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Biochemistry

Bridging of IgE receptors activates phospholipid methylation and adenylate cyclase in mast cell plasma membranes

(Ca^{2+} influx/histamine release/cyclic AMP/anti-receptor antibody)

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ABSTRACT **Bridging of IgE receptors on normal rat mast cells by divalent anti-receptor antibodies induced phospholipid methylation and an increase in intracellular cyclic AMP within 15 sec after the receptor bridging. These biochemical events were followed by Ca^{2+} influx and histamine release. When IgE receptors on isolated plasma membranes were bridged by the antibody, both the increase in the incorporation of [^{3}H]methyl into lipid fraction and the synthesis of cyclic AMP were demonstrated. The synthesis of cyclic AMP in this system was enhanced in the presence of GTP. The results indicated that the bridged IgE receptors are linked to both methyltransferases and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in the plasma membrane. An increase in cyclic AMP prior to receptor bridging suppressed phospholipid methylation in the plasma membrane, Ca^{2+} uptake, and subsequent histamine release. On the other hand, inhibition of phospholipid methylation by (*S*)-isobutyryl-3-deazaadenosine resulted in the suppression of cyclic AMP synthesis in the plasma membrane. These findings suggest that the activation of phospholipid methylation and the activation of adenylate cyclase are mutually regulated.**

Mast cells and basophil granulocytes have receptors for IgE. The bridging of cell-bound IgE antibody molecules by either multivalent antigen or divalent anti-IgE triggers the release of a variety of chemical mediators from the cells (1). In our previous study, it was demonstrated that bridging of receptor molecules on rat mast cells by divalent anti-receptor antibody or its F(ab')$_2$ fragments induced histamine release, whereas the binding of Fab' fragments of the antibody to the receptors failed to do so (2). Subsequent studies have shown that bridging of IgE receptors, either directly by divalent anti-receptor antibody or indirectly by IgE and anti-IgE, stimulated phospholipid methylation and induced an increase in ^{45}Ca influx into mast cells (3, 4). Evidence was obtained that the activation of methyltransferases is intrinsic for ^{45}Ca influx and subsequent histamine release. However, biochemical events triggered by receptor bridging appear to be more complex. Several investigators reported that incubation of rat mast cells with concanavalin A or anti-IgE induced a transient increase in intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) levels (5, 6). The present experiments were undertaken to investigate functional relationships among IgE receptors, methyltransferases, and adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1]. The results show that bridging of IgE receptors activates both methyltransferases and adenylate cyclase on the plasma membranes, and that methyltransferases are involved in the activation of adenylate cyclase.

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MATERIALS AND METHODS

Anti-Receptor Antibodies and Anti-IgE Antibodies. Anti-receptor antibody (anti-RBL), monoclonal rat IgE (IR 162), the IgG fraction of a goat antiserum specific for rat IgE (anti-IgE), and normal rabbit IgG (RGG) were prepared as described (7). The major antibodies in the anti-RBL preparation were directed toward the binding site for IgE in the receptor molecules (8). The F(ab')$_2$ fragments of anti-RBL were obtained by pepsin digestion, and these fragments were split into Fab' fragments by reduction and alkylation (2). Rat IgE was labeled with ^{125}I (New England Nuclear) by the method of McConahey and Dixon (9), using chloramine-T.

Purification of Mast Cells. Mast cells were purified from peritoneal cells of Sprague–Dawley rats (Holtzman, Madison, WI). Two milliliters of peritoneal cells (2–8 × 10^7 per ml) suspended in Tris-A-EDTA buffer (pH 7.6) (4) was layered over 1.5 ml of 22.6% metrizamide (Nyegaard, Oslo, Norway), and were centrifuged at 270 × *g* for 10 min at room temperature. The purity of mast cells recovered from the bottom of the tube was in the range of 91–93% with 97% viability.

