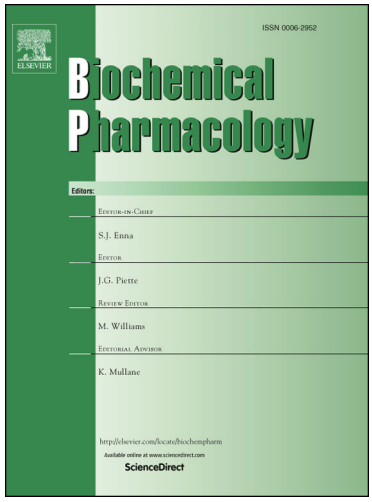

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Development of a novel, sensitive cell-based Corin Assay

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Abbreviations: (pro-)ANP, (pro-)atrial natriuretic peptide; NT-pro-ANP, N-terminal pro-atrial natriuretic peptide; CNG, cyclic nucleotide-gated; CRC, concentration response curve; RFU, relative fluorescence unit; RLU, relative light unit; HTS, high-throughput screening

Abstract

Corin (atrial natriuretic peptide-converting enzyme, EC 3.4.21) is a transmembrane serine protease expressed in cardiomyocytes. Corin exerts its cardioprotective effects via the proteolytic cleavage and activation of pro-atrial natriuretic peptide (pro-ANP) to ANP. We recently described an ANP reporter cell line stably expressing the ANP receptor, a cGMP-dependent cation channel used as a real-time cGMP biosensor, and the Ca^{2+}-sensitive photoprotein aequorin. Here, we describe the generation of a novel reporter cell line expressing the calcium biosensor GCaMP6 instead of aequorin. In contrast to the luminescence-based assay, ANP stimulation of our novel GCaMP6 reporter cell resulted in stable, long-lasting fluorescence signals. Using this novel reporter system, we were able to detect pro-ANP to ANP conversion by purified, soluble wildtype corin (solCorin), but not the active site mutant solCorin(S985A), resulting in left-shifted concentration-response curves. Furthermore, cellular pro-ANPase activity could be detected on HEK 293 cells after transient expression of wildtype corin. In contrast, corin activity was not detected after transfection with the inactive corin(S985A) variant. In supernatants from cardiomyocyte-derived HL-1 cells pro-ANP to ANP conversion could also be detected, while in HL-1 corin knockout cells no conversion was observed. These findings underline the role of corin as the pro-ANP convertase. Our novel fluorescence-based ANP reporter cell line is well-suited for the sensitive detection of corin activity, and may be used for the identification and characterization of novel corin modulators.

Keywords: corin; serine protease; atrial natriuretic peptide; ANP receptor; cGMP; reporter assay

1. Introduction

Corin (atrial natriuretic peptide-converting enzyme, EC 3.4.21) is a cardiac transmembrane serine protease involved in the regulation of blood pressure and cardiac function. In response to high blood pressure, atrial myocytes release immature pro-atrial natriuretic peptide (pro-ANP) from intracellular vesicles into the circulation. During this process, pro-ANP is proteolytically processed by the serine protease corin which gives rise to biologically active ANP [1-4]. ANP binds to and activates the transmembrane guanylate cyclase receptor GC-A, which is highly expressed in vasculature, kidney, central nervous system and adrenal glomerulosa cells. The activated ANP receptor converts intracellular GTP to cGMP. Subsequently, cGMP interacts with its effector proteins including protein kinase G, cGMP-dependent phosphodiesterases and cGMP-dependent ion channels [5-7].

ANP signaling leads to increased sodium and water secretion, vasodilatation, endothelial extravasation, inhibition of the renin-angiotensin system and inhibition of the sympathetic nervous system. In combination, these mechanisms result in blood pressure lowering and have anti-hypertrophic and anti-fibrotic effects on the heart [8-11]. In contrast, impaired corin activity caused by single nucleotide polymorphisms (SNPs) and mutations in the corin gene leading to reduced corin activity correlate with hypertension and cardiac hypertrophy in humans [12-16]. In addition, in acute myocardial infarction patients decreased corin levels correlate with a higher incidence of adverse cardiac events [17, 18].

ANP, BNP and other natriuretic peptide variants have already been exploited as therapeutics for heart failure, however, with limited success. The need for intravenous administration and short half-lifes of the peptides are disadvantages of

these therapeutics [7, 19, 20]. An alternative approach might be the pharmacological enhancement of cardiac corin activity using small molecules. In theory, increased cardiac corin activity could enhance local ANP generation and signaling, which would result in cardioprotection. For example, transgenic overexpression of corin in mice with dilated cardiomyopathy reduces myocardial fibrosis and improves cardiac function [21].

Until now, there is no sensitive assay that enables the identification of novel corin modulators in a high-throughput format. Previous corin activity assays relied on ANP and N-terminal pro-ANP (NT-pro-ANP) extraction, radioimmunoassay (RIA) and gel filtration [22], detection of the NT-pro-ANP peptide using commercially available ELISA assays [23-25], or immunoprecipitation followed by Western Blot analysis [26-28] to detect corin-mediated pro-ANP cleavage.

Previously developed biochemical assays might be suitable for the search for corin activity modulators. However, in these assay very high concentrations of non-specific, fluorescently labelled, short peptide protease substrates were used, which are not related to the natural pro-ANP corin cleavage site [29, 30].

In this publication, we present a novel cell-based corin assay which detects the cleavage and activation of pro-ANP by soluble, purified corin (solCorin) or corin expressing cells. As a readout, we use a reporter cell line that stably expresses the ANP receptor GC-A, the cyclic nucleotide-gated ion channel CNGA2 and the calcium biosensor protein GCaMP6. Binding of ANP to the ANP receptor leads to increased intracellular cGMP synthesis which is detected by opening of the CNG channel used as the cGMP biosensor. The influx of calcium ions into the cells results in long-lasting fluorescence signals mediated by GCaMP6.

2. Materials and Methods

2.1 Generation of a stable ANP reporter GCaMP6 cell line

A luminescence-based ANP receptor cell line was generated as described previously [31]. Accordingly, a stable GCaMP6 expressing CHO cell line was co-transfected with plasmid constructs encoding the bovine CNGA2 channel (**GB ID: X55010**) and rat GC-A (**GB ID: NM_012613**). Stably transfected clones were obtained by geneticin selection. Positive clones were identified by ANP stimulation (Bachem, Weil am Rhein, Germany) and were purified once by the limited dilution technique (data not shown). One clonal cell line was selected for further experiments.

The newly generated ANP reporter cell line was cultured at 37°C and 5% CO_2 in DMEM F12 (PAN Biotech, Aidenbach, Germany), supplemented with 10% (v/v) inactivated fetal calf serum (Merck KGaA, Darmstadt, Germany), 2x Glutamax (4 mM), 1.4 mM sodium pyruvate, 18 mM sodium bicarbonate, 10 mM HEPES, 50 units/ml penicillin, 50 µg/ml streptomycin, and 1 mg/ml geneticin (all Thermo Fisher Scientific, Waltham, MA, USA).

2.2 Luminescence and fluorescence measurements

Luminescence measurements were performed as previously described [31]. Briefly, luminescence-based ANP reporter cells were loaded with 5 µg/ml coelenterazine (p.j.k. GmbH, Kleinblittersdorf, Germany) in Tyrode (130 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgCl_2$, 4.8 mM $NaHCO_3$ at pH 7.4) at 37°C and 5% CO_2 in the absence (**incubation mode**) or presence (**kinetic mode**) of 2 mM calcium ions.

