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Research Article

Activation of CD47 receptors causes histamine secretion from mast cells

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Abstract. Mast cells play pivotal roles in allergic and inflammatory processes *via* distinct activation pathways. Mucosal and serosal mast cells are activated by the IgE/FcεRI pathway, while only serosal mast cells are activated by basic secretagogues. We show that CD47 receptors are expressed on rat peritoneal mast cells. 4N1K, a peptide agonist of CD47, rapidly caused exocytosis. Such exocytosis required increased intracellular calcium and was inhibited by pertussis toxin and an antibody against the βγ dimer of a G_i protein. Cooperation with integrins and glycosylphosphatidylinositol-anchored proteins was necessary, since anti-integrin antibodies and pretreatment with phosphatidylinositol-phospholipase C reduced exocytosis. Depletion of membrane cholesterol inhibited exocytosis and decreased CD47 in lipid rafts, consistent with a CD47/integrin/G_i protein complex being located in rafts. An anti-CD47 antibody inhibited exocytosis induced by 4N1K and by mastoparan and spermine, suggesting that basic secretagogues might target CD47. We propose that 4N1K-stimulated mast cell exocytosis involves a CD47/integrin/G_i protein complex.

Keywords. CD47, integrin-associated protein, mast cell exocytosis, inflammation, basic secretagogues.

Introduction

Mast cells are tissue dwelling cells that play a pivotal role in allergic reactions and take part in other pathophysiological conditions such as innate and acquired immunity, autoimmune diseases, inflammation, wound healing, fibrosis and tumors [1, 2]. Activated mast cells release stored and *de novo*-synthesized mediators including histamine, cytokines, leukotrienes, prostaglandins and proteases [3]. Both mucosal and serosal (or connective tissue) mast cells are activated by interaction of antigen to IgE bound to the high-affinity IgE receptor FcεRI [4]. In addition, serosal mast cells, such as human skin and rat peritoneal mast cells, are also activated by a variety of compounds generically known as basic secretagogues [5], *via* an IgE-independent pathway. These compounds include endogenous and venom peptides (*e.g.*, substance P, bradykinin, anaphylatoxin C3a and mastoparan), drugs (*e.g.*, neomycin and cannabinoids, defensins), and natural or synthetic polyamines (*e.g.*, spermine and compound 48/80) [5–7]. Although many attempts to identify candidate receptors for these compounds have been made, none have been described until recently. It was thus proposed that after penetration into mast cells in an apparently receptor-independent fashion, basic secretagogues interact directly with G proteins to induce exocytosis [5–9]. The inhibitory effects of pertussis toxin pretreatment and specific blocking antibodies against G_i protein subunits, together with stimulation of

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purified G proteins by mastoparan and other basic peptides, support this hypothesis [7, 9, 10].

Nevertheless, the possible involvement of membrane receptors accounting for basic secretagogue-induced mast cell activation has never been formally excluded [5]. Indeed, newly discovered G protein-coupled receptors (GPCR) called Mas-related genes (Mrg) have been recently suggested as candidate receptors for basic secretagogues [11], based on the correlation between cell expression levels and induced responses. Furthermore, trimeric G proteins may also transduce the activation of some non-heptahelical receptors [12]. This has been extensively demonstrated for CD47 (or integrin-associated protein, IAP) [13–15]. CD47, a 50-kDa transmembrane glycoprotein, is a member of the immunoglobulin superfamily that was also termed IAP because it co-purifies and interacts with integrins [16, 17]. CD47 has a single Ig-V like domain heavily glycosylated at its N terminus, five transmembrane domains and an alternatively spliced cytoplasmic C terminus. CD47 is ubiquitously expressed and, based on its modulatory interactions with several types of integrins [18–20], it has been proposed that CD47 and integrin form a complex having seven transmembrane helices that mimics GPCR [18]. CD47 also mediates cell-cell interactions *via* SIRP (signal regulatory protein) family receptors [21] and cell-extracellular matrix interactions *via* thrombospondin family members [22–24]. Thrombospondin is an endogenous ligand for CD47 [20, 23], and peptide 4N1K, widely used as a specific CD47 agonist, corresponds to the C-terminal binding motif of thrombospondin [23–25].

CD47 is implicated in many different physiological or pathophysiological processes [18], including infection [26], inflammation [27, 28], cell spreading, proliferation and apoptosis [20, 27, 29]. Given that CD47 is functionally coupled to trimeric G_i proteins [13–15], has pro-inflammatory effects [27] and is expressed in a variety of mast cells [30–32], we tested whether exocytosis of mast cells is triggered by activating CD47. Here, we show for the first time that stimulation of CD47 by peptide 4N1K rapidly induces secretion of histamine from rat peritoneal mast cells. Pretreatment with a specific anti-CD47 monoclonal antibody (mAb) or pertussis toxin blocked secretion induced by 4N1K. The interaction of activated CD47 with G proteins was confirmed by the inhibition of histamine release upon pretreatment with mAb directed against the Gβγ dimer of G_i proteins. CD47-mediated secretion required cooperation with integrins, as mAb directed against β integrin subunits decreased histamine release. Interestingly, pretreatment with an anti-CD47 mAb also caused inhibition of secretion induced by two reference basic secretagogues, mastoparan and spermine, suggesting that CD47 might be a receptor candidate for basic secretagogues. We also found that membrane cholesterol was required for CD47-mediated exocytosis and that a glycosylphosphatidylinositol (GPI)-anchored protein was important in maintaining the functional integrity of the CD47/integrin/G_i-protein complex.

Materials and methods

Antibodies and reagents. Peptides 4N1K, K-4NGG and 4N1–2 were from Bachem (Bubendorf, Switzerland). 4N1K and 4NGG were also synthesized in the lab by Dr. H. de Rocquigny, and were verified by mass spectroscopy. Anti-CD47 (clone OX101), anti-CD48 (clone OX45), anti-CD18/β2 integrin (clone 6G2) and anti-CD61/β3 integrin (clone F11) mAbs were from Serotec (Oxford, UK). Anti-CD29/β1 integrin mAb (clone Ha2/5) was from BD Pharmingen Biosciences (San Diego, CA, USA). Anti-Gβ mAb (directed against the C-terminal 20 amino acids of mouse G protein β1 subunit, with broad specificity to mouse, rat and human Gβ1–4 subunits) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fura-2/AM and 2-aminoethoxydiphenyl borate (2-APB) were from Calbiochem (San Diego, CA, USA). Alexa Fluor® 568 goat anti-mouse IgG was from Invitrogen (Paisley, UK). All other reagents were from Sigma (St. Louis, MO, USA).

