Disruption of cytoskeletal integrity impairs G\textsubscript{i}-mediated signaling due to displacement of G\textsubscript{i} proteins

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1 integrins play a crucial role as cytoskeletal anchorage proteins. In this study, the coupling of the cytoskeleton and intracellular signaling pathways was investigated in \(\beta1\) integrin deficient (\(-/-\)) embryonic stem cells. Muscarinic inhibition of the L-type \(\text{Ca}^{2+}\) current (\(I_{\text{Ca,L}}\)) and activation of the acetylcholine-activated \(K^{+}\) current (\(I_{\text{k,ACH}}\)) was found to be absent in \(\beta1\) integrin \(-/-\) cardiomyocytes. Conversely, \(\beta\) adrenoceptor-mediated modulation of \(I_{\text{Ca}}\) was unaffected by the absence of \(\beta1\) integrins. This defect in muscarinic signaling was due to defective G protein coupling. This was supported by deconvolution microscopy, which demonstrated that \(G_{i}\) exhibited an atypical subcellular distribution in the \(\beta1\) integrin \(-/-\) cardiomyocytes. A critical role of the cytoskeleton was further demonstrated using cytochalasin D, which displaced \(G_{i}\) and impaired muscarinic signaling. We conclude that cytoskeletal integrity is required for correct localization and function of \(G_{i}\)-associated signaling microdomains.

Introduction

Heart function, in particular the rhythm (chronotropy) and force of contraction (inotropy), is regulated by \(\beta\)-adrenoceptors and muscarinic type 2 (M2)\textsuperscript{*} receptor–mediated activation/inhibition of L-type \(\text{Ca}^{2+}\) current (\(I_{\text{Ca,L}}\)) (chronotropy and inotropy) and acetylcholine-activated \(K^{+}\) current (\(I_{\text{k,ACH}}\)) (chronotropy) (Sakmann et al., 1983). The signaling cascade involved in the modulation of \(I_{\text{Ca}}\) has been described in detail (for review see Trautwein and Hescheler, 1990). In vivo (Redfern et al., 1999) and in vitro studies have suggested that pertussis toxin sensitive G proteins of the \(G_{i}\) family mediate muscarinic depression of \(I_{\text{Ca}}\) (Wickman and Clapham, 1995; Ye et al., 1999; Chen et al., 2001) and activation of \(I_{\text{k,ACH}}\) (Sowell et al., 1997). The involvement of NO in M2 receptor–mediated depression of \(I_{\text{Ca}}\) in adult heart is controversial (Han et al., 1998; Vandecasteele et al., 1999). We previously demonstrated that the mechanism of muscarinic regulation of \(I_{\text{Ca}}\) switches during embryonic development from a NO-mediated depression of basal \(I_{\text{Ca}}\) to direct \(G_{i}\)-mediated lowering of adenylyl cyclase (AC) activity in late stage cardiomyocytes (Ji et al., 1999).

Given that cytoskeleton-associated membrane receptors have been implicated in several heart disorders (for review see Towbin, 1998), we determined whether \(\beta1\) integrin receptors modulate ionic conductances critical for heart function. Since \(\beta1\) integrin \(-/-\) mice died shortly after implantation, chimeric mice or in vitro differentiated embryonic stem (ES) cell–derived \(\beta1\) integrin \(-/-\) cells (Fassler et al., 1996) were used. Previous studies revealed several abnormalities in \(\beta1\) integrin–deficient cells including migration and homing defects of haematopoietic cells (Hirsch et al., 1996), alterations in vasculo- and angiogenesis (Bloch et al., 1997), and severely disturbed cardiomyogenesis (Fassler et al., 1996). Although the biophysical characteristics of ionic conductances of \(\beta1\) integrin \(-/-\) ES cell–derived cardiomyocytes appeared normal, the action potentials generated by deficient cardiomyocytes exhibited a primitive phenotype at all stages of differentiation (Fassler et al., 1996). Furthermore,
β1 integrin−/− ES cell–derived cardiomyocytes exhibited abnormalities in cytoskeletal integrity. Herein, we investigated the role of β1 integrins in hormonal regulation of ICa using β1 integrin−/− cardiomyocytes.

