Volume 314, number 3, 241–245 FEBS 11872 December 1992

Phosphorylation of smg p21B in rat peritoneal mast cells in association with histamine release inhibition by dibutyryl-cAMP

Keiji Izushi, Taihei Shirasaka, Manabu Chokki and Kenji Tasaka

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700, Japan

Received 26 October 1992

IP_3 formation and histamine release from rat peritoneal mast cells stimulated by compound 48/80 were dose-dependently inhibited by Bt_2cAMP. These inhibitions were restored to the control level in the presence of H-8, a protein kinase A inhibitor. The 22 kDa protein in mast cells was revealed as a markedly phosphorylated protein by incubating with Bt_2cAMP, and this phosphorylation was also diminished by H-8. The 22 kDa phosphoprotein of rat mast cells comigrated with phosphorylated smg p21B, purified from human platelets and phosphorylated by protein kinase A in cell-free system, in both one- and two-dimensional PAGE analysis. Moreover, 22 kDa protein in mast cells was identified as smg p21B by immunoblot analysis using an antibody against smg p21B. From the present study, it became clear that smg p21B is phosphorylated by means of protein kinase A system in rat peritoneal mast cells, and it was assumed that phosphorylated smg p21B plays some important role in the suppression of IP_3 formation and histamine release from rat peritoneal mast cells.

smg p21B; Protein phosphorylation; Protein kinase A; Histamine release; Rat mast cell

1. INTRODUCTION

It is known that protein phosphorylation plays some critical roles in the regulation of various cellular responses and functions. So far, many protein kinases have been identified, and protein kinase A and C in particular have been shown to be involved in a wide variety of cell functions [1,2]. We first demonstrated the existence of vimentin, an intermediate filament protein, in rat peritoneal mast cells, and that this protein was phosphorylated after stimulation with some histamine releasers. Further, we found that this phosphorylation proceeded in the presence of protein kinase C [3]. By contrast, the substrate protein(s) of protein kinase A and its physiological functions in rat mast cells remains to be identified. In platelets, prostacyclin has been shown to increase the cAMP level through stimulation of adenylate cyclase, which is effective in leading the inhibition of platelet functions [4]. Moreover, the activation of protein kinase A elicited by increased intracellular cAMP increases the phosphorylation of several proteins having molecular weights of 240, 50, 24 and 22 kDa [5,6].

In rat peritoneal mast cells, it is known that an increase in cAMP level is effective in inhibiting histamine release [1,7,8]. One possible mechanism of cAMP in inhibitory reaction is assumed to be prevention of Ca^{2+} release from intracellular Ca store [9]. Although the inhibitory effect of cAMP on histamine release from rat mast cells is exerted in association with the activation of protein kinase A, the exact mechanism of the interaction between cAMP and protein kinase A is not known. To clarify this point, the present investigation was carried out.

Correspondence address: K. Tasaka, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700, Japan. Fax: (81) (862) 55-7456.

Abbreviations: protein kinase A, cyclic AMP-dependent protein kinase; protein kinase C, Ca^{2+}/phospholipid-dependent protein kinase; G protein, GTP-binding protein; Bt_2cAMP, $N^0,O^{2'}$-dibutyryl cyclic AMP; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; GDS, GDP dissociation stimulator; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PGE_1, prostaglandin E_1.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

The catalytic subunit of protein kinase A was purified from bovine heart according to the method of Beavo et al. [10]. smg p21B was purified to near homogeneity from human platelet membranes [11]. Compound 48/80 and Bt_2cAMP were obtained from Sigma Chemical Co. (St Louis, MO, USA). ATP and H-8 were purchased from Yamasa Shoyu Co. (Chiba, Japan) and Seikagaku Kogyo Co. (Tokyo, Japan), respectively. [^{32}P]Orthophosphoric acid (specific activity 370 TBq/ mmol) and [γ-^{32}P]ATP (specific activity 222 TBq/mmol) were purchased from DuPont/New England Nuclear and Amersham Corp., respectively. Other materials and chemicals were obtained from commercial sources.

