Ca^{2+} oscillation and c-fos gene expression induced via muscarinic acetylcholine receptor in human T- and B-cell lines

Takeshi Fujii · Koichiro Kawashima

Abstract We previously reported that blood acetylcholine (ACh) mainly originates from T-lymphocytes and that muscarinic (Ms) ACh receptor mRNA is expressed in both T- and B-lymphocytes. In the present study, we used confocal laser scanning microscopy and fluo-3, a calcium-sensitive indicator, to investigate the effects of Ms-ACh receptor agonists on the intracellular free Ca^{2+} concentration ([Ca^{2+}]_i) in single cells from human T-cell (CEM) and B-cell (Daudi) lines, which we used as models of lymphocytes. In both cell lines, stimulation of Ms-ACh receptors with ACh (0.1–100 μM), bethanechol (100 μM), carbachol (100 μM) or oxotremorine-M (Oxo-M; 0.1–100 μM) induced [Ca^{2+}]_i-dependent increases in fluo-3 fluorescence, which in the presence of extracellular Ca^{2+} were followed by oscillations in [Ca^{2+}]_i that persisted for at least 10 min. All effects were completely blocked by atropine (1 μM), an Ms-ACh receptor antagonist. In both cell lines Oxo-M (100 μM) up-regulated expression of c-fos mRNA in an extracellular Ca^{2+}-dependent manner. Again, the effect was blocked by 1 μM atropine. These results provide the first evidence that stimulation of Ms-ACh receptors induces Ca^{2+} oscillations and up-regulates c-fos gene expression in T- and B-lymphocytes, which is consistent with the notion that ACh released from T-lymphocytes triggers nuclear signaling via Ms-ACh receptors.

Key words Acetylcholine · Ca^{2+} oscillation · Muscarinic receptor · B-lymphocytes · T-lymphocytes · c-fos

Introduction

The involvement of acetylcholine (ACh) in the regulation of interactions between the nervous and immune systems was proposed by Maslinski (1989), although it was demonstrated somewhat earlier that muscarinic (Ms) ACh receptors are present on lymphocytes (Zalcman et al. 1981; Adem et al. 1986), and that Ms-ACh receptor agonists modulate lymphocyte function and metabolism (Illiano et al. 1973; Strom et al. 1974; Maslinski et al. 1988).

The genes for five Ms-ACh receptor subtypes (M1-M5) have been cloned (Bonner et al. 1987, 1988); the M1, M3 and M5 subtypes are coupled to phosphoinositide signaling pathways, whereas the M2 and M4 subtypes are linked to the adenylate cyclase system (Hulme et al. 1990). Further, the expression of several subtypes has been observed in human mononuclear leukocytes (Costa et al. 1995; Hellström-Lindahl and Nordberg 1996; Fujino et al. 1997; Sato et al. 1999) and in human leukemic cell lines (Kaneda et al. 1993; Sato et al. 1999).

We previously demonstrated that ACh is present in the blood of several mammals, including humans (Fujii et al. 1997; Yamada et al. 1997; Kawashima et al. 1998); that the gene encoding choline acetyltransferase (ChAT; E.C. 2.3.1.6), which catalyzes ACh synthesis (Tucek 1988), is expressed in human leukemic T-cell lines; and that human T-cell lines, but not B-cell lines, possess high ChAT activity and high intracellular ACh content (Fujii et al. 1999). Blood ACh may therefore originate primarily from T-lymphocytes and interact with ACh receptors on themselves and other blood cells, serving as a neuroimmunomodulator.

Ligand binding to transmitter receptors coupled to phosphoinositide signaling pathways activates phospholipase C and induces rapid, transient increases in the intracellular free Ca^{2+} concentration ([Ca^{2+}]_i) via inositol-1,4,5-trisphosphate (IP_3)-induced release of Ca^{2+} from intracellular stores in the endoplasmic reticulum (ER; Berridge and Irvine 1989). Such changes in the [Ca^{2+}]_i, which are known to serve as excitatory and/or inhibitory signals within cells (Berridge 1990), are readily investigated using Ca^{2+}-sensitive fluorescent probes, e.g. fluo-3 and fura-2 (Tsien et al. 1982; Kao et al. 1989; Minta et al. 1989; Okamoto et al. 1995). In that regard, Kaneda et al. (1993) used selective agonists to show that stimulation of...
M3 receptors on populations of fura-2-loaded Jurkat cells, a T-cell line, causes transient elevations in \([\text{Ca}^{2+}]_i\). Digital imaging of \([\text{Ca}^{2+}]_i\), enables analysis of intracellular events within individual cells, which is particularly useful for resolving early activation and asynchronous events that may be unresolved when cell populations are studied (Berridge 1990; Yamada et al. 1993). The oscillations evoked in a variety of cell types by prolonged exposure to an agonist are a clear example (Berridge and Irvine 1989; Berridge 1990; Wacholtz and Lipsky 1993; Kasai et al. 1997).

