Chapter 11

Detection of Non-neuronal Acetylcholine

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Abstract

The biological role of acetylcholine and the cholinergic system has been revisited within the last 25 years. Acetylcholine and the pivotal components of the cholinergic system (high affinity choline uptake, choline acetyltransferase and its endproduct acetylcholine, muscarinic and nicotinic receptors, cholinesterases) are expressed by more or less all mammalian cells, i.e., cells not innervated by neurons at all. Moreover, acetylcholine and cholinergic binding sites have been described in plants. Acetylcholine is even detected in bacteria and algae and thus represents an extremely old signaling molecule on the evolutionary time scale. The following chapter summarizes the detection of acetylcholine beyond neurons with particular emphasis on the presence of acetylcholine in so-called primitive organisms. Finally, an overview is given about the detection in mammalian non-neuronal cells. The existence of the non-neuronal cholinergic system has identified an important new target to illuminate the pathophysiological background of acute and chronic inflammatory diseases as well as heart diseases and cancer.

Key words Non-neuronal acetylcholine, Non-neuronal cholinergic system, HPLC combined with bioreactors and electrochemical detection, Evolution, Bacteria, Plants, Unicellular organisms, Epithelial–mesothelial–endothelial and immune cells, Signaling via muscarinic and nicotinic receptors

1 Introduction

Even in our modern day and age textbooks as well as academic education are presenting acetylcholine as a neurotransmitter mediating the communication between neurons, interneurons and innervated effector cells such as muscle fibers and glandular cells. An actual upload (http://en.wikipedia.org/wiki/acetylcholine [1]) describes acetylcholine as follows: "*acetylcholine has functions both in the peripheral nervous system* (*PNS*) *and in the central nervous system* (*CNS*) *as a neuromodulator*." An actual research in PubMed shows 150-fold more references in favor of the key words "acetylcholine and neurotransmitter" than given for the term "non-neuronal acetylcholine." Of course, our knowledge about acetylcholine and its biological functions has substantially increased in the last 130 years, when for the first time acetylcholine was extracted from the brain and called at first neurin and later on synthesized as acetylcholine [2, 3].

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It is fascinating today to realize that all the biological systems/functions in which acetylcholine, released from central, parasympathetic, and peripheral intramural neurons as well as motoneurons, is involved by stimulating at least 11 different subunits of nicotinic receptors and five subtypes of muscarinic receptors. Thus, acetylcholine acts as neurotransmitter in the motoric and sensoric (i.e., thermal, pain, taste) system; acetylcholine is involved in complex integrative neuronal functions like memory, learning and sexual activity; acetylcholine, as a neurotransmitter of autonomic neurons, controls the cardiovascular, respiratory, gastrointestinal, and urogenital system. However, this summarizes only the role of acetylcholine within the nervous system, and the role of acetylcholine as a general signaling molecule beyond neurons has to be considered additionally.

To discriminate acetylcholine not synthesized by neuronal cells and not mediating nervous impulses but acting as autocrine/paracrine signaling molecule from acetylcholine acting as neurotransmitter, the nomenclature "non-neuronal acetylcholine" and "non-neuronal cholinergic system" has been introduced in 1998 and 1999 [4, 5]. One should consider that the existence of acetylcholine independent of neurons has been known for a long time before (for review see ref. [6]). Unfortunately, the scientific community has forgotten the first experiments by Ewins and Dale who investigated the effect of an extract of the ergot fungus (*Claviceps purpurea*) on the blood pressure in 1914 [7, 8]. Ergot grows on rye particularly during rainy periods in spring and can induce serious intoxications (called "St. Anthony's Fire") which were known during the Middle Ages and were based on the vasoactive effects of the ergot alkaloids. When Ewins and Dale investigated the hemodynamic effects of an extract of this fungus, they found a depressor effect [7, 8]. Later on they could attribute this depressor effect to acetylcholine [8]. In conclusion, the first experiments illuminating a biological role of acetylcholine, "the blood pressure lowering substance", the molecule was extracted from fungi, i.e., from non-neuronal organisms. Some years later (1921) Otto Loewi presented the first experimental evidence for the neurotransmitter role of acetylcholine, when he used a pair of isolated frog hearts [9]. The first heart with the nerves attached was stimulated and the second was used as detector to demonstrate the released substances from the first one. He postulated the so-called "Vagus-Stoff" or "parasympathin" acting as humoral transmission of nervous impulses [9] and 5 years later the vagus-substance was identified as acetylcholine [10]. Later on (1963) Whittaker stated that "acetylcholine occurs in non-nervous tissues and is so widely distributed in nature to suggest a non-nervous function of it" [11] and Koelle speculated about acetylcholine representing a phylogenetically very old molecule, which, in primitive organisms such as plants and unicellular organisms, might be involved in the

