Differential sensitivity of anti-IgM-induced and NaF-induced inositol phospholipid metabolism to serine protease inhibitors in BAL17 B lymphoma cells

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A BAL17 B lymphoma cell line bearing μ and δ chains on its surface behaves in a similar manner to normal mature B cells in terms of initial biochemical transmembrane signalling [Mizuguchi, Beaven, Ohara & Paul (1986) *J. Immunol.* **137**, 2162–2167; Mizuguchi, Yong-Yong, Nakabayashi, Huang, Beaven, Chused & Paul (1987) *J. Immunol.* **139**, 1054–1059]. Therefore the effects of protease inhibitors on increases in inositol phospholipid metabolism and intracellular free calcium concentration ($[Ca^{2+}]_i$) were examined. We show that the serine protease inhibitors Tos-Phe-CH₂Cl (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-, TPCK) and Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one; TLCK) inhibit anti-IgM-mediated accumulation of inositol phosphates in a dose-dependent manner. InsP₃ production induced by anti-IgM is also inhibited by pretreatment with Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl. Tos-Lys-CH₂Cl- or Tos-Phe-CH₂Cl-mediated inhibition is not overcome by high concentrations of anti-IgM. Moreover, anti-IgM-mediated increases in $[Ca^{2+}]_i$ are inhibited by pretreatment of the cells with these inhibitors. However, increases in inositol phospholipid metabolism caused by NaF, an activator of guanine-nucleotide-binding proteins (G-proteins), are approx. 10-fold more resistant to Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl inhibition compared with anti-IgM-induced changes. Furthermore, NaF-induced increases in $[Ca^{2+}]_i$ are not inhibited by Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl pretreatment, suggesting that the inhibitors act at a step proximal to phospholipase C activation. The Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl treatment does not change the membrane IgM density as measured by flow cytometry, indicating that the active site of the inhibitors is distal to the membrane IgM molecule. These results indicate that serine proteases may be involved in coupling the receptor cross-linkage to G-protein.

INTRODUCTION

Receptor–ligand interactions have been shown to induce inositol phospholipid hydrolysis, probably through activation of phospholipase C, in various cell types (Berridge, 1987). Murine B cells also show PtdIns-(4,5)P₂ hydrolysis after stimulation with anti-Ig antibodies to yield InsP₃ and diacylglycerol (Mizuguchi *et al.*, 1987; Cambier & Ransom, 1987). Ins(1,4,5)P₃ causes an increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) by releasing Ca²⁺ from endoplasmic reticulum, and diacylglycerol stimulates protein kinase C (Berridge, 1987; Nishizuka, 1987). Guanine-nucleotide-binding (G) proteins have recently been proposed to be involved in the coupling of receptor cross-linkage of membrane Ig to increased inositol phospholipid metabolism in B cells as well as in other cell types (Gold *et al.*, 1987; Harris & Cambier, 1987; Harnett & Klaus, 1988; Casey & Gilman, 1988). Thus, Gold *et al.* (1987) showed that anti-Ig acts synergistically with guanosine 5'-[γ -thio]triphosphate (GTP[S]) to cause inositol phospholipid hydrolysis in permeabilized B cells. In agreement with these findings, we have demonstrated that NaF, an activator of G-

proteins, causes increases in inositol phospholipid metabolism in B cells (Mizuguchi *et al.*, 1987). These early biochemical events appear to be associated with biologically important events such as the enhancement of class II molecule expression, entry into G₁ and S phase induced by receptor cross-linkage of membrane Ig (Mizuguchi *et al.*, 1986a,b; Klaus *et al.*, 1987).

Participation of protease is suggested in initial triggering in some cell types (Kishimoto *et al.*, 1979; Lerner *et al.*, 1982; Ku *et al.*, 1983; Duque *et al.*, 1983; Utsunomiya & Nakanishi, 1986). Thus, Kishimoto *et al.* (1979) have shown that anti-Ig-stimulated serine protease is responsible for the generation of cytoplasmic factors, leading to the increased phosphorylation of non-histone nuclear proteins in rabbit B cells. Duque *et al.* (1983) showed that membrane depolarization is inhibited by Tos-Phe-CH₂Cl (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one; TPCK) in neutrophils. To analyse the molecular mechanism of serine protease action in B cells, we took advantage of the fact that NaF stimulates B cells to induce an increase in inositol phospholipid metabolism, probably through the activation of G-protein.

