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# Differential modulation of cardiac potassium channels by Grb adaptor proteins

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ABSTRACT

Scaffolding growth factor receptor-bound (Grb) adaptor proteins are components of macromolecular signaling complexes at the plasma membrane and thus are putative regulators of ion channel activity. The present study aimed to define the impact of Grb adaptor proteins on the function of cardiac $K^+$ channels. To this end channel proteins were coinjected with the adaptor proteins in *Xenopus* oocytes and channel activity analyzed with two-electrode voltage-clamp. It is shown that coexpression of Grb adaptor proteins can reduce current amplitudes of coexpressed channels. Grb7 and 10 significantly inhibited functional currents generated by hERG, Kv1.5 and Kv4.3 channels. Only Grb10 significantly inhibited KCNQ1/KCNE1 $K^+$ channels, and only Grb7 reduced Kir2.3 activity, whereas neither Grb protein significantly affected the closely related Kir2.1 and Kir2.2 channels. The present observations for the first time provide evidence for a selective and modulatory role of Grb adaptor proteins in the functional expression of cardiac $K^+$ channels.



## Introduction

The Grb7 (growth factor receptor-bound protein 7) family represents a group of SH2 domain-containing cytosolic adaptor proteins, consisting of three members, Grb7, 10 and 14 [1,2]. Grb7 family proteins are differentially expressed in a variety of human tissues with highest expression of Grb10 in skeletal muscle and pancreas, and high expression in cardiac muscle and brain [3,4].

The physiological functions of the Grb7 family members are incompletely understood. However, it is likely that these proteins act as negative regulators in several physiological processes [5] and the presence of a Pleckstrin homology (PH) domain through its interaction with phospholipids may contribute to Grb's membrane targeting. Specifically, Grb10 has been shown to modulate the activity of plasma membrane-localized Kv1.3 channels [6].

The present study explored whether Grb7 or Grb10 participate in the regulation of cardiac $K^+$ channels. For this, cRNA encoding voltage-gated $K^+$ channels hERG, Kv1.5, Kv4.3, KCNQ1/KCNE1, and inward-rectifier $K^+$ channels Kir2.1, Kir2.2 and Kir2.3 channels, was injected into *Xenopus* oocytes either alone or together with cRNA encoding Grb7 or Grb10 and the activity of the respective $K^+$ channels was determined by two-electrode voltage-clamp. As a result, hERG and other Kv channel currents were strongly downregulated by coexpression of both Grb7 and Grb10, whereas KCNQ1/KCNE1 was only mildly downregulated by Grb10. Inhibitory effects of coexpressed Grb proteins on Kir2 channels in general were only weak, but reached statistical significance in case of Grb7 and coexpressed Kir2.3 currents. Interestingly, Grb7 was also able to stimulate Kir2.2 channels.

## Materials and methods

*Molecular biology*. Template cDNAs encoding hKir2.x, hERG, hKv1.5, hKv4.3, hKCNQ1, hKCNE1 and hGrb were linearized with an appropriate restriction enzyme (New England Biolabs, MA, USA) and cRNA was synthesized from 1 μg of linearized DNA by *in vitro* transcription (mMessage mMachine T7 kit or mMessage mMachine SP6 kit, Ambion, Applied Biosystems, Germany). cRNA concentrations were evaluated by photospectrometry and transcript quality was verified by agarose gel electrophoresis.

*Oocyte isolation*. Ovarian lobes were harvested from *Xenopus laevis* frogs anesthetized with a 0.17% tricaine solution. Oocytes were treated for 120 min with collagenase (1 mg/ml, Worthington, type II) in ND96-$Ca^{2+}$-free solution containing (in mM): 96 NaCl, 2 KCl, 2 $MgCl_2$, 5 HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid) (pH 7.6) to remove follicle cells, and then stored at 17 °C in ND96 containing gentamycin (50 mg/L), theophylline

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(0.5 mM) and Na-pyruvate (2.5 mM). For characterization of cardiac $K^+$ channels, each oocyte was injected with 1 ng Kir2.x cRNA or 4 ng cRNA encoding the other channels followed by coinjections with 3 ng cRNA encoding Grb7 or 10.

