The neuropeptide head activator induces activation and translocation of the growth-factor-regulated Ca^{2+}-permeable channel GRC

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SUMMARY

The neuropeptide head activator stimulates cell proliferation of neuronal precursor and neuroendocrine cells. The mitogenic signaling cascade requires Ca^{2+} influx for which, as we show in this paper, the growth-factor-regulated Ca^{2+}-permeable cation channel, GRC, is responsible. GRC is a member of the transient receptor potential channel family. In uninduced cells only low amounts of GRC are present on the plasma membrane but, upon stimulation with head activator, GRC translocates from an intracellular compartment to the cell surface. Head activator functions as an inducer of GRC translocation in neuronal and neuroendocrine cells, which express GRC endogenously, and also in COS-7 cells after transfection with GRC. Head activator is no direct ligand for GRC, but its action requires the presence of a receptor coupled to a pertussis-toxin inhibitable G-protein. Heterologously expressed GRC becomes activated by head activator, which results in opening of the channel and Ca^{2+} influx. SK&F 96365, an inhibitor specific for TRP-like channels, blocks Ca^{2+} entry and, consequently, translocation of GRC is prevented. Head activator-induced GRC activation and translocation are also inhibited by wortmannin and KN-93, blockers of the phosphatidylinositol 3-kinase and of the Ca^{2+}/calmodulin-dependent kinase, respectively, which implies a role for both kinases in head-activator signaling to GRC.

Key words: Head-activator signaling, Ion-channel trafficking, Receptor-mediated calcium entry, GRC, VRL-1, PI3-K, CaMK, TRP-like channel

INTRODUCTION

Cell proliferation of neuronal precursor and neuroendocrine cells is stimulated by the neuropeptide head activator (HA). HA acts as mitogen in the G_2/mitosis transition, an effect that is measurable 1-3 hours after HA application as an increase of cells in mitosis (Hampe et al., 1999; Hampe et al., 2000). Responsive cell lines include the double-hybrid rat-mouse neuroblastoma-glioma cell line NH15-CA2 (Ulrich et al., 1996), the human neuronal precursor cell line NT2 (Hampe et al., 2000), and the neuroendocrine cell line BON (Kayser et al., 1998), which is derived from a human pancreas carcinoid. HA signal transduction for mitotic stimulation is mediated by a pertussis-toxin-sensitive G-protein, and requires Ca^{2+} influx, downregulation of adenylyl cyclase and hyperpolarization of the membrane potential (Kayser et al., 1998; Ulrich et al., 1996). The latter is achieved by K^+ efflux, for which a Gardos-type Ca^{2+}-regulated K^+ channel is responsible (Kayser et al., 1998).

Growth factors stimulate cell proliferation by inducing Ca^{2+} entry into cells. Recently, a new Ca^{2+}-permeable channel was cloned from mouse spleen that controls proliferation of cells responsive to insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF) and serum. It was named growth-factor-regulated channel (GRC) by Kanzaki et al. (Kanzaki et al., 1999). Rat and human homologues were described by Caterina et al. (Caterina et al., 1999) and called vanilloid-receptor like (VRL-1) because of their similarity to the capsaicin receptor VR1. Both GRC and VRL-1 have almost identical biophysical and pharmacological properties and represent orthologues of the same channel. GRC belongs to the transient receptor potential (TRP) channel family members of which allow Ca^{2+} entry into cells at hyperpolarized membrane potentials (Clapham et al., 2001; Harteneck et al., 2000). GRC contains six transmembrane domains, a pore loop, a cytoplasmic N-terminus with three ankyrin repeats, and a cytoplasmic C-terminus. CHO cells transfected with GRC reacted to IGF-1, PDGF and serum by translocation of this channel from an intracellular pool to the plasma membrane and by an increase in intracellular Ca^{2+} concentration. The effect of IGF-1 and PDGF on GRC translocation was indirect and required activation of the phosphatidylinositol 3-kinase (PI3-K) (Kanzaki et al., 1999). Capsaicin did not affect GRC channel properties, but high noxious heat activated the channel (Caterina et al., 1999). So far it is unclear which endogenous ligands or second

messengers are responsible for GRC activation. Expression of VRL-1/GRC in sensory neurons of dorsal-root ganglia, but also in other organs, such as spleen and lung, hint at multiple functions (Caterina et al., 1999).

Here, we show that HA-responsive cells contain GRC, that the channel translocates to the cell surface after HA application, and that Ca^{2+} influx through this channel is triggered by HA. HA does not bind directly to GRC, but requires the presence of a signaling receptor and respective second messengers. We propose a model how the signaling cascade induced by HA may lead to the activation of GRC as a Ca^{2+}-permeable channel and how its trafficking from intracellular stores to the plasma membrane is regulated.

MATERIALS AND METHODS

Cell culture and transfection

NT2 cells (Pleasure and Lee, 1993) were cultured in Opti-MEM, BON cells (Evers et al., 1994) in Dulbecco's Nut-Mix F-12, both supplemented with 5% fetal calf serum (FCS), NH15-CA2 (Heumann et al., 1979) and COS-7 cells in DMEM, CHO-K1 cells in HAM's F-12, all three supplemented with 10% FCS. To all media, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM Hepes (pH 7.2) were added. For experiments with HA, cells were cultured overnight in serum-free defined medium containing 5 μg/ml insulin, 30 μg/ml transferrin, 20 μM ethanolamine, 30 nM sodium selenite, 1 μM sodium pyrurate, 1% non-essential amino acids, and 1 mM glutamine in the respective basal media.

COS-7 and CHO cells were transfected by electroporation with 10 μg pcDNA3 into which nothing (mock), mouse GRC, mouse GRC-FLAG or human SorLA cDNAs were cloned. For electrical recordings, cells were microinjected with 50 ng/μl GRC-pcDNA3 and 5 ng/μl EGFP-N1-pcDNA3, the latter to facilitate detection of cells with successful expression.

