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Acetylcholine released from T cells regulates intracellular Ca^{2+}, IL-2 secretion and T cell proliferation through nicotinic acetylcholine receptor

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ABSTRACT

Aims: T lymphocytes synthesize acetylcholine (ACh) and express muscarinic and nicotinic ACh receptors (mAChR and nAChR, respectively) responsible for increases in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Our aim in the present study was to assess whether autocrine ACh released from T lymphocytes regulates their physiological functions.

Main methods: MOLT-3 human leukemic cell line and murine splenocytes were loaded with fura-2 to monitor $[Ca^{2+}]_i$ changes in the absence or presence of several AChR antagonists, including mecamylamine, methyllycaconitine and scopolamine. Real-time PCR and ELISA were performed to measure interleukin-2 (IL-2) mRNA and protein levels.

Key findings: T lymphocytes constitutively produce sufficient amounts of ACh to elicit autocrine changes in $[Ca^{2+}]_i$. These autocrine ACh-evoked $[Ca^{2+}]_i$ transients were mediated by nAChRs and then influx of extracellular Ca^{2+}. Mecamylamine, a nAChR inhibitor, suppressed not only these $[Ca^{2+}]_i$ transients, but also IL-2 release and T cell proliferation.

Significance: Here, we confirmed that T lymphocytes utilize ACh as a tool to interact with each other and that autocrine ACh-activated nAChRs are involved in cytokine release and cell proliferation. These findings suggest the possibility that nAChR agonists and antagonists and smoking are able to modulate immune function, which in turn suggests the therapeutic potential of immune activation or suppression using nAChR agonists or antagonists.

1. Introduction

Within immune organs such as the spleen and lymph nodes, acetylcholine (ACh) plays a key role in the regulation of immune function, including the proliferation and differentiation of immune cells and cytokine release [1–4]. $CD4^+$ T cells express choline acetyltransferase (ChAT), the ACh-synthesizing enzyme, as well as mediatophore, a homooligomer of a 16-kDa subunit homologous to the proteolipid subunit c of vacuolar H^+-ATPase (V-ATPase), which may mediate ACh release [1,5,6]. This suggests that $CD4^+$ T cells are a primary source of ACh within the immune system. In addition, phytohemagglutinin (PHA), a T cell activator that stimulates the T-cell receptor (TCR)/CD3 molecule complex, enhances ChAT gene and enzyme expression, leading to increased ACh production [7]. The intracellular ACh content of T cells thus appears to be relevant to their immune status.

Muscarinic and nicotinic ACh receptors (mAChR and nAChR, respectively) and acetylcholinesterase (AChE), an ACh-degrading enzyme, are expressed in various immune cells, including T and B cells, macrophages, and dendritic cells [2,8]. It is therefore likely that ACh synthesized and released from T cells acts as a cell-to-cell mediator via mAChRs and nAChRs expressed on the T cells themselves and/or other nearby immune cells. All five mAChR subtypes (M_{1-5}) are expressed in human mononuclear lymphocytes and cultured T cell lines [8]. M_1, M_3 and M_5 are coupled to $G_{q/11}$ proteins, which activates phospholipase C (PLC) to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DG), leading to IP_3-mediated Ca^{2+} release from intracellular Ca^{2+} ($[Ca^{2+}]_i$) stores [9]. In T cells, for example, oxotremorine-M (Oxo-M), a mAChR agonist, elicits a transient increase of $[Ca^{2+}]_i$, followed by sustained $[Ca^{2+}]_i$ oscillations [10,11]. T cells also express neuronal nAChR subunits [8]. nAChRs are either hetero- or homopentamers that form ion channels permeable to Na^+, K^+ and Ca^{2+} to varying degrees, depending on the subunit combination. Nicotine evokes increases in $[Ca^{2+}]_i$ in T cells [12,13]. Thus, both nAChRs and mAChRs expressed in T cells are capable of mediating increases in $[Ca^{2+}]_i$.

