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RESEARCH ARTICLE

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Effects of fostriecin on β_2 -adrenoceptor-driven responses in human mast cells

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ABSTRACT

As part of the intracellular processes leading to mast cell and basophil activation, phosphorylation of key substrates is likely to be important. These processes, mediated by phosphatases, are responsible for regulating phosphorylation. The aim of the present study was to determine effects fostriecin – a selective inhibitor of PP2A (protein phosphatase-2) – on β_2 -adrenoceptor-driven responses in human mast cells. Here, the effects of fostriecin (PP inhibitors) on the inhibition of histamine release from HLMC, on β -adrenoceptor-driven responses in mast cells and on desensitization were investigated. Long-term incubation (24 h) of mast cells with fostriecin (10^{-6} M) resulted in a significant ($p < 0.001$) reduction in the maximal response (from 41.2 ± 3.0 to 29.9 ± 4.2 %) to salbutamol following fostriecin treatment. The results showed that fostriecin pretreatment significantly attenuated the inhibitory effects of salbutamol. Overall, the present study suggested that PP2A has an important role in regulating mast cell β_2 -adrenoceptors.

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KEYWORDS

Desensitization; histamine; mast cell; fostriecin

Introduction

In the past decade, there has been a considerable increase in attempts to clarify the biological roles of protein phosphatases, and their potential importance in the context of health and disease (Yu et al. 2016). Protein phosphatases that dephosphorylate either tyrosine or serine and threonine constitute the two main families and are referred to as protein tyrosine phosphatases (PTP) and protein serine/threonine phosphatase (PP), respectively (Cohen et al. 1991). Protein tyrosine phosphatases form a large family of still largely uncharacterized enzymes (Kuropka et al. 2016). The level of tyrosine phosphorylation in normal cells is determined by the balanced activity of protein tyrosine kinases (PTK) and PTP. It was previously shown that the adrenoceptors expressed by human lung mast cells (HLMC) and that mediate inhibition of mediator release are β_2 -adrenoceptors and that these receptors may be readily desensitized (Chong et al. 2002).

Decreased responsiveness to stimulation arises with most cell surface receptors when exposed to an agonist. Short-term exposure (few seconds) to β_2 -adrenoceptor agonists results in receptor phosphorylation by both cAMP-dependent protein kinase A (PKA) and G-protein receptor kinase (GRK), whereas long-term exposure to β_2 -adrenoceptor agonists includes downregulation of surface receptor numbers (which involves internalization and degradation of the receptors; Carter & Hill, 2005) and results in changes in the transcription of β -adrenoceptors or mRNA stability (Yanagawa et al. 2016). A reduction in β_2 -adrenoceptor mRNA after incubation with β_2 -adrenoceptor agonists are consistent with decreased synthesis of β_2 -adrenoceptors after prolonged agonist contact; this amount is independent of continued occupation of β_2 -adrenoceptors on the cell surface (Chong et al. 2003). Studies indicated that GRK-catalyzed phosphorylation of G-protein-coupled receptors (GPCR) followed by β -arrestin binding are key steps in the internalization of receptors or in

receptor desensitization. Based on those findings, β -arrestin over-expression promoted sequestration of the β_2 -adrenergic receptor (Sibley et al. 1986). This, in turn, suggested that β_2 -adrenergic receptor internalization played a key role in receptor dephosphorylation and resensitization. It has been termed the GPCR phosphatase (PP) and at least *in vitro*, is active against not only the GRK-phosphorylated β_2 -adrenergic receptor but also the α_2 -adrenergic receptor and rhodopsin.

Previous work from our laboratory has shown that long-term exposure of HLMC to β -agonists attenuated the subsequent ability of the agonists to inhibit histamine release (Chong et al. 2003; Bastan et al. 2014). As phosphorylation of β_2 -adrenoceptors is perhaps essential in inducing desensitization in many cells, including mast cells, this study sought to investigate the importance of phosphorylation events in mast cells by targeting some of their protein phosphatases. Specifically, the main aim here was to investigate effects of fostriecin a selective inhibitor of PP2A – on β_2 -adrenoceptor-driven responses in human mast cells *in vitro*. Comparisons of effects from fostriecin with those from okadaic acid, an inhibitor of PP2A (protein phosphatase-2) and PP1 (protein phosphatase-1), on the functional desensitization of β -adrenoceptor-mediated responses in mast cells *in vitro* were also investigated.

