Stimulation of 14-3-3 Protein and Its Isoform on Histamine Secretion from Permeabilized Rat Peritoneal Mast Cells

Toshihiro FUJII* and Takayuki UEEDA

Department of Kansei Engineering, Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan. Received May 20, 2002; accepted August 8, 2002

The effect of the 14-3-3 protein, an adaptor protein of intracellular signal pathways, on histamine release from rat peritoneal mast cells was investigated. The exogenous 14-3-3 protein from bovine brain increased the Ca^{2+} -dependent histamine release from permeabilized mast cells, but only slightly affected the non-permeabilized cells. Partial amino acid sequences showed that the bovine brain 14-3-3 protein contained 14-3-3 β , γ and ζ isoforms, and that these recombinant isoforms were prepared. Among them, 14-3-3 ζ was an active species while the 14-3-3 β and γ were inactive for histamine release from the permeabilized mast cells. Approximately 15% of the histamine release was stimulated by 14-3-3 ζ at 2.5 μM , and half-maximal stimulation occurred at 1 μM . Treatment of the mast cells with wortmannin or staurosporine completely inhibited the stimulatory effect on histamine release caused by Ca^{2+} or Ca^{2+} /14-3-3 ζ , and genistein partially inhibited both stimulatory effects. PD 98059, however, had little effect on the histamine release. These results suggest the possibility that 14-3-3 ζ is associated with signal transduction for degranulation of the mast cells.

Key words 14-3-3 ζ ; mast cell; histamine release; permeabilization; calcium; phosphorylation

The 14-3-3 protein is a family of conserved, dimeric proteins with a monomeric molecular mass of about 30 kDa, and is ubiquitously expressed in various mammalian tissues.^{1,2)} At least nine isoforms, including α , β , γ , ϵ , ζ , τ , σ , δ and η , are known, and the isoforms of α and δ are phosphorylated forms of β and ζ , respectively. The 14-3-3 protein has been reported to regulate numerous cellular processes, including signal transduction, exocytosis, cell cycle and apoptosis by interaction with a variety of regulatory proteins controlling signal pathways. The target proteins include tyrosine and tryptophan hydroxylases,^{3,4)} protein kinase C,^{5,6)} Raf-1,⁷⁾ phosphatidylinositol 3-kinase (PI3-kinase),⁸⁾ Cdc25 phosphatase⁹⁾ and a Bcl-2 family, Bad.¹⁰⁾

Mast cells and blood basophils release histamine and other potent inflammatory mediators in response to the cross-linking of IgE receptors under acute allergic and inflammatory conditions.^{11,12)} It is also generally believed that calcium and phosphorylation signalings are closely associated with the exocytosis of mast cells. The function of protein kinase C and Ca^{2+} /calmodulin on the secretory process of mast cells has been examined in detail.^{13,14)} Burgoyne and co-workers reported that 14-3-3 protein stimulated catecholamine release from digitonin-permeabilized adrenal chromaffin cells in a Ca^{2+} -dependent manner.^{15–17)} However, little is known about the effect of 14-3-3 protein on the secretion of mast cells. The use of permeabilized cells has provided a means of identification of soluble proteins and signal transmission pathways that are related to the exocytotic process. Previously, we reported that the smooth muscle calcium-binding protein 11 (calyculin) prevented Ca^{2+} -dependent histamine release from permeabilized rat peritoneal mast cells.¹⁸⁾

In this paper, we report that exogenous 14-3-3 protein can stimulate Ca^{2+} -dependent histamine release from permeabilized mast cells. Among the isoforms of 14-3-3 protein, 14-3-3 ζ was an active species, and its enhancement was completely inhibited by staurosporine and wortmannin.

MATERIALS AND METHODS

Materials Staurosporine and ionophore A23187 were purchased from Sigma. Genistein, wortmannin, PD 98059 and peroxidase-conjugated goat anti-rabbit IgG+IgM immunoglobulins were purchased from Wako Pure Chemical Industry.

