Muscarinic Acetylcholine Receptors on Rat Thymocytes: Their Possible Involvement in DNA Fragmentation

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ABSTRACT—Several studies have shown that the nervous (and hormonal) system controls immune functions. In the present study, we examined the presence of muscarinic acetylcholine receptors and the effect of carbachol on DNA fragmentation in adult rat thymocytes. Rat thymocytes possessed high affinity binding sites for the muscarinic antagonist [3H]3-quinuclidinyl benzilate (QNB). The average number of binding sites per cell was 3000, and the equilibrium dissociation constant of [3H]QNB on intact cell was approximately 80 nM. The binding was inhibited by an M1- and M3-selective antagonist, 4-diphenylacetoxy-N-methylpiperidine methiodine (4-DAMP). Hydrocortisone (100 mg/kg, s.c.) treatment of rats for 2 days prior to sacrifice increased the average number of [3H]QNB binding sites on thymocytes by 82±33%. The gel electrophoresis of DNA extracted from carbachol-treated thymocytes revealed a ladder pattern typical of intranucleosomal fragmentation. The addition of oxotremorine-M also induced DNA fragmentation and the effects of muscarinic agonists were inhibited by the addition of atropine or 4-DAMP. The results suggest the existence of muscarinic receptors and the possible involvement in apoptosis in thymocytes.

Keywords: Thymocyte, Muscarinic receptor, Hydrocortisone, DNA fragmentation

Recent studies have shown the presence and regulatory functions of several neurotransmitters in the immune system (1, 2). The vagus nerve, whose major neurotransmitter is acetylcholine, enters the thymus gland early in embryonic development and remains throughout the life span of the animal (3–6). It has been demonstrated that choline acetyltransferase and muscarinic acetylcholine receptors exist in mouse thymus (6), although the physiological roles of muscarinic receptors in thymocytes are unknown. The cell types (CD4−CD8−, CD4+CD8−, CD4−CD8+ or CD4+CD8+) of peripheral T cells are controlled by the selection of T cell clones in the thymus; positive and negative selection of cells are generally considered. Glucocorticoids are known to induce apoptotic death in thymocytes (7–9). In the present study, we examined the presence of muscarinic receptors and their regulation by hydrocortisone treatment and examined the effect of muscarinic receptor stimulation on DNA fragmentation in thymocytes from adult rats.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (5 weeks) were obtained from NRC Haruna Corp. (Gunma). They were fed a commercial feed ad libitum.

Materials
[3H]3-Quinuclidinyl benzilate (QNB) (43.5 Ci/mmol) was purchased from NEN Products (Boston, MA, USA). Atropine and hydrocortisone succinate were purchased from Wako Pure Chemical Corp. (Osaka). 4-Diphenylacetoxy-N-methylpiperidine methiodine (4-DAMP), carbachol and oxotremorine-M were obtained from Research Biochemicals, Inc. (Natick, MA, USA). [11-2[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX), proteinase-K and RNase A were purchased from Boehringer Mannheim (Mannheim, Germany).

Treatment with hydrocortisone
Male Dawley rats, 5-weeks-old, were s.c. treated with hydrocortisone 100 mg/kg (dissolved in saline). At 48 hr after the injection, the animals were sacrificed by decapi-
Isolation of thymocytes

Control and hydrocortisone-treated rats were sacrificed by decapitation and their thymuses were removed. Stem cell suspensions were obtained by teasing the tissue through nylon mesh (300 x 300 μm) in modified Krebs-Ringer buffer (KRB) (119 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15 mM glucose, 10 mM HEPES, pH 7.2). The cells were washed three times by centrifugation (300 x g, 2 min, 4°C) and resuspended in the buffer.

