Original article

Glucocorticoids inhibit degranulation of mast cells in allergic asthma via nongenomic mechanism

Background: Glucocorticoids (GCs) are the most potent anti-inflammatory agents available for allergic diseases including asthma, which are routinely believed to need several hours to take effect through regulating gene expression. Our previous report had shown that GCs could inhibit allergic asthma within 10 min, which the classical mechanism could not explain.
Objective: To confirm the existence and verify the sites of GCs' rapid action, we investigated nongenomic effects of GCs on degranulation of mast cells in allergic asthma.
Methods: The GCs' rapid action on airway mast cells deregulations was evaluated in the allergic asthma model of guinea pigs by the computer-assisted morphometry. Using whole-cell patch clamp and fluorometric assay, we examined GCs' nongenomic effect on IgE-mediated exocytosis and histamine release of rat basophilic leukaemia-2H3 mast cells. Employing the flash photolysis technique, we studied the role of Ca^{2+} signal in the GCs' nongenomic effect.
Results: Inhaled GCs significantly inhibited airway mast cells degranulation in the allergic asthma model of guinea pigs within 10 min. *In vitro*, GCs could rapidly inhibit IgE-mediated exocytosis and histamine release of mast cells, and neither GC nuclear receptor antagonist nor protein synthesis inhibitor could block the rapid action. We further demonstrated that GCs' nongenomic effect was not through direct action on secretory machinery, but was mediated by a reduction in the $[Ca^{2+}]_i$ elevation.
Conclusions: The study suggested for the first time that nongenomic pathway was involved in GCs' rapid inhibition on allergic asthma, and raised the possibility of new therapeutic strategies for allergic diseases including asthma.

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Glucocorticoids (GCs) are the most potent anti-inflammatory agents available for allergic diseases including asthma. Over the past decades, it was widely assumed that GCs work solely through regulating gene expression, which needs several hours to take effect. There have been many studies about the mechanisms of GCs' action on inflammatory cells in asthma through classic genomic mechanisms. These involve interference with binding of the intracellular GC–GC receptor complex to specific DNA sequences, as well as activation/transcription factors such as activator protein-1 (AP-1) and nuclear factor (NF)-κB, eventually reduce synthesis of mediators (1–3).

In addition to these classical 'genomic' mechanisms, there is increasing recognition of GCs' actions that are independent from modulating gene expression and for this reason defined as 'nongenomic' (4). We have proposed for the first time that membrane-receptor mediated rapid effects of GCs exist on mammalian neurons (5). Nongenomic effects of GCs are well studied in neuroendocrinology, although GCs have been employed mainly in anti-inflammatory and immuno-suppressive conditions.

Our recent experiments showed that GCs could inhibit allergic asthma within 10 min, which inferred the possible existence of a new pathway of GCs independent of genomic mode (6). Immunoglobulin (Ig)E-mediated degranulation of mast cell plays a crucial role in the attack of allergic reaction. Cross-linking of the high affinity IgE receptor by antigen induces exocytosis in mast cells, finally resulting in a cascade of biochemical reactions, and attack of allergic reaction (7). Which of these pathways is involved in the GCs' rapid effects, and how GCs could exert its rapid effects remain unclear.

To confirm the existence and verify the sites of GCs' rapid action, we investigated nongenomic effects of

Abbreviations: GCs, glucocorticoids; GC-BSA, bovine serum albumin conjugated corticosterone; RU486, 17β-hydroxy-11β-(a-dimethylaminophenyl)-17-α-(1-propynyl) oestra-4, 9-dien-3one; ATI, actidione; Cm, membrane capacitance.

GCs on mast cells degranulation in allergic asthma *in vivo* and *in vitro*. The results show for the first time that GCs could rapidly inhibit IgE-mediated exocytosis of mast cells in allergic reaction through reduction in $[Ca^{2+}]_i$ elevation independent of traditional genomic mode.

Methods

Allergic asthma model

Male Hartley guinea pig (200–250 g, China) was sensitized by an injection of 0.5 ml suspension (5 mg ovalbumin mixed with 50 mg aluminum hydroxide in saline, intra-peritoneal) plus 1 ml suspension (10 mg ovalbumin in saline, intra-muscular). After 30 days, 3 mg/ml budesonide in hydroxypropyl methylcellulose (HPMC) vehicles was administered by inhalation for 5 min, and then guinea pig was challenged with an aerosol of ovalbumin (suspended in saline, 10 mg/ml) for 25 s (6, 8). Throughout our experiment, inhalations were realized by a Pari-Master nebulizer (Pari Respiratory Equipment, Sternberg, Germany) through a tracheal cannula. A single model of nebulizer was used to control possible differences in drug delivery. The HPMC vehicle and saline were used as control, and hence 24 animals were averagely divided into three groups. All procedures were approved by the Ethics Committee for Animal Experiments of SMMU.

Morphology

Preparation of sample. Lung tissue was collected 3 min after antigen challenge and immediately fixed with 4% paraformaldehyde. After embedded in paraffin, the middle sections of right upper lobe (bronchi of mean internal diameter $217 \pm 37.6 \ \mu m$, $n = 112$) were cut consecutively at 4 μm. Subsequently, the specimens were stained with 1% toluidine blue to obtain metachromatic staining of mast cell granules (9).

