In previous study, we demonstrated the existence of casein kinase II-like ectokinase activity on rat basophilic leukemia 2H3 (RBL-2H3) cells (Immunol. Let. 68, 369, 1999), and to determine the role of ectokinase in the degranulation of RBL-2H3 cells, in this study we investigated the effect of casein kinase II substrate peptide on the degranulation by the cells. Casein kinase II peptide (RRRDDDSDDD) dose-dependently (IC50 = 50 M) inhibited β-hexosaminidase release by IgE-sensitized antigen-stimulated RBL-2H3 cells, whereas casein kinase II peptide analogue (RRRDDDADDD) only partially inhibited β-hexosaminidase release by antigen-stimulated RBL-2H3 cells. Preincubation of RBL-2H3 cells with 200 μM of casein kinase II peptide significantly inhibited external 130 kDa protein phosphorylation, and casein kinase II peptide inhibited the sustained increase in cytosolic calcium ion concentration in response to antigen stimulation. Our findings suggest that casein kinase II acts as an ectokinase in RBL-2H3 cells and that casein kinase II peptide acts as a competitor for phosphorylation of ectoprotein, which involves degranulation and a transmembrane influx of Ca2+ by IgE receptor cross-linking. Another casein kinase II inhibitor, GT copolymer, also inhibited the degranulation of antigen-stimulated RBL-2H3 cells. Thus, the modulator of casein kinase II activity seems to be a good tool for the inhibitor of signal transduction in RBL-2H3 cells.

Key words —— ectokinase, casein kinase II peptide, rat basophilic leukemia 2H3 cells, degranulation

INTRODUCTION

The release of histamine and other inflammation mediators by tissue mast cells and basophils is the primary event in a variety of acute allergic and inflammatory conditions. In our previous paper, we reported that the ectokinase inhibitor K252b decreased histamine secretion by rat basophilic leukemia 2H3 (RBL-2H3) cells and human basophils, suggesting the presence of ectokinase and its involvement in degranulation.1) We also confirmed the presence of casein kinase II-like ectokinase activity.2) Several studies have demonstrated the role of protein kinases on the outer surface of a variety of mammalian cells, including HeLa cells,3) T-lymphocytes,4,5) brain neuron,6) and neuroblastoma cells,7) but few studies have investigated the role of the ectokinases on mast cells and basophils.

In the present study, we used casein kinase II peptide as a specific inhibitor of ectokinase to investigate the role of the ectokinases on RBL-2H3 cells.

MATERIALS AND METHODS

Reagents —— Mouse anti-dinitrophenyl (DNP) IgE mAb (IgE-53-569) and dinitrophenylated bovine serum albumin (DNP-BSA) were prepared as described previously.8) Casein kinase II peptide (CK II peptide; RRRDDDSDDD) and casein kinase II analogue (CKII peptide analogue; RRRD-DDADDD) were synthesized by Biologica Co. (Nagoya, Japan). Random copolymer poly-GT [L-glutamic acid (GluNa) : L-tyrosine (Tyr) = 4 : 1] was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fura-2-AM was obtained from Dojindo (Kumamoto, Japan). All other reagents were of the highest commercial grade.

Cells —— RBL-2H3 cells, donated by Dr. R. P.
Siraganian, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS).

Piperazine-N'-N''-bis(2-ethane sulfonic acid) (PIPES) Buffer ——— The buffer used had the following composition: 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1.0 mM CaCl₂, 5.5 mM glucose, 0.1% BSA, and 10 mM piperazine-N′-N″-bis(2-ethane sulfonic acid) (PIPES, pH 7.4).

Measurement of Degranulation ——— Degranulation of RBL-2H3 cells was monitored by measuring released β-hexosaminidase activity as described previously.1,8 For antigen stimulation, DNP-specific IgE primed RBL-2H3 cells were preincubated for 10 min with various concentrations of CKII peptide, CKII peptide analogue, or randomcopolymer GT, and then stimulated with 10 µg/ml of antigen (DNP₂-BSA). After 30 min, the medium was collected, and 0.2% Triton X-100 was added to the cells. β-Hexosaminidase released into the medium and within the cells (Triton X-100 extract) were determined by colorimetric assay with p-nitrophenyl-2-acetamide-2-deoxy-β-glucopiranoside. β-Hexosaminidase release is expressed in the form of activity released into the medium as a percentage of the total activity.

Measurement of Cytosolic Free Calcium Concentration ([Ca²⁺]i) ——— RBL-2H3 cells (6 × 10⁵ cells/ml) were sensitized with anti-DNP-IgE (50 nM) and then loaded with fura-2-acetoxymethyl (fura-2-AM) as described previously.8) After removing both free dye and unbound IgE by centrifugation, the cells were resuspended in 1.5 ml of PIPES buffer. Fluorescence measurements were made in a 1-cm quartz cuvette with a Shimadzu RF-5000 spectrometer.8) Fura-2 loaded cells were excited at 355 nm or 362 nm, and fluorescence emission was observed at 500 nm.

