

# Role of protein phosphatases inhibitors on the histamine release and the functional desensitization in human lung mast cells

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**Background:** The  $\beta_2$ -adrenoceptor agonist, isoprenaline, is an effective inhibitor of histamine release from human lung mast cells (HLMC). Since phosphorylations of the  $\beta_2$ -adrenoceptors are probably important in inducing desensitization, we sought to investigate the importance of phosphorylation events by targeting protein phosphatases (PPs) in mast cells. To this end, the effects of the inhibitor of on the functional desensitization of  $\beta$ -adrenoceptor-mediated responses in mast cells were investigated. **Materials and Methods:** In this study the effects of PP inhibitors on the inhibition of histamine release from HLMC, on  $\beta$ -agonists in mast cells and on desensitization were investigated. **Results:** Long-term exposure of mast cells to both isoprenaline and salbutamol substantially reduced the extent to which isoprenaline inhibited histamine release. Pretreatments of up to 24 h with inhibitors alone had no effect on immunoglobulin E-mediated histamine release. Shorter ( $\leq 4$  h) pretreatments had little effect on the activity of isoprenaline and salbutamol to inhibit histamine release from mast cells. **Conclusion:** Collectively, these data suggest that PP has an important role in regulating mast cell  $\beta_2$ -adrenoceptors.

**Key words:** Desensitization, histamine, mast cell, protein phosphatases,  $\beta_2$ -adrenoceptors

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## INTRODUCTION

$\beta$ -adrenoceptor agonists are potent bronchodilators, that have also been shown to inhibit the release of histamine and eicosanoids from dispersed human lung, skin and tonsillar mast cells and to inhibit the increase in levels of circulating mediators after antigen exposure of asthmatic subjects *in vivo*.<sup>[1]</sup> Our laboratory has shown that the  $\beta$ -adrenoceptors expressed by human lung mast cells (HLMC) and that mediate inhibition of mediator release are  $\beta_2$ -adrenoceptors<sup>[2]</sup> and that these receptors may be readily desensitized.

Desensitization or reduced responsiveness against stimulation, occurs with most cell surface receptors (such as  $\beta$ -adrenergic receptors) when exposed continuously or repeatedly to an agonist. Short-term exposure (a few seconds) to  $\beta_2$ -adrenoceptor agonists results in receptor phosphorylation by both cAMP-dependent protein kinase A (PKA) and G-protein receptor kinase (GRK), whereas more prolonged exposure results in changes in transcription of

$\beta$ -adrenoceptors or mRNA stability.<sup>[3]</sup> Long-term desensitization includes downregulation of surface receptor number, which involves internalization, and degradation of receptors.<sup>[4]</sup> The reduction in  $\beta_2$ -adrenoceptor mRNA after incubation with  $\beta_2$ -adrenoceptor agonists is consistent with decreased synthesis of  $\beta_2$ -adrenoceptors after prolonged agonist exposure, and this measurement is independent of continued occupation of  $\beta_2$ -adrenoceptors on the cell surface.<sup>[5]</sup> Several lines of evidence now indicate that GRK-catalyzed phosphorylation of G-protein coupled receptors (GPCRs) followed by  $\beta$ -arrestin binding are crucial steps in the internalization of receptors or receptor desensitization. Thus,  $\beta$ -arrestin overexpression promotes sequestration of the  $\beta_2$ -adrenergic receptor. Sibley *et al.*<sup>[6]</sup> suggested that  $\beta_2$ -adrenergic receptor internalization plays 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  $\beta_2$ -adrenergic receptor but also the  $\alpha_2$ -adrenergic receptor and rhodopsin.<sup>[7]</sup> Since phosphorylations of the  $\beta_2$ -adrenoceptors are probably

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important in inducing desensitization, we sought to investigate the importance of phosphorylation events by targeting protein PPs in mast cells.

