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Glyceraldehyde 3-phosphate dehydrogenase serves as an accessory protein of the cardiac sarcolemmal K_{ATP} channel

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Cardiac sarcolemmal ATP-sensitive K^+ (K_{ATP}) channels, composed of Kir6.2 and SUR2A subunits, are regulated by intracellular ATP and they couple the metabolic status of the cell with the membrane excitability. On the basis of previous studies, we have suggested that glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may be a part of the sarcolemmal K_{ATP}-channel protein complex. A polypeptide of ~42 kDa was immunoprecipitated with an anti-SUR2A antibody from guinea-pig cardiac membrane fraction and identified as GAPDH. Immunoprecipitation/western blotting analysis with anti-Kir6.2, anti-SUR2A and anti-GAPDH antibodies showed that GAPDH is a part of the sarcolemmal K_{ATP}-channel protein complex *in vivo*. Further studies with immunoprecipitation/western blotting and the membrane yeast two-hybrid system showed that GAPDH associates physically with the Kir6.2 but not the SUR2A subunit. Patch-clamp electrophysiology showed that GAPDH regulates K_{ATP}-channel activity irrespective of high intracellular ATP, by producing 1,3-bisphosphoglycerate, a K_{ATP}-channel opener. These results suggest that GAPDH is an integral part of the sarcolemmal K_{ATP}-channel protein complex, where it couples glycolysis with the K_{ATP}-channel activity.

Keywords: heart; K_{ATP} channels; glyceraldehyde 3-phosphate dehydrogenase; SUR2A; Kir6.2

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INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels are gated by intracellular ATP and are viewed as a link between cellular metabolism and membrane excitability. In the heart, the opening of these channels has been suggested to protect against myocardial infarction, mediate ischaemic preconditioning and promote survival of cardiomyocytes that are exposed to different kinds of metabolic stresses (Crawford *et al*, 2003; Budas *et al*, 2004).

Structurally, the cardiac subtype of K_{ATP} channels are heteromultimers composed of Kir6.2 subunit, an inwardly rectifying K^+-channel core primarily responsible for K^+ permeance, and SUR2A, a regulatory subunit implicated in ligand-dependent regulation of the channel gating (Inagaki *et al*, 1996). A full understanding of how K_{ATP} channels sense the metabolic conditions of the cell is still under investigation. From reports of channel regulation until now, the intracellular ATP/ADP ratio would seem to be the most important factor in the regulation of sarcolemmal K_{ATP} channels, where ATP and ADP act as endogenous blockers and openers of the channels (reviewed by Dzeja & Terzic, 1998; Jovanović & Jovanović, 2004). In this regard, it has been suggested that there is a close functional relationship between adenylate kinase and creatine kinase phosphotransfer defining the directionality of nucleotide exchange within the sarcolemmal K_{ATP}-channel vicinity. It has been proposed that activation of K_{ATP} channels by adenylate kinase (by $AMP + ATP \leftrightarrow 2ADP$) may be counteracted by creatine kinase (by $ADP + phosphocreatine \leftrightarrow ATP + creatine$), as a system for ATP production and scavenger of the products of adenylate kinase catalysis, thereby keeping K_{ATP} channels closed (Carrasco *et al*, 2001). It has been shown that both adenylate kinase and creatine kinase are physically associated with sarcolemmal K_{ATP} channels, where they can synchronously act as anchoring sites for the cellular phosphotransfer network (Carrasco *et al*, 2001; Crawford *et al*, 2002a). More recently, it has been shown that, besides ATP and ADP, 1,3-bisphosphoglycerate, a product of reaction catalysed by the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), also regulates the activity of K_{ATP} channels. It has been suggested that regulation of 1,3-bisphosphoglycerate could provide a direct link between glycolysis and membrane excitability in the heart independently of ATP (Jovanović & Jovanović, 2005). As 1,3-bisphosphoglycerate is short-lived *in vivo* and abruptly converted into 3-phosphoglycerate, a compound without direct effects on K_{ATP} channels (S.J. & A.J., unpublished results), it is likely that a close

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spatial relationship between GAPDH and K_{ATP}-channel subunits is required if 1,3-bisphosphoglycerate-mediated gating of K_{ATP} channels is to be of any physiological importance. A close proximity of two proteins in the intracellular *in vivo* environment probably requires a physical interaction between these proteins. Consequently, this prompted us to test the hypothesis that GAPDH and K_{ATP}-channel subunits in the heart interact physically with each other and to examine whether this interaction could have a role in the regulation of channel behaviour.

