Gs and adenylyl cyclase in transverse tubules of heart: implications for cAMP-dependent signaling

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Laflamme, Michael A., and Peter L. Becker. Gs and adenylyl cyclase in transverse tubules of heart: implications for cAMP-dependent signaling. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1841–H1849, 1999.—The transverse tubules are highly specialized invaginations of the cardiac sarcolemmal membrane involved in excitation-contraction (EC) coupling. Several proteins directly involved in EC coupling have been shown to reside either in the transverse tubular membrane or in closely associated structures. With the use of immunofluorescence microscopy, we have found that Gs and adenylyl cyclase, key elements in the β-adrenergic signal transduction cascade, are essentially homogeneously distributed throughout the transverse tubular network of isolated rat ventricular myocytes. Gs, in particular, was much more abundant within the transverse tubular membrane than in the peripheral sarcolemma. Furthermore, both proteins are also present in the intercalated disk region. The location of these elements of the cAMP-signaling cascade within a few micrometers of every inotropic target suggests that control and action of this second messenger is quite local. Furthermore, a similar distribution is likely for negatively inotropic receptor systems that oppose Gs-linked receptors at the level of adenylyl cyclase. Thus, in addition to their role in EC coupling, transverse tubules appear to be the primary site for signaling by inotropic agents.

signal transduction; immunolocalization

The mammalian cardiac action potential is transduced to a calcium signal at specialized junctions between the sarcolemmal and sarcoplasmic reticulum (SR) membrane compartments (8). The bulk of these junctions reside along the transverse tubules (T tubules), invaginations of the sarcolemma that permit the rapidly propagating electrical signal to penetrate deep within the cell. By allowing release of the more slowly diffusing activator calcium to occur very close to the contractile proteins, T tubules ensure a more rapid and synchronous onset of contraction. The second messenger cAMP is arguably the most important modulator of cardiac contractility, not only regulating the proteins directly involved in excitation-contraction (EC) coupling but also regulating proteins that shape the resulting calcium transient. The synthesis of cAMP is modulated by a variety of external hormones and neurotransmitters, including the sympathetic agonist norepinephrine, acting at cell surface receptors. These, in turn, regulate the activity of the enzyme adenylyl cyclase via heterotrimeric GTP-binding proteins (10).

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Materials and Methods

Antibodies. Gs was detected with rabbit polyclonal antiserum (Biodesign International, Kennebunk, ME) raised against a peptide corresponding to the carboxy terminus (amino acid residues 385–394) of the α-subunit of the rat Gs (30). Other polyclonal antiserum against the same epitope have also been shown to recognize both the 45- and 52-kDa isoforms of Gsα (16, 30). The polyclonal antibody was diluted 1:100 for immunofluorescence studies. For control experiments, the primary antibody was incubated with a peptide corresponding to that used for immunization (Calbiochem, San Diego, CA) for 1 h at 22°C before use at a concentration of 20 mg/l. For immunoblots, this antibody was used at 1:500 dilution.
competed off with 5 mg/l immunizing peptide for control experiments.

Adenylyl cyclase was detected with a recently available rabbit polyclonal IgG (R-32, Santa Cruz Biotechnology; no. SC-1701) raised against a peptide corresponding to a 33-amino acid sequence near the carboxy terminus (amino acid residues 1019–1051) of the rat adenylyl cyclase type V. This region only differs by one to three amino acid residues from rodent adenylyl cyclase types VI and VII (25, 35), the major isoforms in myocardium (13). Because of the expense and difficulty in synthesizing a 33-residue control peptide, we instead elected to employ two overlapping peptides corresponding to the overall sequence that together would cover all the likely epitopes targeted by the polyclonal antibody (NH2-GPVYAVGIVARKPOQDIWGNTV-COOH and NH2-YDIWGNTVNASRMDSVTG-COOH). These peptides were synthesized and analyzed by the Winship Microchemical Facility (Emory University). The antiserum was employed at dilutions of 1:100 and 1:250 for immunofluorescence and immunoblotting, respectively. The blocking peptides were combined and incubated for 1 h at 22°C with the primary antiserum in 10-fold excess (corresponding to 10 mg/l and 20 mg/l for immunofluorescence and immunoblotting, respectively).