2.2. *Histamine release from rat peritoneal mast cells*

Rat peritoneal mast cells were isolated from the abdominal cavity of male Wistar rats (300–400 g) to more than 95% homogeneity as previously described [12]. The mast cells were suspended in physiological salt solution (PSS) consisting of 154 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 10 mM HEPES/NaOH (pH 7.4) and 5.6 mM glucose. The cell suspension (5×10^4 cells/100 μl) was incubated for 5 min at 37°C

and 100 μl of test drugs were added. After incubation with test drugs for 15 min at 37°C, 50 μl of compound 48/80 were added to make a final concentration of 0.5 μg/ml and the incubation proceeded for 10 min at 37°C. The amount of histamine in the supernatant and residual histamine in the cells were measured by automated fluorometric assay [13].

2.3. *Measurement of IP_3 content in rat peritoneal mast cells*

Rat mast cells (3×10^6 cells/100 μl) were treated with test drugs for 15 min at 37°C, and then the cells were stimulated with compound 48/80 for 5 s as previously described [14]. The reaction was terminated by the addition of 50 μl of 30% trichloroacetic acid. The mixture was placed on ice for 30 min and centrifuged at 15,000 rpm for 10 min at 4°C to remove the acid-insoluble materials. The supernatant (250 μl) was neutralized with an equal volume of ice-cold Freon-amine [12]. IP_3 contents in the aqueous upper phase were measured by means of IP_3 assay kit (Amersham).

2.4. *Protein phosphorylation in rat peritoneal mast cells*

Purified rat mast cells were labeled with ^{32}P (0.5 mCi/ml) in phosphate-free Dulbecco's modified Eagle's medium (Nissui, Japan) for 30 min at 37°C, and washed twice with PSS as previously described [3]. ^{32}P-labeled rat mast cells (10^6 cells/100 μl) were incubated for 5 min at 37°C, and then, 100 μl of test drugs were added and incubated for another 15 min. The reaction was terminated by the addition of an equal volume of SDS-sample buffer containing 150 mM Tris-HCl (pH 6.8), 4% SDS, 4% 2-mercaptoethanol, 20% glycerol and 0.02% Bromophenol blue, and the mixture was heated for 3 min in a boiling water bath. The sample was subjected to SDS-PAGE using 10–20% polyacrylamide gradient slab gel (Daiichi Pure Chemicals Co., Japan) [15]. After electrophoresis, the gel was stained with Coomassie brilliant blue, destained, dried and exposed to an X-ray film (Hyperfilm-MR, Amersham). The autoradiograms were scanned with a laser densitometer (Molecular Dynamics) to quantify the relative radioactivity.

For two-dimensional PAGE, the reaction was stopped by the addition of an equal volume of an isoelectric focusing lysis buffer containing 9 M urea, 2% (w/v) Nonidet P-40, 2% (v/v) ampholytes (pH range 3.5–10) and 5% 2-mercaptoethanol. This sample was subjected to two-dimensional PAGE by the method of O'Farrell [16] using 2% (v/v) ampholytes (pH range 3.5–10) in the first dimension and 10–20% polyacrylamide gradient gel in the second dimension.

2.5. *Protein phosphorylation of smg p21B in cell-free system*

Phosphorylation of smg p21B (220 ng of protein) was carried out by the catalytic subunit of protein kinase A (50 ng of protein) in a reaction mixture (50 μl) containing 50 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 4 mM dithiothreitol, 100 μg/ml bovine serum albumin, 0.15% sodium cholate and 25 μM [γ-^{32}P]ATP (0.1 μCi/50 μl) for 10 min at 30°C. The reaction was terminated by the addition of an equal volume of SDS-sample buffer or isoelectric focusing lysis buffer as described above. The samples were subjected to one-dimensional or two-dimensional PAGE, followed by autoradiography.