We report that β1 integrin−/− cardiomyocytes lacked muscarinic signaling, whereas β-adrenoceptor–mediated stimulation of ICa remained intact. This specific signaling defect was found to be related to spatial displacement of G proteins of the Gi-family.

Results
Muscarinic depression of basal ICa was found to be absent in β1 integrin−/− ES cell–derived cardiomyocytes
As previously reported (Ji et al., 1999), prominent inhibition (44.7 ± 8%; Fig. 1 d) of basal ICa density was observed upon application of the muscarinic agonist carbachol (CCh) (1 μM) (Fig. 1 a) in a large percentage of early developmental stage (EDS) cardiomyocytes (69%, n = 13; Fig. 1 c). Similarly, in the β1 integrin−/− ES cell–derived EDS cardiomyocytes, basal ICa density was depressed in half of the cells tested by 44 ± 8% (n = 10). In contrast, β1 integrin−/− cells did not exhibit CCh-mediated inhibition of basal ICa (n = 37) (Fig. 1 b, c, and d). To rule out that a shift in the voltage dependence of ICa underlies the CCh effect after the step to 0 mV, the current–voltage (I/V) relationship was defined in the presence and absence of CCh (n = 5). As depicted in Fig. 1 f, no CCh-mediated depression of ICa was observed at the chosen potentials.

β-adrenoceptor–mediated stimulation of ICa was preserved in β1 integrin−/− cardiomyocytes
The absence of muscarinic signaling could be explained by perturbed intracellular signaling components including AC, cAMP, or protein kinase A. Therefore, we determined whether additional regulatory pathways, and in particular the β-adrenoceptor–mediated stimulation of ICa, were defective in β1 integrin−/− cardiomyocytes. All wild-type (wt) late developmental stage (LDS) cardiomyocytes exhibited an increased current density (41.4 ± 6.8%, n = 11; Fig. 2, a and d) after prestimulation with the β-adrenoceptor agonist isoprenaline (ISO) (0.1 μM). Similarly, ISO increased the ICa density by 64.6 ± 12% (n = 20) in all β1 integrin−/− cardiomyocytes tested (Fig. 2, b and e). This ISO-induced upregulation of ICa was accompanied by a slight left shift in the I/V relationship (n = 5). However, superfusion with varying concentrations (1–10 μM) of CCh after β-adrenergic prestimulation did not suppress ICa in these cardiomyocytes (n = 20; Fig. 2, c and e). By contrast, CCh-inhibited ISO prestimulated ICa by 37.3 ± 7% (n = 11; Fig. 2 c) in 82% of wt LDS cells.

To address whether the signaling defect was caused by a quantitative reduction of muscarinic receptors, [3H]QNB saturation binding studies were performed. There were no differences in the Bmax values for β integrin−/− (33.7 ± 1.3 fmol/mg membrane protein) and wt ES cells (33.3 ± 3.1 fmol/mg membrane protein); therefore, a change in total muscarinic binding sites was excluded. Furthermore, immunocytochemical studies proved that Goi, is expressed in both, wt (Fig. 2 f) and β1 integrin−/− (Fig. 2 g) cardiomyocytes.

Figure 1. Muscarinic modulation of basal ICa is absent in β1 integrin−/− ES cell–derived cardiomyocytes. (a) Time course of peak ICa in a representative wt EDS ES cell–derived cardiomyocyte. Prominent inhibition of basal ICa was observed after application of carbachol (1 μM). The CCh effect could be reversed by washout. Each data point in the time course was evoked by a 20-ms depolarization from a holding potential of −50 mV to a test potential of 0 mV. The upper trace indicates the holding current. (b) A representative β1 integrin−/− EDS cardiomyocyte demonstrates the absence of the CCh-induced inhibition of basal ICa (50 ms depolarization from −50 mV to 0 mV). The time of current recordings (1 and 2, inset) is indicated. (c) Percentage of wt and β1 integrin−/− cardiomyocytes displaying CCh-induced inhibition of basal ICa. (d) ICa density in wt cells with CCh response. (e) ICa density in β1 integrin−/− cells in absence and presence of CCh. (f) Traces (left) and I/V relationship (right) of Iα, recorded in an ES cell–derived EDS β1 integrin−/− cardiomyocyte (1, control; 2, in presence of CCh). ICa was evoked by 50-ms depolarizations from −40 mV to +40 mV in 10-mV increments (holding potential, −50 mV). *Indicates statistical significance (paired t test, p-value < 0.05).