Dihydropyridine receptor (DHPR) was detected with a mouse monoclonal (IgG2a) antibody (Affinity Bioreagents, Golden, CO) against the α2-subunit of rat skeletal muscle DHPR, a subunit also common to the cardiac channel isoform (34). The antibody was used at a 1:150 dilution. For control experiments, the secondary antibody was tested without inclusion of the primary.

Immunofluorescent labeling. Rat ventricular myocytes were isolated as described (11), filtered, and allowed to gravity settle. All animal procedures were conducted in accordance with institutional guidelines. Myocytes were prepared for experiments. The secondary antibody was tested without inclusion of the primary.

Immunoblotting. Cardiac protein lysate was prepared by polytron homogenization of rat ventricular strips in SDS/stop reaction buffer [6% (wt/vol) SDS, 15% (vol/vol) glycerol, 30 mmol/l Tris, 3 mmol/l EDTA, 1 mmol/l DTT, pH 7.8]. The lysate was generally boiled and loaded (at 100 µg/lane) onto a denaturing 4–15% SDS/polyacrylamide gradient gel (Jule Biotechnologies), electrophoresed, and transferred to nitrocellulose. The nitrocellulose blot was blocked with 5% powdered milk in TBS [in mmol/l: 138 NaCl, 2.7 KCl, and 10 phosphate buffer with a pH of 7.4. Fixed cells were permeabilized by the addition of 0.3% Triton X-100 in PBS for 10 min and then incubated in an isotonic solution of 50 mmol/l glycine in diluted PBS for 15 min. After gravity settling onto polylysine-coated microscope slides, the cells were washed in PBS, incubated in 3% wt/vol BSA in PBS for 5 min, and then incubated in blocking medium (3% wt/vol BSA, 10% vol/vol goat serum in PBS) for 1 h. Incubation with the appropriate dilution of the primary antibody in blocking medium was done overnight at 4°C. Cells were repeatedly washed with 3% wt/vol BSA, 10% vol/vol goat serum, and 0.1% Triton X-100 in PBS, and then incubated with FITC-conjugated species-specific anti-IgG F(ab')2 (Jackson Immunoresearch Laboratories) at a 1:75 dilution in blocking medium, for 45 min at 22°C. Cells were repeatedly washed again, attached to cover slips with a proprietary mounting medium (Biomedia), and stored in the dark until use. For dual-labeling of cells with wheat germ agglutinin (WGA) and anti-Gs, the above protocol was modified by washing cells after fixation and then incubating them in 50 mg/l Texas Red-conjugated WGA (Molecular Probes, Eugene, OR) for 30 min at 22°C, and then washing again before permeabilization.

Fluorescent images of labeled cells were acquired with an MRC-600 Bio-Rad confocal microscope. In single labeled cells, the FITC fluorescence was excited with the 488-nm laser line and detected at wavelengths >515 nm. For dual-labeled myocytes, the anti-Gs FITC fluorescence excited with 488-nm light was detected at 520 nm with one photomultiplier tube (PMT) and the Texas Red-conjugated WGA fluorescence was excited with the 568-nm light and detected at wavelengths >585 nm with another PMT. Background and contrast adjustments were performed using Confocal Assistant software. Images were then imported into Lotus freelance software for cropping and montage assembly. With the exception of Fig. 3B (see Fig. 3B legend), images were not otherwise processed.

The quantitative determination of the relative image intensity within the T tubules and over the peripheral sarcolemma in dual WGA- and anti-Gs-labeled cells was performed using NIH Image software. Regions of individual cells where the WGA probe unequivocally indicated abundant peripheral sarcolemma within the optical slice were selected for this analysis. The image intensity at any point consists of a background signal (the signal observed in the absence of a cell), the specific fluorescence of the probe labeling the structure under study, and nonspecific cell fluorescence. The specific probe fluorescence of the T tubular band was quantified by determining the mean intensity along a line positioned over a T tubular band and subtracting the mean intensity along a parallel line centered between two adjacent T tubular bands (which corrects for background and nonspecific cell fluorescence). The estimation of the probe-specific signal at the edge of the cell required a modified correction to account for the 50% drop in nonspecific cell fluorescence in this region. We separately determined the nonspecific cell fluorescence by measuring the signal between two T tubular bands close to the edge and subtracting the background value (estimated from a cell-free region of the image). We then measured the signal along a line positioned over the bright edge of the cell and subtracted both the background signal and one-half of the nonspecific cell fluorescence. This procedure was performed on images of each probe (WGA and anti-Gs). The average of the surface-to-T tubular ratios in each cell was then averaged to arrive at the final estimate for each probe.
phosphatase activity, as represented by the reactivity at 100 kDa. These bands persisted even when both the primary and secondary antibodies were omitted (not shown), indicating that they represented reaction with NBT/BCIP substrate alone.