*Electrophysiology*. A Turbo-TEC10CX amplifier was used to record currents at room temperature (at 22–23 °C) in oocytes 2–4 days after injection with cRNAs using standard two-electrode voltage-clamp technique (TEVC). The pipettes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ. For all experiments the TEC-integrator (set to 2–3 ms) was used to enable good clamp performance of currents and the clamp performance was always controlled via the TEC-PI-controller. Data were Bessel filtered at 500 Hz (Kir2.x) or 2 kHz (voltage-gated channels) and stored at a sample rate of 2–3 kHz. The standard recording solution ND96 for voltage-gated channels contained in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES and was equilibrated to pH 7.5. The recording solution KD60 (in mM: 60 KCl, 38 NaCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES and was equilibrated to pH 7.5) was used to record Kir2.x. Data acquisition was performed using an IBM compatible computer with Pentium 3 processor, a Digidata 1322 A/D interface and pClamp 8 software (Axon Instruments).

Data were analyzed utilizing Clampfit 8 (Axon Instruments) and Origin 6 (Microcal) software. Data are expressed as arithmetic means ± SEM and statistical analysis was made by ANOVA. $p < 0.05$ was considered as statistically significant. "*n*" denotes the number of independent experiments.

## Results

Grb7 was originally identified by us as a binding protein of the carboxy-terminal tail of renal inward-rectifier $K^+$ channel ROMK (Kir1.1) in a yeast-two hybrid screen, and it reduced the amplitude of the coexpressed ROMK channel in *Xenopus* oocytes [7]. This result prompted us to evaluate the effects of Grb7 and Grb10 proteins on the related cardiac inward-rectifier channels of the Kir2 family. Kir2.x constructs were either expressed alone or together with the respective Grb protein in *Xenopus laevis* oocytes, and after 2 days voltage-clamp recordings were performed in high $K^+$ solution, KD60.

The Kir2.1 channel generated strongly inwardly rectifying $K^+$ currents that, in contrast to the related Kir1.1, were not significantly affected by any of the two coinjected Grb proteins (Fig. 1A). Grb10 apart from a slight tendency to reduce Kir2.2 currents (Fig. 1B), but which did not reach statistical significance, also had no effect on coexpressed Kir2.3 currents (Fig. 1C). In contrast, coexpression of Grb7 significantly decreased Kir2.3 currents and, surprisingly slightly increased the Kir2.2 currents (Fig. 1B). While Grb adaptor proteins were able to change current amplitudes, kinetics of coexpressed Kir channels were not markedly altered.

After demonstrating differential effects on several inward-rectifiers, in a second set of experiments, we studied the effects of Grb7 and Grb10 on outwardly rectifying cardiac channels hERG, Kv1.5, Kv4.3 and KCNQ1/KCNE1.

Coinjection of KCNQ1 and KCNE1 generated slowly activating, non inactivating channels, typical for the cardiac slow delayed rectifier $I_{Ks}$ conductance. When additionally coexpressed, only Grb10 significantly decreased KCNQ1/KCNE1 currents. Coinjected $K^+$ channels with Grb, changes in current amplitude were not accompanied by obvious changes in kinetics. $I_{Ks}$ currents contrastingly remained virtually unaffected by coexpression of Grb7 (Fig. 2).

Currents carried by the rapid delayed rectifier hERG were markedly and significantly reduced by both, Grb7 and Grb10 (Fig. 3A). At the same amount of injected cRNA inhibition of hERG currents reached about 50% with Grb7, but was almost complete with coexpressed Grb10. The hERG kinetics were not obviously modulated by the Grbs.