Measurements in incubation mode were started 6 min after agonist application immediately before adding calcium ions. Measurements in kinetic mode were directly started upon agonist addition. Aequorin luminescence was measured by using a charge-coupled device (CCD) camera (Hamamatsu Corporation, Shizuoka, Japan) in a light tight box.

Fluorescence measurements were performed on black, clear-bottom 384-well microtiter plates (Greiner Bio One, Frickenhausen, Germany). Either 2500 cells/well or 1500 cells/well of the newly generated GCaMP6 reporter cell line were plated and were cultured for 24 h or 48 h, respectively. After removal of the cell culture medium, reporter cells were incubated for 20 min with Tyrode containing a black masking dye in the absence (**incubation mode**) or presence (**kinetic mode**) of 2 mM calcium ions. Measurements in incubation mode were started 6 min after agonist application immediately before adding calcium ions. Measurements in kinetic mode were directly started upon agonist addition. Measurements were done on a FLIPR Tetra® (Molecular Devices, Sunnyvale, CA, USA) or a proprietary fluorescence plate reader equipped with an integrated dispenser unit. IBMX (0.2 mM; Merck KGaA, Darmstadt, Germany) was used in all experiments to prevent cGMP degradation by endogenous phosphodiesterases.

2.3 Cloning of solCorin, pro-ANP-Flag-His_6 and ANP-Flag-His_6

The form of corin used in these studies is referred to as the soluble enterokinase-activatable form of corin (solCorin). SolCorin is comprised of residues 124-1042 and has an engineered internal enterokinase site that is used to activate the protein [29]. The genes for solCorin and the active site mutant solCorin(S985A) were synthesized

(GeneArt, Thermo Fisher Scientific, Waltham, MA, USA) and cloned into a modified pcDNA3.4 mammalian expression vector. The vector was designed to append an N-terminal fusion tag to the protein comprised of the IgK secretion signal, a polyhistidine sequence, a twin Strep-tag, followed by a TEV protease cleavage site. The gene for pro-ANP (residues 26-151) was synthesized (GeneArt) with a C-terminal tag comprised of a flag sequence and a polyhistidine sequence, and cloned into the pET21 bacterial expression vector. To produce just the ANP-Flag-His_6, the gene for this construct was synthesized (GeneArt) with an N-terminal SUMO sequence followed by a TEV protease cleavage site in order to remove the SUMO protein during purification.

2.4 Expression, purification and activation of solCorin, pro-ANP-FLAG-His and ANP-FLAG-His

The mammalian expression vectors were transfected into HEK293 cells (Invitrogen, Carlsbad, CA, USA) using polyethyleneimine, and the cells left to grow for 6 days. The cells were removed from the conditioned media by centrifugation and the filtered media passed over a 5 mL Ni-charged HisTrap column (GE Healthcare, Chicago, IL, USA). Bound protein was eluted by PBS+150 mM NaCl containing 1 M imidazole. Combined fractions containing the eluted protein were diluted 2-fold with PBS+150 mM NaCl and loaded on to a 5 mL StrepTrap column (GE Healthcare). Bound protein was eluted with PBS+150 mM NaCl containing 2.5 M desthiobiotin. To activate solCorin, enterokinase (NEB Inc., Ipswich, MA, USA) was added in a 1:100 (v:v) ratio of enterokinase to solCorin and the solution left to dialyze overnight against 20 mM Tris/Cl pH 8, 50 mM NaCl, and 1 mM $CaCl_2$ at 4˚C. The dialyzed sample was passed

over a Superdex 200 column (GE Healthcare) equilibrated with PBS+150 mM NaCl. To identify the most enzymatically active fractions that came off the sizing column, 100 nM of solCorin from each fraction was incubated with 10-20 µM pro-ANP-Flag-His_6 for 1 hour at RT before stopping the reaction with SDS-PAGE gel loading buffer. Analysis of which fractions gave the most cleaved pro-ANP correlated with the fractions with the most enzymatically active protein. These fractions were combined, pooled, and stored at -80˚C.

Recombinant pro-ANP(26-151)-FLAG-His_6 and SUMO-ANP-Flag-His_6 were transformed into BL21(DE3) cells (Invitrogen). Cells were grown at 37˚C until induction point, induced with IPTG, and then left to grow overnight at 18˚C. The cells were extracted by centrifugation and the pellets stored at -80˚C until use. The cells were lysed using a microfluidizer (Avestin), and the solution clarified by centrifugation. Pro-ANP(26-151)-Flag-His_6 protein was purified on a HisTrap column in a similar manner as described for solCorin. The protein was then passed over a Superdex 75 column equilibrated in PBS+150 mM NaCl. The fractions for pro-ANP(26-151)-FLAG-His_6 were combined and dialyzed into 50 mM Tris/Cl pH 8, 50 mM NaCl and then further purified over a HiTrapQ (GE Healthcare) ion exchange column. The protein was dialyzed into PBS, concentrated, and then stored at -80˚C until use. SUMO-ANP-Flag-His_6 was initially purified in a similar manner using a 5 mL HisTrap column. The eluted protein was dialyzed to remove the imidazole, and then incubated at 4˚C overnight with TEV protease in a 1:100 w:w ratio. The protein was passed back over the 5 mL HisTrap column, where the SUMO was located in the flow through and the ANP-Flag-His_6 was eluted from the column with imidazole. The eluted fractions were combined, dialyzed into PBS, and then store at -80˚C.

2.5 Detection of solCorin activity using the ANP reporter cell line

Different enzyme concentrations of purified solCorin or inactive variant solCorin(S985A) were incubated with 3 µM pro-ANP-FLAG-His in 50 µL assay buffer (150 mM NaCl, 25 mM Hepes, 0.1% BSA, 1 mM $CaCl_2$, pH 7.4) at 37°C for 0 min, 10 min or 90 min. Samples were rapidly frozen and stored at -20°C for later use. Serial dilutions of the samples were prepared in Tyrode containing 2 mM calcium ions and 0.03% BSA.

2.6 Detection of cellular corin activity on transiently transfected HEK 293 cells

HEK 293 cells were cultured for 24 hours at 37°C and 5% CO_2 in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FCS (Merck KGaA, Darmstadt, Germany), 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and 1x non-essential amino acid solution (all Thermo Fisher Scientific, Waltham, MA, USA). Transient transfection of HEK 293 cells was performed on 6-well microtiter plates (200,000 cells/well) using the GenJet™ *in vitro* transfection reagent according to the manufacturer's protocol (SignaGen Laboratories, Rockville, MD, USA). Incubation experiments were performed 1 day after transfection. Cells were washed twice with Opti-MEM™ (Thermo Fisher Scientific) and 600 nM pro-ANP-FLAG-His was added to the cells in a volume of 1.5 ml in Opti-MEM™ with 0.1% BSA. Supernatant samples were taken at different time points and were immediately frozen at -20°C for later use. Serial dilutions of the samples were prepared in Tyrode containing 2 mM calcium ions and 0.03% BSA.