Materials and methods

Buffers

Phosphate-buffered saline (PBS) containing 137 mM NaCl, 8 mM Na_2HPO_4 , 2.7 mM KCl and 1.5 mM KH_2PO_4 was used in these studies. PBS-bovine serum albumin (BSA) was PBS also containing 1 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 1 mg BSA/mL and 15 μg DNase/mL. PBS-human serum albumin (HSA) was PBS additionally supplemented with 1 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose and 30 μg HSA/mL. The pH of all PBS buffers

was 7.3. All materials for the buffers were purchased from Sigma (Poole, UK).

Preparation of inhibitors and stimuli

Protein phosphatase inhibitor isoprenaline was made up as a 10 mM stock in 0.05% (w/v) sodium metabisulfite (in normal saline). Okadaic acid was made up as a 0.5 mM stock in 10% dimethyl sulfoxide (DMSO). Fostriecin was made up as 1 mM solutions in 10% and 100% methanol. Salbutamol was prepared as a 10 mM stock in distilled water. All materials for the inhibitors were purchased from Sigma (Poole, UK). Each drug was diluted to desired levels in buffer just before use.

The stimulus used in mediator release experiments was polyclonal goat anti-human IgE prepared according to manufacturer's instructions (Sigma Chemical Co., Poole, UK). The lyophilized powder was reconstituted in 2 mL ultra-pure H₂O and the solutions were stored at -20 °C. Preliminary experiments indicated the vehicles used to prepare the drugs had no effect on histamine release or on the protein PP assays.

Isolation of human lung mast cells (HLMC)

Mast cells (HLMC) were isolated from human lung tissue using the method of Ali and Pearce (1985). In brief, the lung tissue was obtained from surgical resections of patients were undergoing surgery for carcinoma. All patients gave written informed consent. None had overt pulmonary inflammatory diseases such as sarcoidosis, tuberculosis, etc. at the time of sample collection. There were an equal number of males and females from whom samples were obtained. All of these protocols had received the approval of the Local Research Ethics Committee.

In each case, macroscopically-normal tissue (10–40 g) was excised at least 2 cm from the tumor. Each tissue sample was chopped vigorously for 10 min in a small volume of PBS buffer and then washed through a nylon mesh (100 µm pore size; Incamesh, Warrington, UK) with PBS buffer. The tissue was then placed in PBS-BSA (10 mL/g tissue) containing Collagenase Ia (0.1 mg/mL; Sigma) and placed in a 37 °C water bath. After 90 min with constant stirring, the mixture (containing some mast cells) was separated from nondigested tissue by filtration through 100-µm pore nylon mesh. The filtrate was then diluted with PBS-BSA buffer and further dissociated with a syringe. The mixture was then passed again over 100-µm nylon mesh. After collection, all filtrates were pooled, centrifuged (120 × g, 8 min, 25 °C), and the final pellet reconstituted in PBS-BSA. Cells were counted with a hemocytometer; mast cells were visualized by removing aliquots and staining subsequently prepared slide with Alcian blue stain (Gilbert & Ornstein, 1975). Based on these analyses, HLMC levels in the preparations were calculated. Of the total cells, 3–13% were mast cells. This method routinely yielded 2–9 × 10⁵ mast cells/g tissue HLMC for use in the histamine release experiments.