In this paper, we show that the serine protease

Abbreviations used: Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (TLCK); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK); $[Ca^{2+}]_i$, intracellular free calcium concentration; G-protein, guanine-nucleotide-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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inhibitors Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one; TLCK) inhibit the anti-IgM-mediated increases in inositol phospholipid metabolism, whereas NaF-induced increases are fairly resistant to these inhibitors. This indicates that serine protease is involved in receptor-mediated transmembrane signalling in B cells. The role of proteases in B cell activation will be discussed.

MATERIALS AND METHODS

B lymphoma cells

BAL17 B lymphoma cells, $\mu^+\delta^+\kappa^+$, were maintained in a culture medium consisted of RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% (w/v) fetal calf serum, kanamycin (50 μ g/ml), L-glutamine (2 mM) and 2-mercaptoethanol (50 μ M). BAL17 cells also express Fc receptors and class II molecules.

Reagents

Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl were obtained from Nakarai Chemicals (Kyoto, Japan); and NaF and AlCl₃ were from Wako Pure Chemical Industries (Osaka, Japan). Goat-anti-(IgM heavy chain) specific antibody (anti-IgM) was prepared as described (Sieckmann *et al.*, 1978) and digested by pepsin at pH 4.0, 37 °C to make the F(ab')₂ fragment. Goat anti-IgG_{2a} antibody was obtained from Southern Biotechnology Associates (Birmingham, AL, U.S.A.).

Measurement of accumulation of inositol phosphates

Accumulation of inositol phosphates was measured by the method previously described (Mizuguchi *et al.*, 1987). BAL17 cells [(5–10) $\times 10^5$ /ml] were loaded overnight with 20 μ Ci of *myo*-[³H]inositol/ml of minimum essential medium (Nissui Pharmaceutical Co., Tokyo, Japan), followed by washing. ³H-labelled cells were resuspended at a density of 2×10^7 in 5% fetal calf serum/RPMI 1640 containing 10 mM-LiCl to inhibit inositol-1-phosphatase. Cells (0.2 ml) were pretreated with Tos-Phe-CH₂Cl or Tos-Lys-CH₂Cl for 10 min and then stimulated with anti-IgM or NaF. Reactions were stopped by the addition of 750 μ l of chloroform/methanol (1:2, v/v). This mixture was separated into two phases by the addition of 250 μ l of chloroform and 250 μ l of 0.1 M-HCl. The upper phase of each sample was removed and the chloroform phase was washed twice with 200 μ l of the upper aqueous phase from a mixture of chloroform, methanol and 100 mM-sodium cyclohexane-1,2-diaminetetra-acetate (16:8:5, by vol.). The aqueous phase and the washings were pooled and applied to a Dowex-1 column (formate form). The column was washed with 4 ml of water and 1.5 ml of 5 mM-sodium tetraborate/60 mM-sodium formate. InsP₁, InsP₂ and InsP₃ were sequentially eluted with 0.1 M-formic acid/0.2 M-ammonium formate, 0.1 M-formic acid/0.4 M-ammonium formate, and 0.1 M-formic acid/1 M-ammonium formate respectively.

Determination of [Ca²⁺]_i

BAL17 cells (5×10^6 /ml) were loaded by incubation with 5 μ M-fura-2/AM (Dogan, Kumamoto, Japan) for 30 min at 37 °C in RPMI-1640 containing 1% fetal calf serum. After loading, the cells were washed with phosphate-buffered saline (0.01 M-phosphate buffer/0.15 M-NaCl, pH 7.4) and resuspended in phosphate-buffered saline containing 1 mM-Ca²⁺. For pretreatment

with protease inhibitor, Tos-Phe-CH₂Cl or Tos-Lys-CH₂Cl was added 10 min before the termination of loading and again added to the anti-IgM-stimulated cultures. Stopped-flow fluorescence of the fura-2-loaded cells was measured with a Union Giken stopped-flow spectrophotometer RA-450 system (Nakanishi & Tsuboi, 1978). The sensitivity of the stopped-flow measurement was improved by data accumulation in an on-line computer. The experimental trace was derived from six to ten accumulated individual stopped-flow measurements.