2.7 Generation of HL-1 corin knockout cells by CRISPR/Cas9

Mouse HL-1 cardiomyocytes, derived from a transgenic line using the ANP promoter to drive expression of the SV40 large T antigen [32], were chosen for their atrial features, ability to be passaged indefinitely and corin expression [33]. CRISPR guide sequences to mouse corin exon 2 designed to induce frameshift mutations between residues Ser96 and Lys104 (CTTGAGAGCAGACAGGAGCG, TTGAGAGCAGACAGGAGCGT, GCAGACAGGAGCGTGGGCGA, TTAGCAGTCACCAGCTTCTG) were individually cloned into the PX459 puromycin selective vector pSpCas9(BB)-2A-Puro V2. Corin PX459 guides were transfected using Lipofectamine LTX Plus (Thermo Fisher Scientific, Waltham, MA, USA). Following puromycin selection, 8 clones per guide were picked and expanded. HL-1 clones were genotyped using gDNA exon 2 PCR products to identify frameshift mutations using the NEB PCR cloning kit (n=24 colonies each). HL-1 clones were designated corin KOs after finding only loss of function mutations (clones 2C, 3D, 3G and 4B). A clone containing a heterozygous frameshift at amino acid Ser96 was used as a passage and process control HL-1 cell with partially preserved corin function (clone 3E).

2.8 Detection of cellular corin activity on HL-1 cells

HL-1 cells were cultured at 37°C and 5% CO_2 in Claycomb medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (Merck KGaA, Darmstadt, Germany), and 2 mM L-glutamine. HL-1 wildtype and HL-1 corin knockout cells (generated by CRISPR/Cas9) were plated at a density of 50,000

cells/well on 96-well microtiter plates (Greiner Bio One, Frickenhausen, Germany) for one day. Cells were washed twice with Opti-MEM™ and 300 nM pro-ANP-FLAG-His was added in a volume of 200 µl to the cells in Opti-MEM™ with 0.1% BSA. Supernatants were taken at different time points and were immediately frozen at -20°C for later use. Serial dilutions of the samples were prepared in Tyrode containing 2 mM calcium ions and 0.03% BSA.

2.9 Statistical analysis

The GraphPad Prism Software (version 7.0, GraphPad Software Inc., San Diego, CA, USA) was used for curve fitting and calculation of the half-maximal effective concentrations (EC_{50} values). EC_{50} values were determined from twenty-six (figure 3), five (figure 4), three (figure 5), or six (figure 6) independent experiments performed in quadruplicate. The data are expressed as mean values ± standard error of the mean (SEM).

3. Results

3.1 A GCaMP6-based ANP reporter cell line produces stable, long-lasting fluorescence signals upon ANP stimulation

We previously generated a luminescent ANP reporter cell line stably expressing the rat ANP receptor, the CNGA2 channel and the calcium-dependent photoprotein aequorin [31]. This cell line allows the real-time detection of ANP-stimulated cGMP generation. Binding of ANP to its receptor leads to an increase of intracellular cGMP, which in turn opens the CNGA2 channel. The diffusion of calcium ions into the cell triggers an aequorin-mediated luminescence signal.

Stimulation of this cell line with ANP for 6 minutes followed by calcium addition in the microplate luminometer (incubation mode) leads to luminescence signals that peak and decline within 20 seconds (Figure 1A). Likewise, in the presence of calcium ions, addition of ANP in the luminometer (kinetic mode) stimulates fast but transient luminescence signals (Figure 1B).

Fast signal kinetics are well-suited for the characterization of ANP receptor pharmacology and ligand characterization. However, the transient luminescence signals might not be optimally suited for the detection of corin activity. In preliminary experiments using our luminescence-based ANP reporter cell line, specific corin activity became detectable after rather long incubation times. In addition to that, corin activity was found to be enhanced by calcium ions (unpublished data). In the presence of calcium, corin-mediated pro-ANP cleavage cannot be monitored by using the luminescence-based ANP reporter cell line due to the transient nature of the luminescence signals.

To overcome this problem, we generated a novel ANP reporter cell line stably expressing the rat ANP receptor, the CNGA2 channel and the fluorescent calcium indicator protein GCaMP6 [34]. To characterize our newly developed reporter cell line, stimulation of the cell line was performed with increasing ANP concentrations in the absence of calcium ions (incubation mode). Measurements were started upon calcium addition, and maximal fluorescence signals were observed after about 90 seconds (Figure 1C). These signals stayed stable until the end of the measurement. In kinetic mode, stimulation with 1 nM and 0.3 nM ANP led to maximal fluorescence signals after about 4 minutes which stayed stable until the end of the measurement. Stimulation with lower ANP concentrations resulted in steadily increasing fluorescence signals over 5 minutes (Figure 1D).

The fluorescence signals elicited by submaximal stimulation of the reporter cell line with 0.1 nM ANP or below remained stable over a period of at least 45 minutes (Figure 2A). In contrast, maximal stimulation of the cells with 1 nM ANP or even higher concentrations resulted in fluorescence signals which reached maximal levels after about 5 minutes and started to slowly decline thereafter (Figure 2B).

3.2 The ANP reporter GCaMP6 cell line is a sensitive tool for the detection of natriuretic peptides

To compare the sensitivity of this newly generated ANP reporter cell line with the luminescence-based ANP reporter cell line, both cell lines were incubated with serial dilutions of ANP, ANP-FLAG-His and pro-ANP-FLAG-His. All agonists stimulated both reporter cell lines with similar efficacy (Figure 3). On the luminescence-based reporter cell line, ANP showed the highest potency (EC_{50} = 0.1 ± 0.01 nM), followed

by ANP-FLAG-His (EC_{50} = 0.41 ± 0.05 nM) and pro-ANP-FLAG-His (EC_{50} = 8.8 ± 1.3 nM). Stimulation of the novel GCaMP6 fluorescence-based reporter cell line resulted in EC_{50} values of 0.11 ± 0.02 nM (ANP), 0.38 ± 0.07 nM (ANP-FLAG-His) and 7.7 ± 1.4 nM (pro-ANP-FLAG-His).

The finding that the full-length pro-ANP peptide was able to fully stimulate both ANP reporter cell lines, albeit with reduced potency, was somewhat unexpected. However, similar findings have been described by other groups as well [35, 36].

Taken together, these experiments show that the ANP reporter GCaMP6 cell line is as sensitive to ANP stimulation as the luminescence-based ANP reporter cell line. In both cell lines, the EC_{50} values for the pro-ANP-FLAG-His peptide were found to be about 20-fold higher compared to the EC_{50} values for ANP-FLAG-His. Therefore, the differential sensitivity of the reporter cell lines for these two natriuretic peptides should allow the detection of corin-mediated pro-ANP to ANP conversion. Moreover, the stable, long-lasting fluorescence signals of the ANP reporter GCaMP6 cell line should be well-suited to monitor slow enzymatic processes.

3.3 Detection of corin activity using the ANP reporter GCaMP6 cell line

To test this hypothesis, different concentrations of solCorin or of the catalytically inactive mutant solCorin(S985A) were incubated with pro-ANP-FLAG-His and samples were taken after different time points. Next, serial dilutions of the samples were measured in kinetic mode on the ANP reporter GCaMP6 cell line (Figure 4). In this experimental setup, the conversion of pro-ANP-FLAG-His to ANP-FLAG-His by solCorin was expected to result in a left-shift of the concentration-response curves (CRC) and a reduction of the EC_{50} values with increasing incubation time.

