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MODULATION OF MAST CELL RESPONSES TO ADENOSINE BY AGENTS THAT ALTER PROTEIN KINASE C ACTIVITY

DIANA L. MARQUARDT* and LINDA L. WALKER
Department of Medicine, University of California San Diego Medical Center, San Diego, CA 92103, U.S.A.

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Abstract—The acute incubation of mouse bone marrow-derived mast cells with low concentrations of agents known to activate protein kinase C [phorbol myristate acetate (PMA), 1,2-dioctanoyl-*sn*-glycerol (diC8), and 1-oleoyl-2-acetyl-glycerol (OAG)] caused an enhancement of β-hexosaminidase release stimulated by the calcium ionophore A23187. Higher concentrations of protein kinase C activators tended to inhibit A23187- or antigen-induced preformed mediator release. All concentrations studied induced a striking mast cell hyporesponsiveness to the mediator release augmenting effect of adenosine. Agents that have been reported to block protein kinase C activity [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and sphingosine] demonstrated diverse responses in this system. Up to 100 μM H-7 failed to affect mast cell β-hexosaminidase release in the presence or absence of PMA and secretagogue. Sphingosine (10 μM) was a potent inhibitor of antigen- or A23187-induced mediator release as well as adenosine responsiveness. Sphingosine also blocked the effects of PMA noted above in a dose-dependent fashion. The generation of leukotriene C_4 (LTC_4) by stimulated mast cells surprisingly was not affected by concentrations of diC8 that significantly inhibited granule-associated mediator release. Translocation of protein kinase C activity from the cytosol to the mast cell membrane was evident in cells briefly pretreated with A23187, adenosine alone, and diC8 in the presence of Tyrode's buffer, A23187, or adenosine. These findings lend further support to the contention that signal transduction from mast cell adenosine receptors to processes that regulate degranulation may involve protein kinase C.

The mechanism of potentiation of preformed mediator release from mouse bone marrow-derived mast cells by adenosine is slowly becoming clearer. In many cases, agents that increase mast cell cyclic AMP concentrations inhibit histamine release [1], but the addition of adenosine to stimulated mast cells enhances both cyclic AMP levels and mediator release [2, 3]. Cholera toxin and pertussis toxin, agents known to interact with G proteins and modulate cyclic AMP metabolism [4], exhibit only very modest effects on mast cell adenosine responsiveness [5, 6], suggesting that another second messenger may be more important in adenosine receptor signal transduction in mast cells. The phorbol ester 4β-phorbol 12β-myristate 13α-acetate (PMA†) induces a translocation of mast cell protein kinase C activity from the cytosol to the membrane and eventually a degradation of membrane-associated protein kinase C activity. These agents also induce a striking hyporesponsiveness to the mediator release-enhancing effects of adenosine, even at PMA concentrations that fail to alter A23187- or IgE-mediated β-hexosaminidase release [7]. Although the observed effects of PMA on mediator release correlate with changes in protein kinase C activity, phorbol esters are known to have many biochemical actions that could influence mast cell adenosine responsiveness. For example, phorbol esters can inhibit the recycling of the asialoglycoprotein receptor to the cell surface in Hep G_2 cells [8], thus limiting receptor expression and function.

To better characterize the role of protein kinase C activation in the ability of exogenous adenosine to augment the stimulated release of mast cell granule-associated mediators, the reversible protein kinase C activators, 1-oleoyl-2-acetyl-glycerol (OAG) and 1,2-dioctanoyl-*sn*-glycerol (diC8), and the putative protein kinase C inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and sphingosine, were studied in the presence or absence of secretagogues, phorbol esters, and/or adenosine. The release of β-hexosaminidase, the generation of leukotriene C_4 (LTC_4), and the translocation of protein kinase C activity from cytosol to membrane were examined under various conditions to establish the importance of protein kinase C activation in mast cell adenosine responsiveness.

MATERIALS AND METHODS

Chemicals. The following were purchased from the manufacturers indicated: adenosine, *N*-acetyl-β-D-glucosamide, 2-mercaptoethanol, PMA, OAG, aprotinin, leupeptin, and phenylmethylsulfonylfluoride (PMSF) (Sigma Chemical Co., St. Louis,

* Correspondence address: Diana Marquardt, M.D., UCSD Medical Center, H-811-G, 225 Dickinson St., San Diego, CA 92103.

