[Frontiers in Bioscience 9, 2063-2085, September 1, 2004]

EXPRESSION OF NON-NEURONAL ACETYLCHOLINE IN LYMPHOCYTES AND ITS CONTRIBUTION TO THE REGULATION OF IMMUNE FUNCTION

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TABLE OF CONTENTS

1. ABSTRACT

Lymphocytes express most components of the cholinergic system including acetylcholine (ACh), muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively), choline acetyltransferase (ChAT), high affinity choline transporter and acetylcholinesterase. ACh and mAChR agonists elicit intracellular Ca^{2+} signaling, up-regulation of *c-fos* expression and nitric oxide synthesis within T and B cells probably via M_3 and M_5 mAChRs. Stimulation of nAChRs with ACh or nicotine causes a rapid and transient Ca^{2+} signaling in T and B cells, probably via *alpha*7 nAChR subunit-mediated pathways. Phytohemagglutinin- or antigen-induced T cell activation via cell surface molecules (e.g., T cell receptor/CD3 complexes) enhances lymphocytic cholinergic transmission by up-regulating ChAT and M_5 mAChR expression. It is thus likely that a local lymphocytic cholinergic system is involved in regulating immune function. This idea is supported by the findings that lymphocytic cholinergic activity

Table 1. Functional and biochemical effects induced by mAChR and nAChR agonists in lymphocytes (12)

1.	Enhancement of cytotoxicity
2.	Increase of cGMP synthesis
3.	Activation of DNA synthesis
4.	Activation of proliferation
5.	Increase of spontaneous motility
6.	Activation of RNA synthesis
7.	Increase in membrane fluidity
8.	Inhibition of cAMP synthesis
9.	Activation of selective nonadherent cell proliferation
10.	Inhibition of PHA-induced DNA synthesis
11.	Inhibition of PHA-induced INF-*gamma* synthesis
12.	Inhibition of IgM secretion
13.	Activation of IP_3 synthesis
14.	Increase of $[Ca^{2+}]_i$
15.	Activation of PHA-induced IL-2 production
16.	Enhancement of IL-2 receptor mRNA expression
17.	Enhancement of *c-fos* gene expression

IP_3, inositol trisphosphate; PHA, phytohemagglutinin; IL-2, interleukin-2; IgM, immunoglobulin M; INF-*gamma*, interferon-*gamma*; $[Ca^{2+}]$ intracellular free calcium ion concentration

is altered in animal models exhibiting immunological abnormalities. In addition, it appears likely that during interactions mediated by cell surface molecules T cells communicate via ACh with thymic epithelial cells and vascular endothelial cells, which also express ChAT and nAChRs or mAChRs. This interaction leads to T cell selection and maturation in the thymus and local vascular smooth muscle relaxation. Collectively, these data provide a compelling picture in which lymphocytes constitute a cholinergic system that is independent of cholinergic nerves, and which is involved in the regulation of immune function and local circulation.

2. INTRODUCTION

Acetylcholine (ACh) is familiar to most of us as an important neurotransmitter found in the central and peripheral cholinergic nervous systems; indeed, it was the first compound ever recognized as a neurotransmitter. It is now known, however, that ACh and ACh-synthesizing activity are expressed ubiquitously among widely diverse organisms that include eubacteria, fungi, various kinds of plants, and lower animals such as sponges and nematodes, and higher animals such as insects and vertebrates (1-3). Moreover, Yamada *et al.* (4) recently found that several strains of archaea also express certain levels of ACh and ACh-synthesizing activity. One notable finding is that the tips of bamboo shoot (*Phyllostachys bambusoides),* which grow very rapidly and express ACh at a level of about 440 *micro*g/g wet weight, which is about 80 to 100 times higher than the level in rat striatum, the region containing the highest amount of ACh in the brain (1, 5). Taken together, these findings support the notion that ACh has been expressed by organisms since origin of life and that it is locally involved in the regulation of cellular function.

Knowledge about the synthesis, storage, metabolism and actions of ACh has been derived mostly from studies of the mammalian nervous system. ACh is synthesized by choline acetyltransferase (ChAT, EC 2.3.1.6) from acetyl coenzyme A (AcCoA) and choline taken up by the high affinity choline transporter (CHT1) (6) in the central cholinergic nerves, and by ChAT and, to a lesser extent, carnitine acetyltransferase (CarAT, EC 2.3.1.7) in the periphery (7, 8). The synthesized ACh is transported from the cytosol into synaptic vesicles by the vesicular ACh transporter (VAChT) (9). After which stored ACh is released by exocytosis mediated by a rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (10). Upon release, ACh acts on either muscarinic or nicotinic ACh receptors (mAChR and nAChR, respectively) on the targets and is then rapidly hydrolyzed by acetylcholinesterase (AChE, EC 3.1.1.7) or butyrylcholinesterase, also known as cholinesterase (ChE) into choline and acetate.

It is now clear that ACh, ChAT, and various mAChRs and nAChRs subtypes are all expressed in non-neuronal mammalian tissues and organs, including lymphocytes (see reviews by Fujii and Kawashima (11) and Kawashima and Fujii (12-14)); mucocutaneous epithelia (see reviews by Grando (15, 16)); gastrointestinal, respiratory and urogenital epithelial cells (see reviews by Wessler *et al.* (2, 3, 17, 18); placenta (see a review by Sastry (19); amniotic epithelial cells (20-22) and vascular endothelial cells (23-26). This wide distribution makes it apparent that ACh is a ubiquitous cell signaling molecule regulating the homeostasis of a variety of cell functions (27).

The data indicating that AChRs are expressed in lymphocytes have been accumulating since the early 1970s (for review, see Kawashima & Fujii (12)). ACh and both mAChR and nAChR agonists elicit a variety of functional and biochemical effects in lymphocytes (Table 1) (12). On the basis of these findings, the notion of cross-talk between the cholinergic nervous system and the immune system was postulated and accepted widely. However, considering the enzymatic and physicochemical fragility of ACh, as well as the absence of histochemical and anatomical evidence of cholinergic innervations of the immune system, one would have to conclude that it is highly unlikely that ACh released from cholinergic nerve endings interacts directly with AChRs expressed on lymphoid cells. In this review, we will discuss the evidence for the expression of a non-neuronal cholinergic system in lymphocytes, its regulatory mechanisms, possible physiological functions, and the relationship between lymphocytic cholinergic activity and immune function in immune deficiency models.

3. EXPRESSION OF CHOLINERGIC COMPONENTS IN LYMPHOCYTES

Lymphocytes express most cholinergic components found in the nervous system: 1. AChRs (mAChRs and nAChRs); 2. ACh; 3. ChAT; 4. VAChT; 5. CHT1 and 6. AChE.

Table 2. Receptor binding analyses supporting the presence of mAChR on lymphocytes and thymocytes (12)

Species	Cell Types	Ligand
Mouse	Lymph node lymphocytes	[^{3}H]QNB
	Splenic lymphocytes	[^{3}H]QNB
	Thymocytes	[^{3}H]QNB
Rat	Blood lymphocytes	[^{3}H]QNB
	Lymph node lymphocytes	[^{3}H]QNB
	Splenic lymphocytes	[^{3}H]QNB
	Thymocytes	[^{3}H]QNB, [^{3}H]NMPB, [^{3}H]NMS, [^{3}H]ACh
Human	Blood lymphocytes	[^{3}H]QNB, [^{3}H]NMS
	Thymocytes	[^{3}H]QNB

NMS, N-methylscopolamine; NMPB, 4-N-methylpiperidinyl benzilate; QNB, quinuclidinyl benzilate

Table 3. Expression of mAChR subtypes in human MNLs, lymphocytes, and leukemic cell lines

Cell source and references	Cell type	M_1	M_2	M_3	M_4	M_5
MNLs and T cells						
Costa *et al.* (32)	MNLs	-	-	+	+	+
Hellstrom-Lindahl & Nordberg (33)	MNLs	-	-	+	+	+
Sato *et al.* (34) (3M, 4F)	MNLs	+a	+a	+a	+	+
Ricci *et al.* (35)	MNLs	-	+	+	+	+
Purified T cells						
Hellstrom-Lindahl & Nordberg (33)	T	-	-	+	+	+
Peripheral blood lymphocytes						
Fujino *et al.* (36)	T, B	+	+	-		
Tayebati *et al.* (37)		-	+	+	+	+
Cell lines						
Kaneda *et al.* (38)	Jurkat: T	-	-	+		
Hellstrom-Lindahl & Nordberg (33)	Peer: T	-	-	+	-	+
Sato *et al.* (34)	HL-60: Pro	-	-	-	+	+
	CEM*: T	+	-	+	+	+
	MOLT-3: T	-	-	+	+	+
	HPB-ALL: T	-	-	+	+	+
	HUT-78: T	+	+	+	+	+
	Jurkat: T	-	-	-	+	+
	Daudi: B	-	+	+	+	+
	BALL-1: B	+	-	-	+	+
	NALM-6: B	-	-	-	+	+

M, male; F, female; Mo, monocytes; MNLs, mononuclear leukocytes; Pro, promyelocytic leukemic cells;-, negative;+, positive. CEM*, CCRF-CEM; +a, 5 out of 10 samples

3.1. AChRs

Both mAChRs and nAChRs are expressed in lymphocytes, and expression of nAChRs has recently been detected in the thymus.

3.1.1. mAChRs in lymphocytes

Expression of mAChRs has been confirmed in lymphocytes prepared from blood, lymph node, spleen and thymus of mouse, rat and human using ligand binding techniques (Table 2) (for review, see Kawashima & Fujii (12)). In addition, five distinct mAChR subtypes (M_1-M_5), acting via two different second messenger signaling systems, have been identified by molecular cloning (28-30). The M_1, M_3 and M_5 mAChR subtypes are coupled to $G_{q/11}$, which, upon stimulation, mediates activation of phospholipase C (PLC), leading to increases in $[Ca^{2+}]_i$. The M_2 and M_4 mAChR subtypes are coupled to $G_{i/o}$, which, upon stimulation, mediates inhibition of adenylyl cyclase, leading to declines in cAMP production (31).