regulation of transport processes [12]. Moreover, important contributions in the last century showed the synthesis of acetylcholine in bacteria, algae, yeast, fungi, protozoa, nematodes, sponges, and plants [4–6, 13–22] Thus, acetylcholine is as far as we know one of the oldest signaling molecules in the evolutionary process.

The present chapter is focused on the detection of non-neuronal acetylcholine. In the last years important review articles have been published to describe this topic in more detail [4–7, 23–35]. Moreover, in 2002, 2006, 2011, and 2014 international conferences on non-neuronal acetylcholine were held [36–38].

2 Detection Methods for Acetylcholine

For decades the most sensitive, but less specific, method for determination of acetylcholine was the bioassay, such as the leech longitudinal muscle, the guinea pig small intestine, the frog rectus abdominis muscle, and cat blood pressure. Using these detector systems together with specific antagonists the lower detection limit for acetylcholine amounted to about 0.2–5 ng (corresponding to about 1–20 μmol) [6]. In addition acetylcholine can be detected by gas chromatography combined with a preceding chemical transformation of the quaternary ammonium compound acetylcholine or by ion-pair extraction and using a nitrogen selective detector [39]. The detection limit for these methods is around 50–100 pmol acetylcholine.

Later on in the 1980 decade acetylcholine is detected by HPLC combined with bioreactors, i.e., an analytical column separates acetylcholine from choline and thereafter acetylcholine is converted by immobilized acetylcholinesterase to choline which reacts with immobilized choline-oxidase to H_2O_2 and betaine; H_2O_2 can then be detected either by luminescence or by electrochemical detection [40, 41]. The HPLC method was further optimized in the following years by using microbore columns with an internal diameter of 1 mm and attained a sensitivity of about 10–50 fmol/20 μl ([4], see also Fig. 1a). Finally, a radioimmunoassay for acetylcholine has also been established [42]. Very recently a highly sophisticated method has been developed which can visualize non-neuronal acetylcholine in the epithelial cell layer of the mouse small intestine [43]; matrix-assisted laser absorption/ionization (MALDI-TOF) imaging mass spectrometry has been optimized for the cellular detection and visualization of acetylcholine.

The increase in the detection limit was very important for progress in understanding the non-neuronal cholinergic system, because mammalian non-neuronal cells contain considerably less acetylcholine than neurons. The non-neuronal cells (see Table 3) do not concentrate acetylcholine in high quantities within small vesicles, where acetylcholine is highly concentrated and stored by