Muscarinic signaling was restored in β1 integrin rescue ES cell–derived cardiomyocytes
To confirm that the muscarinic signaling defect was evoked specifically by the absence of the β1 integrin gene, we next investigated the hormonal modulation of ICa in β1 integrin rescue ES cell–derived cardiomyocytes. As depicted in Fig. 3 a, muscarinic receptor activation inhibited basal ICa by 32.3 ± 6% (Fig.
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3 d) in almost half (42%, n = 19; Fig. 3 d) of the EDS cells. In 36% (n = 33; Fig. 3 c) of the LDS β1 integrin rescue cardiomyocytes, CCh (1-10 μM) suppressed ISO prestimulated I_{Ca} by 21 ± 6% (Fig. 3, b and d). The reduction in muscarinic inhibition and altered morphology of β1 integrin reconstituted cells (see Fig. 5 i) suggests that the rescue was incomplete.

Hence, functional responses suggest that β1 integrin deletion resulted in a selective loss of muscarinic modulation of both, basal and ISO-prestimulated I_{Ca}, which could be restored upon reexpression of β1 integrins.

Nitric oxide and atrial natriuretic peptide–mediated depression of I_{Ca} is functionally intact in β1 integrin^{−/−} cardiomyocytes

We next asked whether other signaling pathways involved in the regulation of I_{Ca} besides the β-adrenoceptor were affected by the β1 integrin deficit. Previous work in our group demonstrated constitutive nitric oxide synthase (NOS) 2 activity in EDS cells (Ji et al., 1999). Consistent with this observation, the NOS inhibitor N-methyl-L-arginine (L-NMMA, 0.2 mM) enhanced I_{Ca} by 30 ± 4% in the majority (75%, n = 8) of β1 integrin^{−/−} EDS cells tested (Fig. 3 e). Similarly, as described for wt cardiomyocytes (Ji et al., 1999; Maltsev et al., 1999), application of the nonselective phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (0.2 mM) increased the I_{Ca} density by 67.4 ± 7% (n = 7) in β1 integrin^{−/−} EDS cardiomyocytes. The PDE2 subtype-specific antagonist erythro-9-(2-hydroxy-3-nonyl)adenine (30 μM) also increased I_{Ca} density by 28.4 ± 5% (n = 7) in β1 integrin^{−/−} EDS cardiomyocytes. Together, these findings demonstrate that these principal signaling cascades remained intact in β1 integrin^{−/−} cardiomyocytes. By contrast to the missing CCh effect,
atrial natriuretic peptide (ANP; rat fragment 3–28, 0.1 M) depressed basal I_{Ca} density by 31.9/3.9% (n = 8) in integrin−/− cardiomyocytes (Fig. 3 f) as previously reported for wt ES cell–derived cardiomyocytes (Ji et al., 1999). The defect in muscarinic signaling was found to reside at the G protein level. M2 receptor expression and key intracellular signaling cascades appeared intact in integrin−/− cardiomyocytes. Therefore, we next examined whether the signaling defect was related to dysfunction of the pertussis toxin–sensitive G protein G_{i}. We previously reported that intracellular application of GTP-γ-S in guinea pig cardiomyocytes resulted in a slight stimulation of I_{Ca} (Hescheler et al., 1987), due to the opposing action of G_{s} and G_{i} proteins. Fig. 4 demonstrates that GTP-γ-S induced a small increase in I_{Ca} by 35.9/18% (n = 11; Fig. 4, c and d) in wt LDS cardiomyocytes. Similarly, in integrin-reconstituted ES cell–derived cardiomyocytes, GTP-γ-S induced a small increase (46 ± 18.5%, n = 4) of I_{Ca} (data not shown). By contrast, GTP-γ-S caused a significant augmentation of I_{Ca} density (130.4 ± 37%, n = 12; Fig. 4, b–d) in integrin−/− LDS cardiomyocytes that otherwise declined steadily in the absence of GTP-γ-S (Fig. 4 a). This suggests that the predominant action of GTP-γ-S is to activate G_{i} and that the integrin-related signaling defect is due to a disturbance in G protein signaling.