Reverse transcription-polymerase chain reaction (RT-PCR), Northern blot analyses and immunocytochemical techniques using monoclonal antibodies (mAb) against specific mAChR subtypes have all been used to detect the expression of specific mAChR subtypes in lymphocytes (Table 3) (32-38). M_3, M_4 and M_5 mAChRs have been identified in almost all human peripheral blood lymphocytes tested, whereas expression of M_1 and M_2 mAChRs varies among individual subjects (34). Immunohistochemical studies have shown that the M3 subtype is the most strongly expressed mAChR, followed in order by the M5, M4 and M2 subtypes (37). Which is in line with binding experiments in human mononuclear leukocytes (MNLs), showing the relative density of the mAChR subtypes

Table 4. Expression of nAChRs subtypes in human thymocytes, MNLs and lymphocytes

Cell source		***Alpha* subunit**								***Beta* subunit**		
References	**Cell type**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**	**2**	**3**	**4**
MNLs & thymocytes												
Mihovilovic & Roses (49)	MNLs		-							-		-
	Thymocytes		+							-		
Mihovilovic *et al.* (50, 51)	MNLs		-		+							-
	Thymocytes		+		+							+
Hiemke *et al.* (44)	$CD4^+$ T		+a	+								
Navaneetham (52)	T						-					
Sato *et al.* (34) (3M, 4F)	MNLs	+	-	-	+	+b	+			+c	-	-
Benhammou (53)	MNLs		+	+			+			+		+
Lustig *et al.* (48)	MNLs							+	+			-
Villiger *et al.* (54)	MNLs						+					-
Kuo *et al.* (55)	MNLs	+	-	-	+		+			-		-
Cell lines												
Battaglioli *et al.* (56)	Jurkat: T		+									
	MOLT-4: T		+									
Sato *et al.* (34)	CEM*: T	-	+	-	+	+	+			-	-	+
	MOLT-3: T	-	+	-	+	+	-			-	-	+
	HPB-ALL: T	+	-	-	+	+	+			-	-	+
	HUT-78: T	+	-	-	+	+	-			-	-	-
	Jurkat: T	-	-	-	-	-	-			-	-	+
	Daudi: B	+	-	-	+	+	+			-	-	-
	BALL-1: B	-	-	-	+	+	-			+	-	+
	NALM-6: B	-	-	-	+	-	-			+	-	+

F, female; M, male; MNLs, mononuclear leukocytes; T, T cells; B, B cells; -, negative; +, positive; CEM*, CCRF-CEM; +a, 8 out of 10 samples; +b, 2 out of 7 samples; +b, 3 out of 7 samples

to be $M_3>M_5>M_4>M_2$ in healthy subjects (35). That expression of M_2 and, to a lesser extent, M_5 was more strongly expressed in MNLs from asthmatic patients than from healthy subjects, while expression of M_4 was unchanged (35), suggests the pattern of expression of mAChR subtypes may vary with the physiological conditions of the subject. Costa *et al.* (39) detected mRNAs encoding the M_3 and M_4 mAChR subtypes in rat lymphocytes. Whether the pattern of expression of mAChR subtypes in lymphocytes differs among mammalian species is not yet known.

3.1.2. nAChRs in lymphocytes

The expression of nAChRs in human, rabbit and rat lymphocytes and thymocytes has been demonstrated in the binding studies using labeled nicotine and *alpha*-bungarotoxin (*alpha*-BTX), and in immunocytochemical studies (40-46).

nAChRs are located on the plasma membranes of skeletal muscle cells and neurons. They exist as pentamers comprised of one to four distinct subunits (from among the *alpha*, *beta*, *gamma*, *delta* and *epsilon* subunits) forming a ligand-gated ion channels. Upon activation by ACh or other agonists, membrane depolarization and excitation are mediated by a rapid increase in the membrane permeability to Na^+, K^+ and Ca^{2+}. From among these subunits, at least two copies of the *alpha* subunit are always present; multiple binding sites for ACh are formed at the interfaces of each *alpha* subunit and a neighboring subunit.

The heterogeneity among nAChRs was revealed by molecular cloning (30). In skeletal muscle, the so called muscle type nAChRs contain four distinct subunits within the pentameric complex: $(alpha1)_2/beta1/delta/gamma$ or $(alpha1)_2/beta1/delta/epsilon$. nAChRs in embryonic or denervated muscle contain a *gamma* subunit, whereas an *epsilon* subunit replaces the *gamma* in innervated, adult muscle. By contrast, nAChRs in the central nervous system, the so called neuron type nAChRs, are comprised of only *alpha* and *beta* subunits; six *alpha* (*alpha*2-*alpha*7) and three *beta* (*beta*2-*beta*4) subtypes have been identified in the mammalian nervous system. In addition, the *alpha*8 subtype is expressed in the visual areas of the avian brain (47), and the *alpha*9 and *alpha*10 subtypes have been detected in the hair cells of the mammalian inner ear (48). The *alpha* subunits also can be subdivided according to their sensitivity to *alpha*-BTX: *alpha*1 and *alpha*7, perhaps also *alpha*8 and *alpha*9, are *alpha*-BTX-sensitive, while *alpha*2- *alpha*6 are *alpha*-BTX-insensitive.

Recent molecular biological and immunochemical studies have confirmed the expression of various nAChR subunits in human thymocytes, MNLs, lymphocytes and leukemic cell lines (Table 4) (49-56). So far, only neuron type nAChR subunits have been detected in human lymphoid cells. Expression of mRNAs encoding the *alpha*2, *alpha*5 and *alpha*7 subunits in human MNLs has been confirmed in most studies. While expression of the *alpha*3, *alpha*4, *beta*2 and *beta*4 subunits was variable among the studies, and the *beta*3 subunit was undetectable in MNLs and cell lines. Using co-immunoprecipitation assays and Western blotting, Benhammou *et al.* (53) found that nAChRs in human circulating lymphocytes are likely

Table 5. Blood ACh contents in several mammalian species

Species	ACh content (pmol/ml)	No of samples
Cattle	360.5 +/- 59.7	7
Chimpanzee	21.5 +/- 2.60	6
Dog	1.37 +/- 0.23	10
Goat	4.05 +/- 0.93	5
Horse	93.8 +/- 16.3	5
Human	8.66 +/- 1.02	30
Pig	11.7 +/- 1.58	5
Rat	1.43 +/- 0.20	10
Rabbit	24.5 +/- 6.14	7
Sheep	2.04 +/- 0.20	5

Values are means +/- SEM. Arranged from the data presented in Kawashima *et al.* (64, 65), Fujii *et al.* (66, 67) and Yamada *et al.* (68)

to be composed in part from *alpha*2/*beta*4, *alpha*4/*beta*2 and *alpha*7 subunits, and Lustig *et al.* (48) recently reported expression of the *alpha*9 and *alpha*10 subunits in human peripheral blood lymphocytes. Further studies will be necessary to determine whether the expression pattern of nAChR subunits varies within subjects depending on physiological conditions or genetic predisposition.

Mihovilovic *et al.* (50) detected expression of the *alpha*3, *alpha*5 and *beta*4 subunits in human thymocytes. Moreover, they found that the levels of expression of the *alpha*3 and *beta*4 transcripts differed between immature ($CD4^+CD8^+$) and mature ($CD4^+CD8^-$ and $CD4^-CD8^+$) thymocytes, and that there was no expression of *alpha*3 and *beta*4 transcripts in peripheral blood MNLs. Kuo *et al.* (55) detected expression of the *alpha*2, *alpha*3, *alpha*4, *alpha*5, *alpha*7, *beta*2 and *beta*4 subunits in murine fetal and neonatal thymus, and the *alpha*3, *alpha*5, *alpha*7, *beta*2 and *beta*4 subunits in immature T cells. It is thus likely that the pattern of expression of nAChR subunits changes during the course of T cell maturation. Although no expression of muscle type nAChR subunits has been detected in human thymocytes or lymphocytes, Toyabe *et al.* (46) found the muscle-type *alpha*1 subunit to be expressed in murine lymphocytes. Whether the patterns of lymphocytic expression of nAChR subunits differ among mammalian species remains unknown.

3.1.3. nAChRs in the thymus

Thymus tissue consists of thymocytes (lymphoid cells) and thymic stromal cells (non-lymphoid cells), which include both myoid cells and thymic epithelial cells. All three cell types express various nAChRs. Expression of mRNAs encoding the neuron type *alpha*3, *alpha*5 and *beta*4 nAChR subunits (49-51, 57, 58), as well as the muscle type *alpha*1 subunit (59), has been detected in human thymus tissues and in cultured thymic epithelial cells. In addition, Kuo *et al.* (55) detected the expression of the *alpha*2, *alpha*3, *alpha*4, *alpha*5, *alpha*7, *beta*2 and *beta*4 subunits in cultured murine fetal thymic organ, and Navaneetham *et al.* (60) found expression of mRNAs encoding the muscle type *alpha*1, *epsilon*, *beta*1 and *gamma* nAChR subunits in all or most thymus tissue samples from 20 healthy subjects and 13 myasthenia gravis patients. The *delta* subunit mRNA was detectable in only 2 of the healthy subjects. Apparently, thymic stromal cells express not only neuron type but also muscle type nAChR subunits. One must therefore be cautious when interpreting data obtained from experiments using whole thymus tissue; under those conditions it is not presently possible to identify which thymic cell type expresses a given subunit.

3.2. ACh

The presence of ACh in blood has been a matter of controversy since the 1920s (for review, see Kawashima & Fujii (12)). However, the development of a highly sensitive, specific and simple radioimmunoassay (RIA) for ACh by Kawashima *et al.* (61) has done much to resolve that question. Because this RIA can accommodate sample sizes of up to 400 *micro*L and has a sensitivity of 3 pg (about 20 fmol)/tube, it can determine ACh concentrations as low as 7.5 pg/mL (62). Using this RIA, considerable amounts of ACh has been detected in the blood and plasma of various mammalian species (Table 5) (63-68): cattle have the highest blood ACh content; the ACh content of human blood is comparable to that of chimpanzee, pig and rabbit. It is noteworthy that there is considerable variation in blood ACh among human subjects, but it does not vary within a given subject (65). The physiological significance of high blood ACh content is not yet known.

Because of the high AChE and ChE activities in blood, the presence of ACh in lymphocytes had, until recently, been beyond imagination for most of us. Using the aforementioned RIA, Kajiyama *et al.* (69, 70) and Kawashima *et al.* (65) detected, for the first time, the localization of ACh in rabbit buffy coat cells and in human circulating MNLs. In humans, about 60% of blood ACh is localized in MNLs, suggesting that blood ACh originates mainly from lymphocytes. A considerable amount of ACh has also been detected in cells from human leukemic T cell lines, but not in cells from human leukemic B cell lines (71, 72) (Table 6), though Rinner *et al.* (73) detected ACh in both T and B cells from rats, with a greater amount in T cells. It thus appears that lymphocytic ACh is localized mainly in T cells, at least in mammals. The presence of ACh demonstrated by RIA in human MNLs (65) and human leukemic T cell lines (71) was subsequently confirmed by HPLC-ECD (74, 75).

