

Selective Regulation of H₁ Histamine Receptor Signaling by G Protein-Coupled Receptor Kinase 2 in Uterine Smooth Muscle Cells

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Histamine stimulates uterine contraction; however, little is known regarding the mechanism or regulation of uterine histamine receptor signaling. Here we investigated the regulation of G $\alpha_{q/11}$ -coupled histamine receptor signaling in human myometrial smooth muscle cells using the inositol 1,4,5-trisphosphate biosensor pleckstrin homology domain of phospholipase- δ 1 tagged to enhanced green fluorescent protein and the Ca²⁺-sensitive dye Fluo-4. Histamine addition caused concentration-dependent increases in inositol 1,4,5-trisphosphate and [Ca²⁺]_i in the ULTR human uterine smooth muscle cell line and primary human myometrial cells. These effects were completely inhibited by the H₁ histamine receptor antagonist, diphenhydramine, and were unaffected by the H₂ histamine receptor antagonist, cimetidine. ULTR and primary myometrial cells were transfected with either dominant-negative G protein-coupled receptor kinases (GRKs) or small interfering

RNAs targeting specific GRKs to assess the roles of this protein kinase family in H₁ histamine receptor desensitization. Dominant-negative GRK2, but not GRK5 or GRK6, prevented H₁ histamine receptor desensitization. Similarly, transfection with short interfering RNAs (that each caused >70% depletion of the targeted GRK) for GRK2, but not GRK3 or GRK6, also prevented H₁ histamine receptor desensitization. Our data suggest that histamine stimulates phospholipase C-signaling in myometrial smooth muscle cells through H₁ histamine receptors and that GRK2 recruitment is a key mechanism in the regulation of H₁ histamine receptor signaling in human uterine smooth muscle. These data provide insights into the *in situ* regulation of this receptor subtype and may inform pathophysiological functioning in preterm labor and other conditions involving uterine smooth muscle dysregulation. (*Molecular Endocrinology* 22: 1893–1907, 2008)

THE HUMAN UTERUS contains large numbers of mast cells that are located in close proximity to the uterine myometrial smooth muscle layer (1, 2). This suggests that the myometrium may be a target tissue for the factors released on mast cell degranulation. Mast cells play an important role in allergic responses and during inflammation (3, 4). When stimulated, mast cells release a wide variety of inflammatory mediators such as cytokines, proteases, proteoglycans, and histamine (5, 6). Previous studies have shown that mast cell degranulation can induce myometrial contraction in both pregnant and nonpregnant women (7), and this effect was inhibited by the H₁ histamine receptor antagonist S(+)-chlorphenamine maleate, suggesting

that histamine may be the causative agent. Indeed, like a number of other mediators released during mast cell degranulation, histamine can directly stimulate uterine smooth muscle contraction (8–11). In addition, degranulation of mast cells present in the cervix can lead to H₁ histamine receptor-mediated cervical contractions (12). Furthermore, allergen-stimulated mast cell degranulation has been shown to induce H₁ histamine receptor-mediated preterm labor in guinea pigs (13) and the birth of stillborn pups, whereas pre-treatment of the mothers with the H₁ receptor antagonist, ketotifen, abrogated the histamine effect and the pups were born normally at term (13). Collectively, these studies suggest that histamine signaling may play an important role in the induction of myometrial contraction not only in response to allergic or infectious stimuli, but also conceivably in the normal parturition process. H₁ histamine receptors are G protein-coupled receptors (GPCRs) that stimulate the generation of the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (14, 15). IP₃ production leads to release of Ca²⁺ from intracellular stores, initiating the process of smooth muscle contraction (16, 17). Constant or repeated stimulation of GPCRs can lead to the attenuation of downstream

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Abbreviations: [Ca²⁺]_i, Intracellular Ca²⁺ concentration; eGFP-PH_{PLC δ} , PH domain of PLC δ 1 tagged to enhanced green fluorescent protein (eGFP); GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IP₃, inositol 1,4,5-trisphosphate; PH, pleckstrin homology; PKC, protein kinase C; PLC, phosphoinositide-specific phospholipase C; siRNA, small interfering RNA.

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signaling events through receptor-regulatory processes involving covalent modification and temporary or permanent removal from the plasma membrane (18, 19). In the case of the H₁ histamine receptor, earlier investigations showed that exposure to phorbol esters [that cause activation of conventional and novel protein kinase C (PKC) isoenzymes] could attenuate IP₃ and Ca²⁺ signaling (20–22); however, whether PKC acutely regulates agonist-mediated H₁ histamine receptor regulation in native systems has yet to be established (21).

G protein-coupled receptor kinase (GRK) proteins initiate homologous GPCR desensitization by phosphorylating agonist-activated receptors on serine and threonine residues to promote the subsequent binding of arrestin proteins to the GPCR (19, 23, 24), physically blocking further interaction of receptor and effectors (24, 25). Previous studies have shown that a wide variety of kinases are able to phosphorylate the H₁ histamine receptor (26, 27). More recently, Iwata *et al.* (31) have highlighted a role for endogenous GRK2 in the regulation of recombinant H₁ histamine receptors in human embryonic kidney 293 cells, and have shown that, like a number of other GPCRs (28–30), H₁ histamine receptors can be regulated by phosphorylation-dependent and -independent mechanisms.

At present little is known regarding the molecular mechanism regulating endogenous H₁ histamine receptor responsiveness in native tissues. Our study provides evidence that human ULTR and primary myometrial cells express functional H₁ histamine receptors. Furthermore, we provide evidence indicating that endogenous GRK2 plays a key role in the regulation of endogenous H₁ histamine receptor signaling, suggesting that similar regulation of this receptor occurs *in vivo*.

RESULTS

Characterization of Histamine Receptor Signaling in ULTR Smooth Muscle Cells

Cells treated with a range of histamine concentrations for 30 sec produced transient concentration-depen-

dent increases in intracellular Ca²⁺ ([Ca²⁺]_i), with an EC₅₀ value of 977 ± 50 nM (supplemental Fig. 1, A and B, published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). To assess the ability of histamine to activate phosphoinositide-specific phospholipase C (PLC) signaling, ULTR cells were transfected with the pleckstrin homology (PH) domain of PLCδ1 tagged to enhanced green fluorescent protein (eGFP-PH_{PLCδ}) biosensor. Additions of histamine for 30 sec caused concentration-dependent translocations of eGFP-PH_{PLCδ} to the cytoplasm (EC₅₀, 6.1 ± 1.2 μM; supplemental Fig. 1, C and D), indicative of PLC activation and IP₃ production (32, 33).

To determine which histamine receptor subtype(s) activates IP₃ and Ca²⁺ signaling, Fluo4-loaded ULTR or primary myometrial cells were challenged with histamine (10 μM) for 30 sec. When the [Ca²⁺]_i level had returned to baseline, either the H₁ receptor antagonist diphenhydramine (10 μM) or the H₂ receptor antagonist, cimetidine (10 μM), was added. After 5 min, cells were subjected to a second 30-sec histamine (10 μM) challenge. Addition of diphenhydramine completely inhibited histamine-stimulated Ca²⁺ signaling, whereas cimetidine had no effect (Table 1). In addition, an identical protocol was applied to cells transfected with eGFP-PH_{PLCδ}. Application of diphenhydramine completely blocked histamine-stimulated PLC activity, whereas cimetidine was without effect (Table 1). These data suggest that histamine-stimulated IP₃ and Ca²⁺ signaling are mediated predominantly or exclusively through the H₁ histamine receptor subtype.

H₁ Histamine Receptor Desensitization and Resensitization

Initially, H₁ receptor desensitization was assessed while applying short pulses of a maximal concentration of histamine (100 μM, for 30 sec) with 4-min wash-out periods between agonist challenges. Under these conditions, little attenuation of histamine-stimulated IP₃ or Ca²⁺ responses could be observed (data not shown). These data agree with our previous findings

Table 1. Effects of H₁ and H₂ Histamine Receptor Antagonists on Histamine-Stimulated IP₃ and [Ca²⁺]_i Signaling in ULTR and Primary Myometrial Smooth Muscle Cells

Antagonist	ULTR Cells		Primary Myometrial Cells	
	IP ₃ (R2/R1, %)	[Ca ²⁺] _i (R2/R1, %)	IP ₃ (R2/R1, %)	[Ca ²⁺] _i (R2/R1, %)
Diphenhydramine	8.3 ± 8.0 ^a	0 ± 0 ^a	0 ± 0 ^a	0.54 ± 0.25 ^a
Cimetidine	110 ± 12	107 ± 2	100 ± 6	94.4 ± 1.9

Cells were either transfected with eGFP-PH_{PLCδ} or loaded with Fluo4-AM as described in *Materials and Methods*. Cells were challenged with histamine for 30 sec (R1: 100 μM for IP₃, 10 μM for Ca²⁺ responses), followed by a 5-min wash period during which cells were perfused with either diphenhydramine (10 μM) or cimetidine (10 μM). Cells were then rechallenged with histamine (R2: 100 μM for IP₃, 10 μM for Ca²⁺ responses). Data are expressed as means ± SEM for the percent response obtained after antagonist treatment (R2) compared with the response before antagonist addition (R1) for six to eight cells for IP₃ and 56–135 cells for Ca²⁺ experiments, respectively, performed in at least four separate experiments. Inhibition of H₁ histamine receptors attenuated both IP₃ and [Ca²⁺]_i responses to histamine.