Computer-assisted cytophotometry. Images of the mast cells were registered through a CCD camera attached to the light microscope, and interfaced with a computer through a Video spigot card (Furi Computer Co., Shanghai, China). Measurements of transmittance were carried out using a National Institutes of Health 1.49 image analysis program, standardized using the same bright-field background illumination (10).

IgE-mediated exocytosis of mast cells *in vitro*

Immune serum. Male Sprague Dawley rats (250 ± 25 g, BK Inc., Shanghai, China) were sensitized by subcutaneous injection of 1 ml saline containing 1 mg ovalbumin and 3.975 mg aluminum hydroxide (BP Institute, Shanghai, China). At the same time, 0.5 ml of *Bordetella pertussis* vaccine (BP Institute) containing 2×10^{10} heat-killed organisms was given intraperitoneally as an adjuvant. Ten days later, half of the antigen and *Bordetella pertussis* vaccine were injected subcutaneously as a booster. After 4 weeks, rats were killed to obtain immune serum. The level of specific IgE measured by enzyme-linked immunosorbent assay was 32.9 μg/ml in the pooled immune serum (11).

Sensitization and stimulation of mast cells. Rat basophilic leukaemia (RBL)-2H3 cells were grown on dishes at 37°C, 5% CO_2 in DMEM containing 10% heat-inactivated FBS, 45 mM sodium bicarbonate and 5 mM L-glucose (12). After sensitized with immune serum 50% (v/v) at 37°C for 12 h, the cells (1×10^5/ml) were washed and activated by antigen (200 μg/ml ovalbumin) at 37°C (13).

Electrophysiology

Solutions used for electrical recording. The RBL-2H3 cells were transferred to the recording chamber in a standard external solution (14, 15). For Ca^{2+} uncaging experiments, we used concentrated buffers containing 250 mM Cs-glutamate, 80 mM HEPES (pH 7.2). We added different concentrations of caged-Ca^{2+}, fura-2, furaptra (Molecular Probes, Eugene, OR, USA), $CaCl_2$ or adenosine triphosphate (ATP) to the concentrated buffer. The resulting mixtures were diluted for appropriate osmolarity (310 mosm). The DM-nitrophen (DMNP-EDTA) containing internal solutions consisted of (in mM): 110 Cs-glutamate, 5 DM-nitrophen, 4 $CaCl_2$, 2 MgATP, 0.3 GTP, 0.3 furaptra, 35 HEPES. The basal $[Ca^{2+}]_i$ was measured to be 100–300 nM by fura-2.

Patch-Clamp recording. The whole cell patch-clamp was used to measure the membrane capacitance (Cm) (14, 15). The patch pipettes were made from borosilicate glass and were pulled in two stages using a PP-83 vertical puller (Narishige, Tokyo, Japan). An EPC-9 patch-clamp amplifier was used together with Pulse + Pulsefit 8.0 software (HEKA Electronics, Lambrecht, Germany). We employed the Lindau-Neher technique implemented under the sine + dc mode, which is controlled by the lock-in extension of the Pulse software. 1000 Hz and $\pm$ 20 Mv sinusoid voltage stimulation was superimposed on a holding potential of −60 Mv. The capacitance traces were imported into IGOR Pro (WaveMetrics, Portland, OR, USA) for further analysis. Only recordings that fulfilled the following criteria were included in the analysis: (i) membrane conductance (Gm) < 1 Ns and minimum series conductance (Gs) > 50 Ns (ii) the changes in Cm did not accompany concurrent changes in Gs or Gm.

Determination of histamine release

Histamine was measured fluorimetrically using the method of Shore et al., as modified by Kremzner (16). In the supernatants, *o*-phthaldialdehyde was added directly to the sample after alkalinization with 0.4 M NaOH. The same procedure was used for the pelleted cells, after extraction with 0.1 M HCl. Histamine release was expressed as a percentage of the total amount remained in the cells plus supernatants. Spontaneous histamine release, less than 5%, was subtracted from all values.

$[Ca^{2+}]_i$ measurements and flash photolysis

Flashes of UV light and fluorescence excitation light were generated as described by Xu (17). $[Ca^{2+}]_i$ of cells was monitored with a photomultiplier-based system using a monochromatic light source (TILL Photonics, Munich, Germany) tuned to excite furaptra fluorescence at 340 and 380 nm. Emission was detected from 450 to 550 nm with a photomultiplier whose analogue signals were sampled and processed by the Fura extension of the Pulse software package (HEKA, Lambrecht, Germany). $[Ca^{2+}]_i$ was calculated from the fluorescence ratio R. To obtain stepwise increases in $[Ca^{2+}]_i$, short flashes of ultraviolet light from a xenon arc flash lamp (Rapp Optoelectronics, Hamburg, Germany) were applied to the whole cell loaded with caged-Ca^{2+} through patch pipette.

Reagents and chemicals

Salts and other reagents were obtained from Sigma (St Louis, MO, USA) unless otherwise mentioned. HPMC (4000 cps) vehicle was prepared to suspend budesonide for nebulization by the method of AstraZeneca (Sweden).

Statistical analysis

All results were expressed as mean value ± SEM. Comparisons of experimental and control groups were analysed by Student's *t*-test. *P*-values less than 0.05 were regarded as statistically significant. Analyses were performed using SAS software (SAS Institute, Cary, NC, USA).