RESULTS AND DISCUSSION

Co-immunoprecipitation assays, if executed carefully and with the required precautions, have the potential to provide strong evidence about physical interaction between proteins (Harlow & Lane, 1999). Here, we have used an antibody raised against the SUR2A K_{ATP}-channel epitope to immunoprecipitate a cardiac membrane fraction. This antibody and strategy were described in our previous reports and found suitable to identify proteins forming sarcolemmal K_{ATP} channels (Ranki *et al*, 2001; Crawford *et al*, 2002a,b). Coomassie blue staining of the anti-SUR2A immunoprecipitate showed several proteins, most of which had been previously identified, including lactate dehydrogenase (LDH), Kir6.2, α-actin, creatine kinase, β-myosin and SUR2A (Fig 1A; Crawford *et al*, 2002a,b). One of the proteins that was clearly visible on the gel, but was not identified in previous studies, was p42 (protein of an estimated size of 42 kDa). The appearance of these polypeptide bands was blocked by incubation with the corresponding antigenic peptide, and these proteins did not precipitate if the membrane fraction was probed with a non-K_{ATP}-channel antibody (Crawford *et al*, 2002b). To identify the nature of p42, we have used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis. This method is based on enzymatic digestion of proteins from Coomassie-blue-stained gels and sequencing of the resulting peptide by mass spectrometry (Shevchenko *et al*, 1996). MALDI-TOF analysis has identified p42 as GAPDH (Fig 1B), suggesting that GAPDH was immunoprecipitated by an anti-SUR2A antibody. However, immunoprecipitations could have nonspecific contaminants (Harlow & Lane, 1999), and, therefore, we have used extreme care to provide further evidence that the presence of GAPDH in the immunoprecipitate was not artefactual. First, we have shown that there is no crossreactivity between the anti-SUR2A antibody and purified GAPDH itself (data not shown), thus excluding the possibility that GAPDH was directly precipitated with our anti-SUR2A antibody. Second, western blot analysis has shown that GAPDH is present in immunoprecipitate obtained not only with an anti-SUR2A but also with an anti-Kir6.2 antibody (Fig 2). As these two antibodies target structurally unrelated proteins, it is not likely that GAPDH would appear in both precipitates as a contaminant. Third, when two proteins are immunoprecipitated with antibodies raised against one or another protein, this is usually considered to be strong evidence of a physical association between these proteins (Harlow & Lane, 1999). Therefore, the reciprocal co-immunoprecipitation experiment was carried out, in which the anti-GADPH antibody was used to immunoprecipitate both Kir6.2 and SUR2A subunits from cardiac membrane fraction. As seen in the lower panel of Fig 2, both Kir6.2 and SUR2A could be specifically co-immunoprecipitated with the anti-GADPH antibody but not with the control IgG