† Abbreviations: PMA, 4β-phorbol 12β-myristate 13α-acetate; LTC_4, leukotriene C_4; OAG, 1-oleoyl-2-acetyl-glycerol; PMSF, phenylmethylsulfonylfluoride; PS, phosphatidylserine; DAG, diacylglycerol; DNP, dinitrophenyl; diC8, 1,2-dioctanoyl-*sn*-glycerol; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; and EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

MO); diC8 and phosphatidylserine (PS) (Avanti Polar Lipids, Inc., Birmingham, AL); H-7 (Seikagaku America, St. Petersburg, FL); sphingosine and calcium ionophore A23187 (Calbiochem, La Jolla, CA); [^{3}H]LTC_4 radioimmunoassay kit (New England Nuclear, Boston, MA); RPMI 1640, penicillin–streptomycin, L-glutamine, Minimum Essential Medium (MEM) nonessential amino acids, and fetal calf serum (GIBCO, Grand Island, NY); [^{32}P]ATP and [^{35}S-α]ATP (Amersham, Arlington Heights, IL).

The following were donated: mouse hybridoma antidinitrophenyl (DNP) IgE antibody and dinitrophenyl bovine serum albumin antigen (Drs. Futong Liu and David Katz, Quidel, La Jolla, CA).

Culture of mouse bone marrow-derived mast cells. Bone marrow from femurs of BALB/c mice (Simonsen Laboratories, Gilroy, CA) was cultured in supplemented RPMI [9], 10% fetal calf serum and 20% WEHI-3 supernatant [10]. Cells were passaged weekly, were used between 3 and 8 weeks of culture, and were more than 90% pure viable mast cells.

Assay of mast cell β-hexosaminidase release. To assess the release of β-hexosaminidase, a granule-associated, preformed mediator whose release parallels that of histamine [11], mouse bone marrow-derived mast cells were passively sensitized with anti-DNP IgE (1 μg/10^6 cells) for 30 min at 37° in Tyrode's buffer and challenged with DNP–BSA antigen (200 ng/3 × 10^5 cells/400 μL) or A23187 (1 μg/mL) for 10 min. Cells were centrifuged at 200 *g* for 10 min, and supernatant and pellet β-hexosaminidase activities were determined as previously described [12]. In some experiments, sphingosine, PMA, adenosine, H-7, and/or OAG were added at the times shown before or after secretagogue.

Assay of mast cell LTC_4 release. The release of LTC_4, a newly-generated mast cell mediator, was assessed in separate experiments performed in the same manner as above. Supernatant fractions obtained after a 10-min challenge were stored at −20° for up to 4 weeks before assay with a commercially-available LTC_4 radioimmunoassay kit that exhibits some cross-reactivity with LTD_4 and LTE_4. However, mouse bone marrow-derived mast cells have been shown by high performance liquid chromatography to synthesize almost exclusively LTC_4 by lipoxygenation of arachidonic acid [13].

Assessment of mast cell protein kinase C activity. Protein kinase C was measured by modifications of the methods of Manger *et al.* [14] and White and Metzger [15]. Mast cells (1–2 × 10^7) were washed twice in Tyrode's buffer lacking divalent cations and twice in complete Tyrode's buffer, brought to a volume of 1 mL, incubated at 37° in medium containing diC8 (2.5 μM) or adenosine (10 μM), and challenged with 1 μg A23187 when appropriate for 10 min. Reaction mixtures were placed on ice, 2 mL of ice-cold buffer was added, and tubes were centrifuged in the cold for 3 min at 200 *g*. Pellets were lysed with 200 μL ice water, aspirated repeatedly through a 27-gauge needle, and mixed with 800 μL of cold extraction buffer (20 mM Tris–Cl, 0.33 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.5 μg/mL leupeptin, 100 μg/mL, aprotinin, and 2 mM PMSF). Membranes were centrifuged for 60 min at 4° at 100,000 *g*; the resulting supernatants (cytosol samples) were saved and pellets (membrane samples) resuspended in 1 mL extraction buffer and sonicated for 15 sec. Triton X-100 was added to each to a final concentration of 0.15%, and the protein kinase C activity in 50 μL aliquots was assessed using [^{32}P]ATP as detailed elsewhere [15]. In some experiments a [^{35}S-γ]ATP thiophosphorylation assay [16] was compared to the [^{32}P]ATP assay, yielding somewhat more consistent data and more counts per minute, so the former assay became the standard in these experiments.