We previously measured the intracellular ACh content and its release in T lymphocytes using a specific and sensitive radioimmunoassay (RIA) that enabled us to measure pg-order quantities of ACh [14]. Using that approach, however, it is difficult to observe real-time events, like release of autocrine ACh. Therefore, our aim in the present study was

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to monitor $[Ca^{2+}]_i$ changes elicited via mAChRs and nAChRs expressed in T cells in order to better understand ACh-related autocrine events. Our findings indicate that autocrine ACh acts via nAChRs to regulate interleukin-2 (IL-2) release and T cell proliferation.

2. Materials and methods

2.1. Cell culture

C57BL/6J mice were obtained from SLC. The protocols used in this study were approved by the Ethical Committees of Doshisha Women's College of Liberal Arts (No. 11073). Splenocytes were isolated from C57BL/6J mice after lysing red blood cells for 2 min at room temperature in red blood cell lysing buffer (Sigma-Aldrich). MOLT-3 cells and murine splenocytes were incubated in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. To culture murine splenocytes, 2-mercaptoethanol (100 μM) was added to the medium. Mouse splenocytes were stimulated with plate-bound anti-CD3ε monoclonal antibody (5 μg/ml) and soluble anti-CD28 antibody (1 μg/ml).

2.2. Ca^{2+} imaging

MOLT-3 cells and murine splenocytes (4.0×10^5 cells) were seeded into glass-bottom dishes coated with poly-D-lysine and incubated in RPMI 1640 containing 10% FBS at 37 °C, 5% CO_2 for 1 h. After replacing the medium with Tyrode buffer containing 10 mM HEPES, cells were incubated for 30 min with 5 μM fura-2-AM in Tyrode buffer containing 0.1% bovine serum albumin (BSA) at room temperature. Fura-2 within cells was excited using 340 nm and 380 nm illumination from a TILL Monochromator Polychrome IV (TILL Photonics), and the emitted 510-nm fluorescence was detected using a microscope equipped with 20× objective lens operated using InCytlm^2 (Intracellular Imaging Inc). Ratios of 510 nm fluorescence excited at 340 nm and 380 nm ($R_{340/380}$) were calculated to assess changes in $[Ca^{2+}]_i$. R_{mean} represents the mean R values calculated over 5-min periods. Spontaneous increases in $[Ca^{2+}]_i$ were counted when the max R/R_{mean} values were ≥ 1.5. The frequency of spontaneous $[Ca^{2+}]_i$ transients was evaluated by counting >200 cells in each sample. In some cases, the cells were pretreated for 10 min with mecamylamine (100 μM), methyllycaconitine (MLA, 10 μM), scopolamine (10 μM), nicardipine (1 μM) or YM58483 (5 μM) before the observation. By themselves, antagonists had no effect on $[Ca^{2+}]_i$. Removal of extracellular Ca^{2+} was achieved using Ca^{2+}-free Tyrode buffer containing EGTA (1 mM). Images were obtained at 2-s intervals for 5 min and were analyzed using image J (NIH). The graph was created by analyzing >4 samples.

2.3. Real-time PCR

MOLT-3 cells (5×10^6 cells/well) were seeded into a 6-well plate and incubated in RPMI 1640 supplemented with 7% FBS at 37 °C, 5% CO_2. Total mRNAs were extracted using Sepasol RNA II Super (Nacalai tesque), and cDNAs were prepared through reverse transcription using a Prime Script RT reagent Kit (Takara Bio) in a S1000 Thermal Cycler (Bio-rad). Real-time PCR analysis was conducted using CYBR premix Ex taq II, FAM-labeled probes and predesigned primers (Takara Bio) with a Thermal Cycler Dice Real Time System (Takara Bio). The primer pairs were as follows: for α4 nAChR subunit (HA188413), 5′-CGGACATCGTCCTCTACAACAAGT-3′ and 5′-AACAGGTGGGCCTTGGTCA-3′; for α7 nAChR subunit (HA164722) 5′-TGGCCAGATTTGGAAA CCAGA-3′ and 5′-AGTGTGGAATGTGGCGTCAAAG-3′; for α9 nAChR subunit (HA166995) 5′-AATCATGCCGGCCTCAGAA-3′ and 5′-ATCA GGGCCATCGTGGCTA-3′; for β2 nAChR subunit (HA100887) 5′-AGACTCCCATTCACCGACCTTG and 5′-CTATCACACTGACCGCCTGGAC-3′; for IL-2 (HA244194), 5′-ACCCAGGGACTTAATCAGCAATA-3′ and 5′-GAAATTCTACAATGGTTGCTGTCTC-3′; and for GAPDH (HA067812), 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-TGGTGAAGACGCCAGTGGA-3′. The graph was created by analyzing three samples.

