Immuonochemical evidence for a unique GPI-anchored carbonic anhydrase isozyme in human cardiomyocytes

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Knüppel-Ruppert, Anja Sylvia, Gerolf Gros, Wolfgang Harringer, and Hans-Peter Kubis. Immunochemical evidence for a unique GPI-anchored carbonic anhydrase isozyme in human cardiomyocytes. Am J Physiol Heart Circ Physiol 278: H1335–H1344, 2000.—To clarify the controversial question of cell-specific distribution of carbonic anhydrase (CA) in the heart, endothelial cells and cardiomyocytes were isolated from porcine and human hearts and were characterized with cell-specific markers. CA activity was found in the microsomal fraction of both cell types. It was shown by Triton X-114 phase separation that both cell types possess a membrane-bound form of CA. These CAs share the same mechanism of membrane-anchoring via glycosylphosphatidylinositol (GPI), which excludes identity with transmembrane isoforms CA IX or CA XII. Western blotting analysis of human microsomes with anti-human CA IV antibodies revealed a marked difference in immunoreactivity. Endothelial CA activity resulted in 11-fold stronger CA IV bands compared with identical amounts of myocytic CA activity, indicating that cardiac endothelium and cardiomyocytes possess immunologically distinct forms of CA. We conclude that in human hearts CA IV is associated with the endothelium, whereas most of the CA in myocytes is not identical with one of the known CA isozymes. This suggests that cardiomyocytic CA is a novel isozyme.

carbonic anhydrase activity; membrane-bound isozyme; por
cine heart; human heart

CARBONIC ANHYDRASE (CA) catalyzes the reversible hydration of carbon dioxide. At least nine enzymatically active mammalian CA isozymes have been identified (32, 36), differing in, e.g., kinetic parameters, inhibitor binding, tissue distribution, and subcellular localization. The presence of CA in heart muscle is well established (34). The aim of this work was to provide the basis for an effort to understand the function of CA in the heart by determining the identity and localization of the isozymes that contribute to cardiac CA activity. Some aspects of the subcellular localization of CA in the heart are already known and exhibit some contrast to other striated muscles: Heart muscle lacks any form of cytosolic CA but possesses high levels of membrane-bound CA activity, as demonstrated in mem-

brane fractions from rabbit hearts by Geers et al. (9).

Bruns and Gros (2) subfractionated membranes of bovine hearts and found CA activity enriched predominately in the sarcolemmal fraction. However, the distribution of cardiac CA among the different cell types of the heart remains unclear. In both above-mentioned investigations, tissue homogenates were used, and detected activities were attributed to cardiomyocytes, which represent ~80% of the mass of the myocardium (25). But because cardiac CA is membrane bound, cell-surface relations are important rather than mass relations. According to Piper (25), the surface area of nonmyocytes may exceed the myocytic surface area. Additionally, sarcolemmal membranes prepared by commonly used procedures were shown to be enriched in endothelial cell markers (29, 35), indicating that the measured CA activity must not necessarily be attributed to myocytes or myocytes alone.

Further investigations designed to clarify the cellular origin of cardiac CA activity chose histochemical or immunohistochemical approaches but led to contradictory results: Bruns and Gros (2) examined cryosections from left ventricles of rabbits with the fluorescent CA inhibitor dansylsulfonamide (DNSA). CA-positive staining occurred at the borders of the myocytes and at some intramyocytic structures. CA activity was therefore attributed to the sarcolemma and sarcoplasmic reticulum of the myocytes. On the other hand, immunohistochemical studies at the light microscopic level by Sender et al. (31) indicated that cardiac CA is located exclusively in endothelial cells, because anti-human lung CA IV antibodies detected only capillaries in human ventricular cryosections. In contrast, immunoelectron microscopic investigations by Sender et al. (30) demonstrated reactivity with anti-CA IV at endothelial as well as cardiomyocytic membranes. Application of the Hansson technique (11) to ferret heart sections by Vandenberg et al. (37) demonstrated CA activity at both endothelial cell membranes and myocytic sarcolemmal membranes. Thus the histochemical studies could not clarify unequivocally if cardiac CA is located in endothelial cells, in cardiomyocytes, or in both cell types. One possible explanation for the apparent inconsistencies seemed to be the existence of more than one membrane-bound CA isofrom in the heart muscle.

In 1982 Whitney and Briggle (38) purified for the first time a membrane-bound form of CA from bovine lungs. It was termed CA IV and has long been thought to
represent the only membrane-bound CA isozyme. It had an apparent molecular mass of 52 kDa on SDS gel electrophoresis, containing at least 20% carbohydrate. The human CA IV isozyme was purified in 1990 by Zhu and Sly (42) from human lungs. Its molecular mass of 35 kDa on SDS electrophoresis is the lowest among mammalian CA IV isozymes because it is the only non-glycosylated CA IV (15, 42). The cDNA of human CA IV was cloned and characterized (20), and the 9.5-kb gene was mapped to chromosome 17q (19). From the nascent precursor, the NH2-terminal signal peptide is cleaved off in the endoplasmic reticulum, as well as the COOH-terminal hydrophobic domain, which is released by a preassembled glycosylphosphatidylinositol (GPI) anchor (21), which accounts for CA IV localization on the cell surface (18). Another difference that distinguishes CA IV from all known soluble CA isoforms is its unique property of being stable in 1–5% SDS solutions due to two disulfide linkages that stabilize the conformation of the NH2-terminal domain (33, 42).

