Localization of the Kv1.5 K^+ Channel Protein in Explanted Cardiac Tissue

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Abstract

The cloned Kv1.5 K^+ channel displays similar kinetics and pharmacology to a delayed rectifier channel found in atrial myocytes. To determine whether the Kv1.5 isoform plays a role in the cardiac action potential, it is necessary to confirm the expression of this channel in cardiac myocytes. Using antibodies directed against two distinct channel epitopes, the Kv1.5 isoform was localized in human atrium and ventricle. Kv1.5 was highly localized at intercalated disk regions as determined by colocalization with connexin and *N*-cadherin specific antibodies. While both antichannel antibodies localized the Kv1.5 protein in cardiac myocytes, only the NH_2-terminal antibodies stained vascular smooth muscle. The selective staining of vasculature by this antiserum suggests that epitope accessibility, and perhaps channel structure, varies between cardiac and vascular myocytes. Kv1.5 expression was localized less in newborn tissue, with punctate antibody staining dispersed on the myocyte surface. This increasing organization with age was similar to that observed for connexin. Future work will address whether altered K^+ channel localization is associated with cardiac disease in addition to changing with development. (*J. Clin. Invest.* 1995. 96:282–292.) Key words: immunolocalization • antibodies • intercalated disk • voltage-gated potassium channel • myocardium

Introduction

The electrically excitable cells of the heart display a complex and unique action potential resulting in part from the differential expression of various ion channel isoforms. Probably the most diverse group of ion channels within the heart are the K^+ channels responsible for repolarization. At least five functionally distinct voltage-gated K^+ currents have been described in mammalian heart (1, 2). However, the presence of multiple overlapping currents in cardiac myocytes complicates the study of individual K^+ channels. While the traditional approach uses a combination of holding potential modulation, ion substitution, and pharmacological dissection to eliminate all but the current of interest, molecular cloning and cDNA expression technologies have permitted the study of a single cloned isoform in the expression system of choice. However, this approach poses a new challenge in terms of correlating the cloned channels with specific cells and endogenous currents.

At least six different, *Shaker*-like, K^+ channels have been cloned from rat (3), mouse (4), ferret (5), or human heart (6, 7) and functionally expressed in heterologous expression systems. Of these, the channel designated Kv1.5 is the most cardiac specific from both a developmental and tissue-specificity standpoint (2, 8). Kv1.5 has been cloned also from skeletal (9) and smooth muscle (10), indicating that this isoform is expressed in a variety of muscle cell types. Upon expression of the Kv1.5 clone in either mouse fibroblasts (11), HEK 293 cells (7), or *Xenopus* oocytes (12), this cDNA produces a delayed rectifier-like current with kinetic properties and pharmacology similar to an endogenous cardiac myocyte current described by Boyle and Nerbonne in rat atrium (13) and by Nattel and co-workers in human atrial cells (14). However, the mere resemblance in kinetics is not sufficient to correlate this clone with the endogenous current. The pharmacology is also insufficient since many voltage-gated channels share pharmacological properties. A greater problem is the finding that *Shaker*-like K^+ channel proteins form heteromeric structures both in heterologous expression systems (15–18) and in vivo (19, 20). These heteromeric channels have functional and pharmacological properties distinct from the respective homomeric channels. There is also the more recent finding that function-altering beta subunits exist which can convert cloned channels with delayed rectifier properties into ones that rapidly inactivate (21). Therefore, correlation of Kv1.5 with an endogenous current ultimately requires the modulation of the native current by interventions specific for the Kv1.5 isoform, such as function-altering, isoform-specific antibodies, antisense cRNA approaches, dominant-negative strategies, or gene knockouts in transgenic animals. However, before any of these approaches are undertaken, it is prudent to first confirm that the Kv1.5 channel protein is present in cardiac myocytes.

We report here the localization of the hKv1.5 channel protein in human atrial and ventricular myocardium obtained from newborn and adult patients. In the adult tissue, the channel was not evenly distributed over the myocyte surface but rather was localized in regions of high density within the intercalated disks. This pattern changed with development, with a more dispersed staining pattern observed in newborn tissue as compared with adult myocardium. While two distinct antichannel antibodies localized the Kv1.5 protein in cardiac myocytes, only one stained vascular smooth muscle. The selective staining of vascular smooth muscle cells by the antibodies suggests that channel structure or subunit composition varies between cardiac and vascular myocytes. These data indicate that the human Kv1.5 potassium channel is a true myocyte channel as opposed to being expressed in endothelial or glial cells. Studies focusing on altered Kv1.5 expression in diseased human myocardium are now possible.

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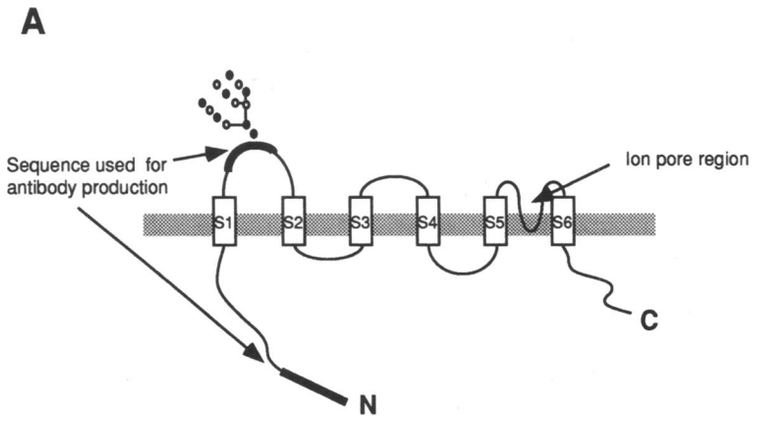

B

N terminal epitope

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HKv1.5   VPLENGGAMTVRGGDEARAGCGQATGGELQCPPTAGLSDGPKEPAPRGRAQR DADSG
RKv1.5   VPLENGSAMTLRGGGEAGASCVQTPRGECGCPPTSGLNNQSKETLLRGRTTLEDANQG

         VRPLPPLPDPGVRPLPPLPEELPRPRRPPPEDEEEEGDPGLGTV-EDQALGTA-SLH
         GRPLPPMAQ           ELPQPRRLSAEDEEGEGDPGLGTVEEDQAPQDAGSLH
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S1-S2 epitope

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HKv1.5      LLRHPPAPHQPPAPAPGANGSGVM A
RKv1.5      LLRHPPVPPQPPAPAPGINGS-VSGA
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Figure 1. Choice of epitopes for antibody production. (*A*) Epitope location within the Kv1.5 channel amino acid sequence. The intracellular NH_2 terminus and the extracellular sequence between S1 and S2 were chosen for antibody production. (*B*) Comparison of Kv1.5 epitope sequence between human and rat. Regions of nonidentity are indicated by the shaded boxes.