[³H]QNB binding assay

[³H]QNB binding to rat thymocytes was examined as described by Maslinski et al. (10, 11) with slight modifications. The thymocyte cell suspension (2 x 10⁶ cells in KKB, 200 μl) was preincubated for 5 min at 20°C with atropine (final concentration of 100 μM) (nonspecific binding) or vehicle (total binding). [³H]QNB was added to yield indicated concentrations in the range of 1.5–50 nM, and the incubation was prolonged for 20 min at 20°C. We could not detect the specific binding at higher concentrations of [³H]QNB, over 50 nM, because the non-specific binding was very large. The cells were collected by filtration on Whatman GF/C filters. Filters were washed rapidly 5 times with 2 ml of ice-cold KRB. The rinsed filters were dried and the bound radioactivity was determined by liquid scintillation spectrometry.

Detection of fragmented DNA

DNA fragmentation was assayed as previously reported (7, 12, 13). Two milliliters of the thymocyte cell suspension (2 x 10⁶ cells) in KRB were incubated in a shaking bath with or without 100 μM carbachol for 30 min at 37°C. The reaction was terminated by the addition of 6 ml of the lysis buffer (5 mM EDTA, 0.5% Triton X-100, 10 mM Tris-HCl, pH 7.4). Under these conditions, not cell culture, DNA fragmentation was observed after 30 min, and the effect of carbachol was remarkable at 30 min. After centrifugation (10,000 x g, 15 min, 4°C), the supernatant was treated with 250 μg/ml of protease-K. DNA and RNA were extracted with a phenol-chloroform mixture and precipitated with ethanol. Samples were treated with 40 μg/ml of RNase A and electrophoresis on 1.5% agarose gel. The fragmented DNA in the gel was stained with ethidium bromide.

Statistical analyses

Two-way analysis of variance was utilized for statistical significance between the control and treated groups.

RESULTS

Muscarinic antagonist binding to intact rat thymocytes was examined with [³H]QNB as the ligand. Binding of [³H]QNB (50 nM) to thymocytes was examined at 4°C, 20°C and 30°C. The specific binding became maximum between 5–10 min of incubation and was sustained at 20°C (Fig. 1). The specific binding was low at 4°C (data not shown). At 37°C, the specific binding reached a maximum between 5–10 min of incubation and declined thereafter, although the maximal specific binding was similar to that at 20°C. A similar decline in specific binding but not in nonspecific binding was reported previously (11). Specific binding of [³H]QNB at 20°C was partially but significantly (30±8%, n=3) inhibited by 10 μM 4-DAMP (an M1- and M3-selective antagonist). Addition of the same concentrations of M1-antagonist (pirenzepine) and M2-antagonist (AF-DX), however, inhibited [³H]QNB binding less than 5±3% and 4±4%, respectively (n=3). Addition of 10 μM oxotremorine-M, a muscarinic agonist, also inhibited [³H]QNB binding 40±10%. Even at 20 μM, oxotremorine-M and 4-DAMP inhibited [³H]QNB binding partially (33±8% and 39±6%, respectively). The reason for this is not clear at this point. No specific binding of [³H]pirenzepine to thymocytes was found upon incubation under the same experimental condi-

![Fig. 1](image-url). Time course of [³H]QNB binding to rat thymocytes at 20°C. The thymocyte suspensions were incubated with 50 nM [³H]QNB for the indicated time in the presence (●) or absence (○) of 100 μM atropine. The [³H]QNB bindings in the presence and absence of 100 μM atropine at 2 min were not constant, and thus the data were not shown.
ditions (data not shown). Figure 2 shows Scatchard analysis of \[^{3}H\]QNB binding to the thymocytes. \[^{3}H\]QNB binding to the thymocytes did not show saturation at higher concentrations of over 100 nM, as previously described (6). The \(K_d\) value was in the range of 80–100 nM, whereas the \(B_{\text{max}}\) was determined to be 2,800–3,200 sites/cell.

Hydrocortisone treatment (100 mg/kg), s.c., 2 days prior to sacrifice, significantly inhibited the increase in body weight (Table 1). The treatment also caused a marked decrease in the thymus size. The calculated ratio of thymus weight/body weight in hydrocortisone-treated rats was significantly smaller compared with that in control rats (Table 1). Hydrocortisone treatment increased the \(B_{\text{max}}\) of \[^{3}H\]QNB binding (Fig. 2). The \(K_d\) in hydrocortisone treatment was in the range of 70–110 nM, whereas the \(B_{\text{max}}\) was 4,300–6,000 sites/cell. Similar experiments were repeated three times. Hydrocortisone treatment significantly increased the \(B_{\text{max}}\) of \[^{3}H\]QNB binding sites by about twofold without any change in the body weight (Table 1).