Mediator release

Mediator release experiments were performed in PBS-HSA buffer. Mast cells were incubated with or without an immunosuppressant for 5 min, unless otherwise indicated in the text, before the challenge with stimulus. Preliminary experiments indicated that extending the incubation time from 5–60 min did not alter inhibitory effects of the drugs. Mediator release was initiated

immunologically with anti-IgE. Concentrations of anti-IgE were chosen that elicited ≈30% of total histamine content from the cell preparations. In HLMC, this was a fixed concentration (1:300) of anti-IgE. Stimulus-induced secretion was allowed to proceed for 25 min at 37 °C after which the cells were pelleted by centrifugation (400 × g, 25 °C, 4 min). Histamine released into supernatant was determined using the automated fluorometric assay of Siraganian (1974). Total histamine content was determined by lysing aliquots of cells (i.e. 20,000 mast cells/sample) with 1.6% perchloric acid for 25 min at 37 °C. Cells incubated in buffer alone served as a measure of spontaneous histamine release [which ranged from 2–8% of total histamine content]. None of the drugs used in this study influenced spontaneous histamine release (data not shown; pilot study). Histamine release was expressed as a percentage of total histamine content after subtracting for spontaneous histamine release. All assays were performed in duplicate.

Statistical analysis

Data are expressed as means ± SEM. Maximal responses (E_{max}) and potencies (pEC_{50}) by nonlinear regression analysis were determined using by Prism software (GraphPad, San Diego, CA). Nonlinear regression was used to generate a line or a curve, as if every value of Y was a random variable, and also used logarithmic functions. To determine if there was any difference in responses after treatments with drugs, repeated measures analysis of variance was performed. To establish whether drug treatments caused statistically significant effects, either paired *t*-tests or a two-way analysis of variance (ANOVA) followed by a Dunnett's test was performed. A *p* values <.05 was accepted as significant.

Results

Effects of PP inhibitors on IgE-mediated histamine release

Effects of PP inhibitors on inhibition of histamine release from HLMC incubated (2 h) with increasing concentrations of fostriecin or okadaic acid (OA) and then exposed to anti-IgE to induce histamine release was assessed. Fostriecin (10⁻⁹–10⁻⁶ M) failed to affect the histamine release from the mast cells, whereas OA (10⁻¹⁰–10⁻⁶ M) effectively inhibited histamine release (i.e. from ≈20% vs. control at 10⁻¹⁰–10⁻⁸ M to >80% versus control at 10⁻⁶ M) (Figure 1).

Effects of PP inhibitors on mast cell β-agonists

To evaluate the effects of fostriecin on isoprenaline inhibition, cells were incubated for 2, 4 or 24 h (Figure 2) with fostriecin (10⁻⁶ M) before incubation with isoprenaline (10⁻¹⁰–10⁻⁵ M) and then challenged by anti-IgE. Treatments with fostriecin had no significant effect on either the efficacy or the potency of isoprenaline.

In a similar set of experiments, the inhibitory response of HLMC to salbutamol, after a 4-h treatment with fostriecin was investigated. Salbutamol inhibition of histamine release was not affected to a significant degree after treatment with fostriecin. However, after a longer (24 h) incubation of the mast cells with fostriecin (10⁻⁶ M), there was a significant (*p* <.001) reduction in maximal response (from 41.2 [±3.0] to 29.9 [±4.2]%) to salbutamol following fostriecin treatment (Figure 3).

The effect of OA on β-agonist inhibition of histamine release from HLMC was also investigated (data not shown). Cells were

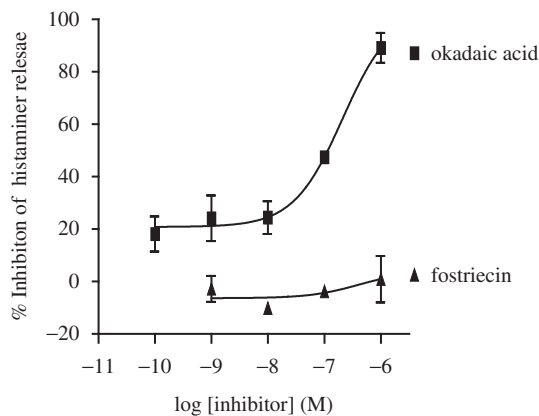


Figure 1. Effect of foscricin and okadaic acid on histamine release from HLMC. Cells were incubated for 2 h with increasing concentrations of foscricin or okadaic acid (OA) before challenge with an optimal releasing concentration of anti-IgE (1:300) for a further 25 min to induce histamine release. Results are expressed as the percent inhibition of the control histamine release values which were $27 \pm 3\%$ (in foscricin studies) and $35 \pm 4\%$ (in OA studies). *Significant inhibition versus control. Values shown are means \pm SEM, $n = 4$ /regimen (foscricin) and $n = 12$ /regimen (OA).