In fact, samples collected after 10 min or 90 min incubation of 1 nM solCorin with pro-ANP-FLAG-His generated lower EC_{50} values compared to samples collected at the beginning of the reaction when measured on the ANP reporter GCaMP6 cell line (Figure 4A, EC_{50} (0 min) = 5.5 ± 3.2 nM, EC_{50} (10 min) = 4.4 ± 2.3 nM, EC_{50} (90 min) = 1.7 ± 0.8 nM). We observed an even stronger time-dependent reduction of the EC_{50} values when the solCorin concentration was increased to 10 nM (Figure 4B, EC_{50} (0 min) = 12.1 ± 4.4 nM, EC_{50} (10 min) = 2.5 ± 0.6 nM, EC_{50} (90 min) = 0.4 ± 0.1 nM). The fastest time-dependent reduction of the EC_{50} values was observed at a solCorin concentration of 100 nM (Figure 4C, EC_{50} (0 min) = 11.7 ± 4.1 nM, EC_{50} (10 min) = 0.8 ± 0.2 nM, EC_{50} (90 min) = 0.4 ± 0.1 nM). In contrast, no significant CRC left-shift was visible when incubating 100 nM catalytically inactive solCorin(S985A) with pro-ANP-FLAG-His (Figure 4D). These experiments show that our novel fluorescence-based ANP reporter cell line can be used to monitor the activity of recombinant solCorin.

3.4 Detection of cellular corin activity on transiently transfected HEK 293 cells

Next, we wanted to know if the activity of wildtype corin located on the surface of cells could also be detected. Therefore, we added pro-ANP-FLAG-His to HEK 293 cells transiently overexpressing human wildtype corin, human corin plus PCSK6, or the catalytically inactive human corin(S985A) variant. Supernatant samples were taken at 0 min, 30 min, 90 min and 120 min, respectively (Figure 5).

Time-dependent leftward shifts of the pro-ANP-FLAG-His concentration response curves were observed when performing the experiment with corin-overexpressing HEK 293 cells (Figure 5A). The control sample (0 min incubation) stimulated the ANP

reporter GCaMP6 cell line with an EC_{50} value of 21.3 ± 6.9 nM, whereas the 30 min, 90 min and 120 min samples generated EC_{50} values of 4.6 ± 1.5 nM, 2.1 ± 0.8 nM and 1.8 ± 0.9 nM, respectively. A somewhat stronger leftward-shift of the pro-ANP-FLAG-His CRC was observed when corin and PCSK6 overexpressing cells were incubated with pro-ANP-FLAG-His (Figure 5B). In this case, samples tested after incubation of 0 min, 30 min, 90 min and 120 min generated EC_{50} values of 10.0 ± 0.9 nM, 2.8 ± 1.0 nM, 1.2 ± 0.5 nM and 1.0 ± 0.4 nM, respectively.

Almost no leftward shifts were detected when pro-ANP-FLAG-His was incubated on HEK 293 cells transfected with the catalytically inactive corin(S985A) variant (Figure 5C, EC_{50} (0min) = 13.2 ± 1.5 nM, EC_{50} (90min) = 17.9 ± 10.3 nM) or empty vector as control (Figure 5D, EC_{50} (0min) = 11.4 ± 0.7 nM, EC_{50} (90min) = 8.9 ± 0.9 nM).

3.5 Detection of corin activity on cardiomyocyte-derived HL-1 cells

Next, we were interested to see if a cell line with endogenous corin expression can also be used to monitor cellular corin activity. Therefore, several cell lines were tested for corin expression via quantitative PCR (data not shown). In line with a previous publication [33], we were able to identify corin expression in the cardiomyocyte-derived HL-1 cell line. This cell line is derived from murine atrial tumor cardiomyocytes. For the generation of an appropriate negative control, we generated HL-1 corin knockout cell clones with heterozygous or complete knockout of the corin alleles, using the CRISPR/Cas9 technology targeting the second coding exon. Complete knockout of the corin alleles was verified by genomic sequencing of only frameshift mutations (data not shown).

Next, we incubated wildtype HL-1 cells and the newly generated HL-1 heterozygous and complete corin knockout cell clones with pro-ANP-FLAG-His. Supernatant samples were collected at different time points and serial dilutions of these samples were measured on the ANP reporter GCaMP6 cell line in kinetic mode (Figure 6). When performing the experiment with the wildtype HL-1 cell line, supernatant samples collected after 1 h and 4 h incubation generated significantly lower EC_{50} values than samples collected at the beginning of the incubation. Respective EC_{50} values of 6.2 ± 1.3 nM (0 h), 2.6 ± 0.8 nM (1 h), and 0.6 ± 0.1 nM (4 h) were observed (Figure 6A). This effect was less pronounced when using a heterozygous corin knockout clone (clone 3E) of the HL-1 cell line. After 0 h, 1 h and 4 h incubation, EC_{50} values of 8.2 ± 2.0 nM, 3.9 ± 1.3 nM, and 1.7 ± 0.5 nM were determined, respectively (Figure 6B). In contrast, a left-shift of the corresponding CRCs was barely detectable when using a corin knockout HL-1 clone (clone 2C) with complete knockout of all corin alleles (EC_{50} (0 h) = 7.2 ± 2.4 nM, EC_{50} (1 h) = 5.9 ± 1.7 nM, EC_{50} (4 h) = 4.8 ± 1.3 nM (Figure 6C). To further strengthen these results, several HL-1 clones with complete knockout of the corin alleles were analyzed in parallel. All clones with complete corin knockout yielded similar results, i.e. left-shifts of the corresponding CRCs were barely detectable (Figure 7).

In summary, these experiments show that our newly generated fluorescence-based ANP reporter cell line is a valuable tool for the detection of the proteolytic activity of endogenously expressed corin on the cardiomyocyte-derived HL-1 cell line.

4. Discussion

Human genetic data and results from experimental heart failure models revealed that the proteolytic activity of the cardiac protease corin has significant impact on cardiovascular pathophysiology. Therefore, corin represents an interesting pharmacological target and activation of corin by small molecules might be a new therapeutic strategy for cardiac diseases. Here, we present a novel cell-based corin assay suitable for the identification of corin modulators.

For the development of the corin assay, we compared a previously generated luminescent ANP reporter cell line [31] with a newly generated GCaMP6 fluorescence-based ANP reporter cell line. Both cell lines proved suitable for the detection of ANP, pro-ANP-FLAG-His and ANP-FLAG-His and are equally sensitive. Specific activation of the ANP reporter cell line has been characterized thoroughly by testing a number of different active and inactive peptide ligands. The assay system is specifically activated by members of the natriuretic peptide family [31].

As expected, ANP has the highest potency on the GC-A receptor, followed by ANP-FLAG-His and pro-ANP-FLAG-His. In both cell lines, the EC_{50} values of pro-ANP-FLAG-His are about 20-fold higher compared to the EC_{50} values for ANP-FLAG-His. We consider this factor to be large enough for an assay that monitors corin-mediated conversion of pro-ANP-FLAG-His to ANP-FLAG-His.

The ANP reporter GCaMP6 cell line exhibits a slightly slower signal generation compared to the luminescent ANP reporter cell line. The GCaMP6 molecule is suitable to monitor calcium transients within milliseconds [34]. Thus, the GCaMP6 reaction is not the time-limiting factor. This implies that the calcium influx is slower in the fluorescent reporter cell line compared to the luminescent reporter cell line. Lower

expression levels of the CNGA2 channel or the ANP receptor might account for this effect.