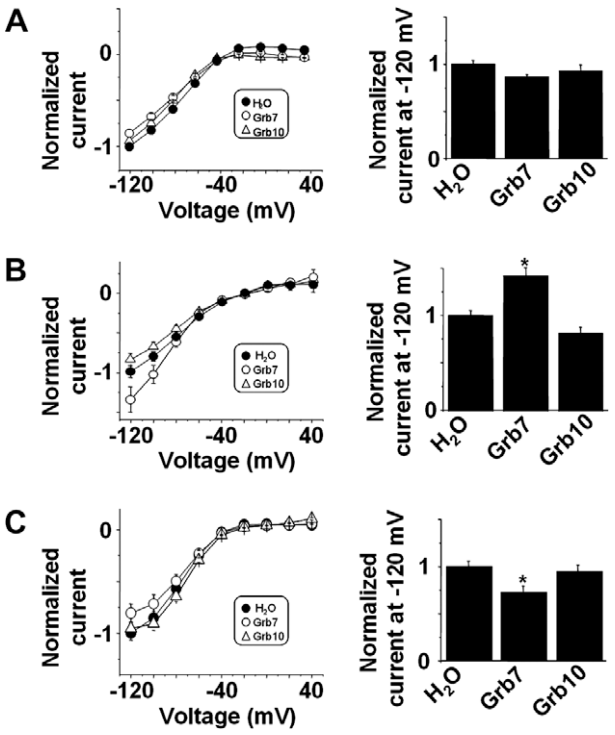


**Fig. 1.** Grb proteins differentially modulate coexpressed Kir2.x channels. *Xenopus* oocytes were injected with Kir2.1 (A), Kir2.2 (B) and Kir2.3 (C) either alone or in combination with Grb7 or Grb10. Kir2.x currents were measured 2 days after injection in KD60, and effects of coexpressed Grb proteins on Kir2 current amplitudes were analyzed at −120 mV. No difference in current amplitude of Kir2.1 channels could be observed in presence of any Grb protein, while Kir2.2 currents were significantly increased and Kir2.3 currents decreased by coexpressed Grb7 adaptor protein. Arithmetic means ± SEM. * indicates a statistically significant difference ($p < 0.05$); $n = 15–31$.

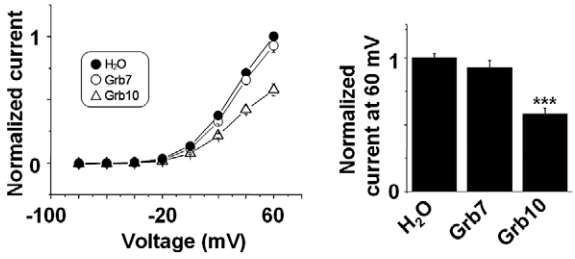


**Fig. 2.** Heteromeric KCNQ1/KCNE1 currents are decreased by Grb10 but not Grb7 coexpression. KCNQ1/KCNE1 $K^+$ channels were recorded 3 days after injection either with or without Grb7 or Grb10. Currents were measured after stepping the potential from −80 to +60 mV. A significant inhibition of currents was only observed in presence of Grb10. *** indicates a statistically significant difference ($p < 0.001$); $n = 25–30$.

Similar to hERG, the ultrarapid delayed rectifier Kv1.5 also was sensitive to Grb7 and 10 coexpression. Again, inhibition by Grb10 was stronger than by Grb7 (Fig. 3B).

Both, Grb7 and Grb10, also strongly reduced currents generated by the transient outwardly rectifying Kv4.3 channel, and inhibition was equally potent for both Grb proteins (Fig. 3C). Again, as was the case with coinjected $I_{Ks}$ channels, changes in current amplitude were not accompanied by obvious changes in kinetics.

## Discussion

The present study reveals that scaffolding proteins of the growth factor receptor-bound (Grb) protein family modulate the