Isolation and purification of mast cells. Male Wistar rats (300–500 g) were anesthetized before bleeding. Rats were injected intraperitoneally with 10 ml HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, 10 mM HEPES and NaOH to pH 7.4) supplemented with 0.1 % BSA. Peritoneal fluid was collected after gentle abdominal massage and centrifuged for 3 min at 180 *g*. The pellet of mixed peritoneal cells was resuspended in the same buffer and mast cells were purified by centrifugation for 10 min at 220 *g* on a discontinuous BSA gradient (30 % and 40 %, w/v). The pellet was then resuspended and mast cells were examined under a light microscope for viability (>95 %) and purity (>97 %) using Trypan blue and toluidine blue, respectively. Rats were raised in the animal house facilities in the Faculty of Pharmacy and used in accordance with Institutional policies (No. D-67-218-26, Direction Départementale des Services Vétérinaires du Bas-Rhin).

Quantification of mast cell exocytosis. Purified mast cells (2×10^4 cells/assay) were pre-incubated for 5 min at 37°C before challenge with different secretagogues

for 10 min at 37°C. Reactions were stopped by adding ice-cold buffer. Exocytosis of mast cells was quantified by determining the amount of secreted histamine by spectrofluorimetry, as previously described [33]. Values for stimulated histamine release in the supernatant were expressed as a percentage of the total cellular histamine content obtained after cell lysis [supernatant/(supernatant+pellet) ×100] and were corrected for the basal release of histamine (similarly calculated) that occurred in the absence of any stimulus. Basal histamine release was less than 5% of total content.

Measurement of intracellular calcium. Intracellular calcium was measured from Fura-2-loaded mast cell suspensions using a spectrofluorimeter (Hitachi F-2000) essentially as previously described [34]. Briefly, mast cells (1×10^6 cells/ml) were incubated with 1 μM Fura-2/AM for 15 min at room temperature in HEPES buffer. Cells were then washed twice in HEPES buffer and re-suspended in the same buffer at 1×10^6 cells/ml in a 1-cm quartz cuvette. Cells were continuously stirred and sequentially excited at 340 and 380 nm for 1-s periods at room temperature; emitted fluorescence was measured at 510 nm. Ca^{2+} concentrations were calculated as described elsewhere [35].

RNA extraction and RT-PCR. Total RNA was extracted from mast cells with PureZOL™ reagent (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendations. Reverse transcription was done using 500 ng total RNA with the SuperScript™III First-strand synthesis system (Invitrogen, Paisley, UK) according to manufacturer's protocol. Amplification was assessed using 1 μl RT products in a mixture containing 200 μM of each dNTP, 0.5 μM oligonucleotide primer, 1× Phusion HF buffer and 0.02 U/μl Phusion DNA polymerase (Finnzymes, Espoo, Finland). PCR primers 5'-CTCTGTGTGCTGAAGGGGGG-3' (forward) and 5'-GGGACGCGCAACAGACATT-3' (reverse) were used to detect rat hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA, and 5'-ACTGAAGGGAGGTGACGGACTGTAACT-3' (forward) and 5'-ATTTTTTATTACGTAAATTACAGCTGC-3' (reverse) for CD47 mRNA. The presence of different CD47 isoforms (in rats: IAP5, IAP6, IAP7) were evaluated using a common forward primer 5'-ACGCTTCTGGTCTTGGCC-3', and specific reverse primers 5'-CATTCATCATCCCTTTCG-3' for IAP5 (corresponding to human form 4), 5'-TCAGTT ATTCGTTAAGGG-3' for IAP6 (human form 3), and 5'-TCAGTTATTCCTAGGAGG-3' for IAP7 (human form 2) [36]. Cycling parameters were: 98°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles, followed by a final elongation at 72°C for 5 min. PCR products were run on 2% agarose gels stained with 1 μg/ml ethidium bromide. As a negative control, cDNA was omitted from the reaction mixture, with no observed amplified band. This ensures that no exogenous contamination was present in our experiments.

Immunofluorescence microscopy. Purified mast cells were allowed to adhere on glass coverslips for 1 h at 37°C. Cells were fixed for 10 min at 4°C with 4% paraformaldehyde/PBS. Nonspecific binding sites were blocked with 2% BSA/PBS for 1 h at room temperature under gentle agitation. Mast cells were incubated with a primary Ab directed against CD47 (10 μg/ml) and/or cholera toxin B subunit-FITC (20 μg/ml) for 1 h at room temperature under gentle agitation. Mast cells were then incubated for 1 h at room temperature with the secondary Ab (Alexa Fluor® 568 goat anti-mouse IgG, 1 μg/ml) under gentle agitation. Coverslips were mounted and observed using an epifluorescence microscope (Nikon Diaphot) or a confocal microscope (Bio-Rad 1024, Kr/Ar laser, excitation 488 nm, emission 522 and 585 nm; Nikon Eclipse TE300, 40x Plan Apo CFI-Fluor oil immersion objective, n.a. 1.3). Image J software (NIH freeware) was used to analyze individual images from z-stacks and to create merged (overlay) images [37].

Determination of reactive oxygen species

Mast cells (10^6/mL) were pre-incubated for 5 min at 37°C. 100 μl aliquots were stimulated with 100 μM 4N1K for 5 min. Reactive oxygen species (ROS) were immediately determined by a chemiluminescent assay as previously described [38].

Statistical analysis. Results are presented as mean ± SEM of at least three independent experiments. Statistical analysis was done using ANOVA. Significant differences are represented throughout as $*p<0.05$, $**p<0.01$ and $***p<0.001$.

Results

Expression of CD47 on rat peritoneal mast cells. CD47 has been shown to be expressed on human skin mast cells [30]. Skin mast cells, like peritoneal mast cells, belong to the serosal mast cell class, and respond to basic secretagogues [39]. We first demonstrated that CD47 was expressed on rat peritoneal mast cells by immunostaining (Fig. 1A). The presence of mRNA encoding CD47 was also verified (Fig. 1B). CD47 is alternatively spliced and, in man, this alternative

splicing results in the expression of four isoforms differing by the length of the C-terminal domain, while in rats only three isoforms are expressed [40]. Here, the three different CD47 isoforms were all expressed in rat peritoneal mast cells (Fig. 1B).