Recently two additional membrane-bound CAs have been identified and termed CA IX (22, 23, 27) and CA XII (36). Both CA IX and XII are catalytically active, have overexpression in certain cancers in common, and, in contrast to CA IV, are attached to the membrane via transmembrane peptide sequences and are thus integral membrane proteins. This study is addressed to the controversial question of cell-specific distribution of CA in the heart and intends to ascertain whether one or more isoforms contribute to membrane-bound cardiac CA activity. In contrast to histochemical approaches, we wanted to investigate the cell-specific CA distribution by direct determination of CA activity in isolated cardiac endothelial cells and cardiomyocytes. Additionally, the cardiac CAs of endothelial cells and cardiomyocytes were to be characterized in some more detail to answer the question of whether CA IV is the only CA isoenzyme or if there is another CA isoenzyme contributing to cardiac CA activity.

**MATERIALS AND METHODS**

Materials. Collagenase type IV and BSA V were purchased from Sigma (St. Louis, MO). Modified Eagle’s medium nontissue culture grade (EMC) and basal medium Eagle’s amino acids (100×) and 10-kDa Protein Ladder were obtained from Life Technologies (Eggenstein). Nitrocellulose membrane (Porablot NCL, 0.45 µm pore size) was a product of Macherey & Nagel (Düren, Germany). Polyclonal rabbit anti-human von Willebrand factor (vWF) antibody was purchased from DAKO (Glostrup, Denmark). Monoclonal IgG1 mouse anti-human endothelial constitutive nitric oxide synthase (eNOS) antibody and human aortic endothelial lysate were bought from Transduction Laboratories (Lexington, KY). Horse heart peroxidase (HRP)-linked sheep anti-mouse Ig antibody, HRP-linked donkey anti-rabbit Ig antibody, and the enhanced chemiluminescence (ECL) system were acquired from American Life Science (Arlington Heights, IL). HRP-conjugated goat anti-rabbit IgG antibody was purchased from Sigma. Phosphatidylinositol-specific phospholipase C (PI-PLC) from Bacillus cereus was from Sigma. Enzymatic Deglycosylation Kit and Deglycosylation Enhancement Kit were purchased from Bio-Rad (Hercules, CA). All other chemicals were purchased either from Sigma, Merck (Darmstadt, Germany), or Serva (Heidelberg, Germany).

Cell separation. Cardiomyocytes and cardiac endothelial cells were isolated from freshly explanted human and porcine heart ventricles. Human ventricles were obtained from transplant recipients (age: 54–60 yr, male, ischemic heart disease). Pigs (2–5 mo of age) were anesthetized with thiopental sodium (15 mg/kg iv), and supplemental doses were given to maintain a sufficient level of anesthesia. After administration of heparin sodium (500 U/kg iv), hearts were rapidly excised and placed immediately in ice-cold oxygenated cardioplegic solution. These experiments had been approved by the local authority. The cell isolation procedure was that modified after J acobson et al. (13). A main branch of the left coronary artery was perfused for 5–10 min at a rate of 27 ml/min with Ca2+-free buffer A containing 60 mM NaCl, 16 mM KCl, 3.25 mM MgSO4, 1.2 mM KH2PO4, 11 mM glucose, 80 mM mannitol, 5 mM pyruvate, 10 mM taurine, 5 µM adenosine, 50 µM EGTA, 20 mM HEPES, 10 mM NaHCO3, 1 ml/100 ml amino acids, 1 mg/ml BSA, and 150 IU/l insulin, equilibrated with 95% O2-5% CO2 to pH 7.3–7.4 at 37°C. Enzymatic digestion was then initiated by perfusion with buffer B containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5 mM glucose, 5 µM adenosine, 20 mM HEPES, 10 mM NaHCO3, 1 ml/100 ml amino acids, 1 mg/ml BSA, 150 IU/l insulin, and 100 mg collagenase type IV per 250 ml of buffer in the case of porcine tissue, and 250 mg of collagenase per 250 ml in the case of human tissue. Ca2+ was added gradually over several minutes to a final concentration of 250 µM. Perfusion conditions were the same as with buffer A. Cell disaggregation was monitored by light microscopy of samples aspirated from a cut edge of ventricle tissue. Digestion required 30 min (young pig hearts) up to 60 min (fibrotic human ventricles). Subsequently, the softened tissue was abraded with a scalpel, and the cells were further disaggregated while being immered for another 15 min in buffer B supplemented with 2% BSA at 37°C, whirled up by a stream of gas (95% O2-5% CO2). The cell suspension was then filtered through a nylon filter with a 300-µm mesh to remove tissue debris (26). Repeated centrifugation at 30 g for 3 min in collagenase-free buffer C containing 20 mg/ml BSA and 20 mM NaHCO3 saturated with 95% O2-5% CO2 at 4°C (pH 7.3–7.4), otherwise identical to buffer B, yielded cell suspensions enriched in either cardiomyocytes (contained in the pellet) or endothelial cells (in the supernatant). The latter were finally sedimented at 250 g for 10 min. Morphology and purity were controlled with phase-contrast microscopy. Cells were stored at −80°C.