Unlike adult cardiomyocytes, EDS and LDS cells did not respond to agonists of other G_{i}-coupled receptors including adenosine or somatostatin. Therefore, we used GTP-γ-S-
mediated activation of $I_{K,ACL}$, to examine $G_i$-coupling pathways. Although $I_{K,ACL}$ could be activated in wt ($n = 14$) and $\beta 1$ integrin rescue cells ($n = 4$, data not shown), no GTP-$\gamma$S-evoked activation of $I_{K,ACL}$ could be observed in $\beta 1$ integrin $^{-/-}$ cardiomyocytes ($n = 14$), consistent with the effect on $I_{Ca}$ regulation.

$G_{Go}$ distribution is altered in $\beta 1$ integrin $^{-/-}$ ES cell-derived cardiomyocytes

Immunostaining was used to investigate the patterns of $G$ protein expression. Antibodies against $G_s$ subunits were used because of the large number of possible $\beta$ and $\gamma$ subunits that could be involved in receptor coupling. Differences in the cellular distribution of $G_s$ and $G_{Go}$, $G_{Go}$, the M2 receptor, and L-type $Ca^{2+}$ channels (VDCC) (data not shown) were found in wt cells. The vast majority of murine embryonic (68.2 ± 11.7%, $n = 489$) and wt ES cell-derived (64.0 ± 24.0%, $n = 210$) cardiomyocytes displayed a network-like pattern of $G_s$, whereas relatively few (20.8 ± 3.6%, $n = 441$) $\beta 1$ integrin $^{-/-}$ cardiomyocytes displayed this pattern. Consistent with this finding, nearly all cells in whole embryoid bodies (EBs) (90.8 ± 7.9%, $n = 1,000$, five experiments) exhibited a linear network-like distribution pattern of $G_s$, whereas few (10.5 ± 6.1%, $n = 800$, 4 experiments) $\beta 1$ integrin $^{-/-}$ EBs had this pattern.

We next investigated the subcellular distribution of these $G$ proteins at higher resolution using deconvolution microscopy. Cardiomyocytes, which were identified by $\alpha$-actinin staining (Kolossov et al., 1998), exhibited a linear- and network-like distribution pattern of $G_s$ in wt (Fig. 5 a) cells and diffuse staining of $G_s$ in the wt (Fig. 5 c) cells. $\beta 1$ integrin $^{-/-}$ ES cell-derived cardiomyocytes exhibited diffuse staining for $G_s$ (Fig. 5 b) and $G_o$ (Fig. 5 d). Consistent with our functional results, a network-like distribution was also observed in the $\beta 1$ integrin rescue cells (Fig. 5 e), whereas $G_o$ was diffusely distributed throughout all cells (data not shown). In addition to distinct patterns of $G_s$, distribution, $\beta 1$ integrin $^{-/-}$ ES cell-derived cardiomyocytes had different shapes as compared with the wt (Fig. 5, g and h) (Fassler et al., 1996). $\beta 1$ integrin rescue cells showed a partial morphological reversal (Fig. 5 i).

Together, these findings are consistent with the electrophysiological results, which suggest that $G_s$ underlies the muscarinic signaling defect.

$G_{Go}$ colocalizes with focal contact proteins

A similar network-like distribution of $G_o$, has been reported for $\beta 1$ integrin focal adhesion-associated molecules such as talin and vinculin (Kostin et al., 1998; Imanaka-Yoshida et al., 1999). We have identified a unique architecture for $G_o$ in the $\beta 1$ integrin $^{-/-}$ cells and found that talin and vinculin exhibit a similar diffuse distribution as $G_o$ (data not shown). We subsequently analyzed the interaction between $\beta 1$ integrins, talin, and vinculin with $G_o$ using deconvolution and electron microscopy. These approaches failed to demonstrate a close colocalization of $\beta 1$ integrins and $G_o$ (Fig. 6 a). Deconvolution microscopy did reveal, however,
spatial colocalization between Goαi and vinculin and talin (Fig. 6, b and c) in wt cells. Electron microscopy of double immunogold–labeled cells further revealed a relatively close colocalization between Goαi and vinculin (40–150 nm) in embryonic (Fig. 6 d) and adult mouse heart (e). Vinculin and Goαi were labeled with 5- and 15-nm gold particles, respectively. The distance between pairs of gold particles was 40–150 nm. Bars: (a and b) 6 μm; (c) 2.5 μm; (d and e) 90 nm.