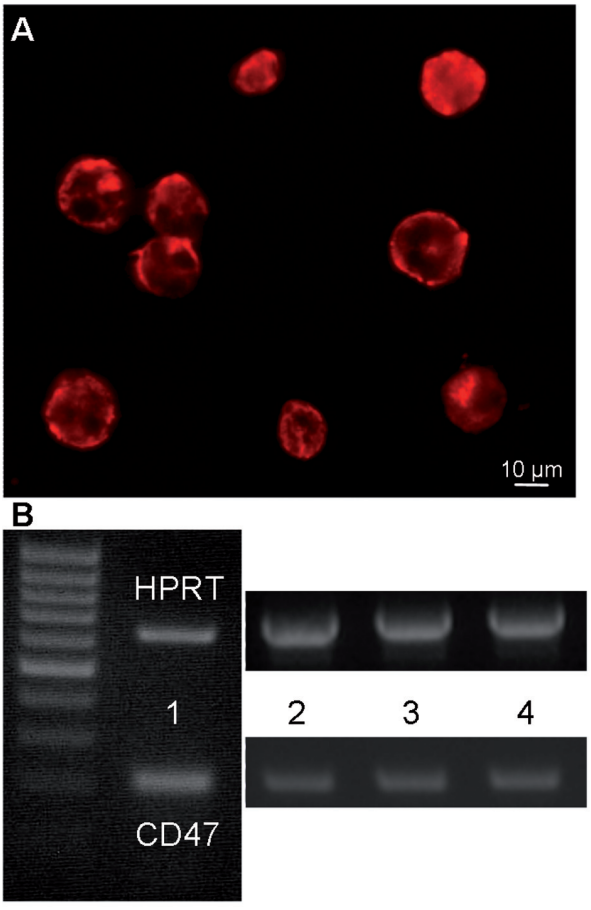

Figure 1. CD47 expression in rat peritoneal mast cells. (*A*) CD47 was immunocytochemically detected on mast cells. (*B*) CD47/integrin-associated protein (IAP) mRNA expression was analyzed by first strand cDNA PCR using primers for all CD47 isoforms (lane 1) or specific primers for IAP5 (lane 2), for IAP6 (lane 3) and IAP7 (lane 4).

Activation of CD47 by 4N1K stimulates mast cell exocytosis and involves G proteins. Histamine secretion from mast cells challenged with peptide 4N1K, a widely used CD47 agonist [19, 23–25], occurred in a concentration-dependent manner (Fig. 2A). A maximal effect of 65 % release of total histamine content was reached at 300 µM. Greater 4N1K concentrations were not used because of lack of peptide solubility. Pretreatment of cells with OX101, a well-characterized function blocking anti-CD47 mAb [21], decreased 4N1K-stimulated secretion (Fig. 2C). 4N1K-induced histamine release was unaffected by pretreatment with a control isotype IgG1 (anti-CD48 mAb OX45; Fig. 2D). Basal histamine secretion from cells in the absence of 4N1K was unaffected by pretreatment with the anti-CD47mAb (Fig. 2E), indicating that CD47 was not constitutively activated.

The time course of histamine release induced by 100 µM 4N1K was rapid, with a plateau response being reached within 10–20 s (Fig. 2F). These rapid kinetics confirm that the CD47-mediated pathway is different from the IgE/RFcε pathway (for which stimulated exocytosis reaches a maximum only after a few minutes [4]), and are similar to those observed for basic secretagogues [5].

In addition to histamine, we also examined whether 4N1K was capable of liberating other mast cell mediators. We assessed the production of ROS using a luminescent assay. Stimulation of mast cells with 100 µM 4N1K for 5 min significantly enhanced ROS production (from 5162±585 RLU to 11 515±1013 RLU; n=3).

Since several CD47-mediated effects depend on G_i protein signaling [13–15], mast cells were pretreated with pertussis toxin. This resulted in a strong dose-dependent inhibition of 4N1K-induced histamine release (Fig. 2A). Next, permeabilized cells were pretreated with an anti-Gβγ mAb since, in mast cells, the only substrates of pertussis toxin are G proteins of the G_{i2} and G_{i3} subtypes [7, 9, 33]. Again, a concentration-dependent inhibition of 4N1K-induced secretion was obtained (Fig. 2B), with essentially complete inhibition at the highest used mAb concentration. This is the first demonstration of CD47 being coupled to the βγ dimer of G proteins.

The Gβγ dimer is known to interact with PLCβ, thereby leading to IP_3 production and Ca^{2+} release from intracellular stocks located in the endoplasmic reticulum. To test whether CD47-mediated exocytosis is dependent on a rise in intracellular Ca^{2+}, mast cells were pretreated with caffeine, which acts on IP_3 receptors. This resulted in a concentration-dependent inhibition of 4N1K-induced histamine release (Fig. 3A). Similar effects were obtained for cells pretreated with 2-APB (Fig. 3B), initially described as an IP_3 receptor antagonist [41] but subsequently reported to also block TRP channel-mediated Ca^{2+} influx [42]. To prevent simulated rises in intracellular Ca^{2+}, mast cells were pretreated with BAPTA-AM [43]. Chelation of intracellular Ca^{2+} by BAPTA resulted in dose-dependent inhibition of 4N1K-stimulated secretion (Fig. 3C). Consistently, we also confirmed that 4N1K application induces a rapid increase in intracellular Ca^{2+} by measurements done on Fura-2-loaded mast cell suspensions (Fig. 3D). Finally, chelation of extracellular Ca^{2+} using EGTA had no effect on 4N1K-induced histamine release (not