^a *P* < 0.01; one-way ANOVA and Dunnett's *post hoc* test.

with the M₁ muscarinic acetylcholine receptor expressed in hippocampal neurons (34) and suggest the presence of a significant H₁ histamine receptor reserve in the smooth muscle cells studied here.

As described previously, an alternative experimental protocol was adopted (34): cells were challenged with an approx. EC₅₀ concentration (1 μ M for Ca²⁺ and 10 μ M for IP₃ experiments) of histamine for 30 sec before (R1) and after (R2) addition of a maximal histamine concentration (R_{max}: 100 μ M, for 60 sec). Comparison of histamine-stimulated IP₃ and Ca²⁺ responses before (R1) and after (R2) the desensitizing pulse of histamine demonstrated a marked reduction in R2 responses (see Figs. 1 and 2). Reduced R2/R1 ratios are interpreted as an indication of receptor desensitization. In ULTR cells the reduction in R2 relative to R1 caused by the R_{max} histamine exposure was maximally decreased by 54% when assessed using Fluo4 (Fig. 1), and by 60% when assessed using the eGFP-PH_{PLC δ} biosensor (Fig. 2) measured 5 min after R_{max}. Extension of the second wash period after application of R_{max} histamine revealed a time-dependent resensitization of both the histamine-induced IP₃ and Ca²⁺ responses (Figs. 1C and 2C). Indeed, the histamine-stimulated R2 IP₃ response was fully restored 15 min after R_{max} histamine addition, whereas the histamine-stimulated Ca²⁺ response recovered within 10 min (Fig. 1, B and C).

Effects of Inhibiting Endogenous GRK Activities on H₁ Histamine Receptor Signaling

To examine whether histamine H₁ receptor signaling is regulated by endogenous GRKs, we inhibited specific GRK isoenzymes by overexpressing catalytically inactive, dominant-negative GRK mutants. This approach has been successfully used to cause specific inhibition of endogenous GRK activities (19). ULTR cells were cotransfected with eGFP-PH_{PLC δ} (0.25 μ g) and 0.75 μ g of either pcDNA3 (vector control), D110A,K220R GRK2 (30), K215R GRK5, or K215R GRK6 (34, 35). The D110A,K220R double-mutant form of GRK2 was chosen for these experiments to prevent the phosphorylation-independent inhibition of PLC signaling caused by this isoenzyme through G $\alpha_{q/11}$ -binding (30, 31). Cells were subjected to the standard desensitization (R1/R_{max}/R2) protocol with 5-min washes between each histamine addition. Control experiments, cotransfecting monomeric red fluorescent protein and eGFP-tagged dominant-negative GRKs, indicate that more than 90% cotransfection of cells occurs (data not shown). In the presence of pcDNA3, the reduction in R2 relative to R1 caused by the R_{max} histamine exposure was approximately 50% and similar to the degree of desensitization previously observed (Fig. 3, A and F). Overexpression of K215R GRK5 or K215R GRK6 had no effect on H₁ receptor desensitization (Fig. 3, C, D, and F). However, in cells expressing D110A,K220R GRK2, R2 and R1 responses were almost identical (Fig. 3, B and F). In addition, a non-G α_q -binding, kinase-active GRK2 mu-

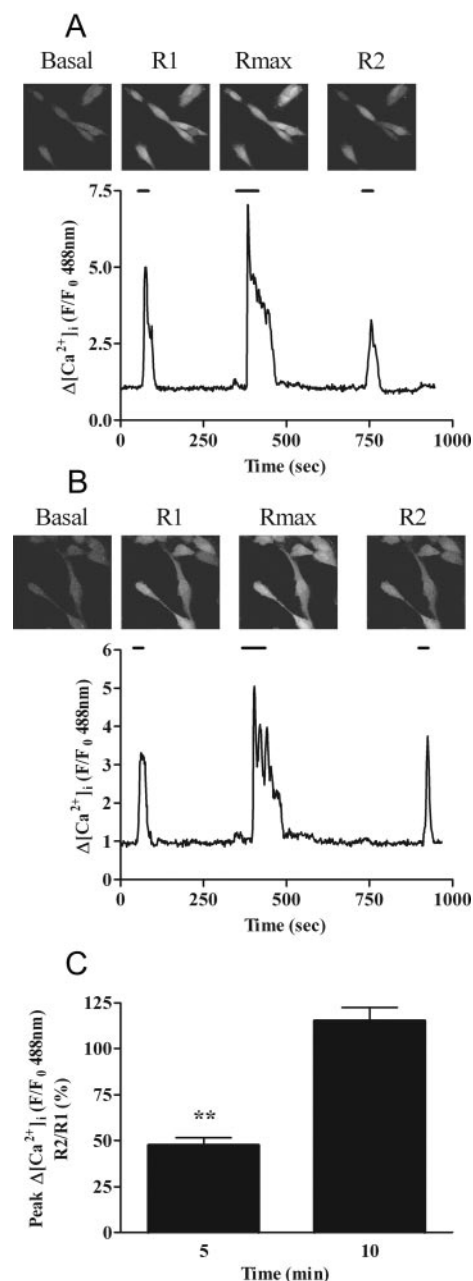


Fig. 1. Desensitization and Resensitization of H₁ Histamine Receptor-Stimulated Ca²⁺ Signaling in ULTR Cells

Cells loaded with the Ca²⁺-sensitive dye Fluo4-AM (3 μ M) were subjected to the desensitization protocol (R1, R2 = 1 μ M histamine, for 30 sec; R_{max} = 100 μ M histamine, for 60 sec). A, Representative images and trace showing the decrease in R2 response compared with R1 when assessed 5 min after addition of the maximal histamine pulse (R_{max}). B, Representative images and trace showing recovery of the R2 response to a similar level to R1 when the delay between R_{max} and R2 was increased to 10 min. Histamine was applied for the times indicated by the bars. C, Cumulative data showing the extent of H₁ histamine receptor desensitization measured 5 min (n = 23 cells) or 10 min (n = 18 cells) after the R_{max} addition. Data are expressed as means $\pm$ SEM for the percent change in R2 relative to R1, from at least four separate experiments. **, $P < 0.01$; R2/R1 ratio for 5 min- vs. 10-min delay after R_{max}.

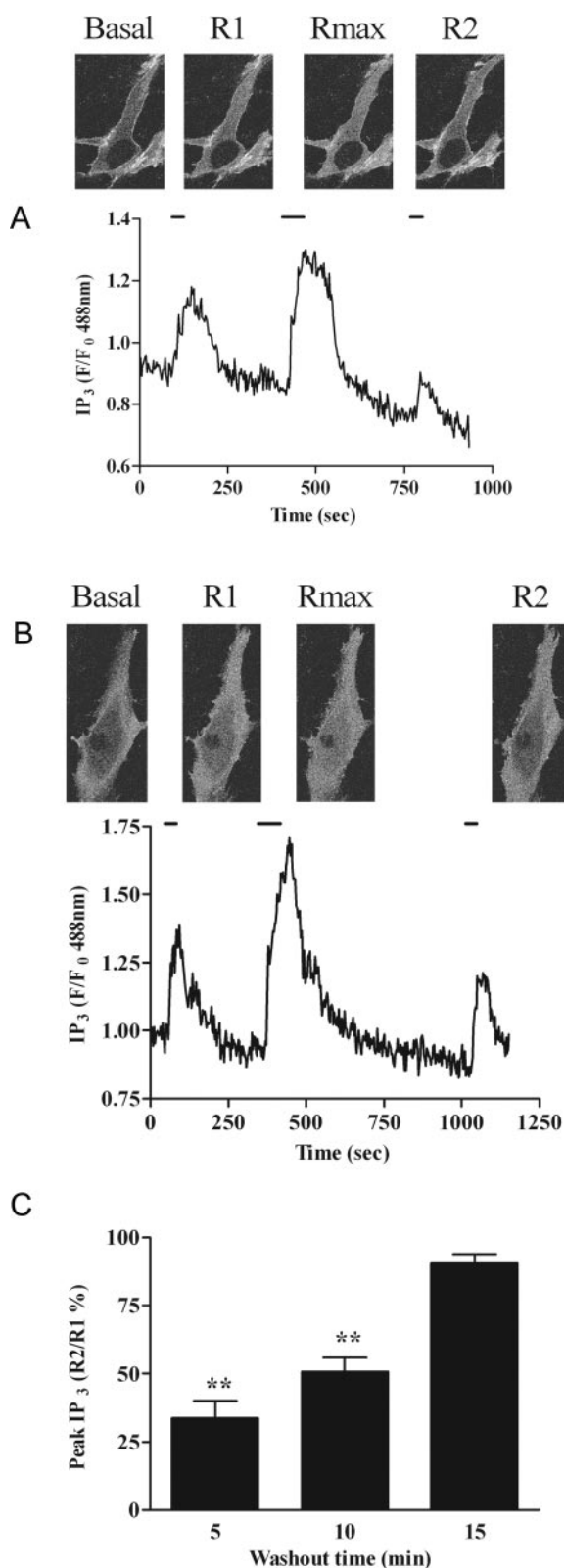


Fig. 2. Desensitization and Resensitization of H₁ Histamine Receptor-Stimulated PLC Signaling in ULTR Cells