PCR analysis of GRC in NT2, BON and NH15-CA2 cells

Two protein motifs conserved in GRC and VR1 (Caterina et al., 1997) were chosen to design oligonucleotides for PCR amplification, namely the sequence LLQ(DE)KWD, located five amino acids in front of the first transmembrane domain, and FKFTIGMG at the end of the pore loop to yield 5′-TTITT(GT)CAGGA(GT)AAGTGGGAT-3′ as sense and 5′CCCATICC(GT)ATIGTGAA(CT)TT(AG)AA-3′ as antisense primers. As templates we used cDNA from BON and NH15-CA2 cells and a commercial cDNA library from uninduced NT2 cells (Stratagene). The PCR profile was 94°C 30 seconds, 45°C 30 seconds, 72°C 45 seconds for 30 cycles, followed by re-PCR under the same conditions.

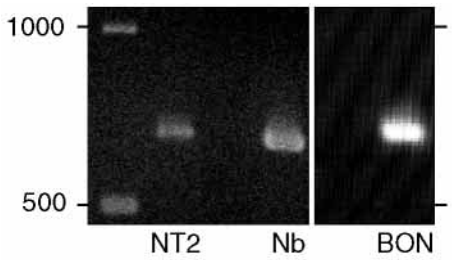

Fig. 1. PCR amplification of putative GRC- and VR1-channel cDNAs from the HA-responsive cell lines NT2, NH15-CA2 (Nb), and BON. No PCR products were visible after 30 cycles, but reamplification showed bands of the expected sizes (681 bp for mouse, 690 bp for human GRC).

Immunostaining and confocal analysis of GRC translocation

NH15-CA2 and BON cells and transfected CHO and COS-7 cells were plated on chamber slides in serum-containing medium for 24 hours. Analysis was performed after subsequent growth in defined medium for 24 hours. HA was monomerized prior to use (Bodenmüller et al., 1986). Signal transduction was inhibited by pretreating cells for 20 minutes with 200 ng/ml pertussis toxin (Sigma), 10 μM SK&F 96365 (Biomol), 10 μM KN93 (Calbiochem), 10 μM roscovitin (Calbiochem), or 100 nM wortmannin (Calbiochem), before HA was added. To demonstrate immunoreactivity on the plasma membrane, living cells were treated with the primary antisera diluted in defined medium for 20 minutes at 37°C. After washing with PBS, cells were fixed with 4% paraformaldehyde in 7% acetic acid and 7% glycerol for 30 minutes at 21°C. To show translocation, cells were first fixed for 5 minutes in ice-cold 1% acetic acid in ethanol, before the primary antisera were added (Kanzaki et al., 1999). The GRC antiserum (Kanzaki et al., 1999) was diluted 1:2000, the FLAG antibody (FLAG M2; Sigma) 1:440 and the SorLA antiserum against the fibronectin domain 1:2000 (Hampe et al., 2000). For confocal analysis cy2- or cy3-labeled secondary antisera were used, the transfection efficiency was controlled with alkaline phosphatase-conjugated secondary antibodies.

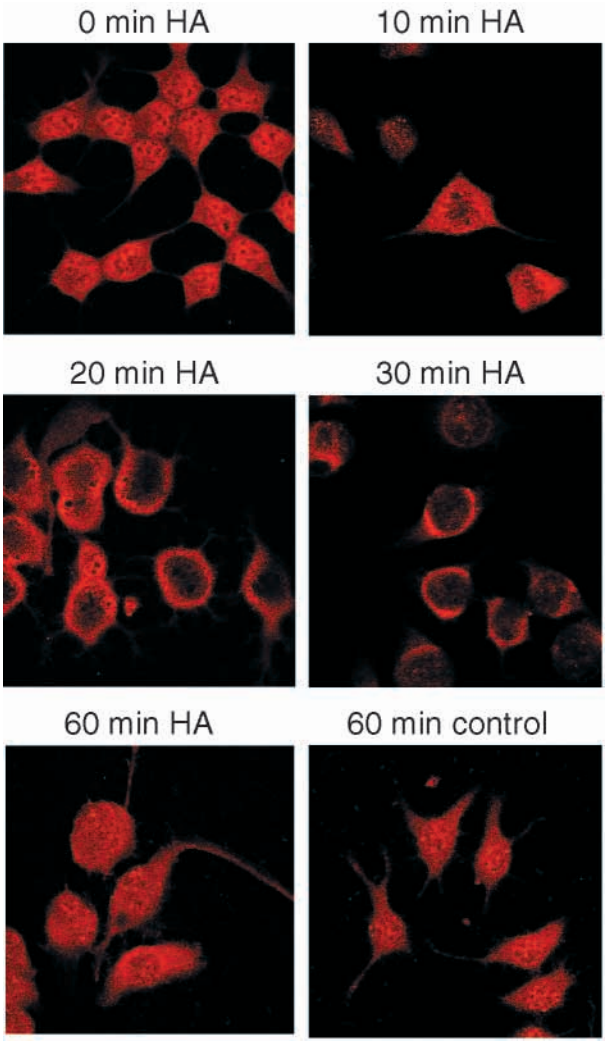

Fig. 2. HA-induced translocation of GRC in NH15-CA2 cells. NH15-CA2 cells were treated for the times indicated with 2 nM monomerized HA, permeabilized by fixation in ice-cold 1% acetic acid in ethanol, and immunostained with the antiserum against GRC.

Membrane preparation, solubilization and HA crosslinking

Cells were harvested by treatment with 2 mM EDTA in PBS for 10 minutes, collected by centrifugation, and ultrasonicated in a Tris-HCl (pH 7.4) buffer containing 2 mM EDTA and a protease-inhibitor cocktail (complete; Roche Molecular Biochemicals). After centrifugation at 100,000 ***g***, the membrane pellets were either used for western blotting or solubilized with 1% Nonidet P-40 in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl by incubation for 30 minutes at 4°C with occasional syringe pipetting. The 100,000 ***g*** supernatant was diluted tenfold in buffer without detergent, and FLAG-tagged GRC was immunoprecipitated with anti-FLAG agarose (Sigma) overnight at 4°C with overhead tumbling. Non-bound material was removed by washing three times with Tris-buffered saline. The various fractions were mixed with sample buffer without heating and subjected to 12% polyacrylamide gel electrophoresis. After semi-dry blotting immunoreactivity was visualized by enhanced chemiluminiscence (Pierce).