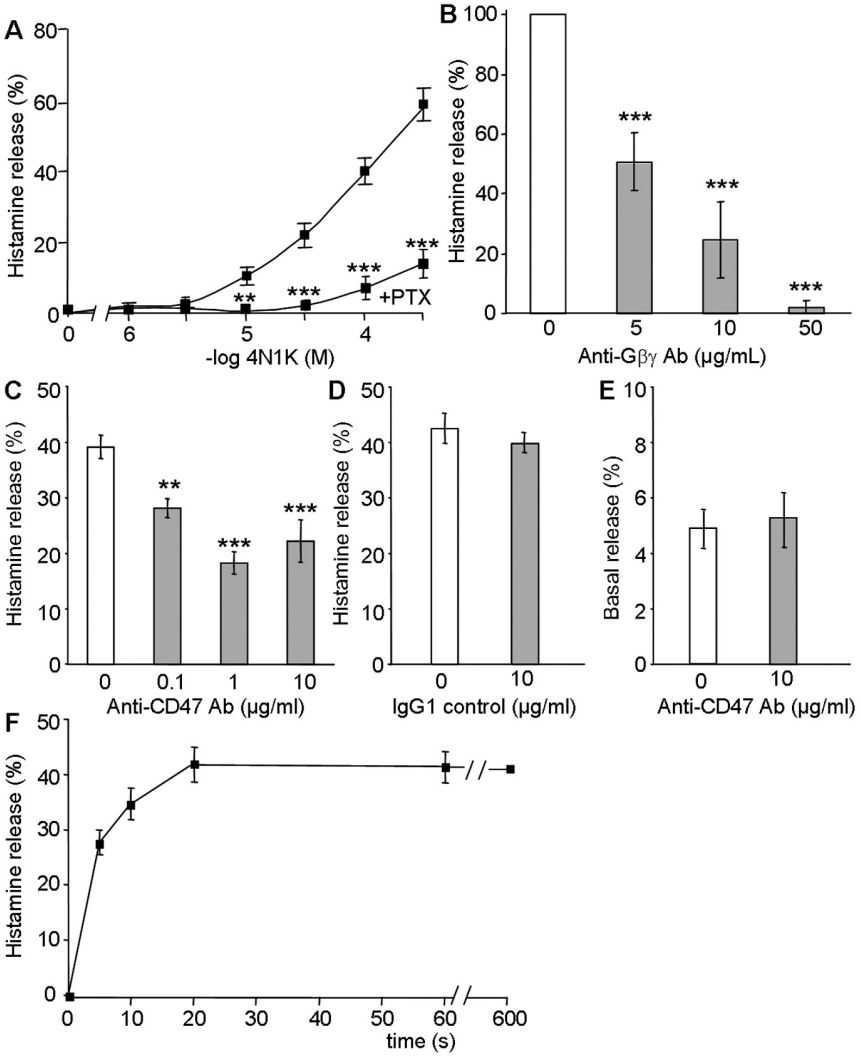

Figure 2. 4N1K-stimulated histamine secretion in rat peritoneal mast cells. (*A*) Cells were pretreated with pertussis toxin (PTX, 50 ng/ml) or not and then stimulated by increasing 4N1K concentrations for 10 min (n=6). Exocytosis was assessed by measuring released histamine. (*B*) Effect of an mAb directed against the βγ dimer of trimeric G proteins on mast cells stimulated with 4N1K. Cells were permeabilized with streptolysin O (2500 U/ml) as described previously [33], incubated for 2 min at 37°C with the indicated concentrations of mAb and then stimulated with 100 µM 4N1K for 2 min (n=3). Results were normalized because of the irregular increases in passive histamine release following permeabilization of mast cells. As a control, we previously reported that specific mAb directed Gαt and Gαs subunits were without effect in permeabilized rat peritoneal mast cells [33]. (*C*) Effect of OX101, a blocking anti-CD47 mAb, on secretion induced by 100 µM 4N1K (n=5). Cells were pre-incubated (1 h, 37°C) with the indicated mAb concentrations and then stimulated for 10 min. (*D*) Pretreatment (1 h, 37°C) with an isotype control mAb (IgG1) was without effect on 100 µM 4N1K-induced histamine release (n=5). (*E*) Pretreatment with the anti-CD47 mAb (1 h, 37°C) was without effect on basal secretion from non-stimulated cells (n=5). (*F*) Time course of 4N1K-induced release of histamine. Cells were pre-incubated for 5 min at 37°C then stimulated with 100 µM 4N1K for the indicated times (n=4). Data are mean ± SEM of n independent experiments (*p<0.05, **p<0.01, ***p<0.001).

shown). Taken together, these data indicate that CD47-mediated secretion requires a rise in intracellular Ca^{2+} and does not depend on Ca^{2+} influx, similar to basic secretagogues that induce IP_3 production in mast cells [6, 33, 34, 44].

CD47-mediated exocytosis involves cooperation with integrins and GPI-anchored proteins in lipid rafts. Since CD47 has been widely reported to interact with integrins [18], we thus determined if integrins cooperate with CD47 in mast cell exocytosis. Pre-incubation of cells with anti-β1 integrin mAb resulted in approximately 50% inhibition of 4N1K-induced secretion (Fig. 4). Greater inhibition was not obtained by increasing mAb concentrations (not shown). Maximal inhibitory effects against 4N1K-induced histamine release obtained for pre-incubation with anti-β2 and anti-β3 integrin mAb were 30% and 35%, respectively

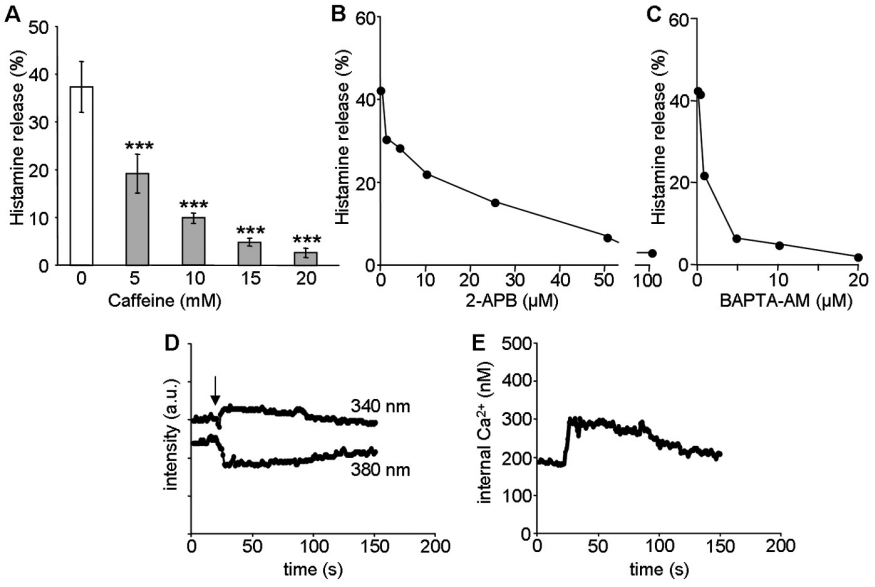

Figure 3. (*A*) Effect of pre-incubation (20 min, 37°C) with increasing concentrations of caffeine on secretion induced by 100 μM 4N1K (10 min, 37°C; n=4). (*B*) Effect of pretreatment (10 min, 37°C) with increasing concentrations of 2-aminoethoxydiphenyl borate (2-APB) on histamine release induced by 100 μM 4N1K (n=3). (*C*) Effect of pretreatment (10 min, 37°C) with increasing concentrations of BAPA-AM on secretion induced by 4N1K (n=3). Data are mean ± SEM of three to four independent experiments (***p<0.001). Error bars in (*B*) and (*C*) are smaller than the symbols. (*D*) Changes in emitted fluorescence from Fura-2-loaded mast cells upon stimulation (arrow) with 100 μM 4N1K (left panels) and corresponding calculated increase in intracellular Ca^{2+} (right panel). Traces are representative from four independent experiments.