Methods

Antiserum preparation and affinity purification. The NH_2-terminal and S1-S2 linker regions on the human Kv1.5 K^+ channel (Fig. 1) were chosen for antibody production. These epitopes were predicted to be antigenic in the rabbit since the sequence shows some species variation when comparing channels cloned from human and rat. However, the sequence in these regions is confined to the Kv1.5 isoform and was predicted to produce isoform-specific antibodies. For amino acid sequence comparisons between Kv1 K^+ channel subfamily isoforms see references 1, 3, and 22. Blast searches of the GenBank and EMBL databases indicated that only the mouse, rat, and canine Kv1.5 K^+ channels showed significant identity with the NH_2-terminal epitope, while only the mouse and rat homologues showed significant identity with the S1-S2 epitope. Antiserum against the S1-S2 epitope (Fig. 1) was produced in rabbits using a synthetic peptide, LLRHPPAPHQPPAPAPGANGSGVMA, as described previously (23). Rabbit antiserum against the NH_2-terminal 112 amino acids was produced by using PCR to amplify the NH_2-terminal region of Kv1.5 from VPLEN . . . to . . . TASLH as described previously in detail (24). The use of VENT polymerase (New England Biolabs, Beverly, MA) and a low cycle number guarded against reaction-induced sequence error. The forward PCR primer sequence, 5′ GAGAGAATTCTGGTGCCCCTGGAGAACGGCGGTG3′, and the reverse primer, 5′GGACAAGCTT*TCA*GTGCAGGGACGCCGTGCCCAG3′, included either EcoRI or HinDIII restriction enzyme sites on the 5′ ends (underlined sequence). The reverse primer also included an in frame stop codon (italicized sequence). The PCR fragment was digested with the restriction enzymes, gel purified, ligated into the pGEX vector (Pharmacia, LKB Biotechnology, Piscataway, NJ), and transformed into *Escherichia coli* strain UT481 as described previously (24, 25). The resulting construct was purified and sequenced by dideoxy methods to check for any unwanted mutations. Construct-containing bacteria were induced to produce glutathione transferase (GSH-T)[1] fusion protein containing the NH_2-terminal Kv1.5 channel sequence, which was then purified by affinity chromatography on GSH-Sepharose (Sigma Immunochemicals, St. Louis, MO), emulsified with complete Freund's adjuvant, and injected into rabbits according to established protocols (25, 26). The antisera were tested by immunofluorescent staining of Kv1.5-transfected L cells as described below. Affinity purification was performed using native GSH-T and GSH-T channel fusion proteins covalently bound to Sepharose as described previously (24). Briefly, the antiserum was passed first over the GSH-T–Sepharose columns to remove antibodies against the carrier protein. The eluate was then passed over the fusion protein column and specifically bound antibody eluted with low pH.

Tissue preparation. Explanted human heart tissue was obtained at the time of heart transplantation from neonates with otherwise inoperable congenital heart disease or from adults with cardiac myopathies with and without coronary artery disease. In one case normal tissue was collected from a donor that did not meet the transplantation requirements. The patients ranged in age from 1 mo to 67 yr. Most commonly, samples of the left ventricular free wall and right and left atrial appendage were obtained. While the immunolocalization presented in this paper is derived from diseased organs, regions showing scarring or obvious ischemic damage were not used. The tissue was placed in cryomolds, embedded in Tissue Tek (Baxter, Stone Mountain, GA), then flash

1. *Abbreviations used in this paper:* CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate hydrate; GSH-T, glutathione transferase.

frozen in an isopentane bath. Alternatively, some samples were slowly frozen at − 80°C. The human specimens were stored at −80°C until used for the immunohistochemical studies. Male Sprague-Dawley rats were anesthetized with intraperitoneal injections of pentobarbital and decapitated, and the hearts were removed. The hearts were flushed with ice cold phosphate-buffered saline (PBS) (0.9% NaCl in 0.01 M NaH_2PO_4 [pH 7.4]), cut into two to three sections, placed in cryomolds, and frozen and stored at −80°C in Tissue Tek embedding medium.

Photomicroscopy. Samples were analyzed using either a Leitz DM microscope equipped with standard epifluorescence and a 35-mm camera or a Zeiss LSM410 laser scanning confocal microscope equipped with 488 and 543 nm laser excitation. All images obtained from the confocal microscope were directly digitized, stored on hard disk, and printed later using a Tektronix Phaser IISDX color printer. Images obtained on Kodak Ektachrome 400 film from the Leitz microscope were digitized by optical scanning with a Nikon Coolscan and later printed. The filter sets used on the Leitz microscope did not fully separate the BODIPY and CY3 fluorophores used in these studies. Therefore, all images of dual staining with these compounds used the Zeiss 488 nm laser in conjunction with the appropriate filters for excitation and detection of the BODIPY signal while the 543 nm laser was used to detect the CY3 fluorophore. This approach completely separated the two signals. The black and white images presented were recorded originally as color images on film but were digitized in greyscale. Additional details are provided in the figure legends.

Western blot analysis of Kv1.5-expressing L cell membranes. Cell membrane preparation and Western blot analysis were performed as described previously in detail (24). The construction of a Kv1.5 truncation lacking the 57 COOH-terminal amino acids has been described previously (27). Basically, native and truncated Kv1.5- and sham-transfected cell membranes were prepared and 60 μg of protein was fractionated on a 10% polyacrylamide SDS gel. After electrophoretic transfer to polyvinyldifluoride, the membrane was incubated with the S1-S2 or NH_2-terminal antisera, 1:500 and 1:1,000 dilutions, respectively. Bound primary antibody was detected with a 1:10,000 dilution of alkaline phosphatase–conjugated goat anti–rabbit IgG and the Western Light protein detection kit according to the manufacturer's protocol (Tropix Inc., Bedford, MA).

Immunofluorescence staining of transfected tissue culture cells. Functional expression of the human Kv1.5 cDNA in mouse L cells and the preparation of sham-transfected cells have been described previously in detail (11). L cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin with 0.25 mg/ml G418, under a 5% CO_2 atmosphere. The transfected L cells were grown on poly-L-lysine–coated, UV-sterilized glass cover slips, and incubated overnight with 4 μM dexamethasone to induce Kv1.5 channel expression. The cells were not fixed, and the coverslips were not removed from their tissue culture dishes until ready for microscopic examination to prevent cell detachment. To reduce bacterial contamination during the experiment, all incubations included 0.08% sodium azide, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, and 0.05 mg/ml gentamicin. The cover slips were washed gently with PBS and then incubated for 1 h 15 min at room temperature with primary antiserum diluted 1:1,000 in PBS plus 10% goat serum and 0.25% saponin. The cells were taken through 3 2-min washes with PBS plus 10% goat serum, then incubated for 30 min at room temperature with biotin-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 in PBS plus 1.5% goat serum. Another wash series with PBS was followed by a final incubation for 30 min at room temperature with CY3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:200 in PBS plus 1% BSA. The coverslips were then wet mounted in PBS on hanging drop glass slides and examined immediately. Channel-free, sham-transfected cells served as controls for background staining levels.

Immunostaining of heart tissue cryosections. 10-μm cryosections of both human and rat heart were cut using a Bright Cryostat (model B2435; Hacker Instruments Inc., Fairfield, NJ). The following wash and blocking steps were performed in Columbia staining jars while the antibody and fluorochrome incubations were performed in a humidity chamber. The sections were collected on gelatin-coated coverslips, dried for 5–10 min at room temperature, and washed three times, 10 min each, in PBS. The tissue was then treated with 0.5% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate hydrate) in PBS with three solution changes, 10 min each. The tissue was washed again with PBS (3 × 10 min) to remove CHAPS, and then blocked overnight at 4°C in PBS containing 10% goat serum and 5% nonfat dry milk. After the overnight block, the tissue was incubated in primary antiserum diluted in PBS plus 10% goat serum, 0.5% CHAPS, and 0.25% saponin, for 1.5 h at room temperature. The tissue was washed three times for 10 min each with PBS plus 10% goat serum and 0.5% CHAPS, followed by 1 10-min wash with PBS alone. Endogenous avidin and biotin were blocked using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendations. The sections were then incubated with biotin-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:200 in PBS plus 1.5% goat serum for 30 min at room temperature. The tissue was washed (3 × 10 min) with PBS plus 0.5% CHAPS and incubated with CY3-conjugated streptavidin (Jackson ImmunoResearch Laboratories) diluted 1:200 in PBS containing 0.5% CHAPS and 1% BSA for 45 min at room temperature. The coverslips were washed a final time with PBS plus 0.5% CHAPS and, if no further staining was to be performed, mounted on glass slides using Aqua-PolyMount (Polyscience, Warrington, PA) and allowed to dry overnight before microscopic examination.