RESULTS

Distribution of G5α. Labeling of rat ventricular myocytes with rabbit polyclonal antiserum raised against a peptide corresponding to the carboxy terminus of the α-subunit of G5 (30) revealed a distinctly T tubular pattern for this protein (Fig. 1B). The specificity of this antibody for G5α was confirmed by Western immunoblotting (Fig. 1A), in which it was found to label a 52-kDa protein from cardiac homogenate. This observation is similar to that reported by Nash et al. (21), who used an antibody against the same epitope. This corresponds to the larger isoform of G5α (27), which, at least in rat hepatocytes, appears to couple more strongly to β-adrenergic receptors (36). This reactivity could be specifically blocked by preincubating the primary antibody with excess immunizing peptide. The antiserum also recognized the smaller 45-kDa isoform of G5α in rat brain homogenate (not shown), a tissue in which it is more abundantly expressed.

The confocal microscope image in Fig. 1B illustrates the typical and very reproducible staining pattern observed with this antiserum on isolated cardiomyocytes. Even at this relatively low resolution, prominent labeling of T tubular bands (periodicity of ~2 µm) and the intercalated disk regions was apparent. In contrast, images of equivalently treated cells in which the primary antibody had been preincubated with the immunizing peptide were faint and lacked any evident T tubular pattern (not shown). Figure 1C demonstrates the T tubular localization of G5 at a higher magnification. As with the earlier anti-G5 example, there appears to be abundant staining of both the T tubular and intercalated disk membranes. In 51 cells examined, all T tubular bands showed roughly similar staining, with no evidence for regional variations in the density of G5 molecules either along the length of the myocyte or at different focal planes.

The density of G5 also appears to be far lower in the peripheral sarcolemma than in the T tubule. Indeed, in many cases the outer surface of anti-G5-labeled cells could not be discerned in the fluorescence image. However, an assessment of specific membrane density is confounded by differences in the amount of membrane contributing to the signal from these two compartments. These differences reflect the relative sizes of these two compartments and variations in their orientation with respect to the plane of the confocal image. To better contrast the density of G5 within the peripheral sarcolemmal and T tubular compartments, we quantitatively assessed the specific anti-G5 labeling to that of WGA in dual-labeled cells. WGA binds to N-acetyl-D-glucosamine and sialic acid moieties distributed homogeneously across all membrane surfaces (33), thus providing an estimate of the amount of sarcolemmal membrane contributing to particular pixels within the image. We used Texas Red-conjugated WGA, permitting simultaneous imaging with the FITC-labeled G5. Figure 2 shows simultaneously acquired confocal images of the G5 (Fig. 2A) and WGA (Fig. 2B) distribution in a representative dual-labeled myocyte. We restricted our assessment to those regions where the WGA labeling pattern at the peripheral edge of the cell was very intense, indicating an optimal orientation of the surface membrane to the optical plane (i.e., ~90°). From measurements of the image intensity within 90 T tubular bands and the peripheral sarcolemmal segments between them (from 7 dual-labeled cells), the ratio of specific peripheral sarcolemma anti-G5 staining to specific T tubular staining was found to be 0.69 ±

