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CHARACTERIZATION OF THE PROSTAGLANDIN E RECEPTOR EXPRESSED ON A CULTURED MAST CELL LINE, BNu-2cl3

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Abstract—Interleukin 3-dependent BNu-2cl3 mast cells, mucosal type-like mast cells, exhibited a specific high-affinity binding site for [^{3}H]prostaglandin (PG) E_2. The binding was completely displaced by M&B 28767, an EP_3-selective agonist, but not by EP_1- or EP_2-selective ligands, indicating that the PGE_2 binding site is of the EP_3 subtype PGE receptor. Whereas the EP_3 subtype is presumed to be coupled to inhibition of adenylate cyclase in various tissues and cells, in BNu-2cl3 cells PGE_2 had no ability to inhibit adenylate cyclase activity, while it induced concentration-dependent stimulation of phosphoinositide metabolism and caused an increase in the intracellular free Ca^{2+} concentration in a pertussis toxin-sensitive manner. PGE_2 by itself did not evoke histamine release from the cells, but it markedly stimulated histamine release in concert with ionomycin, a Ca^{2+} ionophore. The PGE_2-stimulated release was also comletely blocked by pertussis toxin. Thus, the PGE receptor expressed on BNu-2cl3 mast cells is of the EP_3 subtype and is linked to phosphoinositide metabolism via a pertussis toxin-sensitive G protein, and this activation leads to histamine release.

Prostaglandin (PG)‡ E_2 exerts a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membranes [1, 2]. PGE receptors are pharmacologically divided into three subtypes, EP_1, EP_2 and EP_3 [3], and these subtypes are suggested to differ in their signal transduction; they are presumed to be coupled to the stimulation of phospholipase C, and the stimulation and inhibition of adenylate cyclase, respectively [3, 4]. Among these subtypes, EP_3 has been suggested to be involved in inhibition of gastric acid secretion [5], neurotransmitter release in central and peripheral neurons [6], lipolysis in adipose tissue [7], and sodium and water reabsorption in kidney tubulus [8, 9] through suppression of the intracellular cyclic AMP (cAMP) level.

Mast cells are considered to play a pivotal role not only in allergic reactions but also in a number of inflammatory disorders [10]. Mast cells are histochemically divided into two types, the connective tissue type and the mucosal type. The former reside in the skin or peritoneal cavity, contain heparin and a large amount of histamine, and produce PGD_2 exclusively as an arachidonic acid metabolite. In contrast, the latter are present in the gastrointestinal mucosa, contain chondroitin sulfate and a lower amount of histamine, and produce leukotrienes as well as PGD_2. Mast cells exhibit, as their main function, the secretion of histamine and other mediators of allergic reactions [11]. The secretory response is elicited via IgE receptors, and an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) appears to be the primary trigger for histamine release from the cells. In contrast, an increase in the cellular cAMP level inhibits the antigen-induced increase in Ca^{2+} uptake by mast cells [12]. PGE_1 and PGE_2 have been shown to elevate the intracellular cAMP level and to inhibit histamine release from connective tissue mast cells in response to stimulation of immunoglobulin E (IgE) receptors [13, 14], suggesting that this type of mast cells expresses the PGE receptor EP_2 subtype, which plays an important role as a negative feedback regulator of histamine release from the cells. Recently, we cloned a cDNA for the mouse EP_3 receptor from mouse mastocytoma P-815 cells, neoplastic mast cells resembling mucosal mast cells [15], which suggested that the EP_3 receptor is expressed in mucosal mast cells and plays a role in the function of the cells. However, biochemical approaches to PGE receptors in mucosal mast cells are hampered by the lack of a convenient preparation source. Interleukin 3 (IL-3)-dependent bone marrow-derived mast cells are phenotypically very similar to mucosal mast cells and have been used to characterize this type of mast cells [10]. Thus, we characterized PGE receptors in an IL-3-dependent mast cell line, BNu-2cl3, as a model of mucosal mast cells. The present study showed that BNu-2cl3 mast cells express the EP_3 receptor linked to phosphoinositide metabolism via a pertussis toxin-sensitive heterotrimeric GTP-binding protein (G protein), and the activation of this receptor leads to histamine release.