(Fig. 4). Pretreatment with an isotype control IgG1 (anti-CD48 mAb OX45) was again without effect on 4N1K-induced secretion (not shown). Cells were also pretreated with 1 μg/ml anti-CD47 mAb and then with 1 μg/ml anti-β1 or anti-β3 integrin mAb. The inhibition of 100 μM 4N1K-induced histamine release was not significantly greater than observed for the anti-β integrin mAb alone (not shown). These data indicate that mast cell exocytosis provoked by 4N1K involves β integrin subunits and are consistent with a CD47/integrin complex mimicking GPCR [18].

Since GPI-anchored proteins interact with integrins [45], we tested whether a GPI-anchored structure is implicated in CD47-mediated secretion. Mast cells were pretreated with phosphatidyl inositol-phospholipase C (PI-PLC) that catalyzes disruption of the GPI anchor [46]. PI-PLC pretreatment resulted in an approximately 50% inhibition of 4N1K-induced histamine release (Fig. 5). This suggests that a GPI-anchored protein belongs to a macromolecular complex responsible for the effects of 4N1K in mast cells. Cells were also pretreated with 1 μg/ml anti-CD47 mAb and then with 1 U/ml PI-PLC. The inhibition of 100 μM 4N1K-induced histamine release was not significantly greater compared to pretreatment with PI-PLC alone (not shown).

Since cholesterol is a crucial plasma membrane component in maintaining the functional integrity of

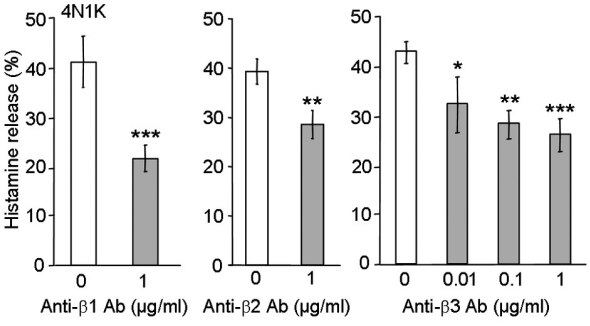

Figure 4. Effects of blocking antibodies against β1, β2 and β3 integrin subunits on the secretory response of rat peritoneal mast cells induced by 100 μM 4N1K. Cells were pre-incubated 1 h at 37°C with the indicated mAb concentrations and then stimulated for 10 min. Data are mean ± SEM of four independent experiments (*p<0.05, **p<0.01, ***p<0.001).

the signaling complex formed by CD47/integrin/G_i protein [47], we determined the role of cholesterol in CD47-mediated mast cell activation. Cells were pretreated with methyl-β-cyclodextrin (MβCD), resulting in a concentration-dependent inhibition of 4N1K-induced secretion (Fig. 6A). Since lipid rafts are also involved in the exocytotic process [48], a potential inhibitory effect of MβCD on secretory granule fusion with the plasma membrane was assessed by pretreating cells with MβCD prior to

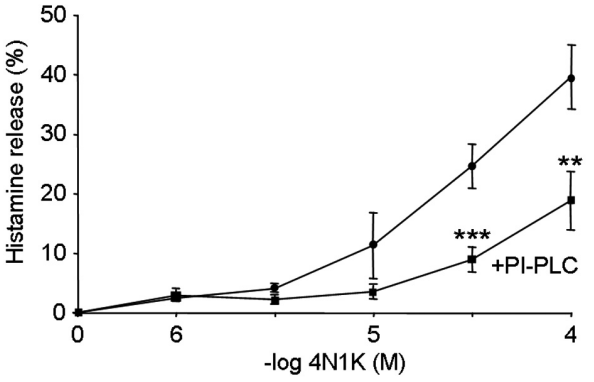

Figure 5. Effects of pretreatment of rat peritoneal mast cells with phosphatidylinositol-phospholipase C (PI-PLC; 1 U/ml, 3 h, 37°C) on the secretory response induced by increasing concentrations of 4N1K (10 min stimulation, 37°C). Data are mean ± SEM of four independent experiments (**$p<0.01$, ***$p<0.001$).

stimulation with the Ca^{2+} ionophore A23187 [34]. No significant inhibition of A23187-induced exocytosis was found after pretreatment with MβCD (Fig. 6B). To verify that CD47 is co-localized in lipid rafts, confocal microscopy was done on fixed mast cells stained for CD47 and for GM1 gangliosides (Fig. 6C–F). GM1 gangliosides are particularly enriched in detergent-resistant lipid raft microdomains and are ligands for cholera toxin B subunits [49, 50]. Resting cells (Fig. 6C, D) and 100 μM 4N1K-stimulated cells (Fig. 6E, F) were pretreated (Fig. 6D, F) or not (Fig. 6C, E) with MβCD. A clear decrease in co-localization of CD47 with GM1 gangliosides was observed for MβCD pretreated cells (Fig. 6D, F). Thus, our data are consistent with 4N1K-induced histamine release from mast cells requiring co-localization of CD47, integrins, G_i proteins and GPI-anchored proteins in lipid rafts.

CD47: A candidate receptor for basic secretagogues?