Immunogen block of tissue staining. Preabsorption of S1-S2 linker antibody with peptide immunogen was used to demonstrate binding specificity of this antibody. Staining was performed as described above, except that alternate cryostat sections were stained with S1-S2 anti-Kv1.5 primary antiserum that had been preincubated for 2 h at room temperature with 150–200 nmol of the S1-S2 peptide per milliliter of diluted antiserum (1:1,000). The control slices were stained with S1-S2 anti-Kv1.5 antiserum that underwent the same 2-h incubation at room temperature, minus the S1-S2 peptide. Preabsorption of NH_2-terminal antibody with GSH-T fusion protein construct was used to demonstrate binding specificity of this antibody. Alternate slices were stained with NH_2-terminal anti-Kv1.5 antiserum, or affinity-purified antibody, that had been preincubated overnight at 4°C with 40 nmol of the NH_2-terminal fusion protein construct (112 NH_2-terminal amino acids coupled to bacterially synthesized GSH-T per milliliter of antisera (diluted 1:1,000) or affinity-purified antibody (diluted to 2 μg/ml). The control slices were stained with NH_2-terminal anti-Kv1.5 antiserum or affinity-purified antibody that underwent the same overnight incubation, either minus the fusion protein construct or with the GSH-T carrier alone. Identical results were obtained in both controls.

Colocalization of Kv1.5, connexin, and N-cadherin antibody binding. Mouse anti–connexin-43 was obtained from Zymed Laboratories (South San Francisco, CA), and rabbit anti–pan cadherin was obtained from Sigma Immunochemicals. These antibodies produced well characterized staining patterns when used separately (28–31) (data not shown). For the connexin colocalization, the immunostaining with the anti-Kv1.5 antibody was performed as described above, but instead of immediate microscopic examination the tissue was incubated overnight at 4°C with the anticonnexin primary antibody (diluted 1:500–1:5,000) in PBS plus 1.5% goat serum. The tissue was taken through 3 10-min washes with PBS containing 10% goat serum and 0.5% CHAPS, then washed 3 times, 10 min each, with PBS containing 0.5% CHAPS alone. A final incubation of the tissue was in BODIPY FL goat anti–mouse IgG fluorophore (Molecular Probes, Inc., Eugene, OR) diluted 1:100 in PBS plus 0.5% CHAPS and 1% BSA. After a final wash (3 × 10 min), the slides were mounted and examined. Standard controls were included to determine the staining levels of the secondary antibodies in the absence of primary antibodies and to confirm that the secondary antibodies did not crossreact between species in terms of detecting the primary rabbit and mouse antibodies. Staining with individual antibodies was also performed to confirm that the staining patterns observed in the single- and double-stained sections were identical.

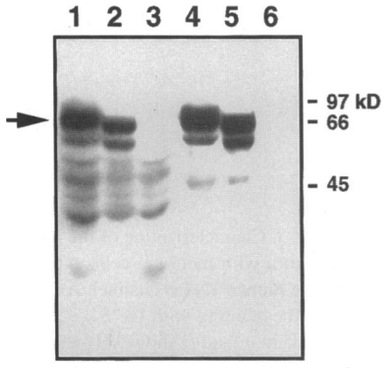

Figure 2. Western blot analysis of Kv1.5-expressing L cells. Cell membranes were prepared, size fractionated by SDS gel electrophoresis, and transferred to filters as described in Methods. Lanes *1* and *4* contain membranes from native hKv1.5-expressing cells and show the binding of S1-S2 and NH_2-terminal antibody, respectively. Lanes *3* and *6* show the binding of the same antibodies to membranes prepared from sham-transfected cells. Lanes *2* and *5* show the binding of the S1-S2 and NH_2-terminal antibodies, respectively, to membranes prepared from cells expressing a hKv1.5 channel lacking the 57 COOH-terminal amino acids. The arrow indicates the position of the full-length hKv1.5 protein.

Since the anticadherin antibodies and both of the Kv1.5 antisera were raised in rabbit, this colocalization required additional blocking steps as described previously in detail for same-species, dual immunohistochemistry (32). The immunostaining with the anti-Kv1.5 antibody was performed as described above, followed by a 1-h room temperature blocking incubation with a rabbit serum that did not stain heart above background levels. This step saturated any remaining rabbit IgG binding sites on the secondary antibody used to detect the Kv1.5 antibody staining. The tissue was then washed with PBS plus 0.5% CHAPS (3 × 10 min) and incubated for 1 h at room temperature with goat anti-rabbit IgG $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories) diluted 1:30 in PBS plus 1.5% goat serum. This blocking step masked F(c) regions on any bound rabbit IgG. The wash was repeated and the tissue was incubated overnight at 4°C with the cadherin antibody (diluted 1:2,000–1:8,000) in PBS plus 1.5% goat serum. The tissue was washed with PBS containing 10% goat serum and 0.5% CHAPS (3 × 10 min) and then washed again with PBS containing 0.5% CHAPS alone (3 × 10 min). The final incubation of the tissue was in BODIPY FL goat anti-rabbit IgG fluorophore (Molecular Probes, Inc.) diluted 1:100 in PBS plus 0.5% CHAPS and 1% BSA. A final wash series was performed (3 × 10 min) and the slides were mounted and examined. Several control slices were included to determine that the blocking steps outlined above were successful, i.e., that the last secondary antibody did not bind to available first round primary or secondary antibodies and that the blocking antibodies did not recognize any proteins in heart. Single staining was also performed to compare the staining patterns with those obtained in the double-stained sections.

Results

Characterization of the Kv1.5 channel in transfected tissue culture cells. Western blot analysis of transfected L cell membranes was performed to characterize the two antisera as shown in Fig. 2. Both the S1-S2 and NH_2-terminal antibodies specifically detected the Kv1.5 channel protein (64,000 M_r, see *arrow*) in hKv1.5-expressing cells (lanes *1* and *4*) but not in sham-transfected cells (lanes *3* and *6*). Lanes *2* and *5* contained membranes from cells expressing a truncated form of hKv1.5 (27) lacking the 57 COOH-terminal amino acids. The band detected by both antibodies had the predicted increase in electrophoretic mobility for the truncated channel.