## MATERIALS AND METHODS

### Buffers

Phosphate-buffered saline (PBS) was employed in these studies. PBS contained (mM): NaCl 137; Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 8; KCl 2.7; KH<sub>2</sub>PO<sub>4</sub> 1.5. PBS-bovine serum albumin (BSA) was PBS which additionally contained: CaCl<sub>2</sub>·2H<sub>2</sub>O 1 mM; MgCl<sub>2</sub>·6H<sub>2</sub>O 1 mM; glucose 5.6 mM; BSA 1 mg/ml; DNase 15 µg/ml. PBS-human serum albumin (HSA) was PBS additionally supplemented with: CaCl<sub>2</sub>·2H<sub>2</sub>O 1 mM; MgCl<sub>2</sub>·6H<sub>2</sub>O 1 mM; glucose 5.6 mM; HSA 30 µg/ml. The pH of all PBS buffers was titrated to 7.3.

### Preparation of inhibitors and stimuli

Salbutamol was prepared as a 10 mM stock solution in distilled water. Isoprenaline was made up as a 10 mM stock solution in 0.05% (w/v) sodium metabisulfite made up in 0.9% (w/v) sodium chloride. The stimulus used in mediator release experiments was polyclonal goat antihuman immunoglobulin E (IgE) which was prepared according to the manufacturer's instructions. The lyophilized powder was reconstituted in 2 ml of ultra-pure H<sub>2</sub>O. All these stock solutions were stored at -20°C. Lyophilized polyclonal goat antihuman IgE antibody, was reconstituted in distilled water and stored at 4°C. The drugs were diluted to the desired concentration in buffer just before use. Preliminary experiments indicated that the vehicles used to prepare the drugs had no effect on histamine release or protein PP assays.

### Isolation of human lung mast cells

Mast cells were isolated from human lung tissue by a modification of the method described by Ali and Pearce.<sup>[8]</sup> Macroscopically normal tissue from lung resections of patients was obtained with the approval of the Local Research Ethics Committee. The tissue was chopped vigorously for 10 min with scissors in a small volume of PBS buffer and then washed over a nylon mesh with PBS buffer to remove lung macrophages. The tissue was reconstituted in PBS-BSA (10 ml/g of tissue) containing collagenase Ia and agitated by using a water-driven magnetic stirrer immersed in a water bath set at 37°C. The supernatant (containing some mast cells) was separated from the tissue by filtration over nylon mesh. The collagenase-treated tissue was then reconstituted in a small volume of PBS-BSA buffer and disrupted mechanically with a syringe. The disrupted tissue was then washed over nylon gauze with PBS-BSA. The pooled filtrates were sedimented, the supernatant discarded and the pellets reconstituted in PBS-BSA (100 ml). The pellet was washed twice more.

Mast cells were visualized by microscopy using an alcian blue stain. Mast cells prepared in this manner were used in mediator release experiments.

### Mediator release

Mediator release experiments were performed in PBS-HSA buffer. Mast cells and basophils 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 up to 60 min did not alter the inhibitory effects of drugs. Mediator release was initiated immunologically with anti-IgE. Concentrations of anti-IgE were chosen that elicited about 30% of the total histamine content from cell preparations. In HLMC, this was a fixed concentration (1:300) of anti-IgE but in basophils, which show greater variability in their responses to anti-IgE, the concentration (1:30,000-1:3000) varied from donor to donor. Alternatively, cells were activated nonimmunologically with ionophore A23187 (0.2 µM). Stimulus-induced secretion was allowed to proceed for 25 (mast cells) or 45 (basophils) min at 37°C after which time the cells were pelleted by centrifugation (400×g, room temperature, 4 min). Histamine released into the supernatant was determined by the modified automated fluorometric. Total histamine content was determined by lysing aliquots of the cells with perchloric acid at a final concentration of 1.6%. Cells incubated in buffer alone served as a measure of spontaneous histamine release, which ranged from 2% to 8% of the total histamine content. None of the drugs used in this study influenced spontaneous histamine release. Histamine release was thus expressed as a percentage of the total histamine content after subtracting the spontaneous histamine release. All assays were performed in duplicate.

### Data analysis

Data are expressed as means ± standard error of mean. Maximal responses ( $E_{max}$ ) and potencies ( $pEC_{50}$ ) by nonlinear regression analysis were determined using by GraphPad Prism software (version 4) (nonlinear regression 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 whether there was any difference in the responses after treatments with drugs, repeated measures analysis of variance was performed. In order to establish whether drug treatments caused statistically significant effects, either paired *t*-tests or ANOVA, followed by Dunnett's test, was performed.

