The Non-neuronal Cholinergic System of Human Skin

Authors
H. Kurzen[1], I. Wessler[2], C. J. Kirkpatrick[2], K. Kawashima[3], S. A. Grando[4]

Affiliations
[1] Department of Dermatology, University Medical Center Mannheim, University of Heidelberg, Heidelberg, Germany
[2] Institute of Pathology, Johannes Gutenberg-University, Mainz, Germany
[3] Department of Pharmacology, Kyoritsu College of Pharmacy, Tokyo, Japan
[4] Department of Dermatology, University of California Davis, Sacramento, USA

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Abstract

In human skin both resident and transiently residing cells are part of the extra- or non-neuronal cholinergic system, creating a highly complex and interconnected cosmos in which acetylcholine (ACh) and choline are the natural ligands of nicotinic and muscarinic receptors with regulatory function in both physiology and pathophysiology. ACh is produced in keratinocytes, endothelial cells and most notably in immune competent cells invading the skin at sites of inflammation. The cholinergic system is involved in basic functions of the skin through autocrine, paracrine, and endocrine mechanisms, like keratinocyte proliferation, differentiation, adhesion and migration, epidermal barrier formation, pigment-, sweat- and sebum production, blood circulation, angiogenesis, and a variety of immune reactions. The pathophysiological consequences of this complex cholinergic "concert" are only beginning to be understood. The present review aims at providing insight into basic mechanisms of this highly complex system.

Introduction

Numerous studies performed in recent years have firmly established the human skin as not only a target but also an active source of various neurotransmitters and hormones. The extra- or non-neuronal adrenergic and cholinergic systems have begun to attract increasing attention as regulators of skin physiology and pathophysiology [1–4].

In 1921 Otto Loewi and Henry Dale identified acetylcholine (ACh) as a principal neurotransmitter, a discovery that was rewarded with the Nobel prize for physiology and medicine in 1936. In the following years, most advances were made by the description of ACh action in the central nervous system and by the characterization of its nicotinic (nAChR) and muscarinic (mAChR) receptors [5]. ACh is synthesized from choline and coenzyme A by choline acetyltransferase (ChAT), which is the rate-limiting step in ACh *de novo* synthesis and it is degraded by acetylcholinesterase (AChE). The first hint towards a non-neuronal production of ACh in the skin came in 1983 from studies on salivary glands of rats, which continued to produce large amounts of ACh despite prior denervation [6]. Six years later ACh production was found in blood cells of rabbits [7] and today, ACh production and expression of its receptors have been shown in a wide variety of organisms from protozoa and plants to humans, thus supporting the hypothesis that ACh is a universal cytotransmitter which has only secondarily become specialized in the nervous system. In humans, different tegumental cells covering the inner and outer surfaces of the human body and most notably various immune cells are part of the non-neuronal cholinergic system [8].

The non-neuronal cholinergic system has been implicated in numerous functions in the skin such as growth and differentiation, adhesion and motility, barrier formation, sweat and sebum secretion as well as modulation of the microcirculation. An important role in human disease, especially in inflammatory disorders such as acne vulgaris or atopic eczema is emerging together with a wealth of new data on its physiological role in maintaining skin homeostasis [4,9]. In human skin both resident and transiently residing cells are part of this system, creating a highly complex and interconnected cosmos in which ACh is the main player with regulatory roles in both physiology and pathophysiology [10]. The aim of this review is to provide insights into basic mechanisms of ACh action

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Correspondence
H. Kurzen
Department of Dermatology · Venereology and Allergology · University Medical Center Mannheim · Ruprecht-Karls University of Heidelberg · Theodor-Kutzer-Ufer 1-3 · 68135 Mannheim · Germany
Tel.: +49/621/383 22 80
Fax: +49/621/383 38 15
Hjalmar.Kurzen@haut.ma.uni-heidelberg.de; Hjalmar.Kurzen@nexgo.de

Table 1 AChR selectivity of cholinergic ligands (modified from Alexander et al. [121, 122])

	Nomenclature		Agonists	Antagonists
nAChR		subunits	ACh, CCh	
Heterooligomers	$\alpha 1^*$	$\alpha\beta\delta\varepsilon$	ACh, CCh, Epi	αBtx, Tub, Str, Suc, Dec Hex
	$\alpha 3^*$	$\alpha 3\beta 2 \pm \alpha 5$	Epi > Nic > ACh	κBtx > Hex, CtxMII > Mec > Tub > Atrop
		$\alpha 3\beta 4 \pm \alpha 5$	Epi > Cyt = Nic > ACh	κBtx, Hex CtxAuIB > Mec > Tub
	$\alpha 4^*$	$\alpha 4(\beta 2/\beta 4) \pm \alpha 5$	Epi > Cyt = Sub	DβE > Tub > Mec
	α10α9	α10α9	ACh	αBtx > Str Atrop, Nic, Mus
Homooligomers	$\alpha 7^*$	$\alpha 7_5$	Cho > Nic	KyA > αBtx > Str
	$\alpha 9^*$	$\alpha 9_5$	Cho > ACh	αBtx > Str, Atrop, Nic, Mus
mAChR			ACh, CCh, Mus, Met	Atrop, Scop
	M_1		AC-42, Des	Gly (11), MT7 (9.8), 4-DAMP (9.2) Trip (8.8), Pzp (8.5)
	M_2		BCh	Trip (9.4), AFDX384 (9.0), Hmn (8.3), 4-DAMP (8.4), Pzp (6.7)
	M_3		Cho, L-689	Gly (11), 4-DAMP (9.3), Dar (8.9), Hmn (6.4), Tio (kinetic selectivity),
	M_4		McN-A343	4-DAMP (9.4), Hmn (8.8), MT3 (8.7), Pzp (8.1), Dar (8.0)
	M_5		Mus > ACh	4-DAMP (9.0), Dar (8.1), Pzp (7.1)

ACh: acetylcholine; Atrop: atropine, BCh: bethanechol; αBtx: α-bungarotoxin; κBtx, κ-bungarotoxin; CCh: carbachol; Cho: choline, Ctx: α-conotoxin; Cyt: cytisine; Dar: darifenacine; Dec: decamethonium; DβE: dihidro-β-erythroidine; Des: desmethylclozapine; Epi: epibatidine; Gly: glycopyrrolate; Hex: hexamethonium; Hmn: himbacine; KyA: kynurenic acid; Mec: mecamylamine; Mus: muscarine; Met: metacholine; MT3 and MT7: mamba toxins 3 and 7; Nic: nicotine; Pil: pilocarpine; Pzp: pirenzepine; Scop: scopolamine; Sub: suberyldicholine; Suc: succinylcholine; Str: strychnine; Tio: tiotropium: Tub: d-tubocurarine. Values in parantheses denote antagonist apparent affinities (pKB). Glycopyrrolate selectivity according to Haddad et al. [123]. Kinetic selectivity of tiotropium at the M_3 according to Disse et al. [115]. A convincing subtype selectivity for muscarinic agonists has so far not been established.

and shed light into possible interconnections of the different components of the non-neuronal cholinergic system of the skin.