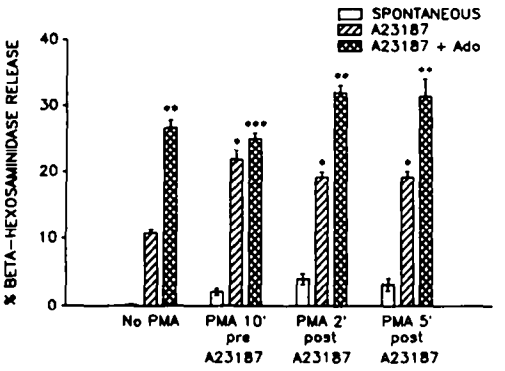

Fig. 1. Time–course of effects of addition of PMA on stimulated β-hexosaminidase release. PMA was added to mast cells before or after buffer alone (□), A23187 (▨), or A23187 and 10 μM adenosine (■). Reactions were terminated 10 min after A23187 addition, and total cell β-hexosaminidase release was determined. Total cell β-hexosaminidase measured spectrophotometrically at 410 nm averaged 1.22 ± 0.09 (mean ± SE, N = 12) O.D. units per 3 × 10^5 cells. Shown are means ± SE of four experiments performed in duplicate. Key: (*) significantly greater values than control cells in the absence of PMA ($P < 0.05$); (**) statistically significant potentiation of β-hexosaminidase release by adenosine ($P < 0.05$); and (***) significantly less potentiation of β-hexosaminidase release by adenosine than in non-PMA-treated cells ($P < 0.01$).

Statistical analysis of data. Statistical significance was determined using the paired, two-tailed Student's *t*-test. Results are expressed as means ± standard errors (SE) unless otherwise specified.

RESULTS

Effects of putative protein kinase C activators on mast cell β-hexosaminidase release. Pretreatment of mouse bone marrow-derived mast cells with PMA, a tumor-promoting agent that stimulates protein kinase C, has been shown to inhibit mast cell responsiveness to adenosine [7] but synergistically enhance the release of β-hexosaminidase induced by A23187 [7, 17]. When PMA was added to cells after A23187 challenge, the ability of adenosine to potentiate mediator release was restored and the ability of PMA to enhance A23187-stimulated β-hexosaminidase release was diminished modestly (Fig. 1).

Two diacylglycerol analogs that have been shown to activate protein kinase C, diC8 and OAG, were used in mast cell mediator release experiments. When cells were stimulated with DNP–BSA antigen

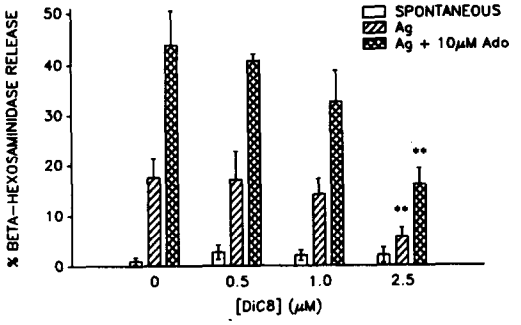

Fig. 2. Effect of diC8 on antigen- and adenosine-stimulated β-hexosaminidase release. Mast cells were incubated with the concentrations of diC8 shown for 5 min and challenged with buffer (□), DNP-BSA antigen (▨), or antigen and 10 μM adenosine (■). Mean (±SE) total β-hexosaminidase release was determined from five experiments performed in duplicate. Key: (**) significantly less β-hexosaminidase release than in comparable non-diC8-treated cells ($P < 0.005$).

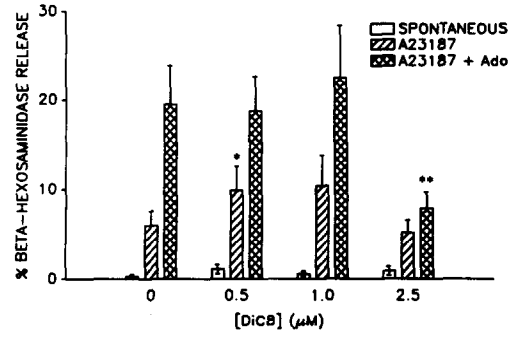

Fig. 3. Effect of diC8 on A23187- and adenosine-stimulated β-hexosaminidase release. Mast cells incubated for 5 min with diC8 were challenged with buffer alone (□), A23187 (▨), or A23187 and 10 μM adenosine (■. Mean (±SE) total β-hexosaminidase was determined from three experiments performed in duplicate. Key: (*) significant increase in β-hexosaminidase release compared to cells in the absence of diC8 ($P < 0.01$), and (**) significant decrease in β-hexosaminidase release compared to cells in the absence of diC8 ($P < 0.025$).