Preparation of 14-3-3 Protein The bovine brain 14-3-3 protein was prepared by the method of Morgan and Burgoyne¹⁵⁾ with slight modification. Fresh tissues (200 g) were homogenized in 2 volumes of 20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid, pH 6.5, 139 mM potassium glutamate, 5 mM MgCl_2 , 2 mM EGTA, 2 mM 2-mercaptoethanol (2-ME) and 2 mM ATP using a Waring blender. The suspension was centrifuged at 4000 $\times g$ for 5 min at 4 °C. The obtained supernatants were dialyzed against buffer A (10 mM imidazole-HCl, pH 7.3, 1 mM 2-ME, 1 mM NaN_3 and 1 mM EGTA) and loaded onto a DEAE-Sepharose column ($\phi 3.5 \times 11$ cm) equilibrated with buffer A. Fractions containing the 14-3-3 protein were collected, dialyzed against buffer A + 1 M $(\text{NH}_4)_2\text{SO}_4$ and loaded onto a phenyl-Sepharose 4B column ($\phi 1.5 \times 15$ cm) equilibrated with buffer A + 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with the same buffer, and the bound protein was eluted with a linear 1—0 M $(\text{NH}_4)_2\text{SO}_4$ gradient. The eluted 14-3-3 protein-rich fractions were then collected and dialyzed against buffer A. The sample was applied to a QMA (Waters) column equilibrated with buffer A and then eluted with a linear 0—600 mM NaCl gradient. The 14-3-3 fractions were collected, dialyzed against buffer A + 1 M $(\text{NH}_4)_2\text{SO}_4$ and re-loaded onto a phenyl-Sepharose 4B column ($\phi 0.8 \times 3$ cm) equilibrated with buffer A + 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with the same buffer, and the bound protein was eluted with a linear 1—0 M $(\text{NH}_4)_2\text{SO}_4$ gradient. The eluted 14-3-3 fraction was concentrated and applied to an AcA-54 column ($\phi 3 \times 85$ cm) equilibrated with buffer A containing 100 mM NaCl. The 14-3-3 protein was then collected and stored at -80 °C. The yield of purified 14-3-3 protein was about 1 mg per 100 g of tissues.

The full-length cDNAs of 14-3-3 β , γ and ζ were cloned

* To whom correspondence should be addressed. e-mail: fujii1@giptc.shinshu-u.ac.jp

by reverse transcriptase-polymerase chain reaction (RT-PCR). The total RNA was extracted from the adult rat brain (Wistar) with ISOGEN containing phenol and guanidine isothiocyanate (Nippon Gene KK) according to the manufacturer's protocol. The sense primers of 14-3-3 β , γ and ζ , including the Nde I restriction site, are 5'-AAAGAACCATATGACCATGGACAAA-AGTGA-3', 5'-TTAATTAACATATGTGGACCGGAGCAAC-3' and 5'-TAGAAATCATATGATATAAATGAGCTGG-T-3', respectively. The antisense primers of 14-3-3 β , γ and ζ , including the EcoR I restriction site, are 5'-GGCGAATTCAAAGATCGAAAGTCCACAT-3', 5'-AAAAGAATTCTGTGAGGCTGCTACTCCGAT-3' and 5'-ATTGAATTCAACAAAAAACTATCTGTGGG-3', respectively. The double-stranded cDNAs amplified by RT-PCR (30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min) were inserted into the pAED4 plasmid vector and transformed into *E. coli* (JM109) for amplification.¹⁹ The recombinant DNAs of pAED4-14-3-3 β , γ and ζ were transformed into the *E. coli* host strain BL21 (DE3) and identified by restriction analysis. Each recombinant 14-3-3 isoform was expressed in BL21 (DE3) that was cultured at 37 °C in LB medium containing 0.5 mg/ml ampicillin. After adding isopropyl-1-thio- β -D-galactopyranoside to a final concentration of 0.1 mM, the cells were incubated for another 4 h. Cells were suspended in buffer A and subjected to disruption with a sonicator (30 s $\times$ 30 times). Each 14-3-3 isoform was purified by the previously described method.