Protein determination. Protein was determined after precipitation with trichloroacetic acid using the Lowry method (Sigma procedure P 5656).

Cell-specific markers. Characterization of cell homogenates with endothelial cell-specific markers was done by Western blotting with antibodies against eNOS for porcine cells or antibodies against von Willebrand factor (vWF) for human cells, respectively. A lane with 10-kDa Protein Ladder served as molecular mass marker in each gel run. eNOS. Porcine homogenate proteins were subjected to SDS-PAGE, using a 7% polyacrylamide separating gel and a 5% stacking gel, and then transferred to a nitrocellulose membrane using a semidry blotting system at 0.8 mA/cm2 for 2 h. The nonspecific binding was blocked with 10% nonfat dry milk in PBS and 0.025% Tween 20. For immunostaining the anti-eNOS antibody was used at 1:250 dilution, detected with...
Myosin heavy chain-1β. As a cardiomyocyte marker we determined myosin heavy chain-1β (MHC-1β) per homogenate protein with an electrophoretic technique designed to distinguish between MHC isoforms as described by Kubis and Gros (14). This SDS-PAGE achieves a high resolution in the molecular mass range of interest (ca. 220 kDa) using a slab gel with a 3.5% stacking gel and a 6.6% separating gel (pH 6.8) followed by a second 6.6% stacking (pH 6.8) with a second 8.8% separating gel (pH 8.8). The four gel sections contain increasing glycerol concentrations (0%, 4.8%, 14.7%, and 30.6% glycerol, respectively). A marker lane with a myosin extract of human cardiomyocytes was present in each gel run. Gels were silver stained according to Heukeshoven and Dernick (12).

Marker quantification. All specific marker bands were scanned, and their optical densities were quantified (OD·mm²/mg protein) using the Image Master System of Pharmacia Biotech (Uppsala, Sweden). The marker signal of each of the two cell types isolated from one heart (endothelial cell vs. cardiomyocyte) was expressed in percentage of the total signal obtained as a sum of signals from both cell types, i.e., 100% is defined as the sum of marker band densities (OD·mm² per 1 μg of endothelial cell protein + OD·mm² per 1 μg of cardiomyocyte protein) obtained from the same heart under identical electrophoretic and staining conditions.

Preparation of microsomes. Microsomes were prepared to enrich membrane-bound CA. Cell suspensions were homogenized in ice-cold 5 mM histidine, pH 7.5 with an Ultra-Turrax (Janke und Kunkel, Staufen, Germany) for 5 times 10 s on ice-water and then centrifuged 1 h at 105,000 g (TFT 70.38 Rotor, TGA-50 Ultracentrifuge, Kontron Instruments, Munich, Germany). The pellet was homogenized (12 times 15 s on ice) in 250 mM sucrose and 10 mM histidine, pH 7.5, and was centrifuged at 11,000 g for 30 min. The pellet was discarded. The supernatant was centrifuged at 103,000 g for 1 h. The supernatant, which contained soluble proteins, was discarded. The pellet (microsomal membranes) was resuspended in 5 mM histidine, pH 7.5, and resedimented at 103,000 g for 1 h. The final pellet (washed microsomal membranes) was diluted (1.4 to 1:10 wt/vol) with 5 mM histidine, pH 7.5, and stored at −80°C.

Determination of CA activity. CA activity was measured in washed microsomal membranes using Maren’s modified micromethod (3, 17). The principle idea is to determine the time required for reacidification following the addition of alkaline barbital buffer to a CO₂-saturated assay volume. Because CO₂ hydration is accelerated in the presence of CA activity, the time needed to reach the point when the pH indicator phenol red changes its color from red back to yellow is reduced by the enzyme. CA activity (A) is defined using the uncatalyzed reaction time (t₀) (absence of CA) and the catalyzed reaction time (A = t₀/t − 1).

Thus a CA activity of 1 unit corresponds to that CA concentration that reduces the uncatalyzed CO₂ hydration time to one-half. The specific CA activity is the number of CA units divided by the protein concentration. Samples were measured in the presence of 0.1% Triton X-100 to make also accessible all CA that might be enclosed inside microsomal vesicles. For a second determination, samples were incubated with 0.2% SDS before the assay to inactivate any soluble CA. The data obtained with Triton X-100 (data not shown) and with SDS were not significantly different; all values of CA activity presented below are results of CA measurements with Triton X-100.

Triton X-114 phase separation. Triton X-114 phase separation was used to partition proteins according to hydrophobicity (1, 8). A mixture of 100 μl of microsomal membranes and 190 μl of 2.2% (wt/vol) Triton X-114 in 300 mM NaCl and 20 mM Tris-HCl (pH 7.4, 4°C) was incubated at 31°C for 4 min and centrifuged at 2,000 g for 3 min. The supernatant was mixed with 100 μl of 4.5% (wt/vol) Triton X-114 in 150 mM NaCl and 10 mM Tris-HCl (pH 7.4, 4°C). After 4 min of incubation at 31°C, the centrifugation step was repeated. The final supernatant (termed water phase) and the combined pellets (termed Triton phase) were assayed for their amount of CA activity (U·ml). The amount of CA activity is the sample volume multiplied by the number of CA units (a value proportional to CA concentration). The distribution of activity in the different phases was expressed in percentage of the total amount of CA activity.