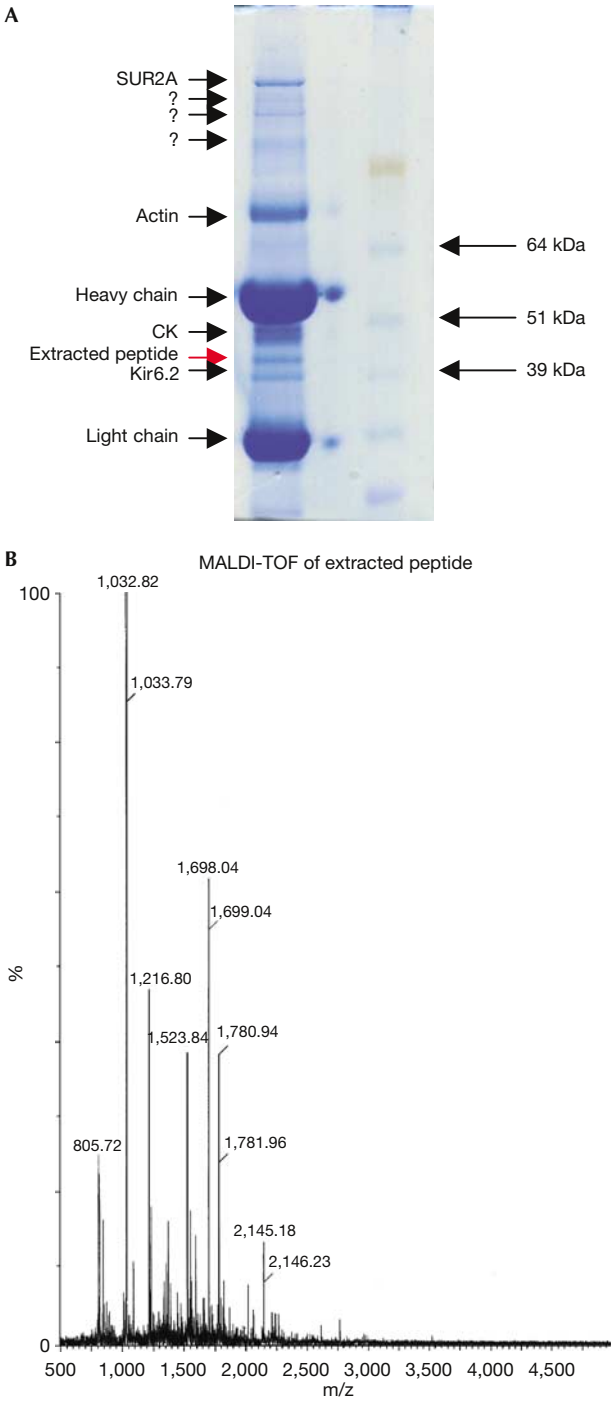

Fig 1 | Identification of peptide p42 (estimated size ~42 kDa) found in the immunoprecipitate of cardiac membrane fraction. (**A**) Coomassie blue stain of immunoprecipitate pellets obtained from cardiac membrane fraction precipitated with an anti-SUR2A antibody. The position of p42 peptide is marked by a red arrow. (**B**) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) of tryptic mass fingerprint obtained from ~42 kDa migrating protein (identified as glyceraldehyde 3-phosphate dehydrogenase).

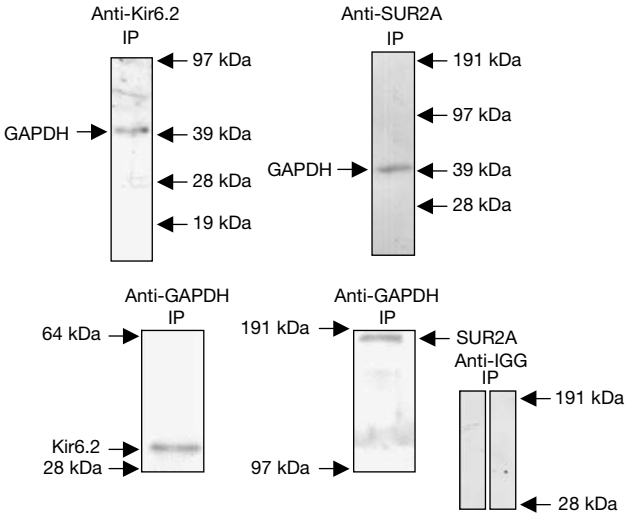

Fig 2 | Glyceraldehyde 3-phosphate dehydrogenase co-immunoprecipitates with the sarcolemmal cardiac K_{ATP}-channel protein complex and vice versa. Western blotting of anti-Kir6.2 and anti-SUR2A immunoprecipitate (IP) of cardiac membrane fraction with an anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody and western blotting of anti-GAPDH IP with anti-Kir6.2 and anti-SUR2A antibodies. Bottom right: western blotting of anti-IGG IP with anti-Kir6.2 (left lane) and anti-SUR2A (right lane) antibodies.

antibody (Fig 2). Taken together, these results strongly suggest that GAPDH associates physically with a protein that is a part of the cardiac K_{ATP}-channel protein complex. As a cardiac K_{ATP}-channel protein complex *in vivo* is composed of more proteins than Kir6.2 and SUR2A subunits (Carrasco *et al*, 2001; Crawford *et al*, 2002a,b), these results per se did not prove that GAPDH has a physical interaction with the K_{ATP}-channel subunits themselves.