Stimulation of T-cell receptors (TCR) on T-lymphocytes by mitogenic stimulation leads to elevations in cytosolic \(\text{Ca}^{2+}\) that spread to the nucleus, where the increased intranuclear \(\text{Ca}^{2+}\) leads to activation of transcription regulators, including c-fos, and modulation of lymphocyte function (Jain et al. 1992). Thus, a more thorough understanding of the relationship between the changes in \([\text{Ca}^{2+}]_i\) and c-fos gene expression induced by Ms-ACh receptor activation in single T- and B-lymphocytes should provide important new information about the function of ACh in the regulation of the immune system. In the present study, we used confocal laser scanning fluorescence microscopy to examine the effect of Ms-ACh receptor activation on \([\text{Ca}^{2+}]_i\), in cell lines serving as models of T- and B-lymphocytes. In addition, c-fos mRNA expression was analyzed using semi-quantitative analysis of reverse transcription-polymerase chain reaction (RT-PCR). Our findings represent the first evidence that Ms-ACh receptor-mediated \(\text{Ca}^{2+}\) oscillations up-regulate c-fos gene expression in both T- and B-lymphocytes.

### Materials and methods

**Chemicals and reagents.** RPMI 1640 medium was purchased from Nissui Seiyaku (Tokyo, Japan). Cover-glass bottom culture dishes coated with poly-\(\alpha\)-lysine (no. P35GC-0-14-C) were obtained from MatTek (Ashland, Mass., USA). Fluo-3 AM was obtained from Dojindo (Kumamoto-ken, Japan). ACh iodide and atropine sulfate were from Wako Pure Chemicals (Osaka, Japan). Carbacol chloride was from Sigma Chemical (St. Louis, Mo., USA). Bethanechol chloride, oxotremorine-M (Oxo-M) and thapsigargin (TGN) were purchased from Research Biochemicals International (Natick, Mass., USA). The c-Fos Primer Set for RT-PCR was prepared using 20 \(\mu\)g of total RNA, 50 pmol random primer p(dN)\(6_m\) and reverse transcriptase (RT), as described elsewhere (Fuji et al. 1998, 1999). As a negative control, RT was omitted from the reaction mixture in some cases. Using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn., USA), 5% of the resultant cDNA was then amplified in a 50-\(\mu\)l reaction volume containing 25 pmol of forward and reverse primers and AmpliTaq DNA polymerase. The amplification reaction was carried out for 27–35 cycles using a 30-s denaturation, 94°C for 45 s; annealing, 60°C for 45 s; and extension, 72°C for 1.5 min) followed by a 15-min final extension at 72°C. The PCR products present in 10 \(\mu\)l of the reaction mixture were then separated according to size on 3% NuSieve GTG agarose gels (FMC Bioproducts, Rockland, Me., USA) and visualized by ethidium bromide staining.

**Cell lines and culture.** Two cell lines were used as models of lymphocytes: the human acute lymphoblastic T-cell line, CEM; and the human leukemic B-cell line, Daudi. Both cell lines express mRNA encoding several Ms-ACh receptor subtypes (Table 1; Sato et al. 1999). The cells were maintained in culture flasks (Corning 2511-75; Corning, N.Y., USA) in RPMI 1640 medium supplemented with 7% heat-inactivated FCS at 37°C under a humidified atmosphere of 5% \(\text{CO}_2\) in air.

**Confocal imaging of \([\text{Ca}^{2+}]_i\).** in human leukemic cell lines. \([\text{Ca}^{2+}]_i\) was imaged in single cells using confocal laser scanning microscopy and the \(\text{Ca}^{2+}\)-sensitive fluorescent indicator, fluo-3 (Kao et al. 1999). Briefly, CEM and Daudi (106 cells) were washed once with fresh RPMI 1640 and then incubated with 3 \(\mu\)M fluo-3/AM in RPMI 1640 for 30 min in a 37°C water bath. Once loaded with fluo-3, the cells were diluted 1:9 with RPMI 1640, incubated for an additional 30 min, washed with Tyrode-HEPES buffer (20 mM HEPES, 137 mM NaCl, 3.3 mM KH2PO4, 1 mM CaCl2, 0.7 mM MgCl2, 5 mM glucose at pH 7.4) to remove free fluo-3/AM, transferred to cover-glass bottom culture dishes, and incubated for at least 30 min more.