PI-PLC treatment. Membrane linkage of endothelial and myocytic CA was analyzed comparing the release of CA from the membrane by PI-PLC. One-hundred microliters of washed microsomal membranes (130–390 μg of protein) were suspended in 105 μl of 100 mM Tris-SO₄ buffer, pH 7.5, containing 0.1% deoxycholate and 575 mU PI-PLC. Controls were suspended in the same buffer without PI-PLC. After incubation at 37°C for 1.5 h with rotation at 500 min⁻¹ on a thermostixer (model 5436, Eppendorf, Germany), the samples were centrifuged at 100,000 g for 50 min. The supernatant and the resuspended pellet were assayed for their respective amount of CA activity as described above. The soluble CA in the supernatant and the membrane-bound CA in the pellet were expressed in percentage of the total amount of CA activity in both the pellet and supernatant.

CA IV immunoblotting. Immunoblotting of human microsomes with anti-CA IV antibodies was performed to compare the immunoreactivity of CA IV of endothelial versus myocytic CA isoforms. Microsomal samples from endothelial cells and cardiomyocytes, containing equal quantities of CA activity, were separated by SDS-PAGE (using a 15% polyacrylamide separating gel and a 5% stacking gel), followed by electroblotting of proteins. The immunostaining was done as described above for the determination of cell-type markers using a peroxidase-linked oligosaccharides from glycoproteins. Blotting and detection procedure were as described for CA IV immunoblotting. Successful deglycosylation was monitored by a shift in
RESULTS

Characterization of cell preparations. Judged by light microscopy, separation of cell types yielded two clearly distinct cell suspensions enriched in either cardiomyocytes or endothelial cells. Figure 1 shows phase-contrast microscopy of the cell preparations obtained from a human left ventricle. It shows the typical cell distribution after cell isolation and separation. The endothelial cell preparation contained endothelial cells among other small cells and some small or fragmented myocytes. Occasional contaminating red blood cells were found exclusively in the endothelial cell fraction. The cardiomyocyte preparation consisted of large myocytes of variable shape and usually showed negligible contamination by other cells.

Characterization with endothelial cell-specific markers. The distribution of endothelial cells among endothelial cell and cardiomyocyte preparations, respectively, was confirmed by the distribution of established endothelial cell markers that were quantified by immunoblotting. The antibody against vWF regularly detected vWF antigen in freshly isolated human heart endothelial cells. Porcine heart endothelial cells were positive for eNOS antigen. To be independent of variable antigen expression or antigen loss during the isolation procedure (16, 24), we standardized the marker signal: 100% was defined as the sum of marker band densities [OD·mm² per 1 µg of endothelial cell protein + OD·mm² per 1 µg of cardiomyocyte protein].

The specific endothelial cell marker distribution is shown (Table 1) to be similar for all hearts investigated: when equal amounts of porcine homogenate protein were compared, eNOS was found to be present by >90%, in one case by 80%, in the endothelial cell preparation. In the case of human hearts, vWF was found to be present by at least 90% in the endothelial cell preparation. This marker distribution confirms that endothelial cell preparations were strongly enriched in endothelial cells. On the other hand, all cardiomyocyte preparations were very low in endothelial cell marker, indicating that cardiomyocytes were well purified and almost free of endothelial cell contamination.

Characterization with cardiomyocyte-specific marker. The distribution of the specific cardiomyocyte marker

| Table 1. Distribution of specific cell type markers among EC and CM preparations |
|--------------------------------|--------------------------------|
| eNOS, %* | MHC-1b, %* |
| EC | CM | EC | CM |
| Porcine heart | Specific EC marker | Specific CM marker |
| 1 | 97.7 | 2.3 | 12.8 | 87.2 |
| 2 | 79.8 | 20.2 | 48.3 | 51.7 |
| 3 | 94.9 | 5.1 | 24.8 | 75.2 |
| 4 | 97.3 | 2.7 | 29.4 | 70.6 |
| Human heart | Specific EC marker | Specific CM marker |
| 1 | 90.9 | 9.1 | 4.9 | 95.1 |
| 2 | 92.7 | 7.3 | 16.1 | 83.9 |

*100% is defined as the sum of marker band densities [OD·mm² per 1 µg of endothelial cell protein + OD·mm² per 1 µg of cardiomyocyte protein] obtained from the same heart under the same electrophoretic and dyeing conditions. Specific endothelial cell marker distribution: when equal amounts of porcine homogenate protein are compared, endothelial nitric oxide synthase (eNOS) is found by 80% or more in the EC preparation. In case of human hearts, von Willebrand factor (vWF) is found by at least 90% in the EC preparation. Specific cardiomyocyte marker distribution: comparing equal amounts of human homogenate protein, myosin heavy chain-1b (MHC-1b) is found by at least 80% in the CM preparation. In porcine hearts, MHC-1b in CM varies between 52% and 87%.