![Fig. 1. Immunolabeling of G5 in cardiac ventricular myocytes. A: Western blot probed with a polyclonal antibody to α-subunit of G5 in absence (lane 1) or presence (lane 2) of immunizing peptide. Without peptide, antiserum detects a 52-kDa protein (arrow) corresponding to molecular mass of large isoform of G5α. Top, top of gel (i.e., bottom of lane well). B and C: confocal images of 2 different myocytes labeled with anti-G5 and detected with a FITC-conjugated secondary antibody, at 2 different magnifications. Note predominance of T tubular (and intercalated disk) labeling relative to that over peripheral sarcolemma. These images are representative of 51 cells from 3 different rats. Scale bar in all images, 10 µm.](http://ajpheart.physiology.org/)
cance for GS within the T tubular membrane requires a general sarcolemma. The density of GS molecules may be nearly 14-fold greater in the T tubular membrane than in the peripheral sarcolemma. Although anti-GS image (A) lacks appreciable fluorescent intensity over the peripheral sarcolemma, labeling with WGA (B) confirms that a significant amount peripheral sarcolemma was indeed contained within the optical slice (arrow). Scale bar, 10 µm. Image pairs are representative of 16 dual-labeled cells.

0.11 (means ± SE). However, an assessment of the WGA labeling strongly suggests that this value overstates the abundance in the peripheral sarcolemma. The specific labeling ratio in the corresponding locations within the simultaneously acquired WGA images was found to be 9.6 ± 1.62. Indeed, by assuming that the WGA-labeling ratio truly reflects membrane distribution, these two estimates taken together suggest that the density of Gs molecules may be nearly 14-fold greater in the T tubular membrane than in the peripheral sarcolemma.

Distribution of adenylyl cyclase. A functional significance for Gs within the T tubular membrane requires an overlapping distribution for other signal transduction elements. Previous cytochemical studies using an insoluble enzymatic reaction product have disagreed on whether or not adenylyl cyclase has a T tubular distribution (29, 31). Recently, Gao et al. (9) used immunofluorescence techniques to assess the distribution of adenylyl cyclase in rabbit ventricular myocytes and concluded that adenylyl cyclase was distributed rather homogeneously within the transverse tubular system.

With the use of a different polyclonal antibody against adenylyl cyclase, we were able to confirm that this protein has a similar transverse tubule localization in rat ventricular myocytes. In addition, we show that this protein is also located in the intercalated disk region.

We employed rabbit polyclonal antiserum raised against a peptide identical to a 33-amino acid sequence contained within the carboxy-terminal domain of the rat adenylyl cyclase type V (25), the predominant isoform of this enzyme in adult rat cardiomyocytes (5). Because the peptide covers a region that is highly homologous with most other isoforms of adenylyl cyclase, this antibody should label all expressed isoforms. The major mammalian cardiac isoforms of adenylyl cyclase have been found to run on SDS-PAGE gels over a broad molecular mass range of ~120–160 kDa (9, 18, 19, 23). The specificity of the adenylyl cyclase polyclonal antiserum was confirmed by Western immunoblotting (Fig. 3A), in which it was found to label a number of appropriately sized, high-molecular-weight species in whole heart extract. All of the high-molecular-weight bands were competed away by preincubating the primary antibody with blocking peptide, as was a much fainter unidentified 60-kDa protein, which may represent a proteolytic fragment (18). A representative immunolabeling pattern obtained with this antiserum is contained in Fig. 3B. Whereas the specific anti-adenylyl cyclase signal intensity was quite a bit lower than that with anti-Gs, consistent with the estimated two orders of magnitude fewer molecules per cell (24), it is nonetheless evident that the cyclase was present in the T tubular membrane and the intercalated disks (Fig. 3B). This labeling pattern was not observed when the primary antibody was preincubated with excess blocking peptide (Fig. 3C). As with Gs, specific adenylyl cyclase reactivity was found in every T tubule, without evidence of clustering or regional variations. In contrast with the localization of Gs, adenylyl cyclase also appeared to be present in the peripheral sarcolemma although the much lower specific signal relative to background staining makes quantitative assessments problematic.