3.3. ChAT

Using the Fonnum method (76), Kajiyama *et al.* (69, 70) demonstrated the presence of both ACh-synthesizing activity and ACh in rabbit buffy coat cells and in human peripheral blood MNLs, which consisted mainly of lymphocytes. Rinner *et al.* (77) also detected ACh-synthesizing activity in lymphocytes from rat thymus, spleen and blood, and in mouse and human lymphoid cell lines.

3.3.1. ChAT expression in human lymphocytes

Fujii *et al.* (78) used RT-PCR and Western blot analyses to demonstrate the expression of ChAT mRNA and protein in the MOLT-3 human leukemic T cell line used as a model of lymphocytes (Figure 1) (78, 79). Notably, the sequence of the RT-PCR product was completely identical to nucleotide positions 322-973 of human brain ChAT cDNA. Subsequently, expression of

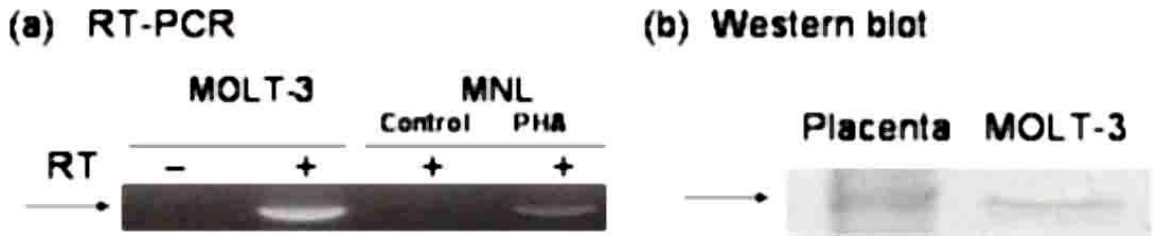

Figure 1. Expression of ChAT mRNA and protein in the MOLT-3 human leukemic T cell line and in human blood MNLs (78, 79). (a) RT-PCR analysis of ChAT mRNA expression in MOLT-3 cells and MNLs. The expected size of the ChAT mRNA RT-PCR product was 652 bp. (b) Detection of ChAT protein in MOLT-3 cells by Western blot analysis; placenta served as a positive control.

Table 6. ACh content, ChAT and CarAT activities, and ChAT mRNA expression in human MNLs and leukemic cell lines, and rat lymphocytes

Species and cells	Cell type	ACh content pmol/10^6 cells	ChAT Activity pmol/mg protein/min	CarAT activity pmol/mg protein/min	ChAT mRNA
Human					
MNLs	T, B, Mo	0.3	27.5	NT	+
CEM	T	12.6 +/- 0.6 (4)	2.9 +/- 0.2 (7)	22.8 +/- 4.6 (7)	+
HSB-2	T	36.2 +/- 3.5 (7)	1.4 +/- 0.1 (11)	58.3 +/- 15.3 (3)	+
Jurkat	T	8.2 +/- 0.4 (4)	4.3 +/- 0.8 (3)	17.2 +/- 1.9 (6)	+
MOLT-3	T	251.5 +/- 34.9 (7)	22.4 +/- 3.0 (4)	53.3 +/- 5.6 (3)	+
MOLT-4	T	38.8 +/- 5.9 (8)	8.0 +/- 1.0 (5)	NT	+
BALL-1	B	ND (4)	0.4 +/- 0.2 (3)	NT	-
Daudi	B	1.2 +/- 0.1 (4)	1.2 +/- 0.1 (4)	125.6 +/- 44.2 (3)	-
NALM-6	B	0.04+/- 0.01 (9)	0.1 +/- 0.02 (4)	NT	-
U937	Mo	0.02+/- 0.01 (4)	0.2 +/- 0.03 (8)	NT	-
Rat					
Lymphocytes	T	9.94			+
	B	3.24			

Values are means +/- SEM. Number of experiments is shown in parenthesis. ND, not detectable. NT, not tested. Mo, monocytes. Rearranged from the data presented in Fujii *et al.* (71, 72) and Rinner *et al.* (73)

ChAT mRNA was confirmed in human MNLs (79), rat lymphocytes (73) and other human leukemic T cell lines (72) (see Table 6).

Rinner *et al.* (73) reported that rat peripheral $CD4^+$ (helper) T cells contain about twice as much ACh as $CD8^+$ (cytotoxic) T cells. While there is no doubt that T cells are a major source of ACh in the lymphocytic cholinergic system (72), which subpopulations of T cells have the ability to produce ACh is not yet clear in humans. To address that issue, Fujii *et al.* (80) analyzed human $CD4^+$ and $CD8^+$ T cells separated from human peripheral blood MNLs, and detected expression of ChAT mRNA in unstimulated $CD4^+$ T cells, but not in unstimulated $CD8^+$ T cells (Figure 2). Furthermore, phytohemagglutinin (PHA), a T cell activator, up-regulated expression of ChAT mRNA only in $CD4^+$ T cells. It appears, therefore, that $CD4^+$ T cells synthesize ACh using ChAT, at least in humans, and that this ACh synthesis is regulated, at least in part, by immunological stimulation.

When ACh-synthesizing activity was determined by the Fonnum method (76), both CarAT and ChAT were found to contribute to the synthesis of ACh from choline and AcCoA. With that method, therefore, it is advisable to use specific inhibitors of ChAT and CarAT (e.g., bromoACh and bromoacetylcarnitine, respectively) to ascribe the ACh-synthesizing activity to either of the two enzymes (7, 8, 81). In human T and B cell lines, Fujii *et al.* (72) detected varying levels of CarAT activity, but no correlation was observed between ACh content and CarAT activity (see Table 6). No expression of ChAT mRNA was detected in B cell lines. Using immunohistochemical analysis, however, Tayebati *et al.* (37) detected expression of ChAT in human peripheral B cells, and Rinner *et al.* (72, 77) detected ACh-synthesizing activity and some ACh in B cells. It therefore remains unclear whether, in addition to T cells, B cells also have the capacity to synthesize ACh via ChAT, and whether CarAT contributes to the synthesis of ACh in T and/or B cells.

3.3.2. ChAT mRNA molecular species expressed in lymphocytes

Expression of five distinct ChAT mRNA species (R-, N0-, N1-, N2- and M-type), which likely differ with respect to translation efficiency and stability, has been detected in human cholinergic neurons (82). MOLT-3 human leukemic T cells express only N2-type mRNA, while CEM cells express N2-, M- and, to a lesser extent, N1-type mRNA (83). PHA significantly increases expression of N2-type mRNA in MOLT-3 cells and induces low levels of N0-type mRNA expression in CEM cells (83). The PHA stimulation increases cellular ACh content and release in MOLT-3 cells, but not CEM cells (71)

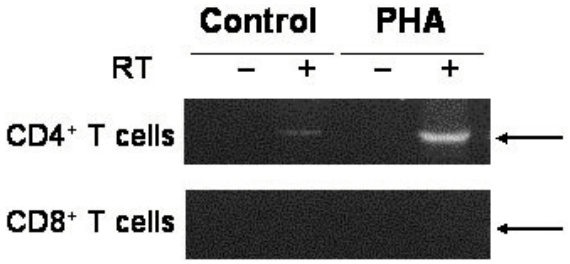

Figure 2. Expression of ChAT mRNA in human peripheral blood $CD4^+$ cells. The expected sizes of the ChAT mRNA RT-PCR product was 652 bp (arrow) (80). No expression of ChAT mRNA was observed in $CD8^+$ T cells. PHA, phytohemagglutinin, a T cell activator.

suggests that the level of N2-type mRNA is a key determinant of lymphocytic ChAT activity and ACh content (see Table 6). No R-type mRNA is expressed in either MOLT-3 or CEM cells. The relation between the inability of lymphocytes to express R-type mRNA and their low capacity to synthesize ACh, as compared to cholinergic neurons, remains to be clarified.

3.3.3. Expression of ChAT mRNA in thymic epithelial cells

The presence of ACh-synthesizing activity in thymocytes was first reported by Rinner *et al.* (77), and Mihovilovic and Butterworth-Robinette (58) recently detected expression of ChAT mRNA in the TE750 thymic epithelial cell line. Together, these findings suggest the possibility that ACh is synthesized not only by thymocytes but also by thymic epithelial cells, and that ACh released from these cells plays a role in the regulation of maturation, selection and differentiation of T cells during the interaction of immature T cells with thymic epithelial cells (see section 5.2).

3.4. VAChT

In the cholinergic neurons, the VAChT gene is located within the first intron of the ChAT gene (9, 84-87), and expression of VAChT is co-regulated with that of ChAT (10, 88-91). Using human MNLs, Fujii *et al.* (79) attempted to determine whether VAChT gene expression is induced along with ChAT when T cells are immunologically stimulated with PHA. They found that whereas PHA up-regulated expression of ChAT mRNA and potentiated ACh-synthesizing activity and ACh synthesis, there was no expression of VAChT mRNA in PHA-stimulated MNLs. On the other hand, Tayebati *et al.* (37) detected VAChT immunoreactivity in human peripheral blood T and B cells. Although no structures resembling synaptic vesicles have ever been observed histologically in lymphocytes, the possibility that ACh in T cells is localized within a storage apparatus cannot be ruled out; that nicotine causes an increase in plasma ACh and a decrease of the ACh content of blood cells in rabbits suggests release of ACh from blood cells to plasma via a depolarization-dependent pathways (64). On the other hand, it may be that ACh in T cells is synthesized when necessary and then directly released without storage. Further studies will be needed to confirm the role, if any, of VAChT and ACh storage vesicles in lymphocytes.