Fig. 1. Phase-contrast microscopy of cell preparations obtained from a human left ventricle immediately after cell isolation and separation. A: endothelial cell preparation (EC); B: cardiomyocyte preparation (CM).
MHC-1β was not equally uniform (Table 1). Comparing identical amounts of human homogenate protein, it has been found that MHC-1β is present by at least 80% in the cardiomyocyte preparation. In the case of porcine hearts, MHC-1β is present by 52–87% in cardiomyocytes and is always higher, and in most cases markedly higher, than in endothelial cell preparations.

The marker determinations confirmed successful cell separation especially for human hearts. In porcine hearts cardiomyocyte preparations are well purified and show little contamination with endothelial cells, whereas endothelial cell preparations show a varying degree of contamination with cardiomyocyte fragments.

CA activity in endothelial and cardiomyocytes. Provided that only one cell type contains CA activity, one would expect that CA activity distribution would resemble the distribution of the marker for the respective cell type. Figure 2 shows the specific CA activity (U·ml·mg protein⁻¹) measured in microsomes as a function of enrichment in endothelial cells, expressed in percentage of specific endothelial cell marker signal (eNOS and vWF, respectively, data from Table 1). As shown by the filled triangles in Fig. 2, the specific CA activity in two human endothelial cell preparations with a high percentage of endothelial cell marker is slightly but not significantly above the specific CA activity of the corresponding cardiomyocyte preparations with a low percentage of endothelial cell marker. In the four porcine hearts studied, specific CA activity is spread over a wide range, and there is clearly no correlation between specific endothelial cell signal and average specific CA activity.

Figure 3 shows the specific CA activity (U·ml·mg protein⁻¹) in microsomes as a function of cardiomyocyte enrichment, expressed as percentage of the total specific cardiomyocyte marker signal (MHC-1β, data from Table 1). In human heart cells, specific CA activity is slightly higher in the two endothelial cell preparations with a low percentage of cardiomyocyte marker than in the corresponding cardiomyocyte preparations. Again in porcine cells there is clearly no correlation between specific CA activity and the presence of cardiomyocytes. The cause of the rather large interindividual variation of CA activities in the porcine data seen in Figs. 2 and 3 is not clear; part of it may be due to age-related developmental differences in the group of pigs studied.

In summary, specific CA activity does not correlate with the presence of either endothelial cells or cardiomyocytes, but is, although highly variable in its absolute value, on average present at similar magnitude in both cell types. This is not compatible with only one cell type containing CA activity. Therefore, we conclude that both cardiac endothelial cells and cardiomyocytes possess CA activity.

Endothelial and myocytic CA is membrane bound. In Triton X-114 phase separation, proteins are distributed according to their relative hydrophobicity. We subjected microsomes from porcine and human cardiac endothelial and cardiomyocytes to Triton X-114 phase separation to analyze the distribution of their CA activity between membranes and cytosol (1). As a control we used membrane-free red blood cell lysate from rats, which is known to contain soluble CA only. As shown in Fig. 4 the phase distribution of CA activity was the same for both species and for both cardiac endothelial as well as myocytic CA activity; CA partitions into the Triton phase by >90%. In contrast, the activity of CA in red blood cells was found mainly in the water phase, as expected for cytosolic enzymes. It may be noted that partitioning of ~10% of soluble CA into the Triton phase is expected in these experiments.
The data indicate that cardiac endothelial as well as myocytic CAs represent membrane proteins. An anchoring mechanism of membrane-bound CA from endothelial cells and cardiomyocytes is a GPI linkage. When microsomal membranes from human cardiomyocytes were treated with PI-PLC, 75% of the membrane-bound CA was released into the supernatant as shown in Fig. 5. This degree of release is comparable to the 78% release of CA from endothelial cell membranes of human hearts. The CA activity of control membranes without PI-PLC treatment was found mainly in the pellet (cardiomyocytes: 83%/endothelial cells: 95%) as expected.

The experiments performed with membranes from porcine heart cells show comparable results: 67% of myocytic and 73% of endothelial CA activity are released by PI-PLC treatment. Thus the membrane anchoring of CA in both investigated cell types and both species is sensitive to PI-PLC of Bacillus cereus, indicating the same mechanism of anchoring to the membrane via GPI.

Different immunoreactivity toward anti-CA IV antibodies. To compare the immunoreactivity of endothelial and myocytic CA, we subjected identical amounts of CA activity to immunoblotting. No difference in resulting CA bands should be apparent, provided that both cell types contain the same CA isozyme. Figure 6 shows immunoblotting of human heart microsomes with anti-human CA IV antibodies. Identical CA activity amounts from endothelial cells and cardiomyocytes were applied to the gel. Antiserum raised to human lung CA IV reacted strongly with a protein of ~32 kDa from endothelial microsomes. Activity of 0.12 U·ml endothelial CA resulted in CA IV bands of 11.9 OD·mm² (±2.0 SD). The same CA activity of 0.12 U·ml from myocytes resulted in faint CA IV bands of 1.1 OD·mm² (±0.47 SD). (Exposure time of X-ray film to luminescence was 30 s.) This means that endothelial CA produces 11-fold stronger CA IV bands compared with equal activity of myocytic CA. This result was confirmed with three different heart preparations, all shown in Fig. 6. These marked differences in immunoreactivity indicate that human cardiac endothelial cells and cardiomyocytes possess different CA isozymes.