Discussion

In this study, we provide evidence that β1 integrin deletion results in a selective loss of muscarinic signaling in cardiomyocytes. This signaling defect is clearly related to the lack of β1 integrins because reconstitution with β1 integrins reversed the altered morphology and the muscarinic signaling in β1 integrin−/− ES cells.

Evidence for Gi dysfunction in β1 integrin−/− cardiomyocytes

To date, β integrins have been shown to play an important role primarily in tyrosine kinase–mediated signaling (see for review Ruoslahti, 1997). Furthermore, integrin-dependent cell anchorage regulates the efficiency of signaling from G protein–coupled receptors (Short et al., 2000). In β1 integrin−/− cardiomyocytes, except for an absence of muscarinic regulation of ICa, and IK,ACh, no other signaling defects could be detected. Our functional data pinpoint the signaling defect at the level of Gi, as intracellular dialysis with GTP-γ-S resulted in pronounced enhancement of Gs-mediated stimulation of ICa and defective activation of IK,ACh in the β1 integrin−/− cardiomyocytes. In previous investigations, antibody neutralization of integrins in situ revealed a role for integrins in the modulation of ICa or IK, (Wu et al., 1998). A recent study found that cat cardiomyocytes cultured on laminin-coated cover slips had disturbed cholin-
ergic and β-adrenergic responses (Wang et al., 2000a,b). These data are consistent with our findings and indicate a critical role for integrins in both receptor coupling and ion channel modulation. Clark et al. (1998) suggested that changes in integrin matrix interactions might underlie defects in signaling molecule clustering. Herein, we provide mechanistic evidence that receptor expression remain unchanged upon β1 integrin deletion; however, the altered distribution pattern of G proteins is observed only under conditions that also result in a muscarinic signaling defect.

Role of the cytoskeleton in G protein function

Our work also demonstrates a focal adhesion–related intracellular distribution for G, but not Gαo and Gαs. The idea that components of the cytoskeleton can determine G protein localization and function (for review see Janmey, 1998) has been proposed before by Ueda et al. (1997) and Ibarondo et al. (1995) who found a direct association between the γ12 subunit and between Gαo/Gαs, and actin filaments, respectively. Furthermore, the activation and release of Gαo from the cytoskeleton has been reported (Sarnidahl et al., 1996). Consistent with our functional findings, the subcellular distribution of G is severely altered by the β1 integrin deficiency in cardiomyocytes. Our findings are also consistent with previous studies using antisense approaches (Kleuss et al., 1991, 1993), where, in contrast to G protein protein–mediated receptor–effector coupling could not be reconstituted at all by other G proteins. This is also supported by recent work, where selective G protein β/γ complexes were shown to be involved in the inhibition of neuronal Ca2+ channels (Diverse-Pieriussi et al., 2000). This study suggests that the specificity of signal transduction is not only related to protein–protein interaction, but also to the spatial arrangement of signaling components.

Due to the similar cellular distribution observed for G and that reported for the focal contact–associated proteins talin and vinculin, we tested their spatial relation to one another in cardiomyocytes. In wt cardiomyocytes, Gαo and both talin and vinculin colocalized, whereas they did not in β1 integrin−/− cardiomyocytes. The association between these molecules was further demonstrated by inhibiting the polymerization of G actin to F actin with cytochalasin D (Vasioukhin et al., 2000), further demonstrated by inhibiting the polymerization of G α2 with KOH). For perforated patch clamp recordings, amphotericin B (Sigma-Aldrich). Whole cell or perforated patch measurements yielded similar results. Depolarizing pulses lasting 20 or 50 ms were applied at a frequency of 0.33 and 0.2 Hz, respectively, with an Axopatch 200-A (Axon Instruments, Inc.) or an EPC-9 amplifier (Heka). For most recordings, currents were leak subtracted using a P/0 leak subtraction protocol (trace in Fig. 3, not leak subtracted). Data were acquired at a sampling rate of 2–10 kHz, filtered at 1 kHz, stored on hard disk, and analyzed offline using the ISO (MFK) or Pulse/Pulse-Fit (Heka) software package. Analysis of the (CCh) and the ISO effects was performed as previously described (Ji et al., 1999). Murine embryonic (E11.5, E17.5) ventricular cardiomyocytes were harvested from superovulated mice (Fleischmann et al., 1998). The heart was removed from the embryo, and the cardiomyocytes were isolated by collagenase digestion.