Preparation of Mast Cells, Cell Permeabilization and Histamine Release Mast cells were obtained from the abdominal cavities of male rat (Wistar; 300–400 g), as described previously.¹⁸ The content of the mast cells in the preparation examined by toluidine blue staining was greater than 95%. The freshly isolated mast cells were suspended at a density of $5 \times 10^5 \text{ ml}^{-1}$ in mast cell medium (MCM), which was comprised of 144 mM NaCl, 2.7 mM KCl, 4.7 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 5.6 mM glucose, 10 U/ml heparin and 1 mg/ml bovine serum albumin (pH 7), and then used for each experiment. The purified mast cells (2×10^4 /tube) containing 14-3-3 protein were preincubated in 200 μl of MCM for 5 min at 37 °C and permeabilized by the addition of 12.5 $\mu\text{g/ml}$ β -escin for 10 min at 37 °C. The cell suspension was further incubated for 10 min at 37 °C after adding 0.9 mM CaCl_2 . The reactions were stopped by adding 1.8 ml of ice-cold MCM containing 1 mM EGTA. The mast cells were pelleted, and the histamine released into the medium was assayed using *o*-phthalaldehyde as described by Shore *et al.*²⁰ Histamine release from the mast cells (%) was calculated by the following equation: $[(\text{histamine release}) - (\text{spontaneous release})] / (\text{total histamine} - (\text{spontaneous release})) \times 100$.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Immunoblotting Aliquots of the mast cell lysate and 14-3-3 protein were mixed with concentrated SDS–PAGE sample buffer, then boiled and subjected to SDS–PAGE on 12.5% polyacrylamide gels using the Laemmli buffer system.²¹ Proteins were electrotransferred onto a nitrocellulose membrane (Advantec) in a 25 mM Tris, 192 mM glycine and 20% methanol mixture at 100 V for 1 h. The transferred membrane was blocked with 2.5% TBS (25 mM Tris–HCl, pH 7.2, 50 mM NaCl) and immunoblotted with 1 : 1500 polyclonal antibodies to porcine 14-3-3 protein

for 90 min at 37 °C. The membrane was washed 4 times with TBS-T (TBS containing 0.5% Tween-20) and then immunoblotted with 1 : 30000 peroxidase-conjugated secondary antibodies, goat anti-rabbit IgG (H and L) antibody in TBS-T for 90 min at 37 °C. Detection was performed using the Super Signal CL-HRP Substrate System (Pierce).²²

Amino Acid Sequencing For amino acid sequencing, the 14-3-3 protein and its digest were applied onto SDS–PAGE. Proteins on the gel were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. After Ponceau S staining, the proteins on the PVDF membranes were directly applied to a Shimadzu PPSQ-21 Protein Sequencer.

Statistical Analysis Data are expressed as mean $\pm$ S.E.M. Student's two-sided *t*-test was used to examine the significance of difference among groups.

RESULTS AND DISCUSSION

Histamine Release by 14-3-3 Protein Treatment of rat peritoneal mast cells with 12.5 $\mu\text{g/ml}$ β -escin in the presence of 0.9 mM CaCl_2 caused histamine release (about 10%) which compared with the amount in the absence of CaCl_2 (Fig. 1). When bovine brain 14-3-3 protein was incubated with the permeabilized mast cells, the histamine release increased with increasing amounts of protein added in the presence of Ca^{2+} , but not in the presence of EGTA. Furthermore, we observed that 14-3-3 protein had little influence on non-permeabilized cells which were stimulated by the Ca^{2+} plus ionophore A23187, indicating that the 14-3-3 protein functions after incorporation into the mast cells. The stimulation of Ca^{2+} -dependent histamine release reached about 2–2.5-fold of the original level in the absence of 14-3-3 protein at more than 2.5 μM , and half-maximal stimulation was 0.8–1.2 μM .