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‡ Abbreviations: PG, prostaglandin; IgE, immunoglobulin E; PT, pertussis toxin; IL-3, interleukin 3; IP, inositol phosphate; G protein, heterotrimeric GTP-binding protein; MES, 4-morpholineethanesulfonic acid; and HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MATERIALS AND METHODS

Materials. M&B 28767, butaprost and SC-19220 were gifts from Dr. M. P. C. Caton of Rhone-Poulenc Ltd., Dr. P. J. Gardiner of Bayer UK Ltd., and Dr. P. W. Collins of Searle, respectively. The other agents were obtained from commercial sources, as follows: [5,6,8,11,12,14,15-^{3}H]PGE_2 (154 Ci/mmol), Du Pont-New England Nuclear (Boston, MA, U.S.A.); cAMP [^{125}I] assay system, *myo*-[2-^{3}H]inositol (17.6 Ci/mmol) and iloprost, Amersham Corp. (Arlington Heights, IL, U.S.A.); PGE_2, PGE_1, $PGF_{2\alpha}$, PGD_2 and U-46619, Funakoshi Pharmaceuticals (Tokyo, Japan); pertussis toxin (PT), Seikagaku Kogyo (Tokyo, Japan); forskolin, Sigma (St. Louis, MO, U.S.A.); ionomycin, Calbiochem (San Diego, CA, U.S.A.); and fura-2/AM, Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of reagent grade.

The IL-3-dependent mast cell line, BNu-2cl3, established from splenocytes of mice (BALB/C, nu/nu), was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50% WEHI-3 conditioned medium as a source of IL-3, and maintained at between 2×10^5 and 1×10^6 cells/mL.

[^{3}H]*PGE_2 binding to the membrane fraction.* The membrane fraction was prepared from the BNu-2cl3 cells as described previously [16]. The standard assay mixture was comprised of 2 nM [^{3}H]PGE_2 (30.8 nCi), and 100 μg of the membrane fraction in 100 μL of 20 mM 4-morpholineethanesulfonic acid (MES), pH 6.0, containing 1 mM EDTA and 10 mM $MgCl_2$. After incubation for 1 hr at 30°, the reaction was terminated by the addition of 2.5 mL of the ice-cold buffer, and then the mixture was rapidly filtered through a Whatman GF/C glass filter (Φ 2.4 cm). The filter was washed four times with 2.5 mL of the ice-cold solution, and then the radioactivity associated with the filter was measured by scintillation counting using a toluene solution containing 30% (v/v) Triton X-100 and 2,4-diphenyloxazole. Nonspecific binding was determined using a 1000-fold excess of unlabeled PGE_2 in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

Measurement of [^{3}H]*phosphatidylinositol hydrolysis.* Phosphatidylinositol hydrolysis was measured as described previously [17]. BNu-2cl3 cells (1×10^6 cells) were labeled with [^{3}H]inositol (1 μCi/mL) for 12 hr in inositol-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 300 U/mL recombinant mouse IL-3. The cells were washed twice with a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution comprised of 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 15 mM HEPES, pH 7.4, and then preincubated with the same solution containing 10 mM LiCl for 10 min at 37°. The reaction was started by adding the test agents. After incubation for the indicated times at 37°, the reaction was terminated by the addition of 5% (w/v) trichloroacetic acid. Separation of [^{3}H]inositol phosphates (IPs) was carried out by Bio-Rad AG 1-X8 chromatography essentially as described by Berridge *et al.* [18].