3.5. CHT1

It is generally accepted that choline taken up by the CHT1 is utilized exclusively for ACh synthesis, and that choline uptake by the CHT1 is the rate-limiting step in ACh synthesis catalyzed by ChAT in cholinergic neurons (8). Okuda *et al.* (6, 92) recently cloned cDNAs encoding rat and human CHT1. When Fujii *et al.* (93) investigated the expression of CHT1 in lymphocytes using human leukemic T cell lines as models, they detected expression of CHT1 mRNA in MOLT-3 cells, but not in CEM and Jurkat cells, where levels of ACh synthesis and release are lower than in MOLT-3 cells (72) (Table 6). Consistent with the expression of CHT1, specific binding of [^{3}H]hemicholinium-3 (HC-3), an inhibitor of CHT1, and HC-3-sensitive [^{3}H]choline uptake were also detected in MOLT-3 cells. Moreover, expression of CHT1 protein in MOLT-3 cells has now been confirmed immunocytochemically using a specific antibody (Fujii *et al.,* in preparation). Collectively, these results provide molecular biological, pharmacological and immunocytochemical evidence that MOLT-3 cells express CHT1, and suggest that CHT1 plays a key role in mediating uptake of choline into T cells for ACh synthesis. Still, we cannot rule out the possibility that choline from other sources is also used for ACh synthesis in T cells.

3.6. AChE

The mechanisms involved in the termination of ACh action are quite different from those of other neurotransmitters. Whereas, catecholamines and serotonin are removed from their target sites mainly by a reuptake mechanism via respective transporters, the action of ACh is terminated exclusively by rapid hydrolysis into choline and acetate by AChE at neuromuscular and neuroeffector junctions, and by ChE in plasma, liver and neuronal elements. It is now generally accepted that AChE is expressed not only in cholinergic neurons, but also in other neurons and in non-neural tissues, irrespective of direct cholinergic innervations (e.g., Bellinger *et al.* (94); Nance *et al.* (95)). While AChE is not an essential constituent of cholinergic system, its expression certainly indicates the presence of ACh nearby the cells or tissues. Szelenyi *et al.* (96) detected AChE activity in human peripheral blood T cells, where the activity was augmented by PHA, but not in B cells. However, Ando *et al.* (97) detected expression of mRNAs encoding three different types of AChE (98) in human MNLs and in the CEM human leukemic T cell and Daudi B cell lines (Figure 3). In addition, Tayebati *et al.* (37) found expression of AChE in both T and B cells using Western blot and immunocytochemical techniques. It thus appears that both T and B cell express AChE, but that the level of expression is higher in T cells.

4. ROLES OF AChRs IN THE REGULATION OF LYMPHOCYTE FUNCTION

The lymphocytic cholinergic system operates through ACh released from T cells acting as an autocrine and/or paracrine factor on either mAChRs or nAChRs on

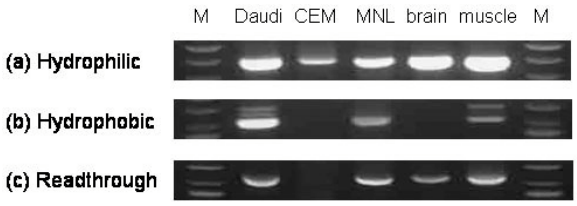

Figure 3. Expression of AChE mRNA in the Daudi human leukemic B cell line, the CEM T cell line, human blood MNLs, human brain and skeletal muscle (97). Expression of (a) hydrophilic, (b) hydrophobic and (c) readthrough types of AChE mRNA (98) was detected by RT-PCR analysis.

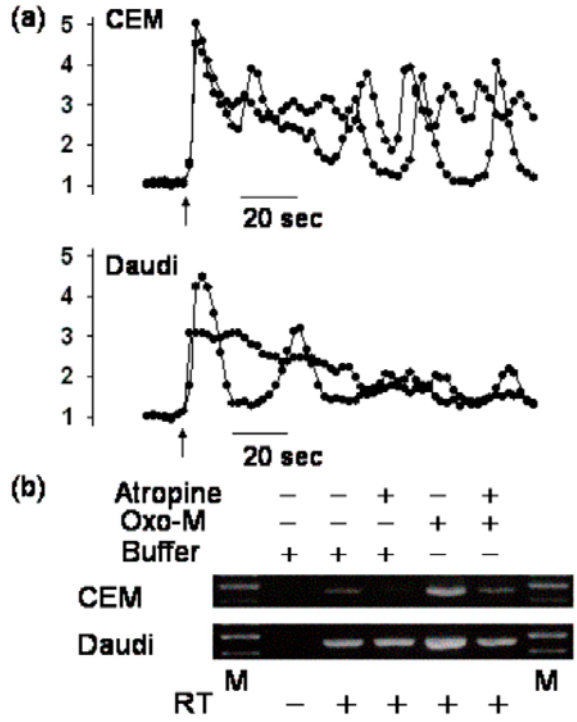

Figure 4. Ca^{2+} concentration ($[Ca^{2+}]_i$)-dependent changes in fluo-3 fluorescence and up-regulation of *c-fos* gene expression by mAChR stimulation with Oxo-M in the CEM human leukemic T cell and Daudi B cell lines (99, 100). (a) Time course of the $[Ca^{2+}]_i$-dependent changes in fluo-3 fluorescence in CEM (upper panel) and Daudi cells (lower panel). Data points were obtained every 2 s. Application of Oxo-M (100 *micro*M) is indicated by the arrow. (b) Effects of atropine, a nonspecific mAChR antagonist, on Oxo-M-induced up-regulation of *c-fos* gene expression in CEM and Daudi cells. The cells were exposed to 100 *micro*M Oxo-M for 1 h in the presence or absence of atropine. Expression of *c-fos* mRNA was analyzed by RT-PCR. M, DNA size standards (expressed in bp); RT, reverse transcriptase.

target cells – e.g., T and B cells and antigen presenting cells, such as macrophages and dendritic cells. Stimulation of mAChRs and/or nAChRs on lymphocytes by ACh or other agonist causes such biochemical and functional changes as enhancement of lymphocyte cytotoxicity, increased cGMP and inositol-1,4,5-triphosphate (IP_3) content, inhibition of cAMP synthesis, and increased $[Ca^{2+}]_i$ (see Table 1). Most of these responses support the idea that the lymphocytic cholinergic system is involved in the regulation of lymphocyte function, acting via mAChRs coupled to PLC or adenylyl cyclase and nAChRs forming ligand-gated ion channels.

Fujii and Kawashima (11, 99-101), Kaneda *et al.* (38) and Kimura *et al.* (102, 103) have all studied the mechanisms involved in mAChR- and nAChR-mediated functional and metabolic changes in lymphocytes in relation to evoked changes in $[Ca^{2+}]_i$.

4.1. mAChRs

4.1.1. Effects of mAChR agonists on $[Ca^{2+}]_i$ and *c-fos* gene expression in lymphocytes

Both ACh and oxotremorine-M (Oxo-M), a non-selective mAChR agonist, induce rapid increases of $[Ca^{2+}]_i$ that are followed by more prolonged Ca^{2+} oscillations in both CEM T cells and Daudi B cells (99, 100) (Figure 4a). In the absence of external Ca^{2+}, no changes were observed in the number of cells exhibiting the initial rise in $[Ca^{2+}]_i$ in response to Oxo-M, but the subsequent Ca^{2+} oscillations were almost completely blocked. Depletion of the Oxo-M-releasable intracellular Ca^{2+} stores using thapsigargin blocked all changes in $[Ca^{2+}]_i$, suggesting that mAChR-mediated increases of $[Ca^{2+}]_i$ and the subsequent Ca^{2+} oscillations are transduced in lymphocytes via IP_3-mediated signaling pathways.

RT-PCR analysis showed that Oxo-M also up-regulates the intranuclear transcription regulator *c-fos* gene expression in both CEM and Daudi cells (Figure 4b). Both the Oxo-M-induced up-regulation of *c-fos* gene expression and the intracellular Ca^{2+} signaling were reversed by 4-DAMP, an M_1, M_3, M_4 and M_5 mAChR antagonist; YM905, an M_1 and M_3 mAChR antagonist; and atropine, a non-selective mAChR antagonist (99-101). On the other hand, neither pirenzepine, an M_1 mAChR antagonist, nor AF-DX 116, an M_2 and M_4 mAChR antagonist, had any effect. Taken together, these findings suggest that ACh and mAChR agonists induce intracellular Ca^{2+} signaling in lymphocytes via M_3 and/or M_5 mAChRs, leading to IP_3-mediated up-regulation of *c-fos* expression, and that activation of DNA and RNA synthesis, as well as the cell proliferation observed after mAChR stimulation in lymphocytes (see Table 1), are in some way related to that up-regulation of *c-fos* expression.

4.1.2. Stimulation of lymphocytic nitric oxide (NO) synthesis by mAChR agonists

NO synthase (NOS) catalyzes the synthesis of NO from L-arginine. Human lymphocytes and leukemic cell lines express the endothelial, neuronal and inducible (ecNOS, nNOS and iNOS, respectively) forms of NOS mRNA and protein, and exhibit NOS activity (for review

see Kawashima and Fujii (12)). Using quantitative fluorescence microscopy and a novel NO-sensitive fluorescent indicator, DAF-2 (104), Kamimura *et al.* (105) demonstrated that Oxo-M enhances NO production in CEM cells expressing mRNAs encoding iNOS and nNOS and the M_1, M_3, M_4 and M_5 mAChRs (Table 3). The effect of Oxo-M on NO production was inhibited by N^G-nitro-L-arginine methyl ester, a NOS inhibitor, and by the mAChR antagonists 4-DAMP, pirenzepine and atropine, which suggests that ACh stimulates NO synthesis by nNOS and/or iNOS in lymphocytes via PLC-coupled M_1, M_3 and/or M_5 mAChR-mediated pathways. This means that the enhanced cytotoxicity and increased cGMP synthesis observed in lymphocytes after mAChR stimulation (see Table 1) are likely related, at least in part, to increased NO production.

4.1.3. Enhancement of interleukin (IL)-2-mediated signal transduction

Fujino *et al.* (36) found PHA to enhance IL-2 production and to up-regulate expression of IL-2 receptor mRNA in human lymphocytes following stimulation with Oxo-M. This suggests that IL-2-mediated signal transduction in lymphocytes is enhanced by mAChR stimulation during immunological reactions. On the basis of that idea, Okuma and Nomura (106) and Nomura *et al.* (107) speculated that ACh acts as an autocrine factor via mAChRs to positively modulate IL-2 signaling and cell growth in human lymphocytes.

Most available data on the effects of mAChR stimulation in lymphocytes and its targets have been derived from acute *in vitro* studies (see reviews by Kawashima and Fujii (12-14); Fujii and Kawashima (11); Maslinski (108)). However, observations of the *in vivo* effects of long-term stimulation or inhibition of mAChRs on lymphocytes will be needed if we are to relate lymphocytic cholinergic activity to the regulation of immune function.