However, if GAPDH activity is to have functional significance on the behaviour of K_{ATP} channels, it is likely that this would require a close spatial relationship between GAPDH and K_{ATP}-channel subunits and, possibly, a physical interaction between these proteins. To test this hypothesis, we have used genes encoding K_{ATP}-channel subunits and A549 cells as a heterologues expression system. A549 cells are cells that do not contain K_{ATP} channels, but express GAPDH (Sundararaj *et al*, 2004). The idea was to express each of the two K_{ATP}-channel subunits and to test whether GAPDH interacts physically with Kir6.2 or SUR2A when expressed alone in A549 cells. This strategy has been successful previously in detecting proteins that are physically associated with K_{ATP}-channel subunits (Crawford *et al*, 2002a,b). When the possibility that GAPDH associates with SUR2A was examined, we immunoprecipitated protein extracts of cells expressing the SUR2A subunit with an anti-SUR2A antibody and probed with an anti-GAPDH antibody and vice versa. The SUR2A subunit was absent from the anti-GAPDH precipitate and GAPDH was absent

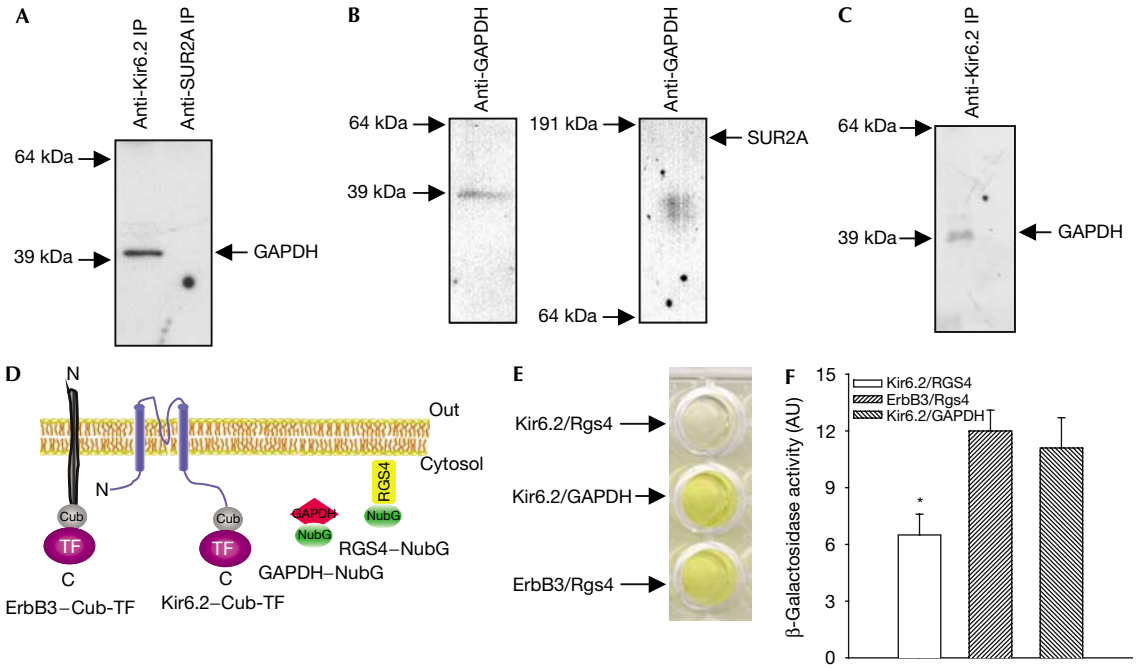

Fig 3 | Glyceraldehyde 3-phosphate dehydrogenase associates with the Kir6.2 but not the SUR2A subunit. (**A**) Western blotting with an anti-Kir6.2 or anti-SUR2A antibody of anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) immunoprecipitate (IP) of A549 cells transfected with Kir6.2 and SUR2A (alone), respectively. (**B**) Western blotting with an anti-GAPDH antibody of anti-Kir6.2 or anti-SUR2A IP of A549 cells transfected with Kir6.2 and SUR2A (alone), respectively. (**C**) Western blotting with an anti-GAPDH antibody of anti-Kir6.2 IP of A549 cells transfected with Kir6.2 (left lane) and 1–170 Kir6.2 (ΔKir6.2) amino-acid fragment (right lane). (**D**) Membrane yeast two-hybrid system (MbYTH) constructs used in this study. The Cub-TF moiety was fused to the cytosolic carboxyl terminus of ErbB3 and Kir6.2. NubG was fused C terminally to GAPDH and RGS4, an ErbB3-interacting protein. (**E**) Liquid β-galactosidase assay of yeast THY. AP4 reporter strain coexpressing different sets of bait and prey proteins (as depicted) tested in MbYTH. Yellow colour is indicative of β-galactosidase activity. (**F**) Quantitative β-galactosidase activity under same conditions as (**E**). Each bar represents mean ± s.e.m. ($n = 10$ for each). *$P < 0.01$. AU, units calculated as $1{,}000 \times OD_{420}/(OD_{600} \times \text{min})$; OD, optical density.