However, the ANP reporter GCaMP6 cell line shows superior signal stability in the presence of calcium ions. This appears particularly suitable for the detection of rather slow enzymatic processes in a cell-based assay format. In both cell lines, binding of ANP to the GC-A receptor stimulates intracellular cGMP synthesis which leads to the opening of the CNGA2 channel and calcium influx into the cell. In the luminescent reporter cell line, binding of Ca^{2+} to aequorin results in the oxidation of coelenterazine followed by the release of photons [37]. The consumption of aequorin in this reaction is probably the major reason for the transient signals of the luminescent ANP reporter cell line. In contrast, binding of calcium to GCaMP6 leads to conformational changes that result in increased fluorescence [34]. As long as the cytosolic calcium concentration stays stable, the conformation of GCaMP6 should not change, resulting in stable fluorescent signals of the ANP reporter GCaMP6 cell line.

Nevertheless, upon strong stimulation of this cell line with high concentrations of ANP, we observed a slow fluorescence decrease over 45 minutes. The slow signal decline might be due to desensitization of the GC-A receptor by dephosphorylation, internalization of the GC-A receptor, inactivation of the CNGA2 channel by calcium-activated calmodulin or activation of calcium transporters [6, 38, 39]. In contrast, submaximal stimulation with ANP resulted in stable, long-lasting fluorescent signals. We therefore opted on developing a cell-based corin assay using our newly established fluorescence-based ANP reporter cell line.

Interestingly, our results show that pro-ANP-FLAG-His is able to directly stimulate both ANP receptor cell lines with unexpected potency. However, this is in line with observations made by other groups. Ichiky et al. could show that pro-ANP is able to stimulate the ANP receptor and intracellular cGMP synthesis in a stably transfected

HEK 293 cell line. In addition, it has also been shown that pro-BNP is able to stimulate the ANP and BNP receptor (GC-A), albeit with reduced potency [35, 36].

To further characterize our newly established reporter assay, we incubated pro-ANP-FLAG-His with soluble corin, corin-overexpressing HEK 293 cells or HL-1 cells with endogenous corin expression and collected samples at different time points. Next, we applied serial dilutions of the samples to the GCaMP6 fluorescence-based ANP reporter cell line. Samples collected at later time points generated lower EC_{50} values compared to samples collected after short incubation times only. Therefore, the leftward shift of the corresponding concentration-response curves was found to be time-dependent. In addition, the amount and speed of the leftward shift increased with increasing the solCorin concentration in the assay from 1 nM to 10 nM or 100 nM. Moreover, no reduction of the EC_{50} values with time was observed when repeating the experiments with catalytically inactive solCorin(S985A), mutant corin(S985A)-overexpressing HEK 293 cells or HL-1 corin knockout cells.

These results show that the enzymatic corin activity located on the surface of cells, as well as of purified soluble corin can be specifically monitored using the ANP reporter GCaMP6 cell line. In addition, the results also show that the assay has a unique sensitivity. Corin activity can already be detected at enzyme concentrations as low as 1 nM. Furthermore, in contrast to published assays, very low concentrations of the natural corin substrate are needed. Biochemical assays require the addition of short, fluorescently labeled substrates in millimolar concentrations [29].

It is most likely that the observed reduction of the EC_{50} values arises from peptide cleavage and removal of the N-terminal pro-ANP sequence (NT-pro-ANP) from pro-ANP-FLAG-His by corin. In fact, mass spectrometric analysis after incubation of purified solCorin with pro-ANP-FLAG-His shows that the enzyme cleaves the peptide

at the published activation site at Arg-98↓Ser-99 of pro-ANP, generating NT-pro-ANP, and the C-terminal peptide ANP-FLAG-His (V. Kaushik, pers. communication; [1]).

Corin is produced as a zymogen and activated by cleavage at Arg-801 by proprotein convertase subtilisin/kexin type 6 (PCSK6) [40]. In our corin assay, we could detect specific corin activity in HL-1 cells without overexpressing PCSK6. Moreover, coexpression of corin with PCSK6 in HEK 293 cells only slightly increased the speed of pro-ANP activation compared to the overexpression of corin only. This is in line with Chen et al. who were able to detect PCSK6 expression in HEK 293 and HL-1 cell lines. Moreover, they could show that activated corin protein is present in corin expressing HEK 293 cells. Overexpression of PCSK6 in HEK 293 cells further increased the fraction of activated corin, however, the effect on pro-ANP-processing was not described. According to that notion, overexpression of PCSK6 might only slightly enhance pro-ANP processing [40].

When comparing the EC_{50} values of pro-ANP-FLAG-His and ANP-FLAG-His on the ANP reporter GCaMP6 cell line, an about 20-fold reduction of the EC_{50} values would be expected after incubation and complete cleavage of pro-ANP-FLAG-His on corin expressing cells. However, this value did not exactly reproduce in the incubation experiments performed with pro-ANP-FLAG-His on HL-1 cells, possibly because the 4 hour incubation time used in the assays were not long enough. This might be related to the low amount of corin on HL-1 cells, since the pro-ANPase activity on these cells seems to be fairly low [26]. In addition, murine corin expressed on HL-1 cells might also not be able to optimally process the human pro-ANP peptide. This is not unlikely, since human and murine pro-ANP show significant differences, especially in their N-terminal (NT-pro-ANP) peptide sequences. Jiang et al. showed that HL-1 cells overexpressing human pro-ANP are able to convert the pro-peptide to

mature ANP [41]. However, details about the kinetics of the reaction are not given. In any case, the assay window observed in our corin assay should be well-suited to identify corin modulators in a high-throughput screening (HTS) format.

To date, only endpoint analysis is described in most publications demonstrating specific cellular corin activity using pro-ANP as the substrate. In contrast, we measured pro-ANP-FLAG-His activation after different time points. Our cell-based corin assay allows a closer examination of enzyme kinetics and shows that processing of pro-ANP by corin is a rather slow enzymatic process. In fact, solCorin exhibited a slower turnover than other serine proteases in a biochemical assay (unpublished data). Thus, corin modulators might increase the corin activity to the level of other proteases. The analysis of enzyme kinetics will be also extremely valuable for the analysis of structure-function relationships of corin mutants.

To our knowledge, this assay is the first cellular corin assay that is suitable for the identification of corin modulators in a 384-well microtiter plate format. While previous biochemical assays might also be amenable to HTS [29], the short fluorescently labeled substrates had almost no sequence similarity with pro-ANP. In contrast, our cell-based corin assay uses the full length pro-ANP peptide and recombinant wildtype or endogenously expressed corin enzyme which mimics the physiological interaction of membrane bound corin with pro-ANP in a cellular context. In addition, our newly developed corin assay requires low levels of recombinant enzyme. If performed at a corin concentration of 10 nM, the corin assay presented in this paper requires only 0.05 pmol purified enzyme. Our data suggest that a further reduction of the corin concentration below 10 nM might also be feasible. In contrast, a 96-well plate biochemical assay requires 5.8 pmol of recombinant corin enzyme per assay point [29].

Another cell-based corin assay that in theory could be used for HTS was recently published. Chen et al. monitored cleavage of the short chromogenic substrate (p-tosyl-Gly-Pro-Arg)2-rhodamine by corin in membrane fractions from human left ventricles in a 96-well plate format. Of course, the low availability of human samples impairs the use of this assay for HTS. Moreover, membrane fractions from corin knockout mice were used as a background control. This might not be the appropriate control because a different distribution of membrane serine proteases in human and mice cardiac tissue cannot be ruled out [30].

In principle, our novel cell-based reporter assay might allow the identification of direct corin activators, as well as indirect modulators of corin activity. Indirect activation of corin on corin expressing cells could be achieved by modulating the activation of corin by PCSK6 [40], corin glycosylation [27, 33] or the inactivation of corin by membrane shedding [42].