Deglycosylation. To determine whether the difference in immunoreactivity reflects differences in the protein structure or is based on differences in glycosylation pattern only, we tested the effect of deglycosylation before immunoblotting of human heart microsomes with anti-human CA IV antibodies (Fig. 7). Identical CA activity amounts from endothelial cells and cardiomyocytes were subjected to the deglycosylation assay. Subsequent CA IV immunoblotting showed an unchanged marked 9- to 11-fold difference between intense CA IV bands from endothelial CA activity and weak bands from myocytic CA activity (exposure time of X-ray film to luminescence was 60 s). This indicates that the difference in immunoreactivity of endothelial CA and myocytic CA isozymes does not originate from different patterns of glycosylation.

DISCUSSION

Cell-specific distribution of CA in the heart. The question of the cell-specific distribution of CA in the heart arose from the apparently inconsistent histochemical and immunohistochemical results (2, 31, 37). We propose that membrane-bound cardiac CA activity is located in both cardiac endothelial cells and cardiomyocytes, because we found comparable specific CA activity in microsomes from both cell types (Figs. 2 and

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**Fig. 4.** Distribution of CA activity after Triton X-114 phase separation. For each cell type the percentage of CA activity in Triton phase (solid bars) and percentage of CA activity in water phase (open bars) is given. 100% is defined as total amount of CA activity found in both phases. Data are given as means ± SD. Number of experiments: human, n = 3; porcine, n = 5; rat, n = 4. Right: as control, distribution of soluble CA isozymes from red blood cells (RBC). Left: cardiac endothelial and myocytic CA activity from both species partitioned by >90% in Triton phase. The predominant partitioning into the Triton phase is characteristic for membrane-bound proteins.

**Fig. 5.** Distribution of CA activity after phosphatidylinositol-specific phospholipase C (PI-PLC) treatment. For each cell type, percentage of CA activity in pellet (solid bars) and percentage of CA activity in supernatant (open bars) is given. 100% is defined as total amount of CA activity found in both phases after centrifugation. When treated with PI-PLC, the cardiac endothelial and myocytic CA activity from both species are found to be more than two-thirds in the supernatant. CA activity of identical control samples without PI-PLC was found by >80% in the pellet.
3). It may be noted that the specific CA activity of "endothelial cells" represents results from a mixture of vascular cells. The exact cell composition is not known, but it seems reasonable to assume that they represent an average of all coronary microvascular cells with endothelial cells constituting the majority, followed by a few pericytes (in myocardium, ~10% of capillary endothelial cells) and even fewer smooth muscle cells (26). In addition to the numerical predominance of endothelial cells, we feel justified in neglecting other small cells, because there is as yet no hint at a CA in these cells in the literature, whereas the localization of CA in capillary endothelial cells is well established by immunohistochemistry (30, 31), demonstrating CA IV in cardiac endothelial cells in the rat and the human heart at the light and electron microscopic level. Therefore, it appeared justified to choose established endothelial cell markers to quantify contamination of cardiomyocytes, because the endothelial cell is then the most probable source of CA contamination in cardiomyocyte preparations.

The marker characterization employed in our study has clearly established the separation of endothelial cells from cardiomyocytes (Table 1). The cardiomyocyte preparations especially show negligible endothelial cell contamination. The results of the present marker determinations did not suggest a need to aim at more extensive purification by using primary culture with the risk of influencing CA expression and reducing cell yield. We have shown that cell suspensions of clearly distinct cell composition, after undergoing the same microsome preparation procedure, show identical specific CA activities not correlated with the presence of one cell type. This is not compatible with localization of CA in only one cell type and clearly indicates that both endothelial cells and cardiomyocytes contribute to the membrane-bound CA activity in the heart. A localization of cardiac CA in endothelial cells and cardiomyocytes is in line with the results of Vandenberg et al. (37), who demonstrated with the Hansson technique that CA is located at the sarcolemma of cardiomyocytes and at the plasmalemma of capillary endothelial cells in ferret hearts. At variance with this evidence are the results of histochemical localization using the fluorescent CA inhibitor DNSA in rabbit ventricular sections, which appeared to show CA in myocytes only (2). The failure of DNSA to stain capillaries might be due to different affinities of endothelial cell CA and cardiomyocyte CA for DNSA, or it may be caused by the presence of erythrocytes in the capillaries because hemoglobin quenches the CA-DNSA emission light (6).

The fact that our CA activity measurements as well as the CA activity-based Hansson technique demonstrated CA in cardiomyocytes (both methods do not discriminate between different isozymes), whereas immunohistochemistry with a specific antibody against CA IV failed to detect CA in cardiomyocytes in cryosections of human hearts (31), leads to the question of whether the CA activity in cardiomyocytes represents a CA isozyme different from the endothelial one. Therefore, we aimed at characterizing the myocytic isozyme in comparison with the endothelial form.