2.6. *Immunological detection of smg p21B*

Rat peritoneal mast cells (10^7 cells) were suspended in 50 μl of 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA and 1 mM PMSF, and incubated for 30 min at 4°C. The detergent-soluble mast cell proteins and purified smg p21B (15 ng of protein) were subjected to SDS-PAGE, and were electrophoretically transferred from gel to a polyvinylidene difluoride membrane (Immobilon, Millipore). The membrane was incubated for 2 h at 37°C in blocking solution (phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 3% skim milk (Difco)). Then, the membrane was incubated with anti-smg p21B antiserum diluted 1:150 in blocking solution for 2 h at 37°C. After the membrane was washed three times with PBS containing 0.1% Tween 20, the incubation was continued with biotinylated goat anti-rabbit IgG (Vector). The immuno-reactive protein was detected with an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector).

3. RESULTS

Rat peritoneal mast cells were incubated with Bt_2cAMP and then stimulated by 0.5 μg/ml of compound 48/80. In control cells, compound 48/80 (0.5 μg/ml) released about 50% of the total histamine, and the amount of IP_3 formed within 5 s increased to approximately 9 times that of the resting cells. As shown in Fig. 1A, Bt_2cAMP induced a dose-dependent inhibition of histamine release from rat peritoneal mast cells. At a concentration of 1 mM, Bt_2cAMP inhibited histamine release by approximately 70%. In order to examine whether or not this inhibitory effect of cAMP is exerted in association with the activation of protein kinase A, the effect of H-8, a protein kinase A inhibitor [17], was investigated. When various concentrations of H-8 were added with 1 mM of Bt_2cAMP during the preincubation period, Bt_2cAMP-induced histamine release inhibition was dose-dependently restored by H-8, and the maximum effect of H-8 was reached at 100 μM (Fig. 1B). When mast cells were exposed to compound 48/80 for 5 s, the intracellular content of IP_3 increased to approx-

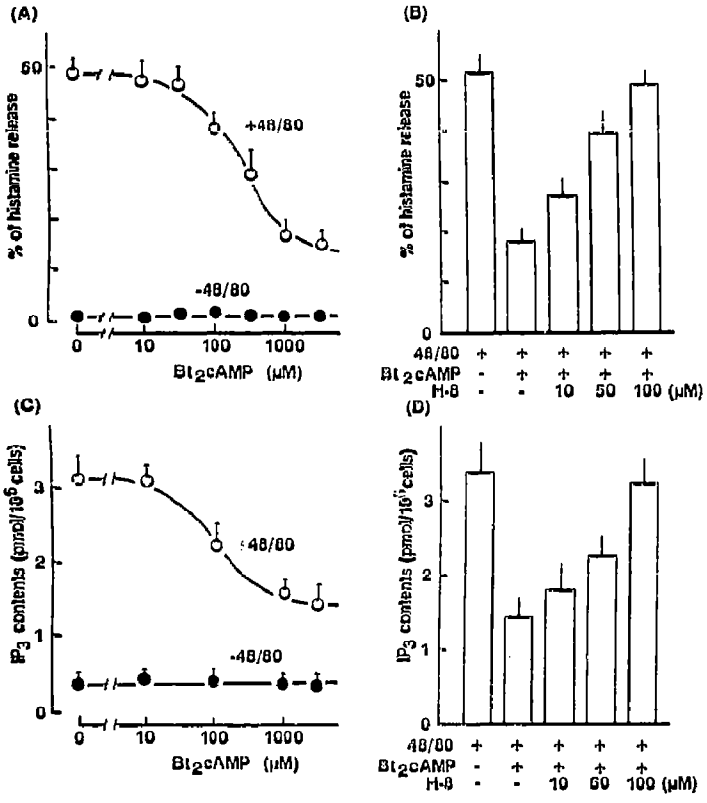

Fig. 1. Effects of Bt_2cAMP and H-8, a protein kinase A inhibitor, on histamine release and IP_3 formation in rat peritoneal mast cells. Histamine release was elicited by compound 48/80 (0.5 μg/ml) for 10 min. (A) The inhibitory effect of Bt_2cAMP on histamine release from rat mast cells. (B) The restoring effect of H-8 on Bt_2cAMP-induced histamine release inhibition. (C) The inhibitory effect of Bt_2cAMP on IP_3 formation in rat mast cells. (D) The restoring effect of H-8 on Bt_2 CAMP-induced inhibition of IP_3 formation. The results indicate the means ± SEM ($n = 4$).