Distribution of DHPR. DHPRs have been shown to be abundantly distributed within the T tubular network of skeletal muscle (6). With the use of indirect immunofluorescence, Carl et al. (4) examined the subcellular localization of the cardiac DHPR, an important target of cAMP-dependent regulation, and found it, too, was largely restricted to the T tubular compartment. In rabbit myocardium, DHPRs had a punctate distribution consistent with clustering at sites of dyadic sarcolemmal-SR couplings intermittently spaced along the length of the T tubule. We were interested in using the distribution of DHPRs as a marker of dyadic junctions to contrast with that observed with Gs and adenylyl cyclase. Figure 4 shows a representative example of the cellular distribution of this channel in rat ventricular myocytes using a monoclonal antibody raised against the α2-subunit of the rat skeletal receptor. In general,
the observed pattern was similar to that reported by Carl et al. (4) in rabbit myocytes: prominent T tubular labeling along the length of the cell, with only rare reactivity at the peripheral sarcolemma or intercalated disks. Images of equivalently treated cells in which the primary antibody was omitted were faint and lacked any evident T tubular pattern (not shown). Notably, although Gs and adenylyl cyclase immunostaining was relatively uniform along the extent of a given T tubular band, the DHPR pattern was distinctly punctate. The strong implication is that Gs and adenylyl cyclase, unlike DHPRs, are distributed more homogeneously along the T tubule. This observation also suggests that the apparent enrichment of Gs that we detected in the transverse tubular membrane did not arise from additional Gs molecules residing within adjacent junctional SR membranes (28).

DISCUSSION

We have used indirect immunofluorescent labeling to determine the subcellular localization of two important elements of the signal transduction cascade employed by the β-adrenergic and other receptor systems coupled to production of cAMP. Consistent with the immuno-gold electron microscopy study of Nash et al. (21) in canine and porcine heart, we show that Gs molecules are considerably more abundant in the T tubular compartment of the cell than in the peripheral sarcolemma, and was also present in the intercalated disk region. We estimated that, in rat ventricular myocytes, Gs is ~14-fold more concentrated in the T tubular membrane than in the peripheral sarcolemma, a higher T tubular concentration than Nash et al. estimated for porcine and canine myocytes. The distribution of Gsα we observed in normal rat myocytes was virtually identical to that observed by Muntz et al. (20) in heart cells from transgenic mice that overexpress this protein. We also found that Gs was coexpressed with its target adenylyl cyclase in the transverse tubular compartment. These observations confirm those reported by Gao et al. (9). Those investigators employed an adenylyl cyclase antibody raised against a different epitope, making it unlikely that this apparent distribution reflects binding to other proteins. We also found that adenylyl cyclase was expressed in the intercalated disk compartment, a feature not noted by Gao et al. (9). Thus the distribution of Gs appears to represent the distribution of functional cAMP-production sites.

Among other advantages, immunofluorescence microscopy allows one to assess the local density of proteins in relation to that of the whole cell. Our results show not only that Gs and adenylyl cyclase were colocalized within the T tubular compartment but that the distribution within this compartment was essentially homogenous throughout the cell. Gs and adenylyl cyclase were found to be expressed in all T tubular bands along the length of all myocytes examined, with no evidence for regional variations that might be expected if, for example, clustering of these proteins were dictated by the positioning of nearby nerve varicosities. In contrast with the punctate localization of DHPRs, Gs and adenylyl cyclase also appear uniformly distributed within individual T tubules.

Nash et al. (21) reported that Gsα was most abundant within the intercalated disks of canine and porcine ventricular myocytes, and Muntz et al. (20) reported a similar localization in this compartment in cells from mice overexpressing Gsα. Our study confirms that this protein is also present in this specialized membrane structure in normal rat ventricular myocytes. Furthermore, our work shows that adenylyl cyclase is also abundant within this compartment.
in the soluble fraction (1, 12). These findings led Buxton and Brunton (3) to propose that cAMP was compartmentalized into different subcellular pools having functionally distinct actions, although precisely how these compartments are delimited remains unclear. Presumably, it would require, among other characteristics, that cAMP pools either be physically segregated or that cAMP have a spatially limited range of action. Recently, Jurevicium and Fischmeister (14) have shown that cAMP from one half of a frog ventricular myocyte (a cell type that lacks T tubules) that was exposed to the β-adrenergic agonist isoproterenol had a very limited ability to increase the calcium current through channels located on the other half of the cell. Their investigation elegantly demonstrated that cAMP does indeed have a limited spatial range of action. These investigators also showed that this range of action could be extended by treating cells with phosphodiesterase inhibitors, demonstrating that cAMP was constrained not by a physical barrier, but rather by a limited lifetime relative to its diffusion rate. Because of the particulars of the preparation and the technique employed, they could not resolve the range of action below ~20 µm. Thus it is unclear whether the actual limit would be of physiological significance in 10- to 15-µm wide mammalian cells.