Before the immunostaining of cardiac tissue, antibodies were first tested for the ability to localize the human Kv1.5 channel expressed in stable mouse L cell lines. Using sham-transfected cells as the measure of true background staining, conditions were established which demonstrated specific antibody binding with a satisfactory signal-to-noise ratio. Specific binding of both the S1-S2 and NH_2-terminal antibodies to hKv1.5-expressing cells is shown in Fig. 3, *A* and *C*, respectively. The channel-expressing cells in Fig. 3 *A* were incubated with the S1-S2 antiserum as described in Methods. Intense antibody binding is seen at the cell surface with moderate intracellular staining. Considerable variability was observed between individual cells, consistent with variations seen in peak current amplitudes at 60 mV under whole cell voltage clamp (0.5–20 nA, average = 1.6 nA) (11). Current amplitudes of several nanoAmperes correspond to several thousand channels per cell or 1–3 channels/μm^2/. Sham-transfected cells (Fig. 3 *B*) showed no detectable surface staining under these conditions, indicating binding specificity. Fixation in 1% paraformaldehyde or acetone prevented detection of cell surface channels (data not shown). The S1-S2 antibody was species specific in that it did not stain L cells expressing the rat Kv1.5 clone, even though equivalent current amplitudes were obtained (data not shown). Since this antibody recognizes an extracellular epitope, staining was also performed with live cells in the absence of membrane-permeabilizing reagents. As expected, a cell surface pattern similar to that shown in Fig. 3 *A* was obtained (data not shown). Fig. 3, *C* and *D*, shows similar results obtained with the NH_2-terminal antibody and the hKv1.5- and sham-transfected cells, respectively. In contrast to the S1-S2 antibody, the NH_2-terminal serum did recognize the rat Kv1.5 channel in transfected L cells (data not shown), an unexpected finding since the NH_2-terminal epitope sequence differed more between the two species than did the S1-S2 linker epitope (see Fig. 1 *B*). The lower magnification shown in Fig. 3 *C* further illustrates the heterogeneity in staining intensity. Immunofluorescent staining of cells expressing the truncated hKv1.5 produced patterns identical to those shown in Fig. 3, *A* and *C* (data not shown).

Localization of the Kv1.5 channel in human myocardium. The Kv1.5-like currents recorded from native human atrial myocytes are ~ 0.4 nA in amplitude at 50 mV (14). The fluorescence signal obtained from the transfected, Kv1.5-expressing tissue culture cells suggested that the immunofluorescence approach used here should detect the endogenous channel protein in sectioned human myocardium. Therefore, unfixed cryosections were prepared from six different adult human atrium and ventricle samples and stained with the S1-S2 antibody as described in Methods. As shown in Fig. 4 *A*, the channel was localized to two types of myocyte surface regions in human left atrial appendage. Binding was detected at both the ends of the myocytes near the intercalated disk and along the sides of some myocytes, especially near points of contact with adjacent myocytes. This labeling pattern was not observed when the peptide antigen was preincubated with the diluted S1-S2 antibody before staining the tissue section (Fig. 4 *C*). The localization observed in adult human ventricular muscle (left ventricular free wall) is shown in Fig. 4 *D* and was similar to that observed in atrium except that little longitudinal staining was seen. Again, the staining was completely blocked by preincubation with the peptide antigen as shown in Fig. 4 *F*. Antibody specificity was confirmed not only by the peptide block but also by the finding that this human specific antiserum did not stain rat or mouse heart above background. In addition, both aldehyde and acetone fixation prevented binding to human myocardium, again consistent with the L cell results. However, to guard against the un-

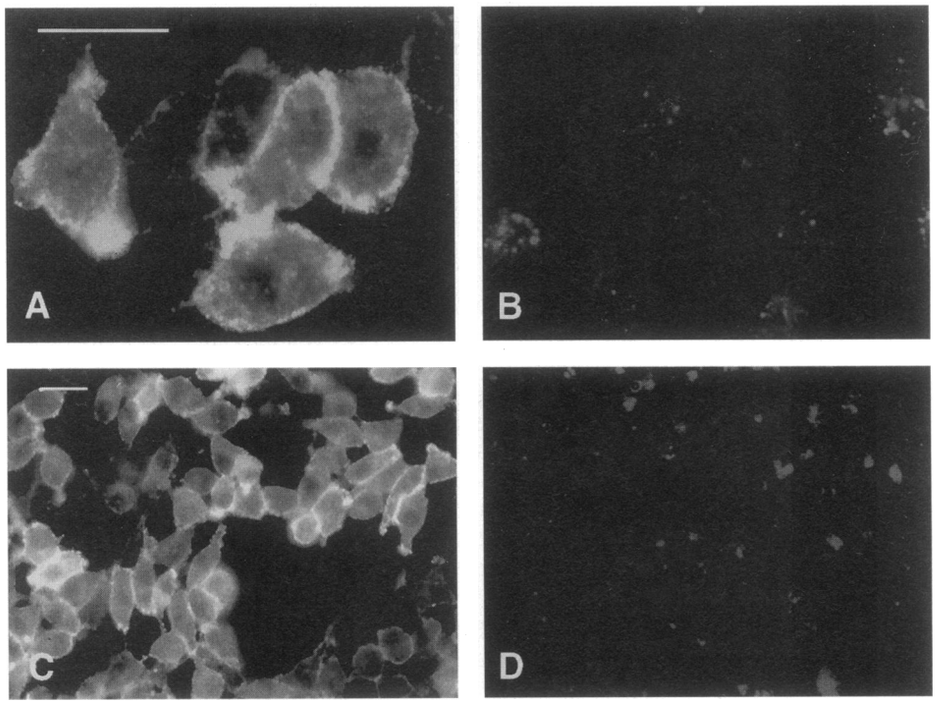

Figure 3. Characterization of the Kv1.5 antiserum with mouse L cells expressing the human Kv1.5 channel. *A* and *B* show the staining with the S1-S2 antibody while *C* and *D* show NH_2-terminal antibody staining. *A* and *C* show antibody binding to human Kv1.5-expressing L cells while *B* and *D* show background binding to sham-transfected cells. Antibody binding and detection was performed with nonfixed L cells permeabilized with 0.25% saponin as described in Methods. The bars represent 25 μm. Channel-binding antibodies were affinity purified from NH_2-terminal serum as described in Methods while the S1-S2 antiserum was used directly.