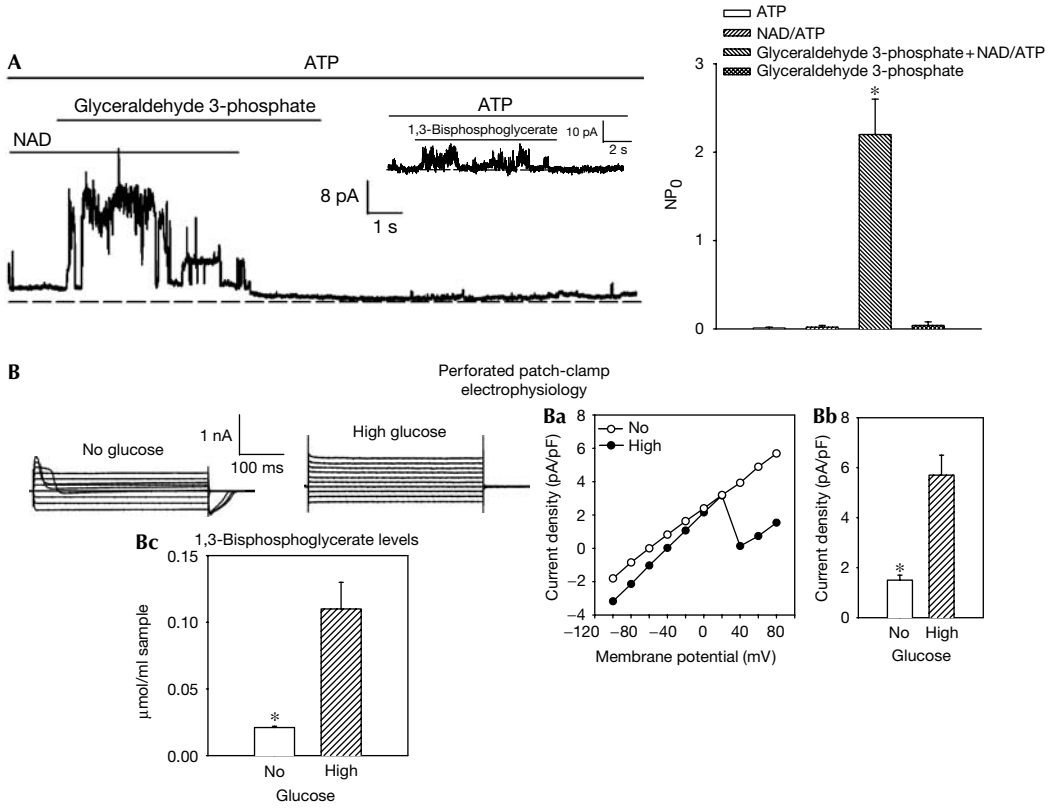

Fig 4 | Glyceraldehyde 3-phosphate dehydrogenase regulates the activity of sarcolemmal K_{ATP} channels, allowing cardiac cells to sense high glucose. (**A**) Left panel: inside-out single-channel recording of K_{ATP}-channel activity in membrane patch treated with ATP (5 mM) alone, ATP (5 mM) plus NAD (20 mM), ATP (5 mM) plus NAD + (20 mM) plus glyceraldehyde 3-phosphate (20 mM) and ATP (5 mM) alone. Holding potential, 0 mV. The dashed line corresponds to zero current levels. Inset: inside-out single-channel recording of K_{ATP}-channel activity in membrane patch treated with ATP (5 mM) alone, ATP (5 mM) plus 1,3-bisphosphoglycerate (20 mM) and ATP (5 mM) alone. Holding potential, 0 mV. The dashed line corresponds to zero current levels. Right panel: channel activity expressed as NP_0 under conditions in the left panel ($n = 6$). *$P < 0.01$ when compared with ATP alone. (**B**) Membrane currents recorded in response to 400-ms-long pulses (from −100 to +80 mV) using perforated patch-clamp electrophysiology and corresponding *I–V* relationships (**Ba**; each point is mean ± s.e.m., $n = 4$ for each). Cardiomyocytes were bathed with Tyrode solution without and with glucose (30 mM). Currents were recorded 5 min after whole-cell configuration of the patch-clamp technique was established. (**Bb**) Current density at +80 mV under conditions in (**B**). Each bar represents mean ± s.e.m. ($n = 4$). *$P < 0.01$. (**Bc**) 1,3-Bisphosphoglycerate concentration in cardiomyocytes after 5 min of incubation in solution without (no) and with (high) 30 mM glucose. Each point represents mean ± s.e.m. ($n = 3$ for each). *$P < 0.01$.

from the anti-SUR2A precipitate, which suggests that there is no physical interaction between the SUR2A subunit and GAPDH (Fig 3). In contrast, in anti-Kir6.2 precipitate obtained from cells expressing Kir6.2, GAPDH was detected (Fig 3). Also, Kir6.2 was found in anti-GAPDH precipitate from cells expressing Kir6.2. These results suggest that Kir6.2 and GAPDH