To crosslink HA to cell surface proteins, cells were incubated for 10-20 minutes at 37°C with the ^{125}I-labeled HA bipeptide (Hampe et al., 1996), washed twice with PBS, and treated with 0.1% 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide-HCl (EDC; Pierce) and 0.1% N-hydroxysulfo-succinimide (sulfo-NHS; Pierce) in PBS for 20 minutes at 37°C.

Electrophysiology

Membrane currents were recorded in the whole-cell configuration (Hamill et al., 1981) or the perforated-patch configuration with nystatin (Horn and Marty, 1988) of the patch-clamp technique. An EPC9 patch-clamp amplifier was used in conjunction with the PULSE stimulation and data acquisition software (HEKA Elektronik). The patch electrodes were made from 1.5 mm diameter borosilicate glass capillaries with resistances of 2.5-4 MΩ when filled with intracellular solution. Data were low-pass filtered at 3 kHz and compensated for both fast and slow capacity transients. Series resistance was compensated by 75-90%. Current traces are shown without correction for leakage, taking into account that GRC shows a substantial inward current. All experiments were performed at room temperature (22-25°C).

The pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM EGTA, 10 mM Hepes and had a calculated free Ca^{2+} concentration of 66 nM. The pH was adjusted to 7.3 with KOH. The standard external solutions contained 130 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 10 mM Hepes, and 10 mM glucose. The NaCl, CsCl and $CaCl_2$ solutions contained 150 mM NaCl or CsCl or 100 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose, buffered to pH 7.3 with NaOH, CsOH or $Ca(OH)_2$. Nystatin was dissolved in DMSO. Its final concentration in the standard pipette solution was 0.2 mg/ml. All chemicals for electrophysiology were purchased from Sigma.

RESULTS

HA-responsive cells contain GRC

To assay for the presence of GRC or related TRP-like channels in HA-responsive cells, two primers were designed based on conserved amino acid motifs in GRC and VR1, namely LLQ(DE)KWD in front of the first transmembrane domain and FKFTIGMG at the end of the pore region. A PCR was performed using cDNA from the HA-responsive cell lines NH15-CA2, BON and NT2 served as templates for PCR amplification. No product was obtained after 30 cycles, but re-PCR revealed bands of the expected sizes (Fig. 1). Subcloning and subsequent sequence analysis showed that NT2 and BON cells contained human GRC, and NH15-CA2 cells contained mouse GRC. Since NH15-CA2 cells are hybrids derived from rat glioma and mouse neuroblastoma, this may indicate that neurons contain GRC, whereas glial cells do not. No amplificates of VR1 or of new TRPs were detected.

HA treatment leads to translocation of GRC to the cell surface

Translocation of GRC from an intracellular pool to the plasma membrane was studied, as previously described (Kanzaki et al., 1999), with an antiserum directed against a peptide in the extracellular loop between the transmembrane domain 5 and the pore region of mouse GRC. We found that treatment of NH15-CA2 cells with 2 nM HA led to the translocation of GRC from an intracellular compartment to the plasma membrane. The effect started to be visible 10 minutes after HA application, peaked at 20-30 minutes, and disappeared after 60 minutes (Fig. 2). No change in the location of GRC was observed within 60 minutes in untreated control cells, when only the medium was renewed (Fig. 2). Incubation of living cells with the GRC antibody showed that very few GRC

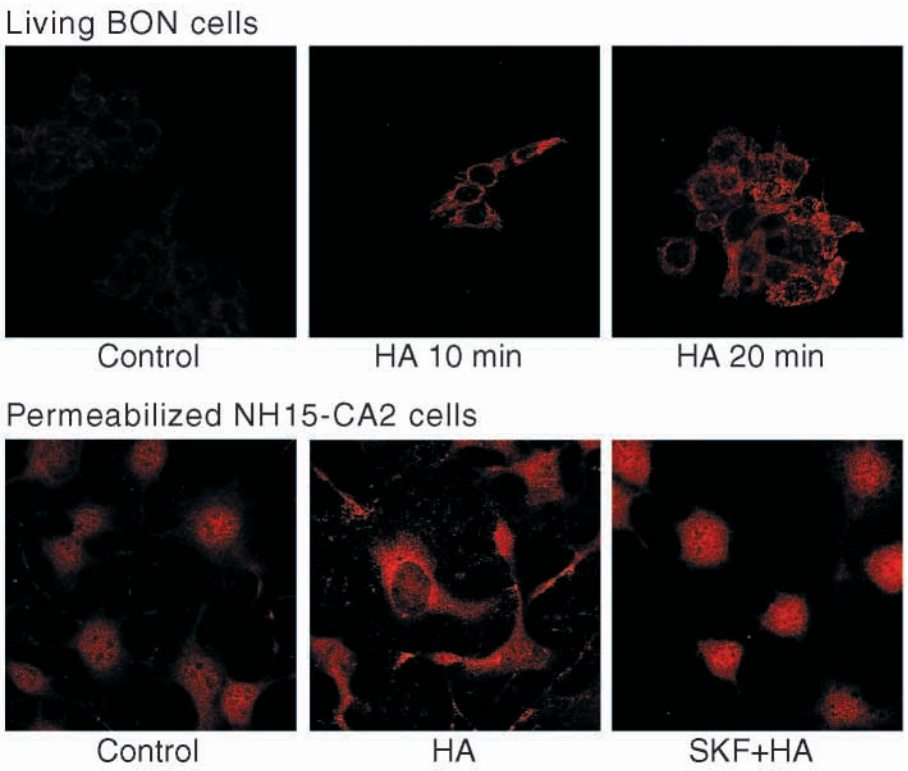

Fig. 3. Stimulation of GRC cell-surface expression by HA and inhibition of translocation by the Ca^{2+}-channel blocker SK&F 96365. BON cells were incubated for 0, 10 and 20 minutes with 2 nM HA, then for 20 minutes at 37°C with the antiserum against GRC (top panel). GRC translocation was blocked if NH15-CA2 cells were pretreated for 20 minutes with SK&F 96365 (10 μM), before HA (2 nM) was added (bottom panel).