4.2. nAChRs

With a special emphasis on the effects of smoking on immune function, the roles of nAChRs in the regulation of lymphocyte activity has been investigated both *in vitro* and *in vivo* (12, 13).

4.2.1. Effects of nAChR agonists on $[Ca^{2+}]_i$ and suppression of nAChR subunit expression by nicotine

Acute nicotine stimulation in human circulating MNLs and human leukemic T and B cell lines elicits rapid and transient increases of $[Ca^{2+}]_i$ that are dependent on the presence of extracellular Ca^{2+} (102, 103, 109). Kimura *et al.* (102) observed that $[Ca^{2+}]_i$ transients elicited by either nicotine or epibatidine, an *alpha*3 *alpha*4, and *alpha*7 subunit agonist, in CEM cells expressing mRNAs for the *alpha*3, *alpha*5, *alpha*6 and *alpha*7 nAChR subunits were effectively suppressed by *alpha*-BTX, which indicates that the *alpha*7 nAChR is at least partly responsible for nicotine-induced Ca^{2+} signaling in lymphocytes. The physiological significance of such Ca^{2+} signaling in lymphocytes is not known, however.

Middlebrook *et al.* (110) suggested that nAChRs were involved in the regulation of thymocyte development based on their observation that continuously exposing isolated fetal murine thymus to nicotine increased the numbers of immature T cells while diminishing numbers of mature T cells. Using RT-PCR techniques, Kimura *et al.* (103) found that long-term (1-8 weeks) exposure to nicotine (0.01-10 *micro*M) in CEM cells down-regulates expression of *alpha*7 and other nAChR *alpha* subunits. Moreover, Ca^{2+} signaling evoked by nicotine declined significantly as the duration of nicotine exposure became more prolonged. These findings suggest that nicotine, and most likely smoking, affects immune function by suppressing expression of nAChR subunits involved in Ca^{2+} signaling in lymphocytes.

4.2.2. Effects of systemic nicotine administration on lymphocyte function

Singh *et al.* (111) suggested that T cell proliferation is regulated via nAChR-mediated pathways based on their observations that acute or chronic administration of nicotine inhibited concanavalin A-induced MNL proliferation in rats, and that the effect was reversed by mecamylamine, a nAChR antagonist. Most other data obtained *in vivo* also suggest that continuously exposing T cells to nicotine suppresses immune function by inhibiting proliferative responses (111, 112), or by causing anergy through constitutive activation of protein kinases and depletion of IP_3-sensitive Ca^{2+} stores (113-115). However, these investigators also found that acute nicotine administration increased plasma cortisone levels; that the effect on plasma cortisone was inhibited by mecamylamine; and that the acute effects of nicotine on T cell proliferative responses were abolished in adrenalectomized animals. These findings highlight the importance of bearing in mind the hormonal effects of acutely administered nicotine. Still, they clearly support the idea that ACh and nicotine, perhaps incorporated through smoking, modulate immune function by affecting lymphocyte proliferation.

5. REGULATION OF LYMPHOCYTIC CHOLINERGIC ACTIVITY

Through various cell surface molecules, including cell adhesion molecules (CAMs), lymphocytes can interact directly with targets expressing the respective counter molecules. So far, several cell surface molecules have been shown to be involved in regulating lymphocytic cholinergic activity (13, 14, 80, 116, 117). For example, stimulating T or B cells with their respective activators via cell surface molecules – e.g., T cell receptor (TCR)/CD3 complexes, CD2, CD7 and CD11a on T cells and surface immunoglobulin on B cells – up-regulates ChAT and M_5 mAChR gene expression (71, 73, 79, 80, 116, 117). And activation of G_s protein-coupled EP_4 receptor with prostaglandin E_2 (PGE_2) enhances ACh synthesis (118), while apelin acts via the G_i protein-coupled orphan receptor APJ to suppress ACh synthesis in MOLT-3 cells (119). It thus appears that both immunological and inflammatory processes, and a variety of biologically active substances, are involved in regulating lymphocytic cholinergic activity. The effects

of cytokines and chemokines on lymphocytic cholinergic activity remain to be investigated.

5.1. Cell surface molecules

5.1.1. TCR/CD3 complexes on T cells

PHA activates T cells by binding to TCR/CD3 complexes, which leads to activation of the PLC-IP_3 system (120) and, in turn, activation of protein kinase C (PKC) and/or mitogen-activated protein kinase (MAPK) cascades. Fujii *et al.* (71, 79) and Rinner *et al.* (73) demonstrated that PHA up-regulates ChAT mRNA expression and stimulates ChAT activity, thereby enhancing ACh synthesis and release in the MOLT-3 human T cell line, human MNLs and rat lymphocytes. In analogous fashion, activation of human MNLs using phorbol 12-myristate 13-acetate (PMA), a PKC activator, or A23187 or ionomycin, Ca^{2+} ionophores, up-regulates expression of ChAT mRNA. Notably, the effect is inhibited by FK506, an immunosuppressant calcineurin inhibitor (Watanabe *et al.,* unpublished data), suggesting the involvement of calcineurin-mediated pathways in the regulation of ChAT mRNA transcription in MNLs. In addition, Fujii *et al.* (80) found that PHA specifically up-regulates expression of M_5 mAChR mRNA in T cell lines without affecting M_3 or M_4 mAChR mRNA expression, though non-specific stimulation of lymphocytes using PMA plus ionomycin up-regulated expression of both M_3 and M_5 mAChR mRNAs. Collectively, these findings represent the first demonstration that immunological stimulation leads to activation of lymphocytic cholinergic activity via specific up-regulation of M_5 mAChR and ChAT gene expression, at least in part via PKC and MAPK pathways, perhaps by modulating the activity of transcription factor AP-1.

5.1.2. CD2, CD7 and CD11a on T cells

Antithymocyte globulin-Fresenius (ATG-F), which is comprised of rabbit globulin against human thymocytes, is used as an immunosuppressive after renal allograft (121) and after hematopoietic stem cell transplantation (122) carried out in the treatment of aplastic anemia (123, 124). ATG-F acts via the CD2, CD7 and CD11a cell surface molecules (122), also known as lymphocyte function-associated antigen 2 (LFA-2), an immunoglobulin superfamily member and LFA-1 *alpha*-chain, respectively. Stimulation of these molecules with respective mAbs activates intracellular signaling pathways leading to increases in $[Ca^{2+}]_i$ and modulation of lymphocyte function (125, 126).

Fujii *et al.* (116) found that incubating CEM cells with ATG-F enhances ACh release from the cells within 6 h, which initially led to declines in intracellular ACh content. However, the ACh content of the cells recovered within 48 h as a result of up-regulation of ChAT expression. ATG-F also induced rapid, transient increases in $[Ca^{2+}]_i$, and similar effects were elicited by anti-CD7 mAb, which is consistent with observations made by Ledbetter *et al.* (125) and Leta *et al.* (126) in other cell types and suggests that the increase in ACh release observed during the early stages of the experiments was due to increases in $[Ca^{2+}]_i$ induced by ATG-F via its interaction with CD7. On the other hand, incubation for 48 h with anti-CD11a mAb up-regulated the expression of ChAT mRNA in CEM cells, suggesting ATG-F stimulates ACh synthesis via its interaction with CD11a. ATG-F also up-regulated expression of M_5 mAChR mRNA (116). It thus appears that in T cells surface molecules involved in cell-to-cell adhesion and/or aggregation are involved in regulating lymphocytic cholinergic activity by enhancing synthesis and release of ACh and expression of M_5 mAChR.

5.1.3. Surface immunoglobulin on B cells

Staphylococcus aureus Cowan I (SAC), a B cell activator, initiates a signal transduction cascade involving tyrosine kinase-mediated activation of PLC, which in turn activates PKC and promotes B cell proliferation by binding to surface immunoglobulin. SAC up-regulates M_5 mAChR mRNA expression in Daudi human leukemic B cells, but has no effect on M_3 or M_4 mAChR mRNA expression (117). PMA together with ionomycin also up-regulates M_5 mAChR expression in Daudi cells, suggesting that, as in T cells, PKC is involved in stimulus-evoked up-regulation of M_5 mAChR expression in B cells.

Fujii *et al.* (117) tested whether B cells are involved in the regulation of lymphocytic cholinergic activity using a human circulating MNL fraction consisting mainly of T and B cells and a small number of monocytes. They found that incubating MNLs with SAC for 48 h significantly increased their ACh content and up-regulated ChAT expression. Given that IL-12, a cytokine produced by B cells, is thought to provide help for T cells, these data collectively suggest that cytokines released from activated B cells act on T cells to stimulate ChAT expression and ACh synthesis, which in turn activates lymphocytic cholinergic transmission via M_5 mAChR-mediated pathways in both T and B cells. Since lymphocytic cholinergic stimulation accelerates antibody production by B cells (108), induction of M_5 mAChR expression by immunological stimulation of B cells may lead to enhanced antibody production.

5.2. Biologically active substances

So far, among the vast number of biologically active substances known, only PGE_2 and apelin have been investigated with respect to their capacity to affect lymphocytic cholinergic activity.

5.2.1. PGE_2

Within the immune system, PGE_2 is synthesized mainly by antigen presenting cells, such as macrophages and monocytes (127-129). PGE_2 inhibits T cell proliferation induced by activators, such as PHA, concanavalin A and antibodies against CD3 (130-132), suggesting it contributes to the regulation of immune function by modulating the activities of T cells.

Suenaga *et al.* (118) tested whether ONO-4819, a selective EP_4 receptor agonist, has any ability to modulate lymphocytic cholinergic activity in MOLT-3 cells, which express all four EP receptors (EP_1 - EP_4). They found that PHA significantly enhanced expression of EP_4 receptor mRNA during the first 3-6 h of exposure. ONO-4819 added

to cultures after 3 h of PHA stimulation significantly increased cellular ACh content and release, and up-regulated expression of ChAT mRNA and ChAT activity, but inhibited MOLT-3 cell proliferation. This suggests that PGE_2 released from antigen presenting cells during their interaction with T cells facilitates lymphocytic cholinergic transmission, at least in part through EP_4 receptor-mediated pathways. At present, however, it remains unclear whether the inhibitory effect of PGE_2 on T cell proliferation is mediated directly or indirectly through the enhanced lymphocytic cholinergic transmission.