### Materials

The following were purchased from the sources indicated; antihuman IgE, aprotinin, BSA, collagenase, dimethyl sulphoxide, DNase, Dowex AG-50W, Dowex AG1-X8, dithiothreitol, HSA, leupeptin, Percoll, phenyl methyl

sulphonyl fluoride, ethylenediaminetetraacetic acid, calcium chloride and magnesium chloride (BDH, Poole, UK); isoprenaline, salbutamol, (Sigma Chemical Co., Poole, UK).

## RESULTS

### Effect of $\beta$ -adrenoceptor agonists on histamine release from human lung mast cells

We investigated the effects of the  $\beta$ -adrenoceptor agonists isoprenaline and salbutamol on IgE-dependent histamine release from HLMC. Isoprenaline and salbutamol inhibited histamine release from mast cells in a concentration-dependent manner [Figure 1]. Our results indicate that isoprenaline ( $pD_2$ ;  $8.3 \pm 0.1$ ) was significantly ( $P < 0.05$ ) more potent than salbutamol ( $pD_2$ ;  $6.8 \pm 0.4$ ).

### Desensitization of $\beta$ -adrenoceptor-mediated responses

Mast cells were exposed (24 h) to either salbutamol ( $10^{-6}$  M) or isoprenaline ( $10^{-6}$  M) and then the ability of isoprenaline to inhibit histamine release was assessed. Salbutamol and isoprenaline induced similar levels of functional desensitization [Table 1]. In a further series of experiments, the ability of salbutamol to desensitize itself was evaluated [Figure 2]. Long-term (24 h) incubation of mast cells with salbutamol ( $10^{-6}$  M) caused a significant ( $P < 0.001$ ) reduction in the subsequent ability of salbutamol to inhibit histamine release (control  $E_{max}$  for salbutamol,  $40.0\% \pm 3.6\%$ ;  $E_{max}$  after treatment;  $9.7\% \pm 2.0\%$ ).

### Recovery from functional desensitization

To evaluate the extent of recovery (re-sensitization) after long-term incubation with  $\beta$ -agonists, HLMC were incubated (24 h) with isoprenaline ( $10^{-6}$  M) then the desensitized cells were extensively washed and incubated

in buffer for a further 24 h and following this, the ability of isoprenaline ( $10^{-10}$ - $10^{-5}$  M) to inhibit histamine release from HLMC was determined [Figure 3]. There was an approximately 50% recovery of the inhibitory response of isoprenaline after a 24 h recovery period following desensitizing treatment [Table 2]. The extent of recovery of the maximal isoprenaline inhibition, following salbutamol desensitization, was about 30% [Table 3].

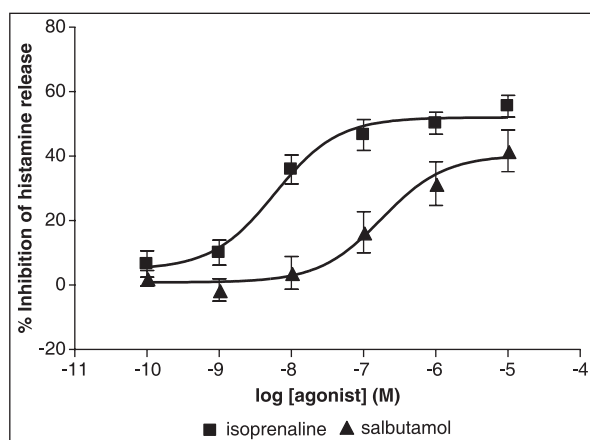
## DISCUSSION

Receptor desensitization has long been recognized as a physiological mechanism aimed at limiting agonist action.<sup>[9]</sup> Desensitization is a complex process that is thought to involve uncoupling, internalization and degradation of receptors. Critical to the desensitization process is phosphorylation of the receptor-mediated both by GRKs and PKA.<sup>[10]</sup> The phosphorylated receptor uncouples from G-protein and can be targeted for internalization. Once internalized, receptors may either be degraded or recycled to the cell surface. The factors that govern whether receptor degradation or recycling occurs may relate to the length and extent of agonist exposure. With respect to recycling of internalized receptors to the cell surface, it appears that the dephosphorylation of the