Pharmacology of AChR

Hitherto, five molecular subtypes of muscarinic AChR, M_1-M_5, have been identified. These receptors are single subunit transmembrane glycoproteins of which the M_2 and M_4 are coupled to G-proteins of the G_i family, leading to inhibition of cAMP synthesis. The M_1, M_3 and M_5 subtypes are coupled to the Gq class of the G-proteins acting on down- stream signals such as phospholipase C or D, consequently regulating intracellular calcium levels [11].

Human nicotinic nAChR are composed of different subunits, i.e. α1–α10, β1–β4, γ, δ and ε, which can be combined to pharmacologically distinct pentameric ion channels. The α1, β1 and δ chains form heteropentamers present at the neuromuscular junction together with the γ (fetal phenotype), and ε (adult phenotype) chains. The neuronal heteropentamers that contain the α3 subunit together with other subunits are also termed $\alpha 3^*$ nAChR. The α7 and α9 subunits form homopentamers and are mainly gating calcium while the $\alpha 3^*$ nAChR are sodium and/or potassium channels [12]. It has been suggested that α9 subunits may form heteromeric nAChR together with α10 subunits [13]. Depending on their subunit composition, the nAChR show different affinities for ACh, choline and other cholinergic compounds like nicotine. Both ACh and choline have been shown to activate the M_3 AChR while all other mAChR are physiologically activated only by ACh [14]. Of the nAChR, the α7 and α9 homopentamers are activated by choline, but not the $\alpha 3^*$ nAChR (**Table 1**). In the past, the question of agonist or antagonist AChR subtype selectivity has contributed considerably to confusion in AChR research. For example, atropine has been viewed as a classical antimuscarinergic substance. Recent studies, however, have demonstrated that nAChRs are also inhibited by atropine, in the rank order α9>α7>α3 nAChR [15, 16]. The α9/α10-nAChRs behave pharmacologically distinct and can be activated neither by nicotine nor muscarine. These classical cholinergic agonists reduce the ACh effects at the α9-nAChR. Like the α7-nAChR, the α9/α10-nAChRs can be blocked by α-bungarotoxin and like the mAChR they can be blocked by atropine. Similar to the AChR present at the neuromuscular junction (αβδε-nAChR) they can be blocked by strychnine [1, 17–20]. In addition, along with their classical orthosteric binding site for ACh and competetive antagonists, mAChRs possess a second, allosteric binding site. Allosteric binding modulates the action of ligands at the orthosteric binding site. This process is designated positive or negative cooperativity. Gallamine is one of the first substances with proven negative cooperativity at the mAChRs. Strychnine, a potent inhibitor of glycine receptors and of the α1 and α9 nAChRs, has been shown to exert positive cooperativity with *N*-scopolamine (a competitive mAChR inhibitor) at the M_2 and M_4 AChRs and a negative cooperativity with ACh at the M_2 and M_3 AChRs [21]. In addition, strychnine has also been shown to activate at least the M_2 and M_4 AChRs at the allosteric binding site independent of natural ligands [22]. This complex binding and activation pattern that can be found for several cholinergic substances and explains different effects of the same substance on the same cells, dependent on the presence or absence of natural or synthetic agonists and antagonists [21–23]. Because of the described highly complex actions and interactions of cholinergic substances, older pharmacological studies have to be interpreted cautiously. Using antimuscarinergic substances, it has to be kept in mind that the so called "selective" binding is lost, if higher concentrations of the respective antagonist are applied. Recent studies using antisense oligonucleotides or siRNA approaches have tried to circumvent these difficulties [24, 25]. The different pharmacological properties of commonly used cholinergic agonists and antagonists are summarized in **Table 1**.

Is endocrine action of ACh mediated via choline?

In the body, choline serves several biological functions. It is the precursor of phosphatidylcholine and sphingomyelin, two phos-

pholipids that serve as components of biological membranes and as precursors for intracellular messengers such as diacylglycerol or ceramide. Choline is also the precursor of ACh and two signaling lipids, platelet-activating factor and sphingosylphosphorylcholine. Furthermore, choline can be enzymatically degraded to betaine and H_2O_2 via choline oxidase. The methyl groups of betaine may then used to resynthesize methionine from homocysteine, thereby providing methionine for protein synthesis and transmethylation reactions [4,26]. Activation of AChRs through choline provides the basis for an endocrine action, while ACh itself is degraded rapidly through AChE, thus acting only in an autocrine or paracrine manner. Choline, usually as part of phosphatidylcholine, is widely available in a number of foods. Dietary intake of choline ranges from 300 to 900 mg a day and the mean serum free choline level is ~35 μM at birth and gradually decreases to ~10 μM after birth [27]. Choline fits the original description of a vitamin and is classified today as an essential nutrient [26]. In many mammals, long term (weeks to months) ingestion of a diet deficient in choline is adequate, however, when limited to methionine and folate leads to hepatic, renal, pancreatic, memory, and growth disorders. Muscle damage also occurs from choline deficiency [28,29].

Mammalian cells in culture require choline for cell division and without it die by apoptosis. Apoptosis-induction via choline deficiency has also been observed in liver epithelial cells where it is associated with cell-cycle arrest and upregulation of p53 and $p21^{WAF1/CIP1}$ as well as with persistent activation of NF-κB. This interesting finding has been interpreted as a possible molecular mechanism by which choline deficiency may promote carcinogenesis [30]. Hypercholinemia has been found to indicate a poor prognosis in patients with acute coronary syndrome. The source of choline whole blood elevation has not been determined and needs further research [31]. Probably because of the hitherto underestimated endocrine action of choline on AChR present in the different non-neuronal cholinergic systems, there are no reports on the effects of choline deficiency or choline excess on skin physiology or the immune system. It has been recently demonstrated that choline is chemotactic to human epidermal keratinocytes [24], and that its downstream signaling of keratinocyte α7 AChR, which involves the Ras/Raf-1/MEK1/ERK pathway coupled to integrin expression, mediates cholinergic regulation of keratinocyte directional migration [24]. It remains to be determined which sources are mainly responsible for choline present in blood and tissues in different physiological and pathological situations and whether variations in choline concentration indeed influence signaling of the different non-neuronal cholinergic systems.