physically associate. Previously, it has been reported that creatine kinase associates physically with the SUR2A subunit and that LDH associates with both Kir6.2 and SUR2A subunits (Crawford *et al*, 2002a,b). Here, no contact was observed between the amino-terminal half of Kir6.2 protein and GAPDH (Fig 3), which suggests that the interaction between Kir6.2 and GAPDH is not by means of the N-terminus. This finding is in accord with previous reports that the N-terminus interacts with another part of the K_{ATP}-channel protein complex, M-LDH (Crawford *et al*, 2002b), and that Kir6.2 and GAPDH interact probably by means of the part of Kir6.2 that is closer to the carboxyl terminus. Thus, these results further confirmed that there is a physical interaction between Kir6.2 and GAPDH. It should be noted that all methods we have used thus far are antibody-based and that was why we decided to test this hypothesis using a non-antibody-based method such as the membrane yeast two-hybrid system (MbYTH; Stagljar *et al*, 1998; Thaminy *et al*, 2003; Iyer *et al*, 2005). This modified form of the YTH assay is one of several hybrid protein approaches that are capable of detecting interactions occuring at cellular membranes, and its principles have been described in detail elsewhere (Stagljar & Fields, 2002; Stagljar, 2003; Iyer *et al*, 2005). To test whether Kir6.2 and GAPDH interact in MbYTH, we used full-length Kir6.2 fused to Cub-TF as bait and GAPDH fused to NubG as prey (Fig 3D). As shown in Fig 3E, coexpression of Kir6.2–Cub-TF with a NubG-tagged GAPDH resulted in the activation of the yeast reporter gene system as exemplified by the strong β-galactosidase activity. Conversely, Kir6.2–Cub-TF did not interact with an unrelated human regulator of G protein signalling (Rgs4) protein fused to NubG, indicating that the Kir6.2–GAPDH interaction is specific. As a positive control in MbYTH, the previously described interaction between the human epidermal growth factor receptor 3 (ErbB3) and Rgs4 was used (Thaminy *et al*,