Isolation of Plasma Membranes. Plasma membranes of purified mast cells were isolated by the method described by Emmelot *et al.* (10) with some modifications. In some experiments, mast cells (2–3 × 10^7 cells) were preincubated with ^{125}I-labeled rat IgE (^{125}I-IgE) at 10 μg/ml at 0°C for 1 hr. The cells were resuspended in 3 ml of 5 mM sodium bicarbonate (pH 8.0) and were disrupted by eight strokes of a motor-drive Teflon-coated pestle in a tissue grinding vessel. After centrifugation, precipitates were homogenized in 0.5 ml of 5 mM sodium bicarbonate and the homogenate was mixed with 2.5 ml of 81% sucrose. Plasma membranes in the homogenate were isolated by flotation through a discontinuous sucrose density gradient. After centrifugation at 73,000 × *g* for 1 hr, plasma membranes were harvested by collecting the top fraction, which included the density 1.14/1.16 g/ml interface and the 1.16/1.18 g/ml interface. The next fraction, including the density 1.18/1.20 g/ml interface, contained mainly mitochrondria. Both membrane and mitochondria fractions were diluted with an appropriate buffer and recovered by centrifugation. Protein concentration in the fractions was determined by phenol reagent (Lowry's method), using human serum albumin as a standard. Sixty to 100 μg of membrane fraction was recovered from 2 × 10^7 purified mast cells.

Purity of Plasma Membranes. The biochemical character-

Abbreviations: cAMP, cyclic AMP; cGMP, cyclic GMP; anti-RBL, anti-receptor antibody; RGG, normal rabbit IgG; 3-deaza-SIBA, (*S*)-isobutyryl-3-deazaadenosine; AdoMet; *S*-adenosyl-L-methionine.

istics of plasma membrane fractions were determined by measuring marker enzyme activities. Monoamine oxidase (11), 5'-nucleotidase (12), and β-galactosidase (13) were assayed by published procedures, using 30–50 μg of membrane fractions. 5'-nucleotidase was enriched 2.8-fold in the plasma membrane fraction (9.1 nmol/mg of protein per 30 min) and monoamine oxidase was enriched 3.1-fold in the mitochondrial fraction (15 pmol/mg of protein per 10 min). The profile of enzyme distribution showed that the plasma membrane fraction was minimally contaminated with mitochondria and lysozomes.

When the membrane fraction was incubated at 4°C overnight with ^{125}I-IgE at 1 μg/ml, a significant amount of IgE (1.75 ng of IgE per 20 μg of membrane) bound to the preparation. The binding of ^{125}I-IgE was inhibited by the presence of unlabeled IgE at 100 μg/ml, but not by normal rat or rabbit IgG at 1 mg/ml, showing that receptors specific for IgE are present in the isolated membrane.

Determination of Phospholipid Methylation. Methylation of phospholipid in purified mast cells was determined by exactly the same procedures as described (4). The lipid fraction of the cells was extracted with chloroform/methanol and [^{3}H]methyl incorporated in the chloroform phase was determined.

In order to determine phospholipid methylation in the plasma membranes, 7–18 μg of the membranes was suspended in 40–50 μl of 50 mM sodium acetate buffer (pH 8.0) containing 5 mM $MgCl_2$ and 200 μM *S*-adenosyl-[*methyl*-^{3}H]-L-methionine (12.9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (New England Nuclear). The suspension was incubated with an appropriate concentration of anti-IgE or anti-RBL at 37°C for 1 hr. The reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid containing 10 mM L-methionine and the precipitates were extracted with 3 ml of chloroform/methanol (2:1, vol/vol) as described (14).

Determination of cAMP and cGMP. Purified mast cells (1–2 × 10^5 cells) suspended in 45 μl of Tyrode's solution (pH 7.0) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM Hepes, 5 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) (Sigma), and 0.5 g of gelatin per liter, were challenged with an appropriate concentration (5 μl) of either anti-IgE or anti-RBL at 37°C. The reaction was terminated by adding 1 ml of acidic alcohol (1 ml of 1 M HCl/100 ml of ethanol) and freezing in dry ice/acetone. The reaction mixture was brought to dryness under nitrogen and residues were dissolved in 1 ml of 50 mM acetate buffer (pH 6.2). cAMP and cGMP were determined by radioimmunoassay (15). Antibodies specific for cAMP or cGMP were described previously (16).

Plasma membrane fraction was suspended in 0.3 ml of 50 mM Tris·HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ and 2.5 mM ATP. Membrane fractions (7–18 μg/40 μl) were incubated at 37°C for 1 hr with 5 μl of either anti-IgE or anti-RBL in the absence or presence of 1 mM GTP, and cAMP in the suspensions was measured by radioimmunoassay.