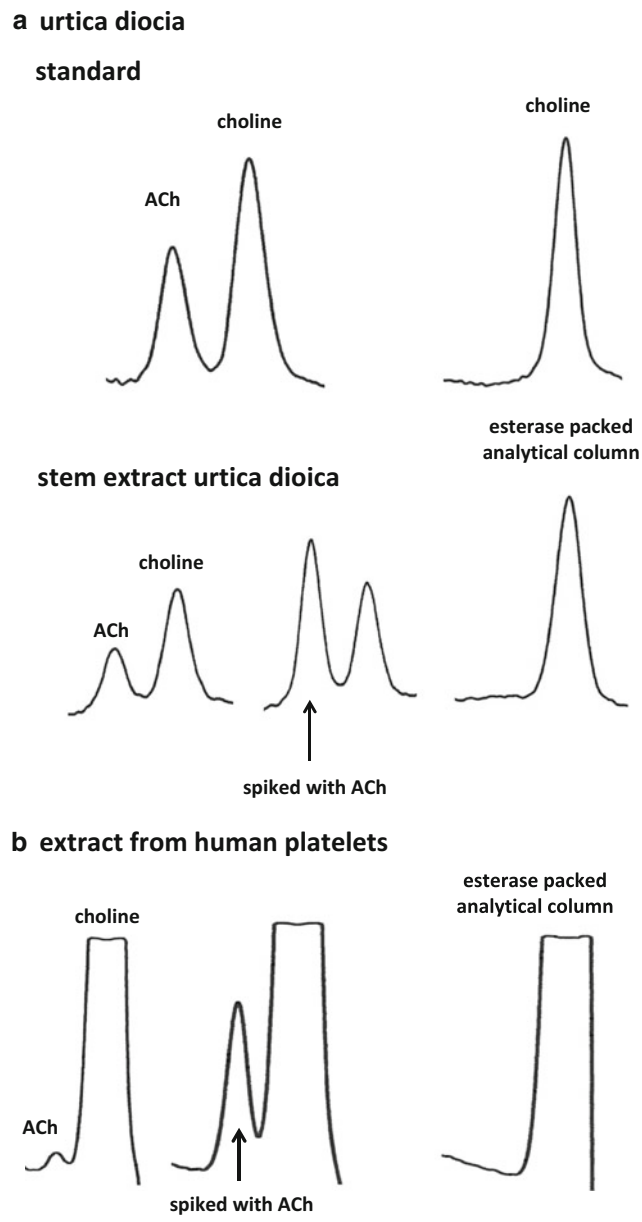

Fig. 1 Detection of non-neuronal ACh by HPLC with bioreactors and electrochemical detection. (**a**) 85 mg of a stem of *Urtica dioica* was placed in 1 ml of 15 vol.% formic acid in acetone and minced with scissors. After standing on ice (30 min) and centrifugation, the supernatant was evaporated to dryness by nitrogen. The dried sample was resuspended in 1 ml of a phosphate buffer, diluted by a factor of 200 and an aliquot (20 μl) was injected onto the HPLC-system (for details see ref. [44]). *First row*: chromatogram of a standard solution containing 1 pmol acetylcholine and choline/20 μl using the regular analytical column or, on the *right hand side*, an analytical column packed with acetylcholine-specific esterase, i.e., under this condition the first acetylcholine peak disappeared. *Second row*: chromatogram of the extract from *Urtica dioica*; the second chromatogram shows the same sample spiked with 0.6 pmol acetylcholine/20 μl; still only one peak appears at the retention time corresponding to acetylcholine; third chromatogram shows the same

neurons to generate a super threshold signal upon neuronal activity. In contrast, non-neuronal cells appear to release acetylcholine in small quantities more or less continuously to maintain cellular homeostasis by autocrine and paracrine signaling via muscarinic and nicotinic receptors which are abundantly expressed on more or less all cells. Thus, a very sensitive method is required to detect non-neuronal acetylcholine extracted either from human epithelial cells or human skin by dermal microdialysis [44, 45].

However, one has to consider that HPLC measurement combined with bioreactor and electrochemical detection does not represent a 100 % specific method, as other unknown compounds or other choline-esters can produce peaks with a retention rate similar to that of acetylcholine. Therefore, one has to prove the identity of the acetylcholine peak by spiking the sample with a low quantity of applied acetylcholine and by using an acetylcholinesterase-packed analytical column. Under this condition the acetylcholine peak must disappear and the choline peak should increase correspondingly. A typical example is shown in Fig. 1a using extracts of leaves of *Urtica dioica* and for reference a standard sample containing 1 pmol/20 μl of both acetylcholine and choline. Figure 1b demonstrates the presence of acetylcholine in human platelets.