The distribution of Gs and adenylyl cyclase throughout the T tubular network indicates that the apparatus for synthesizing cAMP in response to external signals resides very close to the targets involved in modulating contractility. Thus, although our data do not further resolve the spatial limits to cAMP, they do indicate that cAMP could have a range of action as small as 1 or 2 µm and yet retain access to essentially all targets of β-adrenergic modulation. Such a tight, local action could permit the cell to more finely regulate the inotropic level in different domains within the cell. Furthermore, it suggests that different compartments of this second messenger that regulate distinct functions could be demarcated in large part by the proximity of the receptor and transduction apparatus to their cellular targets.

Although the extensive membrane folding within this compartment (32) makes a quantitative assessment difficult, it is nonetheless clear that the intercalated disk has its own complement of these proteins. This suggests that cAMP modulation of elements that reside there is not only important (2) but perhaps regulated independently from inotropic targets.

The second messenger cAMP is utilized by several surface receptor types in cardiac cells, although there have long been disturbing discrepancies between the ability of certain agents to elevate cAMP and to alter contractility (12). For example, both β-adrenergic agonists and prostaglandin E can activate adenylyl cyclase, yet when compared at doses that yielded equivalent total cell cAMP elevations, only β-adrenergic stimulation produced an increased contractility (3, 12). β-Adrenergic agonists were more effective at elevating cAMP in a crude particulate fraction of homogenized myocytes, and the cAMP in this fraction more tightly correlated with inotropic changes than did cAMP levels in the soluble fraction (1, 12). These findings led Buxton and Brunton (3) to propose that cAMP was compartmentalized into different subcellular pools having functionally distinct actions, although precisely how these compartments are delimited remains unclear. Presumably, it would require, among other characteristics, that cAMP pools either be physically segregated or that cAMP have a spatially limited range of action. Recently, Jurevicium and Fischmeister (14) have shown that cAMP from one half of a frog ventricular myocyte (a cell type that lacks T tubules) that was exposed to the β-adrenergic agonist isoproterenol had a very limited ability to increase the calcium current through channels located on the other half of the cell. Their investigation elegantly demonstrated that cAMP does indeed have a limited spatial range of action. These investigators also showed that this range of action could be extended by treating cells with phosphodiesterase inhibitors, demonstrating that cAMP was constrained not by a physical barrier, but rather by a limited lifetime relative to its diffusion rate. Because of the particulars of the preparation and the technique employed, they could not resolve the range of action below ~20 µm. Thus it is unclear whether the actual limit would be of physiological significance in 10- to 15-µm wide mammalian cells.

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The recent studies of Nash et al. (21) and Gao et al. (9), along with our own investigation, have added Gs and adenylyl cyclase to a growing list of proteins, including the DHPR (4), the Na+/Ca2+ exchanger (7, 15), the Na+/K+-ATPase (17), dystrophin (22), and the anion exchanger (26), that have been found to be widely distributed throughout the cardiac T tubular system. Because of the unavailability of an antibody of sufficient specificity for cellular immunofluorescence, the localization of the β-adrenergic receptor has not yet been determined. Nonetheless, the distribution of Gs and adenylyl cyclase strongly implies that this receptor, the most abundant and physiologically important Gs-linked receptor type in the heart, also resides within the T tubular compartment. Moreover, these findings would appear to require a T tubular localization for signaling molecules that functionally interact with this
system at the level of adenylyl cyclase, such as muscarinic and purinergic receptors and their respective downstream elements. Thus, in addition to their well-known role in excitation-contraction coupling, transverse tubules appear to be the primary site for signaling by inotropic hormones and neurotransmitters.

We thank H. Bindu Vanapalli and J on D. Hall for technical assistance and Drs. Charles Buck and Ron Abercrombie for helpful advice.

This investigation was supported by grants from the National Institutes of Health and the American Heart Association (Georgia affiliate) to P. L. Becker.

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Received 30 April 1998; accepted in final form 3 June 1999.

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