Impact of ACh on keratinocyte biology

As for other kinds of tegumental cells, resident skin cells like keratinocytes synthesize and degrade ACh [32]. While the α2, α4, α6, β3 nAChR**s** have never been demonstrated in human skin, several studies have identified the presence of α3, α5, α7, α9, α10, β2 and β4 nAChR. In addition, the presence of β1 nAChR mRNA and protein was shown only recently [9, 18, 20, 33,]. There seems to be a highly variable expression of the nAChR in the epidermis, especially of the heterooligomeric species of the $\alpha3^*$-type. Putative influencing factors include age, atopic disposition, smoking habits or minimal trauma. Differences in body site may also explain contrasting results obtained with the same antibodies (e.g. anti-α3, -β2) by different authors. Based on in situ hybridization and double-label immunofluorescence, the α3, α5, β2 and β4 nAChR subunits have been demonstrated in the epidermal basal layer and – to a variable extent – in a single cell layer in the stratum granulosum**.** The homo-oligomeric nAChR subunits α7 and α9 show a clearly distinct distribution within the epidermis. While the α9 AChR are prominent in the basal layer and lowest suprabasal layers, the α7 AChR can be found in the upper stratum spinosum and in the stratum granulosum, co-localizing with the α10 and β1 chain. It is unclear at present whether the β1 chain, alone or together with other subunits, can form a functional AChR receptor in the epidermis. The α9 and α10 subunits form functional AChR**s** in various organs [13,34]. However, in the epidermis α10 expression parallels α7 and β1 expression rather than α9 nAChR subunit expression. In the other compartments of the skin, there is a complete dissociation of the expression patterns for these four subunits (i.e. α7, α9, α10 and β1), indicating that either α10 might be able to form functional receptors with different subunits, or that α10 like β1 might be able to form functional receptors on its own. However, this has never been demonstrated in vitro. Of the mAChR, M_1 and M_4 were found in the suprabasal layers, while M_2, M_3 and M_5 remained restricted to the lower layers [9, 18, 35].

The functional impact of the observed AChR distribution in the epidermis has been examined in a current study [36] using organotypic co-cultures (OTC) as an *in vitro* skin equivalent system. In this system, blocking of all AChR by combined treatment with mecamylamine and atropine or treatment with strychnine (which blocks α9 nAChR) for 7–14 days resulted in complete inhibition of epidermal differentiation and proliferation. Blockage of nAChR with mecamylamine led to a less pronounced delay in epidermal differentiation and proliferation than blockage of muscarinic mAChR with atropine, evidenced by reduced epithelial thickness and expression of terminal differentiation markers such as CK2e, CK10 or ZO1. In OTCs treated with atropine, mecamylamine or strychnine there was an intracellular lipid accumulation already in the lower epidermal layers, indicating metabolic stress and a severely disturbed epidermal barrier. In addition, prominent acantholysis could be observed in the basal and lower suprabasal layers in mecamylamine-, atropine- and strychnine-treated cultures, accompanied by a decreased expression of desmosomal, adherens junction and tight junction proteins. This globally reduced cell adhesion led to cell death via intrinsic activation of apoptosis. In contrast, stimulation of nAChR>mAChR with cholinergic drugs resulted in a significantly thickened epithelium, accompanied by an increase of intercellular lipid content in the corneal layer. In this study, it was demonstrated that ACh is crucial for the development of a stratified epidermis-like epithelium *in vitro*, well in line with the fact that virtually all keratinocyte culture media contain choline in a micromolar range [37], corresponding to human free choline serum levels and protecting keratinocytes from apoptosis as described above. Adding the pharmacological profile for the cholinergic substances used to the distribution of the AChR in the epidermis and OTC of different developmental stages, it is most likely that inhibition of either $\alpha3^*$ or α9 nAChR, which are both expressed in the basal and lower suprabasal layers, is necessary to induce acantholysis. In addition, inhibition of at least the stimulatory M_3 AChR, possibly also the M_5 AChR, which are both found in the basal layer, seems to produce similar effects. On the other hand, predominant inhibition of the M_1 AChR by glycopyrrolate did not lead to acantholysis but to a disturbed

epithelial architecture in the upper epidermal layers, thus interfering with barrier formation. These conclusions are supported by recent findings using knock-out and gene-silencing approaches [38]. In conclusion, terminal differentiation, barrier formation, keratinocyte cell adhesion and proliferation are controlled by both nicotinic and muscarinic AChR.

Does ACh influence the function of cutaneous adnexal structures?

The pilosebaceous unit seems to possess a complex AChR expression pattern that is only beginning to be understood. In the infundibulum, an epidermis-like AChR expression pattern has been demonstrated, with increased immunoreactivity especially for the α5, α10, β2, M_3 and M_5 antisera applied. In the subinfundibular outer root sheath, all AChRs except α9, β1 and M_4 can be found in the basal layer while the α9, M_4 and M_5 AChRs seem to be restricted to the central layer. The α5, α10, β1, β2, M_1–M_4 chains are strongly expressed in the inner root sheath. In the trichocytes forming the hair shaft a strong immunoreactivity of α3, β4, α9, M_2, M_3, M_4 and M_5 sera can been noted, while matrix cells seem to express only the α5, α9, M_3 and M_4 AChR subunits. Up to now, no functional data are available on the impact of ACh on hair follicle biology.

The main manifestation of a reduced sebum production, sebostasis, is dryness of the skin. Increased sebum production, seborrhea, is associated with several skin diseases including acne vulgaris or seborrhoic eczema [39]. Increased sebum production or altered sebum composition may be caused by chronic nicotine exposure on nAChR present in sebaceous glands explaining why smoking negatively influences acne vulgaris [40]. In sebaceous glands, the undifferentiated basal sebocytes express the α3, α9, β4, M_3–M_5 AChRs while the α7, β2, β4, M_2 and M_4 AChR subunits are produced in mature sebocytes. The sebaceous duct shows a particularly strong staining with α5, α7 and M_3 sera. The presence of the nAChR suggests a role for ACh in sebum production and as promoter of sebocyte differentiation. Moreover, an upregulation of the "inhibitory" mAChR M_2 and M_4 in mature sebocytes as compared to undifferentiated sebocytes of the basal seboglandular layers was demonstrated while the "stimulatory" mAChR M_3 and M_5 are both expressed in basal sebocytes [9].