Cytochalasin D treatment

~12 h after dissociation, murine embryonic cardiomyocytes were treated with cytochalasin D for times indicated (15 min–16.5 h). Murine embryonic cardiomyocytes were incubated with 10 μM cytochalasin D and ES cell–derived cardiomyocytes with 20 μM cytochalasin D.

Electrophysiology

Only spontaneously beating single cardiomyocytes were selected for patch clamp recordings as previously described (Ji et al., 1999; Viatchenko-Karpinski et al., 1999). Whole cell or perforated patch measurements yielded similar results. Depolarizing pulses lasting 20 or 50 ms were applied at a frequency of 0.33 and 0.2 Hz, respectively, with an Axopatch 200-A (Axon Instruments, Inc.) or an EPC-9 amplifier (Heka). For most recordings, currents were leak subtracted using a P/0 leak subtraction protocol (trace in Fig. 3, not leak subtracted). Data were acquired at a sampling rate of 2–10 kHz, filtered at 1 kHz, stored on hard disk, and analyzed offline using the ISO (MFK) or Pulse/Pulse-Fit (Heka) software package. Analysis of the (CCh) and the ISO effects was performed as previously described. Only those cells exhibiting a change in ICa, density of >5% after ISO or CCh application were analyzed. The GTPγS effect was compared with the response of untreated cells on the same experimental day. The average change in ICa, density was calculated during the period of 180 s after break in (wt, n = 9; β1 integrin−/−, n = 10; rescue cardiomyocytes, n = 5) and subtracted from the GTPγS response. 

Thus, we propose that β1 integrin deletion results in altered cytoskeletal architecture due to the loss of important anchorage proteins. This causes spatial displacement of Gαi proteins and both a physical and functional disruption of signaling microdomains.

Materials and methods

ES cell preparation

Studies used ES cells of the D3 line (wt) (Doetschman et al., 1985; Wobus et al., 1991), the heterozygous β1 integrin−/+ ES cell line, the β1 integrin−/− ES cell line generated on D3 background (Fassler et al., 1995), and the β1 integrin rescue ES cell line. This line was generated by reexpressing β1 integrins in β1 integrin−/− ES cells under control of the phosphoglycerate kinase promoter. wt and β1 integrin−/+ ES cells were cultured and differentiated into spontaneously beating cardiomyocytes as previously described (Wobus et al., 1991). E11.5 ES cells were derived from EBs plated for 3–4 d and LID cells from EBs 9–12 d after plating. β1 integrin−/− and β1 integrin rescue ES cells were grown on gelatin-coated cover slips without feeder cells in medium supplemented with 5 ng/ml recombinant human leukemia inhibitory factor (Fassler et al., 1996). β1 integrin−/− ES cells were cultured for 3 d in hanging drops and 3 d in suspension before plating on gelatin-coated plates. Because of the delayed onset of beating, β1 integrin−/− cardiomyocytes derived from EBs plated for 5–10 d were defined as EDS, and those cultured for 14–18 d were defined as LID cells (Fassler et al., 1996). β1 integrin rescue ES cells were maintained in presence of G418 (300 μg/ml). Single cardiomyocytes were isolated from clusters of spontaneously beating areas as previously described (Ji et al., 1999). Murine embryonic (E11.5, E17.5) ventricular cardiomyocytes were harvested from superovulated mice (Fleischmann et al., 1998). The heart was removed from the embryo, and the cardiomyocytes were isolated by collagenase digestion.
Data are expressed as mean ± SEM, paired or unpaired Student's t test was used to analyze electrophysiological data, and a p-value of <0.05 was considered significant.