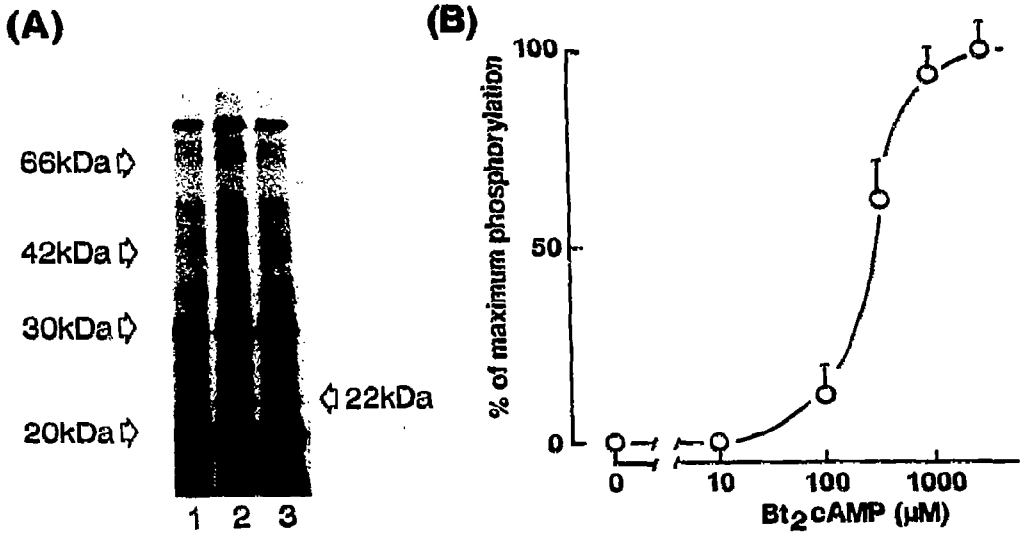

Fig. 2. Protein phosphorylation induced by Bt_2cAMP in rat mast cells. (A) Protein phosphorylation in rat mast cells. (Lane 1) Untreated control; (lane 2) rat mast cells were treated with 1 mM of Bt_2cAMP; (lane 3) rat mast cells were treated with 1 mM of Bt_2cAMP plus 100 μM of H-8. (B) Dose-dependent phosphorylation of 22 kDa protein in rat mast cells induced by Bt_2cAMP. The results indicate the means ±SEM (n = 4).

imately 9 times that of the control. The amount of IP_3 formed by compound 48/80 was also inhibited by pretreatment with Bt_2cAMP at the same concentration range (Fig. 1C). Moreover, Bt_2cAMP-induced inhibition of IP_3 formation was dose-dependently restored by the presence of H-8 as in the case of histamine release (Fig. 1D). These results seem to suggest that the inhibitory effects of cAMP in mast cells might be related to both the amount of activated protein kinase A and the amount of target protein phosphorylated by protein kinase A.

Next, protein phosphorylation in rat peritoneal mast cells exposed to Bt_2cAMP was investigated. As shown in Fig. 2A, when rat mast cells were incubated with 1 mM of Bt_2cAMP, several proteins were phosphorylated. In particular, 22 kDa protein was markedly phosphorylated, as shown in Fig. 2A (lane 2). However, phosphorylation of 22 kDa protein was clearly inhibited in the presence of 100 μM of H-8 (Fig. 2A, lanes 3). Bt_2cAMP-induced phosphorylation of 22 kDa protein increased in a dose-dependent manner (Fig. 2B) and reached a maximum at 1 mM of Bt_2cAMP. The concentration of Bt_2cAMP necessary for protein phosphorylation was the same as the concentration of Bt_2cAMP required in inhibiting histamine release and IP_3 formation, as shown in Fig. 1. The same molecular weight (22 kDa) of phosphorylated protein has been reported in platelet [5,6] and this was identified as smg p21, a ras-related small molecular weight G protein [6]. But, it is not known whether or not smg p21B exists in rat peritoneal mast cells. As shown in Fig. 3A, smg p21B was detected as a single protein band (lane 1) when rat mast cell proteins were tested in the immunoblot analysis using anti-smg p21B antiserum. This protein band had the same molecular weight as that of purified human platelet smg p21B (Fig. 3A, lane 2). The 22 kDa protein phosphorylated in response to Bt_2cAMP in rat mast cells had the same molecular weight as the smg p21B phosphorylated by protein kinase A in cell-free system (Fig. 3B, lane 1 and 2). When these two were mixed and subjected to SDS-PAGE, two proteins comigrated to the same position (Fig. 3B, lane 3). To confirm whether or not these two proteins are identical, the 22 kDa phosphorylated protein in rat mast cells and phosphorylated smg p21B were subjected to two-dimensional PAGE [16]. One radioactive spot was observed as phosphorylated smg p21B (Fig. 4A). Fig. 4C shows the autoradiogram of the protein phosphorylation in rat peritoneal mast cells induced by 1 mM of Bt_2cAMP. The phosphorylated 22 kDa protein