Measurement of ^{45}Ca Influx and Histamine Release. Purified mast cells were suspended in Tyrode's solution (pH 7.0), in which phosphatidylserine at 50 μg/ml (Supelco, Bellefonte, PA) had been dispersed by sonication. After incubation with anti-RBL or anti-IgE at 37°C, ^{45}Ca influx was measured by the methods of Foreman *et al* (17) and histamine content in the supernatants was measured by the automated technique of Siraganian (18).

Chemicals. (*S*)-Isobutyryl-3-deazaadenosine (3-deaza-SIBA) was synthesized as described (19). Theophylline, isoproterenol, propranolol hydrochloride, dibutyryl cyclic AMP, ATP, GTP, and *S*-adenosyl-L-methionine (AdoMet) were purchased from Sigma.

RESULTS

Increase of Intracellular cAMP by Bridging of IgE Receptors. Purified mast cells were challenged with F(ab')$_2$ fragments of anti-RBL at 50 μg/ml, and intracellular cAMP was measured at various intervals. A monophasic rise in cAMP was obtained 15 sec after the challenge (Fig. 1). After a sharp decline, cAMP slightly increased again after 3 min. When the same cells were challenged with either Fab' fragments of anti-RBL or F(ab')$_2$ fragments of RGG, no significant change in cAMP was observed. On the other hand, cGMP increased 2- to 3-fold at 30 seconds after challenge with the F(ab')$_2$ fragments of anti-RBL and returned to the baseline level at 1 min (data not shown).

Because our previous studies demonstrated that incorporation of [^{3}H]methyl groups into phospholipid peaked at 15 sec after the challenge of rat mast cells with anti-RBL or anti-IgE, kinetics of both phospholipid methylation and an initial rise in cAMP were compared, using the same mast cell preparation. The results confirmed that both phospholipid methylation and cAMP reached maximum at 15 sec after challenge and then sharply declined (Fig. 1). The initial increase in cAMP by anti-RBL was more rapid than that induced by stimulation of β-adrenergic receptors. The maximal cAMP concentration was attained at 1 min after 40–100 μM isoproterenol had been added and was comparable to that induced by an optimal concentration of either anti-RBL or anti-IgE. As shown in Fig. 2, 200 μM propranolol, a β-adrenergic antagonist, inhibited an increase in cAMP by isoproterenol but did not affect a cAMP increase by anti-IgE. The results indicated that β-adrenergic receptors might not be involved in the synthesis of cAMP induced by bridging of IgE receptors.

Synthesis of Cyclic AMP and Phospholipid Methylation in Plasma Membranes. To establish that both phospholipid methylation and cAMP synthesis occur in plasma membranes upon bridging of IgE receptors, plasma membranes were isolated from purified mast cells. Aliquots of the membrane fraction (8–18 μg) were incubated with an optimal concentration of F(ab')$_2$ fragments or Fab monomer fragments of anti-RBL at 37°C for 1 hr in the presence of *S*-adenosyl-[*methyl*-^{3}H]-L-methionine, and the incorporation of [^{3}H]methyl into phospholipids was measured. The F(ab')$_2$ fragments of anti-RBL induced a substantial increase in [^{3}H]methyl incorporation into phospholipids, whereas neither Fab' fragments of anti-RBL nor

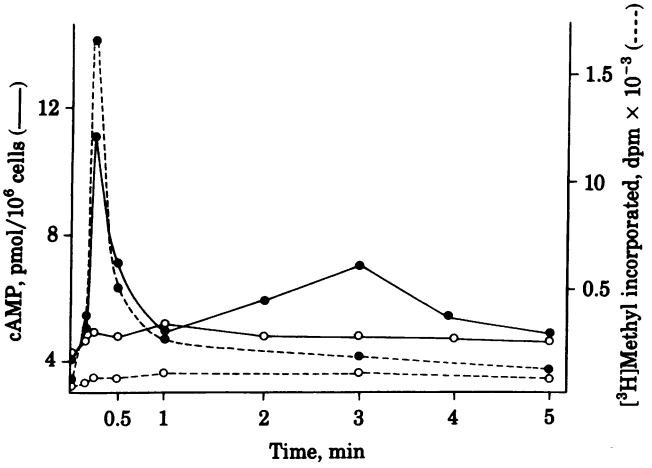

FIG. 1. Time course of changes in intracellular cAMP (——) and phospholipid methylation (----) induced by receptor-bridging. Purified mast cells were challenged with either F(ab')$_2$ fragments (●) or Fab' fragments (○) of anti-RBL at 50 μg/ml. Each point represents the mean of duplicate samples for a single representative experiment.

