Distribution of Kir6.0 and SUR2 ATP-sensitive potassium channel subunits in isolated ventricular myocytes

Singh H[1], Hudman D[2], Lawrence CL[2], Rainbow RD[2], Lodwick D[1], Norman RI [1]

1, Division of Medicine and Therapeutics, University of Leicester, Robert Kilpatrick Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK and 2, Department of Cell Physiology and Pharmacology, University of Leicester, University Road, Leicester LE1 7RH, UK

Author for correspondence: Dr. R.I. Norman, Division of Medicine and Therapeutics, University of Leicester, Robert Kilpatrick Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK. Tel: +44 (0)116 252 3178; Fax: +44 (0)116 252 3273; e-mail: rin1@le.ac.uk

Running Title: K_{ATP} channel subunit localization in ventricular myocytes

Abstract

The subcellular distribution of K_{ATP} channel subunits in rat isolated ventricular myocytes was investigated using a panel of subunit specific antisera. Kir6.1 subunits were associated predominantly with myofibril structures and were co-localized with the mitochondrial marker MitoTracker red (correlation coefficient (cc) = 0.63 ± 0.05). Anti-Kir6.1 antibodies specifically recognized a polypeptide of 48 kDa in mitochondrial membrane fractions consistent with the presence of Kir6.1 subunits in this organelle. Both Kir6.2 and SUR2A subunits were distributed universally over the sarcolemma. Lower intensity antibody associated immunofluorescence was detected intracellularly, which was correlated with the distribution of MitoTracker red in both cases (cc, Kir6.2, 0.56 ± 0.05; SUR2A, 0.61 ± 0.06). A polypeptide of 40 kDa was recognized by anti-Kir6.2 subunit antibodies in Western blots of both microsomal and mitochondrial membrane fractions consistent with the presence of this subunit in the sarcolemma and mitochondria. Similarly, SUR2A and SUR2B subunits were detected in Western blots of microsomal membrane proteins consistent with a sarcolemmal localization for these polypeptides. SUR2B subunits were shown in confocal microscopy to co-localize strongly with t-tubules (cc, 0.81 ± 0.05). Together the results indicate that Kir6.2 and SUR2A subunits predominate in the sarcolemma of ventricular myocytes consistent with a Kir6.2/SUR2A subunit combination in the sarcolemmal K_{ATP} ($sarcK_{ATP}$) channel. Kir6.1, Kir6.2 and SUR2A subunits were demonstrated in mitochondria. Combinations of these subunits would not explain the reported pharmacology of the mitochondrial K_{ATP} ($mitoK_{ATP}$) channel (Lui *et al*. 2001. Mol. Pharmacol. 59:225-230) suggesting the possibility of further unidentified components of this channel.

Introduction

ATP-sensitive potassium (K_{ATP}) channels link the metabolic energy status of cells to membrane potential by responding to the ATP:ADP ratio.[1] Although these channels would normally be closed in cardiac myocytes, during myocardial ischemia, where the cellular ATP:ADP ratio falls substantially, activation of K_{ATP} channels could contribute to cardioprotective mechanisms.[2,3] Functional K_{ATP} channel currents have been demonstrated in both cardiac sarcolemmal[4] ($sarcK_{ATP}$) and mitochondrial ($mitoK_{ATP}$) membranes[5]. The $mitoK_{ATP}$ channel is much more sensitive to the potassium channel opener, diazoxide, and the K_{ATP} channel antagonist, 5-hydroxydecanoate (5-HD) compared to the $sarcK_{ATP}$ channel, which is more sensitive to the blocker HMR1098.[6]

K_{ATP} channels are hetero-octomers ($Kir6.0_4/SUR_4$) formed by four pore forming polypeptides of the inwardly rectifying K^+ channel family Kir6.0 and four sulphonylurea receptor (SUR) subunits of the ATP-binding cassette (ABC) protein superfamily.[7] The pharmacological properties of channels expressed in heterologous systems suggest strongly that the $sarcK_{ATP}$ channel is composed of Kir6.2 and SUR2A subunits.[1] Current flow via Kir6.2 subunits in $sarcK_{ATP}$ channels is integral to mechanisms of action potential shortening. This was shown by the rescue of pinacidil-induced outward current and action potential shortening in ventricular cells from $Kir6.2^{-/-}$ knockout mice by gene transfer of the Kir6.2 subunit.[8] The structure of the $mitoK_{ATP}$ channel has not been elucidated but participation of Kir6.1 is suggested from immunogold labelling of the inner mitochondrial membrane.[9]