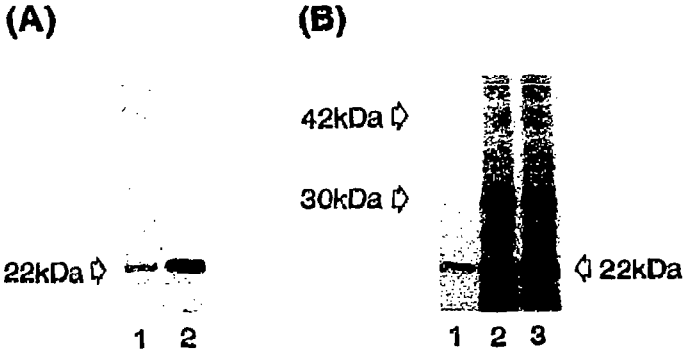

Fig. 3. Immunoblot analysis of smg p21B and Bt_2cAMP-induced protein phosphorylation of rat mast cells. (A) Lane 1, rat mast cell proteins; lane 2, purified human platelet smg p21B. (B) Lane 1, phosphorylated smg p21B by protein kinase A; lane 2, phosphorylated proteins in rat mast cells elicited by 1 mM of Bt_2cAMP; lane 3, phosphorylated proteins in rat mast cells plus phosphorylated smg p21B.

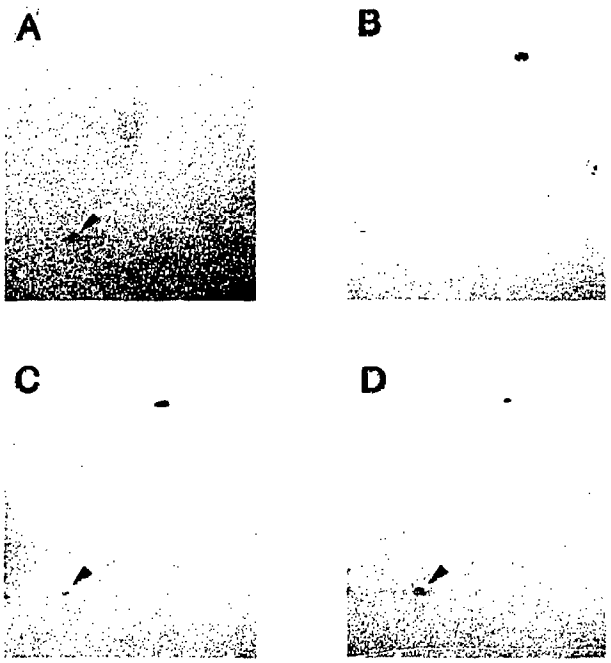

Fig. 4. Two-dimensional PAGE analysis of protein phosphorylation by Bt_2cAMP in rat mast cells. (A) smg p21B phosphorylated by protein kinase A. (B) Protein phosphorylation in rat mast cells in the absence of Bt_2cAMP. (C) Protein phosphorylation in rat mast cells induced by 1 mM of Bt_2cAMP. (D) Phosphorylated protein in rat mast cells elicited by Bt_2cAMP plus phosphorylated smg p21B by protein kinase A. Arrows indicate phosphorylated smg p21B (A), the 22 kDa phosphorylated protein in rat mast cells (C) and the mixture (D: A plus C).