after a 5-min preincubation with diC8, the ability of exogenous adenosine to augment β-hexosaminidase release was decreased modestly in a concentration-dependent manner, and at a concentration of 2.5 μM diC8, both antigen- and antigen and adenosine-induced mediator release were inhibited significantly (Fig. 2). OAG produced nearly identical results at similar concentrations. Higher concentrations of diC8 either completely inhibited β-hexosaminidase release (5 μM) or induced substantial release in the absence of other secretagogues (>10 μM).

When A23187 was used to induce mast cell degranulation, a similar concentration–response for diC8 was observed with the exception that an intermediate concentration of diC8 (0.5 μM) synergistically enhanced A23187-stimulated mediator release (Fig. 3). Thus, PMA and diC8 exhibited strikingly similar

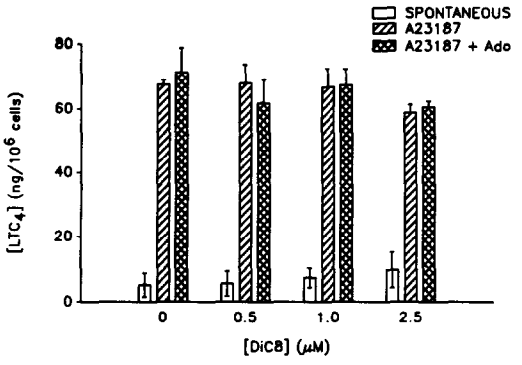

Fig. 4. Effect of diC8 on mast cell LTC_4 production. Cells were preincubated for 5 min with diC8 and challenged for 10 min with buffer (□), A23187 (▨), or A23187 and 10 μM adenosine (■). Depicted are means ± SE of duplicate determinations from three experiments.

acute effects on the release of granule-associated mast cell mediators.

Effect of diC8 on mast cell LTC_4 generation. The generation and release of LTC_4 by activated mast cells appear to be regulated quite differently from preformed mediator release [18]. Concentrations of PMA that dramatically inhibit β-hexosaminidase release fail to affect LTC_4 production [7], and exogenous adenosine is relatively impotent in potentiating LTC_4 generation [19]. The concentrations of diC8 that positively or negatively modulated mast cell β-hexosaminidase release had no demonstrable effect on LTC_4 generation induced by A23187 (Fig. 4), further underscoring the differential regulation of these two mediators.

Effects of putative protein kinase C inhibitors on mast cell β-hexosaminidase release. Several agents have been shown to inhibit protein kinase C activity, although many of these exhibit effects on other protein kinases as well [20]. Two such agents, H-7 and sphingosine, were examined for their effects on mast cell mediator release. Up to 100 μM H-7 was unable to alter either antigen- or A23187-induced mast cell β-hexosaminidase or the observed effects of PMA on stimulated mast cells (data not shown), although its K_i toward protein kinase C is reported to be 6 μM [21]. However, sphingosine induced a concentration-dependent inhibition of antigen- and A23187-stimulated β-hexosaminidase release that was more marked with antigen challenge (Fig. 5). The ability of adenosine to potentiate mediator release was abrogated completely by 10 μM sphingosine (Fig. 5). Higher concentrations of sphingosine (>50 μM) caused a large (>50%) spontaneous leak of β-hexosaminidase.

To assess more directly the possible protein kinase C-related effects of sphingosine on mast cells, sphingosine was added 10 min before and PMA (0.1 μM) was added 5 min before A23187 challenge. PMA produced the expected enhancement of β-hexosaminidase secretion, and adenosine did not potentiate this. Mediator release was decreased below control levels by 10 μM sphingosine, and the effects of PMA were partially inhibited by 1 μM sphingosine