Results

Rapid effects of GCs on airway mast cells degranulation in allergic asthma

To examine whether the degranulation of airway mast cells could be the target for GCs' rapid effects in allergic asthma, guinea pigs were administered budesonide (3 mg/ml

in HPMC vehicles) by inhalation for 5 min before antigen challenge ($n = 8$). Budesonide is one of inhaled GCs with high topical potency, which has been widely used in the treatment of clinical asthma. The HPMC vehicle and saline were used as controls ($n = 8$ each). Lung tissue of animal was collected 3 min after antigen challenge. The middle sections of right upper lobe (bronchi of mean internal diameter 217 ± 37.6 μm, $n = 112$) embedded in paraffin were cut, and stained with toluidine blue. The transmittance of airway mast cell was measured by computer-assisted cytophotometry (Fig. 1A–C), which reflected the quantity of rest granules within mast cells (10). The transmittance is from 0 to 256, and zero means the deepest stain. The transmittance increases, as the stain density becomes weaker, when more granules are secreted. In each experimental group, the transmittance of 88 chosen mast cells, 11 randomly from each of the eight different animals was measured, and the mean transmittance value (± SEM) was then calculated.

The average transmittance value of GCs, HPMC and saline was 45.81 ± 1.08, 63.47 ± 0.77 and 63.77 ± 1.07,
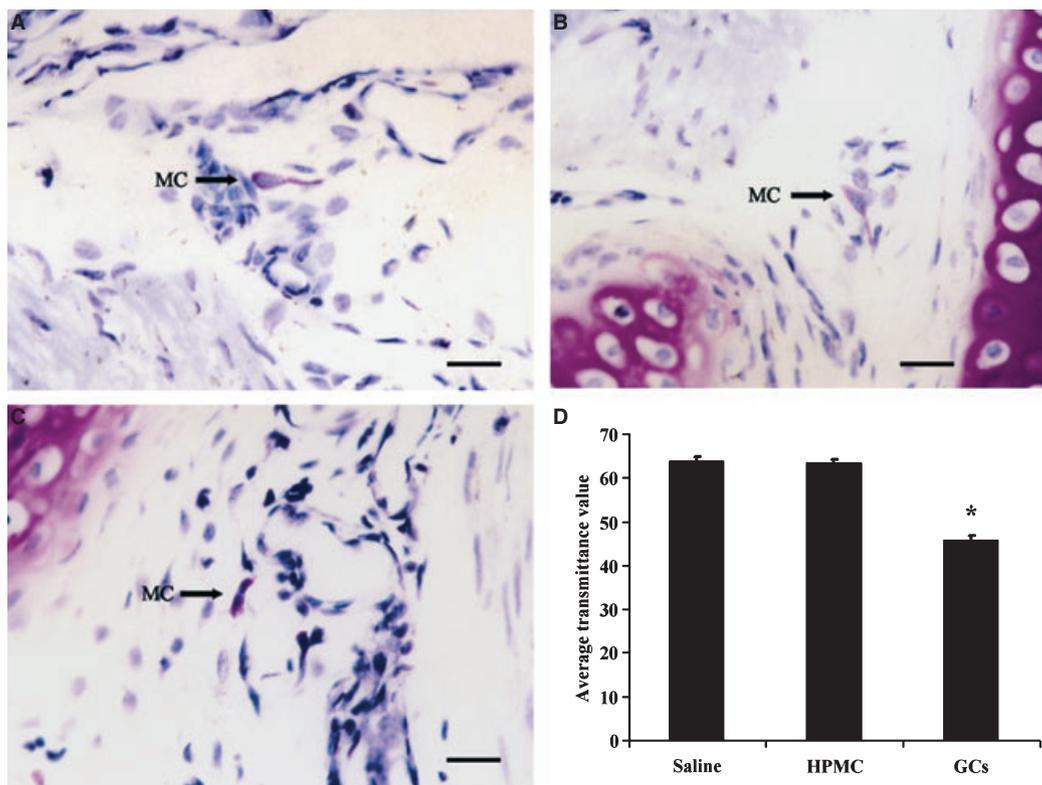
Figure 1. Rapid effects of glucocorticoids (GCs) on airway mast cells degranulation in allergic asthma. Animals were inhaled GCs (budesonide, 3 mg/ml) or control [hydroxypropyl methylcellulose (HPMC) vehicle or saline group] for 5 min before challenged to allergic asthma by antigen, and 4 μm paraffin sections were stained with toluidine blue to measure transmittance value of airway mast cells. (A–C) Light microscopy of airway mast cells of guinea pigs. Bars = 15 μm; MC, mast cell. Mast cells of saline group (A) and HPMC group (B) showed weak metachromatic. Mast cell of GCs group (C) was intensely metachromatic. (D) Average transmittance value of airway mast cells in allergic asthma reaction under saline control, HPMC vehicle and GC. Inhaled GCs (budesonide, 3 mg/ml) before antigen challenge could significantly decrease the average transmittance value of airway mast cells compared with control groups within 10 min. The data are expressed as mean values ± SEM of the average transmittance value (88 chosen mast cells, 11 randomly from each of the eight different animals per group). *$P < 0.05$ in comparison with HPMC vehicle group.

respectively. As shown in Fig. 1D, inhaled budesonide significantly decreased the average transmittance value of airway mast cells in allergic asthma within 10 min ($P < 0.05$). The result suggests that GCs could rapidly inhibit degranulation of airway mast cells in the allergic asthma, which preclude genomic-mediated responses that need several hours to occur.