2003). These results confirm our findings—identified by co-immunoprecipitation assay—and provide strong evidence that Kir6.2 and GAPDH interact physically. In this regard, GAPDH is the first accessory protein found to associate with the Kir6.2 subunit alone.

The physical association between Kir6.2 and GAPDH may have a significant functional consequence on K_{ATP}-channel behaviour, as this enzyme catalyses the reaction in which 1,3-bisphosphoglycerate is produced. 1,3-Bisphosphoglycerate has recently been suggested to be an endogenous K_{ATP}-channel opener that has the ability to activate sarcolemmal K_{ATP} channels even in the presence of high levels of intracellular ATP (Jovanović & Jovanović, 2005). Therefore, we have tested whether GAPDH can regulate the activity of sarcolemmal K_{ATP} channels by virtue of its enzymatic activity. After excision of a membrane patch from guinea-pig ventricular cardiomyocyte, an ATP-sensitive K^+ current was observed (Fig 4A). Addition of NAD+ (20 mM) on the intracellular face of the excised membrane patch did not affect the ATP-induced inhibition of the channel activity (Fig 4A). However, when both NAD+ (20 mM) and glyceraldehyde 3-phosphate (20 mM) were concomitantly in contact with the intracellular side of sarcolemma, K_{ATP} channels opened despite high intracellular ATP (5 mM; Fig 4A). Removal of NAD+ alone resulted in a significant decrease in K_{ATP}-channel activity, indicating that glyceraldehyde 3-phosphate per se did not activate K_{ATP} channels. These results are in accord with the idea that GAPDH is a part of the sarcolemmal K_{ATP}-channel protein complex, where it may activate K_{ATP} channels by producing 1,3-bisphosphoglycerate, a compound that is reported to act as a K_{ATP}-channel opener (Jovanović & Jovanović, 2005). Indeed, when 1,3-bisphosphoglycerate was added on the intracellular face of the excised membrane patch, it induced activation of K_{ATP} channels, which suggests that this intermediate product of glycolysis acts on K_{ATP}-channel proteins or closely associated subunits.

For a long time, it has been proposed that glycolytic ATP may be more important for regulation of K_{ATP} channels than ATP obtained by oxidative phosphorylation (Weiss & Lamp, 1987). On the basis of their data, Weiss & Lamp (1987) have suggested that the glycolytic enzymes must be attached physically to the cardiac K_{ATP} channels to be able to regulate their behaviour in the described manner. The present study is the first to provide direct evidence in favour of this hypothesis. The existence of a glycolytic signalling pathway in regulating K_{ATP} channels probably allows subtlety in the regulation of these channels. A 30 mM portion of glucose was sufficient to activate sarcolemmal K_{ATP} channels and this was associated with increased intracellular levels of 1,3-bisphosphoglycerate (Fig 4C), which suggests that 1,3-bisphosphoglycerate may have a role in K_{ATP}-channel regulation *in vivo*. It is possible that glucose metabolism, independently of ATP production or any other factors, may be transduced into changes in membrane excitability, which make the heart responsive to changes in glucose levels irrespective of the conditions of other metabolic pathways and/or total energy status. This idea would be in accord with our previous findings showing that hyperglycaemia may regulate cardiac membrane excitability by stimulation of glucose transport and production of intracellular 1,3-bisphosphoglycerate (Jovanović & Jovanović, 2005). It seems that physical contact between Kir6.2 and GAPDH secures a close spatial relationship between the pore-forming subunit and 1,3-bisphosphoglycerate-producing enzyme, which, in turn, allows fast sensing of glucose levels.

METHODS

Methods are presented in the supplementary information online. **Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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