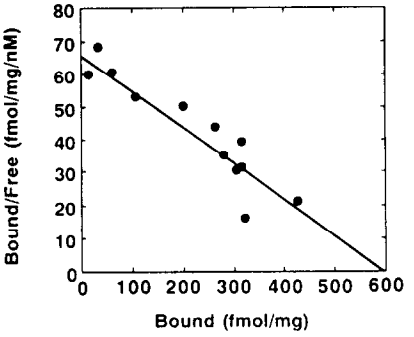

Fig. 1. Scatchard plot for PGE_2 binding to the membrane fraction of BNu-2cl3 cells. The membrane fraction of BNu-2cl3 cells was incubated for 60 min at 30° with increasing concentrations of [^{3}H]PGE_2 (0.25 to 20 nM). Specifically bound [^{3}H]PGE_2 was determined as described under Materials and Methods. The Scatchard plot was transformed from the value of specific [^{3}H]PGE_2 binding. The plot is representative of three independent experiments that yielded similar results.

Measurement of $[Ca^{2+}]_i$. BNu-2cl3 cells (1×10^6 cells/mL) were loaded with 3 μM fura-2/AM for 30 min at 37°. The cells were then washed twice with HEPES-buffered saline containing 0.5% bovine serum albumin and kept at 4° in the same solution until assayed. After centrifugation, the cells (1×10^6 cells) were resuspended in HEPES-buffered saline without bovine serum albumin, and then the fluorescence intensity was measured, at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrophotometer (Jasco CAF-100; Tokyo, Japan) as described previously [19].

Miscellaneous. The cAMP content of the cells and adenylate cyclase activity were determined as described previously [20]. Histamine release from the cells was determined according to the method of Shore *et al.* [21]. Protein concentrations were determined according to Lowry *et al.* [22] with bovine serum albumin as the standard.

RESULTS

[^{3}H]*PGE_2 binding to the membrane fraction of BNu-2cl3 cells.* The membrane fraction of BNu-2cl3 cells exhibited highly specific [^{3}H]PGE_2 binding activity, and the specific binding was more than 80% (data not shown). Scatchard analysis of this binding yielded a dissociation constant of 8.8 nM and a binding maximum of 597 fmol/mg (Fig. 1). The specificity of this binding is shown in Fig. 2A. The binding of [^{3}H]PGE_2 was inhibited by unlabeled PGs in the order of $PGE_2 = PGE_1 >$ iloprost, a stable PGI_2 analogue $> PGF_{2\alpha} > PGD_2 >$ U-46619, a stable thromboxane A_2 agonist. We further characterized the specificity of this [^{3}H]PGE_2 binding using ligands specific for the PGE receptor subtypes. As shown in Fig. 2B, among various PGE analogues,

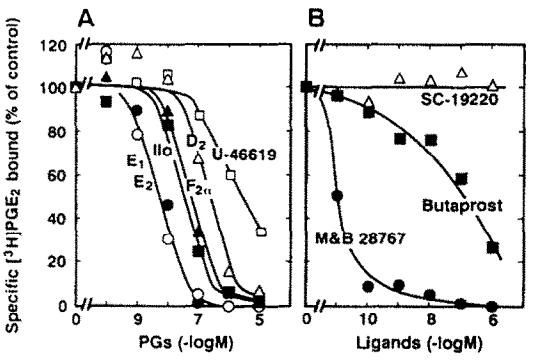

Fig. 2. Binding of [^{3}H]PGE_2 to the membrane fraction of BNu-2cl3 cells. (A) Displacement of [^{3}H]PGE_2 binding by various PGs. Unlabeled PGs were added to the binding assay mixture at the indicated concentrations, and specific [^{3}H]PGE_2 binding was determined as described under Materials and Methods. Key: (○) PGE_1; (●) PGE_2; (■) iloprost; (▲) $PGF_{2\alpha}$; (△) PGD_2; and (□) U-46619. (B) Displacement of [^{3}H]PGE_2 binding by ligands for PGE receptor subtypes. Key: (●) M&B 28767; (■) butaprost; and (△) SC-19220. The results shown are the means for three independent experiments, which varied by less than 5%.

only the EP_3-specific agonist, M&B 28767, caused strong and complete inhibition of the binding. On the other hand, an EP_2-specific agonist, butaprost, required a higher concentration for the inhibition, and an EP_1-specific antagonist, SC-19220, did not inhibit it at all. These results indicate that the PGE receptor expressed on BNu-2cl3 cells is of the EP_3 subtype.