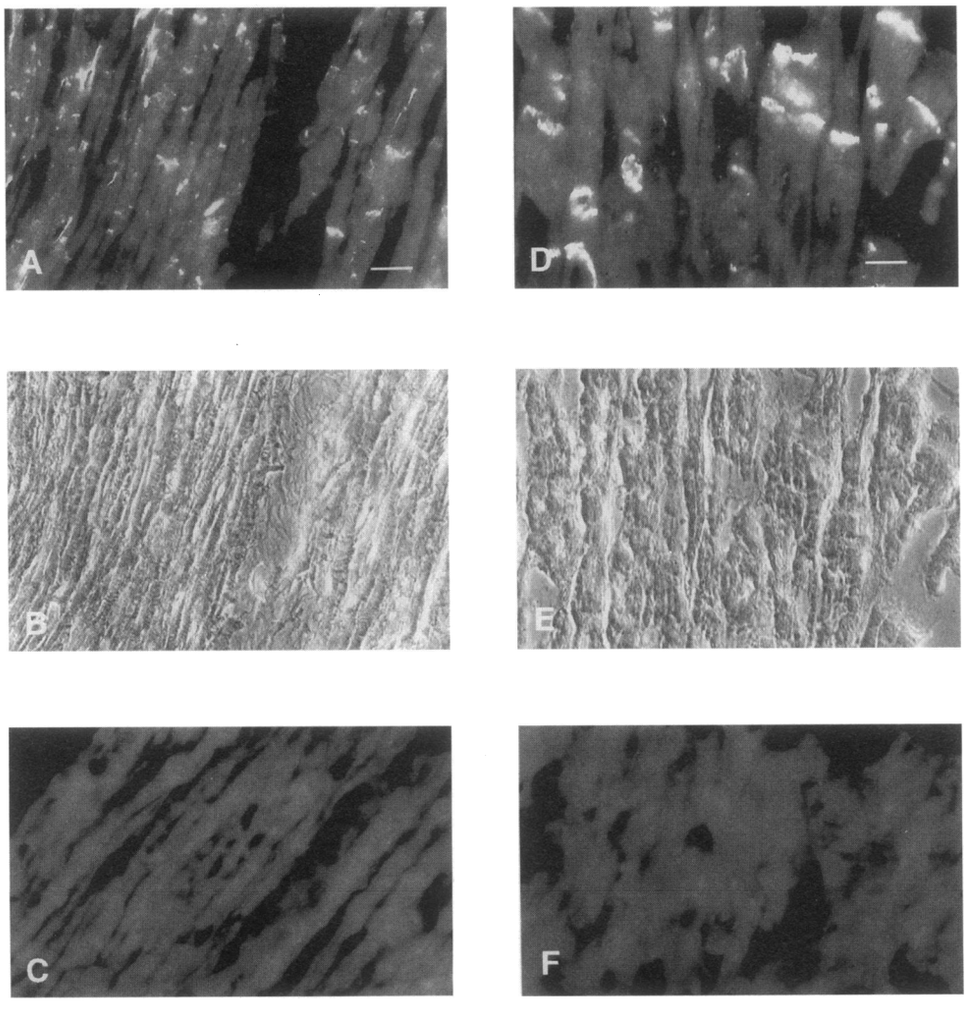

Figure 4. Immunodetection of the Kv1.5 channel protein in human myocardium using the S1-S2 antibody. 10-μm cryosections were prepared from adult human atrium and ventricle and stained with the S1-S2 antiserum as described in Methods. The left column shows the staining of atrial tissue with immunofluorescent detection of primary antibody binding, a phase micrograph of the fluorescent image, and peptide block of the staining to a similar tissue section (*A–C*, respectively). The right column, *D–F*, shows the staining of ventricular tissue in the same sequence. The bars represent 25 μm.

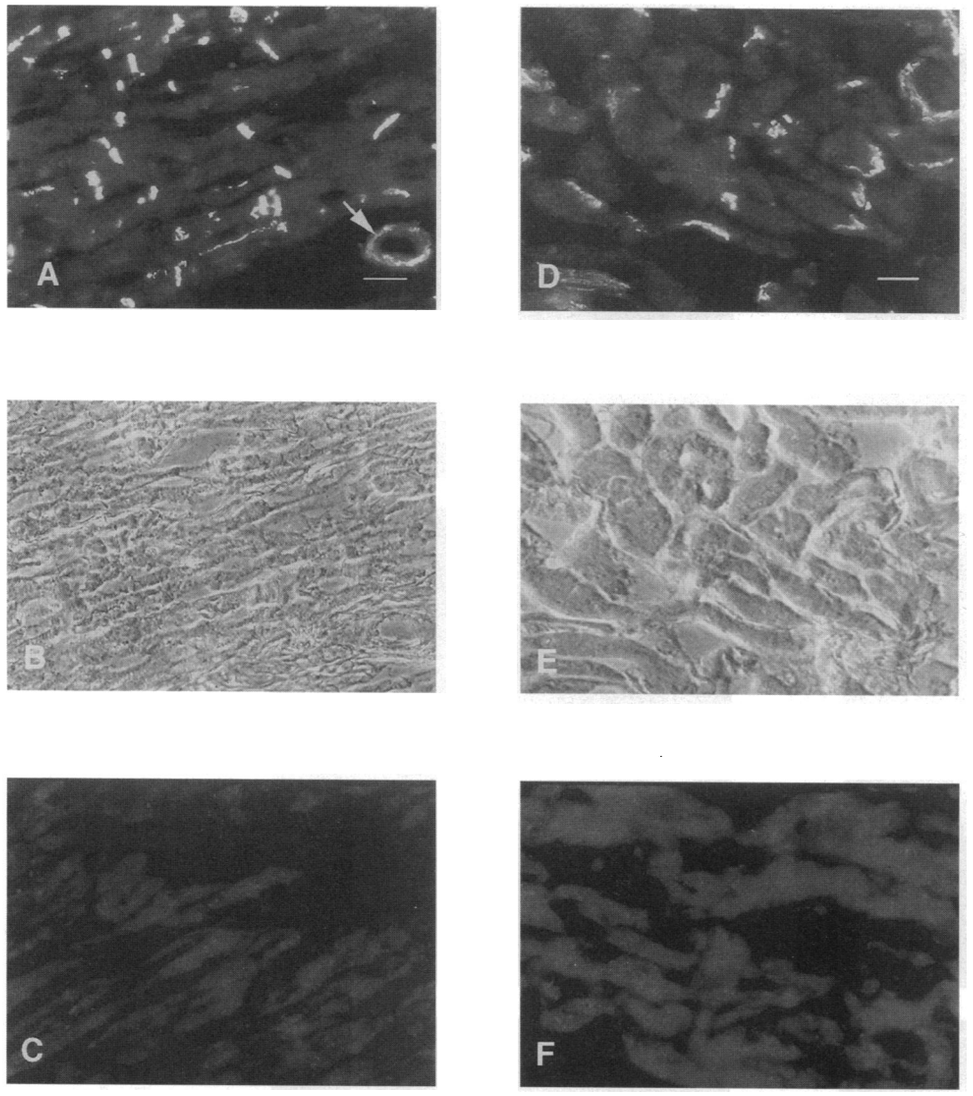

Figure 5. Immunodetection of the Kv1.5 channel protein in human myocardium using the NH_2-terminal antibody. 10-μm cryosections were prepared from adult human atrium and ventricle and stained with the affinity-purified NH_2-terminal antibody as described in Methods. The left side shows the staining of atrial tissue with immunofluorescent detection of primary antibody binding, a phase micrograph of the fluorescent image, and fusion protein immunogen block of the staining to a similar tissue section (*A–C*, respectively). The arrow denotes staining of a blood vessel. The right side, *D–F*, shows the staining of ventricular tissue in the same sequence. The bars represent 25 μm.

likely possibility that the S1-S2 antiserum was crossreacting with a related nonchannel epitope present at the intercalated disk, a second antibody directed against a different region of the Kv1.5 channel was produced.

The NH_2-terminal antibody was produced against the 112 NH_2-terminal amino acids coupled to bacterially synthesized GSH-T as described in Methods. This antibody was affinity purified to reduce staining background and all results obtained with the affinity-purified materials were also obtained with the antiserum. Fig. 5 shows the localization of the Kv1.5 channel in both human atrial and ventricular myocytes (*A* and *D*, respectively). The patterns are essentially the same as those observed with the S1-S2 antibody except that the NH_2-terminal-terminal antibody also stained vascular tissue (Fig. 5 *A*, *arrow*). Examination of antibody binding to human aorta suggests that this binding is to the vascular smooth muscle within the medial layers (data not shown). The staining of both the blood vessels and cardiac myocytes was completely blocked by incubation with the channel sequence containing fusion protein (Fig. 5, *C* and *F*), but not by the GSH-T carrier alone (data not shown). Since the same staining pattern in atrial and ventricular myocytes was observed with two different antisera directed against different regions of the channel, the pattern is representative of the subcellular localization of the Kv1.5 K^+ channel in human cardiac myocytes. The differential staining of blood vessels suggests that epitope accessibility, and therefore channel structure, varies between cardiac and vascular myocytes.