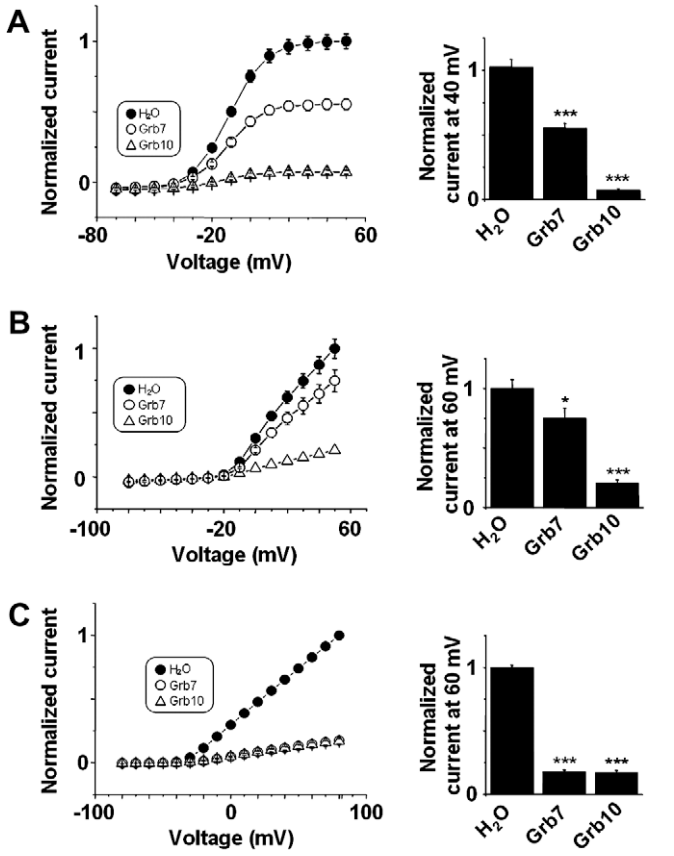


**Fig. 3.** (A) Reduced current for hERG, Kv1.5 and Kv4.3 channels when coexpressed with both Grb adaptor proteins. hERG channels were expressed alone or in combination with Grb7 or Grb10, and currents recorded 3 days after injection. Both adaptor proteins significantly inhibited hERG currents, measured at 60 mV; $n = 23–29$. (B) Kv1.5 was recorded in the absence and presence of coexpressed Grb7 or Grb10. Strong inhibition of Kv1.5 currents was observed upon coexpresssion with Grb10, while Grb7 was less effective. Kv1.5 currents were analyzed at 60 mV; $n = 29–38$. (C) Kv4.3 was recorded in the absence and presence of coexpressed Grb7 or Grb10. Strong inhibition of Kv4.3 currents was observed upon coexpresssion with Grb7 and Grb10. Kv4.3 currents were analyzed at 60 mV; $n = 25–38$. Statistical difference is indicated by $^{*}p < 0.05$) and $^{***}p < 0.001$).

activity of several cardiac $K^+$ channels. The Grb proteins Grb7/10/14 are cellular adaptor proteins which share a conserved multidomain structure [8] with a central PH domain, a carboxy-terminal SH2 domain, and an amino-terminal proline-rich region. They are mainly localized in the cytoplasm, but can also be found at the plasma membrane and at mitochondria. Mostly through their SH2 domains, Grb proteins interact with many cell surface receptor tyrosine kinases and other intracellular signaling molecules that play a role in various cellular functions, like metabolism, migration, survival, and proliferation. Grb10 and Grb14 in particular play a role in insulin signaling by binding to the insulin receptor and inhibition of downstream effectors like PI3K (phosphoinositide 3-kinase) and protein kinase B/Akt [8].

The Grb adaptor protein Grb10 has previously been shown to modulate Kv1.3 $K^+$ channels in the olfactory bulb and brain [6]. When coexpressed with the Src kinase, Grb10 reversed the normal Src-induced inhibition of Kv1.3 currents. It is thought that the proline-rich N-terminal sequence of Grb10 binds to the SH3 region of Src kinase and thereby prevents its binding to the Kv1.3 channel that normally results in tyrosine phosphorylation and reduced channel activity. This regulatory interaction of Kv1.3 channel and Grb protein is of physiological importance as demonstrated by the similar phenotypes of individual knock-out animals. Genetic deletion of the Kv1.3 channel causes an upregulation of Grb10 protein [9] and Kv1.3 (−/−) mice very similar to Grb10-deficient mice present an increased peripheral insulin sensitivity [10,11].

Since Grb10 is also expressed in cardiac tissue [4], it is possible that adaptor proteins by a similar modulation of cardiac $K^+$ channels could participate in the regulation of the cardiac action potential. As a matter of fact, we found that both, Grb7 and Grb10 strongly decreased the activity of hERG, Kv1.5 and Kv4.3. Grb10 in addition effectively reduced KCNQ1/KCNE1 currents. Whereas voltage-gated $K^+$ channels were strongly inhibited, Grb proteins had only weak effects on coexpressed inward-rectifier $K^+$ channels that are important in cardiac membrane excitability. Only overexpressed Grb7 decreased Kir.2.3 channel activity, and increased Kir.2.2 current amplitudes. Grb7 probably does not occur in the heart. The observed regulation of $K^+$ channels thus may be of physiological relevance in tissues where it is coexpressed with these channels, such as kidney, pancreas, intestine, and liver.