For detection of non-neuronal acetylcholine in tissue or cells (freshly isolated or cultured) it is important to homogenize or lyse the cells/tissue in small volumes. For example, pieces of isolated airways or small intestine can be fixed in a Petri dish with the luminal surface facing upwards and a cotton-tipped applicator can gently rubbed for 5 s along the luminal surface. Using this approach the basal membrane of the surface airway epithelium is not penetrated, i.e., the underlying lamina propria remained intact [44]. Likewise, rubbing of the intestinal surface removed tips of villi only, the lamina muscularis mucosae with the underlying cholinergic submucosal plexus remained intact [44]. Corresponding samples can be taken from the lung surface (pulmonary pleura) or from the surface epithelium of oral and vaginal mucosa of volunteers [44]. After rubbing, the cotton-part of the applicator is placed in 1 ml ice-cold 15 % formic acid in acetone (v/v) for 30 min with intermittent vortexing. This medium mediates cell lysis and inactivates all enzymes immediately. Thereafter, the cotton is removed and the medium evaporated to dryness by a smooth nitrogen jet.

Likewise, tissue can be pulverized by means of liquid nitrogen and placed in 1 ml ice-cold 15 % formic acid in acetone (v/v). After standing on ice (30 min) with repeated vortexing, the samples

Fig. 1 (continued) sample using the esterase-packed analytical column; the first acetylcholine peak disappears. (**b**) 40 ml of a concentrate of human platelets were centrifuged and the pellet was analyzed as described above under a; the huge choline peak may be caused by activation of platelets by the separation procedure

can be centrifuged (10 min; 4000 rpm), and the supernatant is evaporated to dryness by a smooth gas jet of nitrogen. This will take about 30 min and within that time samples can again be placed on ice to prevent spontaneous hydrolysis of acetylcholine. The dried sample is resuspended in 300–1000 µl of the mobile phase of the HPLC system (70 mM phosphate buffer with 0.3 mM EDTA; pH 8.5 adjusted). The principle of the detection of acetylcholine by HPLC and the use of bioreactors and electrochemical detection is described above. The use of microbore columns is helpful because the flow-front of H_2O_2 originating from conversion of acetylcholine to choline and betaine is concentrated and then, can produce a measurable current at the working electrode. Moreover, the enzymatic reaction acts as an amplifier, because 1 mol acetylcholine produces 2 mol H_2O_2. It is difficult to use internal standard, therefore acetylcholine content has to be quantified by comparison with external acetylcholine standard which is measured before and after an individual sample.

In the following sections examples are given of the detection of non-neuronal acetylcholine, in which acetylcholine is either measured directly or the expression of one of the synthesizing enzymes is shown. Acetylcholine can be synthesized by choline acetyltransferase (ChAT) or carnitine acetyltransferase (CarAT). Both enzymes have been found to mediate the synthesis of non-neuronal acetylcholine, for example, in plants but also in vertebrates and invertebrates [6, 22, 46–48].

3 Detection of Acetylcholine in So-Called Primitive Organisms Generated Very Early on the Evolutionary Time Scale

Bacteria are regarded as one of the first forms of life on earth, arising about four billion years ago; also archaea represent prokaryotic microorganisms and are thought to have populated the earth about three billion years ago. Using a radioimmunoassay or HPLC combined with electrochemical detection acetylcholine has been detected in bacteria and archaea (see Table 1). An acetylcholine-synthesizing activity has been isolated from extracts of bacteria or archaea, but the properties differ from the mammalian ChAT enzyme. The function of acetylcholine in these microorganisms is unknown so far. However, bacteria show locomotion and it has been shown that motility of two photosynthetic bacteria (*Rhodospirillum rubrum*, *Thiospirillum jenense*) was stopped by 1 mM atropine, an antagonist of muscarinic receptors. Furthermore, physostigmine and other cholinesterase inhibitors also reduced motility [49]. It is probable that the system became desensitized in the presence of cholinesterase inhibitors. Table 1 gives an overview about the presence of acetylcholine in prokaryotic microorganisms and unicellular eukaryotic organisms as well as in fungi and lower