channels were present at the cell surface without HA stimulation, but that GRC immunoreactivity increased strongly after HA application. This is shown for BON cells which, like NH15-CA2 cells, also responded to HA stimulation by GRC translocation (Fig. 3, top panel). We had shown earlier that SK&F 96365, which is a specific blocker of receptor-mediated, store-independent Ca^{2+} entry specific for TRP-like channels, inhibits HA-stimulated entry into mitosis (Kayser et al., 1998). If NH15-CA2 cells were pretreated for 20 minutes with SK&F 96365, no translocation was observed (Fig. 3, bottom panel).

COS-7 cells but not CHO cells transfected with GRC respond to HA with translocation

GRC with or without FLAG-tag was heterologously expressed in COS-7 and CHO cells. Cells were harvested 48 hours after transfection and membrane and soluble fractions analyzed by western blotting. Both COS-7 and CHO cells expressed GRC of the expected size of about 90 kDa. A minor band at 75 kDa probably represents a glyco-isoform. The FLAG-tagged GRC was slightly retarded in its migration pattern. Mock-transfected cells contained no or little GRC. All immunoreactivity was recovered in the membrane fraction (Fig. 4).

Immunocytochemistry revealed GRC expression in 40-60% of COS-7 and 20-40% of CHO cells after transfection of GRC by electroporation (data not shown). Translocation of heterologously expressed GRC and FLAG-GRC after HA treatment was studied by confocal microscopy. Like endogenous GRC in HA-responsive cells, a 30 minute treatment with HA resulted in the translocation of GRC and FLAG-GRC from a perinuclear compartment to a surface location in COS-7, but not in CHO cells (Fig. 5). Mock-transfected COS-7 cells were not stained.

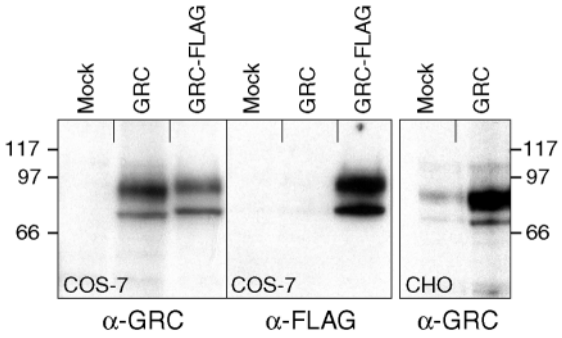

Fig. 4. Expression of GRC and GRC-FLAG in COS-7 and of GRC in CHO cells. COS-7 and CHO cells were transiently transfected by electroporation with the indicated pcDNA3 constructs. GRC in the membrane fraction was made visible by immunostaining with antibodies against GRC or FLAG.

Characterization of GRC as a HA-regulated Ca^{2+}-permeable channel in COS-7, but not in CHO cells

To monitor HA-induced current activation COS-7 cells were injected with cDNA coding for GRC. Increased membrane currents were observed 20 minutes after incubation with HA. COS-7 cells mock-injected with the pcDNA3 vector showed only a small linear background current after HA treatment (Fig. 6A). The I/V curves demonstrate the outward rectifying properties of the current activated by HA (Fig. 6B). To determine the ion selectivity of GRC, membrane currents were recorded with ramp pulses in COS-7 cells expressing GRC, which had been treated with HA for 20 minutes. In external solutions containing CsCl (Fig. 6C) or NaCl (Fig. 6B) the