Analysis of 14-3-3 Isoforms and Preparation of 14-3-3 β , γ and ζ The 14-3-3 protein has been reported to consist of a number of isoforms, including β , γ , ϵ , ζ , η , θ and σ . We examined which of these isoforms are contained in the bovine brain 14-3-3 protein fraction. Since the N terminal

Fig. 1. Histamine Release from Permeabilized Mast Cells by Brain 14-3-3 Protein

Mast cells were preincubated with various concentrations of 14-3-3 protein, as indicated, for 5 min at 37 °C. The cells were permeabilized with 12.5 $\mu\text{g/ml}$ β -escin for 10 min at 37 °C, and then challenged with 0.9 mM CaCl_2 (●) or 0.5 mM EGTA (○) for 10 min. The cells were also stimulated by 0.4 $\mu\text{g/ml}$ ionophore A23187 + 0.9 mM CaCl_2 (▲) at for 10 min 37 °C. Values are expressed as means $\pm$ S.E. of three or four experiments done in duplicate. The molecular weight of the 14-3-3 protein was calculated to be 60000. **p* < 0.05 versus control.

Fig. 2. Western Blot Analysis of Recombinant 14-3-3 Isoforms Using Antibodies against Porcine 14-3-3 Protein

The purified recombinant 14-3-3 β , γ and ζ were separated by 12.5% SDS-PAGE. Proteins were electrophoretically transferred from the gel to a nitrocellulose membrane, then stained with amide black (A) or immunostained with anti-porcine brain 14-3-3 protein antibodies (B). Lane 1, 14-3-3 β ; lane 2, 14-3-3 γ ; lane 3, 14-3-3 ζ . Molecular weight standards in kDa are shown on the left.

Fig. 3. Effects of 14-3-3 Isoforms on Histamine Release from Permeabilized Mast Cells in the Presence and Absence of CaCl_2

Mast cells were preincubated with the recombinant 14-3-3 β (Δ , $\bullet$), γ ($\square$, $\blacksquare$) and ζ ($\circ$, $\blacktriangle$) for 5 min at 37°C. The cells were further permeabilized with 12.5 $\mu\text{g/ml}$ β -escin and challenged with 0.9 mM CaCl_2 ($\bullet$, $\blacktriangle$, $\blacksquare$) or 1 mM EGTA ($\circ$, Δ , $\square$), as shown in the legend of Fig. 1. Values are expressed as means $\pm$ S.E. of three or four experiments done in duplicate. * $p < 0.05$ versus control.

amino acid of the bovine brain 14-3-3 protein was blocked, its fragment with a 25 kDa fragment generated by chymotryptic limited digestion was used for analyzing the partial amino acid sequence by Edman degradation. The 25 kDa fragment begins at R-V(I, V)-S-S-I-E-Q-K-T(E, M, D, S)-(A, G, R, N)-(A, D, N, S)-(E, G)-(K, N)-(K, E)-(K, Q)-(K, Q)-(M, L, I)-(E, A)... which corresponds to the α -helix domain from Arg60-62, as the reported amino acid sequence of 14-3-3 β , γ and ζ isoforms.^{4,23} These data suggest that 14-3-3 β , γ and ζ isoforms, at least, may be members of the bovine brain 4-3-3 protein. Next, we prepared recombinant 14-3-3 β , γ and ζ as described in "Materials and Methods". Immunoblot analysis shows that the three recombinant proteins are cross-reacted with antibodies against porcine brain 14-3-3 protein (Fig. 2). Furthermore, partial amino acid sequence analysis (till 15 amino acids) indicates that each protein is identical to the reported amino acid sequence.²³ We used these proteins in the following experiments.