Cells expressing eGFP-PH_{PLC δ} were subjected to the desensitization protocol (R1, R2 = 10 μ M histamine, for 30 sec; R_{max} = 100 μ M histamine, for 60 sec, as shown by the

tant (^{D110A}GRK2) further decreased the R2 response relative to R1 (Fig. 3, E and F), suggesting that overexpression of GRK2 can enhance H₁ histamine receptor desensitization.

Effects of Depleting GRK Expression on H₁ Histamine Receptor Signaling

To confirm and extend the data obtained using the dominant-negative constructs, we have also studied the effects of GRK knockdown using small interfering RNAs (siRNAs) on H₁ histamine receptor desensitization. Initial experiments indicated that maximal depletion of endogenous GRK2 was achieved 48 h after siRNA transfection (data not shown), and that a maximal depletion of GRK2 protein could be attained at concentrations of siRNA of 10 nM or greater (Fig. 4, A and D). The knockdown caused by the anti-GRK2 siRNA appears to be specific, because expression of GRKs 3, 5, and 6 was unchanged (Fig. 4, A and D). Similar specific knockdowns of GRK3 (Fig. 4, B and E) and GRK6 (Fig. 4, C and F) were achieved using the siRNA approach. Negative control siRNA (1, 10, or 100 nM) used as a transfection control had no effect on GRK2, GRK3, GRK5, or GRK6 expression levels (Fig. 4, A–C).

To examine whether depletion of endogenous GRK2 affects H₁ histamine receptor desensitization, ULTR cells were cotransfected with eGFP-PH_{PLC δ} (0.5 μ g) and negative control siRNA (100 nM), anti-GRK2 (10 nM), anti-GRK3 (100 nM), or anti-GRK6 (10 nM) siRNAs. In the presence of control siRNA, anti-GRK3 or anti-GRK6 siRNAs (Fig. 5, A and C–E), R2 responses were decreased by 50–60% compared with R1, consistent with the extent of receptor desensitization observed in untransfected cells. However, in cells transfected with anti-GRK2 siRNA, R2 and R1 responses were almost identical (Fig. 5, B and E). In agreement with these data, treatment of cells with the anti-GRK2 siRNA also markedly attenuated the desensitization of H₁ histamine receptor-stimulated Ca²⁺ responses compared with cells transfected with negative-control siRNA (supplemental Fig. 2, A and C). These data confirm that GRK2 is a key regulator of H₁ histamine receptor signaling in uterine smooth muscle.

Histamine Receptor Signaling in Primary Myometrial Smooth Muscle Cells

Histamine stimulated increases in IP₃ (Fig. 6D) and [Ca²⁺]_i (Fig. 6B) in primary myometrial smooth muscle

horizontal bars). Representative images and traces showing the extent of H₁ histamine receptor desensitization measured 5 min (A) or 10 min (B) after the R_{max} challenge. C, Cumulative data showing changes in the R₂/R₁ ratio 5, 10, and 15 min after R_{max} challenge. Data are expressed as means $\pm$ SEM for the percent change in R₂ relative to R₁; n = 7 cells for each time point, from at least four separate experiments; **, P < 0.01; R₂/R₁ ratios for 5 min and 10 min vs. 15-min delay after R_{max}.

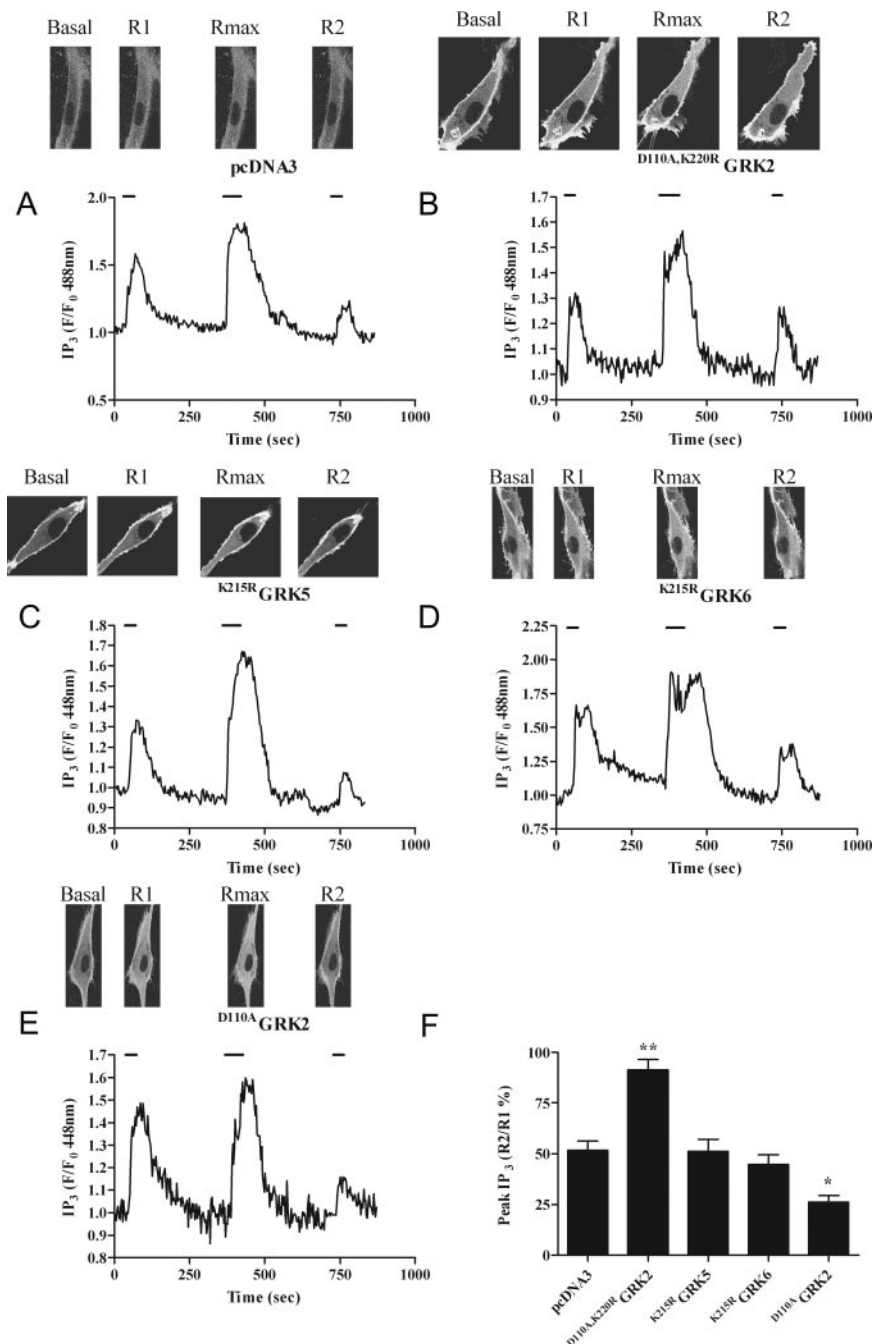


Fig. 3. Effects of Overexpressing Dominant-Negative GRK Mutants on H₁ Histamine Receptor Desensitization in ULTR Cells. Cells were cotransfected with 1:3 ratio of eGFP-PH_{PLCδ} with pcDNA3 (A), D110A,K220R GRK2 (B), K215R GRK5 (C), K215R GRK6 (D), or D110A GRK2 (E) and 48 h later subjected to the standard R1/R_{max}/R2 desensitization protocol. Panels A–E show representative images and traces for the H₁ histamine receptor desensitization protocol (histamine additions, indicated by the horizontal bars were: R1, R2 = 10 μ M; R_{max} 100 μ M histamine) in individual cells expressing the various GRK constructs. Panel F shows cumulative data for changes in R2/R1 ratios in ULTR cells overexpressing the various GRK constructs. Data are presented as means $\pm$ SEM for the percent changes in R2/R1 ratio, for at least 10 cells from five separate experiments. Responses in D110A GRK2- (*, $P < 0.05$) and D110A,K220R GRK2- (**, $P < 0.01$) expressing cells are significantly different from those of vector control, K215R GRK5-, and K215R GRK6-expressing cells.

cells with similar profiles and EC₅₀ values (Fig. 6A) to those previously determined in ULTR cells. The IP₃/[Ca²⁺]_i responses to histamine were completely inhibited by the H₁ receptor antagonist, diphenhydramine

(10 μ M), whereas the H₂ receptor antagonist cimetidine (10 μ M) was without effect (Table 1).