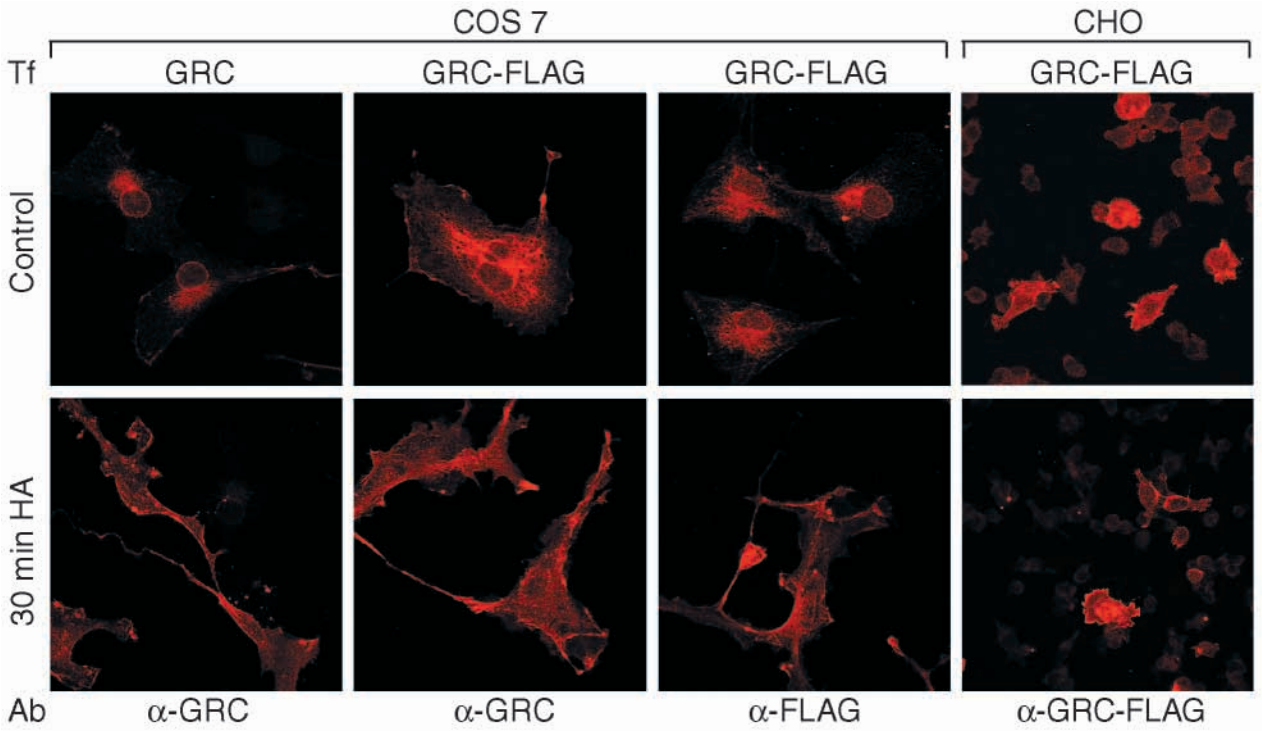

Fig. 5. HA-induced translocation of GRC in COS-7 and CHO cells transfected (Tf) with GRC or GRC-FLAG. Two days after transfection, cells were treated for 30 minutes with 2 nM HA. After fixation they were immunostained as indicated with the antibodies (Ab) against GRC or FLAG.

reversal potential was –2.2±1.2 mV (mean±s.d.; *n*=4) and –6.3±2.1 mV (*n*=8), respectively, indicating that GRC does not discriminate between Cs^+ or Na^+. By contrast, in an external $CaCl_2$ solution there was a shift of the reversal potential to more positive membrane potentials (Fig. 6D). The mean shift was 13.3±5.8 mV (*n*=3) demonstrating that GRC is permeable to Ca^{2+}. Fig. 6E shows that after acute application of HA an inward current was activated in a cell expressing GRC, which slowly increased to steady-state within about 5 minutes (*n*=4). The GRC current could be blocked either with La^{3+} (1 mM; *n*=3) or ruthenium red (10 μM; Fig. 6E; *n*=3). The properties of the GRC-mediated current agree with those reported recently, where CHO cells transfected with GRC had been used to monitor Ca^{2+} influx and GRC channel characteristics after IGF-1 stimulation (Kanzaki et al., 1999). The sensitivity of the HA-induced current to ruthenium red is a characteristic property of GRC that links the channel with native vanilloid receptors of the TRP-like family. CHO cells expressing GRC showed no current activation after HA application (Fig. 6F; *n*=12), although the cells responded to 10% FCS (Fig. 6G; *n*=8). As in COS-7 cells, this current could be blocked by ruthenium red.

HA signal transduction to GRC is indirect

COS-7 cells were incubated with ^{125}I-HA bipeptide. Binding or internalization was stronger in cells transfected with GRC than with vector alone, and grains were more concentrated