appeared as one spot and its position was the same as that of phosphorylated smg p21B. When phosphorylated 22 kDa protein in mast cells and phosphorylated smg p21B were mixed and subjected to two-dimensional PAGE, these two proteins comigrated together showing exactly the same molecular weight and the same isoelectric point (Fig. 4D). These results clearly indicate that smg p21B is located in rat peritoneal mast cells, and that 22 kDa protein, which is phosphorylated by protein kinase A in rat mast cells in the presence of increased cAMP level, is 'smg p21B'.

4. DISCUSSION

It is known that when rat mast cells are treated so as to increase intracellular cAMP contents, histamine release is significantly inhibited [1,7,8]. We also reported that intracellular Ca^{2+} concentrations and histamine release induced by histamine releasers were inhibited by pretreatment with Bt_2cAMP [18]. Moreover, an increase in IP_3 formation elicited by compound 48/80 was also inhibited by pretreatment with Bt_2cAMP (Fig. 1C). As shown in Fig. 1B and D, it is clear that the inhibitory effects of cAMP on histamine release and on IP_3 formation take place in association with protein kinase A activation, since H-8 was effective in reversing these inhibitory reactions. So far, in rat peritoneal mast cells the direct substrate protein(s) for protein kinase A remain to be identified. We found that 22 kDa protein was markedly phosphorylated by treatment with Bt_2cAMP (Fig. 2A and B). However, this phosphorylated protein was not detected in either compound 48/80- or substance P-stimulated rat peritoneal mast cells [3]. In human platelets, four proteins of 240, 50, 24 and 22 kDa have been shown to be phosphorylated in response to PGE_1, a cAMP-elevating agent, and 22 kDa phosphorylated protein was estimated as smg p21B [6]. As shown in one- and two-dimensional PAGE (Figs. 3B and 4), 22 kDa protein phosphorylated in response to Bt_2cAMP in rat peritoneal mast cells comigrates with purified smg p21B; this seems to indicate that these two proteins have the same molecular weights and isoelectric points. Moreover, smg p21B exists in rat peritoneal mast cells as indicated by the immunoblot analysis using anti-smg p21B antiserum (Fig. 3A). Protein kinase A phosphorylates smg p21B mainly at Ser^{179}, which is located between the polybasic region and the geranylgeranylated cysteine residue in the C-terminal region [19]. This C-terminal region is essential to interact with the stimulatory GDP/GTP exchange protein named smg GDS [20]. When smg p21B was phosphorylated by protein kinase A, it markedly increased the action of smg GDS and initiated the conversion from GDP-bound inactive form to the GTP-bound active form [19]. It is known that smg p21B has the same putative effector domain and consensus C-terminal sequences as the ras gene products [11,20]. Therefore, it is possible to assume that smg p21B exerts actions similar to, or antagonistic to, those of the ras gene products [6,21].

Wakelam et al. showed that normal $p21^{N\text{-}ras}$ might be involved in the regulation of receptor-linked phospholipase C activation without affecting the increase of receptor numbers [22]. Lapetina and Reep also reported that ras-like 29 kDa G protein might regulate phospholipase C activity in human platelets [23]. Moreover, the activation of PIP_2-specific phospholipase C was prompted by the addition of small molecular weight G protein [24,25]. It has been reported that IP_3 formation in human platelets was inhibited by pretreatment with prostacyclin, a potent adenylate cyclase activator [4]. IP_3 formation, induced by compound 48/80 in rat mast cells, was also inhibited by the pretreatment with Bt_2cAMP (Fig. 1C), and this inhibitory action was restored by H-8 in a dose-dependent fashion (Fig. 1D). IP_3 promotes Ca^{2+} release from intracellular Ca^{2+} store and released Ca^{2+} is essential for triggering histamine release from rat peritoneal mast cells [9,12,18]. The inhibitory effects (to histamine release and IP_3 formation) of Bt_2cAMP and 22 kDa protein phosphorylation were induced at the same concentration range of Bt_2cAMP.