5.2.2. Apelin

The orphan receptor APJ is coupled to G_i protein, which inhibits adenylyl cyclase activity (133). Its endogenous ligand is apelin, whose cDNA has been isolated from human, bovine, rat and mouse (133-135). *In vivo*, apelin is synthesized as a 77-amino acid preprotein and then cleaved to yield the mature protein comprised of the 36 C-terminal amino acids (apelin-36) (134). Apelin mRNA has been identified in a number of rat tissues, in particular mammary gland during pregnancy and lactation (134). Large amounts of apelin are secreted in bovine colostrum, and it even remains detectable in commercial bovine milk. In mouse spleen cells, apelin partially suppresses cytokine production induced via TCR/CD3-mediated pathways, suggesting apelin modulates immune function in neonates (134).

Horiuchi *et al.* (119) investigated whether apelin is involved in the regulation of lymphocytic cholinergic activity using various human leukemic T and B cell lines as models. APJ mRNA was constitutively expressed in T and B cell lines, among which MOLT-3 and Daudi cells respectively expressed the highest levels of APJ mRNA. Apelin-13, a 13-amino acid synthetic C-terminal peptide that is more active than native apelin-36 (133), up-regulated expression of APJ mRNA in PHA-stimulated MOLT-3 cells, whereas it down-regulated expression of ChAT and interleukin-2 mRNAs (Figure 5). This suggests that apelin down-regulates lymphocytic cholinergic activity during immunological responses by inhibiting cAMP production via activation of the G_i-coupled orphan receptor APJ, which is consistent with the finding that cAMP up-regulates ChAT induction (136).

6. POSSIBLE PHYSIOLOGICAL FUNCTIONS OF THE LYMPHOCYTIC CHOLINERGIC SYSTEM

6.1. Antigen presentation

T cells interact with antigen presenting cells expressing major histocompatibility complex (MHC) class II or MHC class I via the TCR/CD3 complex plus CD4 or CD8, respectively. On the basis of the findings discussed above, it seems likely that antigen presentation activates lymphocytic cholinergic activity by enhancing synthesis and release of ACh and expression of M_5 mAChR. To test this idea, Watanabe *et al.* (137) first isolated splenic MNLs, consisting of mainly T and B cells and a small number of monocytes (macrophages and dendritic cells) from mice one week after immunizing them with incomplete or complete Freund's adjuvant containing dead *Mycobacterium tuberculosis* (IFA and CFA, respectively). The cells were then cultured for 48 h with or without *Mycobacterium tuberculosis* purified protein derivatives (MTPPD). The cellular ACh content and the release of ACh into the conditioned media were significantly greater in MNLs from mice immunized with CFA and cultured in the presence of MTPPD than in the cells cultured in the absence of MTPPD, or in MNLs from mice immunized with IFA and cultured in the presence or absence of MTPPD. These findings support the notion that the interaction of $CD4^+$ or $CD8^+$ T cells via TCR/CD3 with macrophages or dendritic cells respectively presenting MTPPD antigen on their MHC class II or MHC class I up-regulates lymphocytic cholinergic activity by enhancing synthesis and release of ACh by the T cells (Figure 6). Furthermore, because of the close proximity of T cells and antigen presenting cells under these conditions, even a small amount of ACh released from an activated T cell into the microenvironment should be able to interact with AChRs on the target cells before hydrolysis by AChE.

6.2. Interaction of T cells with thymic stromal cells

As mentioned above, the thymus is comprised of thymocytes (lymphoid cells) and myoid and epithelial cells (non-lymphoid cells). Interactions between developing thymocytes and thymic epithelial cells are necessary for maturation of thymocytes into mature T cells, and these interactions are all mediated by CAMs on immature T cells that bind to various molecules expressed on the surface of thymic epithelial cells. Patel *et al.* (138, 139) demonstrated that thymic epithelial cells express various CAMs, cytokine receptors, Apo-1, and MHC-encoded molecules. Activation of thymic epithelial cells by IFN-*gamma* induces marked increases of the expression of surface molecules such as CD54 (intercellular adhesion molecule-1 (ICAM-1)), which interacts with LFA-1, and MHC class I and MHC class II, which interact with CD8 and CD4, respectively. Since both thymic epithelial cells (58) and thymocytes (73) not only synthesize ACh but also express various nAChR subunits (see sections 3.1.2 and 3.1.3), it seems likely that ACh released from thymic epithelial cells and/or thymocytes plays a role in regulating differentiation, maturation and selection of T cells, acting in autocrine and/or paracrine fashion via nAChRs (58, 140) (Figure 7). While observations on the effects of nicotine on T cell maturation support this hypothesis (110), it is yet to be confirmed.

Several lines of evidence support the idea of postsynaptic sympathetic innervations of the thymus (95, 141-145). On the other hand, it is controversial whether there is direct cholinergic innervations of thymic epithelial cells or thymocytes. On the basis of histochemical staining and immunocytochemical labeling with anti-ChAT mAb, Fatani *et al.* (146) reported that there is cholinergic innervations along the blood vessels within the thymic traveculae and the parenchyma, while others have also detected AChE-positive fibers and cells (144, 147-149). Nevertheless, microscopic dissection of the terminal branches of the vagus nerve by Nance *et al.* (95) failed to identify any major branches leading to the thymus, making direct vagal innervations of the thymus unlikely. Moreover, unilateral vagotomy did not alter ChE activity in the thymus.

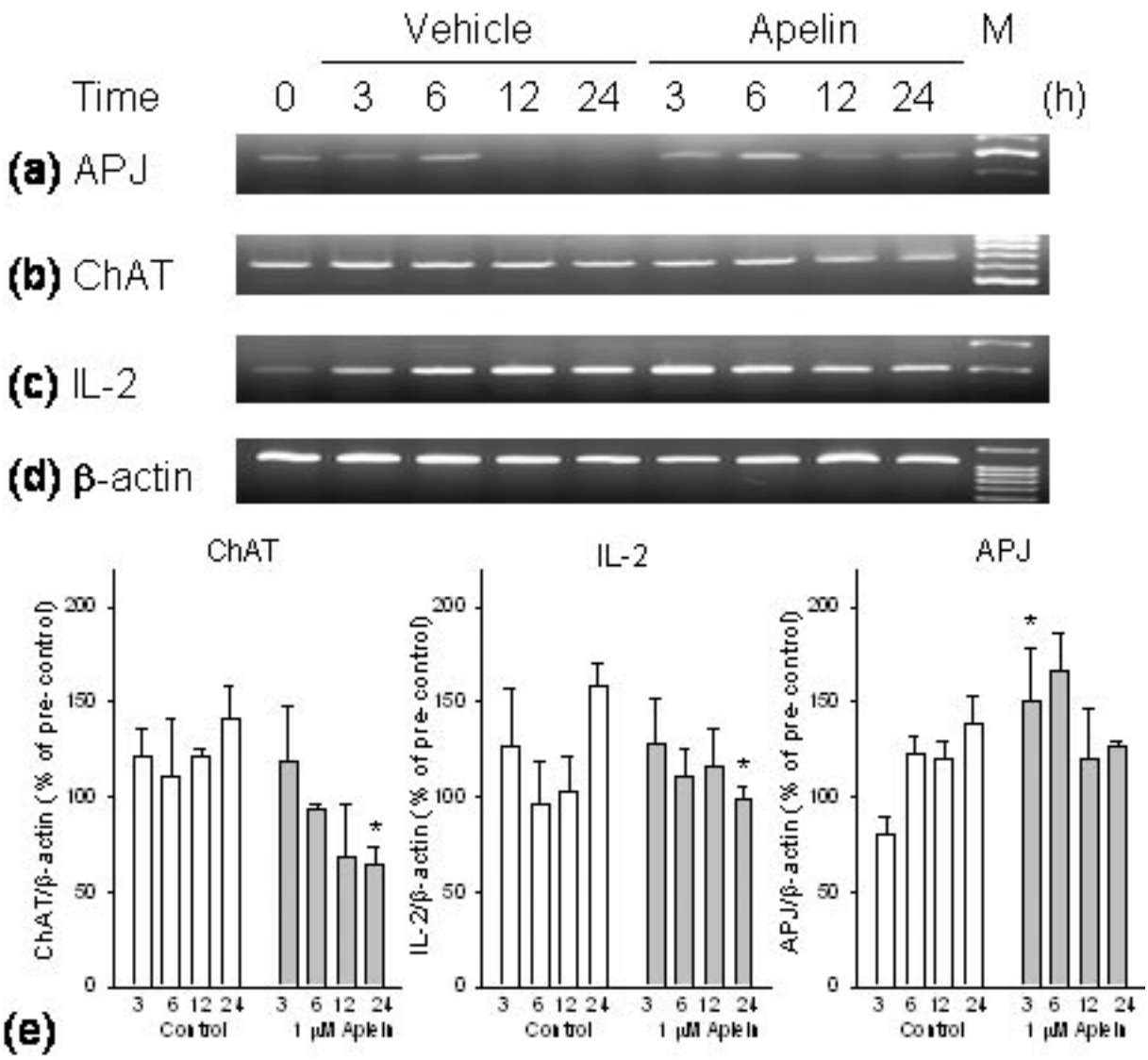

Figure 5. Effects of apelin-13 on APJ, ChAT, IL-2 and *beta*-actin mRNA expression in MOLT-3 cells (119). Cells were exposed to apelin-13 (1 *micro*M) or vehicle (control) for the indicated times. Data representing three independent experiments are shown. The expected sizes of the APJ (a), ChAT (b), IL-2 (c) and *beta*-actin (d) RT-PCR products were 481, 652, 305 and 1128 bp, respectively. (e) The bars represent means +/- SEM (n=3) of ChAT (or IL-2, APJ)/*beta*-actin ratios normalized to pre-treatment values; **P*<0.05 vs. control (3 h of vehicle).

At present, no conclusive evidence for or against direct cholinergic innervations of thymic epithelial cells and thymocytes is available. However, considering the extreme susceptibility of ACh to hydrolysis by AChE and ChE, as well as its physicochemical instability in physiological fluids (see reviews by Kawashima and Fujii (12, 13)), it seems highly unlikely that ACh released from cholinergic nerve endings would interact with receptors expressed on thymocytes.

Electrical stimulation of the vagal fibers running in the recurrent laryngeal nerve has a tonic and phasic facilitatory effect on the mechanism responsible for lymphocytic release from the thymus, which is modulated via nAChRs (150). Since thymus tissue expresses both muscle type (59, 60) and neuron type nAChR subunits (51, 55), it may be that ACh released from vagal fibers directly innervating myoid cells induces contraction of the thymus by acting on muscle type nAChRs, thereby facilitating release of lymphocytes.