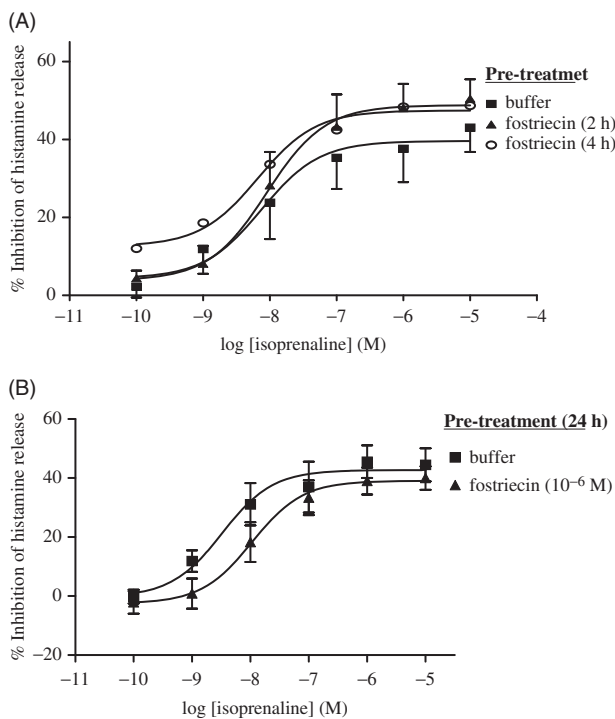


Figure 2. Effect of foscricin on isoprenaline inhibition of histamine release from HLMC. Cells were incubated without (square) or with foscricin (10^{-6} M) for either (A) 2 h (triangles) or 4 h (circles) or (B) 24 h. After incubation, each set of cells was washed and incubated for 10 min with isoprenaline (10^{-10} – 10^{-5} M) before challenge with anti-IgE (1:300) for 25 min to induce histamine release. Values expressed as % inhibition of control histamine release, that is, in (A) $38 \pm 8\%$ (in buffer studies), $40 \pm 5\%$ (in 2-h foscricin studies), and $37 \pm 6\%$ (in 4-h foscricin studies), or in (B) $26 \pm 3\%$ (in 24-h buffer studies) and $30 \pm 4\%$ (in 24-h foscricin studies) following treatments with buffer or foscricin. Values shown are means \pm SEM, $n = 5$ /regimen. All values were significantly different ($p < 0.05$) vs. respective controls.

incubated (24 h) either with buffer or OA (3×10^{-8} M) before incubation with isoprenaline (10^{-10} – 10^{-5} M) and then challenged by anti-IgE (1:300). Longer (24 h) treatment with OA at this low concentration had no effect on inhibition of histamine release by isoprenaline.