Primary myometrial cell H₁ histamine receptors underwent a similar degree of receptor desensitization

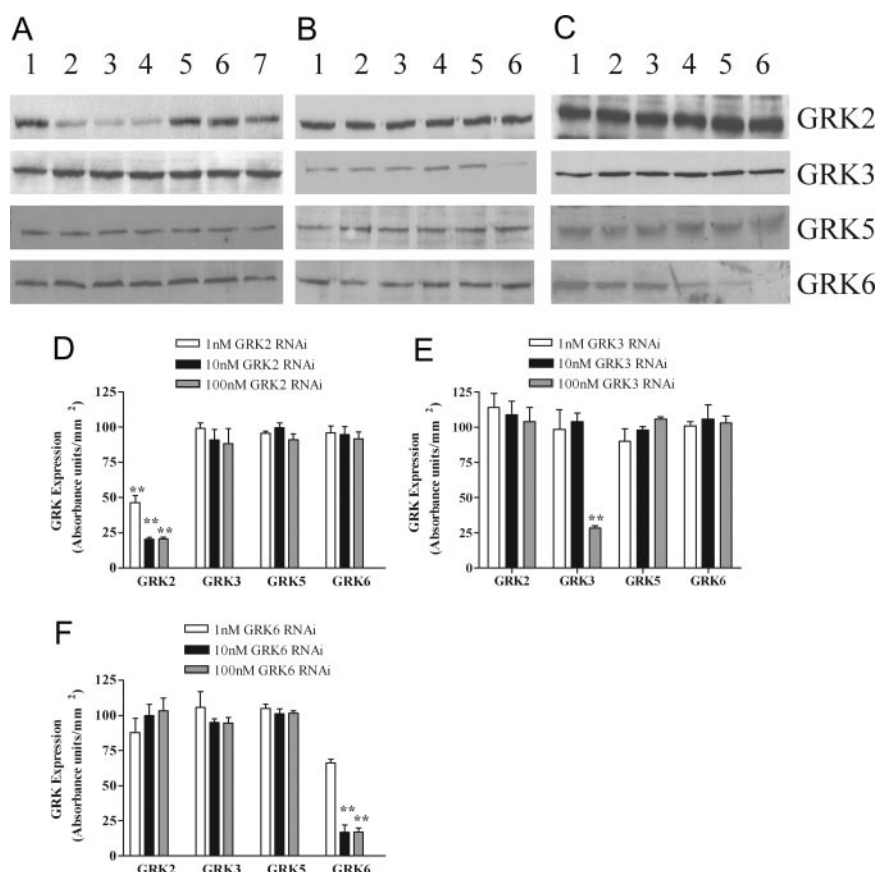


Fig. 4. Effects of GRK2, GRK3, and GRK6 siRNA Treatment on Endogenous GRK Expression in ULTR Cells

Cells were transfected with 1, 10, or 100 nM of negative control, anti-GRK2, anti-GRK3, or anti-GRK6 siRNAs as described in *Materials and Methods*. After 48 h ULTR cells were lysed and 40 μ g of protein was loaded per lane for SDS-PAGE separation and immunoblotting. A, Western blots showing effects of anti-GRK2 siRNA on levels of GRK2, GRK3, GRK5, and GRK6 expression (lane 1: nontransfected; lanes 2–4: 1, 10, or 100 nM anti-GRK2 siRNA; lanes 5–7: 1, 10, or 100 nM, negative control siRNA). B, Western blots showing effects of anti-GRK3 siRNA treatment on levels of GRK2, GRK3, GRK5, and GRK6 expression (lanes 1–3: 1, 10, or 100 nM, negative control siRNA; lanes 4–6: 1, 10, or 100 nM anti-GRK3 siRNA). C, Western blots showing effects of anti-GRK6 siRNA treatment on levels of GRK2, GRK3, GRK5, and GRK6 expression (lanes 1–3: 1, 10, or 100 nM, negative control siRNA; lanes 4–6: 1, 10, or 100 nM anti-GRK6 siRNA). Densitometric analysis was undertaken on all blots. Data shown highlight changes in GRK2, GRK3, GRK5, and GRK6 expression in ULTR cells after anti-GRK2 (D), anti-GRK3 (E), or anti-GRK6 (F) siRNA treatments. Data are presented as means $\pm$ SEM for four separate experiments where GRK isoenzyme expression was compared with nontransfected cells; **, $P < 0.01$ comparing anti-GRK2/anti-GRK3/anti-GRK6 siRNA treatments with respective negative control siRNA treatments. RNAi, RNA interference.

when subjected to a similar protocol ($R1/R_{\max}R2$; where $R1/R2$ is 1 μ M histamine for Ca^{2+} and 10 μ M for IP_3 experiments) used in the ULTR experiments. Thus, the $R2/R1$ ratio was decreased by 58% (Fig. 6, B and F) and 46% (Fig. 6, D and F) for Ca^{2+} and IP_3 responses, respectively. Extension of the wash period between $R_{\max}$ and $R2$ resulted in a time-dependent recovery of both the Ca^{2+} and IP_3 responses to histamine rechallenge (Fig. 6, C, E, and F). These data suggest that the magnitude of H₁ histamine receptor desensitization is similar in primary human myometrial smooth muscle and ULTR cells, whereas H₁ histamine receptor-stimulated IP_3 responses may resensitize somewhat faster in primary myometrial compared with ULTR cells.

Effects of Inhibiting Endogenous GRK Activities in Myometrial Smooth Muscle Cells

To assess the involvement of endogenous GRK isoenzymes in H₁ histamine receptor desensitization in primary cells, human myometrial smooth muscle cells were cotransfected with eGFP-PH_{PLC δ} and pcDNA3, D110A, K220R GRK2 (30, 31), K215R GRK5, or K215R GRK6 (34, 35). After 48 h, cells were subjected to the standard ($R1/R_{\max}/R2$) desensitization protocol with 5-min washes between each histamine addition. Initial control experiments using monomeric red fluorescent protein- and eGFP-tagged dominant-negative GRK constructs indicated more than 90% cotransfection of cells (data not shown). In primary myometrial cells