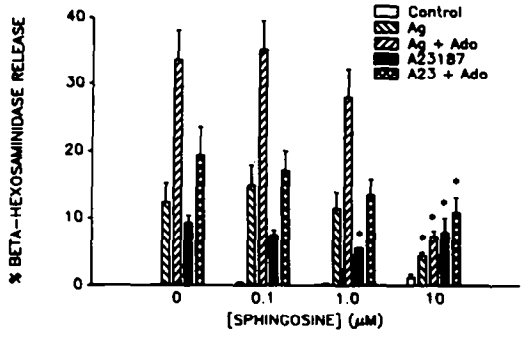

Fig. 5. Effect of sphingosine on mast cell β-hexosaminidase release. Cells were preincubated for 10 min with concentrations of sphingosine as shown followed by challenge with buffer (□), antigen (▨), antigen and 10 μM adenosine (▨), A23187 (■), or A23187 and 10 μM adenosine (■). Means ± SE, determined from four experiments performed in duplicate, are shown. Key: (*) significantly less than comparable values in the absence of sphingosine ($P < 0.01$).

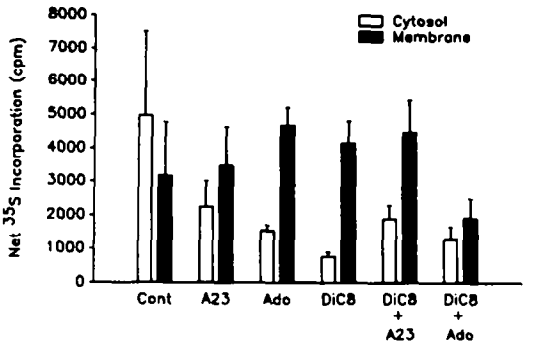

Fig. 7. Alterations in protein kinase C activity in mast cells. Protein kinase C activity in mast cell cytosol (□) and membrane (■) fractions was assessed after a 5-min preincubation with A23187, 10 μM adenosine, 2.5 μM diC8, diC8 + A23187, or diC8 + adenosine by a 35S-thiophosphorylation assay. Depicted are means ±SE of values from four experiments performed in duplicate.

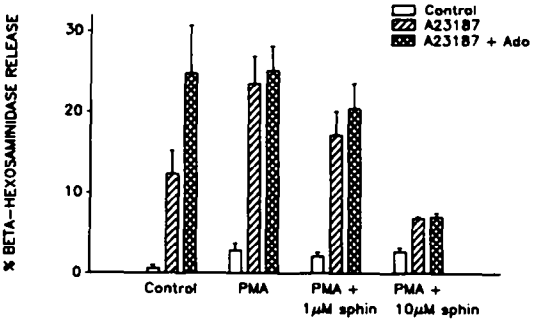

Fig. 6. Suppression of PMA effects by sphingosine. Mast cells were treated for 10 min with sphingosine and 5 min with PMA before challenge with buffer (□), A23187 (▨), or A23187 and 10 μM adenosine (■). PMA induced a significant enhancement of A23187-induced β-hexosaminidase release in the absence of sphingosine only ($P < 0.01$). Adenosine potentiation of β-hexosaminidase release was abrogated by PMA ($P < 0.01$). Sphingosine (10 μM) significantly inhibited the PMA enhancement of mediator release as well as A23187-stimulated mediator release alone ($P < 0.05$). Results are means ± SE of values from five experiments performed in duplicate.

(Fig. 6), consistent with a possible competitive inhibition of PMA and A23187-induced protein kinase C activation by sphingosine.

Effect of diC8 on mast cell protein kinase C activity. To assess directly the ability of diC8 to activate protein kinase C, the translocation of protein kinase C activity from the cell cytosol to the membrane was determined. DiC8 (2.5 μM) induced a striking decrease in cytosolic protein kinase C and an increase in membrane-associated protein kinase C activity (Fig. 7). Interestingly, a similar pattern of protein kinase C activation was seen with 10 μM adenosine pretreatment of the cells, A23187 stimulation, or A23187 stimulation in the presence of 2.5 μM diC8, suggesting that all of these agents may activate protein kinase C and that protein kinase C activation alone does not directly correlate with mast cell secretion, as neither adenosine nor diC8 alone at these concentrations induced mast cell mediator release.