Fluorescence analysis by flow cytometry

Indirect staining was carried out as previously described (Mizuguchi *et al.*, 1986b). The samples were analysed on an EPICS V flow cytometer (Coulter Electronics Inc., Hialeah, FL, U.S.A.). The histograms of fluorescence distribution are plotted as number of cells versus fluorescence intensity (log scale).

RESULTS

We have previously shown that anti-IgM causes an increase in inositol phospholipid metabolism in B cells (Mizuguchi *et al.*, 1986b,c). NaF, in concert with a minute amount of AlCl₃, has been shown to activate G-protein to induce an increase in inositol phospholipid metabolism or an increase in [Ca²⁺]_i in some cell types (Blackmore & Exton, 1986; O'Shea *et al.*, 1987). However, we could not demonstrate any dependence on AlCl₃ of NaF-mediated increases in inositol phospholipid metabolism and [Ca²⁺]_i. It is possible that a trace amount of AlCl₃ is already bound to BAL17 lymphoma cells, or that our cell cultures were contaminated with AlCl₃.

Inhibition by Tos-Phe-CH₂Cl or Tos-Lys-CH₂Cl of the anti-IgM-mediated, but not the NaF-mediated, increase in inositol phospholipid metabolism in BAL17 lymphoma cells

We investigated whether the serine protease inhibitors Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl inhibit the increases in inositol phospholipid metabolism induced by anti-IgM or NaF. As shown in Fig. 1, Tos-Lys-CH₂Cl, a trypsin inhibitor, inhibits anti-IgM-induced accumulation of inositol phosphates with a half-maximal activity at 0.075 mM. However, NaF-induced inositol phosphate production is fairly resistant to Tos-Lys-CH₂Cl with half-maximal inhibition at about 0.920 mM. Tos-Phe-CH₂Cl, a chymotrypsin inhibitor, is a stronger inhibitor of anti-IgM-induced inositol phosphate production, with a half-maximal activity at less than 0.010 mM. As with Tos-Lys-CH₂Cl, NaF-induced inositol phospholipid metabolism is about 10-fold more resistant to Tos-Phe-CH₂Cl pretreatment than anti-IgM-induced inositol phospholipid metabolism. Essentially similar results were obtained using the F(ab')₂ fragment of goat anti-IgM (results not shown). These results indicate that serine protease inhibitors inhibit anti-IgM-mediated inositol phospholipid metabolism at a step proximal to phospholipase C activation.

Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl inhibition of the anti-IgM-mediated increase in inositol phospholipid metabolism is not overcome by high concentrations of anti-IgM

Anti-IgM-mediated accumulation of inositol phosphates increases in a dose-dependent manner. Tos-

Fig. 1. Anti-IgM-mediated accumulation of inositol phosphates is inhibited by Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl pretreatment, whereas NaF-induced accumulation is not

myo-[^3H]inositol-labelled BAL17 cells pretreated with various concentrations of Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl were stimulated with 10 μg of anti-IgM/ml (○) or 30 mM-NaF (□). The accumulation of inositol phosphates was determined by the procedure described in the Materials and methods section. Percentages of control values were calculated as follows: [(c.p.m. in experimental group – c.p.m. without stimulants)/(c.p.m. in control group – c.p.m. without stimulants)] $\times$ 100, where c.p.m. in the control group for anti-IgM and NaF was 1864 and 1159 respectively, and c.p.m. without stimulants was 206; c.p.m. in control irrelevant antibody stimulation of goat anti-IgG2a was 198. The data represent means of three replicates. Relative standard deviations were less than 10%. The identical results were obtained from two other experiments.

Fig. 2. Tos-Lys-CH₂Cl inhibits the accumulation of inositol phosphates induced by various concentrations of anti-IgM, but not by NaF

myo-[^3H]inositol-labelled BAL17 cells pretreated with (▨) or without (□) 0.2 mM-Tos-Lys-CH₂Cl for 10 min were cultured in the presence of various concentrations of anti-IgM or NaF. The accumulation of inositol phosphates was determined by the procedure described in the Materials and methods section. The data represent means $\pm$ S.D. of three replicates.