As a further improvement of our novel cell-based corin assay, the incubation of corin expressing cells with pro-ANP-FLAG-His and detection of fluorescence might be performed on the same plate in the same well. This entails a co-culture of corin expressing cells (e.g. HL-1 cells) with the ANP reporter cell line. The ANP reporter GCaMP6 cell line appears particularly suitable for this assay because of its long-lasting fluorescence signals in the presence of calcium ions. In future experiments it is planned to validate if a co-culture approach can be used for the detection of novel corin modulators in an HTS setting.

In summary, our newly established GCaMP6 fluorescence-based ANP reporter cell line is well-suited for the detection of corin activity, can be used for structure-function studies of corin variants, and might be used for identification of novel corin modulators by HTS. Specific corin activation could represent a novel pharmacological

principle and might become an innovative approach for the treatment of cardiovascular diseases in the future.

Conflict of interests: All authors have no financial interests or potential conflicts of interest to declare.

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Figures

Figure 1: Comparison of ANP reporter cell lines. Measurements were performed in incubation mode (A,C) and kinetic mode (B,D), using luminescence-based (A,B) or GCaMP6 fluorescence-based (C,D) ANP reporter cell lines. ANP stimulation of GCaMP6 reporter cells results in stable, long-lasting fluorescence signals. Results are expressed as relative light units per second (RLU/s) or relative fluorescence units per second (RFU/s) and data from an individual experiment are presented as means with SD (n = 12).

Figure 2: Signal stability of GCaMP6-mediated fluorescence signals. Stimulation of the ANP reporter GCaMP6 cell line with (A) 0.1 nM or (B) 1 nM ANP in the presence of calcium ions (kinetic mode) for 5 - 45 min. Results are expressed as relative fluorescence units per second (RFU/s) and data from an individual experiment are presented as means with SD (n = 4).

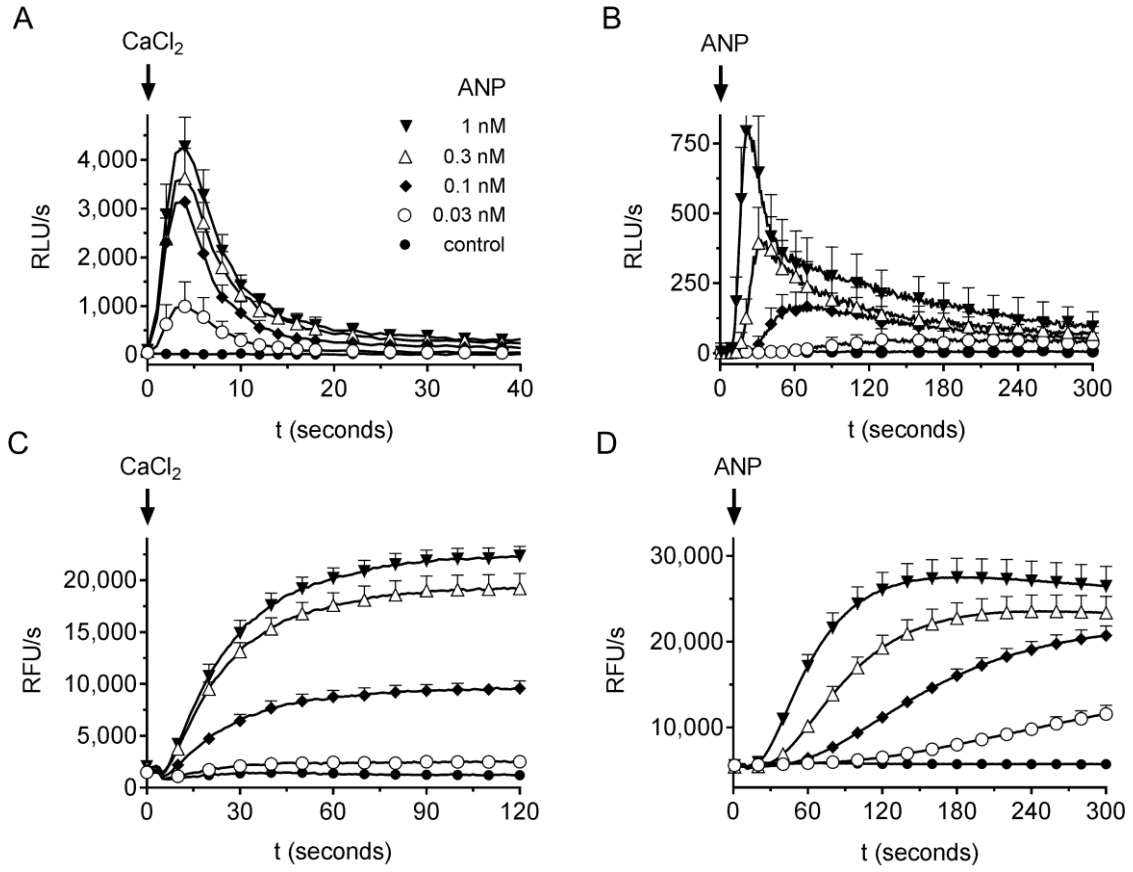
Figure 3: Stimulation of ANP receptor reporter cell lines by natriuretic peptides. Luminescent (A) and fluorescent (B) signals mediated by stimulation with ANP (▼), ANP-Flag-His (○) or pro-ANP-Flag-His (■). Luminescence measurements were performed in incubation mode. GCaMP6 cells were measured over 5 min in kinetic mode. Arrows depict the putative assay window for corin-mediated ~20-fold shift of EC_{50} values. Results are expressed as relative light units (RLU) or relative fluorescence units (RFU) and data from an individual experiment are presented as means with SD (n = 4).

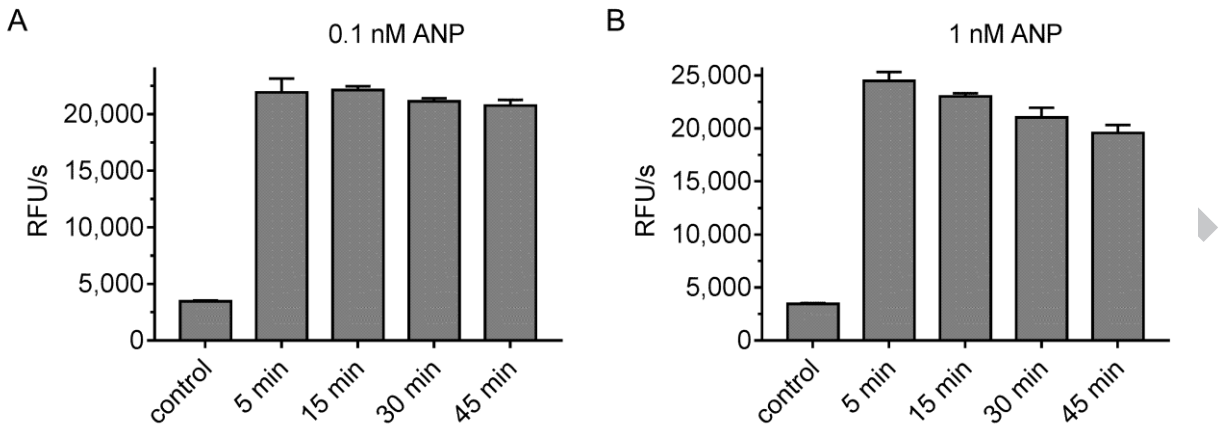
Figure 4: Time- and concentration-dependent pro-ANP conversion by purified solCorin. Wildtype solCorin or inactive solCorin(S985A) mutant were incubated with 3 µM pro-ANP-Flag-His for 0 min (■), 10 min (○) or 90 min (▲). Serial dilutions of the samples were measured in kinetic mode on GCaMP6 cells. Results are expressed as relative fluorescence units (RFU) and data from an individual experiment are presented as means with SD (n = 4).