Cholinergic control of melanocytes and tegumental pigmentation

The roles of melanocytes and endothelial cells in the production of erythema and tanning, respectively, are well-known. Much less is known about the signaling pathways initiating these responses. In certain plants, prokaryotes and eukaryotes, light modulates ACh metabolism, and ACh mediates biologic effects of light on the organism [41,42]. Melanocytes (MC) have been shown to be targets of ACh action by virtue of their AChR expression. Both mAChR (M1–M5) and α1, α3, α5, α7, β1, β2, γ and δ nAChR have been found in cultured and/or normal human MCs [43]. To characterize the second messenger pathways downstream of the melanocyte ACh receptors, $[Ca^{2+}]_i$ measurements were performed using Fura 2 [43]. Stimulation of MCs with micromolar concentrations of carbachol or muscarine induced a peak of $[Ca^{2+}]_i$ in MCs, reaching approximately 10 times the baseline at 100 μM of muscarine. The rise of $[Ca^{2+}]_i$ could be blocked with atropine but not with mecamylamine, suggesting that a ganglionic nAChR subtype was not involved. Regulation of $[Ca^{2+}]_i$ through melanocyte ACh receptors suggests an important physiologic role of the ACh axis in melanocyte biology and skin pigmentation. Indeed, in cultures of human MCs, ACh increases the quantity of Bcl-2 and other cell proteins and decreases tyrosine hydroxylase and DOPA oxidase activities [44].

At the skin level, ACh inhibits the local response of MCs to α-MSH [45], and directly alters vital functions of MCs. Acting through its nicotinic receptors, ACh has been shown to elicit pigmentation. Melanin pigmentation was the predominant finding in oral mucosal lesions at the site of application for 3–6 months of a sublingual tablet containing 2 mg nicotine in a smoking cessation study [46]. The nicotinic effects of ACh, leading to hyperpigmentation, seem to be controlled by its muscarinic effects, mediated by mAChRs. Kurzen and Schallreuter [4] have recently proposed that the melanocyte M2 and M4 subtypes, which are known to inhibit cAMP synthesis, produce a negative feedback on tyrosinase-pigmentation to counteract the α-MSH/MC-1R and catecholamine/β2-adrenergic response in MCs as described by Gillbro and co-workers [47].

Hypothetical role of acetylcholine in mediating cutaneous effects of UV radiation (UVR)

Endogenous NO is generated in human skin in response to both ACh injection and UVR [48], but the cell type producing NO remains unknown. The neural system apparently is not involved since the erythema response to UVB is seen in denervated skin [49]. UVB upregulates NO production in cultured keratinocytes [50] and NO produced by UV-irradiated keratinocytes stimulates melanogenesis. Both UVB- and ACh-induced NO production is mediated by upregulation of the Ca^{2+}-dependent constitutive NO synthase [48,51]. ACh is well known to regulate cutaneous blood flow via NO [52]. Therefore, it can be hypothesized that ACh releases NO from keratinocytes and cutaneous endothelial cells, and this NO then induces erythema and melanogenesis, as proposed in **Fig. 1**.

Vitiligo

The response of MCs to ACh depends on the activity/amount of the ACh-degrading enzyme AChE. The AChE activity is lowered in vitiliginous skin during depigmentation, but returns to normal on repigmentation [53], in keeping with the hypothesis that an enhanced cholinergic activity in vitiliginous skin may be a direct effect of increased local ACh concentration due to either increased secretion of decreased local clearing of ACh [54]. The hypothesis about causative role for ACh in depigmentation in vitiligo was formulated based on finding in the vitiliginous areas of an increase of a) surface temperature, b) sweat production, and 3) bleeding, which was interpreted as an evidence in favor of a local predominance of cholinergic influences, compared to the normal skin areas. Only very recently has it been recognized that AChE activity, but not that of ChAT, is regulated by H_2O_2 [55]. Considering that the outer layer of human skin can be a target for UV-generated H_2O_2 in the millimolar range, this mechanism needs to be taken into account for the regulation of ACh

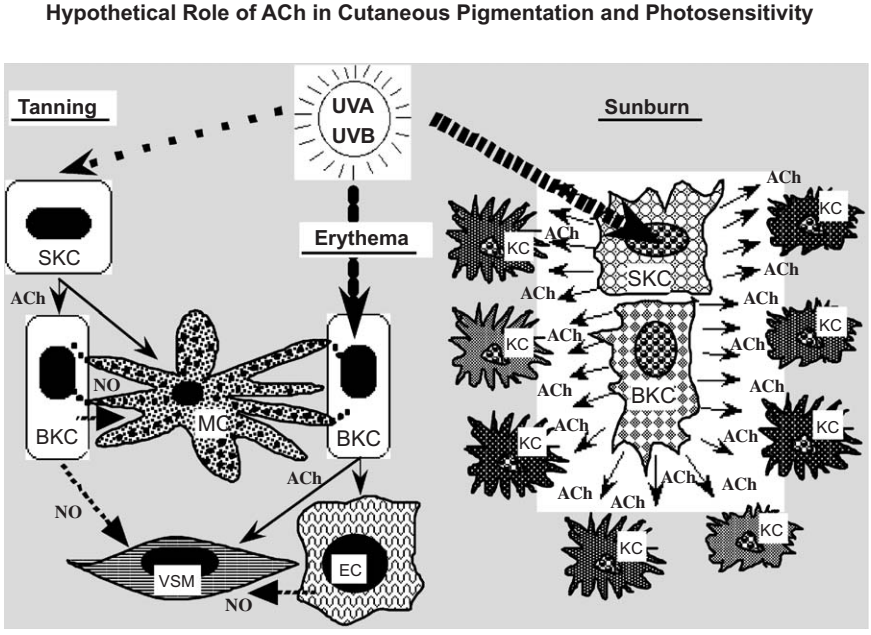

Fig. 1 Hypothetical scheme of ACh involvement in cutaneous UVR effects. The epidermis converts UVR into ACh signals by changing the kinetics of ACh metabolism in keratinocytes. Newly synthesized ACh then activates other skin cells by releasing NO. The cutaneous response involves melanocytes (MC), endothelial cells (EC), and vascular smooth muscles (VSM). The tanning UVR dose releases preformed ACh from suprabasal keratinocytes (SKC) which stimulates NO release from basal keratinocytes (BKC), thus activating melanogenesis and causing erythema. The inhibitory nature of *delayed* effects of ACh on MC [53] may be required to prevent hyperpigmentation. The erythemagenic dose stimulates BKC to release ACh. In addition to its putative *immediate* effect on MC, such as pigment aggregation, ACh, released by BKC, also stimulates NO production by EC, leading to erythema and increased microcirculatory flow [124]. The burning dose exhausts ACh stores and abolishes ACh signaling because it causes ACh receptor desensitization. Keratinocytes deprived of endogenous ACh shrink, loosen their attachments and thus die (a mechanism for blistering?).

homeostasis in skin biology and pathology. In this context, it has been suggested that ACh, as well as millimolar concentrations of H_2O_2, may well account for the described pruritus in active/progressive vitiligo [4].