Table 1
Presence of non-neuronal acetylcholine early in evolution

		Amount	References
Bacteria	*Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *Lactobacillus bacillus*	0.39–55 pmol/10^{10} colony forming units (CFU)	[19, 22, 79]
Archaea	Hyperthermophiles *T. kodakaraensis* Euryarchaeota *S. tokodaii*; *P. calidifontis* Methanogens *M. thermautotrophicus* *M. barkeri* Halophiles *Halobacterium* sp., *H. volcanii*	0.05–1.2 pmol/g	[48]
Algae	Blue-green algae		[21]
Protozoa (Protista)	Paramecium, *Trypanosoma rhodesiense*		[14, 80]
Porifera (Sponge)	*Theonella swinhei*, *Xestospongia sapra*, *Halichondria japonica*, *Halichondria okudai*, *Halochondria permollis*	60–2200 pmol/g	[22]
Mussels	*Gill plates* (*Mytilis edulis*, *Anodonta*)	3.8 nmol/g	[16]
Fungi	Yeast *Saccharomyces cerevisiae* Shiitake mushroom *Lentinus eddoes* Matsutake mushroom *Tricholoma matsutake* *Agaricus bisporus*, *Cantharellus cibarius* Sordariomycetes *Claviceps* (*Ergot fungi*)	900–2800 pmol/g	[7, 22, 24]
"Lower plants"	Moss Spenophyta *Equisetum robustum*	2–124 nmol/g ~2 nmol/g	[50, 81]

plants, i.e., in biological systems (including the gill plates of mussels) not regulated by neurons. All these biological systems are created very early during evolution.

4 Detection of Acetylcholine in the Plant Kingdom

Table 2 gives an overview of the expression of acetylcholine in the plant kingdom, i.e., multiple examples for the expression of acetylcholine independent of any existing neuronal system. Obviously, acetylcholine is expressed in lower and higher plants. It seems inevitable that the list will be enlarged in the future. One has to consider that our knowledge about the biological function of acetylcholine is very scanty, and a systematic analysis of the expression and biological role of acetylcholine within the plant kingdom is lacking. Reports have indicated that the synthesis of acetylcholine in plants may be regulated by light [50]. One very interesting observation is that the acetylcholine content is very high in rapid growing plants like *bamboo*, *helianthus* and *Urtica dioica*. Moreover, it has been shown that at least in *Urtica dioica* acetylcholine is involved in the regulation of water homeostasis and photosynthesis [24]. Particularly, 1 μM atropine reduced the intracellular space, the cell vacuole, and cell size and mediated proliferation of the thylakoid membrane [24, 34]. In conclusion, also in the plant kingdom binding sites for acetylcholine exist, which can be blocked by atropine.

5 Detection of Acetylcholine or Positive Anti-ChAT Immunoreactivity in Mammalian Non-neuronal Cells

To demonstrate the existence of non-neuronal acetylcholine in mammalian cells without any doubt, a contamination of neuronal acetylcholine has to be excluded in the respective samples. Thus, it has to be shown conclusively that acetylcholine is synthesized by cells not innervated at all by neurons and that these cells/tissues cannot take up acetylcholine which may be released from possible adjacent neurons. The following findings demonstrate without any doubt that isolated cells or tissue which lacks any cholinergic innervation synthesize and release acetylcholine:

(a) Acetylcholine synthesis has been demonstrated in various cultured cells (keratinocytes, airway epithelial cells, and cardiomyocytes) and cell lines like leukemic T-cells (MOLT-3), embryonic stem cells (CGR8), colon (Caco-2; H508) or lung (H82) cancer cell lines [26, 51–60]. All these cultured cells are free of any neuronal input. Release of acetylcholine was demonstrated also from cultured bovine arterial endothelial cells [61].