Colocalization of the hKv1.5 channel with intercalated disk proteins. Conformation that the Kv1.5 channel was localized in the intercalated disk region required colocalization of Kv1.5 with other proteins known to be associated with the disk. The two proteins chosen for colocalization with the Kv1.5 channel were cadherin and connexin-43. The antibodies against *N*-cadherin recognize a variety of cadherin subspecies composing the fascia adherins region of the intercalated disk. The connexin-43 antibody recognizes the gap junction channels present near the fascia adherins. These two antibodies were applied to human atrial and ventricular tissue sections after staining with the S1-S2 antibody. The fluorescent signals were collected separately using 543 and 488 nm laser excitation with the appropriate emission filters as described in Methods and shown in Fig. 6. Kv1.5 antibody binding was detected with CY3-coupled streptavidin and therefore appears red, while the cadherin and connexin signals were localized with BODIPY-labeled anti–rabbit

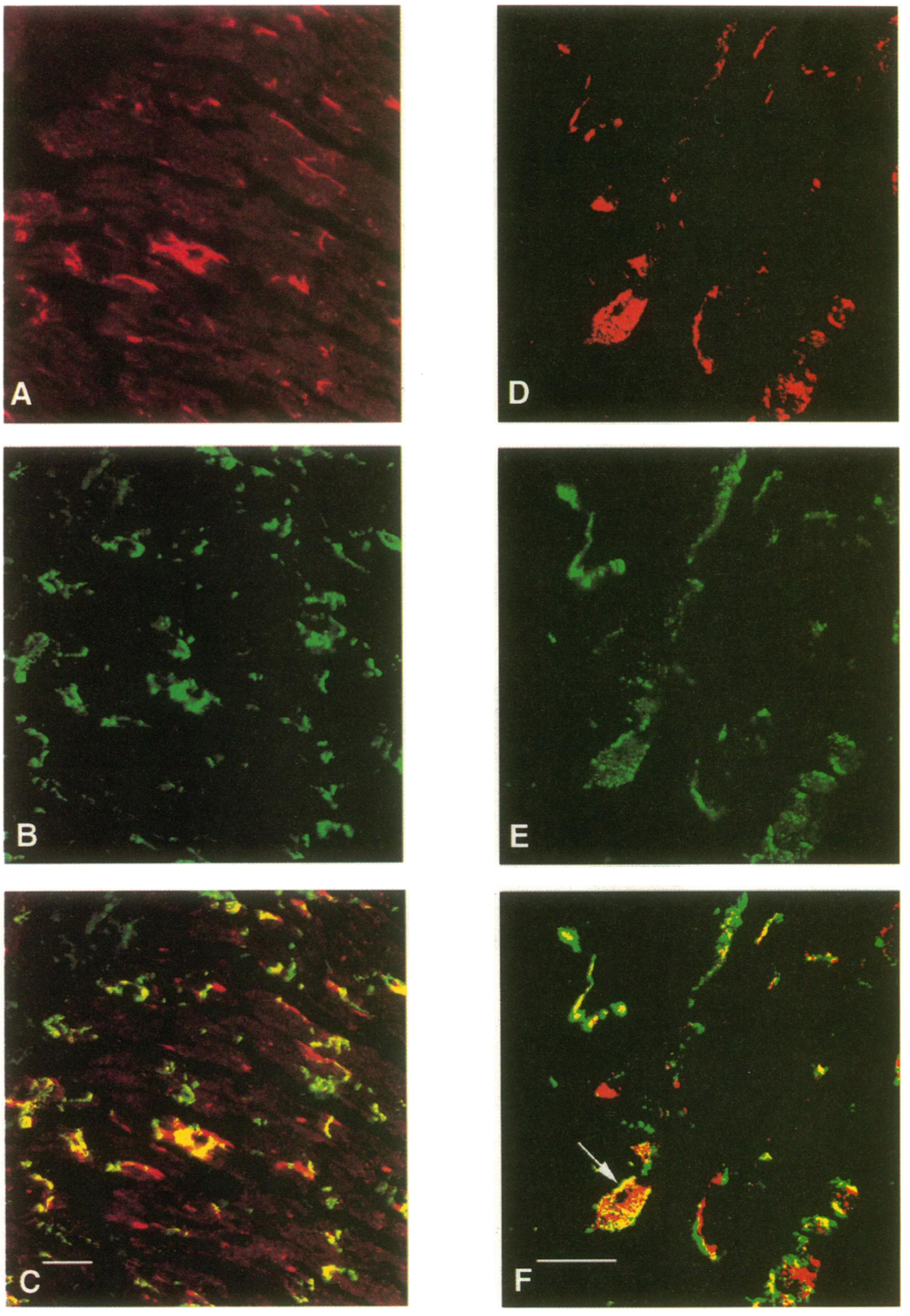

Figure 6. Localization of the Kv1.5 channel to the intercalated disk region. Colocalization with connexin- and cadherin-specific antibodies. The detection of bound anticadherin antibody and S1-S2 anti-Kv1.5 antibody is presented individually and together in *A–C*, respectively. The detection of bound anticonnexin antibody and S1-S2 anti-Kv1.5 antibody is presented individually and together in *D–F*, respectively. In both cases the bound Kv1.5 antibody was detected with CY3-conjugated streptavidin (*red*) and the connexin and cadherin antibodies were detected with BODIPY-conjugated IgGs (*green*). The fluorescent signals were collected separately using 543 and 488 nm laser excitation with the appropriate emission filters as described in Methods. The cadherin colocalization was performed with atrial sections while ventricular tissue was used for the connexin localization. The arrow in *F* denotes an intercalated disk in cross-section. The bars represent 25 μm.

or anti–mouse IgG and appear green. The detection of bound S1-S2 and cadherin antibodies in human adult atrial sections is presented individually and together in Fig. 6, *A–C*, respectively. As shown in Fig. 6 *C*, the red signal locating the channel is often associated with the green signal marking the cadherin location. Where complete colocalization is seen, the observed color is yellow due to the combination of the two primary signals. The patterns are not identical and often the two signals are not associated. Similar results were obtained when the Kv1.5 channel was colocalized with connexin in atrium (data not shown).

Human adult ventricular sections were stained also with both intercalated disk markers and the Kv1.5 antibody. Fig. 6, *D–F*, shows the staining of ventricular sections with S1-S2