Nongenomic effects of GCs on exocytosis of mast cells *in vitro*

IgE-mediated exocytosis of mast cell plays a crucial role in the attack of allergic reaction including asthma, and we investigated GCs' rapid effects on exocytosis of mast cells *in vitro*. The RBL-2H3 mast cells were sensitized with immune serum, and then we applied whole-cell patch-clamp technique to detect the change of membrane capacitance, which could evaluate exocytosis of RBL-2H3 cells (18). RBL-2H3 cell line was cloned from rat mast cell and was widely used in the study on biological character of mast cells (19).

To obtain the dose–response curve for GCs' effects, RBL-2H3 cells were sensitized with immune serum, and then incubated with corticosterone at various concentra-tions for 7 min before antigen challenge. As shown in Fig. 2A, the increments of membrane capacitance (ΔCm) among antigen (Ag) (200 μg/ml ovalbumin), Ag + GCs (corticosterone, 10^{-8} M), Ag + GCs (corticosterone, 10^{-6} M) and no Ag were 2.8621, 1.9172, 1.5219 and 0.073 pF respectively. We plotted the percentage of inhibition values against GCs' concentrations in Fig. 2B, 10^{-11} M ($n = 17$), 10^{-10} M ($n = 22$), 10^{-9} M ($n = 13$), 10^{-8} M ($n = 16$), 10^{-7} M ($n = 19$), 10^{-6} M ($n = 27$), 10^{-5} M ($n = 21$) and 10^{-4} M ($n = 22$). The results showed that corticosterone from 10^{-8} to 10^{-4} M could significantly inhibit IgE-mediated exocytosis in RBL-2H3 cells within 15 min.

We carried out a second series of experiments to assess nongenomic mechanism involved in the GCs' rapid effects. Bovine-serum albumin conjugated cortico-sterone (GC-BSA) could mimic the rapid effects of corticosterone but could not go through cell membrane within 30 min, and hence is considered to be a useful tool for studying nongenomic effects (20, 21). The sensitized cells were preincubated with corticosterone or GC-BSA at 10^{-6} M for 7 min prior to antigen addi-tion. The percentage of control under corticosterone
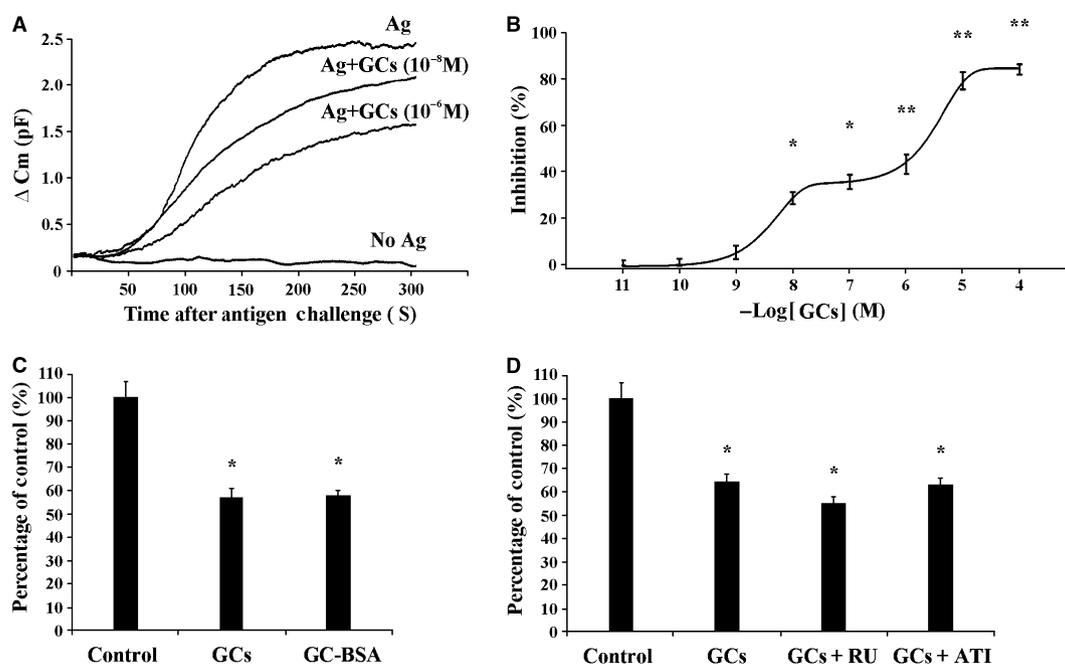
Figure 2. Nongenomic effects of glucocorticoids (GCs) on exocytosis of rat basophilic leukaemia (RBL)-2H3 cells. (A) Traces of membrane capacitance of RBL-2H3 cells under antigen (Ag) (ovalbumin, 200 μg/ml), Ag + GCs (10^{-8} M), Ag + GCs (10^{-6} M) and no Ag (without antigen challenge) were plotted. The sensitized RBL-2H3 cells were incubated with corticosterone at the indicated concentration or control vehicle for 7 min before antigen or control vehicle was added, and membrane capacitance was measured to evaluate exocytosis of RBL-2H3 cells. (B) Dose–response curve of GCs' inhibition on exocytosis of RBL-2H3 cells. Corticosterone (10^{-11}–10^{-4} M) could dose-dependently inhibit antigen-induced exocytosis in RBL-2H3 cells with 15 min. (C) Summary of the rapid effects of GCs and GC-BSA expressed as the percentage of control. Bovine serum albumin conjugated corticosterone (GC-BSA) has the similar inhibitory effects on exocytosis of RBL-2H3 cells as corticosterone within 15 min. (D) Normalized inhibition of the increments of membrane capacitance under control, GCs (corticosterone, 10^{-7} M), GCs + ATI (actidione, 10^{-4}M), GCs + RU (RU486, 10^{-6}M). RU486 or actidione could not block the rapid inhibitory effects of GCs on exocytosis of RBL-2H3 cells. The data are expressed as mean values ± SEM. *$P < 0.05$, **$P < 0.01$ in comparison with control group.