Table 2
Presence of non-neuronal acetylcholine in the plant kingdom

Family	Genus, *Species*	Amount	References
Amaranthaceae	Spinacia *Spinacia oleracea*	~3–7 nmol/g	[21, 50]
Anthophyta	Arabidopsis *Arabidopsis thaliana* Eggplant *Solanum melongena* Bamboo shoot *Phyllostachys bambusoides* *Phyllostachys pubescens*	23.7 pmol/g 416 nmol/g 2.9 μmol/g 0.6–1.7 μmol/g	[22]
Apocynaceae	Amsonia *Amsonia angustifolia*		[82]
Araceae	Arum *Arum specificum*, *Arum maculatum*	~1.8 nmol/g	[24]
Asteraceae	Helianthus *Helianthus annuus* Senecio *Senecio vulgaris*	3–8 μmol/g ~6.5 nmol/g	[21, 50]
Brassicaceae	Capsella *Capsella bursa-pastoris* Sinapis *Sinapis alba*	~4.8 nmol/g	[24]
Bryophyta	Moss *Conocephalum conicum* *Polytrichum*, *Brachythecium*	0.03–8.0 nmol/g	[22, 24, 50, 83]
Coniferophyta	Cedar *Cryptomeria japonica* Hinoki *Chamaecyparis obtuse* Pine *Pinus thunbergii* Podocarp *Podocarpus macrophyllus*	120–343 pmol/g	[50]
Cucurbitales	Cucurbita *Cucurbita pepo*	3–10 nmol/g	[84]
Fabaceae	Phaseolus *Phaseolus vulgaris*, *Phaseolus aureus* Pisum *Pisum sativum*	~100 ng/g 1–8 nmol/g	[50, 85]
Moraceae	*Malayan jack-fruit Artocarpus integra*	564 μg/g	[84]
Plantaginaceae	Digitalis *Digitalis ferruginea*	1.6 mg/50 g pulverized leaves	[86]
Pterophyta	Fern *Pteridium*, *Gleichenia glauca*	0.07–1.6 nmol/g	[22]
Rosaceae	Fragaria *Fragaria vesca* Crataegus *Crataegus specificus*	~5.4 nmol/g	[24, 87]
Sphenophyta	Horsetail *Equisetum arvense* *Equisetum robustum*	38 pmol/g ~2.8 nmol/g	[22, 24]
Urticaceae	Urtica *Urtica dioica* *Girardinia heterophylla*	~0.5 μmol/g	[24, 88, 89] Fig. 1a

(b) Cultured epithelial cells isolated from the airways of monkeys release acetylcholine into the supernatant [62]. In organic-cation-transporter knockout mice, i.e., a condition limiting the release of non-neuronal acetylcholine, airway epithelial acetylcholine content was doubled, which indicates an in vivo release of acetylcholine from these cells [63, 64]. Likewise, the release of non-neuronal acetylcholine becomes evident by the inhibitory

effect of nicotine receptor antagonists on the migration of cultured airway epithelial cells [65].

(c) The placenta of various species (human, monkey, cow, rabbit, rat, mouse), an organ free of cholinergic neurons, synthesizes, stores and releases acetylcholine [6, 15, 66–70].

(d) ChAT mRNA and ChAT protein have been demonstrated in most of these cells.

(e) In vivo release of acetylcholine from human skin has been demonstrated by dermal microdialysis. Botulinum toxin blocks neuronal acetylcholine release but does not inhibit acetylcholine release from the human skin [45].

In conclusion, convincing experimental data have been published since the third decade of the last century about the presence of non-neuronal acetylcholine in mammalian cells. Nevertheless, in the following decades the scientific community has focussed more or less exclusively on the role of neuronal acetylcholine in the brain and peripheral nervous system. Possibly, the brain and neurons may have drawn more attraction than apparently less specified non-neuronal cells, although the regulation and communication of these cells and their respective networks is already extremely complex. It is fascinating that epithelial or immune cells communicate by the same molecules and cholinergic receptors as do neurons in the brain. Both, the specific cholinesterase and the pseudocholinesterase, play an important role to clearly separate both systems (non-neuronal vs. neuronal) in vivo. This is operating because specific cholinesterase represents the enzyme with the highest turnover rate created by nature and because of the abundant presence of both enzymes in mammalian organisms thus limiting neuronal acetylcholine to act at hot spots only.

Table 3 gives an overview about the expression of non-neuronal acetylcholine in various mammalian cells. Accordingly, acetylcholine has been detected directly after extraction from these cells/tissues or positive anti-choline acetyltransferase (ChAT) immunoreactivity has been found. However, in the case of using the method of immunohistochemistry alone some caution is required, because false positive staining has been found with antibodies directed against muscarinic receptors in corresponding knockout mice [71].