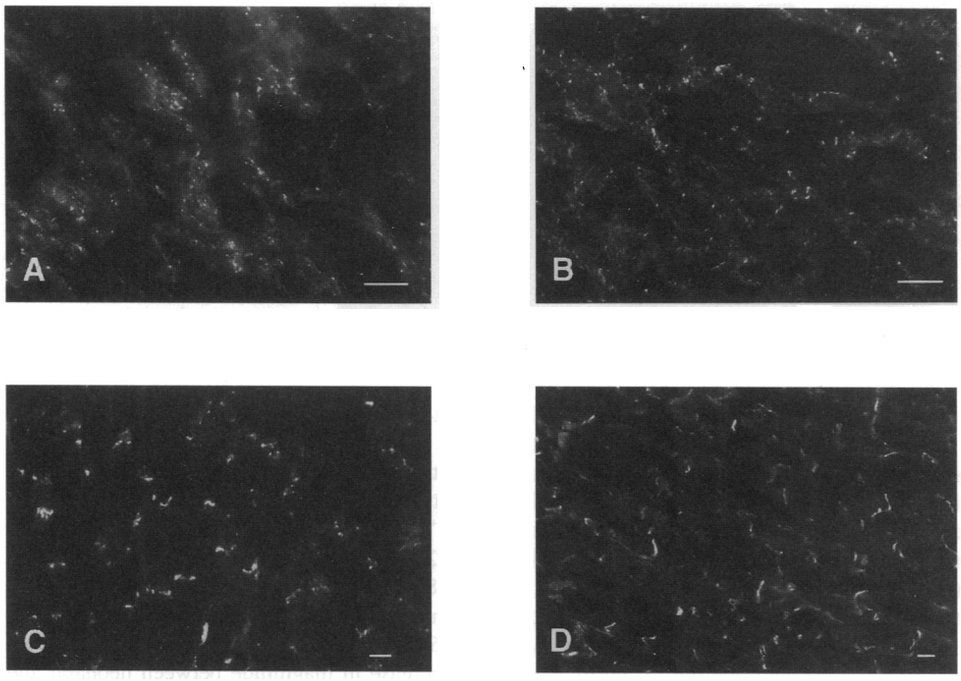

Figure 7. Comparison of Kv1.5 immunolocalization between newborn and adult myocardium. *A* and *B* show the staining observed with ventricular tissue from a 2-mo-old patient, using anticonnexin and the NH_2-terminal anti-Kv1.5 channel antibodies, respectively. *C* and *D* were stained with the same antibodies except that adult (36 yr old) tissue was used. The bars represent 25 μm.

Kv1.5 antiserum and the connexin antibody, individually and together. As shown in Fig. 6 *F*, the extent of colocalization between the intercalated disk marker and Kv1.5 in ventricle is much greater than that observed in atrium. In ventricle almost all the channels were associated with the connexin. Note the disk captured in cross section (*arrow*) in Fig. 6 *F*, illustrating the close association between the Kv1.5 and gap junction channels. When cadherin and Kv1.5 were colocalized in ventricle, the tight association between the two signals was also seen (data not shown).

The Kv1.5 localization pattern changes with development. The data presented in Fig. 6 confirm that the Kv1.5 channel is tightly associated with intercalated disk proteins in both human adult atrium and ventricle. Since newborn myocardium from both human (28) and rat (33) has a poorly defined disk structure compared with the adult, and Kv1.5 is localized near the disk, the localization of the channel in newborn myocardium was examined. Fig. 7 shows the localization of connexin and Kv1.5 in newborn ventricle (*A* and *B*, respectively). Both proteins are localized in a diffuse pattern on the cell surface, in some cases almost outlining the cell. The connexin localization shown in Fig. 7 *A* is similar to the patterns described previously for the localization of connexin in rat and human newborn heart (28, 33). In contrast to the diffuse pattern in Fig. 7, *A* and *B*, Fig. 7, *C* and *D*, shows the staining of adult human ventricle with the connexin and Kv1.5 antibody, respectively. In these two panels the standard intercalated disk pattern is seen, indicating that the Kv1.5 localization changes during development, probably in parallel with the connexin protein.

Localization of the Kv1.5 channel in rat heart. While the S1-S2 antibody did not recognize the rat homologue of Kv1.5 after expression in tissue culture cells, the NH_2-terminal antibody did. Therefore, this antibody was used to stain rat ventricular cryosections as shown in Fig. 8 *A*. The intercalated disk pattern was obtained in addition to staining of blood vessels. Staining of both cell types was blocked by the appropriate fusion protein (data not shown). A human atrial cryosection stained with the NH_2-terminal antibody is shown in Fig. 8 *B* for comparison.

Discussion

The Kv1.5 K^+ channel is expressed in human and rat cardiac myocytes at the intercalated disk. The immunofluorescence data presented in Figs. 4 and 5 clearly show that the Kv1.5 channel is expressed in both atrial and ventricular myocytes. A similar pattern of expression was observed in rat heart as shown in Fig. 8. While it is difficult to quantitate the fluorescent signal, the amount of channel expressed in atrium and ventricle of both species appeared similar, although at times it appeared as though more staining occurred in atrium. One possibility is that as channel density increases the efficiency of antibody binding decreases, making it difficult to compare fluorescence intensities between different cardiac regions. In rat heart the Kv1.5 transcript is equally distributed between these two regions based on Northern blot (3) and RNase protection analysis (34). Northern analysis suggests that the Kv1.5 channel is more abundant in human atrium than in ventricle (2, 6) while a PCR-based analysis suggests the levels are similar between human atrium and ventricle (7). The steady state level of the Kv1.5 transcript in rat heart is altered by several interventions. Kv1.5 channel mRNA is downregulated as a function of hypertrophy (35) and levels decreased eightfold in ventricles of adrenalectomized animals (36). Dexamethasone treatment of these animals increased ventricular Kv1.5 mRNA 50-fold, with no effect on mRNA levels in atrium (36). Perhaps mRNA levels in human tissue are also dynamic and sometimes reduced in explanted tissue. In any case, it is not clear that protein expression should always follow mRNA levels. Detection of Kv1.5 protein by Western analysis in equal amounts in human atrium and ventricle would support the immunofluorescence data. However, Western analysis of human atrial and ventricular membrane and

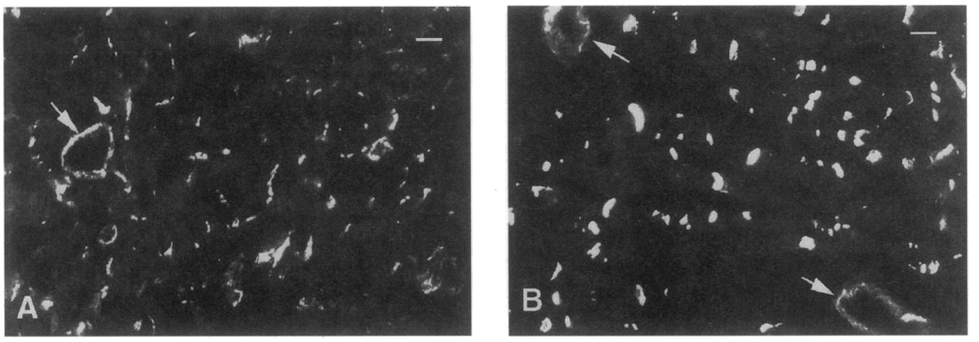

Figure 8. Immunolocalization of the Kv1.5 channel in rat heart. Adult rat heart cryosections were prepared and stained with the NH_2-terminal affinity-purified antibody as described in Methods (*A*). Cryosections from human atrium stained with the same antibody are shown for comparison (*B*). The structures indicated with the arrows represent blood vessels. The bars represent 25 μm.

whole tissue preparations were not successful despite repeated attempts.

Immunolocalization data on rare membrane proteins must be evaluated critically. Since channel proteins are often present at surface densities of several molecules per square micron, the slightest epitope crossreactivity with a more abundant membrane protein will produce the anticipated fluorescence signal. One region of the cardiac myocyte that is enriched in protein and thus is a good candidate for producing such nonspecific signals is the intercalated disk. It is for these reasons that we have relied on the localization of the Kv1.5 channel with two antibodies made against distinct regions of the channel, the NH_2-terminal and the S1-S2 linker. The likelihood that both antibodies are crossreacting with related epitopes associated with the intercalated disk is remote, especially since the S1-S2 antibody is species specific. It does not recognize the rat homologue of Kv1.5 and does not stain rat heart. The NH_2-terminal antibody does recognize the channel from both species in transfected cells and does stain rat and human heart.