The cholinergic system of dermal fibroblasts: regulation of cell-cycle progression and apoptosis

High AChE activity in human dermis [56] suggested the existence of a non-neuronal cholinergic system in dermal fibroblasts (DFs). The results of RT-PCR, western blotting and immunofluorescence assays showed that human DFs respond to ACh via classical ACh receptors. At different *in vitro* and *in vivo* conditions, DFs may express $\alpha3\beta2(\beta4)\pm\alpha5$, $\alpha7$, and $\alpha9$ nAChRs [20], and M_2, M_4, and M_5 mAChR subtypes coupled to the regulation of $[Ca^{2+}]_i$ levels [57]. These findings are consistent with early reports that both anti-mAChR antibody [18,58] and muscarinic drugs [59] react specifically with DFs.

Nicotinic and muscarinic effects on fibroblast proliferation had also been reported [60,61]. To elucidate the biological functions of nAChRs expressed in DFs, the nicotinic effects on transcription and translation of the genes encoding the cell cycle and apoptosis regulators were measured in *in vitro* experiments [62]. DFs stimulated with nicotine showed increased levels of the p21, cyclin D1, PCNA, Ki-67, caspase 3 and bcl-2 mRNA transcripts. These effects were largely blocked in the presence of mecamylamine – an antagonist preferentially ligating the "ganglionic" type of nAChRs. These results suggested that the role of the ACh-gated ion channels involves the contribution of the $\alpha3$ subunit, i.e., $\alpha3\beta2(\beta4)\pm\alpha5$, in the nicotinergic control of DFs.

Quantitative RT-PCR and western blotting assays were used to measure alterations in the expression of genes coding for the cell cycle and apoptosis markers in DFs from neonates delivered by $\alpha3+/-$ mice [62]. Compared to wild type DFs, the $\alpha3-/-$ DFs showed decreased mRNA levels of p21, PCNA, cyclin D1, Ki-67 and bcl-2, and increased mRNA levels of p53, bax and caspase 3. Functional deletion of $\alpha3$ nAChR with receptor-specific antisense oligonucleotides resulted in characteristic changes in the cell cycle gene expression, which were similar to those observed in DFs from $\alpha3$ knockout mice. The changes in the cell cycle progression of murine DFs lacking $\alpha3$ were found to be just the opposite to those observed in human DFs treated with nicotine, suggesting that DF $\alpha3$-containing nAChRs mediate, at least in part, the effects of nicotine on DFs.

Fibroblast nicotinic receptors control tissue remodeling

Nicotine has been reported to alter extracellular matrix reorganizational properties of DFs [63]. To determine the role of fibroblast nAChRs in mediating cutaneous effects of nicotine, the expression of collagen I$\alpha1$, elastin and MMP-1 were measured in cultured human and murine DFs [62]. Nicotine increased all studied parameters, and mecamylamine abolished these alterations, indicating that they resulted from stimulation of an $\alpha3^*$-made nAChR. A quantitative analysis of collagen I$\alpha1$, elastin and MMP-1 in DFs grown from $\alpha3-/-$ mice showed a 1.3-fold decrease of both the mRNA and the protein levels of elastin, compared to $\alpha3+/+$ DFs. The mRNA level of collagen I$\alpha1$ was not altered in $\alpha3-/-$ DFs. Surprisingly, the mRNA and protein levels of MMP-1 and the protein level of collagen I$\alpha1$ were increased in $\alpha3-/-$ DFs, with MMP-1 mRNA exceeding the control level by 24-fold [62]. Thus, nicotine may alter elastin production through the signaling pathways downstream from $\alpha3^*$ nAChR, whereas changes in the collagen I$\alpha1$ and MMP-1 gene expression may be mediated by other type(s) of nAChRs expressed in DFs. In support of this concept, mRNA transcripts of collagen I$\alpha1$, elastin and MMP-1 are decreased in the skin of $\alpha7$ knockout mice [64].

Cutaneous toxicity of nicotine

Epidemiological studies point to a significant correlation between tobacco smoke and alterations in tissue remodeling,

such as premature skin aging, i. e., thin, dry, pale, rough and wrinkled, or simply "cigarette," skin [65]. Tobacco smoke contains at least 4000 chemicals, and it has been proposed that nicotine is one of the key constituents causing adverse health effects (reviewed in [66]). Smoking down-regulates collagen synthesis in skin, which is considered as one etiologic factor for accelerated skin aging [67]. The mechanism may involve up-regulated expression of MMP-1, MMP-2 and MMP-3 [68]. The *in vitro* exposure experiments have demonstrated that nicotine significantly increases both degradation of type I collagen and collagen production [63, 69]. The expression of the tissue inhibitor of MMP-1 and -3 mRNAs remained unchanged [70].

Recently it has been demonstrated that nAChRs expressed by non-neuronal cells not only mediate pharmacological effects of nicotine in these locations but also are a target themselves for deleterious effects of nicotine [71, 72]. Long-term exposure to nicotine alters gene expression of nAChR subunits, which modifies nicotinic pharmacology of the exposed cells. Thus, for example an overexposure to nicotine alters both the ligand-binding kinetics and the subunit composition of nAChRs [62]. The changes in the $\alpha3\alpha5\alpha7\beta2$ and $\beta4$ nAChR subunit gene expression are found at both the mRNA and protein levels. Since exposure to nicotine increases the filaggrin content in keratinocytes [73], and since overstimulation of nAChRs produces antagonist-like effects due to receptor desensitization [71], the exhaustion of the nAChR-mediated regulatory pathway of moisturizing factor production may offer a novel explanation of the early appearance of premature aged skin in tobacco users [74]. Thus, some of the pathobiologic effects of tobacco products on extracellular matrix turnover in the skin may stem from nicotine-induced alterations in the physiologic control of the genetically determined program of growth and tissue remodeling in the dermis as well as alterations in the structure and function of fibroblast nAChRs.