$(10^{-6}$ M) and GC-BSA $(10^{-6}$ M) was $56.9 \pm 4.21\%$ $(n = 21)$, $57.8 \pm 2.3\%$ $(n = 13)$ respectively (Fig. 2C). The results showed that GC-BSA has the similar rapid inhibitory effects on exocytosis of mast cells as corticosterone.

To confirm the nongenomic mechanism involved in GCs' rapid effects, we studied the influence of 17β-hydroxy-11β-(a-dimethylaminophenyl)-17-α-(1-propynyl) oestra-4, 9-dien-3one (RU486) and actidione (ATI) on rapid effect of GCs. RU486 is the specific antagonist of classic GCs nuclear receptor, which could exclude the traditional genomic mechanism mediated by GCs nuclear receptor (21, 22). Actidione is the protein synthesis inhibitor, which could block the protein synthesis by genomic mechanism pathway (4, 21, 23). The sensitized cells were preincubated with RU486 $(10^{-6}$ M) for 30 min or ATI $(10^{-4}$ M) for 3 h, and then incubated with corticosterone $(10^{-7}$ M) for 7 min prior to antigen addition. The percentage of control under GCs, GCs + RU486 and GCs + ATI $(10^{-4}$ M) was $64.5 \pm 3.0\%$ $(n = 21)$, $55.1 \pm 2.7\%$ $(n = 14)$ and $63.1 \pm 2.9\%$ $(n = 16)$ respectively, and there was no significant difference between them (Fig. 2D). The results showed that the

rapid inhibitory effects of GCs on exocytosis of mast cells did not depend on the protein synthesis or classic GCs nuclear receptor pathway, which further verified the existence of nongenomic mechanism.

Nongenomic effects of GCs on histamine release from mast cells *in vitro*

Histamine is thought to be a major mediator released from activated mast cells in the allergic reaction (24), and then we investigated GCs' rapid effects on IgE-mediated histamine release of RBL-2H3 cells *in vitro*. RBL-2H3 cells were sensitized with immune serum, and then incubated with corticosterone at various concentrations for 7 min prior to antigen addition. After cells were stimulated by antigen for 8 min, the reaction was stopped in ice, and histamine release was detected. The histamine release percentage of control, GCs 10^{-9}M, GCs 10^{-8}M, GCs 10^{-7}M, GCs 10^{-6} M GCs 10^{-5}M and GCs 10^{-4}M were $36.76 \pm 1.59\%$ $(n = 8)$, $39.31 \pm 2.04\%$ $(n = 8)$, $34.19 \pm 1.29\%$ $(n = 8)$, $30.30 \pm 1.94\%$ $(n = 8)$, $27.90 \pm 1.52\%$ $19.62 \pm 0.76\%$ and $18.26 \pm 0.80\%$ $(n = 8)$ respectively (Fig. 3A). The results
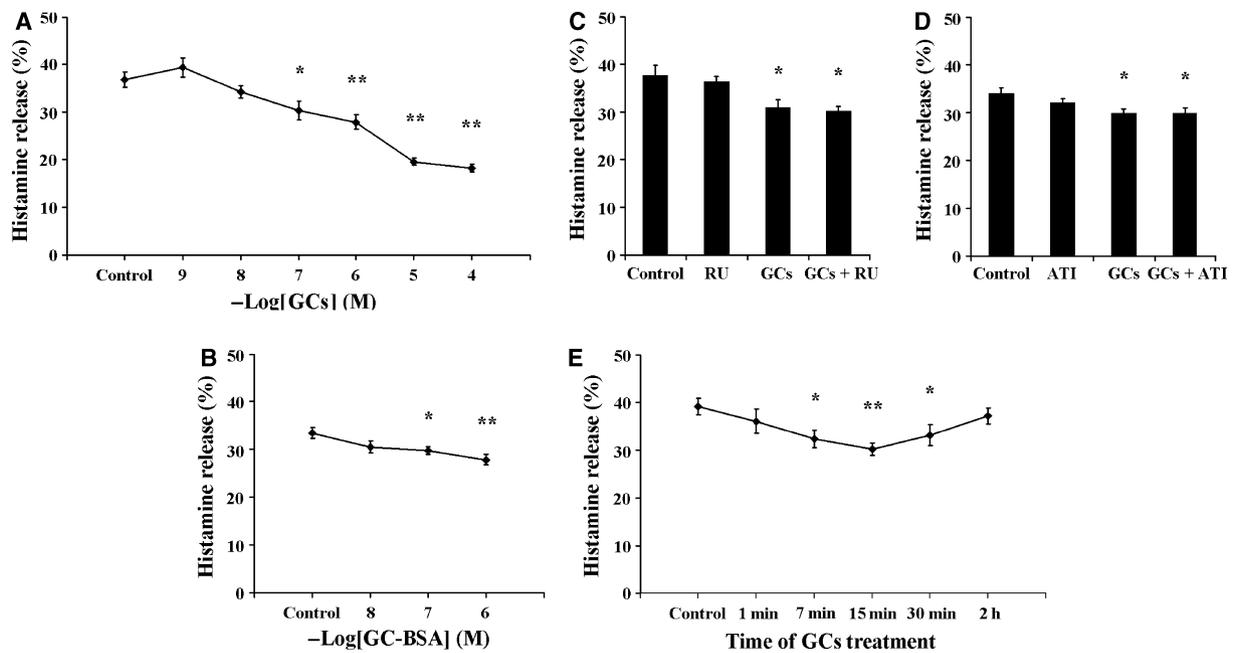
Figure 3. Nongenomic effects of glucocorticoids (GCs) on histamine release from rat basophilic leukaemia (RBL)-2H3 cells. (A) Dose–response curve of corticosterone's rapid inhibition on histamine release of RBL-2H3 cells. The sensitized RBL-2H3 cells were incubated with corticosterone $(10^{-4}$–10^{-9} M) or control vehicle for 7 min before antigen (200 μg/ml) was added. After RBL-2H3 cells were stimulated by antigen for 8 min, histamine release percentage was detected. The GCs could inhibit histamine release in dose-dependent manner within 15 min. (B) Bovine serum albumin conjugated corticosterone (GC-BSA) has the similar inhibitory effects as corticosterone within 15 min. (C) Histamine release percentage of control, GCs (corticosterone, 10^{-7} M), RU (RU486, 10^{-6} M), and GCs $(10^{-7}$ M) + RU(10^{-6} M). RU486 cannot block the rapid inhibitory effects of GCs. (D) Histamine release percentage of control, ATI (actidione, 10^{-4} M), GCs (corticosterone, 10^{-7} M) and GCs $(10^{-7}$ M) plus ATI $(10^{-4}$ M). Actidione cannot block the rapid inhibitory effects of GCs. (E) Time-course curve of GCs' inhibition on histamine release. The sensitized RBL-2H3 cells were incubated with corticosterone $(10^{-7}$ M) for the indicated times or control vehicle for 7 min before antigen was added. After RBL-2H3 cells were stimulated by antigen for 8 min, histamine release percentage was detected. The data are expressed as mean values $\pm$ SEM of histamine release percentage. *$P < 0.05$, **$P < 0.01$ in comparison with control group.

