from early in the last centrury, but its physiological role has been questioned since that time. The expression of non-neuronal acetylcholine in more or less all cells gives a convincing explanation for the simultaneous expression of the degradating enzyme. Thus, the widespread hydrolyzing activity prevents non-neuronal acetylcholine from acting as a hormone. The effects of non-neuronal acetylcholine are closely limited to the area of its synthesis and release which allows a fine tuning of some few individual cells only.

Taken together it is evident that the non-neuronal cholinergic system represents a most widely expressed and highly effective system created by nature to regulate or modulate basic cell functions:

1) Acetylcholine is formed from two key precursor molecules, choline and acetyl-CoA, which are present in every cell. Choline is available from cell membrane phospholipids, i.e., synthesis and breakdown of acetycholine may target the properties of the phospholipid bilayer and vice versa. Acetyl-CoA plays a key role in metabolic pathways (Krebs cycle, cholesterol and steroid synthesis, metabolism of carbohydrate, fatty acid, and amino acids (for example tyrosine, glycine, threonine)). Synthesis and breakdown of acetylcholine may target metabolic pathways of an individual cell and vice versa.

2) Acetylcholine activates multiple, if not all cellular effector systems, via nicotinic and muscarinic receptors and possibly also via direct protein interactions. Translocation of the cation acetylcholine across a cell membrane mediates an electrical current which can change the electrical property of the cell membrane.

3) Cholinesterase, the most effective enzyme created by nature so far, is ubiquitously expressed in non-neuronal cells, thereby terminating the action of acetylcholine in the

immediate vicinity of its synthesis and release.

Functional role of non-neuronal acetylcholine in humans

Our knowledge about the biological functions of the non-neuronal cholinergic system is growing steadily. Non-neuronal acetylcholine leaves the cell and via auto-/paracrine mechanisms can regulate basic cell functions like gene expression, proliferation, differentiation, cytoskeletal organization, cell-cell contact (tight and gap junctions, desmosomes), locomotion, migration, ciliary activity, electrical activity, secretion and absorption (2 – 5). Epithelial surfaces, the boundary between the individual and its immediate environment, represent an area of extreme relevance for maintaining homeostasis. Epithelial and mucosal surfaces are responsible for important biological functions such as secretion, absorption and the exterior defences against the penetration of microbes such as viruses, bacteria, fungi and protozoa. Multiple cellular functions which are regulated by the non-neuronal cholinergic system contribute to epithelial defense, for example, the formation of tight junctions, secretion processes (mucus, sebaceous gland, cilia lining), ciliary activity and local immune responses. Acetylcholine, via muscarinic receptors, can modulate the glycoconjugate profile of the epithelial glycocalyx (49), thus facilitating the binding of bacteria to mucus glycoconjugates and therewith limiting deeper bacterial invasion. Immune responses are produced primarily by local leukocytes, and increasing experimental evidence suggests that non-neuronal acetylcholine is involved in these immune functions (50).

Acetylcholine receptor antagonists cause substantial alterations of the shape and cell-cell contact of epithelial and endothelial cells. The cells shrink, retract their intermediate filaments and detach from each other (2, 4, 39). These findings suggest that non-neuronal acetylcholine by regulating the shape and motility of surface cells controls the physical barrier function of inner (airways, intestine) and outer surfaces (skin). It has been demonstrated recently that cytoplasmic myosin is regulated by muscarinic receptors in non-muscle cells by a process involving RhoA and protein kinase C (51). Via this pathway non-neuronal acetylcholine could control the cytoskeleton and cell properties like proliferation, adhesion, locomotion and migration. Proliferative effects of acetylcholine have been found in glial cells, epithelial cells, splenocytes, thymocytes and different cultured cell lines (2 – 5).

Pathophysiological aspects

Our knowledge of whether the non-neuronal cholinergic system plays a role in the pathogenesis of diseases is very scanty. However, the high impact of the non-neuronal cholinergic system on phenotype functions of the different cells (epithelial, endothelial and immune cells) implicates cellular dysfunction in the case of a cholinergic dysfunction. It has been found that in neurodermitis, the content of actylcholine in the superficial skin cell layers is increased 15-fold compared to healthy volunteers (52). Also the release of acetylcholine from the buccal mucosa was increased in neurodermitis patients (52). Enhanced levels of acetylcholine modify the activity of immune cells, can impair the physical barrier function and can induce itching, thus contributing to acute or chronic inflammation. Glucocorticoids substantially reduce epithelial acetylcholine, a pathway that can contribute to the anti-inflammatory effect of these compounds (19).

The possible dysfunction of the non-neuronal cholinergic system in diseases like inflammation, dementia, atherosclerosis, local and systemic infection, and finally in cancer has to be clarified in future experiments.

Conclusions

Even in the 21th century, in which efforts are being made to define cell functions by a molecular approach at the level of gene expression, it is fascinating to revise the role of the "old fashioned molecule" acetylcholine. Plants are equipped with receptors sensitive to atropine and tubocurarine, which is not a surprising finding because the antagonists represent alkaloids. Obviously, the muscarinic and nicotinic receptors have been developed in the plant kingdom and subsequently were used in animalia as well. At least in *Urtica dioica*, acetylcholine is involved in the regulation of photosynthesis and water balance.

The vast majority of human cells synthesizes acetylcholine, an observation which explains the ubiquitous expression of acetylcholine-sensitive receptors and cholinesterase. Acetylcholine, via nicotinic and muscarinic receptors and possibly also via a direct protein interaction, can regulate most of the cellular signalling pathways and appears to be involved in the regulation of basic cell functions like gene expression, proliferation, differentiation, cytoskeletal organization, cell-cell contact (tight and gap junctions, desmosomes), locomotion, migration, ciliary activity, electrical activity, secretion and absorption. The non-neuronal cholinergic system may also interfere with biochemical cell properties via a change of the availability of both precursor molecules, choline and acetyl-CoA. It is time to intensify the research on the non-neuronal cholinergic system and to consider the involvement of this system in the pathogenesis of diseases.

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