Fluorescent images of living cells in Tyrode-HEPES buffer were acquired using a Leica TCS NT system (Leica) equipped with an argon-ion laser. Fluo-3-loaded cells were excited at 488 nm, and the fluorescent emission above 530 nm was imaged using a 40× Leica PL FLUOTAR objective (N.A. 1.0). Confocal images (512×512 pixels) were obtained at a rate of 1 every 2 s. Ms-ACh receptor agonists and/or atropine, an antagonist, were added directly to the culture dishes and flupicine was added at least 5 min prior to addition of agonists. To remove extracellular \(\text{Ca}^{2+}\), normal Tyrode-HEPES buffer was replaced with \(\text{Ca}^{2+}\)-free buffer, in which EGTA was substituted for CaCl2 on an equimolar basis.

The methods for using fluo-3 to estimate relative changes of \([\text{Ca}^{2+}]_i\), are well established (Kao et al. 1989; Minta et al. 1989; Okamoto et al. 1995; Kasai et al. 1997). Briefly, for each cell studied, basal fluorescence (\(F_0\)) was calculated from three sequentially acquired images as the mean fluorescent emission from the total area of the cell prior to the addition of any agonist. Cells were considered to be responding to stimulation when fluorescence intensity increased at least twofold over \(F_0\) (Okamoto et al. 1995; Kasai et al. 1997). \([\text{Ca}^{2+}]_i\)-dependent changes in fluorescence (\(F\)) were measured in at least 100 individual cells in each microscopic field, after which the data were normalized to \(F_0\) (\(F_0/F_0\) ratio) in order to control for variations in basal fluorescence.

**Preparation of total RNA for analysis of c-fos mRNA expression.** To prepare RNA for analysis of c-fos mRNA expression, cultured cells were exposed to 100 \(\mu\)M Oxo-M in fresh RPMI 1640 for 1 h at 37°C in the presence or absence of 1 \(\mu\)M atropine. To remove extracellular \(\text{Ca}^{2+}\), RPMI 1640 was replaced with \(\text{Ca}^{2+}\)-free Tyrode-HEPES buffer. The cells (5×106 cells) were then transferred to centrifuge tubes (Corning 25311) and pelleted at 300 \(g\) for 8 min at 4°C, after which the pellet was washed three times with 10 ml of phosphate-buffered saline. Total RNA was directly extracted from the pellets using RNA zol B as previously described (Fuji et al. 1998, 1999). RNA concentrations were determined by absorbance at 260 nm.

**First-strand cDNA synthesis and semi-quantitative RT-PCR amplification of c-fos mRNA expression.** First-strand cDNA was prepared using 20 \(\mu\)g of total RNA, 50 pmol random primer p(dN)\(6_m\) and reverse transcriptase (RT), as described elsewhere (Fuji et al. 1998, 1999). As a negative control, RT was omitted from the reaction mixture in some cases. Using a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, Conn., USA), 5% of the resultant cDNA was then amplified in a 50-\(\mu\)l reaction volume containing 25 pmol of forward and reverse primers and AmpliTaq DNA polymerase. The amplification reaction was carried out for 27–35 cycles using a 30-s denaturation, 94°C for 45 s; annealing, 60°C for 45 s; and extension, 72°C for 1.5 min) followed by a 15-min final extension at 72°C. The PCR products present in 10 \(\mu\)l of the reaction mixture were then separated according to size on 3% NuSieve GTG agarose gels (FMC Bioproducts, Rockland, Me., USA) and visualized by ethidium bromide staining.

### Table 1 Expression of muscarinic acetylcholine (ACh) receptor subtypes and the capacities for ACh synthesis of two human leukemic cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Types of lymphocytes</th>
<th>Muscarinic ACh receptors</th>
<th>ACh synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>T-lymphocyte</td>
<td>m1, m3, m4, m5</td>
<td>Positive</td>
</tr>
<tr>
<td>Daudi</td>
<td>B-lymphocyte</td>
<td>m2, m3, m4, m5</td>
<td>Negative</td>
</tr>
</tbody>
</table>

\(^{a}\)Data from Sato et al. 1999  
\(^{b}\)Data from Fuji et al. 1999