2.4. ACh measurement

MOLT3 cells (1×10^6 cells) were incubated with PHA (10 μg/ml) for 24 h. The culture supernatant was then collected and assayed for released ACh. To prepare ACh extracts, aliquots (1 ml) of the culture supernatant were mixed with 500 μl of 1.2 N perchloric acid, after which the solution was kept on ice for 15 min and then centrifuged at 15,000 × g for 30 min at 4°C. The ACh content of the extracts was determined with a RIA using [^{3}H]ACh (specific activity: 2.81 TBq/mmol, GE Healthcare) and antiserum against ACh raised in a rabbit immunized with choline hemiglutarate-BSA conjugates [14]. ACh content was determined by RIA. The sensitivity of the assay is 3 pg/tube (about 20 fmol/tube), and its cross-reactivity with choline, phosphatidylcholine and phosphorylcholine is <0.012%. Detailed procedures for the determination of ACh in the extract have been described elsewhere [15]. The graph was created by analyzing four samples.

2.5. Enzyme-linked immunosorbent assay (ELISA)

IL-2 release from MOLT-3 cells and murine splenocytes (1×10^5 cells) was evaluated using a sandwich ELISA. Monoclonal anti-human and mouse IL-2 antibodies (2 μg/ml) were coated onto 96-well plates as the capture antibodies. After blocking with 0.5% BSA in PBS containing 0.5% Tween 20, the diluted samples and standards of recombinant human and mouse IL-2 were incubated for 1 h at room temperature. Plates were then incubated with biotin-conjugated anti-human and mouse IL-2 antibodies (1 μg/ml) as the detection antibody for 1 h at 37 °C and reacted with streptavidin-conjugated horseradish peroxidase, followed by *o*-phenylenediamine. The reaction was terminated by addition of 0.5 M H_2SO_4. Absorbance was measured at 450 nm. The graph was created by analyzing three samples.

2.6. Cell proliferation assay

MOLT cells (3×10^3 cells) and murine splenocytes (5×10^5 cells) were seeded into 96-well plates and incubated for 4 days. Cell numbers were determined using cell count reagent SF (Nacalai tesque) according to the manufacturer's instructions, by measuring absorbance at 450 nm (SpectraMax M5 Microplate Reader). The graph was created by analyzing three experiments, each of which was carried out using three samples.

2.7. Statistical analysis

Data are presented as means ± S.E.M. All representative experiments were repeated three times. Statistical analysis was performed using SigmaPlot (Systat Software Inc.). Differences between two groups were evaluated using Student's *t*-test, and between three or more groups using one- and two-way analysis of variance (ANOVA) with post-hoc Dunnett's or Tukey's test, respectively. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Autocrine ACh elicits $[Ca^{2+}]_i$ transients through nAChR activation in MOLT-3 cells

To determine whether release of autocrine ACh occurs in T lymphocytes and modulates their own immune activities, $[Ca^{2+}]_i$ changes in MOLT-3 cells were monitored. The fura-2-loaded cells exhibited one or more apparently spontaneous transient increases in $[Ca^{2+}]_i$ during the 5-min observation periods (Fig. 1A and B). Pharmacological inhibition of nAChR activity using mecamylamine (100 μM), a non-selective