The role of the cholinergic system in endothelial cell biology and angiogenesis

All four components of the non-neuronal cholinergic system are expressed within the endothelium, a tissue which is present ubiquitously in the body including skin. 1) Synthesis of ACh has been shown in cultured endothelial cells of different species including man [75–77]. Positive ChAT-immunohistochemistry and ChAT-mRNA were found in freshly isolated human umbilical cells [78, 79]. 2) Positive immunohistochemistry of the catabolizing enzyme acetylcholinesterase has been demonstrated in brain capillaries [80]. 3) The high affinity choline uptake system supplies the endothelial cell with extracellular choline [81]. 4) Finally, muscarinic and nicotine receptors have been demonstrated on endothelial cells. M_1- and M_3- mAChR are found in most vessels while only the mRNA transcript of the M_2-subtype has been demonstrated in endothelial cells. In the pulmonary circulation it is also likely that the functionally active M_4-subtype is expressed. Nicotine receptor subunits are expressed in a species- and tissue specific-manner: $\alpha3$, $\alpha5$, $\beta2$ and $\beta4$ subunits in endothelial cells of the human aorta [82]; $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, and $\alpha10$ in rat aorta and $\alpha2$ in rat pulmonary trunk [83]; $\alpha3$, $\alpha5$, $\alpha7$, $\beta2$ and ($\beta4$) subunits in bovine brain and rat coronary microvascular endothelial cells [84–86]. These subunits form functionally active homo- or heteropentamers. Taken together, endothelial cells represent a prominent part of the non-neuronal cholinergic system. Thus, these cells synthesize and may release non-neuronal ACh, which by stimulating muscarinic and nicotinic receptors affects endothelial phenotypic functions, such as regulation of vasomotor tone, angiogenesis, infection and immune response.

Endogenous ACh may be involved in the regulation of these phenotypic functions by auto- and paracrine mechanisms. Importantly, applied cholinergic agonists/antagonists can interfere with this system including drugs applied directly on the skin (for example nicotine or scopolamine containing delivery systems). It is widely accepted that endothelial cells contribute to the regulation of perfusion. In vascular tissue acetylcholine via activation of muscarinic receptors (M_3- and M_1-subtypes) is a well-known mediator for the release of nitric oxide, endothelium-derived hyperpolarizing factor and prostanoids. Blood flow, shear stress and local blood pressure may affect endothelial ACh synthesis and release and as a consequence may modulate the release of vasoactive mediators. Milner and colleagues [87] have shown the release of endothelial ACh in response to an increased flow. The endothelium is also an important target for immuno-competent cells, which must penetrate the vascular wall to migrate into the tissue. Adhesion molecules mediate the cross talk between immune and endothelial cells. Kirkpatrick et al. [79] did not find an effect of nicotine (100 nM–100 μM) on the expression of VCAM and E-selectin, but ICAM1 expression was slightly enhanced. In contrast to these results it was reported that nicotine substantially stimulated the expression of VCAM1, ICAM and E-selectin in human umbilical vein endothelial cells (HUVEC) via calcium influx, an effect blockable by mecamylamine and MAPK inhibitors [88–90]. It should also be considered that Saeed and colleagues described an inhibitory effect of nicotine on the expression of adhesion molecules, when the endothelium was stimulated by the Schwarztman reaction *in vivo* or by TNFα *in vitro* [91]. Probably, the effect of nicotine depends on the activation state of the endothelial cells.

Low concentrations of nicotine (0.1 μM) promote the invasion of *E. coli.* bacteria in HUVEC, an effect which could be blocked by α-bungarotoxin [92]. Whether this mechanism can explain the increased microbial infections of heavy smokers remains an open question. Nevertheless, it has convincingly demonstrated that nicotine impairs microvascular permeability: Nicotine increases the blood brain barrier permeability and paracellular permeability and reduces connexin 43 expression and gap-junctional communication [84, 86, 93]. All these findings open new and highly important insights into the fine tuning of endothelial homeostasis by non-neuronal cholinergic mechanisms. Nicotine promotes angiogenesis *in vivo* (0.03 μg/kg) and *in vitro* (100 pM) in a mouse model and accelerates the growth of tumours under the condition of an artificially stimulated neovascularization [94]. In the *in vitro* model stimulated angiogenesis was blocked by mecamylamine or α-bungarotoxin, indicating firstly that an endogenous cholinergic pathway is involved and secondly, that nicotinic receptors of the $\alpha7$-subtype are mediating this effect [95]. Most likely, the proliferative effect of non-neuronal acetylcholine (or applied nicotine) contributes to this mechanism [96]. Such a mechanism may contribute to regeneration and repair of human tissue. However, an overstimulated or impaired non-neuronal cholinergic system may cause a reduction of the endothelial barrier function, an enhanced permeability for signaling molecules and migrating immune cells and as a consequence inflammation and imbalance between proliferation and cell death.

Taken together, the endothelium can regulate its phenotypic functions via the involvement of the non-neuronal cholinergic system, i.e. is independent of cholinergic neurons. Non-neuronal ACh can originate from endothelial as well as from circulating immune cells. Smoking and other pathogenic (exogenous, endogenous) factors target the endothelial non-neuronal cholinergic system which contributes to the pathogenesis of various diseases like atherosclerosis, tumor growth and inflammation.

Cholinergic components expressed in immune cells

Direct measurement of physiologically relevant amounts of ACh in the plasma and blood cells of humans and rabbits (see the review by Kawashima and Fujii [97] and Kawashima et al. [7]) has stimulated investigation of the synthesis of non-neuronal ACh by immune competent cells. While the Langerhans cells residing in follicular and interfollicular epidermis were demonstrated to express AChE [98] accumulated evidence demonstrates that lymphocytes express most of the cholinergic components found in the cholinergic nervous system and is consistent with expression of a non-neuronal cholinergic system in immune cells. For example, T cells produce ACh, ChAT [99] and CHT1. Both T and B cells express AChE and varying levels of mAChRs (M_1, M_2, M_3, M_4 and M_5) and nAChRs ($\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, $\alpha7$, $\alpha9$, $\alpha10$, $\beta2$ and $\beta4$) (reviewed in Kawashima and Fujii [100]).

Regulatory mechanisms affecting lymphocytic cholinergic activity

The T cell activator phytohemagglutinin (PHA) up-regulates ChAT gene expression and enhances synthesis and release of ACh via TCR/CD3 complex activation [101]. Although in the periphery ACh synthesis is catalyzed by both ChAT and carnitine acetyltransferase [102], PHA specifically activates ChAT [101] and M_5 mAChR gene expression [103] in T cells. Similarly, monoclonal antibody-mediated stimulation of CD11a (LFA-1 α-chain) up-regulates ChAT and M_5 mAChR gene expression in CEM T cells [104]. Lymphocytic cholinergic transmission appears to be activated by the interaction of T cells with antigen presenting cells and/or other cell types. Thus, for instance, immunological synapses are formed via the interaction of CD4 and CD8 with MHC class II and MHC class I, respectively, and between LFA-1 and ICAM-1 [100].