Analysis of Phosphorylated Proteins ——— RBL-2H3 cell monolayers in culture flasks were detached with a cell scraper (Falcon #3086), collected by centrifugation, and suspended at 1 × 10⁶ cells in 0.5 ml of PIPES buffer with 2 mM MgCl₂ and 2 mM NaF, pH 7.4. The DNP-specific IgE primed-cells were preincubated in glass test tubes with or without peptide at 37°C for 5 min, and phosphorylation was initiated by addition of [γ³²P] adenosine 5′-triphosphate ([γ³²P]ATP) (5 µM, 2.2 Ci/mmol) and various stimulants. The cells were incubated at 37°C for 10 min, and the reaction was terminated with 1 mM ATP. After washing with PIPES buffer, phosphorylated cells were solubilized with 80 µl of Laemmlli sodium dodecyl sulfate (SDS)-solubilizing buffer, and the phosphorylated proteins (40 µl) were separated by SDS-polyacrylamide gel electrophoresis. The following molecular weight standards were run simultaneously: myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). After the run, the gels were cut off at the position of the tracking dye and fixed. The radioactivity incorporated in each protein band was determined by radioluminography with a Bioimage analyzer BAS 1500 (Fuji Film, Tokyo, Japan).

RESULTS AND DISCUSSION

Ectokinases and phosphorylated proteins have been reported to be present on the outer cell surface of HeLa cells,3) T-lymphocytes,4,5) brain neurons6) and neuroblastoma cells.7) Studies to characterize ectokinases and identify their physiological role and substrates are now in progress,1–7,10) and a few ectokinases have been characterized. One has the catalytic properties of atypical protein kinase C and is present on the cell surface of brain neurons,6) and another is the casein kinase on HeLa cells and T-lymphocytes.3,5,8) However, there have been no reports on basophils and mast cell ectokinases.

In a previous study, we demonstrated the presence of a casein kinase II-like ectokinase on the outer surface of the plasma membrane of RBL-2H3 cells and showed that synthetic peptide substrate could be used to specifically and sensitively monitor casein kinase II.11) In the present study, we used the peptide as a specific inhibitor of ectokinase and examined the role of ectokinase in the degranulation of RBL-2H3 cells. We investigated the effect of CKII peptide and its analogue on β-hexosaminidase release by RBL-2H3 cells after antigen stimulation. As shown in Fig. 1a, CKII peptide dose-dependently inhibited β-hexosaminidase release induced by antigen (IC₅₀ = 50 µM), whereas, as shown in Fig. 1b, CKII peptide analogue only partially inhibited β-hexosaminidase release by antigen-stimulated RBL-2H3 cells. Another casein kinase II inhibitor, 30 µM GT copolymer, also significantly inhibited degranulation by antigen-stimulated RBL-2H3 cells (data not shown). These findings suggest that phosphorylation of ecto-protein by casein kinase II is involved in IgE-receptor-mediated degranulation.
In a previous paper, we reported that K252b inhibited IgE-receptor mediated degranulation of RBL-2H3 cells, and that the major endogenous substrates of ectokinases were 130 kDa, 115 kDa, and 67 kDa proteins. As shown in Fig. 2, 200 $\mu$M of CKII peptide significantly inhibited the phosphorylation of 130 kDa protein (lane 3), and the decrease seemed to be well correlated with the inhibition of $\beta$-hexosaminidase release (Fig. 1). Since IgE Fc receptor I (FceRI) chains are not major substrates of ectokinases in RBL-2H3 cells, molecules associated with or close to the receptor, such as cluster of differentiation (CD) 31, AA4-associated molecules, or ecto-p120 as a cell-surface homologue of human nuclear phosphoprotein p140.12–14 are candidates for the endogenous substrates of casein kinase II.

CKII peptide also significantly inhibited Ca$^{2+}$ influx induced by antigen stimulation (Fig. 3). As shown in Fig. 3a, the [Ca$^{2+}$]i increase after antigen stimulation persisted for at least 200 sec. In contrast, as shown in Fig. 3b, the increase in [Ca$^{2+}$]i in response to antigen stimulation decreased immediately after the addition of 200 $\mu$M of CKII peptide, and sequential addition of calcium ionophore (0.5 $\mu$M ionomycin) induced an additive [Ca$^{2+}$]i elevation. As CKII peptide inhibited the IgE-receptor mediated Ca$^{2+}$ signal and did not affect IgE-receptor bypassed Ca$^{2+}$ response, the receptor-operated Ca$^{2+}$ channels seem to be regulated by partially ecto-type protein kinases.

In conclusion, the results of this study (1) demonstrated inhibition of the degranulation of RBL-2H3 cells by casein kinase II peptide and (2) that the phosphorylation of 130 kDa protein is also inhibited by casein kinase II peptide, (3) suggesting the presence of a functional ecto-phosphorylation system on the surface of RBL-2H3 cells.
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Fig. 3. Effect of CKII Peptide on the Ca^{2+} Response
(a) Fura-2-loaded IgE-primed RBL-2H3 cells (final 6 × 10^5 cells/ml) were preincubated without drug for 2 min at 37°C and then mixed with DNP-BSA (10 µg/ml). (b) Fura-2-loaded IgE-primed RBL-2H3 cells (final 6 × 10^5 cells/ml) were preincubated without drug for 2 min at 37°C, mixed with DNP-BSA (10 µg/ml), and added 200 µM CKII peptide at 140 sec, and then added 0.5 µM ionomycin at 280 sec.