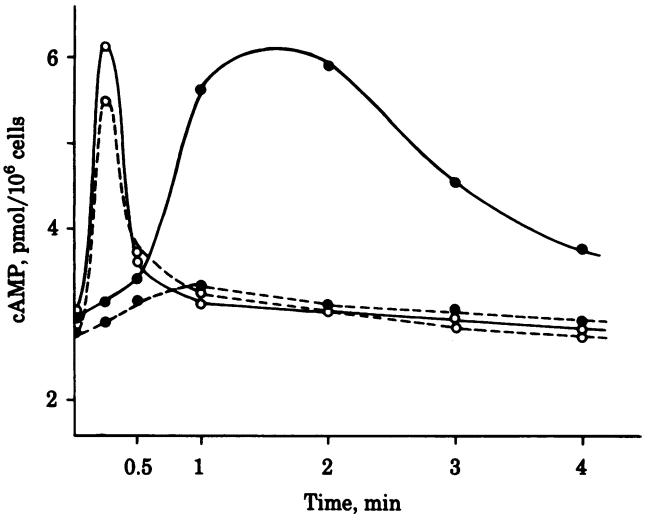

FIG. 2. Effect of propranolol on the increase in cAMP induced by isoproterenol or anti-IgE. Purified mast cells were incubated with rat IgE (100 μg/ml) at 4°C for 30 min, washed, and then incubated with either 40 μM isoproterenol (●) or goat anti-IgE (1.6 μg/ml) (○) in the presence (----) or absence (——) of 200 μM propranolol. Two separate experiments gave similar results.

$F(ab')_2$ fragments of normal RGG induced phospholipid methylation (Fig. 3). When the same membrane preparation was incubated with $F(ab')_2$ fragments of anti-RBL in the presence of 2.5 mM ATP, substantial amounts of cAMP were synthesized in the cell-free system. The addition of 1 mM GTP significantly enhanced the increase in cAMP ($P < 0.05$), suggesting the involvement of coupling factor (GTP factor). The Fab' fragments of anti-RBL and $F(ab')_2$ fragments of normal RGG failed to induce the synthesis of a detectable amount of cAMP even in the presence of 1 mM GTP. Essentially similar results were obtained when membrane preparations were obtained from mast cells saturated with IgE and were incubated with anti-IgE (Fig. 3).

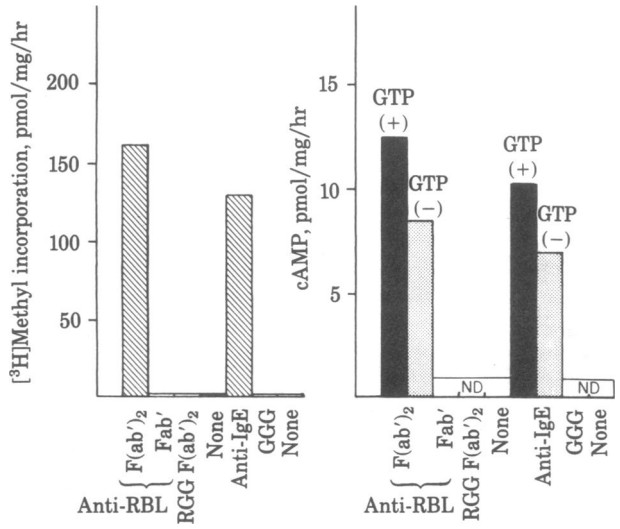

FIG. 3. Stimulation of phospholipid methylation and an increase in cAMP in membrane preparations. Aliquots of plasma membranes were incubated at 37°C for 1 hr with either $F(ab')_2$ or Fab' fragments of anti-RBL or goat anti-IgE. As controls, the same membrane preparation was incubated with the $F(ab')_2$ fragments of normal rabbit IgG (RGG) or goat IgG (GGG) of a comparable concentration. GTP was 1 mM; ND, not detectable. Three more experiments of the same design gave similar results.