Radioligand binding assays
EBs were harvested, and membrane homogenates were prepared. In brief, homogenates were incubated for 3 h at 22°C in 25 mM sodium phosphate (pH 7.4) containing 5 mM MgCl₂. Saturation binding experiments were carried out with [3H]N-quinuclidinyl benzilate ([3H]QNB; 42.0 Ci/mmol; NEN Life Science Products). Binding was performed with saturating concentrations of each radioligand (2 nM), and nonspecific binding was defined as binding in the presence of 2.5 μM atropine. Protein concentrations were determined by the Bradford method.

Immunocytochemistry
Whole EBs, EB and murine embryonic heart-derived isolated cells were incubated overnight with antibodies at 4°C. Antibodies included mouse anti-α-actinin (1:800; Sigma-Aldrich), monoclonal antialtalin or anti-actinulin (1:100; Chemicon International), polyclonal rat anti–mouse β1 integrin (Sigma-Aldrich), rabbit polyclonal anti-M2 receptor (1:2,000; R&D Systems), α/1 (1:200; Alomone Labs), and anti–G protein α-subunits. G-protein data and measured PSFs, calculated from fluorescent beads with a diameter of 1 μm (Molecular Probes, T-14792), were used. A conjugated gradient maximum likelihood deconvolution algorithm with 60 iterations were performed on the image stacks. Output intensities were scaled between gray value 0 and 255. Generally, theoretical and measured PSFs generated comparable results with slightly higher resolution in the resulting images.

Morphological analysis
The distribution pattern of Gαi was analyzed by fluorescence microscopy (ZEIII in ves (EDS, T + 3; LDS, 7 + 11), β1 integrin (E2D, 6 + 10; LDS, 6 + 14), and murine embryonic cardiomyocytes (E11.5/12.5, E17.5). All cardiomyocytes, identified by α-actinin containing were analyzed on each slide (ranging from 49 to 174). Statistical analysis was performed using the unpaired Student’s t test. DIC pictures were taken using an appropriately equipped Axiovert 135 microscope (ZEIII).

Deconvolution microscopy
For each specimen, an image stack (64 planes) was collected using an inverted ZEIII Axiovert 100 M with a ZEIII 63 Planapochromat oil immersion objective. Bandpass filter sets for Cy2 and Cy3 (AHF) without overlap in the fluorescence emission spectrum were used. The thickness of each optical section was 100 nm to assure appropriate optical resolution for the subsequent iterative deconvolution process. A three-dimensional deconvolution algorithm was applied to the image stacks (Schafer, 1997) to increase the image resolution for colocalization (KS 400; ZEIII). Both theoretical point-spread functions (PSFs) based on microscope and specimen data and measured PSFs, calculated from fluorescent beads with a diameter of 1 μm were used. A conjugated gradient maximum likelihood deconvolution algorithm with 60 iterations were performed on the image stacks. Output intensities were scaled between gray value 0 and 255. Generally, theoretical and measured PSFs generated comparable results with slightly higher resolution in the resulting images by using the measured PSFs.

Immunogold labeling
Adult and embryonic mouse or rat heart tissue was perfused with 4% PFA in 0.1 M PBS or with 2% PFA/2% glutaraldehyde at 4°C. After fixation, the tissue was embedded with araldit or LR-white. The tissue grids were treated with 50 mM TBS for 15 min and blocked with 4% BSA in TBS for 1 h at room temperature. Then, primary antibodies against vinculin or talin (1:30–50) or β1 integrin (1:100; PharMingen) and a rabbit polyclonal antibody for Gαi were used at room temperature for 2 h. Grids were rinsed four times with 0.05% Tween-20–TBS, incubated with 5-nm gold-conjugated anti–mouse (or anti–rabbit) IgG (1:30–50) for 1 h, and treated with biotinylated goat anti–rabbit or anti–mouse (1:30; Dako) for 1 h at room temperature. Finally, specimens were incubated with 15-nm gold-labeled streptavidin (1:30; Zymed Laboratories) for 1 h followed by 2% glutaraldehyde fixation for 10 min. For contrast, grids were treated with 2% uranylacetate in 70% ethanol for 20 min and PBS for 7 min.

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