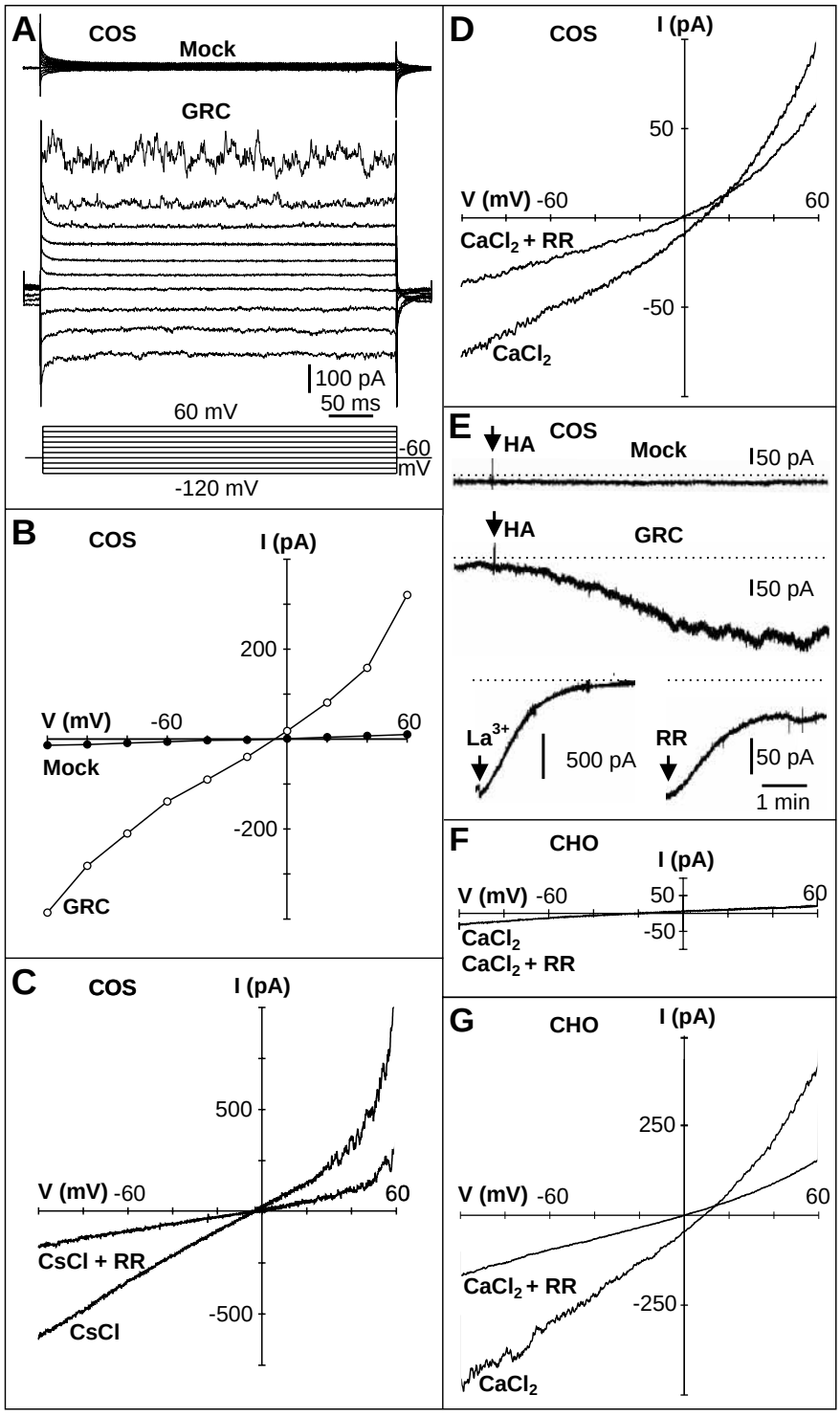

Fig. 6. HA-induced increase of membrane currents in COS-7 (A-E) and in CHO cells (F,G) transfected with GRC-pcDNA3. (A) 400 ms potential steps were applied from –120 mV to 60 mV in increments of 20 mV from a holding potential of –60 mV. Membrane currents recorded in external NaCl solution are shown of a mock-injected COS-7 cell not expressing GRC and of a cell expressing GRC, both after a treatment for 20 minutes with HA. (B) The amplitudes of the membrane currents as determined in A at the end of the pulses were plotted versus the pulse potential. Data points were connected by straight lines (mock, ●; GRC, ○). (C) Activation of GRC by HA was reduced after application of 10 μM ruthenium red (RR) in an external CsCl solution. Membrane currents were recorded with a 250 ms voltage ramp command from –100 mV to 60 mV from a holding potential of –60 mV. (D) HA-activated GRC was Ca^{2+} permeable, if the measurement was performed in an external $CaCl_2$ solution, and the currents were blockable by RR. (E) The membrane current of a cell expressing GRC, but not of a mock-injected COS-7 cell, increased after HA application as measured in the perforated-patch configuration using standard external NaCl solution. Membrane currents activated by HA in cells expressing GRC were blocked by application of 1 mM La^{3+} or 10 μM RR. Dashed lines denote zero current, arrows indicate the time of HA or blocker application (F,G). In CHO cells heterologously expressed GRC was not activated by HA (F), but by serum (G). Membrane currents were elicited as described in (C). In an external $CaCl_2$ solution a CHO cell pretreated with HA for 20 minutes did not exhibit increased membrane currents (F). Pretreatment with 10% fetal calf serum activated GRC-mediated membrane currents, which could be reduced by 10 μM RR (G).

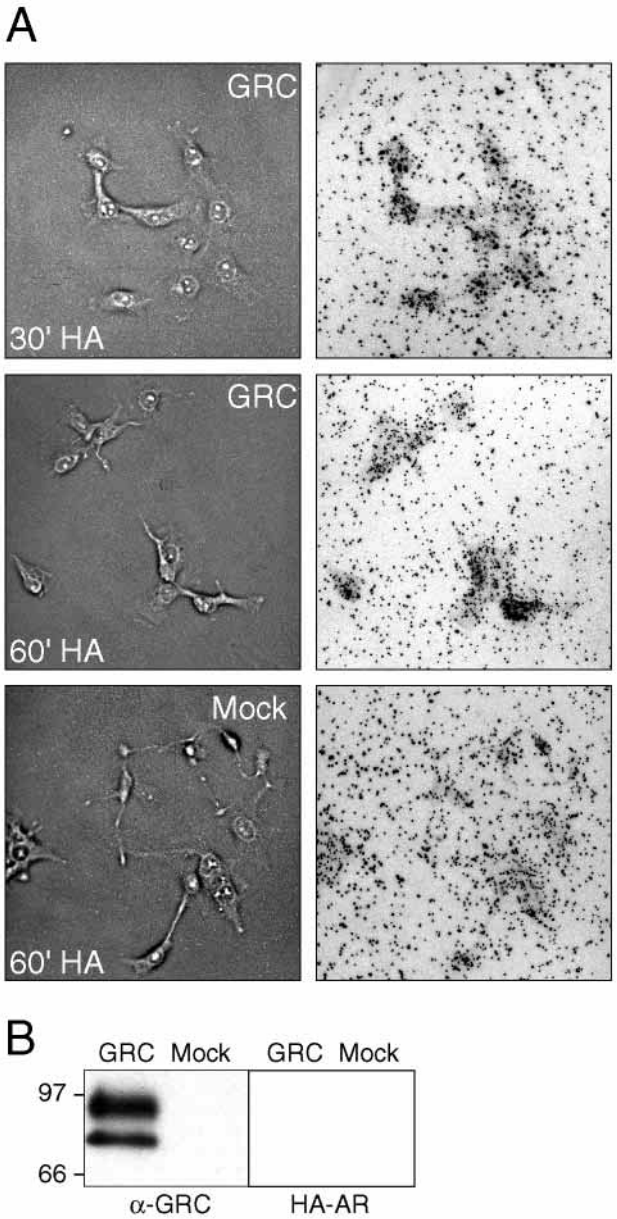

Fig. 7. Binding of HA to GRC-transfected COS-7 cells, but not to GRC itself. (A) Mock- and GRC-transfected COS-7 cells were incubated with ^{125}I-HA bipeptide (1.3×10^6 cpm/ml) for 30 minutes at 37°C. After fixation with 4% formaldehyde in 7% acetic acid and 7% glycerol, slides were covered with dipping film and developed for autoradiography 8 days later. Shown on the left are phase-contrast and on the right bright field micrographs. (B) ^{125}I-HA bipeptide was crosslinked to GRC-FLAG or mock-transfected COS-7 cells by EDC and sulfo-NHS. Membrane fractions were solubilized, and subjected to pull-down with anti-FLAG agarose. GRC was recovered quantitatively in the immunoprecipitate, but contained no radioactive HA. (HA-AR, HA autoradiography).

within cells at 60 minutes compared with 30 minutes (Fig. 7A). To assay whether HA binds GRC directly, FLAG-GRC and mock-transfected COS-7 cells were treated with radiolabeled HA for 30 minutes, followed by crosslinking with 0.1% EDC and 0.1% sulfo-NHS. While FLAG-GRC was recovered quantitatively in an anti-FLAG-agarose pull-down assay, HA