It is possible that smg p21B is phosphorylated by protein kinase A in response to increased intracellular cAMP levels, and that at least some parts of the inhibitory actions of protein kinase A system in rat mast cells are exerted by smg p21B. Although it is not known whether phospholipase C in mast cells is also regulated by small molecular weight G protein, it can be assumed that phosphorylated smg p21B is useful to convert GDP-bound inactive form to GTP-bound active form, and consequently, it may inhibit phospholipase C activation by the antagonistic action to phospholipase C-activated G protein.

In this experiment, we first demonstrated that smg p21B exists in rat peritoneal mast cells and that it is phosphorylated by activation of protein kinase A. Further, it was assumed that smg p21B may play some important role in inhibiting histamine release from rat peritoneal mast cells.

Acknowledgements: We are grateful to Drs. Y. Takai and K. Kaibuchi (Department of Biochemistry, Kobe University School of Medicine, Kobe, Japan) for kindly supply of anti-smg p21B rabbit antiserum and for helpful discussions.

REFERENCES

[1] Nishizuka, Y. (1986) Science 233, 305–312.
[2] Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) Annu. Rev. Biochem. 56, 567–613.
[3] Izushi, K., Fujiwara, Y. and Tasaka, K. (1992) Immunopharmacology 23, 153–161.
[4] Watson, S.P., McConnell, R.T. and Kapetina, E.G. (1984) J. Biol. Chem. 259, 13199–13203.
[5] Haslam, R.J., Davidson, M.L., Davies, T., Lynham, J.A. and McClenaghan, M.D. (1978) Adv. Cyclic Nucleotide Res. 9, 533–552.
[6] Kawata, M., Kikuchi, A., Hoshijima, M., Yamamoto, K., Hashimoto, E., Yamamura, H. and Takai, Y. (1989) J. Biol. Chem. 264, 15688–15695.
[7] Foreman, J.C. (1980) Trends Pharmacol. Sci. 1, 460–462.
[8] Alm, P.E. and Bloom, G.D. (1982) Life Sci. 30, 213–218.
[9] Yoshii, N., Mio, M. and Tasaka, K. (1988) Immunopharmacology 16, 107–113.
[10] Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) Methods Enzymol. 38, 2925.
[11] Ohmori, T., Kikuchi, A., Yamamoto, K., Kim, S. and Takai, Y. (1989) J. Biol. Chem. 264, 1877–1881.
[12] Izushi, K. and Tasaka, K. (1991) Pharmacology 42, 297–308.
[13] Siraganian, R.P. (1974) Anal. Biochem. 57, 383–392.
[14] Tasaka, K., Mio, M., Fujisawa, K. and Aoki, I. (1991) Biochem. Pharmacol. 41, 1031–1037.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[17] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[18] Mio, M., Izushi, K. and Tasaka, K. (1991) Immunopharmacology 22, 59–66.
[19] Hata, Y., Kaibuchi, K., Kawamura, S., Hiroyoshi, M., Shirataki, H. and Takai, Y. (1991) J. Biol. Chem. 266, 6571–6577.
[20] Yamamoto, T., Kaibuchi, K., Mizuno, T., Hiroyoshi, M., Shirataki, H., and Takai, Y. (1990) J. Biol. Chem. 265, 16626–16634.
[21] Ohmori, T., Kikuchi, A., Yamamoto, K., Kawata, M., Kondo, J. and Takai, Y. (1988) Biochem. Biophys. Res. Commun. 157, 670–676.
[22] Wakelam, M.J., Davies, S.A., Houslay, M.D., McKay, I., Marshall, C.J. and Hall, A. (1986) Nature 323, 173–176.
[23] Lapetina, E.G. and Reep, B.R. (1986) Proc. Natl. Acad. Sci. USA 84, 2261–2265.
[24] Baldassare, J.J., Knipp, M.A., Henderson, P.A. and Fisher, G.J. (1988) Biochem. Biophys. Res. Commun. 154, 351–357.
[25] Wang, P., Nishihata, J., Takabori, E., Yamamoto, K., Toyoshima, S. and Osawa, T. (1989) J. Biochem. 105, 461–466.