Signal transduction of the EP_3 receptor in BNu-2cl3 cells. We recently demonstrated that the cloned EP_3 receptor is coupled to the inhibition of adenylate cyclase in Chinese hamster ovary cells expressing this clone [15]. Thus, we first examined whether PGE_2 causes inhibition of adenylate cyclase in BNu-2cl3 cells. However, PGE_2 up to 1 μM had no effect on iloprost-induced cAMP accumulation in the cells, and PGE_2 did not inhibit forskolin-stimulated adenylate cyclase activity in the membrane fraction (data not shown), indicating that the EP_3 receptor is not coupled to the inhibition of adenylate cyclase in BNu-2cl3 cells.

We next examined whether PGE_2 triggers phosphatidylinositol hydrolysis. Figure 3A shows the time courses of the [^{3}H]IP levels following the addition of 1 μM PGE_2. PGE_2 induced a rapid and transient increase in [^{3}H]IP_3 formation within 30 sec, and the level decreased to the basal level by 2 min. This increase was followed by ones in [^{3}H]IP_2 and IP_1, and these levels decreased to the basal levels within 5 min. Figure 3B shows the concentration dependency of the effect of PGE_2 on the [^{3}H]IP_3 level at 30 sec. The formation of [^{3}H]IP_3 was concentration dependent over the concentration range of 10 nM to 1 μM. M&B 28767 also induced [^{3}H]IP_3 formation, but butaprost did not induce it at all, and SC-19220 failed to antagonize the effect of M&B 28767 (data not shown). As phosphoinositide metabolism has been shown to be followed by Ca^{2+} mobilization from the endoplasmic reticulum via IP_3, we measured $[Ca^{2+}]_i$ using the fluorescent probe, fura-2. In the presence of extracellular Ca^{2+}, PGE_2 induced a rapid increase in $[Ca^{2+}]_i$ within 10 sec and then the level decreased gradually, the basal level being regained within 1 min (Fig. 4A). In Ca^{2+}-free medium, PGE_2 induced a rapid increase in $[Ca^{2+}]_i$, but the extent of the maximal increase in $[Ca^{2+}]_i$ was reduced (Fig. 4B). M&B 28767 also caused an increase in $[Ca^{2+}]_i$, but butaprost had no effect on $[Ca^{2+}]_i$ at all, and SC-19220 did not alter the PGE_2-induced increase in $[Ca^{2+}]_i$ (Fig. 4, C–E). When the cells were treated with PT (50 ng/mL for 6 hr), the PGE_2-induced [^{3}H]IP_3 formation and elevation of $[Ca^{2+}]_i$ were almost and completely suppressed, respectively (Table 1). To confirm the effect of the toxin, the membrane fraction of the cells was incubated with the activated toxin and [α-^{32}P]NAD. One PT-sensitive band, corresponding to M_r = 41,000 and thus presumably G_i was labeled in the membrane fraction from the untreated cells, but not in that from PT-pretreated cells (data not shown).