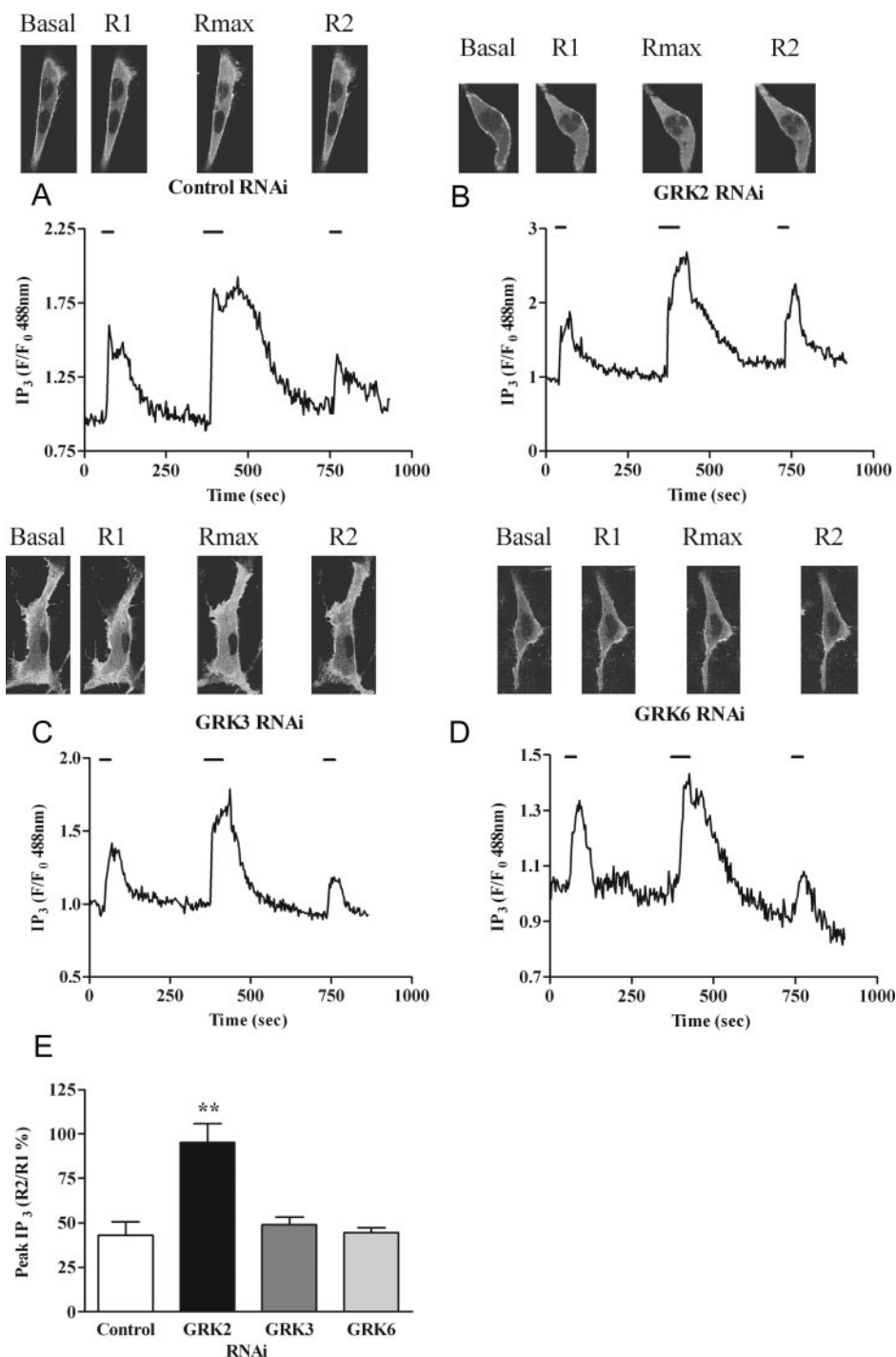


Fig. 5. Effects of Selective Cellular GRK2, GRK3, or GRK6 Depletion by siRNA on H₁ Histamine Receptor Desensitization in ULTR Cells

Cells were cotransfected with eGFP-PH_{PLC δ} and either 10 nM anti-GRK2 or anti-GRK6, or 100 nM anti-GRK3 or negative control siRNAs. After 48 h, receptor desensitization was assessed using the standard desensitization protocol (R1, R2 = 10 μ M histamine for 30 sec; R_{max} = 100 μ M histamine for 60 sec). Representative traces and images showing the effects of negative control (A), anti-GRK2 (B), anti-GRK3 (C), and anti-GRK6 (D) siRNAs on H₁ histamine receptor desensitization. The cumulative data (panel E) show a significant (**, $P < 0.01$) decrease in the extent of H₁ histamine receptor desensitization after depletion of endogenous GRK2. Data are presented as means $\pm$ SEM for the percent change in R2/R1 ratio, for 9–11 cells from at least five separate experiments. RNAi, RNA interference.

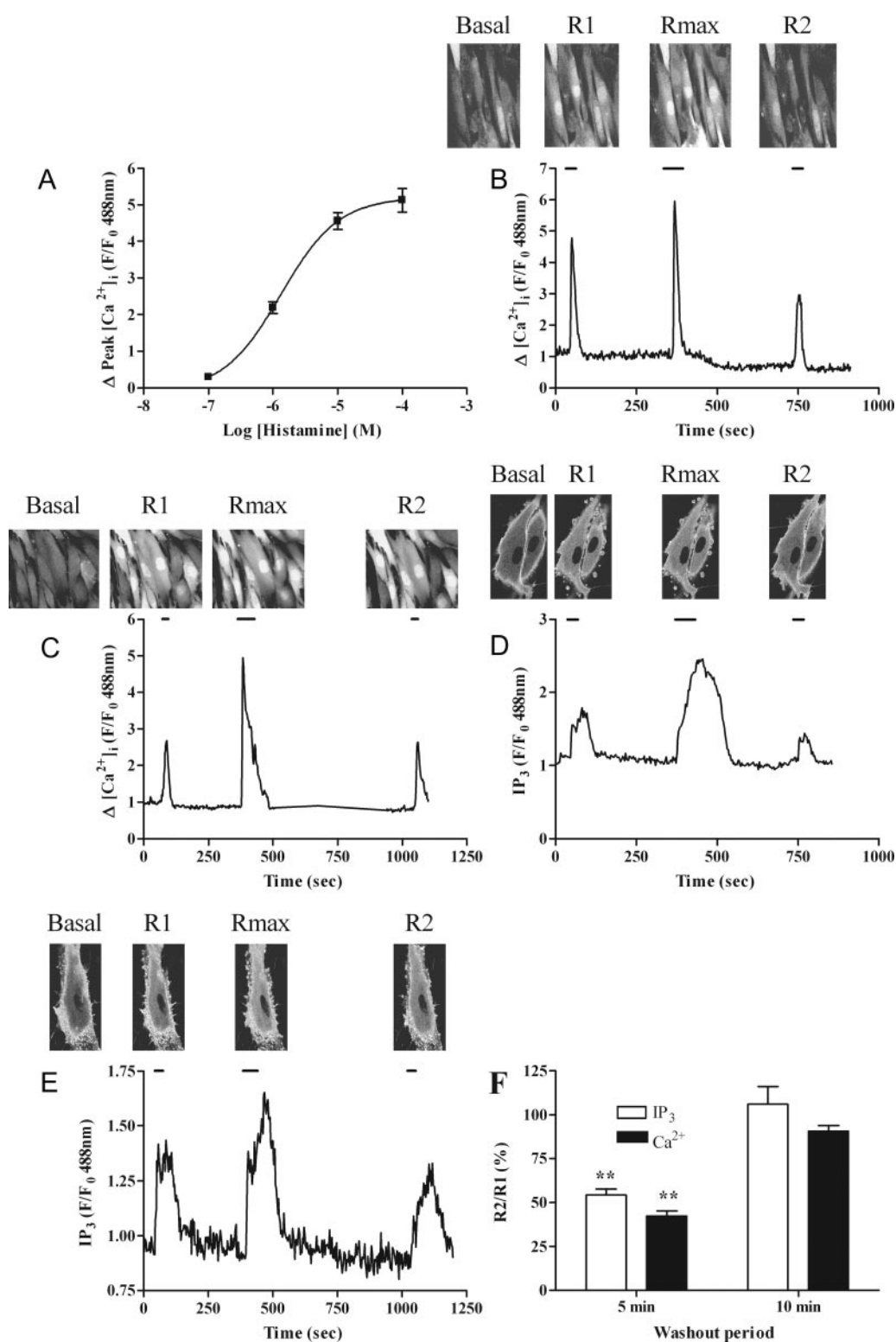


Fig. 6. Desensitization and Resensitization of Histamine-Stimulated Ca^{2+} and IP_3 Signaling in Isolated Primary Human Myometrial Smooth Muscle Cells

Primary human myometrial smooth muscle cells were loaded with Fluo4-AM ($3 \mu\text{M}$) for 60 min. **A**, Concentration-response curve showing $[\text{Ca}^{2+}]_i$ changes after 30-sec additions of histamine (10^{-7} to 10^{-4} M). Data are shown as means $\pm$ SEM for $n = 100$ cells, from six separate experiments. Fluo4-loaded cells were subjected to the standard desensitizing protocol (R1, R2 = $1 \mu\text{M}$ histamine for 30 sec; R_{max} = $100 \mu\text{M}$ histamine for 60 sec). **B**, Representative images and traces showing the decrease in R2 compared with R1 when assessed 5 (**B**) or 10 (**C**) min after commencing washout of an R_{max} histamine addition. Primary

transfected with pcDNA3, responses after the desensitizing histamine exposure were decreased by approximately 50% compared with the preexposure response (Fig. 7, A and C). However, after overexpression of ^{D110A,K220R}GRK2, R2 responses were not significantly different to R1 (Fig. 7, B and C). In contrast, expression of ^{K215R}GRK5 or ^{K215R}GRK6 had no attenuating effect on the R2/R1 ratio (Fig. 7C; see also supplemental Fig. 3, A and B). To increase cellular GRK2 activity a non-Gα_q-binding version of GRK2 (^{D110A}GRK2) was overexpressed. The presence of ^{D110A}GRK2 further extenuated the difference between R1 and R2 responses (Fig. 7C; see also supplemental Fig. 3C). These data suggest that GRK2 is a key regulator of H₁ histamine receptor desensitization in primary human myometrial cells.