CA in endothelial cells and cardiomyocytes is membrane-associated via GPI anchors. Our results with Triton X-114 phase separation (Fig. 4) show a clear difference in the detergent-binding properties between soluble CA from erythrocytes and the investigated CA isozymes from heart cell microsomes. CA isozymes from
both cardiac cell types partition by >90% into the Triton phase, which proves their lipophilic nature. Thus we conclude that endothelial cells and cardiomyocytes from porcine as well as human hearts possess membrane-bound forms of CA. As mentioned above, there are presently three known membrane-bound CA isoforms, the GPI-anchored CA IV with a wide tissue distribution and the transmembrane proteins CA IX and XII, which are expressed in tumors and have a rather limited distribution in normal tissues.

The results from treatment with bacterial PI-PLC demonstrate that endothelial and myocytic CAs from both species are membrane linked via GPI. Figure 5 demonstrates sensitivity to PI-PLC treatment, which released at least two-thirds of the CA activity into the supernatant. Endothelial CA IV is known to be membrane linked via GPI (21, 28, 42). The amount of cardiomyocytic CA released by PI-PLC is comparable to the release of endothelial CA, indicating that both CAs share the same mechanism of GPI-membrane anchoring. The less probable alternative explanation for a release by GPI-phospholipases is interaction with GPI-linked proteins (18). However, the present results are not compatible with either of the membrane-bound CAs in the heart being a transmembrane protein, and therefore they exclude that myocytic CA represents transmembrane CA IX or XII.

Novel CA isozyme in human cardiomyocytes. To investigate whether CA IV, the only known GPI-anchored CA to date, is responsible for myocytic CA activity, we analyzed its immunoreactivity with anti-human CA IV antibodies by immunoblotting. The specificity of the antibody used has been well established, and it has often been shown to detect human CA IV in Western blots (20, 31, 42).

Our results (Fig. 6) reveal that the majority of myocytic CA activity is not CA IV because it failed to react to an extent comparable to that seen with identical amounts of endothelial CA IV activity. Thus CA activity in human cardiomyocytes must predominantly originate from a different and as yet unknown GPI-anchored CA isozyme possessing weak or no immunoreactivity toward anti-CA IV antibodies. Our results confirm that CA IV is associated with cardiac endothelial cells, as indicated by an intense CA IV band in the immunoblot. In contrast, myocytic CA activity resulted in very weak CA IV bands, which were approximately 11 times weaker than those obtained with identical amounts of endothelial CA activity.

Deglycosylation did not change the very weak immunoreactivity of the myocytic isozyme nor the strong immunoreactivity of the endothelial isozyme toward anti-CA IV antibodies (Fig. 7). In the case that the myocytic isozyme was a CA IV protein, modified by oligosaccharide chains, deglycosylation might be expected to have led to an equalization of immunoreactivities of CA from endothelial cells and cardiomyocytes, because it would remove oligosaccharides that previously might have reduced the accessibility of antigenic sites to the antibodies. The possibility that the myocytic isozyme is a glycosylated CA IV was excluded by the results of the deglycosylation experiment, showing neither a detectable change in staining intensity nor in apparent molecular weight. They support that CA in cardiomyocytes differs in protein structure from the endothelial CA IV. The speculation that a putative myocytic CA IV selectively loses immunoreactivity, e.g., because of different exposure to digestion enzymes, is very unlikely. Several other features like enzyme activity, resistance to SDS, and GPI anchoring all have been shown to be preserved. Thus the results from CA IV Western blotting seem to provide evidence for a novel CA isozyme in human cardiomyocytes.

Our findings explain the contradictory results of immunocytochemical studies, because CA IV immunostaining of cryosections detected only endothelial CA IV and thus stained only capillaries (31). In contrast, all isoforms take part in the chemical reaction of the Hansson technique (11, 37), which therefore is indeed expected to also visualize the activity of the new myocytic CA isozyme. Wolfensberger et al. (41) reported a similar phenomenon in human retinal pigment epithelial cells: in immunocytochemistry with anti-CA IV antibodies, positive staining was restricted to the apical membranes, whereas the Hansson technique detected CA activity at apical and basolateral membranes of these cells. The latter may be interpreted to represent a non-CA IV membrane-bound CA.

A controversial discussion regarding the molecular mass of CA IV led to the assumption that there might be more than one GPI-anchored CA isozyme. In 1984 Wistrand (39) found human renal CA IV to have a high molecular mass of 68 kDa by SDS gel electrophoresis, but Wistrand and Knuutila (40) later reported its molecular mass to be 34.4 kDa from SDS electrophoresis and 36.7 kDa from amino acid analysis. Carter et al. (4) detected a single immunostained band at 55 kDa in a range of fetal human tissues. Wolfensberger et al. (41) found in human retinal pigment epithelium a 55-kDa “native” CA IV on SDS-PAGE, which was reduced to 35 kDa in the presence of 0.1% Triton X-100.

Controversial reports in the literature regarding the molecular mass of CA IV and its cellular localization may be interpreted as hints at the existence of more than one membrane-bound CA isozyme. This study provides experimental support for CA activity in cardiomyocytes and confirms by immunoblotting and other methods that most of this CA activity likely does not represent CA IV, CA IX, or CA XII. Our data rather suggest the presence of an as yet unknown CA isozyme, membrane-associated via GPI, in human cardiomyocytes.