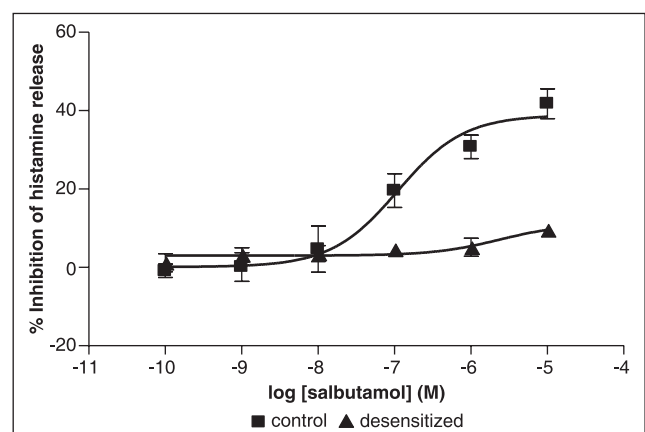
**Table 1:  $E_{max}$  and  $pD_2$  values for isoprenaline for the inhibition of histamine release following desensitization**

Values	Control	Isoprenaline desensitized	Salbutamol desensitized
$E_{max}$ (%)	43.7 $\pm$ 5.0	12.9 $\pm$ 3.9*	17.0 $\pm$ 3.2**
$pD_2$	7.8 $\pm$ 0.22	7.21 $\pm$ 0.45	7.55 $\pm$ 0.35

Values are means  $\pm$  SEM; Long-term treatment with isoprenaline and salbutamol caused statistically significant (\* $P < 0.01$  or \*\* $P < 0.001$ ) reductions in  $E_{max}$  values for isoprenaline; SEM = Standard error of mean



**Figure 1:** Effect of  $\beta$ -adrenoceptor agonists on histamine release from human lung mast cells. Cells were incubated for 10 min either without or with increasing concentrations of isoprenaline or salbutamol before challenge with an optimal concentration of anti-immunoglobulin E (1:300). Histamine release was allowed to proceed for 25 min. Results are expressed as the percent inhibition of the control histamine releases which were ( $41\% \pm 3\%$ ; isoprenaline) and ( $40\% \pm 4\%$ ; salbutamol). Values are means  $\pm$  standard error of mean,  $n = 16$  (isoprenaline) and  $n = 7$  (salbutamol)



**Figure 2:** Functional desensitization of  $\beta_2$ -adrenoceptor mediated responses in mast cells. Human lung mast cells were incubated (24 h) without (control) or with (desensitized) salbutamol ( $10^{-6}$  M) and then the cells were washed extensively. Cells were then incubated with increasing concentrations of salbutamol ( $10^{-10}$ - $10^{-5}$  M) for 10 min before challenge with an optimal releasing concentration of anti-immunoglobulin E (1:300) for histamine release. Values are expressed as the percent inhibition of the control histamine releases which were ( $30\% \pm 2\%$  and  $28\% \pm 3\%$  following 24 h treatments with buffer and salbutamol, respectively). Values are means  $\pm$  standard error of mean from 6 independent experiments

**Table 2:  $E_{max}$  and  $pD_2$  values for isoprenaline following recovery from desensitization**

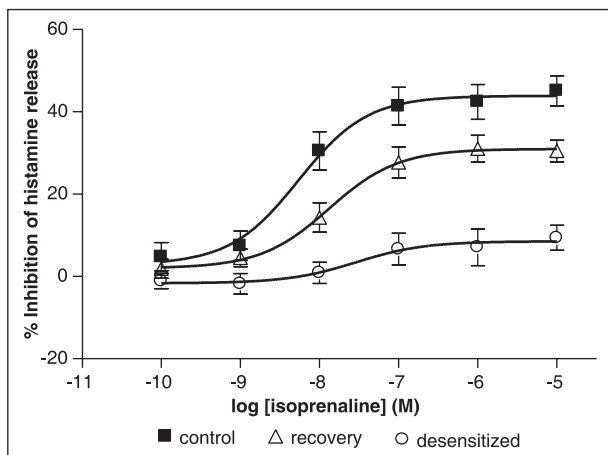
Inhibitor	Control		Recovery		Desensitized	
	$E_{max}$ %	$pD_2$	$E_{max}$ %	$pD_2$	$E_{max}$ %	$pD_2$
Isoprenaline	44.2±4.0	8.2±0.1	31.9±2.8** <sup>a</sup>	7.6±0.2	9.9±3.0** <sup>a</sup>	7.5±0.2*