Since the secretory responses induced by 4N1K and by basic secretagogues share several similarities, we tested the effect of the anti-CD47 mAb OX101 on secretion induced by mastoparan and spermine, two reference basic secretagogues [6]. Pretreatment with the anti-CD47 mAb decreased secretion induced by both mastoparan and spermine (Fig. 7A, B). Again, a

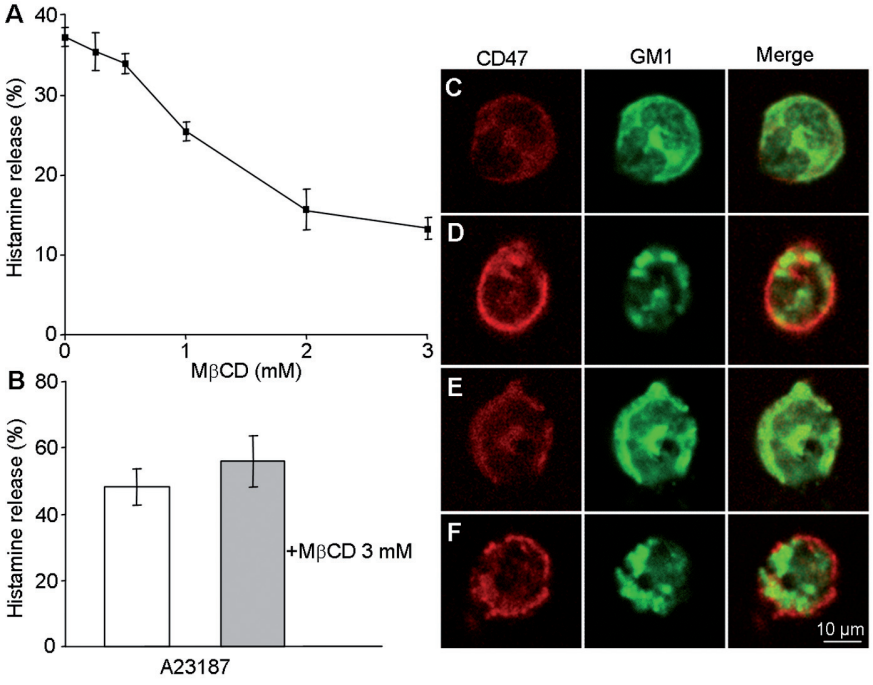

Figure 6. Effects of pretreatment of rat peritoneal mast cells by methyl-β-cyclodextrin (MβCD) on the secretory response induced by 4N1K (*A*) and by the Ca^{2+} ionophore A23187 (*B*). Cells were pre-incubated (10 min, 37°C) with the indicated concentrations of MβCD and then stimulated (10 min, 37°C) by 100 μM 4N1K or 1 μM A23187. Data are mean ± SEM of four independent experiments. (*C–F*) Confocal micrographs from z-stacks of fixed mast cells. CD47 was labeled with Alexa Fluor® 568 (red) and lipid rafts were stained with cholera toxin B subunit-FITC (green), a ligand for GM1 gangliosides that are particularly enriched in detergent-resistant membrane microdomains. The distribution of each marker was analyzed by Image J colocalization software. Also shown is an overlay of each image (merge). Images are representative of at least ten cells from three independent experiments: (*C*) untreated mast cells; (*D*) mast cells pretreated with MβCD (3 mM) for 10 min at 37°C before immunostaining; (*E*) mast cells stimulated for 10 min with 100 μM 4N1K at 37°C before immunostaining; (*F*) mast cells pretreated with MβCD and then stimulated with 4N1K before immunostaining.

control IgG1 (anti-CD48 mAb OX45) had no effect on mastoparan-induced exocytosis (Fig. 7C). Secretion induced by the Ca^{2+} ionophore A23187, which depends on Ca^{2+} influx, was also unaffected by the anti-CD47 mAb (not shown). Furthermore, when mastoparan was used as a secretagogue, similar levels of inhibition were obtained with the anti-β integrin mAb (Fig. 7D) compared to 4N1K-induced secretion (Fig. 4). Lastly, pretreatment with PI-PLC also decreased mastoparan-stimulated histamine release (Fig. 7E). These results suggest that basic secretagogues might trigger mast cell exocytosis *via* CD47 receptors, thus representing a possible receptorial pathway for such positively charged molecules.

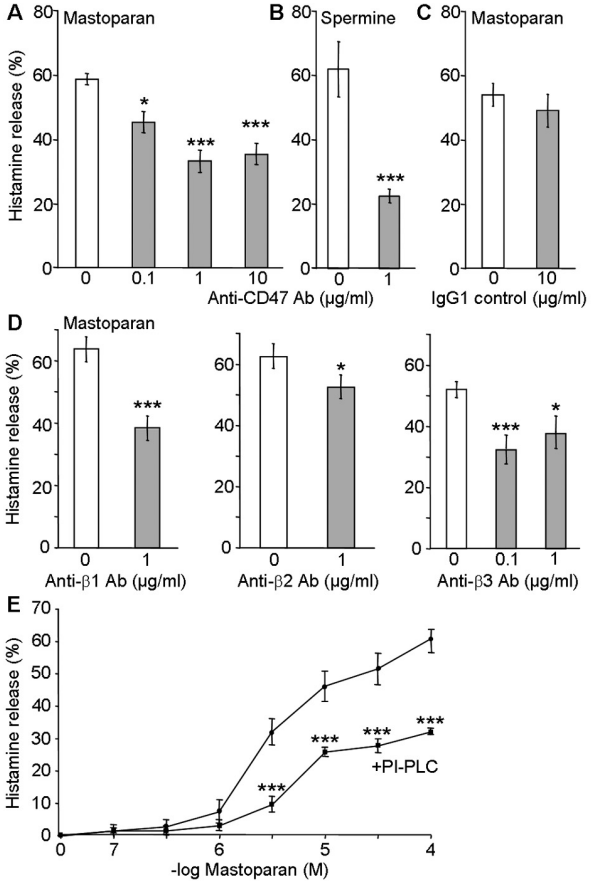

Figure 7. Effects of the anti-CD47 mAb OX101 on the secretory response of rat peritoneal mast cell to 100 µM mastoparan (*A*, n=5) and 100 µM spermine (*B*, n=3). (*C*) Pretreatment with isotype control (IgG1) mAb was without effect on 100 µM mastoparan-induced secretion (n=5). Effects of mAb against β integrin subunits on histamine release induced by 100 µM mastoparan (*D*, n=4). Cells were pre-incubated (1 h, 37°) with the indicated mAb concentrations and then stimulated for 10 min. (*E*) Effects of pretreatment with PI-PLC (1 U/ml, 3 h, 37°C) on the secretory response induced by increasing concentrations of mastoparan (10 min stimulation, 37°C). Data are mean ± SEM of n independent experiments (*p<0.05, ***p<0.001).