DISCUSSION

The observation that PMA abrogates the ability of adenosine to potentiate mouse bone marrow-derived mast cell mediator release [7] led to the consideration that adenosine may exert its effects on mast cells via a protein kinase C-dependent mechanism. Should this be true, one would expect that the addition of adenosine prior to PMA exposure might allow the enhancement of mediator release to be evident and that other modulators of protein kinase C activity would also alter mast cell adenosine responsiveness. To address the first question, a comparison of the times of addition of adenosine and PMA revealed that whereas a 10-min preincubation with PMA followed by A23187 challenge caused an increase in mast cell β-hexosaminidase release and no additional mediator release induced by adenosine, the addition of PMA after A23187 and adenosine uncovered the adenosine enhancement of mediator release (Fig. 1).

Two putative activators of protein kinase C, OAG and diC8, were used in mast cell mediator release experiments and produced nearly identical results. As seen with PMA, diC8 alone at low concentrations (<2.5 μM) failed to induce β-hexosaminidase release from resting mast cells (Figs. 2 and 3), although higher levels of diC8 (10–100 μM) induced an apparently cytotoxic release of granule-associated mediators. DiC8 (2.5 μM) also inhibited the ability of adenosine to potentiate β-hexosaminidase release induced by antigen or A23187. As with PMA, diC8 exhibited different effects depending on the secretagogue used. An inhibition of mediator release induced by antigen was noted with 2.5 μM diC8, whereas A23187-stimulated mediator release was not significantly different in the presence of 2.5 μM diC8. However, 0.5 μM diC8 induced a potentiation of

A23187-stimulated β-hexosaminidase release similar to that induced by PMA and A23187 together.

The fact that diC8 activates protein kinase C but may inhibit antigen-induced mediator release and adenosine responsiveness may be explained by the rapid metabolism of diC8 and its very brief period of protein kinase C activation. In platelets 2.5 μM diC8 is 80% metabolized in 2.5 min [22]. Alternatively, IgE-mediated release systems may exhibit a strict dependence on dynamic protein kinase C activity, whereas calcium mobilizing release systems such as A23187 may be more flexibly influenced by doses and times of addition of protein kinase C modulators [15].

The ability of mouse bone marrow-derived mast cells to generate LTC_4 with antigen or A23187 stimulation was unchanged by concentrations of diC8 that significantly altered β-hexosaminidase release (Fig. 4). Phorbol esters produce similar effects on mast cell mediator secretion [7]. PMA and A23187 together induce the release of LTB_4 but not cyclooxygenase products from neutrophils [23], providing another example of differential activation of mediator generation in inflammatory cells. Adenosine itself has long been example of this phenomenon, enhancing preformed mast cell mediator release but not arachidonic acid metabolite production [19].

The inhibition of β-hexosaminidase release and loss of adenosine response by 10 μM sphingosine (Fig. 5) support a role for protein kinase C in the effect of adenosine on mast cell secretion. The ability of sphingosine to block some of the actions of PMA (Fig. 6) also supports a protein kinase C-dependent mechanism of action of PMA. The concentrations of sphingosine used in these studies were somewhat lower than those required for protein kinase C inhibition in platelets (80 μM) [24] or hepatocytes (200 μM) [25] but similar to those used in other systems [26, 27]. Up to 100 μM H-7 did not block the effect of PMA on mast cells, consistent with an extreme variability of H-7 effectiveness in other cells [15, 27, 28], often requiring 300–400 μM H-7 before protein kinase C inhibition is realized [25, 29].

The measurement of protein kinase C activation by assessing the translocation of protein kinase C activity from the cytosol to the cell membrane presents some problems in that membrane protein kinase C appears to be degraded rapidly. Thus, small changes in the time taken to activate the cells and separate the fractions may result in large fluctuations in the counts incorporated into the cytosol and membrane. In any case, resting mast cells incorporated more ^{35}S counts into the cytosolic than the membrane fraction, and diC8 or A23187 challenge induced a loss of cytosolic activity and an increase in membrane protein kinase C. The translocation of protein kinase C from the cytosol to membrane induced by 10 μM adenosine was particularly interesting in that adenosine alone does not alter mast cell secretion. This finding raises two important points about the mast cell secretory process. First, the activation of protein kinase C does not correlate directly with mast cell exocytosis, suggesting that another signal is necessary to facilitate degranulation, as has been suggested by others [15]. Second, adenosine mimics PMA and diC8 that it can activate protein kinase C but does not of itself induce mast cell preformed mediator release. Furthermore, it appears that the enhancement of granule-associated mediator release by adenosine requires an intact pathway to protein kinase C activation, in that agents that of themselves modulate protein kinase C activity are capable of suppressing adenosine responsiveness.

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