Table 1. Effects of hydrocortisone treatment in vivo on body weight and thymus weight

| Treatment     | Body weight (\% of increase) | Thymus weight (g) | Thymus weight/body weight (\%) |
|---------------|------------------------------|-------------------|-------------------------------|
| Control       | +12.8 ± 1.0                  | 0.525 ± 0.058     | 4.34 ± 0.09                   |
| Hydrocortisone| +6.2 ± 1.7*                  | 0.241 ± 0.120*    | 2.23 ± 0.37*                  |

Rats (155 ± 6 g, \(n=9\)) were s.c. injected with hydrocortisone (100 mg/kg body weight). Body weight was measured 48 hr after the administration of hydrocortisone. The rats were sacrificed, and their thymuses were removed and weighed. The increase in body weight over a period of two days and the ratio of thymus weight/body weight were measured. Data show the mean ± S.E.M. of 4–5 rats. Statistical significance was determined by two-way analysis of variance. Significance: *\(P<0.01\), **\(P<0.001\), versus control.

Table 2. Effects of hydrocortisone treatment in vivo on \[^{3}H\]QNB binding to rat thymocytes

| Treatment   | \(K_d\) (nM) | \(B_{\text{max}}\) (% of control) |
|-------------|--------------|----------------------------------|
| Control     | 95.2 ± 15.2  | 100                              |
| Hydrocortisone | 73.0 ± 13.0  | 182 ± 33.6*                      |

Rats were injected i.p. with hydrocortisone (100 mg/kg) or saline at 48 hr before thymocyte isolation. Thymocytes were incubated with \[^{3}H\]QNB as described in Fig. 2. Data show the mean ± S.E.M. of 4 independent experiments using 1 pair of rats for each. Statistical significance was determined by two-way analysis of variance. Significance: *\(P<0.01\), versus control.
Agarose gel electrophoresis of fragmented DNA isolated from carbachol-treated rat thymocytes. The thymocyte suspensions from control rats were incubated for 30 min at 37°C. The fragmented DNA was extracted as described in Materials and Methods. Lane 1: DNA molecular weight markers, lane 2: no incubation, lane 3: control, lane 4: 1 mM carbachol, lane 5: 1 mM carbachol and 10 μM 4-DAMP, and lane 6: 1 mM carbachol and 10 μM atropine. A typical result of three separate thymocyte preparations is shown.

The fragmentation of nuclear DNA into oligonucleosomal fragments has been linked to chromatin condensation and the formation of apoptotic nuclei in glucocorticoid- (14) and Ca²⁺-ATPase inhibitor thapsigargin-treated thymocytes (13). As shown in Fig. 3, agarose gel electrophoresis of the DNA extracted from 1 mM carbachol-treated thymocytes revealed a ladder pattern typical of intranucleosomal fragmentation. Incubation for 30 min without carbachol did not stimulate DNA fragmentation. A typical DNA cleavage pattern was also observed with 100 μM carbachol. The addition of 10 μM 4-DAMP or atropine inhibited the carbachol-induced DNA fragmentation. Atropine and 4-DAMP had no effect on DNA fragmentation by itself. The treatment of 100 μM oxotremorine-M, an M1- and M3-agonist, for 30 min also induced DNA fragmentation in rat thymocytes (data not shown). These findings suggest that the stimulation of muscarinic acetylcholine receptors caused DNA laddering in rat thymocytes. The apoptotic effect of carbachol in the Ca²⁺-free buffer was smaller than that in the presence of CaCl₂ (data not shown), as described in the effect of adenosine (15).