We found that both peptides K-4NGG and 4NGG (in which the VVM motif is replaced by GGM) stimulated mast cell secretion (Table 1). This effect of these peptides may arise independently of the thrombospondin binding site on CD47, possibly because such peptides mimic basic secretagogues. The potency of basic secretagogues is correlated to their net positive charge and deleting positively charged residues (lysine and arginine) from peptides decreases their triggering effect on mast cells [51]. Consistently, the truncated peptide 4N1–2 (RFYVVM), which lacks the lysine (K) residue at its C-terminal, had much weaker effects (Table 1). This suggests that positively charged 4N1K-related peptides might drive mast cell secretion independently of the VVM motif because they resemble basic secretagogues.

Table 1. Exocytosis induced by 4N1K-related peptides.[a]

Compounds (mM)	Sequence	Exocytosis (%)
4N1K	RFYVVMWK	
0.1		38.3 ± 2.5
0.3		57.6 ± 5.1
K-4NGG	KRFYGGMWK	
0.1		51.5 ± 2.0
0.3		62.6 ± 3.4
4NGG	RFYGGMWK	
0.1		55.3 ± 1.8
0.3		51.4 ± 2.2
4N1–2	RFYVVM	
0.1		5.5 ± 2.6
0.3		28.5 ± 2.5

[a] Cells were pre-incubated 5 min at 37°C and then stimulated with peptides at the indicated concentrations for 10 min. Data are mean ± SEM of at least three independent experiments.

Discussion

We have demonstrated that CD47 is expressed in rat peritoneal mast cells that belong to the serosal (or connective tissue) mast cell class. Similarly, CD47 was reported to be expressed in human dermal mast cells [30] that belong to the same class of mast cells. All isoforms of CD47 were about equally expressed in rat mast cells, suggesting that a specific isoform cannot be associated with mast cell exocytosis. This differs from a previous study [36] showing that IAP5 (human form 4) and IAP6 (human form 3) were closely associated with memory consolidation in rats, while IAP7

(human form 2) was the major isotype implicated in signal transduction in rat astrocytes.

4N1K stimulation of CD47 leads to mast cell histamine secretion; this is the first evidence of a role for CD47 in mast cells. The activation of mast cells results in the development of an inflammatory reaction, in agreement with previous studies showing that CD47 is implicated in many inflammatory processes. CD47 was discovered as a plasma membrane molecule that co-purified with the integrin αvβ3 in leukocytes and placenta [16]. Indeed, CD47, in cooperation with integrins, mediates cytokine production in inflammatory cells like monocytes [27] and neutrophils [26]. However, CD47 was reported to inhibit cytokine production in an integrin-independent fashion in macrophages and dendritic cells [28, 52].

Taken together, it may be that pro-inflammatory processes mediated by CD47 require cooperation with integrins, and that CD47 acting *via* an integrin-independent pathway mediates anti-inflammatory reactions. Thus, differences in the involvement of integrins in the function of CD47 may be a determinant in the switching of immune cell responses from pro-inflammatory to anti-inflammatory. CD47-mediated histamine secretion requires interaction with integrins (Fig. 4). β1, β2 and β3 integrin subunits are all expressed in rat peritoneal mast cells [53, 54], and α4β1 and α2β1 have been shown to contribute to the development of inflammatory responses of mast cells *in vivo* [55, 56]. Our results therefore reinforce the notion that integrins have a key role in inflammatory processes induced by mast cells and may, therefore, constitute a new area of investigation.

An integrin/CD47 complex has been proposed [18] to mimic heptahelical receptors in activating G_i proteins (Fig. 8). In agreement, we found that CD47 signaling in mast cells also involves coupling to G_i protein, with the βγ dimer (Fig. 2B) being responsible for mast cell exocytosis *via* mobilization of intracellular Ca^{2+} stores (Fig. 3). While previous reports have identified the βγ dimer as the target of mastoparan and spermine in mediating mast cell exocytosis [33], this is the first description of CD47 coupling to a G protein βγ dimer. Lipid rafts, plasma membrane microdomains enriched with glycosphingolipids, cholesterol and GPI-anchored proteins [46], function as platforms regulating the induction of signaling pathways [57]. Cholesterol depletion following MβCD pretreatment resulted in decreased CD47-mediated exocytosis (Fig. 6A), consistent with cholesterol being necessary to maintain the functional integrity of the CD47/integrin/G protein complex [47]. In agreement, our confocal micrographs indicate that CD47 localization in lipid rafts is clearly decreased after MβCD pretreatment (Fig. 6C–F). This strongly indicates that CD47-mediated exocytosis in mast cells requires CD47 localization in lipid rafts (Fig. 8), as also described for CD47-mediated T cell activation [58]. Experiments using two combinations of inhibitors (anti-CD47 mAb and anti-β1/anti-β3 mAb or PI-PLC) show that there was no additive inhibitory effect, with a maximal inhibition of about 50 % being observed. A possible explanation may be

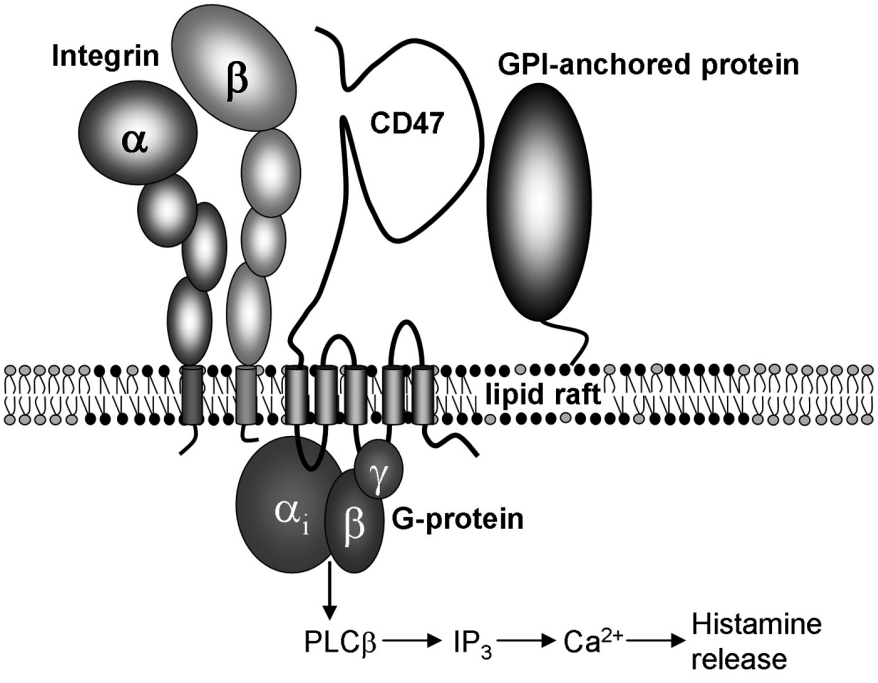

Figure 8. Schematic representation of the CD47/integrin/GPI-anchored protein complex located in cholesterol-enriched lipid rafts proposed to result in activation of G_i proteins and histamine secretion in rat peritoneal mast cells stimulated by 4N1K and basic secretagogues.