Inhibition of Phospholipid Methylation by Increase in cAMP Levels. In view of previous observations that antigen-induced histamine release from basophil granulocytes and mast cells was inhibited by increasing intracellular cAMP levels (20–22), experiments were carried out to study the possible regulatory role of cAMP on phospholipid methylation. Purified mast cells were preincubated with L-[methyl-^{3}H]methionine for 30 min at 37°C, and aliquots of the cells were treated with various concentrations (10 μM to 8 mM) of theophylline for 5 min. Intracellular cAMP levels after the treatment are shown in Fig. 4. An aliquot of the treated cells was then incubated with an optimal concentration of $F(ab')_2$ fragments of anti-RBL or anti-IgE, and the incorporation of [^{3}H]methyl into phospholipid, ^{45}Ca influx, and histamine release were measured after 15-sec, 2-min, and 5-min incubation, respectively. It is apparent in Fig. 4 that preincubation of mast cells with theophylline inhibited phospholipid methylation, and the inhibition paralleled intracellular cAMP levels. Pretreatment of mast cells with theophylline inhibited ^{45}Ca influx and subsequent histamine release in the same dose–response fashion. In keeping with this observation, dibutyryl cAMP (200 μM to 4 mM) added to mast cells also inhibited all three of the reactions in a similar dose–response fashion. Although β-adrenergic receptors may be coupled to adenylate cyclase, intracellular increase in cAMP by isoproterenol differed depending on cell preparation employed; cAMP rise was transient in some cell preparations. However, when isoproterenol (2–200 μM) gave a persistent increase in cAMP level, all of the anti-RBL-induced phospholipid methylation, ^{45}Ca uptake, and histamine release were inhibited. The effect of cAMP on phospholipid methylation was confirmed by using membrane preparations. When plasma membranes were incubated with $F(ab')_2$ of anti-RBL in the presence of various concentrations of dibutyryl cAMP (200 μM to 4 mM), phospholipid methylation in the plasma membrane was inhibited in a quantitative manner (Fig. 5). These findings indicated that an increase in intracellular cAMP before challenge inhib-

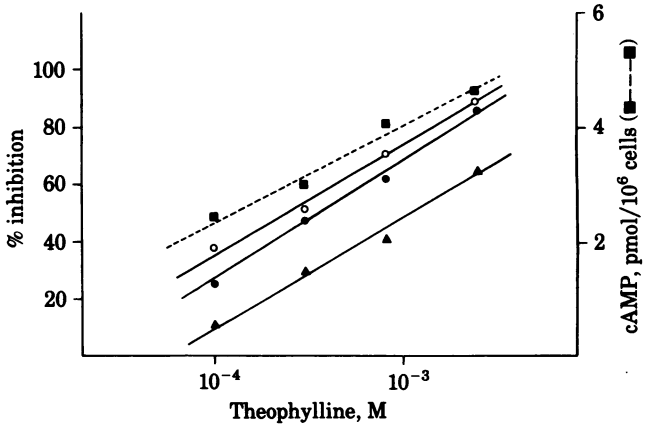

FIG. 4. Inhibition of anti-RBL-induced [^{3}H]methyl incorporation (●——●), ^{45}Ca uptake (○——○), and histamine release (▲——▲) by theophylline. Mast cells were preincubated with theophylline at 37°C for 5 min and then challenged with divalent anti-RBL. cAMP levels in theophylline-treated mast cells (■---■) are included in the figure. Each point depicts the mean of duplicate measurements. The [^{3}H]methyl incorporation, ^{45}Ca uptake, and histamine release in the absence of theophylline were 2685 ± 102 dpm per 1.2×10^5 cells, 2951 ± 125 cpm per 10^6 cells, and 22%, respectively. The level of cAMP in untreated mast cells was 1.9 pmol per 10^6 cells. Two more experiments of the same design gave similar results.