Staphylococcus aureus Cowan I up-regulates expression of M_5 mAChR mRNA in Daudi B cells and up-regulates expression of ChAT in mononuclear leukocytes (MNLs), thereby increasing their ACh content [103]. Thus, cytokines released from activated B cells appear to act in an autocrine/paracrine fashion to stimulate ChAT expression and ACh synthesis by T cells, which in turn activates lymphocytic cholinergic transmission via M_5 mAChRs in both T and B cells.

Roles of ACh in the regulation of lymphocyte function

The biochemical and functional changes induced by stimulation of lymphocytic mAChRs and/or nAChRs include enhanced cytotoxic activity, increased cGMP and inositol-1,4,5-triphosphate (IP_3) content, inhibition of cAMP synthesis and increased intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). ACh and mAChR agonists induce rapid increases in $[Ca^{2+}]_i$ followed by Ca^{2+} oscillations in both CEM T cells and Daudi B cells [97, 100, 105–108]. RT-PCR analysis showed that mAChR agonists also up-regulate *c-fos* expression in both CEM and Daudi cells. Pharmacological analysis using various mAChR-specific antagonists revealed that ACh induces Ca^{2+} signalling in lymphocytes via M_3 and/or M_5 mAChRs, leading to IP_3-mediated up-regulation of *c-fos* expression, and that M_1 mAChRs are involved in the differentiation of CD8+ T cells into cytotoxic T cells [109]. Nicotinic cholinergic signaling also appears to be involved in the regulation of lymphocyte function. In human MNLs and leukemic T and B cell lines, nicotine acutely elicits influxes of extracellular Ca^{2+} that mediate rapid and transient increases of $[Ca^{2+}]_i$. That this response is effectively suppressed by α-bungarotoxin in CEM cells indicates the nicotinic signal is transduced via $\alpha7$ nAChRs [100, 108]. In addition, chronic nicotine modifies immune function by inhibiting proliferative responses or by causing anergy via constitutive activation of protein kinases and depletion of IP_3-sensitive Ca^{2+} stores. Finally, the altered lymphocytic cholinergic activity seen in animal models exhibiting immunological abnormalities is consistent with the involvement of a local lymphocytic cholinergic system in the regulation of immune function (reviewed in Kawashima and Fujii [100]).

Possible interaction of immune cells with vascular endothelial cells (VECs) and keratinocytes (KCs) through non-neuronal ACh

ACh may play an intermediary role in the dialogue between immune competent and tissue cells regulating immune function and local circulation [100]. During CAM-mediated interactions, T cells and VECs are believed to use ACh to communicate reciprocally via mAChRs on both cell types, and possibly nAChRs on T cells. Kawashima and Fujii proposed that the interactions between T cells and VECs facilitate ACh synthesis and release in both cell types, leading to vascular smooth muscle relaxation and erythema. Potentiation of NO synthesis during the interaction is believed to evoke local vascular smooth muscle relaxation, thereby facilitating extravascular migration of T cells. ACh released from T cells, and possibly VECs, may also be involved in regulating production of TNF-α, which in turn acts on nAChRs in T cells (reviewed by Kawashima and Fujii [100]).

In addition to synthesizing ACh and expressing mAChRs and nAChRs [1], KCs have the ability to secrete cytokines and chemokines that facilitate lymphocyte recruitment to the skin. Furthermore, KCs also express MHC class II and adhesion molecules (ICAM-1) under the influence of lymphocyte-derived cytokines such as IFN-γ and IL-17 [110]. Immunological synapses formed between T cells and KCs through the interaction of CD4 with MHC class II and LFA-1 with ICAM-1 should facilitate synthesis and release of ACh in both T cells and KCs, which should in turn act as an autocrine/paracrine factor on their own mAChRs and/or nAChRs, leading to skin lesions through modification of KC differentiation, cell cycle progression, adhesion and apoptosis (**Fig. 2**).

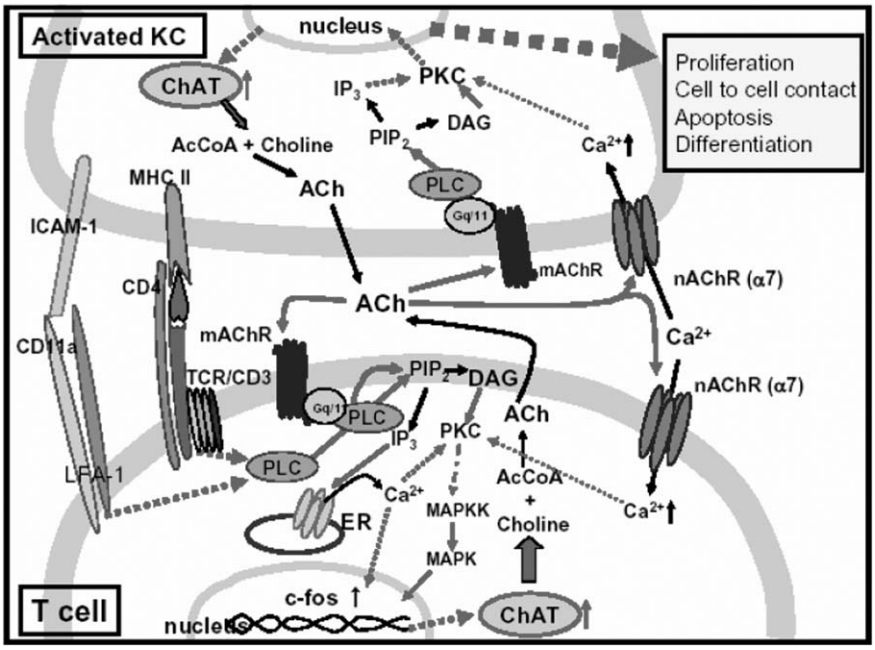

Fig. 2 Schematic diagram illustrating the numerous transduction and regulatory pathways that affect and are affected by the lymphocytic cholinergic system during the interaction of T cells with activated keratinocytes expressing MHC class II and ICAM-1. ACh: acetylcholine; AcCoA: acetyl coenzyme A; ChAT: choline acetyltransferase; DAG: diacyl glycerol; ER: endoplasmic reticulum; ICAM-1: intercellular adhesion molecule-1; IP_3: inositol-1,4,5-trisphosphate; KCs: keratinocytes; mAChR: muscarinic ACh receptor; MAPK: mitogen activated protein kinase; MAPKK: MAPK kinase; MHC: major histocompatibility complex; nAChR: nicotinic ACh receptor; PKC: protein kinase C; PIP2: phosphatidyl inositol-4,5-diphosphate; PLC: phospholipase C; TCR: T cell receptor.