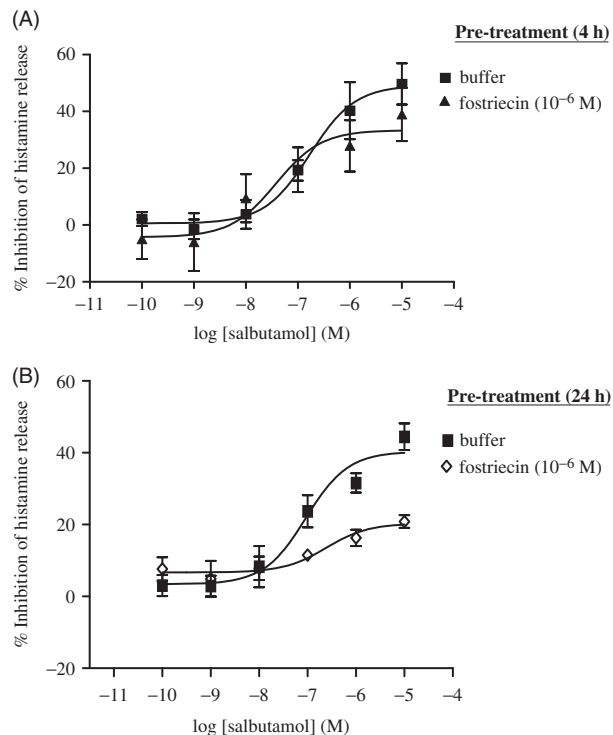


Figure 3. Effect of foscricin on salbutamol inhibition of histamine release from HLMC. Cells were incubated (A) 4 h or (B) 24 h with buffer (control) or foscricin (10^{-6} M). After incubation, cells were washed and then incubated with salbutamol (10^{-10} – 10^{-5} M) before challenge with anti-IgE (1:300) for 25 min to induce histamine release. Results are expressed as % inhibition of control histamine release, that is, in (A) $26 \pm 6\%$ (in buffer studies) and $23 \pm 5\%$ (in foscricin studies), and in (B) $40 \pm 4\%$ (in buffer studies) and $37 \pm 5\%$ (in foscricin studies) following treatments with buffer or foscricin. Values are means \pm SEM, $n = 6$ /regimen.

Effect of PP inhibitors on desensitization

To evaluate whether desensitization induced by β -agonists could be influenced by foscricin, HLMC were treated with either buffer or foscricin (10^{-6} M) and with/without isoprenaline (10^{-6} M) for 24 h (Figure 4). Isoprenaline (10^{-6} M) pretreatment reduced maximal responses to 10^{-10} – 10^{-6} M isoprenaline (prior to anti-IgE stimulation) by almost $>50\%$ (vs. level seen in cells pretreated with buffer, that is, $46 \pm 2\%$ inhibition versus release from control cells that only saw anti-IgE) across nearly all the re-treatment doses. Foscricin pretreatment alone (10^{-6} M) had little effect on functional desensitization with levels lower (albeit not significant) than levels of inhibition seen with buffer pretreated cells across nearly all the doses of isoprenaline used pre-anti-IgE. Effects from the combined pretreatment with foscricin and isoprenaline yielded inhibition levels nearly midway between those of either agent alone. Effects of OA (3×10^{-8} M) on desensitization were also investigated (data not shown). Though 24-h pretreatments with OA had no effect on the ability of isoprenaline to inhibit histamine release, it significantly enhanced the extent of isoprenaline-induced desensitization (data not shown).

In another series of assays, HLMC were treated with buffer or foscricin (10^{-6} M) and with/without salbutamol (10^{-6} M) for 24 h (Figure 5) before salbutamol (10^{-10} – 10^{-5} M) retreatment for 10 min and then challenge with anti-IgE (1:300) to induce histamine release. As expected, salbutamol pretreatment significantly ($p < .001$) reduced subsequent inhibition of histamine release from mast cells by salbutamol (i.e. vs. cells that had been pretreated with buffer; maximal inhibition in the latter

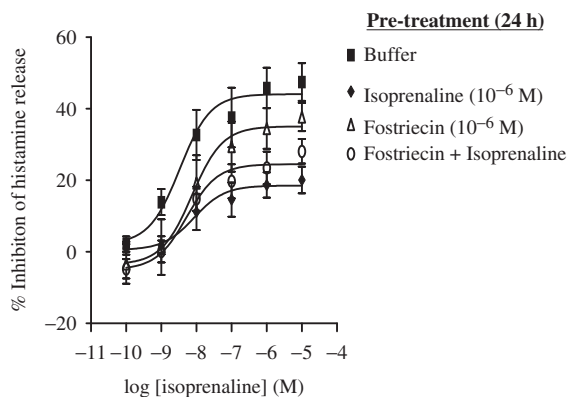


Figure 4. Effect of fostriecin on isoprenaline-mediated functional desensitization. Cells were incubated (24 h) ■ without (control) or ◆ with isoprenaline (10^{-6} M) or △ fostriecin (10^{-6} M) or ○ fostriecin and isoprenaline combined. After incubation, cells were washed and then incubated with isoprenaline (10^{-10} – 10^{-5} M) for 10 min before challenge with anti-IgE (1:300) to induce histamine release. Values are expressed as % inhibition of control histamine release, that is, $46 \pm 2\%$ (in buffer studies), $44 \pm 3\%$ (in isoprenaline studies), $42 \pm 3\%$ (in fostriecin studies) and $39 \pm 2\%$ (in fostriecin + isoprenaline studies) following treatment with buffer, isoprenaline, fostriecin, or isoprenaline + fostriecin. Values shown are means \pm SEM, $n = 7$ /regimen.