Effects of Depleting GRK Expression in Myometrial Smooth Muscle Cells

To confirm and extend the findings obtained using the dominant-negative constructs, we investigated the effects of siRNAs directed at GRK2, GRK3, and GRK6. Because we could only achieve low transfection efficiencies in human myometrial smooth muscle cells using conventional lipofection methods, we employed the Amaxa nucleofection technique (see *Materials and Methods*). Preliminary transfections with eGFP showed more than 50% cell transfection using this method (data not shown). Negative control siRNA had no effect on endogenous GRK expression, whereas GRK2-, GRK3-, and GRK6-targeted siRNAs selectively depleted their respective GRK targets by more than 75% (Fig. 8).

To assess the roles that GRK isoenzymes play in H₁ histamine receptor desensitization, primary myometrial cells were cotransfected with eGFP-PH_{PLCδ} and either negative control, anti-GRK2, anti-GRK3, or anti-GRK6 siRNAs. Cells were subjected to the standard (R1/R_{max}/R2) desensitization protocol with 5-min washes between each histamine addition. In agreement with previous data in ULTR cells, negative control, anti-GRK3, or anti-GRK6 siRNAs did not affect the extent of H₁ histamine receptor desensitization caused by exposure to histamine (100 μM, 60 sec; Fig. 9C; see also supplemental Figs. 3, D and E, and supplemental Figs. 4 and 6), whereas siRNA knockdown of GRK2 completely prevented receptor desensitization (R2 response was 113 ± 7%; of R1; Fig. 9, A–C; see also supplemental Fig. 5). These data again indicate that GRK2 is the key GRK isoenzyme regulating

H₁ histamine receptor signaling in both primary human myometrial smooth muscle and ULTR cells.

DISCUSSION

Preterm birth is a major challenge in perinatal health-care (36). Accumulating evidence indicates that myometrial cells express histamine receptors, and H₁ histamine receptors can mediate uterine contraction (7–12). Mast cells resident within the myometrium can degranulate, releasing a cocktail of mediators including histamine (1–6, 13). Thus, it has been proposed that mast cell activation during pregnancy may be one cause of preterm birth (7, 11, 12). Therefore, understanding the regulation of H₁ histamine receptors within the myometrium is of potential clinical significance.

In the present study, the immortalized human uterine smooth muscle ULTR cell line and primary human myometrial cells have been used to investigate the regulation of this GPCR subtype. Histamine stimulated similar patterns of IP₃ production and [Ca²⁺]_i via the H₁ histamine receptor subtype in ULTR and primary myometrial cells, consistent with previous reports (8, 9, 11). Using a single-cell fluorescence imaging protocol similar to that used previously by us to investigate muscarinic receptor desensitization (30, 34, 35), we have provided evidence that brief (60 sec) exposure to a high concentration of histamine can cause substantial receptor desensitization, as evidenced by an attenuated response to an approximately EC₅₀ concentration of agonist given after (compared with one given before) the desensitizing histamine pulse. In addition, we have shown that both IP₃ (measured using the eGFP-PH_{PLCδ} biosensor) and Ca²⁺ (measured using Fluo4) can be used to assess receptor desensitization in this cell background. Thus, provided an appropriate submaximal histamine concentration is used for R1/R2 stimulations (1 μM for Ca²⁺; 10 μM for IP₃), comparable signal attenuations can be observed using either readout. In both ULTR and primary myometrial cells the H₁ histamine receptor desensitization (caused by the 60-sec desensitizing pulse of histamine) was transient because within 10–15 min of the desensitizing histamine pulse, differences between the R2 and R1 responses were no longer evident.

A variety of previous studies have investigated H₁ histamine receptor desensitization, internalization, and down-regulation, principally in recombinant systems.

myometrial cells were transfected with eGFP-PH_{PLCδ} for 48 h before the desensitization protocol was carried out (see above). Representative images and traces showing the extent of H₁ histamine receptor desensitization measured at 5 (D) or 10 (E) min after commencing washout of an R_{max} histamine addition. F, Cumulative data showing the extent of H₁ histamine receptor desensitization measured 5 or 10 min after R_{max}. Data are expressed as means ± SEM for percent change in R2 relative to R1 responses, for n = 37–106 cells from at least four experiments (Ca²⁺ data), and n = 6–11 cells, from at least five separate experiments (IP₃ data). In all cases, cells derived from at least three patient donors were used; **, P < 0.01 for 5 vs. 10 min R2/R1 values.

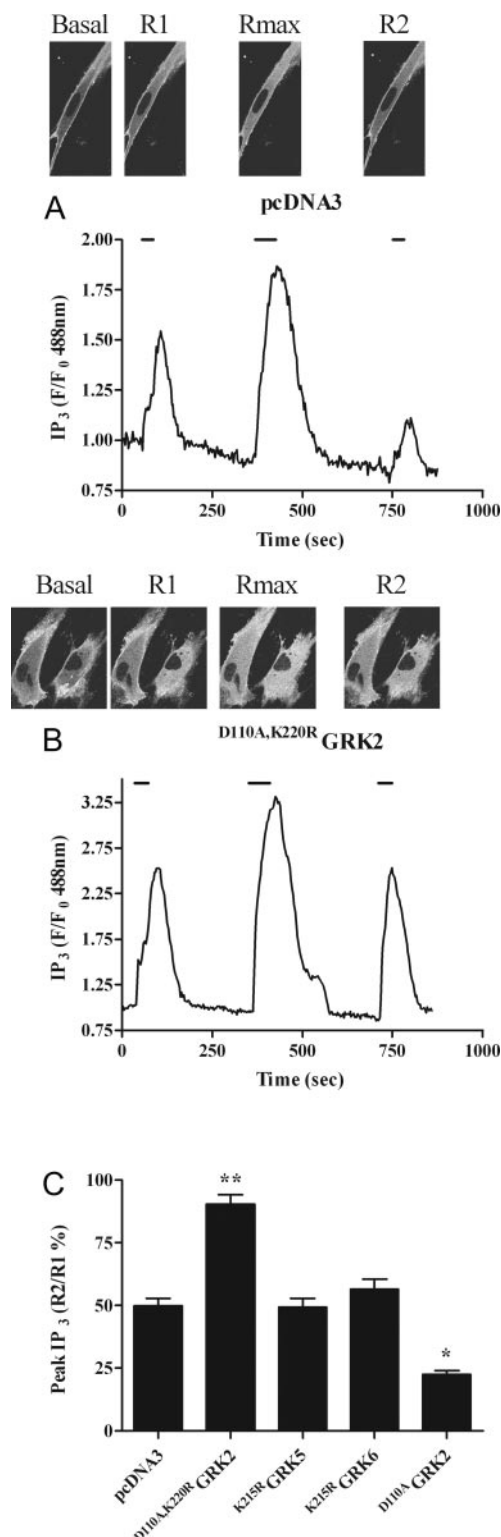


Fig. 7. Effects of Overexpressing Dominant-Negative GRK Mutants on H_1 Histamine Receptor Desensitization in Primary Human Myometrial Cells

Cells were cotransfected with 1:3 ratio of eGFP-PH_{PLC β 8} with pcDNA3 (A), D110A,K220R GRK2 (B), K215R GRK5, K215R GRK6, or D110A GRK2 (see supplemental Fig. 3, A–C) and 48 h later subjected to the standard R1/R_{max}/R2 desensitization protocol. Panels A and B show representative images

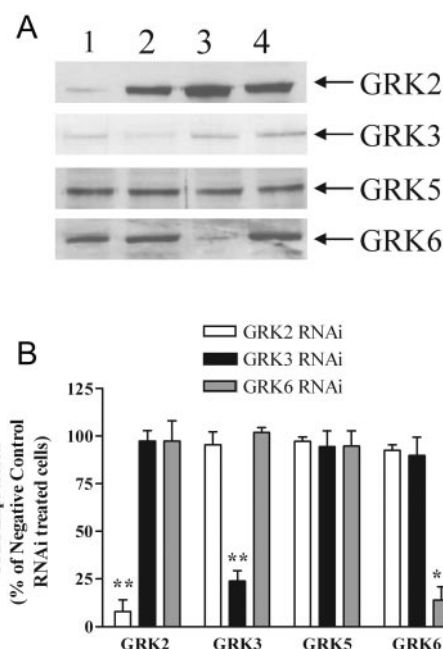


Fig. 8. Effects of GRK2, GRK3, and GRK6 siRNA Treatment on Endogenous GRK Expression in Primary Human Myometrial Cells

Cells were transfected with anti-GRK2 (10 nM), -GRK3 (100 nM), -GRK6 (10 nM), or negative control (100 nM) siRNA as described in *Materials and Methods*. After 48 h cells were lysed, and 40 μ g of protein was loaded per lane for SDS-PAGE separation and immunoblotting. A, Representative blots showing effects of anti-GRK siRNA treatment on endogenous GRK expression: lane 1, anti-GRK2; lane 2, anti-GRK3; lane 3, anti-GRK6; lane 4, negative control. B, Densitometric analysis was undertaken on all blots. Data shown highlight changes in GRK2, GRK3, GRK5, and GRK6 expression in primary myometrial cells after anti-GRK2, -GRK3 or -GRK6 siRNA treatments. Data are presented as means $\pm$ SEM for three experiments from cells derived from three separate patient donors. **, $P < 0.01$ comparing anti-GRK2/anti-GRK3/anti-GRK6 siRNA treatments with respective negative-control siRNA treatments. RNAi, RNA interference.