Cholinergic modulation of immune responses

In addition to the mostly sympathetic hard-wiring of lymphatic organs by the autonomic nervous system [111, 112], which is a prerequisite for a direct activation of, e. g., lymphocytes in lymph nodes, extraneuronal "neurotransmitter" and local hormone systems have been recognized in recent years. A central player in this "inflammatory reflex" is the cholinergic system [113]. The autonomic cholinergic system, in part represented by the vagal nerve, transmits information bidirectionally from the peripheral immune organs to the brain and back, thus detecting local inflammatory reactions, e.g., in response to microbial invasion. This sensory input has been called the sixth sense. Consequently, dissecting the vagal nerve has serious consequences e.g. for the detection of bacterial infections. Intraperitoneal injection of Il-1 or endotoxin fails to induce fever after vagotomy. In contrast, electrical stimulation of the vagal nerve inhibits TNF-α production in the liver, spleen and heart observed during ischemia, shock or endotoxinemia. Many other in vitro data support a potent immune-modulating capacity of ACh. In macrophages, the inhibitory effect on TNF-α, Il-1 or Il-6 production seems to be mediated, at least in part, through the α7 nACh-R. The role of other AChR present on macrophages is still under investigation. In human alveolar macrophages, ACh has been found to stimulate chemotactic activity on neutrophils, monocytes and eosinophils. This chemotactic effect has been suggested to be predominantly mediated by leukotriene B4 [114]. A combination of different anticholinergic substances (4-DAMP effective, pirenzipine ineffective) that were able to inhibit the observed ACh effects, led the authors to conclude that the responsible AChR expressed on alveolar macrophages could be the M_3mAChR. However, recent studies demonstrated that 4-DAMP does not discriminate between M_3 and M_5 AChR. In our own studies, we found M_5 to be the predominant mAChR on human blood-derived macrophages (HK, unpublished observation).

Many anticholinergic substances such as ipratropium or tiotropium, currently in clinical use for the treatment of chronic obstructive pulmonary disease (COPD), have been shown to exert anti-inflammatory effects, supposedly through inhibition of the mAChR subunits on alveolar macrophages [115].

Nicotine has been shown to reduce IL-2 and TNF-α release from PBMC significantly but not quite as potently as prednisolone. In addition, transdermal application of nicotine reduces the irritant contact eczema induced by SDS and similarly the UVB-induced sunburn reaction (reviewed in [4]). These nicotine effects may in part be explained by its ability to suppress the migration of leukocytes to an inflammation/infection site. The decreased inflammation correlates with lower chemotaxis/chemokinesis of peripheral blood mononuclear cells (PBMC) toward formyl-methionyl-leucyl-phenylalanine and monocyte chemoattractant protein-1 without affecting the density of their respective receptors. Thus, because nicotine suppresses leukocyte migration, it might contribute to the delayed wound healing and increased incidence of respiratory infections among smokers [116]. Another potential disease-modulating effect was found in Chlamydia pneumoniae (Cpn)-infected immune cells. Lymphocytes and macrophages are susceptible to Cpn infection, which has been shown to alter their expression levels of IL-10, IL-12 and TNF-α in a time-dependent fashion. Nicotine treatment of the Cpn-infected cells up-regulated IL-10, but not TNF-alpha and IL-12, and also resulted in significant down-regulation of TGF-β1 production which was marked in the Cpn-infected control cells. The combined action of nicotine and Cpn on cytokine production may have an impact in chronic inflammatory diseases [117].

Interaction of systems

It is well known that the release of ACh from cholinergic neurons is modulated by a battery of receptors located on the varicosities. For example, noradrenaline inhibits the release of ACh from myenteric neurons via α2-adrenoceptors and vice versa acetylcholine reduces the release of noradrenaline via presynap-

tic inhibitory muscarinic receptors. In addition various kinds of neuronally localized receptors (adenosine receptors, 5-hydroxytryptamine receptors, opioid receptors, P2X- and P2Y-receptors, prostanoid receptors) modulate the release of neuronal ACh. It is unknown, whether the release of non-neuronal ACh is regulated likewise. In the human placenta the release of non-neuronal ACh is stimulated by nicotine receptors. Moreover, in the human placenta it has been shown that the release of non-neuronal acetylcholine is mediated via organic cation transporters, subtype OCT1 and OCT3, the latter also known as non-neuronal catecholamine uptake 2 [118]. The cation transporters are widely expressed and multiple interactions with endogenous substrates as well as with applied drugs are possible [119]. Thus multiple interactions between endogenous compounds as well as xenobiotics and the release of non-neuronal acetylcholine can emerge. For example, noradrenaline and adrenaline reduced the release of non-neuronal ACh in the human placenta via substrate inhibition at the cation transporter. Thus, circulating catecholamines may interfere with the release of non-neuronal acetylcholine at this common target via substrate competition, i.e., on the basis of a receptor-independent pathway. Quinine, like many as drugs, is a strong inhibitor of OCTs and reduces the release of non-neuronal acetylcholine which may explain its atropine-like actions [120]. For further research it is important to identify those drugs which interfere with the release of non-neuronal acetylcholine in attempt to find new therapeutical targets and to reduce possible side effects of the current therapy.

It is also possible that non-neuronal ACh released from epithelial cells modifies the functions of immune cells migrating into the mucosa and vice versa. The action radius of non-neuronal ACh is not known. Can ACh released from fibroblasts, fat cells or eccrine glands within the skin cross the basal membrane and attain at all epidermal cell layers? We assume a very restricted area of action, because of the ubiquitously expressed esterases. The ACh specific esterase represents the most effective enzyme created by nature so far. In vascular tissue, however, it is possible that non-neuronal ACh released from adherent immune cells interacts with endothelial cells. Also within the microvascular space (lung, intestine) a direct interaction between endothelial and epithelial acetylcholine appears possible.

Perspective

In the last 10–15 year , a wealth of data has emerged, describing different roles of extra- or non-neuronal ACh in different organs, most notably a highly complex setting of active players and targets and possible bystanders in the cholinergic concert. In the skin, not only epidermal keratinocytes are the main players, but in addition, as described, most other components permanently or transiently residing in the skin. Whether it is mostly autocrine and paracrine or also endocrine actions of ACh/choline and the AChRs which predominate in different pathobiological scenarios still remains to be elucidated. Bridges will have to be built to the autonomic cholinergic system and most importantly to the different components of the immune system.

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