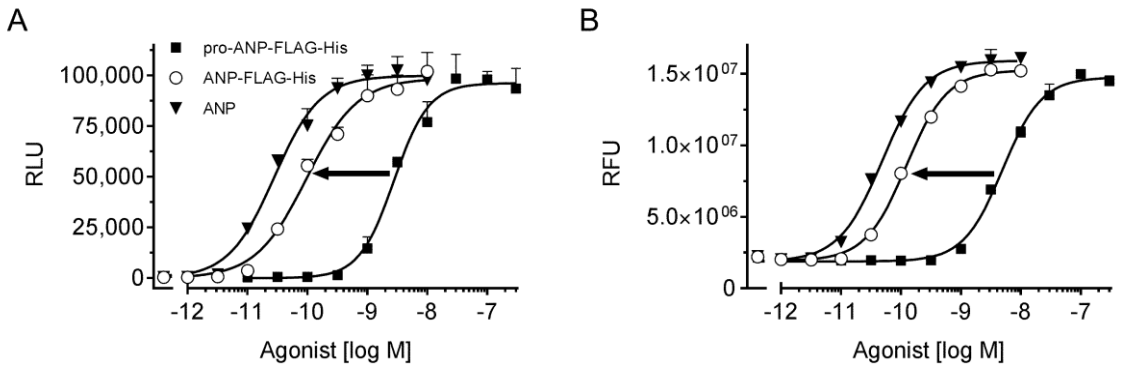
Figure 5: Detection of corin activity on transfected HEK 293 cells. Pro-ANP conversion on HEK 293 cells transiently transfected with human corin (A), corin plus PCSK6 (B), inactive corin(S985A) mutant (C) or empty vector (D). Cells were incubated with 600 nM pro-ANP-Flag-His for 0 min (■), 30 min (○), 90 min (▲) or 120 min (♦). Serial dilutions of the supernatant samples were measured in kinetic mode on GCaMP6 cells. Results are expressed as relative fluorescence units (RFU) and data from an individual experiment are presented as means with SD (n = 4).

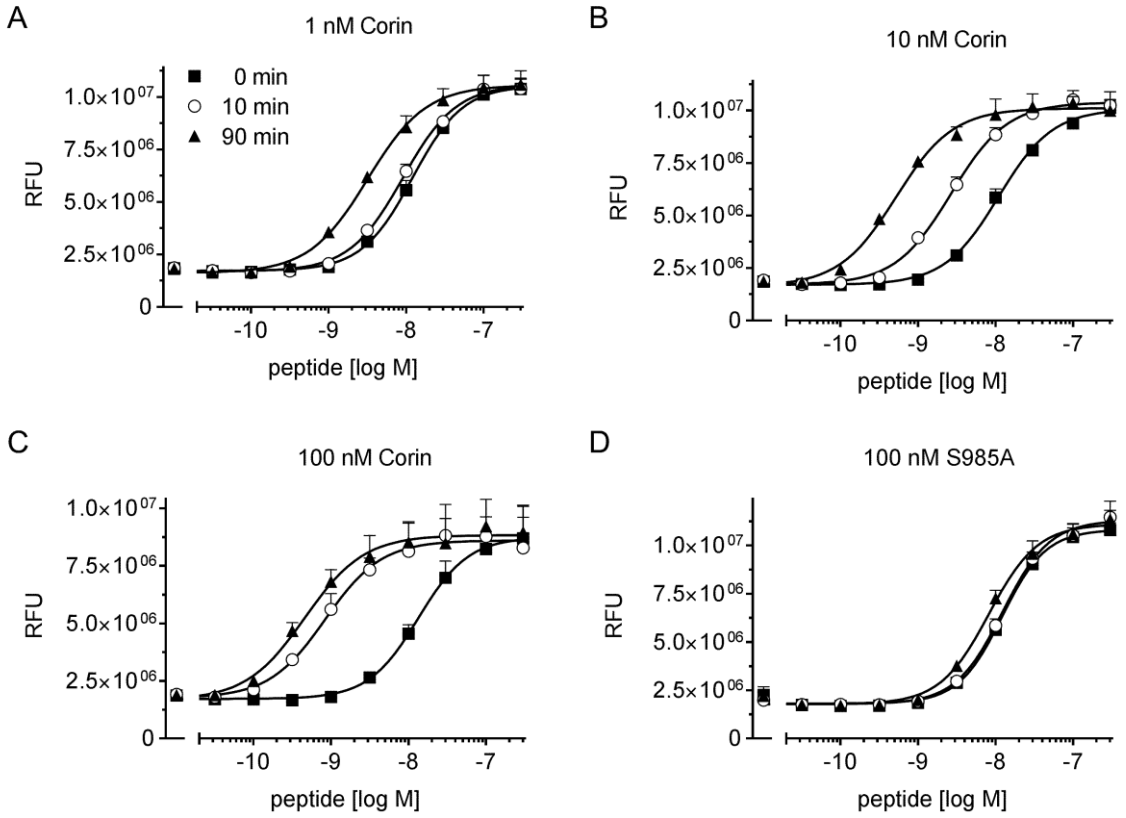
Figure 6: Detection of corin activity on cardiomyocyte-derived HL-1 cells. Pro-ANP conversion by cardiomyocyte-derived HL-1 wildtype cells (A), or HL-1 heterozygous knockout clone 3E (B) and complete corin knockout clone 2C (C). Cells were incubated with 300 nM pro-ANP-Flag-His for 0 h (■), 1 h (○) or 4 h (▲). Serial dilutions of the supernatant samples were measured in kinetic mode on GCaMP6 cells. Results are expressed as relative fluorescence units (RFU) and data from an individual experiment are presented as means with SD (n = 4).