Effect of PGE_2 on histamine release from BNu-2cl3 cells. The main function of mast cells is the secretion of histamine [11]. We thus examined the effect of PGE_2 on histamine release from BNu-2cl3 cells. As shown in Table 2, whereas 1 μM PGE_2 by itself exhibited no significant ability to stimulate histamine release from the cells, PGE_2 clearly promoted it in concert with 0.1 μM ionomycin; at this concentration, ionomycin itself did not evoke histamine release. This increased release caused by PGE_2 with ionomycin was inhibited completely by PT pretreatment. M&B 28767 also reproduced this stimulatory action, but butaprost did not when used with ionomycin. These results indicate that the activation of the EP_3 receptor stimulates the histamine release from BNu-2cl3 cells through G_i in concert with Ca^{2+} mobilization.

DISCUSSION

The objective of this study was to characterize the PGE receptor expressed on an IL-3-dependent mouse mast cell line, BNu-2cl3. The membrane fraction of BNu-2cl3 cells exhibited PGE_2 binding specificity for PGE_1, PGE_2 and the EP_3-specific agonist M&B 28767 (Fig. 2), indicating that the PGE receptor expressed on BNu-2cl3 cells is of the EP_3 subtype. We have recently cloned the mouse EP_3 receptor from mastocytoma P-815 cells and demonstrated that this receptor is engaged in inhibition of adenylate cyclase [15]. Furthermore, we showed that the EP_3 receptor functionally associates with G_i and inhibits adenylate cyclase activity in a reconstitution system [23]. Adipocytes [24] and cortical collecting tubule cells [9] express the EP_3 receptor, and PGE_2 inhibits adenylate cyclase in these cells. However, the EP_3 receptor was found to be coupled to phosphoinositide metabolism through a PT-sensitive G protein, but not to inhibition of adenylate cyclase in BNu-2cl3 cells (Fig. 3). It was proposed that PT-sensitive G proteins are involved in the activation of phospholipase C in several types of cells [25–27].

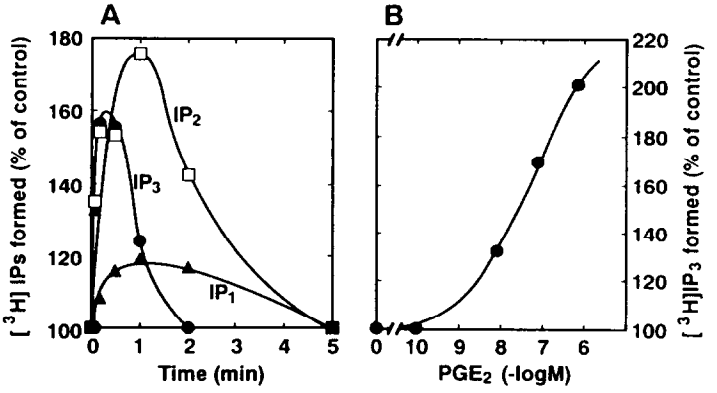

Fig. 3. Effect of PGE_2 on phosphatidylinositol hydrolysis in BNu-2cl3 cells. (A) Time course: cells were labeled with [^{3}H]inositol for 12 hr. After preincubation with 10 mM LiCl for 10 min, the cells were stimulated with 1 μM PGE_2. At the time points indicated, [^{3}H]IPs were extracted and analyzed by AG 1-X8 chromatography as described under Materials and Methods. The values for IP_1 (▲), IP_2 (□), and IP_3 (●) are expressed as percentages of the control at each time point. The dpms in the control (0 time) were 680 ± 20 for IP_1, 160 ± 10 for IP_2, and 230 ± 20 for IP_3; the control values at each time point did not vary significantly. (B) Concentration dependency of IP_3: the cells were stimulated for 30 sec with the indicated concentrations of PGE_2, and IP_3 was determined by measurement of radioactivity. The values are expressed as percentages of the control. The dpm for [^{3}H]IP_3 in the control was 250 ± 60. The results shown are the means for three independent experiments, which varied by less than 5%.