A focus of these studies has been to define the role of PKC in the regulation of this receptor subtype. Thus, key serine/threonine residues within intracellular-facing domains of the receptor have been defined as potential PKC substrates (26, 37) and although this mechanism of regulation is unlikely to mediate acute

and traces for the H_1 receptor desensitization protocol (histamine additions, indicated by the horizontal bars were: R1, R2 = 10 μ M; R_{max} 100 μ M histamine) in individual cells. Panel C shows cumulative data for changes in R2/R1 ratios in myometrial cells overexpressing the various GRK constructs. Data are presented as means $\pm$ SEM for the percent changes in R2/R1 ratio, for 12–18 cells from at least five separate experiments. Responses in D110A GRK2 (*, $P < 0.05$)- and D110A,K220R GRK2 (**, $P < 0.01$)-expressing cells are significantly different from those of vector control, K215R GRK5-, and K215R GRK6-expressing cells.

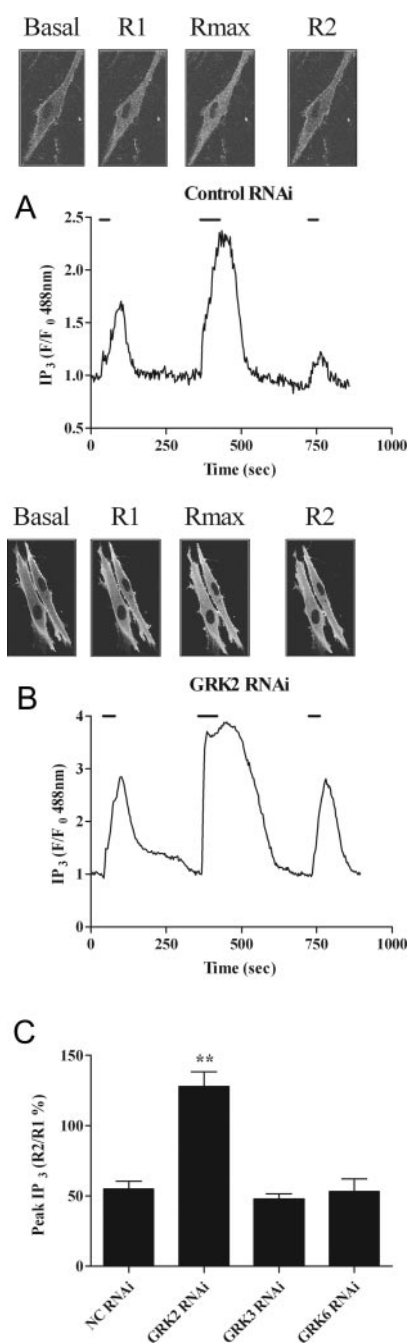


Fig. 9. Effects of Selective Cellular GRK2, GRK3, or GRK6 Depletion by siRNA on H₁ Histamine Receptor Desensitization in Primary Myometrial Smooth Muscle Cells

Cells were cotransfected with eGFP-PH_{PLCδ} and either 10 nM anti-GRK2 or anti-GRK6, or 100 nM anti-GRK3 or negative control siRNAs. After 48 h, receptor desensitization was assessed using the standard desensitization protocol (R1, R2 = 10 μ M histamine for 30 sec; R_{max} = 100 μ M histamine for 60 sec). Representative traces and images showing the effects of negative control (A) and anti-GRK2 (B) on H₁ histamine receptor desensitization (see supplemental Fig. 3, D and E, for cells transfected with anti-GRK3 and anti-GRK6 siRNAs). The cumulative data (panel C) show a significant (**, $P < 0.01$) decrease in the extent of H₁ histamine receptor desensitization after depletion of endogenous GRK2. Data are presented

receptor desensitization, it may play a role in determining the ultimate fate of the receptor (*i.e.* via down-regulation vs. resensitization pathways) (38). In addition, in airways smooth muscle PKC activation may additionally regulate the H₁ histamine receptor at a transcriptional level (22). Evidence for GRK involvement in H₁ histamine receptor regulation has been published only recently. Using a variety of approaches, Iwata *et al.* (31) have provided compelling evidence that the H₁ histamine receptor, transiently expressed in a human embryonic kidney 293 cell background, is preferentially phosphorylated and desensitized by GRK2.

Because there are no specific pharmacological inhibitors of GRKs, we have previously used a variety of molecular biological approaches to define which GRK isoenzymes are responsible for M₁ and M₃ muscarinic acetylcholine receptor desensitization in a variety of cell backgrounds, including GRK overexpression and antisense and dominant-negative strategies, to inhibit endogenous GRK activities (30, 34, 35, 39). Here we have used the latter approach to assess whether overexpression of dominant-negative (kinase-dead) GRK constructs can interfere with the observed acute decrease in H₁ histamine receptor responsiveness. These experiments provided evidence for the D110A,K220R GRK2 construct, which is mutated to prevent both kinase activity and G $\alpha_{q/11}$ binding (30), almost completely preventing receptor desensitization. In contrast, overexpression of kinase-dead GRK5 and GRK6 mutants was without effect. To examine further the possible role of GRK2 in H₁ histamine receptor regulation, we overexpressed this isoenzyme (in fact, a non-G $\alpha_{q/11}$ -binding, D110A GRK2 construct) and found that it could amplify the decrease in receptor responsiveness caused by agonist-stimulation. Together these data point to a key role for GRK2.

Both the dominant-negative and GRK overexpression approaches can be criticized because they might cause off-target effects or lack absolute specificity for the desired GRK isoenzyme. Therefore we have also used a similar siRNA strategy to that employed by Iwata *et al.* (31) to deplete specifically endogenous GRK2, GRK3, and GRK6 in ULTR and primary myometrial cells. As in the dominant-negative experiments, siRNA-mediated depletion of endogenous GRK2 almost completely prevented H₁ histamine receptor desensitization with respect to effects on IP₃ and Ca²⁺ responses in both ULTR and primary myometrial cells. In contrast, siRNA-mediated GRK3 and GRK6 depletions were without effect.

Collectively, these data strongly suggest that GRK2 is the preeminent GRK isoenzyme regulating the initial phase of H₁ histamine receptor desensitization.

as means $\pm$ SEM for the percent change in R2/R1 ratio for between 6–18 cells from at least five separate experiments. In each case, cells derived from at least three patient donors were used. RNAi, RNA interference.

zation in both uterine smooth muscle preparations. However, it remains possible that other GRK isoenzymes (and perhaps other second messenger-regulated protein kinases) play roles in later stages of this regulatory process (e.g. receptor internalization and/or down-regulation).

In addition to providing important new information about the regulation of this GPCR subtype in native cells, our data also add to the existing literature supporting ULTR cells as an appropriate model of primary myometrial cells (40–42).

During pregnancy, GPCRs involved in myometrial contraction and the onset of labor are tightly controlled, so that inappropriate contraction and preterm labor are prevented. Long-term regulation of receptor expression is mediated through the actions of a number of hormonal and cellular signals during gestation. However, such a regulatory system may be unable to respond to rapid changes in the extracellular milieu (e.g. as might be caused by mast cell degranulation) that cause receptor activation. Rapid attenuation of receptor signaling under these circumstances is likely to be advantageous during pregnancy where more prolonged receptor activation might cause the onset of labor. The molecular processes involved in myometrial smooth muscle activation and deactivation are poorly understood, but are likely, at least in part, to involve the desensitization and down-regulation of important uterotonic receptors. It is interesting in this context to note that GRK protein levels alter during pregnancy, with GRK2 and GRK6 levels, in particular, being markedly enhanced in the pregnant myometrium (43, 44). These findings, combined with the data presented here, suggest that H₁ histamine receptor regulation may be tightened as pregnancy proceeds, highlighting a potentially important role for GRK proteins in the regulation of labor.