DISCUSSION

Previously, we reported the presence of M3 subtype muscarinic acetylcholine receptors and receptor-mediated increases in the cytoplasmic concentration of Ca²⁺ in Jurkat, a human leukemic helper T lymphocyte line (16). Binding parameters of [³H]QNB in Jurkat cells were $K_d = 14.1$ nM and $B_{max} = 45,370$ sites/cell using intact cells. In thymocytes from adult rats, the $K_d$ was in the range of 80–100 nM and $B_{max}$ was approximately 3,000 sites/cell. Thus, binding affinity was lower and $B_{max}$ was small in rat thymocytes compared with those in Jurkat cells. Similar levels of [³H]QNB binding sites/cell in thymocytes have been reported in rats (11) and mice (6,
The results presented here suggest that one of the subtypes of muscarinic receptors in rat thymocytes is presumed to be the M3 subtype, since 4-DAMP (M1- and M3-selective antagonist) inhibited [3H]QNB binding effectively and pirenzepine (M1-selective antagonist) did not show an inhibitory effect, although it was difficult to determine the subtypes of muscarinic receptors by [3H]-QNB binding due to a low level of binding sites.

Glucocorticoids are known to have many effects in vivo. Hydrocortisone treatment significantly inhibited the increase in body weight (Table 1), because of its metabolic effects specifically on skeletal muscle tissues. Glucocorticoids are also known to induce apoptotic death in thymocytes (7, 8), and in our experiments hydrocortisone treatment caused a marked decrease in the thymus weight (Table 1). The effect of hydrocortisone on the thymus did not seem to be derived from its metabolic effects, because the ratio of thymus weight/body weight in hydrocortisone-treated rats was significantly small compared with that in control rats. Maslinski et al. (10) reported that maximal binding of [3H]QNB for the entire thymocyte population in hydrocortisone-treated rats was two times higher than that for thymocytes in normal rats. In our experiments, hydrocortisone treatment increased [3H]-QNB binding sites twofold (Table 2). The major population of thymocytes that are induced to undergo apoptotic death by glucocorticoids appear to be CD4+CD8+ double-positive immature cells (9, 17). It was also reported that 2-chloroadenosine, an agonist to A2-adenosine receptors, selectively depleted a human thymocyte population that was found mainly in the CD4+CD8+ double positive immature thymocyte population (15). It has to be determined which population of rat thymocytes is sensitive to muscarinic stimulation.

In Jurkat cells, the stimulation of M3 muscarinic receptors was accompanied by an increase in the cytosolic-free Ca^2+ concentration ([Ca^2+]i) (16). A sustained increase in [Ca^2+]i of rat thymocytes was followed by DNA fragmentation and a loss of cell viability (12). In human thymocytes, adenosine stimulated DNA fragmentation by Ca^2+-mediated mechanisms (15). Thus, it is possible to speculate that the increase in [Ca^2+]i, following stimulation of muscarinic receptors modulates DNA fragmentation of rat thymocytes, although we could not measure the change in [Ca^2+]i in the thymocytes using the Ca^2+ indicator fura-2/aceotxymethyl ester. In addition to the direct effect (7-9), glucocorticoid seems to have an ability to enhance the muscarinic receptor-mediated apoptosis by up-regulation of the receptors.

We confirmed the existence of [3H]QNB binding sites and its up-regulation by hydrocortisone in rat thymocytes. The following new findings were obtained in our present study: 1) [3H]QNB binding sites in rat thymocytes are presumed to be the M3 subtype and 2) stimulation of muscarinic receptors (maybe M3 subtype) induced DNA fragmentation. However, the biochemical basis and physiological roles of M3 muscarinic receptors in rat thymocytes are not well established because the inhibitory effects of 4-DAMP on [3H]QNB binding and carbachol-induced DNA fragmentation were partial, not complete. Costa et al. (19) suggested that the mRNAs for M3, M4 and M5 receptors were detected in human lymphocytes. The characteristics of muscarinic receptors in rat thymocytes should be clarified in the future. It would be very interesting to know which subpopulations of thymocytes bear muscarinic receptors.

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