showed that corticosterone could dose-dependently inhibit antigen-induced histamine release from mast cells within 15 min.

We examined the rapid effect of GC-BSA on histamine release. The sensitized cells were preincubated with GC-BSA from 10^{-8} to 10^{-6} M for 7 min prior to antigen addition. The histamine release percentage of control, GC-BSA 10^{-8}M, GC-BSA 10^{-7}M and GC-BSA 10^{-6}M were $33.43 \pm 1.10\%$ ($n = 7$), $30.52 \pm 1.20\%$ ($n = 7$), $29.78 \pm 0.85\%$ ($n = 7$) and $27.83 \pm 1.04\%$ ($n = 7$) respectively (Fig. 3B). The results showed that GC-BSA had the similar rapid inhibitory effects as corticosterone.

The RU486 and ATI were also applied to confirm the nongenomic mechanism. We preincubated the sensitized cells with RU486 (10^{-6}M) for 30 min or ATI (10^{-4} M) for 3 h, and then incubated with corticosterone (10^{-7} M) for 7 min prior to antigen addition. There was no significant difference in histamine release between GCs and GCs + ATI and GCs + RU486 (Fig. 3C,D). The results showed that RU486 or ATI could not block the rapid inhibitory effects of corticosterone on histamine release from mast cells.

We further examined the time-course of GCs' effect. The sensitized cells were incubated with corticosterone (10^{-7} M) at various time intervals prior to antigen addition. The histamine release percentage of GCs' incubation by 1, 7, 15 and 30 min and 2 h were $36.11\% \pm 2.53\%$, $32.41\% \pm 1.84\%$, $30.25\% \pm 1.33\%$, $33.13\% \pm 2.19\%$ and $37.21\% \pm 1.67\%$ ($n = 8$, each) respectively (Fig. 3E). A significant decrease in histamine release was detected from 7 min of GCs' incubation, and peaked at 15 min. But the GCs' inhibition was reduced with 30-min incubation and was weaken with 2-h incubation.

Nongenomic effects of GCs on Ca^{2+} signal

Ca^{2+} acts as an important second messenger in mast cells upon activation (25). We investigated GCs' rapid effects on the dynamics of intracellular calcium elicited by antigen in RBL-2H3 cells. Antigen stimulation induced rapid increase in $[Ca^{2+}]_i$ (Fig. 4B), and pretreatment with 10^{-5} M corticosterone significantly reduced the $[Ca^{2+}]_i$ elevation. Simultaneous measurement of exocytosis demonstrated similar extent of inhibition (Fig. 4A). The mean increments of $[Ca^{2+}]_i$ were 755 ± 35 nM ($n = 36$) and 233 ± 23 nM ($n = 23$) for control cells and GCs-treated cells with corticosterone (Fig. 4D), whereas ΔCm was 2.715 ± 0.409 pF ($n = 36$) and 0.569 ± 0.137 pF ($n = 23$) respectively (Fig. 4C).