Lys-CH₂Cl (0.2 mM) or Tos-Phe-CH₂Cl (0.025 mM) inhibition of the anti-IgM-mediated increases in inositol phospholipid metabolism is not overcome by high concentrations of anti-IgM. In contrast, NaF-mediated increases at every dose were not inhibited by Tos-Lys-

CH₂Cl or Tos-Phe-CH₂Cl pretreatment (Figs. 2 and 3). These results further indicate the differential sensitivity to protease inhibitors of the increases in inositol phospholipid metabolism induced by anti-IgM and NaF.

Fig. 3. Tos-Phe-CH₂Cl inhibits the accumulation of inositol phosphates induced by various concentrations of anti-IgM, but not by NaF

myo-[³H]inositol-labelled BAL17 cells pretreated with (■) or without (□) 0.025 mM-Tos-Phe-CH₂Cl were stimulated with various concentrations of anti-IgM or NaF as shown in Fig. 2. The data represent means $\pm$ S.D. of three replicates.

Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl inhibit InsP₃ production

Receptor-mediated phospholipase C activation is followed by hydrolysis of PtdIns(4,5)P₂, which results in the production of InsP₃. Therefore we checked whether Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl can inhibit InsP₃ production induced by anti-IgM. BAL17 cells labelled with *myo*-[³H]inositol were stimulated with anti-IgM (30 µg/ml) or NaF (30 mM). Significant increases in the accumulation of InsP₃, InsP₂ and InsP₁ induced by anti-IgM were observed 5 min after stimulation, and these increases were inhibited by both Tos-Lys-CH₂Cl (0.2 mM) and Tos-Phe-CH₂Cl (0.025 mM) pretreatment. On the other hand, the inhibitors had no effect on NaF-induced increases (Table 1). In contrast, a control irrelevant antibody (goat anti-IgG2a antibody) did not cause an increase in accumulation of inositol phosphates (results not shown). All of these results indicate that pretreatment with the inhibitors abolishes anti-IgM-induced PtdIns(4,5)P₂ hydrolysis, probably through diminished phospholipase C activation.

Increases in [Ca²⁺]_i are inhibited by Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl

Increases in [Ca²⁺]_i after receptor-ligand interaction have been shown to be coupled to an increase in inositol phospholipid metabolism in some cell types (Berridge, 1987). We asked whether this increase in [Ca²⁺]_i, measured using a stopped-flow spectrophotometer, is also inhibited by pretreatment with protease inhibitor. As shown in Fig. 4, the anti-IgM-mediated increase is

Table 1. Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl inhibit anti-IgM-mediated production, but not NaF-mediated production of InsP₁, InsP₂ and InsP₃

The *myo*-[³H]inositol-labelled BAL17 cells pretreated with Tos-Lys-CH₂Cl (0.2 mM) or Tos-Phe-CH₂Cl (0.025 mM) are stimulated with anti-IgM (30 µg/ml) or NaF (30 mM) for 5 min. Results are means $\pm$ S.D. of three replicates from a single experiment. Essentially similar results were obtained from two other experiments.

Stimulants $\pm$ inhibitor	Accumulation of [³ H]inositol phosphates (c.p.m.)		
	InsP ₁	InsP ₂	InsP ₃
Expt. 1			
Medium	187 $\pm$ 29	87 $\pm$ 8	87 $\pm$ 6
Anti-IgM	442 $\pm$ 9	319 $\pm$ 7	291 $\pm$ 5
Anti-IgM + Tos-Lys-CH ₂ Cl	272 $\pm$ 15	180 $\pm$ 9	144 $\pm$ 8
NaF	299 $\pm$ 23	167 $\pm$ 14	160 $\pm$ 7
NaF + Tos-Lys-CH ₂ Cl	308 $\pm$ 7	191 $\pm$ 7	166 $\pm$ 3
Expt. 2			
Medium	125 $\pm$ 6	98 $\pm$ 9	97 $\pm$ 18
Anti-IgG2a	120 $\pm$ 13	103 $\pm$ 15	102 $\pm$ 8
Anti-IgM	336 $\pm$ 20	303 $\pm$ 18	280 $\pm$ 15
Anti-IgM + Tos-Phe-CH ₂ Cl	224 $\pm$ 27	198 $\pm$ 13	146 $\pm$ 20
NaF	219 $\pm$ 2	182 $\pm$ 27	155 $\pm$ 10
NaF + Tos-Phe-CH ₂ Cl	236 $\pm$ 14	183 $\pm$ 14	153 $\pm$ 3