Autonomic innervations of the spleen appear to be relatively sparse. Nevertheless, direct contacts between noradrenergic sympathetic fibers and lymphocytes, as well as with other cells of the immune system, have been reported in the rat spleen (151). Cholinergic innervations of the rat spleen is less likely, as AChE-positive staining colocalized with norepinephrine in noradrenergic nerves and persisted even after vagal denervation (94).

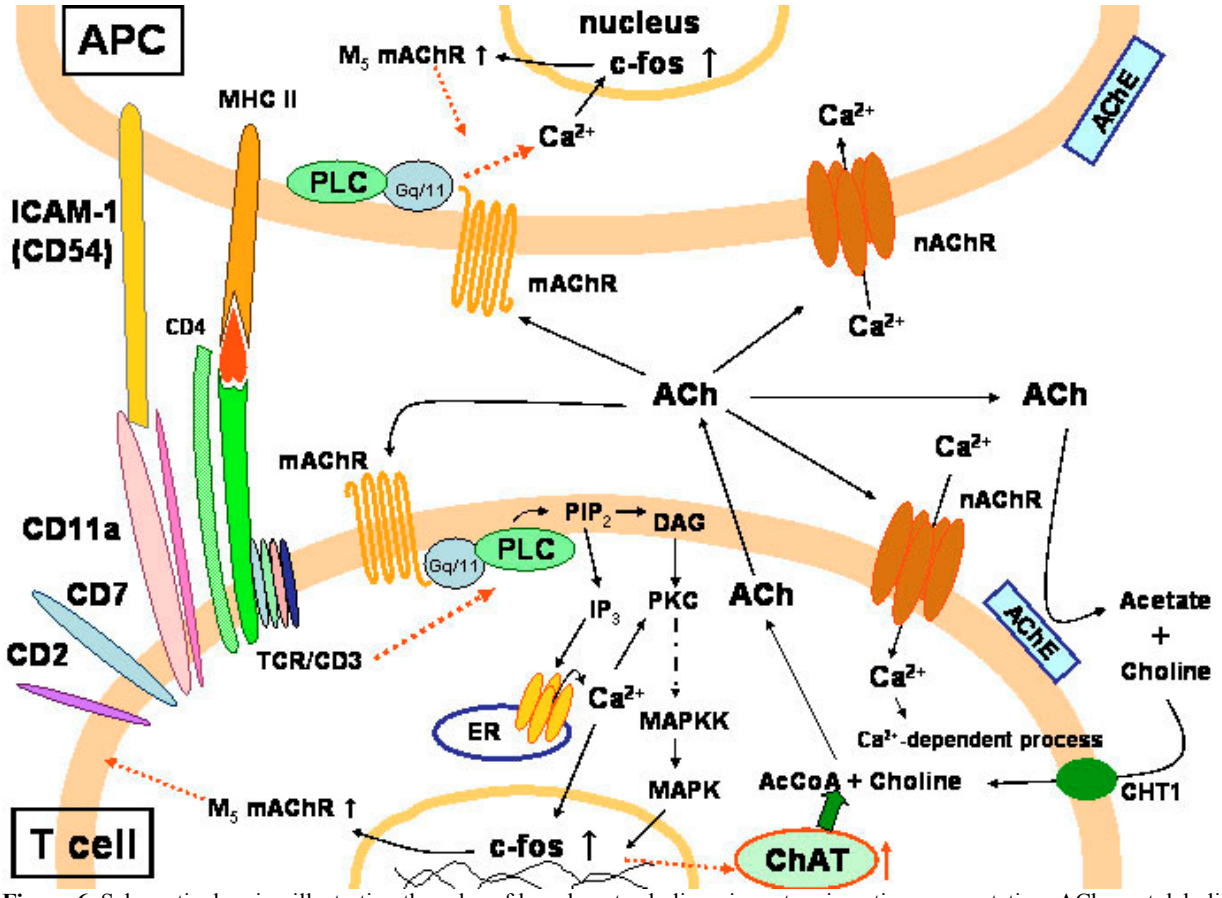

Figure 6. Schematic drawing illustrating the roles of lymphocyte cholinergic system in antigen presentation. ACh, acetylcholine; AChE, acetylcholinesterase; AcCoA, acetyl coenzyme A; APC, antigen presenting cell; ChAT, choline acetyltransferase; CHT1, high-affinity choline transporter; DAG, diacylglycerol; ER, endoplasmic reticulum; ICAM-1, intercellular adhesion molecule-1; IP_3, inositol-1,4,5-trisphosphate; LFA-1, leukocyte function-associated antigen-1; mAChR, muscarinic ACh receptor; MAPK, mitogen activated protein kinase; MAPKK, MAP kinase kinase; MHC II, major histocompatibility complex class II; TCR, T cell receptor.

6.3. Interaction of T cells with vascular endothelial cells

T cells express various CAMs through which they interact with vascular endothelial cells; these include very late antigen-4 (VLA-4), a major adhesion receptor that interacts with vascular CAM-1 (VCAM-1); CD4, which interacts with MHC class II; LFA-1, which interacts with ICAM-1; and CD2, which interacts with LFA-3 (152, 153). Kokura *et al.* (153) found that interactions between T cells and postanoxic endothelial cells via VLA-4-/VCAM-1 potentiated the synthesis of tumor necrosis factor-*alpha* (TNF-*alpha*), leading to enhanced endothelial CAM expression.

Both T cells and vascular endothelial cells have the ability to synthesize ACh (see sections 3.2 and 3.3) (23-26) and to express mAChRs (see Table 3; Kan *et al.* (154) and Elhusseiny *et al.* (155)); expression of nAChRs has been confirmed in T cells (see Table 4), and is suggested in vascular endothelial cells (156). This suggests that CAM-mediated interactions between T cells and endothelial cells facilitate ACh synthesis and release in both cell types (Figure 8). During CAM-mediated interactions, T cells and vascular endothelial cells may therefore communicate reciprocally via ACh, which stimulates mAChRs on both T cells and vascular endothelial cells, and possibly nAChRs on T cells. The fact that mAChR stimulation potentiates NO synthesis in both T cells and vascular endothelial cells (105, 157) suggests the possibility that potentiation of NO synthesis during the interaction evokes local vascular smooth muscle relaxation, thereby facilitating extravascular migration of T cells. We further suggest that ACh released from T cells, and possibly from vascular endothelial cells, plays a role in regulating production of TNF-*alpha*, which in turn acts on nAChRs in T cells.

Synthesis of TNF in inflammatory cells (e.g., macrophages) appears to be regulated, at least in part, via nAChR-mediated pathways (158, 159). TNF concentrations in the conditioned medium of lipopolysaccharide (LPS)-stimulated macrophages were suppressed in the presence of either ACh or nicotine, and that suppression was reversed by addition of *alpha*-conotoxin, an inhibitor of *alpha*-BTX-sensitive nAChRs. On the basis of their observation that electrical stimulation of the efferent vagus nerve suppressed serum TNF concentrations, which protected rats

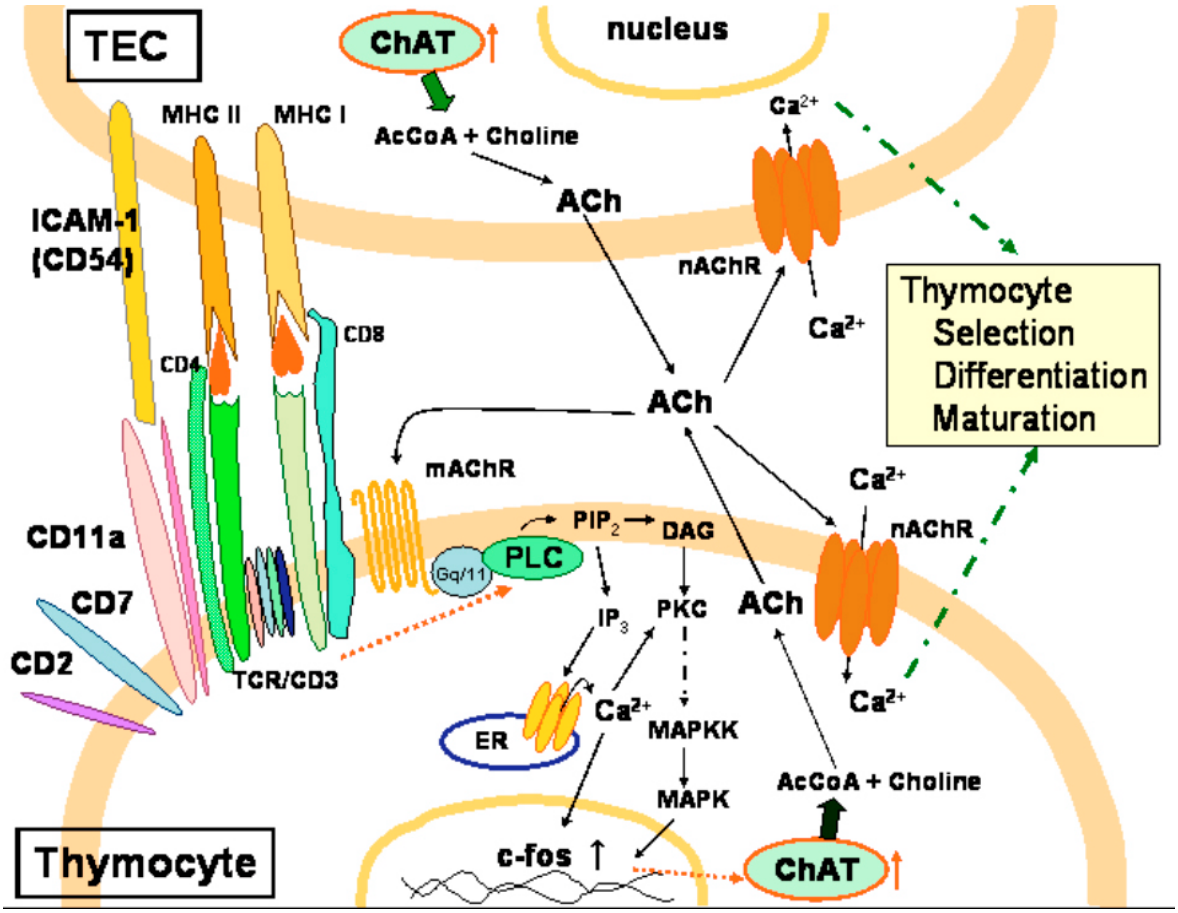

Figure 7. Schematic drawing illustrating roles of the lymphocyte cholinergic system in thymocyte selection, differentiation and maturation. ACh, acetylcholine; AcCoA, acetyl coenzyme A; ChAT, choline acetyltransferase; ER, endoplasmic reticulum; ICAM-1, intercellular adhesion molecule-1; IP_3, inositol-1,4,5-trisphosphate; mAChR, muscarinic ACh receptor; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MHC, major histocompatibility complex; nAChR, nicotinic ACh receptor; TEC, thymic epithelial cells.