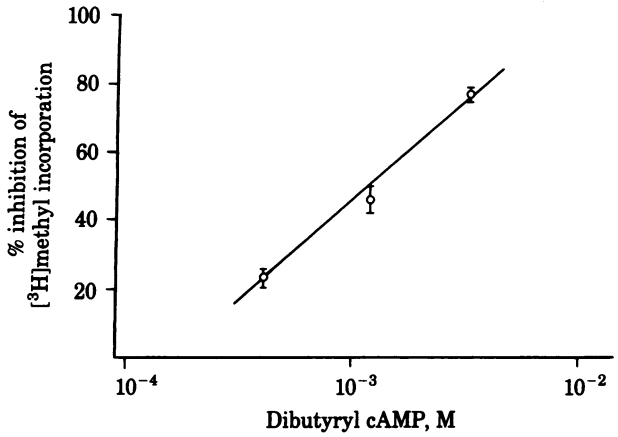

FIG. 5. Inhibition of phospholipid methylation in the membrane preparation by dibutyryl cAMP. Aliquots of plasma membranes were incubated with $F(ab')_2$ fragments of anti-RBL at 50 μg/ml in the presence of various concentrations of dibutyryl cAMP at 37°C for 1 hr. Error bars indicate SD. [^{3}H]Methyl incorporation in the membrane in the absence of dibutyryl cAMP was 138.7 pmol/mg of protein per hr. A repeated experiment gave comparable results.

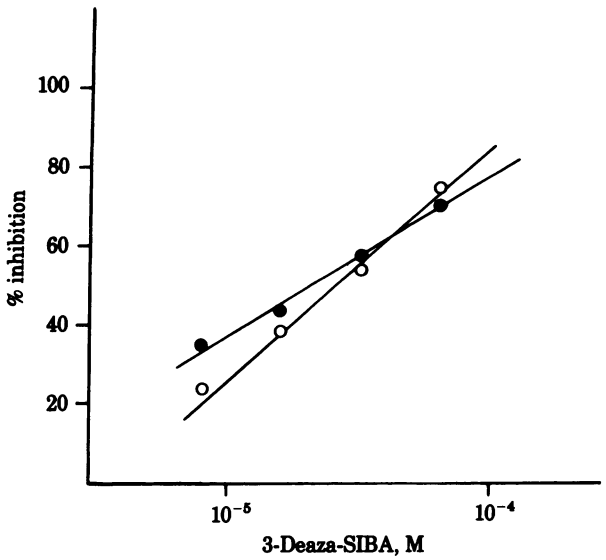

FIG. 6. Concomitant inhibition of phospholipid methylation (●) and cAMP synthesis (○) in the plasma membrane by 3-deaza-SIBA. Aliquots of plasma membranes were incubated with $F(ab')_2$ fragments of anti-RBL at 50 μg/ml in the presence of various concentrations of 3-deaza-SIBA. In the absence of 3-deaza-SIBA, 142 pmol/mg of protein per hr of [^{3}H]methyl incorporation and 13.1 pmol/mg of protein per hr of cAMP synthesis was induced. Two more experiments gave identical results.

ited phospholipid methylation and subsequent ^{45}Ca uptake and histamine release.

Possible Role of Methyltransferases in cAMP Synthesis. Finally, we studied possible participation of phospholipid methylation in the activation of adenylate cyclase induced by IgE receptor bridging. Purified mast cells were preincubated at 4°C with various concentrations of 3-deaza-SIBA, and were challenged with the $F(ab')_2$ of anti-RBL. Measurement of phospholipid methylation and cAMP at 15 sec after the challenge confirmed that 3-deaza-SIBA at 10–200 μM inhibited anti-RBL-induced phospholipid methylation in a dose–response fashion. In contrast, 3-deaza-SIBA at less than 30 μM gave no significant effect on cAMP rise. However, 100 μM 3 deaza-SIBA, which inhibited phospholipid methylation by 80–95%, suppressed the cAMP rise by 30–40%.

The effect of 3-deaza-SIBA on the synthesis of cAMP was confirmed by using a membrane preparation. When a membrane preparation was incubated with anti-RBL in the presence of 3-deaza-SIBA at 10–100 μM, both phospholipid methylation and cAMP synthesis were inhibited in an identical dose–response fashion (Fig. 6). In order to prove that the suppression of cAMP synthesis by 3-deaza-SIBA is indeed due to inhibition of the methylation, we studied the possible countereffect of AdoMet on 3-deaza-SIBA. The results showed that the suppressive effect of 3-deaza-SIBA (16–64 μM) on anti-RBL-induced cAMP synthesis was reversed when 4 mM AdoMet was added to the system. Essentially identical results were obtained when cAMP synthesis by the plasma membranes was stimulated by NaF. It was found that the activation of adenylate cyclase by 5 mM NaF was inhibited by 40 μM 3-deaza-SIBA and that the inhibition was reversed by the addition of 4 mM AdoMet.