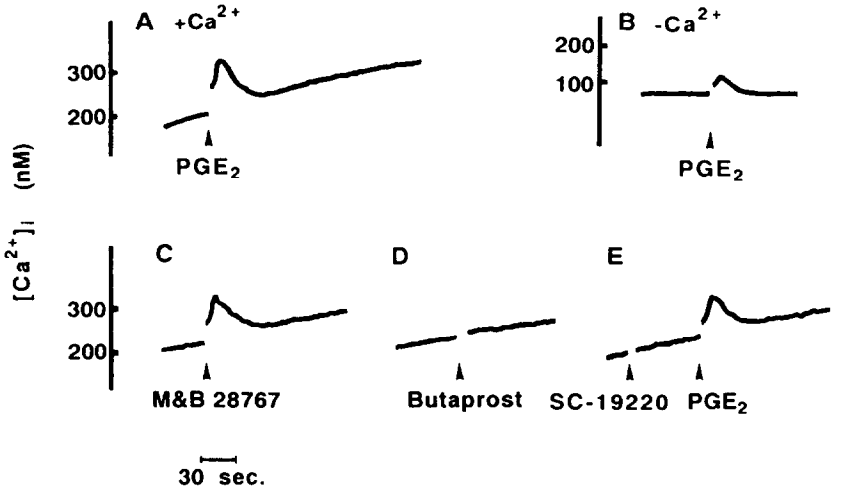

Fig. 4. Effects of PGE_2 and ligands specific for PGE receptor subtypes on $[Ca^{2+}]_i$. The extracellular Ca^{2+} concentration was adjusted to 2 mM (A, C, D and E); the extracellular ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) concentration was adjusted to 0.3 mM without the addition of $CaCl_2$ (B). Fura-2-loaded cells (1×10^6 cells) were stimulated with 1 μM PGE_2 (A and B), M&B 28767 (C), or butaprost (D). After the cells had been preincubated for 1 min at 37° with 1 μM SC-19220, they were stimulated with 1 μM PGE_2 (E). The agents were added at the times indicated by the arrows. The recordings shown are representative of three independent experiments that yielded similar results.

Both G_i and G_o have been shown to reconstitute stimulation of phospholipase C by fMet-Leu-Phe in membranes prepared from PT-treated differentiated HL-60 cells [28]. It is, therefore, clear that G_i can activate phospholipase C as well as inhibit adenylate cyclase, but an effector coupled with G_i would be selected in tissues or cells. Direct activation of G_i by compound 48/80 or mastoparan has been shown to stimulate phosphoinositide metabolism and Ca^{2+} mobilization in mast cells [29, 30], indicating that the effector transduced by G_i is phospholipase C in mast cells. Thus, the EP_3 receptor exerts the

Table 1. Effect of PT treatment on PGE_2-induced $[^3H]IP_3$ formation and the increase in $[Ca^{2+}]_i$

Treatment	$[^3H]IP_3$ (% of control)	$\Delta[Ca^{2+}]_i$ (nM)
None	222 ± 12	127 ± 4.2
PT	127 ± 20*	ND†

Cells were treated with or without 50 ng/mL PT for 6 hr. The cells labeled with [^{3}H]inositol or fura-2-loaded cells were stimulated with 1 μM PGE_2. $[^3H]IP_3$ at 30 sec after the stimulation was determined as described under Materials and Methods. The values shown represent percentages of the control. The control value for $[^3H]IP_3$ was 227 ± 22 dpm. The peak $[Ca^{2+}]_i$ level induced by PGE_2 was determined as described under Materials and Methods. Values are the means ± SEM for three independent experiments.

* Significant difference between PT treatment and no treatment, $P < 0.01$.

† ND, not detected.