GCs do not have an effect on the exocytotic machinery directly

Photolysis of caged-Ca^{2+} has been used to generate well-defined $[Ca^{2+}]_i$ increases, and thus provides a tool in the
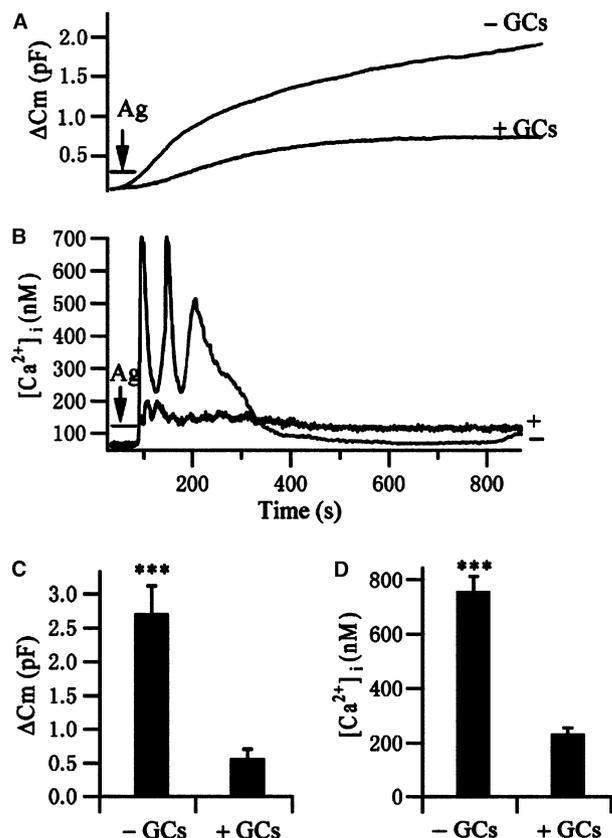
Figure 4. Glucocorticoids (GCs) inhibit exocytosis by affecting the Ca^{2+} signaling. Comparison of IgE-mediated change of membrane capacitance (A) and $[Ca^{2+}]_i$ responses (B) between GCs (Corticosterone, 10^{-5} M) pretreated ($+$GCs), and untreated cell ($-$GCs). The peak of $[Ca^{2+}]_i$ and increments of membrane were calculated from the antigen stimulation. The recording duration was longer than 800 s. Summary of the averaged increments of membrane capacitance (C) and $[Ca^{2+}]_i$ (D) between GCs pretreated and untreated cells. The data are expressed as mean values $\pm$ SEM. ***$P < 0.001$ in comparison with untreated control ($-$GCs) group.

study of Ca^{2+}-dependent processes (17). Using the technique, we checked whether GCs could exert additional effects on the process of exocytosis independent of its action on Ca^{2+} signaling in RBL-2H3 cells. We compared the time course of Cm increases elicited by step-like $[Ca^{2+}]_i$ elevations generated by flash photolysis (Fig. 5A). The Cm traces display an initial, rapid exocytotic burst followed by a slower sustained phase. Detailed analysis of the exocytotic burst in response to flash photorelease of Ca^{2+} from RBL-2H3 cells were obtained by fitting the exocytotic burst with double exponential. The initial burst component is believed to represent the fusion of the readily releasable granules, and the sustained component reflects the recruitment of the readily releasable granules. We observed no significant difference between control and GCs-treated cells. The size of readily-releasable pool in the absence and presence of
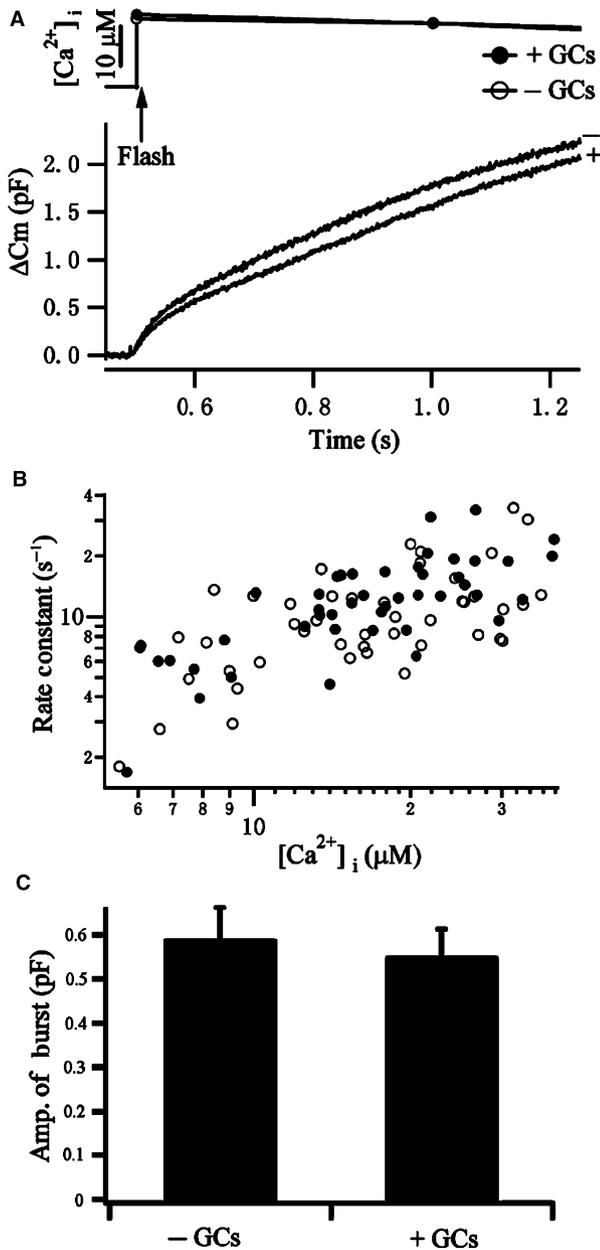
Figure 5. Glucocorticoids (GCs) have no direct effects on the secretory machinery. (A) Example responses of membrane capacitance elicited by similar postflash $[Ca^{2+}]_i$ with (open circles) or without (filled circles) GCs-treatment. (B) The rate constants between pretreated GCs and unpretreated GCs are similar. The rate constants in the absence and presence of GCs were plotted *vs* postflash $[Ca^{2+}]_i$. (C) The amplitude of the burst component is unaltered by GCs treatment. The data are expressed as mean values ± SEM.