from endotoxin-induced shock, Borovikova *et al.* (158) and Tracey (159) postulated the existence of cholinergic anti-inflammatory pathways. Since activation of TCR/CD3, CD2, CD7 or CD11a up-regulates lymphocytic cholinergic activity (see section 5.1) (71, 73, 79, 116), the interaction of T cells and vascular endothelial cells via VLA-4-/VCAM-1 should also either facilitate or suppress ACh synthesis in the T cells. This suggests that ACh released from T cells, and possibly from vascular endothelial cells, plays a role in regulating TNF-*alpha* synthesis by acting on T cell nAChRs. Consistent with that idea, Kokura *et al.* (153) reported TNF-*alpha* synthesis to be enhanced by the interaction of T cells with vascular endothelial cells. At present, however, there is little direct information as to whether ACh released from T cells during their interaction with vascular endothelial cells facilitates or suppresses TNF-*alpha* synthesis. But, again, given that there is no cholinergic innervation of the vascular endothelial cells and given ACh's lability in physiological solution and susceptibility to hydrolysis by AChE, it seems highly unlikely that ACh originating from cholinergic nerves acts directly on either T cells or vascular endothelial cells.

7. LYMPHOCYTIC CHOLINERGIC ACTIVITY IN ANIMAL MODELS WITH IMMUNE ABNORMALITIES

Using the spontaneously hypertensive rat (SHR), an immune deficiency model (for review see Takeichi (160)), and the MRL/MpJ-lpr/lpr (MRL-lpr) mouse, an immune accelerated model (161, 162), Fujimoto *et al.* (163, 164) obtained evidence to suggest that changes in lymphocytic cholinergic activity are related to immune dysfunction.

7.1. SHR

The SHR is a well-established hypertension model derived from the Wistar Kyoto rat (WKY) (165) and is also known to exhibit immune deficiencies resulting from the emergence of a natural thymocytotoxic autoantibody, an age-related decline of T cell function to various stimuli, and morphological changes in the thymus and spleen (160). Fujimoto *et al.* (163) discovered that the

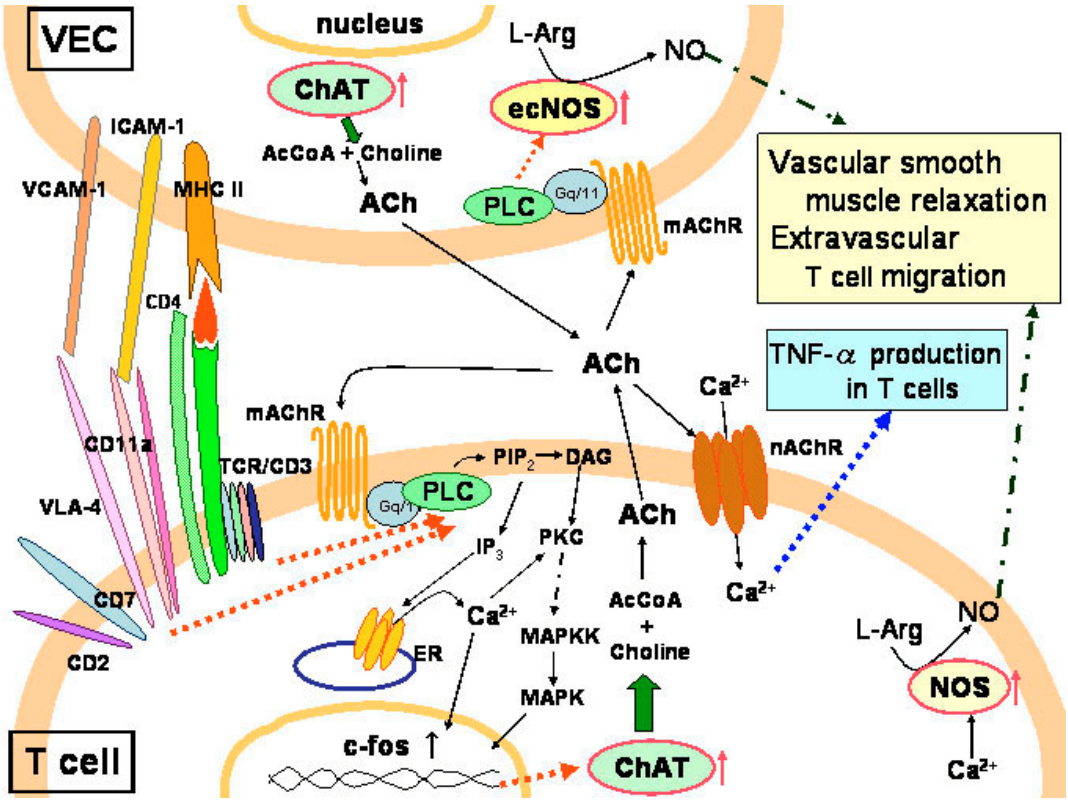

Figure 8. Schematic drawing illustrating the roles of the lymphocyte cholinergic system in vascular smooth muscle relaxation. ACh, acetylcholine; AcCoA, acetyl coenzyme A; L-Arg, L-arginine; ChAT, choline acetyltransferase; ER, endoplasmic reticulum; ICAM-1, intercellular adhesion molecule-1; IP_3, inositol-1,4,5-trisphosphate; mAChR, muscarinic ACh receptor; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MHC II, major histocompatibility complex class II; NO, nitric oxide; ecNOS, endothelial NO synthase; PLC, phospholipase C; PKC, protein kinase C; TCR, T cell receptor; TNF-*alpha*, tissue necrosis factor-*alpha*; VCAM-1, vascular CAM-1; VEC, vascular endothelial cells; VLA-4, very late antigen-4.

ACh contents of the blood, MNLs, thymus and spleen in 5- to 20-week-old SHRs are all significantly lower than in age-matched WKYs, as is expression of ChAT mRNA in circulating MNLs, making it likely that the suppressed lymphocytic cholinergic activity reflects an immune deficiency related to T cell dysfunction.

7.2. MRL-lpr mice

The MRL-lpr mouse spontaneously develops a lupus-like autoimmune syndrome, the symptoms of which include nephritis due to production of antinuclear antibodies associated with massive lymphadenopathy related to expansion of a unique T cell subset expressing Thy-1, CD3 and B220 (161, 162). Fujimoto *et al.* (164) found that the ACh contents of the blood, thymus and spleen of 20-week-old MRL-lpr mice were significantly greater than in age-matched MPL/MpJ-+/+ (wild type) and BALB/c (control) mice, although no changes in lymphocytic cholinergic activity were detected at 5 and 10 weeks, when an autoimmune syndrome was yet not evident.

8. CONCLUSIONS AND PERSPECTIVES

It is now evident that lymphocytes express the components needed to constitute an independent, non-neuronal cholinergic system, and that lymphocytic cholinergic activity is regulated, at least in part, by immunological responses and is therefore a reflection of immune function. Still, the precise function and physiological significance of the lymphocytic cholinergic system remain unknown. Particularly important in the future will be *in vivo* studies of the roles of lymphocytic cholinergic system in the regulation of cytokine, chemokine and antibody production. The results of those studies should provide us with important clues about immune regulatory mechanisms and contribute to the development of new drugs with novel mechanisms of action with which to modulate immune function.

9. AKNOWLEDGMENTS

The authors wish to thank the faculty and graduate students of the Department of Pharmacology, Kyoritsu College of Pharmacy for their technical assistance. Our research is supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (No. 14370037) and by a Grant from the Smoking Research Foundation.

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Abbreviations: AcCoA: acetyl coenzyme A, ACh: acetylcholine, AChE: acetylcholinesterase, AChRs: ACh receptors, APC, antigen presenting cell, ATG-F: antithymocyte globulin-Fresenius, *alpha*-BTX: *alpha*-bungarotoxin, $[Ca^{2+}]_i$: intracellular free Ca^{2+} concentration, CAM: cell adhesion molecule, CarAT: carnitine acetyltransferase, CFA: complete Freund's adjuvant, ChAT: choline acetyltransferase, ChE: cholinesterase, CHT1: high affinity choline transporter, DAG: diacylglycerol, ER: endoplasmic reticulum, ICAM: intercellular adhesion molecule, IFA: incomplete Freund's adjuvant, IL-2: interleukin-2, IP_3: inositol-1,4,5-triphosphate, L-Arg: L-arginine, LFA: lymphocyte function-associated antigen, mAb: monoclonal antibody, MAPK: mitogen-activated protein kinase, MAPKK: MAPK kinase, MHC: major histocompatibility complex, mAChRs: muscarinic ACh receptors, MNLs: mononuclear leukocytes, MTPPD: *Mycobacterium tuberculosis* purified protein derivatives, nAChRs: nicotinic ACh receptors, NO: nitric oxide, NOS: NO synthase, ecNOS: endothelial NOS, iNOS: inducible NOS, nNOS: neuronal NOS, Oxo-M: oxotremorine M, PHA: phytohemagglutinin, PLC: phospholipase C, PKC: protein kinase C, PMA: phorbol 12-myristate 13-acetate, RIA: radioimmunoassay, RT: reverse transcriptase, RT-PCR: reverse transcription-polymerase chain reaction, SAC: *Staphylococcus aureus* Cowan I, SHR: spontaneously hypertensive rat, TCR: T cell receptor, TEC: thymic epithelial cells, TNF: tumor necrosis factor, VAChT: vesicular ACh transporter, VCAM-1, vascular CAM-1, VLA-4: very late antigen-4, WKY: Wistar Kyoto rat.

Key Words: Acetylcholine, Acetylcholinesterase, Apelin, Antigen presentation, B cell, Calcium, C-fos, Choline, Choline Acetyltransferase, High affinity choline transporter, Lymphocyte, Muscarinic receptor, Nicotinic receptor, Nitric oxide, PGE_2, Phytohemagglutinin, Radioimmunoassay, Spontaneously hypertensive rat, T cell, Thymic epithelial cell, Thymocytes, Vascular endothelial cell, Review

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