Voltage-clamp recordings have detected outward currents in human atrial myocytes that resemble Kv1.5 when expressed in heterologous systems (14), an observation consistent with the localization of the Kv1.5 protein to human atrial myocytes. However, even though the present study finds the Kv1.5 protein in human ventricular myocytes, corresponding whole cell voltage-clamp currents have not been found in these cells (37). Since Kv1 family members can have their function and pharmacology significantly altered by heterotetramer formation (15) and beta subunit composition (21), it is possible that the Kv1.5 protein forms a homomeric channel in atrium while forming a heteromeric channel in ventricle, perhaps in association with function altering beta subunits. The I_{to} current observed in human ventricular cells differs from that observed in atrium (37). Perhaps this ventricular I_{to} is due to a heteromeric or beta subunit–associated Kv1.5 channel while the atrial delayed rectifier current is due to homomeric Kv1.5. Recently, we cloned additional beta subunits from human ventricle that alter the inactivation kinetics of Kv1.5 after coexpression in *Xenopus* oocytes (England, S. K., V. N. Uebele, H. Shear, K. Jayaveera, P. B. Bennett, and M. M. Tamkun, manuscript submitted for publication). It will be important to determine whether these beta subunits colocalize with the Kv1.5 channel in human ventricle but not in atrium.

The cell surface distribution of Kv1.5 varies with development. Fig. 7 indicates that the localization of the Kv1.5 channel in human myocardium changes drastically as a function of patient age. In newborn myocardium (1 or 2 mo old) the channel is distributed in a punctate manner over the cell surface with little localization into structures resembling the intercalated disk of the adult. While developmental issues have not been examined in human heart, several studies have compared the cardiac myocyte K^+ currents found in fetal, neonatal, and adult animals. Inward rectifier K^+ current in the rabbit increases > 2-fold between fetal days 21 and 28 and 1.6-fold between the neonate and adult stages (38). The cardiac myocytes of neonatal dogs lack the transient outward current which does not appear until approximately day 60 (39). In the rabbit these transient outward currents not only increase in magnitude between neonatal and adult stages but they also show kinetic differences, suggesting that either different isoforms are expressed or that there is age-specific modulation of the same isoform (40).

The distribution of gap junctions, as visualized by staining with connexin-specific antibodies, is also poorly organized early in life relative to the adult pattern (Fig. 7, *A* and *C*, and reference 28). Perhaps the adult architecture of the heart in terms of cell–cell communication is not mature until most of the organ growth has been accomplished. Only then is the stable intercalated disk pattern observed in the adult established. A complex intercellular coupling network may be incompatible with rapid cardiac growth and development. The distribution and number of gap junction proteins appear to differ between normal human myocardium and hypertrophied and ischemic tissue (29–31), suggesting altered intercellular communication between the myocytes of diseased tissue. It has been suggested that this breakdown in the ordered gap junction structure is responsible for the arrhythmogenic nature of diseased tissue. An alternative explanation involves altered expression or distribution of the Kv1.5 channel. Ischemic conditions do alter voltage-gated K^+ channel expression in the canine model. Myocytes isolated from the infarct border zone show at least a fourfold reduction in the transient outward current as compared with cells isolated from control animals (41). It will be interesting to determine the distribution of Kv1.5 antibody staining in ischemic regions of human myocardium. While the immunolocalization presented in this paper is derived from diseased organs, regions showing obvious ischemic damage were not used.

The Kv1.5 K^+ channel structure may vary between vascular muscle and the cardiac myocyte. While the S1-S2 antibody did not show specific staining of blood vessels, the NH_2-terminal antibody stained what appear to be vascular smooth muscle in both human and rat heart (Fig. 8). This finding was not completely unexpected since the Kv1.5 mRNA is found in rat thoracic aorta (3), arguing for the presence of this channel in the vasculature. Kv1.5-like currents exist in rabbit and canine vascular smooth muscle at a cell surface density greater than that found in human cardiac myocytes (14, 42, 43). The canine

homologue of Kv1.5 has been cloned recently from colonic smooth muscle and found to be abundantly expressed in both gastrointestinal and vascular smooth muscle (10). In addition, Koren and co-workers (44) have reported localization of the rat Kv1.5 protein to the cardiac vasculature. These investigators have reported also that both rat cardiac myocytes and vascular smooth muscle cells contain the Kv1.5 mRNA based on in situ hybridization (44). Therefore, even though the localization to the human vasculature reported here was performed with only a single antiserum, it is likely that this channel is expressed in vascular smooth muscle. Perhaps the S1-S2 and NH_2-terminal antibodies, which recognize very different epitopes, do not both stain the vessels because the channel structure differs between cell types, with the S1-S2 epitope being exposed only in the myocyte. The vascular myocyte could have a heterotetramer subunit composition that is incompatible with S1-S2 antibody binding. Alternatively, since the S1-S2 epitope contains an N-linked glycosylation site, perhaps altered glycosylation in the vasculature blocks antibody binding. Region-specific masking of ion channel epitopes has been documented. For example, two monoclonal antibodies recognizing different NH_2-terminal epitopes on the skeletal muscle voltage-gated Na^+ channel differentially stain the surface membrane and T-tubular system (45). It appears that the epitope for one antibody is accessible in both membrane systems while the other is exposed only on the surface membrane. If such heterogeneity can occur within the same cell, it is likely to also occur between cells. Another explanation for the differential staining of vessels with the Kv1.5-specific antibodies is that an undiscovered channel exists in the vasculature which shares sequence identity with Kv1.5 in the NH_2-terminal epitope.

Functional implications for K^+ channel expression at regions of cell–cell contact. The Kv1.5 channel in both rat and human is concentrated at regions of cell–cell contact, near the intercalated disk protein cadherin and adjacent to connexin. Given the length constants in the cardiac myocyte there is no functional reason to concentrate the Kv1.5 channel, or another voltage-gated ion channel, at the regions of intercellular communication. However, there is no reason why the channels cannot be concentrated here. Voltage-gated sodium channels have been reported to be concentrated here (46) even though the inward current in a neighboring myocyte is capable of depolarizing the adjacent cell even in the complete absence of Na^+ channels. Also believed concentrated at the disk region are membrane proteins such as IP_3 receptors (47) and, to a certain extent, glucose transporters (48). Perhaps this region of the myocyte is suited best for anchoring channel proteins to the cytoskeleton. The membrane cytoskeleton may be simply more stable in this region. Alternatively, the biosynthetic mechanisms for inserting the nascent channels into the surface membrane may concentrate channel protein here for reasons unrelated to channel function. The Kv1.5 channel may exist at low density outside the intercalated disk, being evenly distributed over the cell surface. If such surface density was less than one channel per several square microns, detection by the fluorescence technique used here may not be possible given the nonspecific background binding evident in Figs. 4 and 5.

Conclusion. For reasons that are not completely clear, the Kv1.5 K^+ channel is localized at the intercalated disk regions in human and rat myocardium. These data are the first description of the cell surface localization of any voltage-gated K^+ channel in human muscle and confirm that the Kv1.5 channel is likely to play a role in the cardiac action potential. The expression patterns are similar between atrium and ventricle and vary in both regions with development, consistent with the idea that alterations in Kv1.5 expression are in part responsible for changes in myocyte electrical properties as a function of development. The channel is expressed also in vascular smooth muscle, suggesting it may modulate vascular tone. The localization of Kv1.5 expression in specific cardiac regions such as the SA node and conducting system remains to be determined as do the cell-specific changes in expression that occur during ischemia, hypertrophy, and hypertension. A more challenging task will be the determination of the subunit composition of the Kv1.5 protein–containing complex in cardiac and vascular myocytes.

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