that other yet-unidentified proteins participate in the secretory response evoked by 4N1K, and therefore that our suggested CD47/integrin/GPI-anchored protein/G_i protein signaling complex is probably too simple. Note, however, that completion inhibition of 4N1K-induced histamine release was obtained after pretreatment with 50 μg/ml anti-Gβγ mAb (Fig. 2B). This indicates that G proteins represent a convergent and obligate pathway for 4N1K-induced secretion.

Our data suggest that the integrin/CD47 complex might constitute a possible target for basic secretagogues, given the inhibitory effects of anti-CD47 mAb and anti-β integrin mAb on exocytosis induced by mastoparan and spermine (Fig. 7). Indeed, secretory responses induced by CD47 and by basic secretagogues are inhibited by pertussis toxin and an anti-Gβγ mAb, and require rapid internal Ca^{2+} mobilization. Also, CD47 stimulated a significant increase in ROS production, as previously reported for compound 48/80 and substance P in rat peritoneal mast cells [59]. Basic secretagogues are thought to directly enter cells, subsequently activating G proteins and exocytosis [6–9]. Substance P was shown to rapidly penetrate cells leading to mast cell exocytosis within a few seconds. However, when applied intracellularly, exocytosis occurred only after a delay of 100–500 s [60]. On the other hand, it was shown 30 years ago that polymyxin B and compound 48/80 grafted on Sepharose beads induce histamine release [61, 62], clearly indicating that interaction of basic secretagogues with the cell membrane can initiate the secretory response without requiring entry into mast cells. Furthermore, neurotensin-induced mast cell exocytosis was reported to be receptor mediated [63], and Mrg receptors have been recently proposed to be targets of basic secretagogues [11]. Other observations are also consistent with basic secretagogues interacting at the cell surface. For example, removing surface sialic acid residues with neuraminidase [5] and [D-Pro^4, D-Trp^{7,9,10}] SP1–4 antagonizes basic secretagogues at the cell surface [6].

If indeed CD47 is a target for basic secretagogues, this raises the question of whether 4N1K stimulates exocytosis because of its CD47 agonist properties or because 4N1K resembles basic secretagogues. The octapeptide 4N1K (RFYVVMWK) is a widely used CD47 agonist that corresponds to a C-terminal motif of thrombospondin [24, 25], an endogenous CD47 ligand [23]. The sequence VVM was proposed to be a key motif of thrombospondin and 4N1K, responsible for binding to CD47, based on affinity labeling and immunoprecipitation studies and functional cellular responses [24]. Indeed, peptide 4NGG, which corresponds to 4N1K with its VVM motif replaced by GGM, is inactive in promoting CD47-mediated proliferation and migration of smooth muscle cells [64] and apoptosis of breast tumor cells [15], consistent with decreased binding of 4NGG to CD47. However, it should be noted that other 4N1K-derived peptides like 4N3G (RFGVVMWK) and 4N7G (RFYVVMGK) that conserve the VVM motif are only partially active in inducing CD47-mediated cell adhesion and spreading [25]. This strongly indicates that the VVM motif is not solely sufficient for completely activating CD47. Moreover, to our knowledge, specific binding of radiolabeled peptides containing or not the VVM motif to CD47 has never been reported, and, to date, the effects of such peptides have been only assessed using functional cellular responses.

In any case, since the selective anti-CD47 mAb inhibits exocytosis stimulated by mastoparan, spermine and 4N1K, CD47 is clearly involved. It might be possible that basic secretagogues bind either to CD47 at sites distinct from the thrombospondin motif or on other membrane structures interacting with CD47.

While it is widely held that basic secretagogues activate serosal but not mucosal mast cells [5], surprisingly, no explanation exists for this striking difference that has been known for over 50 years. Since CD47 is expressed in mast cells from human skin, gastrointestinal tract and lung [30–32], and also in neoplastic mast cells obtained from patients with systemic mastocytosis [31], it alone cannot account for the difference in sensitivity to basic secretagogues between serosal and mucosal mast cells. We attempted experiments using siRNA-mediated knockdown of CD47 to demonstrate whether CD47 is necessary for responses to basic secretagogues. However, we found that freshly isolated, mature, rat peritoneal mast cells could not be maintained alone in culture for more than 1–2 days, unfortunately precluding siRNA-based experiments in these cells.

We observed that pretreatment of mast cells with PI-PLC, which catalyzes disruption of GPI-anchored proteins [46], decreases both 4N1K- (Fig. 5) and basic secretagogue-induced secretion (Fig. 7E). It was first reported in lymphocytes that GPI-anchored proteins like CD48, CD59 and Thy-1 are associated with $G\alpha_{i2}$ and $G\alpha_{i3}$ [65]. Cholesterol depletion with MβCD decreased CD59-mediated activation [66], consistent with GPI-anchored proteins being located in lipid rafts [46]. A yet-unidentified transmembrane protein present in lipid rafts was proposed to be involved in the recruitment of $G\alpha_{i2}$ at CD59 clusters [66]. We suggest that the CD47-integrin complex might be responsible for the recruitment of G proteins and GPI-anchored proteins in lipid rafts. If indeed a CD47-containing complex plays a role in serosal mast cell secretion induced by basic secretagogues, such a functional complex might not be formed in mucosal mast cells, which are insensitive to these compounds.

In conclusion, we show for the first time that activation of CD47 receptors leads to secretion of histamine from rat peritoneal mast cells. Our data indicate that CD47 interacts with integrins, G proteins and GPI-anchored proteins, forming a complex localized in lipid rafts (Fig. 8). This complex could contribute to the control of inflammation, possibly being implicated in processes like cell proliferation or apoptosis that are influenced by mast cell reactivity.

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