When non-neuronal acetylcholine is released and present in the extracellular space or plasma [26], it will diffuse in close proximity to its source but also to neighboring cells, because in principle the expression level of cholinesterase activity is lower in non-innervated than in innervated cells. For example release of acetylcholine from the isolated placenta can be measured without preceding inhibition of cholinesterase [6, 70]. Consequently, non-neuronal acetylcholine can mediate autocrine and paracrine effects by stimulating muscarinic and nicotinic receptors which are ubiquitously expressed in the majority of cells (for review see ref. [33]).

Table 3
Detection of ACh or positive anti-ChAT immunoreactivity independent of neuronal innervation in mammalian cells

Cell type	Location	References
Epithelial cells	*Airways* (basal, ciliated, secretory and brush cells of the airway mucosa) *Alimentary tract* (mucosa of oral cavity, esophagus, stomach (partially), jejunum, ileum, colon, sigmoid, gall bladder) *Skin* (keratinocytes, eccrine and sebaceous glands) *Urogenital tract* (urothelium, vaginal mucosa, tubuli of kidney, granulosa cells, spermatozoa, embryonic stem cells) *Eye* (cornea) *Placenta* (trophoblast) and *Amnion* *Glandular tissue* (female breast)	[4–6, 15, 33, 44, 51, 52, 90–101]
Endothelial cells	Skin, umbilical vein, pulmonary vessels	[4, 61, 102]
Immune cells	Leukocytes, bone marrow-derived dendritic cells, macrophages, skin mast cells, microglia	[6, 24, 54, 103–105]
Mesothelial cells	Pleura, pericardium, mesenteric root	[4, 44]
Various	Pinealocytes, astrocytes, embryonic stem cells, platelets	[4, 44, 55, 60, 106, 107] Fig. 1b
Mesenchymal cells	Adipocytes (skin), myoblasts and myotubes, smooth muscle fibers, fibroblasts (airways), tendon (tenocytes), cardiac muscle fibers, osteoblast-like cells	[4, 6, 33, 95, 108–114]

6 Conclusion

Research early and late in the last century has substantially broadened our understanding of the role of acetylcholine and the cholinergic system, i.e., choline uptake, synthesizing enzymes, muscarinic and nicotinic receptors, and the inactivating enzymes. This system has been created extremely early on the evolutionary time scale (about three to four billion years ago). Thus, it is not surprising to detect the cholinergic system in plants, unicellular organisms and in more or less all mammalian cells independently of neurons. Neurons have become specialized cells prepared for signaling on the milliseconds time scale. Therefore, neurons have taken an advantage of the already established cholinergic signaling system and have further specialized this system during evolution: storing in specialized organelles (vesicles), triggering vesicular release, establishing of hot spots for nicotinic receptors and acetylcholinesterase. In contrast, the non-neuronal cholinergic system does not mediate cellular communication on the millisecond time scale but establishes a variable cholinergic tone to co-regulate basic cell functions like proliferation, differentiation, cell–cell contact, secretion, and absorption. For example, acetylcholine via muscarinic receptors causes an increase of intracellular calcium within seconds but not within milliseconds [58]. It is important to learn more about the physiological and pathophysiological role of non-neuronal acetylcholine and the non-neuronal cholinergic system. Recent articles have described an important role of the non-neuronal cholinergic system in different diseases [30, 72–77]. Particularly, chronic airway diseases (COPD and bronchial asthma) have been identified to be treated with long acting muscarinic receptor antagonists (aclidinium, glycopyrronium, tiotropium) to induce therapeutic effects beyond bronchodilation. In animal models provoking COPD or asthma these receptor antagonists have been shown to reduce airway inflammation and airway remodeling by suppressing cellular effects of non-neuronal acetylcholine [78]. Moreover, intensive research is required to further illuminate the pathophysiological role of the non-neuronal cholinergic system in other inflammatory diseases, cancer, and heart diseases.

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