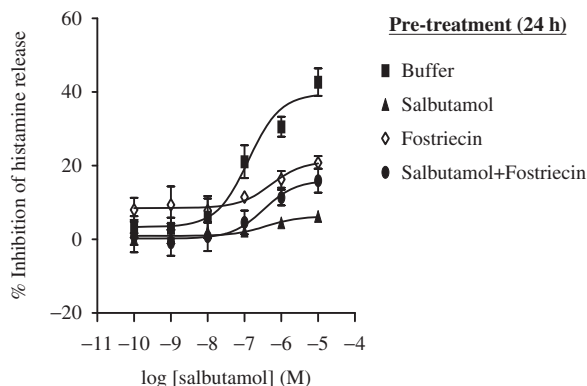


Figure 5. Effect of fostriecin on salbutamol-mediated functional desensitization. HLMC were incubated (24 h) ■ without (control) or △ with either salbutamol (10^{-6} M), or ◇ fostriecin (10^{-6} M) or ● salbutamol + fostriecin. After incubation, cells were washed extensively and then incubated with salbutamol (10^{-10} – 10^{-5} M) for 10 min before challenge with anti-IgE (1:300) to induce histamine release. Values are expressed as % inhibition of control histamine release, that is, $40 \pm 4\%$ (in buffer studies), $37 \pm 6\%$ (in salbutamol studies), $37 \pm 5\%$ (in fostriecin studies), and $36 \pm 7\%$ (in fostriecin + salbutamol studies) after treatment with buffer, salbutamol, fostriecin, or salbutamol + fostriecin. Values shown are means \pm SEM, $n = 8$ /regimen.

was $\approx 40 \pm 4\%$ relative to release by cells stimulated with anti-IgE alone). The effects of fostriecin on desensitization were hard to discern since fostriecin pretreatment significantly ($p < .001$) attenuated inhibitory effects from the subsequent salbutamol re-exposure before anti-IgE administration.

Effects on desensitization

The effects of fostriecin on recovery from isoprenaline-induced desensitization were investigated (Figure 6). Longer (24 h) treatment of the mast cells with isoprenaline (10^{-6} M) caused a 80% reduction in the maximal response to isoprenaline. If cells were allowed to recover from this treatment for 24 h, there was $\approx 50\%$ recovery in responses. However, if recovery was done in the presence of fostriecin (10^{-6} M), the extent of recovery was not as