Effect of 14-3-3 Isoforms on Histamine Release from Permeabilized Mast Cells The histamine release from permeabilized mast cells was examined by adding exogenous 14-3-3 isoforms (Fig. 3). Interestingly, 14-3-3 ζ showed a

Fig. 4. Effect of Kinase Inhibitors on Ca^{2+} - and 14-3-3 ζ -dependent Histamine Release from Permeabilized Mast Cells

Mast cells were preincubated with 14-3-3 ζ (2.5 μM) and the indicated drugs (50 μM) for 5 min at 37°C in the presence of 0.9 mM CaCl_2 . The cells were permeabilized with 12.5 $\mu\text{g/ml}$ β -escin and challenged with 0.9 mM CaCl_2 , as shown in the legend of Fig. 1. $\square$, 0.9 mM CaCl_2 ; $\blacksquare$, 0.9 mM CaCl_2 + 2.5 μM 14-3-3 ζ . C, control; S, staurosporine; G, genistein; W, wortmannin; P, PD 98059. Values are expressed as means $\pm$ S.E. of three experiments done in duplicate. * $p < 0.05$ versus controls.

stimulatory effect on histamine release in the presence of CaCl_2 , but not in the presence of EGTA. On the other hand, both 14-3-3 β and γ had little effect on histamine release in the presence and absence of CaCl_2 . The maximal release by 14-3-3 ζ reached about 40% (substantial amount of 32%) of the total histamine content in the mast cells, and half-maximal release occurred at 1 μM . These values were in good accord with those obtained by brain 14-3-3 protein, as shown in Fig. 1. We postulated that bovine the brain 14-3-3 protein used was a mixture of homo- and hetero-dimeric molecules, while the recombinant 14-3-3 isoforms are individual homodimers. These may affect the different actions on the secretory processes in mast cells.

Effect of Drugs on 14-3-3 ζ -dependent Histamine Release The interactions of 14-3-3 protein with Raf-1, c-Bcr, Cb1, tyrosine hydroxylase and tryptophan hydroxylase were regulated by phosphorylation and dephosphorylation.^{2,23,24} Aitken *et al.*²⁵ have shown that phosphorylated 14-3-3 ζ , as well as 14-3-3 β , exhibits increased potency in the inhibition of the protein kinase C activity. It is also interesting to note that recombinant 14-3-3 ζ is essential for exhibiting Raf kinase activity, and Raf kinase is certainly present in the mast cells.^{24,26}

To determine whether phosphorylation and dephosphorylation were involved in the stimulatory release of histamine by 14-3-3 ζ , mast cells were preincubated with or without kinase inhibitors, such as staurosporine (for serine/threonine kinases), genistein (for tyrosine kinases), wortmannin (for PI3-kinase) and PD 98059 (for mitogen-activated protein kinase kinase) for 5 min at 37°C. After permeabilization by adding β -escin, the mast cells were further incubated in the presence of CaCl_2 for 10 min at 37°C. The Ca^{2+} -dependent histamine release from permeabilized mast cells was suppressed by staurosporine (50 μM) and wortmannin (50 μM). Furthermore, the stimulatory histamine release by 14-3-3 ζ was completely decreased to the original level in the absence of 14-3-3 ζ , when staurosporine or wortmannin was present. The action of 14-3-3 ζ is thus phosphorylation-dependent. Furthermore, 14-3-3 protein has been reported to directly interact with PI3 kinase and inhibits activity in T cells.²⁷ The

addition of genistein, which partially inhibits histamine release induced by Ca^{2+} , did not affect Ca^{2+} plus 14-3-3 ζ -dependent histamine release. PD 98059 had little effect on both histamine releasing conditions. These results suggest that 14-3-3 ζ affects the PI3 kinase related signal transduction pathways other than the mitogen-activated protein kinase pathways, and leads to histamine release at higher intracellular Ca^{2+} concentrations.