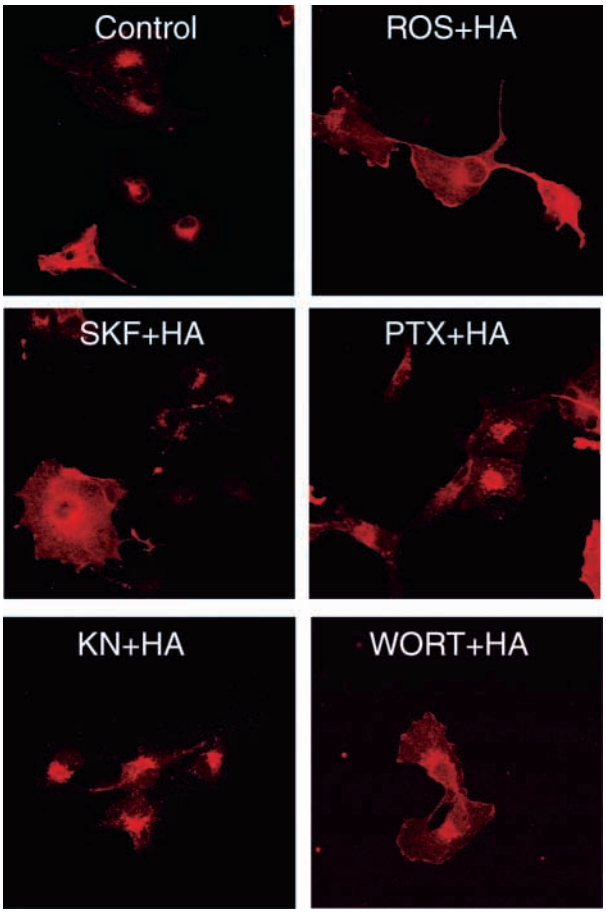

Fig. 8. Second messengers involved in HA signaling to GRC. GRC-transfected COS-7 cells were treated with various inhibitors for 20 minutes at 37°C, before HA was added. Blockage of Ca^{2+} channels by SK&F 93625 (SKF), of inhibitory G proteins by pertussis toxin (PTX), of Ca^{2+}/calmodulin-dependent kinases by KN-93 (KN), and of PI(3)-kinase by wortmannin (WORT) inhibited the HA-induced translocation of GRC, whereas roscovitine (ROS), which blocks cyclin-dependent kinases, had no effect.

remained in the non-agarose bound solubilizate. Only 0.3% of the total radioactivity was co-precipitated with GRC and could not be visualized by autoradiography as crosslinked to GRC (Fig. 7B). Similar pull-down experiments were performed to monitor direct interaction of GRC with heterotrimeric G proteins. Neither G_α nor $G_{\beta/\gamma}$ subunits were detectable in the FLAG-GRC immunoprecipitate.

To find out which second messengers are involved in transducing the HA signal to GRC, GRC-transfected COS-7 cells were pretreated with various inhibitors before HA was added. Fig. 8 shows that GRC translocation was inhibited by pertussis toxin, SK&F 96365, KN-93 and wortmannin, which are blockers of inhibitory G proteins, Ca^{2+} channels, Ca^{2+}/calmodulin-dependent kinases, and PI3-kinase, respectively, but not by roscovitine, which blocks cyclin-dependent kinases. Likewise, the effect of HA on GRC currents was abolished or reduced in the presence of the respective inhibitors (Fig. 9). From this we conclude that a G-protein-coupled receptor is present in COS-7 cells and is required as the first step in HA signaling.

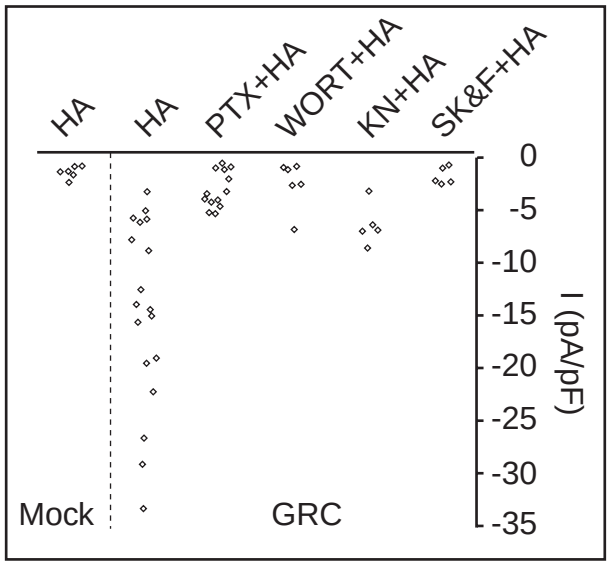

Fig. 9. Inhibition of the HA signaling cascade leading to increased membrane currents. Membrane current densities of mock- and GRC-injected COS-7 cells were recorded at a membrane potential of –80 mV in standard external NaCl solution. The cells were pretreated with the inhibitors as described in Fig. 8, except for PTX, which was preincubated for 30 minutes to 8 hours, and for SK&F 93625, which was added after induction of GRC currents by HA. Each symbol represents one measured cell.

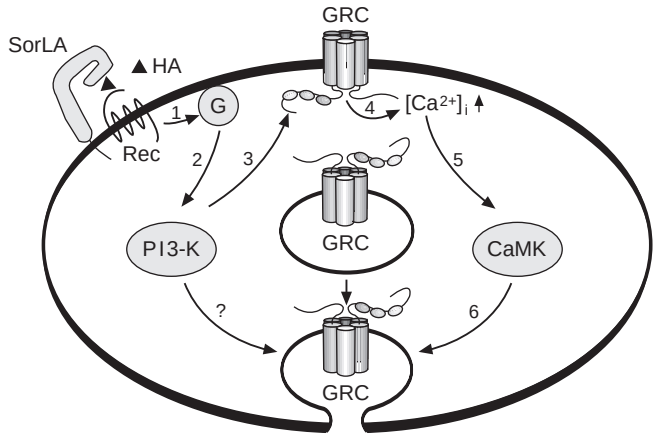

Fig. 10. Model for HA signaling to GRC. After binding HA the HA receptor SorLA together with a postulated second receptor component (Rec) activates a pertussis-toxin sensitive heterotrimeric G protein (1), which interacts via PI3-K (2) with surface GRC (3) to induce Ca^{2+} influx (4). The increase in Ca^{2+} leads to activation of CaMK (5), which possibly together with PI3-K (?), triggers shuttling of more GRC molecules to the plasma membrane (6).