Values are means ± SEM and have been calculated from data in Figure 3. Long-term (24 h) treatment with isoprenaline ( $10^{-6}$  M) and (desensitized) caused statistically significant ( $*P < 0.01$  or  $**P < 0.001$ ) reductions in  $E_{max}$  and  $pD_2$  values for isoprenaline. Following a 24 h recovery from desensitisation treatments, the  $E_{max}$  value for isoprenaline was still significantly ( $P < 0.001$ ) depressed compared to control; However, there was significant ( $P < 0.01$ ) recovery from desensitisation; SEM = Standard error of mean

**Table 3:  $E_{max}$  and  $pD_2$  values for isoprenaline following recovery from desensitization by isoprenaline or salbutamol**

Values	Control	Isoprenaline-treated		Salbutamol-treated	
		Recovery	Desensitized	Recovery	Desensitized
$E_{max}$ (%)	43.7±5	29.1±4.6**	15.6±3.1*	32.2±3.1**	11.9±4.2**
$pD_2$	7.8±0.2	7.4±0.3	7.2±0.4	7.3±0.5	7.2±0.3

Values are means ± SEM Asterisks denote statistically different ( $*P < 0.01$  or  $**P < 0.001$ ) values compared to control  $E_{max}$  and  $pD_2$  values;  $E_{max}$  values for recovery were significantly different to  $E_{max}$  values following desensitization; SEM = Standard error of mean



**Figure 3:** Recovery from the functional desensitization. Cells were incubated either for 48 h in buffer (control), or for 24 h with buffer and then for 24 h with isoprenaline ( $10^{-6}$  M; desensitized), or for 24 h with isoprenaline ( $10^{-6}$  M) and then in buffer for 24 h (recovery). After each 24 h incubation, all sets of cells were washed extensively. After these incubations cells were incubated with increasing concentrations of isoprenaline ( $10^{-10}$ - $10^{-5}$  M) for 10 min and then exposed to anti-immunoglobulin E (1:300) for histamine release. Values are expressed as the percent inhibition of the control histamine releases which were  $41\% \pm 4\%$ ,  $39\% \pm 3\%$  and  $33\% \pm 4\%$  in control, recovery and desensitized sets, respectively. Values are means ± standard error of mean,  $n = 16$

receptor, by a PP2A-like PP, is involved in ensuring receptor re-expression. The primary aim of the present study was to establish whether PP2A is involved in the desensitization/resensitization of  $\beta_2$ -adrenoceptors expressed by HLMC.

In initial studies, the effects of  $\beta$ -adrenoceptor agonists on IgE-mediated histamine release from mast cells were investigated. In accord with previous studies,<sup>[11]</sup> isoprenaline inhibited histamine release in a concentration-dependent manner and was more potent and slightly more efficacious than salbutamol. Thus, as has been previously reported,<sup>[5,12]</sup> salbutamol acts as a partial agonist in this system.

In further studies, the effects of long-term exposure of mast cells to  $\beta$ -agonists on the subsequent ability of isoprenaline to inhibit histamine release were investigated. Long-term exposure of mast cells to both isoprenaline and salbutamol

substantially reduced the extent to which isoprenaline inhibited histamine release. This functional desensitization persisted for quite some time since even after a 24 h recovery period, following desensitizing treatments, the response of mast cells to isoprenaline had recovered by only approximately 50%. These findings are in good agreement with previous studies reported by our group.<sup>[12,13]</sup> Despite the inconclusive nature of some of these experiments, collectively, these data suggest that PP has an important role in regulating mast cell  $\beta_2$ -adrenoceptors. Thus, the effects of foscetecin on  $\beta_2$ -adrenoceptor-driven responses in mast cells need to be investigated. Further work will establish whether this is indeed the case.

## AUTHORS CONTRIBUTION

RB contributed in the conception of the functional research and work and agreed for all aspects of the work. NE writing and, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. AMS contributed in the conception of the work, conducting the study, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. SHZE contributed in the conception of the work, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. PTP contributed in the conception of the work, revising the draft, of the manuscript, and agreed for all aspects of the work

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