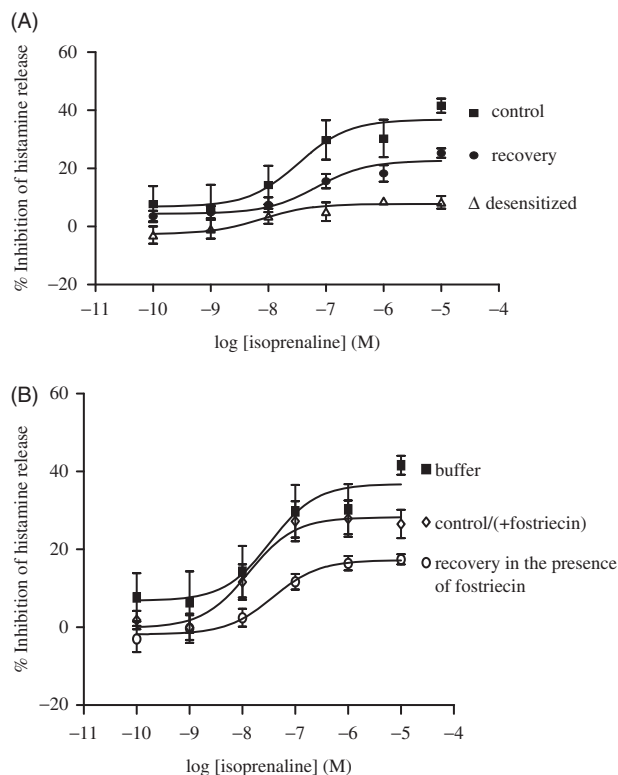


Figure 6. Influence of fostriecin on recovery from desensitization. Cells were incubated (24 h) without (control) or with isoprenaline (10^{-6} M) and then washed and incubated without or with isoprenaline (10^{-6} M) and with or without fostriecin (10^{-6} M) for a further 24 h. The cells were then extensively washed and incubated with isoprenaline (10^{-10} – 10^{-5} M) for 10 min before challenge with anti-IgE (1:300) to induce histamine release. Values are expressed as % inhibition of the control histamine release, that is, in (A) $31 \pm 3\%$ (square), $40 \pm 3\%$ (closed circle), $36 \pm 4\%$ (triangle) and in (B) $31 \pm 3\%$ (square), $35 \pm 4\%$ (diamond) and $38 \pm 4\%$ (open circle). Split panels are provided for clarity. Values shown are means \pm SEM, $n = 10$ /regimen.

great. Though the data suggested fostriecin impeded recovery, it seemed here fostriecin reduced the maximal response to isoprenaline – complicating data interpretation.

A similar trend was observed when the effects of fostriecin on recovery from salbutamol-induced desensitization were investigated (Figure 7). Longer (24 h) treatment of mast cells with salbutamol (10^{-6} M) caused an 87% reduction in the maximal response to salbutamol. If the cells were allowed to recover from this treatment for 24 h, then there was an $\approx 57\%$ recovery in the response to salbutamol. However, if recovery was performed in the presence of fostriecin (10^{-6} M), then the extent of recovery was not as great. However, fostriecin alone reduced the maximal response to salbutamol making interpretation of the data difficult.

Discussion

As part of the intracellular process leading to mast cell activation, the phosphorylation state of key substrates is likely to be important. G protein-dependent and arrestin-dependent functions of many G-protein-coupled receptors (GPCR) can be dissociated pharmacologically by ligands that exhibit functional selectivity or a “bias” favoring one pathway or another. Thus, differential coupling of receptors to G protein- and arrestin-based pathways is one of many mechanisms underlying ligand-directed signaling. Whereas conventional GPCR agonists and antagonists are believed to activate or inhibit all aspects of signaling equally, “biased agonists” have a potential to change the signal output of