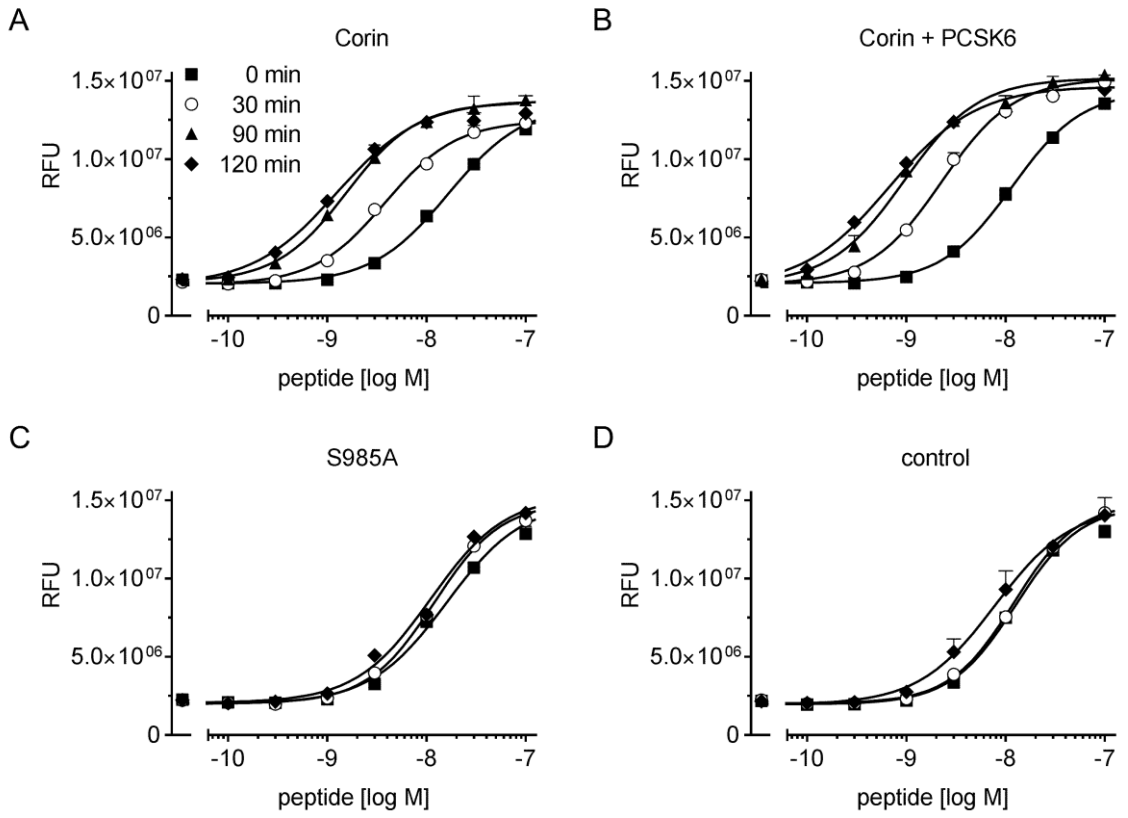
Figure 7: Detection of corin activity on cardiomyocyte-derived HL-1 cells. Pro-ANP conversion by cardiomyocyte-derived HL-1 wildtype cells (A), or HL-1 heterozygous (B) and complete (C-F) corin knockout clones. Cells were incubated with 300 nM pro-ANP-Flag-His for 0 h (■), 1 h (○) or 4 h (▲). Serial dilutions of the supernatant samples were measured in incubation mode on the luminescent ANP reporter cells. Results are expressed as relative luminescence units (RLU) and data from an individual experiment are presented as means with SD (n = 4).

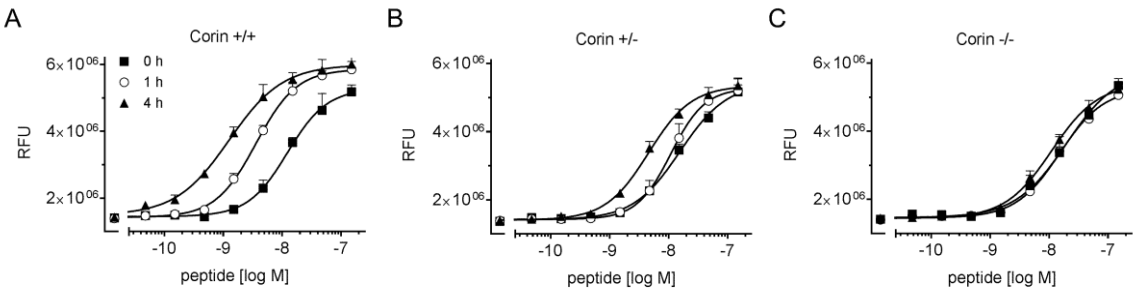


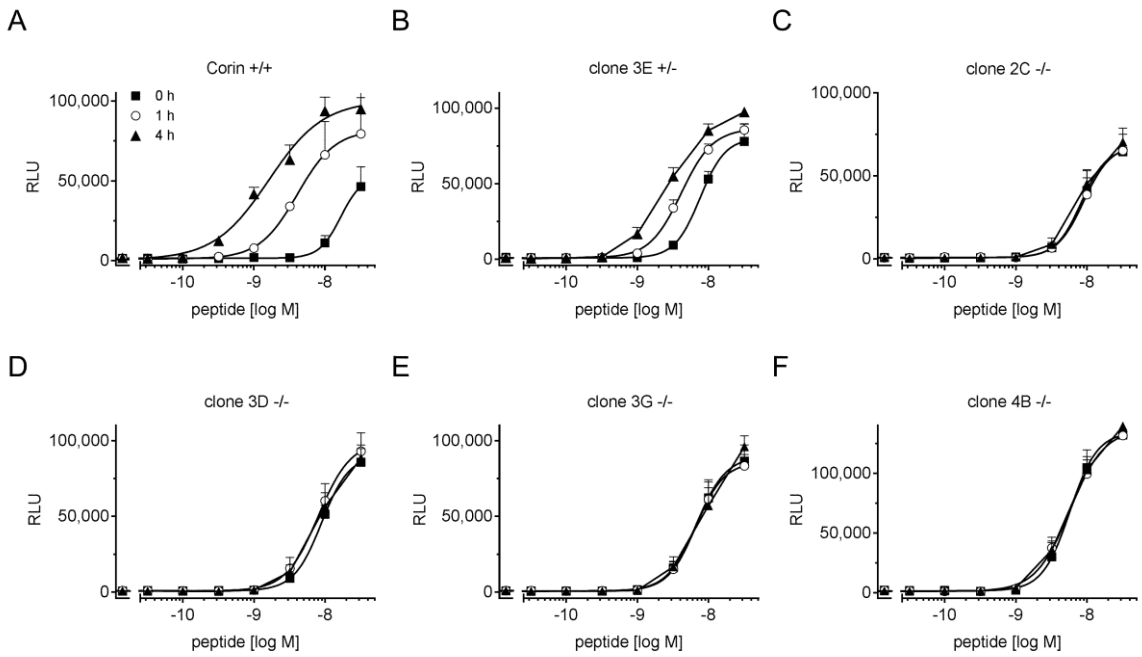


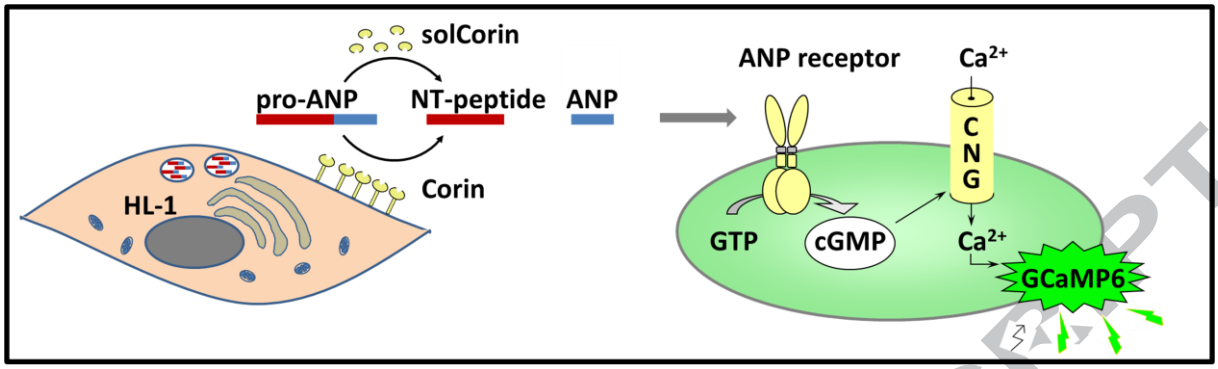












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