Fig. 4. Tos-Lys-CH₂Cl inhibits the increase in [Ca²⁺]_i induced by anti-IgM, but not by NaF

Fura 2-loaded BAL17 cells pretreated with Tos-Lys-CH₂Cl for 10 min were stimulated with (a) anti-IgM (10 µg/ml) or (b) NaF (30 mM) with (b) or without (a) Tos-Lys-CH₂Cl (0.1 mM) for 10 s. The fluorescence intensity after stimulation was monitored using a stopped-flow spectrophotometer.

inhibited by Tos-Lys-CH₂Cl pretreatment, whereas the NaF-induced increase is not altered. The identical pattern of inhibition was observed with Tos-Phe-CH₂Cl pretreatment at a concentration of 0.025 mM (results not shown). These results further indicate that protease inhibitor interferes with a step proximal to phospholipase C activation.

Membrane IgM density is not diminished by Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl treatment

One possible explanation for the differential sensitivity of increased inositol phospholipid metabolism to protease inhibitors is that the amount of IgM molecules is diminished after Tos-Lys-CH₂Cl (0.2 mM) or Tos-Phe-CH₂Cl (0.025 mM) treatment. B-cell surface IgM levels were determined using a flow cytometer. As shown in Fig. 5, the staining profile of Tos-Lys-CH₂Cl-treated cells is essentially the same as that of control cells. Tos-Phe-CH₂Cl treatment also does not alter the membrane IgM density (results not shown). These results suggest that the inhibition occurs at a step distal to the membrane Ig molecule.

DISCUSSION

Our results indicate that anti-IgM-mediated increases in inositol phospholipid metabolism or [Ca²⁺]_i are inhibited by pretreatment of BAL17 cells with the serine protease inhibitors Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl. However, NaF-induced increases are more than 10-fold more resistant to these inhibitors. The Fc_γ receptor is not involved in this inhibition, since essentially similar results were obtained using F(ab')₂ fragment of goat anti-IgM (results not shown). These results indicate that these serine protease inhibitors act at a step proximal to

Fig. 5. Tos-Lys-CH₂Cl treatment does not alter membrane IgM density

BAL17 cells treated with (—) or without (-----) Tos-Lys-CH₂Cl (0.2 mM) were stained with rat anti-(mouse IgM) antibody followed by fluorescein-labelled mouse anti-rat antibody as described in the Materials and methods section.

phospholipase C activation. In agreement with our findings, Duque *et al.* (1983) have already shown that Tos-Phe-CH₂Cl inhibits *N*-formylmethionyl-leucyl-phenylalanine-induced membrane depolarization and superoxide generation in rat neutrophils, although the site at which Tos-Phe-CH₂Cl acts was unclear in their study. Kishimoto *et al.* (1979) have previously shown that anti-Ig treatment stimulates membrane-bound serine protease leading to generation of a cytoplasmic factor, which is involved in non-histone nuclear protein phosphorylation. Our results and those of Kishimoto *et al.* (1979) have clearly demonstrated that serine proteases are involved in anti-Ig-mediated signal transmission.