Two GPI-anchored CA isozymes in cardiomyocytes. In the present Western blots of myocyte microsomes, there is a weak CA IV band. This raises the question whether cardiomyocytes contain only the non-CA IV CA isozyme or if there is CA IV present in addition. There are three possible interpretations of the weak band obtained from myocytic CA activity: 1) it might be due to a weak cross reactivity of the non-CA IV myocytic CA isozyme with anti-CA IV antibodies, 2) the low percentage of vWF found in human cardiomyocyte preparations might...
suggest that it is contamination by endothelial cell membranes that contribute some CA IV to cardiomyocyte preparations, and 3) human cardiomyocytes may contain two membrane-bound CA isozymes, a small amount of CA IV responsible for the CA IV band and a larger amount of a non-CA IV isozyme contributing the bulk of CA activity.

The first and latter interpretations are in line with recent immunohistochemical studies of Sender et al. (30). They demonstrated for the first time CA IV at the myocytes' sarclemma in human and rat heart muscle. However, detection was positive only in semithin and ultrathin sections (30, 31). In cryosections the anti-CA IV antibody failed to detect sarclemmal CA, whereas it reacted strongly with capillaries. This discrepancy was interpreted as a problem of accessibility and density of the antigenic sites. In situ hybridization histochemistry detected CA IV encoding mRNA in human heart cryosections, demonstrating CA IV expression in endothelial cells and cardiomyocytes of human hearts. However, cross reaction with a proposed novel non-CA IV membrane-bound CA isozyme cannot be excluded, because a high percentage of homology on the mRNA level is possible. Furthermore, the results of Sender et al. (30) did not allow any quantitative comparison of CA IV expression in endothelial cells and cardiomyocytes, and therefore, they are not incompatible with our finding that myocytic CA originates predominantly from a non-CA IV membrane-bound isozyme.

Although the immunocytochemical evidence for CA IV in cardiomyocytes is not unequivocal, taken together with the results of in situ hybridization, it appears possible that cardiomyocytes contain two CA isozymes, with the non-CA IV isozyme contributing by far the majority of activity. The functional significance of this second GPI-anchored CA isozyme and a possible role distinct from that of CA IV remains to be investigated.

Functional role of endothelial CA IV. We detected CA activity in vascular cells and confirmed with immunoblotting that this activity corresponds to CA IV. In situ hybridization confirmed CA IV mRNA expression in cardiac endothelial cells (30). All mentioned histochemical studies agreed in attributing endothelial CA to the luminal membrane of endothelial cells. An additional localization at the external capillary membrane was suggested from results with immunocytochemistry on semithin sections (30) and with the Hansson technique (37).

As demonstrated by Forster and Crandall (7) extracellular, endothelial CA IV with an orientation toward the capillary lumen acts by accelerating CO₂ hydration in the plasma and prevents a disequilibrium of H⁺ concentration between red blood cells and plasma to develop during capillary transit. Forster and Crandall (7) have shown that in this way endothelial CA IV accelerates and enhances CO₂ uptake by the blood during capillary transit. Such a role is also supported by the three-dimensional structure of CA IV recently analyzed by Stams et al. (33): The active site of the protein is located away from the membrane, opposite to the GPI-membrane anchor, and this position is stabilized by interaction of the COOH-terminal electropositive surface potential and negatively charged membrane phospholipids. The CA IV on the external capillary surface might contribute to interstitial buffering, where the CO₂-HCO₃⁻ system is the main buffer system for H⁺ leaving tissue cells.

Functional role of CAs in cardiomyocytes. Cardiomyocytes contain GPI-anchored CA, which provides CA activity directly on the external surface of these cells. Surface pH measurements with microelectrodes on superfused Purkinje strands and papillary muscles by DeHemptinne et al. (5) demonstrated the functional presence of CA on the cell surfaces and its functional consequences. CA inhibition was shown to magnify the amplitude of surface pH transients that follow step changes in extracellular propionate concentration. This indicates a function of extracellular sarclemmal CA in attenuating interstitial pH transients by making the CO₂-HCO₃⁻ buffer system rapidly available as a H⁺ source or sink.

In reperfusion experiments after ischemia in ferret hearts the initial rate of intracellular pH recovery is slowed by CA inhibitors, as measured by a shift of 2-deoxy-o-glucose 6-phosphate with 3¹P NMR spectroscopy by Vandenbergh et al. (37). Slowing of intracellular pH recovery was accompanied by slower recovery of left ventricular diastolic pressure. This inhibitory effect was obtained with both membrane-impermeable and -permeable inhibitors, which suggests that it is caused by inhibition of an extracellular CA. This may indicate that interstitial and/or intracapillary H⁺ buffering is important for recovery of intracellular pH after ischemia of the heart, although the precise mechanism remains to be understood.

It may be noted that recent experiments in our laboratory (P. Wetzel, S. Papadopoulos, and G. Gros, unpublished observations) suggest that the extracellular CA of myocytes may be essential for the transport of important substrates across the sarcomemmal membrane.

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