corticosterone was 461 ± 37 fF ($n = 39$) and 447 ± 45 fF ($n = 46$) respectively. Glucocorticoids neither affect the $[Ca^{2+}]_i$ dependence of kinetics of the burst component (Fig. 5B) nor the size of burst components (Fig. 5C).

Discussion

Recently, there were a few more reports about GCs' rapid action on asthma, the mechanism of which were still unclear (26, 27). Here, it was demonstrated that IgE-mediated mast cells degranulation could be one of the targets for GCs' rapid effects on allergic asthma from the morphology. Budesonide inhaled before antigen challenge showed significant inhibitory effects on airway mast cells degranulation in allergic asthma model of guinea pigs within 10 min, precluding genomic responses that usually take several hours.

To further prove the nongenomic mechanism involved in GCs' action, we utilized RBL-2H3 mast cell line *in vitro*. The result showed that corticosterone could rapidly inhibit exocytosis and histamine release independent of the classical GCs receptor or intracellular protein synthesis, which verified the existence of nongenomic mechanism.

However, it was unexpected that corticosterone ($10^{-7}M$) inhibited histamine release merely by 17.6% (Fig. 3A), whereas inhibited ΔCm by 35.5% (Fig. 2B). The timing for measurement of histamine release at 8 min point after antigen challenge comparing with real-time detection of membrane capacitance might answer for the difference, besides the higher sensitivity of patch-clamp technique on the single cell. Moreover, it suggested that there might be other mediators released from the inflammation cells, which also act as the targets of GCs' rapid effect. There has been considerable controversy over GCs' effect on histamine release from mast cells. Grosman reported that preincubation of hydrocortisone and prednisolone almost completely inhibited histamine release within 20 min (28); Vendelin et al. observed no effect of budesonide on histamine release from rat peritoneal mast cells, but reported a 50% reduction in TNF-α release with an hour's 300 nM pretreatment (29). The effects reported in these earlier publications were well-characterized interactions of the classic glucocorticoid receptor, but here we provided certain evidence that a nongenomic mechanism was involved in the rapid effect. And the time-course of GCs' effect shown in our report (Fig. 3E) might be one of the characters of the nongenomic mechanism, which also was helpful to explain previous controversy.

The nongenomic effects could be due to interactions with specific membrane receptor, or/and unspecific physicochemical interaction with cell membrane constituents (4, 21, 30). We have reported that in neuroglial cells and pituitary cells, GC might act on putative membrane receptors, via the pertussis toxin (PTX)-sensitive-G protein-PKC pathway and the PKA pathway to induce a variety of intracellular responses (31, 32). As an increase in intracellular Ca^{2+} has been proposed as an essential trigger for mast cells activation, here we examined the role of intracellular Ca^{2+} signal in GCs' rapid inhibition on IgE-mediated exocytosis of mast cells. Glucocorticoids significantly inhibited the raise of $[Ca^{2+}]_i$ induced by

antigen and caused a similar degree of reduction in exocytosis, suggesting that modulation of Ca^{2+} signalling might plays an important role in the nongenomic action of GCs. Finally, we checked the possibility whether GCs could influence the membrane fusion and the granules in mast cells by the employment of well-controlled Ca^{2+} stimulation generated by intracellular photolysis of caged-Ca^{2+}. Neither the number of the granules in the readily releasable state nor the Ca^{2+}-dependence of exocytosis is found to be altered by GCs. Thus, we concluded that the rapid nongenomic effects of GCs on exocytosis of mast cells were not through direct action on secretory machinery, but were mediated by a reduction in the Ca^{2+} elevation.

It is now clear that steroids can modulate hormone secretion, neuronal excitability, carbohydrate metabolism, behaviour and so on, within seconds or minutes via nongenomic pathways (4, 21). However, the physiological significance of GCs' nongenomic mechanism is poorly understood. In our experiment, GCs could take nongenomic effects on degranulation of mast cells in allergic reaction at the minimum concentration 10^{-8} M, which is approximate to the GCs' physiological concentration in stress *in vivo* (33, 34). It indicates that GCs' nongenomic effects might play an important role in modulating stress and immune response.

Long-term treatment of GCs easily results in serious side-effects such as osteoporosis; hence nongenomic mechanism in GC's rapid inhibition on degranulation of mast cells in allergic reaction provides us a new way to investigate them. Is there a synergic effect between GCs and mast cells stabilizer (cromones) on allergic asthma? A further study might raise the possibility of new therapeutic strategies for allergic diseases including asthma.

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