Acknowledgments We thank Dr. C. Itagaki (Department of Biochemistry, Faculty of Science, Niigata University) for providing the anti-14-3-3 polyclonal antibodies. We also wish to thank Drs. M. Okazaki and G. Taguchi (Gene Research Center, Shinshu University) for helpful suggestions and discussion related to this work. This study was supported by Hokuto Foundation for Bioscience and Grants-in-Aid for COE Research (H10CE2003) and Science Research (C) (14593003) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

- 1) Fu H., Subramanian R. R., Masters S. C., *Annu. Rev. Pharmacol. Toxicol.*, **40**, 617—647 (2000).
- 2) Tzivion G., Avruch J., *J. Biol. Chem.*, **277**, 3061—3064 (2002).
- 3) Ichimura T., Isobe T., Okuyama T., Yamauchi T., Fujisawa H., *FEBS Lett.*, **219**, 79—82 (1987).
- 4) Ichimura T., Isobe T., Okuyama T., Takahashi N., Araki K., Kuwano R., Takahashi Y., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 7084—7088 (1988).
- 5) Toker A., Ellis C. A., Sellers L. A., Aitken A., *Eur. J. Biochem.*, **191**, 421—429 (1990).
- 6) Furukawa Y., Ikuta N., Omata S., Yamauchi T., Isobe T., Ichimura T., *Biochem. Biophys. Res. Commun.*, **194**, 144—149 (1993).
- 7) Fu H., Xia K., Pallas D. C., Cui C., Conroy K., Narsimhan R. P., Mamon H., Collier R. J., Roberts T. M., *Science*, **266**, 126—129 (1994).
- 8) Bonnefoy-Berard N., Liu Y. C., von Willebrand M., Sung A., Elly C., Mustelin T., Yoshida H., Ishizaka K., Altman A., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 10142—10146 (1995).
- 9) Conklin D. S., Galaktionov K., Beach D., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 7892—7896 (1995).
- 10) Zha J., Harada H., Yang E., Jockel J., Korsmeyer S. J., *Cell*, **87**, 619—628 (1996).
- 11) Grant J. A., Dupree E., Goldman A. S., Schultz D. R., Jackson A. L., *J. Immunol.*, **114**, 1101—1106 (1975).
- 12) Ishizaka T., Ishizaka K., *Prog. Allergy*, **34**, 188—235 (1984).
- 13) Izushi K., Tasaka K., *Pharmacology*, **44**, 61—70 (1992).
- 14) Razin E., Szallasi Z., Kazanietz M. G., Blumberg P. M., Rivera J., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 7722—7726 (1994).
- 15) Morgan A., Burgoyne R. D., *Nature (London)*, **355**, 833—836 (1992).
- 16) Roth D., Morgan A., Burgoyne R. D., *FEBS Lett.*, **320**, 207—210 (1993).
- 17) Roth D., Burgoyne R. D., *FEBS Lett.*, **374**, 77—81 (1995).
- 18) Fujii T., Kuzumaki N., Ogoma Y., Kondo Y., *Biol. Pharm. Bull.*, **17**, 581—585 (1994).
- 19) Fujii T., Nakamura K., Ootani H., Koizumi Y., Takahashi K., *J. Biochem. Mol. Biol. & Biophys.*, **5**, 399—405 (2001).
- 20) Shore P. A., Burkhalter A., Cohn V. H., *J. Pharmacol. Exp. Ther.*, **127**, 182—186 (1959).
- 21) Laemmli U. K., *Nature (London)*, **227**, 680—685 (1970).
- 22) Nakamura A., Arimoto M., Fujii T., *Biol. Pharm. Bull.*, **25**, 569—572 (2002).
- 23) Isobe T., Hiyane Y., Ichimura T., Okuyama T., Takahashi N., Nakajo S., Nakaya K., *FEBS Lett.*, **308**, 121—124 (1992).
- 24) Aitken A., Howell S., Jones D., Madrazo J., Martin H., Patel Y., Robinson K., *Mol. Cell Biochem.*, **149—150**, 41—49 (1995).
- 25) Tzivion G., Luo Z., Avruch J., *Nature (London)*, **394**, 88—92 (1998).
- 26) Hirasawa N., Santini F., Beaven M. A., *J. Immunol.*, **154**, 5391—5402 (1995).
- 27) Bonnefoy-Berard N., Liu Y. C., von Willebrand M., Sung A., Elly C., Mustelin T., Yoshida H., Ishizaka K., Altman A., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 10142—10146 (1995).