Anti-IgM-mediated signal transmission could be assessed using BAL17 B lymphoma cells, since these cells behave similarly to normal mature B cells in initial biochemical events. Thus, receptor cross-linkage of μ or δ chains on the cell surfaces of either normal B cells or BAL17 cells causes increases in membrane fluidity, inositol phospholipid metabolism and [Ca²⁺]_i, all of which are inhibited by pretreatment with phorbol myristate acetate (Mizuguchi *et al.*, 1986a, 1988). BAL17 cells, representing mature B cells, do not show growth arrest of the G₁ phase of the cell cycle after receptor cross-linkage, while WEHI-231 B lymphoma cells, representing immature B cells, have shown to be arrested (Boyd & Schrader, 1981; Scott *et al.*, 1986; J. Mizuguchi, N. Utsunomiya, M. Nakanishi, Y. Arato & H. Fukazawa, unpublished work). Furthermore, normal resting B cells show decreased anti-IgM-mediated inositol phospholipid metabolism and [Ca²⁺]_i in the presence of Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl (J. Mizuguchi, N. Utsunomiya, M. Nakanishi, Y. Arata & H. Fukazawa, unpublished work).

A serine protease has been shown to be involved in the mediation of cytotoxic T cells (Masson & Tschopp, 1985; Podack *et al.*, 1985). This serine protease has been analysed using an affinity-labelling reagent and found to have a molecular mass of 28 kDa (Pasternack & Eisen,

1985). The protease involved in signal transduction described here may differ from that in cytotoxic T cells, since the level of the 28 kDa protein is quite low in B cells. Furthermore, the protease activity in cytotoxic T cells is inhibited by di-isopropylfluorophosphoridate, but not by Tos-Lys-CH₂Cl, which we have shown here to inhibit early anti-IgM-mediated signalling events.

G-protein has been shown to exist as a family of subtypes, stimulatory (G_s) and inhibitory (G_i), which modulate adenylate cyclase activity, and transducin, which is involved in the modulation of cyclic GMP concentration in the retina (Casey & Gilman, 1988). A related protein, G_o, has been purified from brain tissues, although the function of G_o remains largely unknown. G-proteins involved in phospholipase C activation can be classified by their sensitivities to cholera toxin or pertussis toxin (Casey & Gilman, 1988). Anti-IgM-mediated inositol phospholipid metabolism has been shown to be enhanced by GTP[S] in B-cell membranes and permeabilized B cells (Harris & Cambier, 1987; Harnett & Klaus, 1988). Anti-IgM-mediated inositol phospholipid metabolism is insensitive to cholera toxin and pertussis toxin (Harnett & Klaus, 1988), indicating that the G-protein involved in this process is different from G_s or G_i. A low molecular mass G-protein (20 kDa) has been shown to participate in inositol phospholipid metabolism (Preiss *et al.*, 1986; Fleischman *et al.*, 1986) and is not the substrate for ADP-ribosylation by bacterial toxins. It is attractive to speculate that a low molecular mass G-protein plays some role in the anti-IgM-mediated signalling pathway.

Some G-proteins have been shown by molecular cloning and biochemical analysis to be composed of three subunits, α , β , and γ , and to be involved in the coupling of receptor cross-linkage to phospholipase C activation in some cell types (Casey & Gilman, 1988). A receptor-ligand interaction has been proposed to lead to the replacement of GDP by GTP in the receptor-G-protein complex on the α subunit. The activated GTP-G α subunit complex dissociates from the G $\beta\gamma$ subunit complex and interacts with effectors such as phospholipase C. The reaction is terminated by hydrolysis of the GTP-G α subunit with GTPase. In contrast, NaF, in the presence of trace amounts of aluminium, is proposed to be complexed as AlF₄, which mimics the role of the γ -phosphate of GTP (Bigay *et al.*, 1987). Thus, AlF₄ activation bypasses the requirement for the interaction of the receptor with G-protein, which would result in nucleotide exchange. There are several possibilities to explain Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl-mediated inhibition of inositol phospholipid metabolism: (1) the receptor density is diminished; (2) the activated form of the GTP-G α subunit complex fails to interact with phospholipase C; (3) the association of the receptor with G-protein is impaired. The first possibility seems unlikely, since Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl treatment does not diminish the level of IgM molecules on BAL17 lymphoma cells as assayed by flow cytometry. The second possibility is also unlikely, since NaF-induced inositol phospholipid metabolism is not altered by Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl treatment. Therefore we favour the possibility that the interaction of the receptor with G-protein is impaired by Tos-Lys-CH₂Cl or Tos-Phe-CH₂Cl treatment.

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