DISCUSSION

The present experiments together with previous observations (4) showed that bridging of IgE receptors on normal mast cells induced not only phospholipid methylation but also a monophasic rise in cAMP. Both the incorporation of [^{3}H]methyl into phospholipid and an intial rise in cAMP reached maximum at 15 sec after receptor bridging and then sharply declined to the baseline levels (Fig. 1). As reported by Lewis *et al.* (6), the initial rise of cAMP was not inhibited by 10 μM indomethacin, a cyclooxygenase inhibitor, which completely suppressed a secondary rise of cAMP at 3 min (data not shown). Although the bridging of IgE receptors on rat mast cells leads to metabolism of arachidonate (23), this process does not appear to be involved in the initial rise in cAMP. Incubation of plasma membranes from mast cells with anti-receptor antibodies resulted in cAMP synthesis. Although β-adrenergic receptors are known to couple with adenylate cyclase, cAMP synthesis induced by bridging of IgE receptors could not be blocked by propranolol, a β-adrenergic antagonist. Furthermore, it is unlikely that an increase in cAMP is due to inhibition of phosphodiesterase, because the membrane preparations did not contain phosphodiesterase. The synthesis of cAMP in the membrane by receptor bridging was markedly enhanced in the presence of GTP, suggesting that bridging of IgE receptors activates adenylate cyclase through coupling factors (guanine nucleotide-binding regulatory protein).

Simultaneous activation of both methyltransferases and adenylate cyclase in plasma membranes upon receptor-bridging indicates a close association between IgE receptors and both of the enzymes: the activation of methyltransferases and adenylate cyclase is mutually regulated. In isolated plasma membranes, 3-deaza-SIBA inhibited both the increase in phospholipid methylation and the synthesis of cAMP in a similar dose–response fashion. The fact that inhibition of cAMP synthesis by 3-deaza-SIBA was reversed by the addition of excess AdoMet indicates that accumulation of *S*-adenosyl-L-homocysteine or its analogues was responsible for the inhibition of not only methyltransferases but also the IgE receptor–adenylate cyclase system. It is not known whether phospholipid methylation is an essential step for the activation of adenylate cyclase or merely enhances the coupling of the receptor to the enzyme through an increase in membrane fluidity (24). NaF activates adenylate cyclase by binding at the GTP site of the coupling factor (25). The activation of adenylate cyclase in the isolated plasma membranes of mast cells by NaF was also inhibited by 3-deaza-SIBA and its inhibition was reversed by AdoMet. Therefore, it is likely that phospholipid methylation is involved

in the function of the coupling factor, probably by changing the composition of annulus lipids.

The early increase in cAMP could be an "off" signal after the histamine releasing cascade already initiated by an increase of phospholipid methylation. The possibility is supported by the fact that an increase in cAMP prior to receptor bridging suppressed phospholipid methylation, Ca^{2+} uptake, and histamine release. On the other hand, a possible role of adenylate cyclase in mediator release can not be excluded. Holgate *et al.* (26) demonstrated a close linkage between IgE-dependent activation of adenylate cyclase and granule secretion from rat mast cells. They showed that stimulation of adenylate cyclase with a purine-modified adenosine analogue augmented IgE-mediated granule secretion, whereas inhibition of adenylate cyclase with a ribose-modified adenosine analogue suppressed the secretion. From these results they speculated that bridging of IgE receptors leads to transmembrane activation of adenylate cyclase, activation of cAMP-dependent protein kinase, and granule secretion.

It is not known whether methyltransferases or adenylate cyclase is the first enzyme to be activated by receptor bridging. Our recent studies provided evidence that chymotrypsin or a trypsin-like proteolytic enzyme is activated upon bridging of IgE receptors on rat mast cells. Various chymotrypsin substrates and chymotrypsin inhibitors, as well as trypsin substrates and inhibitors, inhibited both phospholipid methylation and the initial rise in cAMP induced by receptor bridging (27). The concentration of each reagent required for inhibiting phospholipid methylation was comparable to that required for inhibition of the cAMP rise. The results suggest that membrane-associated proteolytic enzyme(s) is activated by receptor bridging prior to the activation of methyltransferases and adenylate cyclase. Identification of this putative proteolytic enzyme and the relationship of the enzyme to IgE receptors requires further studies.

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