Elevated maternal plasma histamine levels during pregnancy have been linked to several adverse clinical conditions, including preeclampsia (45–48), preterm labor (49), and spontaneous abortion (50–53). The presence of large numbers of mast cells (2, 54), which lie in close proximity to the myometrial smooth muscle layer, has prompted the suggestion that myometrial tissue may be a target for mast cell degranulation, although the factors causing initiation of degranulation and its consequences are presently poorly understood. Comparison of nonpregnant and pregnant patients has demonstrated that mast cell density is significantly increased during pregnancy (55). Additionally, animal experiments have shown that H₁ histamine receptor antagonists can prevent preterm birth and stillbirth, adding weight to the idea that histamine released by uterine resident mast cells may be important in some patient groups susceptible to preterm labor. Indeed, it is interesting to note that labor has been described as an inflammatory process, induced through the production of prostaglandins, cytokines, and perhaps other mediators (56), and to speculate that certain pathological conditions, such as infections

during pregnancy and histamine release as a consequence of mast cell activation, may lead to uterine contraction and preterm labor.

In summary, the present study has identified GRK2 as the preeminent protein kinase terminating H₁ histamine receptor signaling in ULTR and primary myometrial uterine smooth muscle cells. These data highlight the important role for GRK2 in acute H₁ histamine receptor desensitization and potentially the prevention of myometrial cell activation and preterm labor. However, future studies are now required to examine H₁ histamine receptor signaling and desensitization pathways in pregnant primary uterine smooth muscle from term and preterm patients to further test this hypothesis.

MATERIALS AND METHODS

Cell Culture

ULTR cells (57), created using retroviral infection of isolated primary myometrial cells with the E6/E7 open reading frames of the human papilloma virus type 16, were a generous gift from Dr. James K. McDougall (Fred Hutchinson Cancer Research Centre, Seattle, WA). We and others have identified ULTR cells as a good model for the nonpregnant myometrium (40–42). ULTR cells were grown in DMEM, supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml). All cells were maintained under humidified conditions at 37°C, in air/5% CO₂.

Tissue Collection

All protocols for human tissue collection were approved by the University Hospitals of Leicester Research and Development Group, and the Leicestershire, Northamptonshire, and Rutland Research Ethics Committees under reference no. 6816. Uterine samples were obtained at hysterectomy from women undergoing surgery for nonneoplastic indications, e.g. dysfunctional uterine bleeding. All patients provided informed consent.

Primary Cell Culture

Primary human myometrial cells were isolated as described previously (58). Briefly, small samples of human uterine tissue were excised from total hysterectomy samples and transferred in a sterile container to the laboratory on ice where the myometrium was dissected free of the endometrium and serosal surface and any attached vaginal or cervical tissue. The tissue was then cut with sterile scalpels into small (<2 mm) cubes and placed in 20 ml of DMEM-F12 medium (Invitrogen, Paisley, Scotland, UK) containing collagenase (2 µg/ml; Sigma-Aldrich, Poole, Dorset, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin that had been sterilized by filtration through 0.2-µm filters and prewarmed to 37°C. Digestion was continued for 180 min in a reciprocating water bath at 300 rpm. After the digestion period, large pieces of undigested tissue were allowed to settle to the bottom of the tube under gravity for 2 min. The digested cells were transferred to a fresh sterile tube, diluted with DMEM-F12 and collected by centrifugation at 800 × *g* for 5 min at 4°C. The cell pellets were then washed twice, before resuspension in DMEM-F12 containing 100 U/ml penicillin and 100 µg/ml streptomycin, and supplemented with 10% heat-inactivated fetal calf serum

(Invitrogen) and plated onto two T-75 tissue culture flasks. Cells were allowed to attach to the plastic substratum, and the medium was changed every day for 7–10 d until the cultures became confluent. Cells were then subcultured for individual experiments. Cells were not used beyond passage 5.

Western Blotting and Assessment of siRNA Effects on Endogenous GRK Expression

ULTR cells were plated at 150,000 cells per well 24 h before transfection with either negative control, anti-GRK2 siRNA (5'-GGCAGCCAGUGACCAAAAtt-3') anti-GRK3 siRNA (5'-GGAACUUCUUCUGUUCAtt-3'), or anti-GRK6 siRNA (5'-GGACACAAAGGAUCAAGtt-3') (Ambion/Applied Biosystems, Warrington, UK), at final concentrations of 1, 10, or 100 nM. Transfection was achieved using the Interferin transfection reagent (Autogen Bioclear, Calne, UK) according to the manufacturer's instructions. Primary myometrial cells were transfected with anti-GRK siRNAs using the Nucleofection technique (Amaxa Biosystems, Gaithersburg, MD) (59), according to the manufacturer's optimized protocol. Briefly, 1×10^6 cells per reaction were transfected with anti-GRK2 (10 nM), anti-GRK3 (100 nM), anti-GRK6 (10 nM), or negative control siRNAs, before seeding onto six-well plates.

After 48 h ULTR or primary myometrial cells were lysed and subjected to electrophoretic separation exactly as described previously (60). Separated proteins were transferred to nitrocellulose paper, and GRK expression was detected using polyclonal antibodies against GRK2, GRK3, GRK5, and GRK6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein expression was visualized after application of enhanced chemiluminescent reagent and exposure to Hyperfilm (GE Healthcare, Little Chalfont, UK). The relative expression of individual GRK proteins was determined using the GeneGnome image analysis system and software (Syngene, Cambridge, UK).

Measurement of PLC Activity

To assess PLC activity, cells were grown on 25-mm² glass coverslips for 24 h before transfection with the eGFP-PH_{PLC δ} biosensor (0.5 μ g) using Lipofectamine 2000 as per the manufacturer's instructions. After 48 h, agonist-stimulated translocations of eGFP-PH_{PLC δ} were visualized using an Olympus FV500 scanning laser confocal microscope (Olympus Corp., Lake Success, NY) as described previously (30, 34). Briefly, cells were incubated at 37 °C using a temperature controller and microincubator (PDMI-2 and TC202A; Burleigh, UK) and perfused at 5 ml/min in the following buffer [134 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, and 1.3 mM CaCl₂ (pH 7.4)]. Single-cell images were captured using an oil immersion $\times 60$ objective. Cytosolic IP₃ levels were determined as the relative change in fluorescence in an area of interest as described previously (30, 34). Drugs were applied via the perfusion system for the times specified in *Results*. Alterations in IP₃ and [Ca²⁺]_i levels are represented as the change in fluorescence emission (F) divided by the initial basal fluorescence (F₀).

Measurement of Single-Cell Cytosolic Ca²⁺ Levels

After loading with the calcium-sensitive dye Fluo4-AM (3 μ M, 60 min), cells were excited at 488 nm, using an Olympus FV500 scanning laser confocal microscope as described previously (30). Cells were incubated at 37 °C and perfused as described in the previous section. Single-cell images were captured using an oil immersion $\times 60$ objective. Cytosolic Ca²⁺ levels were measured as the relative change in fluorescence detected in an area of interest as described previously (30, 34). Drugs were introduced via the perfusion system for the times specified in *Results*.

Assessment of H₁ Histamine Receptor Desensitization

Receptor desensitization was assessed using a protocol similar to that used previously to study M₁ and M₃ muscarinic acetylcholine receptors (30, 39). Desensitization of histamine-stimulated Ca²⁺ and IP₃ responses was assessed using the following protocol. ULTR or primary human myometrial cells, either loaded with Fluo4-AM or transfected to express eGFP-PH_{PLC δ} (described above), were exposed to 1) an approximate EC₅₀ concentration of histamine termed R1 (1 μ M histamine for Ca²⁺ and 10 μ M histamine for IP₃ experiments); 2) after 5-min washout, receptor desensitization was induced by applying a maximal histamine concentration (100 μ M) for 60 sec, termed R_{max}; 3) a further application of an approximate EC₅₀ concentration of histamine was added either, 5, 10, or 15 min after the R_{max} application. Agonist-induced changes in intracellular Ca²⁺ and IP₃ were measured using an Olympus FV500 scanning laser confocal microscope as described above.

Data Analysis

Concentration-response curves were generated and EC₅₀ values were determined using nonlinear regression analysis software (GraphPad Prism version 3.0; GraphPad Software Inc., San Diego, CA). Data were analyzed using one-way ANOVA, followed by Bonferroni's or Dunnett's *post hoc* test (Excel 5.0; Microsoft, Redmond, WA). Significance was accepted when $P < 0.05$.

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