SorLA/LR11, a member of the VPS10- and LDL-receptor family, has recently been described as a possible HA receptor. SorLA binds HA and exists as a membrane and soluble receptor (Hampe et al., 2000). SorLA contains a single transmembrane domain and its intracellular carboxy tail displays motifs necessary for binding to the inhibitory α subunit of G proteins. To assay whether SorLA is the HA receptor which triggers GRC translocation, we cotransfected CHO cells with GRC and SorLA. As in CHO cells transfected with GRC alone, HA was unable to induce GRC translocation or GRC currents (data not shown). This indicates that CHO cells miss an important component of the HA signaling cascade.

DISCUSSION

The neuropeptide HA requires presence of a G-protein-coupled receptor and Ca^{2+} influx to stimulate mitosis and cell proliferation in neuronal precursor and neuroendocrine cells (Hampe et al., 1999). Recently, GRC was described as a Ca^{2+}-permeable channel which, when heterologously expressed in CHO cells, can be activated by IGF-1, PDGF and serum leading to Ca^{2+} influx (Kanzaki et al., 1999). Since serum contains nanomolar concentrations of HA (Roberge et al., 1984), and since GRC is expressed in neuronal cells (Caterina et al., 1999), it was a good candidate for HA signaling. One intriguing new property of GRC was that its activation was augmented by translocation from an intracellular pool to the plasma membrane, and that this trafficking may amplify the response (Kanzaki et al., 1999).

We found that HA-responsive cells contain GRC and react to HA stimulation with a translocation of GRC to the cell surface. GRC could be visualized on the surface of living cells without HA stimulation, but its density increased considerably after HA application. Maximal translocation was observed in NH15-CA2 cells 30 minutes after HA treatment and returned to control levels after 60 minutes. A similar time course was observed for HA induced inward currents, which showed biophysical and pharmacological properties agreeing with those reported previously for GRC/VRL-1 by other workers (Caterina et al., 1997; Kanzaki et al., 1999). We interpret this to mean that some GRCs are always present at the outer cell membrane, that they may be responsible for the immediate stimulation of receptor-mediated Ca^{2+} entry, but that the sustained Ca^{2+} influx requires recruitment or shuttling of GRC from intracellular stores to the cell surface.

The closest relative of GRC in the TRP family is the vanilloid receptor VR-1, for which capsaicin and amandamide or other endogeneous lipids serve as ligands (Caterina et al., 1997; Hwang et al., 2000; Jung et al., 1999). These ligands are supposed to activate channel opening by directly binding to VR-1. One possible scenario for HA signaling to GRC could therefore be direct binding. To test this hypothesis we transfected COS-7 and CHO cells with GRC and measured translocation and Ca^{2+} influx. Whereas HA was able to trigger a response in COS-7 cells, no effect was obtained in CHO cells, although both reacted with serum. In line with this we found that radioactively labeled HA could bind to COS-7, but not to CHO cells, and that cell proliferation could be stimulated with HA in non-transfected COS-7, but not in CHO cells (data not shown). Binding of HA and internalization was speeded up in the presence of GRC, indicating that the presence of GRC enhances HA's action. Crosslinking of HA to COS-7 cells and subsequent pull-down of FLAG-tagged GRC with FLAG agarose confirmed the notion that HA does not bind directly to GRC.

HA-stimulated translocation of GRC to the cell surface and HA-induced currents were inhibited by SK&F 96365, a

specific blocker of receptor-mediated, store-independent Ca^{2+} entry specific for TRP-like channels (Bennett et al., 2001). SK&F 96365 prevents HA triggered mitosis and cell proliferation (Kayser et al., 1998; Ulrich et al., 1996), and it inhibits the effects of other growth factors on stimulating proliferation of astrocytoma, neuroblastoma and blood cells (Chung et al., 1994; Lee et al., 1993). SK&F 96365 leads to an arrest of cells at the G_2/mitosis transition (Nordström et al., 1992). This suggests that the GRC is involved in the control of cell proliferation, mediating HA's action at this cell-cycle checkpoint.

In the presence of pertussis toxin, the effect of HA on stimulating mitosis and cell proliferation is inhibited (Ulrich et al., 1996). Likewise, pretreatment of GRC-transfected cells with pertussis toxin inhibited HA-induced translocation of GRC to the cell surface and HA-induced currents, indicating that HA signaling for mitosis and GRC translocation and channel activation are mediated by a receptor that is coupled to an inhibitory G protein. No co-immunprecipitation of GRC with G-protein subunits was found, implying indirect activation over other second messengers. SorLA, a single transmembrane receptor that binds HA, contains motifs typical for coupling to inhibitory G proteins (Franke et al., 1997). Cotransfection of CHO cells with GRC and SorLA did not result in conferring responsiveness to HA, indicating that an additional component is missing in CHO cells. Therefore, we postulate that HA together with SorLA binds most likely to a member of the G protein-coupled receptor family, which transmits the signal over an inhibitory G protein to GRC. HA-induced translocation of GRC was blockable by inhibitors of two kinases, namely by KN-93, an inhibitor of the Ca^{2+}/calmodulin-dependent kinases and by wortmannin, a blocker of the PI3-kinase. No effect was found with roscovitin, which blocks the cyclin-dependent kinase CDK1. A model of how these different components may interact is depicted in Fig. 10. After binding its ligand the HA-receptor complex activates a pertussis-toxin-sensitive G protein, which probably interacts via PI3-K with GRC at the cell surface to induce Ca^{2+} entry. The increase in Ca^{2+} concentration activates CaMK, which alone or together with PI3-K triggers translocation of more GRC channels to the cell surface thus leading to a prolonged Ca^{2+} influx.

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