The mechanism by which Grbs affect the functional activity of these ion channels is not known at present. By analogy to previous findings Grbs may either hinder access of regulatory tyrosine kinases to the channel protein [6] or, as suggested by Grb7 and Kir1.1, alter channel activity as a consequence of a direct protein–protein interaction (Bundis, 2006, doctoral thesis). In light of the latter finding, it is intriguing that the tested Kir2 inward-rectifier $K^+$ channels were less strongly regulated than the closely-related Kir1.1 and the even more distantly related voltage-gated $K^+$ channels. As was the case with Kir1.1, Grb7 again affected Kir2.2 and Kir2.3, whereas Grb10 did not. Also in contrast to Kv channels Src-dependent tyrosine phosphorylation of inward-rectifiers has not yet been described [12], which may point to a different mechanism of action of Grb7 and Grb10 on voltage-gated versus inward-rectifying $K^+$ channels. Since activity of Kir channels critically depend on the binding of phosphoinositols, in particular on $PIP_2$, the phospholipid-binding properties of the PH domain of the interacting Grb might be of importance. However, correlation between phosphoinositol binding specificities of individual Grbs and the observed effects on channel activity is difficult, since binding affinity of the Grb7 PH domain to $PIP_2$ seems rather weak and binding specificity of the Grb10 PH domain has not been reported [8]. A further complication in the interpretation of the obtained results is the fact that $K^+$ channels can form heteromultimers. In a heteromultimeric ion channel even a subunit that is not affected by a coexpressed Grb protein when expressed as a homomeric channel, might in fact be so via a coassembled heterologous subunit in the native channel.

Grb proteins also may exert their effects by bringing other regulating proteins into the vicinity of the ion channel. Thus, Grb10 also binds 14-3-3 proteins [13], another family of signaling proteins which bind serine- or threonine phosphorylated proteins and thereby change the activity of the bound ligands, alter their association with other cellular components or change their intracellular localization [14]. 14-3-3 proteins have recently been identified also as interactors of several ion channels, including HERG, Task and inward-rectifiers Kir2.1 and Kir6.2 [15]. Coexpression of 14-3-3 with these $K^+$ channels generally stimulated channel activity by increasing their cell surface expression. Our finding that Grb10 coexpression causes downregulation of HERG and Kir2.1 might suggest that it competes with endogenous oocyte 14-3-3 for binding to the channels and therefore impairs their trafficking to the plasma membrane.

Also Nedd4-2 an ubiquitin protein ligase, that marks proteins for subsequent degradation, can interact with Grb10 [16] via its SH2 domain. While Grb10 itself is not ubiquitinated by Nedd4-2, this interaction could regulate ubiquitination and degradation of membrane proteins bound to the Grb protein. Consistingly, Grb10 overexpression increased VEGRF2 levels in endothelial cells

apparently by inhibition of Nedd4-mediated receptor degradation. Nedd4 regulates ubiquitin-dependent degradation of several ion channels, in particular of ENaC [17]. It is therefore possible that Grb10 also targets cardiac $K^+$ channels to Nedd4-dependent ubiquitination and degradation, which would explain the reduction in channel activity observed in our oocyte expression studies. Insulin is one of the hormones that regulates activity of ENaC, and it has been demonstrated that Akt1/PKB mediates the effect of insulin on ENaC by inhibiting Nedd4-2 [18]. Moreover, when phosphorylated by the structurally similar kinase SGK1, Nedd4-2 on the other hand [19,20], interacts with 14-3-3 regulatory proteins. This interaction then inhibits interaction of Nedd4-2 with ENaC [21,22]. Consistingly, SGK isoforms have previously been shown to positively regulate various ion channels and transporters, including KCNQ1/KCNE1 [23], Kv4.3 [24] and hERG [25].

Future studies clearly are necessary to elucidate the role of Grb signaling adaptors in the regulation of ion channels and in the diseased myocardium, where activity of ion channels is altered. Grb10 can negatively regulate the PI3K/Akt insulin-signaling pathway as overexpressed Grb10 isoforms inhibit the autophosphorylated insulin receptor [26–28]. The PI3K signaling pathway is cardioprotective and reduces heart dysfunction, e.g., after ischemia injury through activation of Akt/PKB [29]. The structurally similar SGK1, another PI3K effector, also promotes cardiomyocyte survival and is dynamically regulated in the heart [30]. PKB and SGKs by inducing interaction of Grb10 with 14-3-3 [13] may thereby overcome the inhibitory effects of the adaptor protein on $K^+$ channel currents.

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