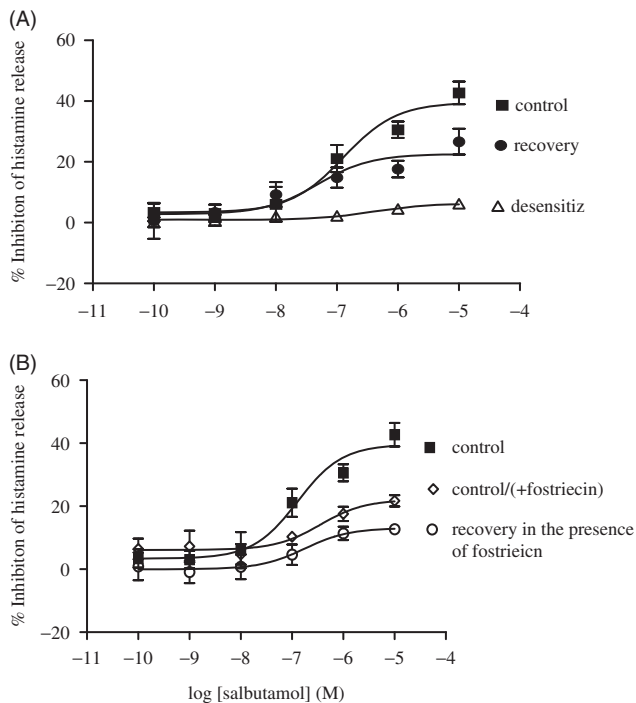


Figure 7. Influence of fostriecin on recovery from desensitization. Initially, cells were incubated 24 h without (control) or with salbutamol (10^{-6} M) and then washed extensively. Cells were then incubated without or with salbutamol (10^{-6} M) and with or without fostriecin (10^{-6} M) for a further 24 h, then extensively washed and incubated with salbutamol (10^{-10} – 10^{-5} M) for 10 min before challenge with anti-IgE (1:300) to induce histamine release. Values are expressed as % inhibition of control histamine release, that is, $40 \pm 4\%$ (square), $38 \pm 7\%$ (closed circle), $36 \pm 7\%$ (triangle), $37 \pm 7\%$ (diamond) and $37 \pm 7\%$ (open circle). Split panels are provided to enhance clarity. Values are means \pm SEM, $n = 7$ /regimen.

a GPCR, thereby controlling not only the quantity but also the quality of efficacy.

Desensitization can be induced by challenge of immune cells like mast cells with IgE-dependent signals. During desensitization, phosphorylated receptors uncouple from G-proteins and can be targeted for internalization. Once internalized, the receptors may either be degraded or recycled to the cell surface. With respect to recycling, it appears that dephosphorylation of the receptor, by a PP2A-like phosphatase, is involved in ensuring receptor re-expression. Our previous studies (Peirce et al. 1997) provided a framework for the study here to determine the role, if any, of PP2A in desensitization/re-sensitization of mast cells. Those studies (and later ones) had shown that mast cells expressed PP2A but that inhibition of PP2A with the highly selective inhibitor fostriecin had no effect on IgE-mediated histamine release (Bastan et al. 2001). Thus, the effects of fostriecin on β_2 -adrenoceptor-driven responses in mast cells were investigated in the present study.

Initially, the effects of pretreatment (up to 24 h) of the mast cells with fostriecin on the subsequent activity of β -agonists were investigated. Pretreatments of up to 24 h with fostriecin alone had no effect on IgE-mediated histamine release. Shorter pretreatments had little effect on the activity of isoprenaline and salbutamol to inhibit histamine release. However, after 24-h treatment with fostriecin, there was a modest reduction in potency of isoprenaline and a rather more substantial attenuation in ability of salbutamol to inhibit histamine release. These findings suggested that there was low-level constitutive phosphorylation of β_2 -adrenoceptors by some kinases in the cells. Inhibition of PP2A by fostriecin prevented dephosphorylation of the

receptors; the implications from this was that as levels of phosphorylated receptors increased in a time-related manner, an increased number of receptors were thus made nonusable. The higher efficacy agonist isoprenaline tolerates this loss in receptors more so than the partial agonist salbutamol. Indeed, this effect of fostriecin on the responses of isoprenaline and salbutamol was reminiscent of studies by Drury et al. (1998) in which proportions of mast cell β_2 -adrenoceptors were inactivated with the irreversible antagonist bromo-acetyl-alprenolol methane (BAAM). Treatments with BAAM affected the response of mast cells to salbutamol to a greater degree than responses to isoprenaline. Although this conjecture is speculative, these data suggested PP2A could regulate β_2 -adrenoceptors in mast cells.

Effects of fostriecin on functional desensitization and resensitization of β_2 -adrenoceptor-mediated responses in mast cells were also investigated. However, as treatment with fostriecin appeared to exert some residual effects on responses to isoprenaline and especially salbutamol, these studies proved difficult to interpret. There was some suggestion that fostriecin acted to prevent recovery from desensitization. Presumably, the mechanism involved in resensitization would be the dephosphorylation of phosphorylated receptors. Inhibition of PP2A, with fostriecin, would prevent the dephosphorylation and thereby impair the recovery at β_2 -adrenoceptor-mediated responses.

Some studies were also performed with okadaic acid (OA) that inhibits PP2A at low concentrations and PP1 at higher concentrations (Takai et al. 1995; Favre 1997). When used at a concentration (30 nM) that should be selective for PP2A and that had no effect by itself on histamine release, OA enhanced the isoprenaline-induced desensitization in mast cells. Again, these data suggest that PP2A has a role in limiting desensitization. Despite the inconclusive nature of some of these experiments, collectively, these data suggest that PP2A has an important role in regulating mast cell β_2 -adrenoceptors. Further work will establish whether this is indeed the case.

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Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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