Table 2. Effect of PGE_2, M&B 28767 or butaprost on histamine release from BNu-2cl3 cells

Addition	Histamine release (% of total)	
	Untreated	PT-treated
None	2.2 ± 0.070	3.23 ± 0.20
PGE_2	2.72 ± 0.24	3.41 ± 0.050
Ionomycin	2.74 ± 0.070	3.92 ± 0.16
PGE_2 + ionomycin	7.27 ± 0.17	3.91 ± 0.10
M&B 28767	2.85 ± 0.11	3.02 ± 0.34
M&B 28767 + ionomycin	7.93 ± 0.36	2.15 ± 0.28
Butaprost	2.47 ± 0.11	
Butaprost + ionomycin	3.32 ± 0.30	

Cells were treated with or without 50 ng/mL PT for 6 hr. The cells (8×10^6 cells/assay) were then incubated for 5 min at 37° with the vehicle, 1 μM PGE_2, 1 μM M&B 28767, 1 μM butaprost, 0.1 μM ionomycin, or the indicated combination. Histamine release was determined as described under Materials and Methods. The values are expressed as percentages of the total cellular histamine (14.0 μg/8×10^6 cells), and are the means ± SEM for triplicate experiments.

inhibition of adenylate cyclase via G_i in adipocytes and cortical collecting tubule cells but, in contrast, it exerts the activation of phospholipase C via G_i in BNu-2cl3 cells.

The main function of mast cells is histamine secretion [11]. Stimulators of histamine release from mast cells, such as IgE, thrombin and fMet-Leu-Phe, stimulate phosphoinositide metabolism and induce sustained uptake of extracellular Ca^{2+} [31, 32]. Histamine release is highly dependent on sustained extracellular Ca^{2+} mobilization although an additional synergistic signal is clearly necessary [33]. Protein kinase C has been proposed to play a role in exocytosis [34]. Low concentrations of Ca^{2+} ionophores can induce substantial increases in $[Ca^{2+}]_i$ without stimulating histamine release, but when they are added together with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), secretion occurs [35], indicating that activation of protein kinase C resulting from phosphoinositide metabolism promotes a synergistic signal with Ca^{2+} for the secretion. A potent stimulator of histamine release, IgE or thrombin, can provide the necessary signals for the stimulatory events, sustained extracellular Ca^{2+} mobilization and activation of protein kinase C [36, 37]. In contrast, although PGE_2 stimulated phosphoinositide metabolism and induced a transient increase in $[Ca^{2+}]_i$, PGE_2 by itself failed to evoke histamine release from BNu-2cl3 cells (Table 2). The lack of ability of PGE_2 to induce histamine release may be due to the lack of sustained Ca^{2+} uptake (Fig. 4). However, PGE_2 could stimulate the release in the presence of ionomycin in a PT-sensitive manner, suggesting that the release is synergistically evoked by the ionophore-induced Ca^{2+} mobilization and the activation of protein kinase C resulting from PGE_2-stimulated phosphoinositide metabolism. Activation of adenosine receptors also provides synergistic signals for histamine secretion in antigen-stimulated RBL-2H3 cells through stimulation of inositol phospholipid hydrolysis [38]. Considering these results, the EP_3 receptor may exert a stimulatory effect on antigen-induced histamine release from mast cells.

IL-3 induces the preferential growth and differentiation of hematopoietic progenitor cells in bone marrow to mast cells, which resemble mucosal mast cells [10]. Recently, a new mast cell growth factor has been proposed to be involved in the differentiation of the progenitor cells to connective tissue mast cells [39, 40], but the developmental relationship of these types of mast cells remains unknown. Connective tissue mast cells appear to express the EP_2 receptor, which suppresses histamine secretion from the cells by elevating the intracellular cAMP level [13, 14]. In contrast, BNu-2cl3 mast cells express the EP_3 receptor, which is coupled to phosphoinositide metabolism, suggesting that mucosal mast cells express the EP_3 receptor. Thus, expression of the two PGE receptor subtypes, EP_2 and EP_3, may be differentially regulated during the development of mast cells, and each receptor type modulates the function of mast cells at different developmental stages; the EP_2 receptor suppresses histamine release from connective mast cells, and the EP_3 receptor promotes it from mucosal mast cells.

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