Exposure of the myocardium to a brief ischemic insult paradoxically protects the myocardium against subsequent prolonged ischemic insult.[10] The involvement of cardiac K_{ATP} channels in ischemic preconditioning (IPC) has been suggested by a number of pharmacological studies which show that potassium channel openers (KCO) can mimic IPC, while K_{ATP} channel antagonists, glibenclamide and 5-HD, abolish or reduce this protection.[2,3] Which of the cardiac K_{ATP} channels is responsible for ischemic preconditioning remains a matter of debate.[3] The involvement of the sarcK_{ATP} channel in IPC was supported by experiments on ischemic ventricular cells from Kir6.2$^{-/-}$ knockout mice which showed the absence of sarcK_{ATP} channel activation and action potential shortening in IPC normally seen in wild type cells.[11] Arguing against this, KCO have been reported to produce cardioprotection and increases in flavoprotein oxidation at concentrations that were without effect on sarcolemmal I_{KATP} or action potential duration.[3] In addition, block of KCO-induced cardioprotection by 5-HD, which is suggested to be selective for the inhibition of mitoK_{ATP} channels, has been shown to occur without effect on sarcolemmal K_{ATP} channel currents.[2,3] These observations favor an involvement of the mitoK_{ATP} channel in the preconditioning response, although an independent effect of K_{ATP} channel modulators on mitochondrial metabolism cannot be ruled out.[12-14]

To understand the different roles of sarcK_{ATP} and mitoK_{ATP} channels in cardiac myocytes, the molecular composition of each needs to be resolved. In this study a panel of isoform specific, site-directed antibodies against K_{ATP} channel subunit peptides was produced to investigate the cellular localization of K_{ATP} channel subunit isoforms in

isolated ventricular myocytes by (i) confocal microscopy and (ii) subcellular fractionation and Western blotting.

Materials and Methods

Preparation of polyclonal antibodies

Antisera were raised to peptides corresponding to the C-terminal domains of rat Kir6.1 (accession number D42145), Kir6.2 (D86039), SUR2A (D83598) and SUR2B (AF087838) K_{ATP} channel subunits. Peptides were synthesized according to Atherton and Sheppard[15] and were composed of the following amino acid residues: Kir6.1; (C)KVQFMTPEGNQCPSES (residues 409-424, Protein and Nucleic Acid Chemistry Laboratory, University of Leicester, UK), Kir6.2; (C)KAKPKFSISPDSLS (residues 377-390, Research Genetics Inc. Huntsville, USA), SUR2A; PNLLQHKNGLFSTLVMTNK(C) (residues 1527-1545, Pepceuticals Ltd., Leicester, UK), SUR2B; ESLLAQEDGVFASFVRADM(C) (residues 1528-1546, Pepceuticals Ltd)[15]. N- or C-terminal cysteine residues were added to the peptides to facilitate conjugation[16] to ovalbumin (Kir6.1) or Keyhole Limpet Haemocyanin carrier protein and were not part of the channel subunit sequences. Antisera were raised against peptide carrier protein conjugates in New Zealand White Rabbits (Kir6.1, University of Leicester; Kir6.2, Research Genetics Inc.; SUR2A and SUR2B, Pepceuticals Ltd.). Antibody titre was estimated by ELISA using microtitre plates coated with 1 μg/ml peptide.[17]

Western blotting

Twenty five microlitres of *in vitro* translated subunit protein reaction[17] or ventricular myocyte proteins (11 μg protein/ml) were denatured, separated by sodium dodecyl sulfate polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE)[18] and transferred to nitrocellulose membranes (Hybond-C Super, Amersham Biosciences UK Limited, Little Chalfont, UK) with a wet-blot technique.[19] Membranes containing protein standards were stained with amido black stain (0.1 % amido black stain, 45 % methanol, 10 % acetic acid) and destained in 1 % acetic acid. Membranes for immunodetection were incubated with 10 % dried milk solution (10 % w/v dried milk powder, 10 mM Tris-HCl (pH 7.5), 0.9 % NaCl, 0.05 % Tween 20). Individual lanes were isolated in a Decaprobe (Hoefer Scientific Instruments, San Francisco, USA) and treated with anti-K_{ATP} channel subunit antiserum diluted 1:250 in dried milk solution for 16 h at 4^{o}C. Membranes were washed with 10 mM Tris-HCl (pH 7.5), 0.9 5 NaCl, 0.05 % Tween 20, 0.25 % N-lauryl sarcosine, 0.25 % nonidet P40 and incubated with horseradish peroxidase conjugated anti-rabbit immunoglobulin (Sigma) diluted 1:1000 for 2 h at room temperature. After washing, photoluminometric detection was with Western blot reagent ECL (Amersham Biosciences, Frieberg, Germany) and data were recorded on Kodak X-ray film.

Immunoprecipitation

In vitro translated K_{ATP} channel subunit proteins (5-10 μl) solubilized in 1 % Triton X-100 were immunoprecipitated from a final reaction volume of 100 μl containing 20 mM

Tris-HCl (pH 7.5), 10% bovine serum albumin, 500 mM KCl with 1 μl of anti-K_{ATP} channel subunit antiserum as described previously.[17] Immunoprecipitates were subjected to SDS-PAGE[18] on 7 % polyacrylamide mini gels for 1.5 h at 120 V. Following electrophoresis, gels were prepared for autoradiography as described previously.[17]

HEK 293 cell transfection

Human embryonic kidney (HEK) 293 cells stably expressing SUR2A alone or SUR2A/Kir6.2[20] (a gift from Dr. A. Tinker, University College London, London, UK) were transiently transfected with either Kir6.1 or SUR2B subunits cloned in the expression vector pIRES-EGFP (Clontech, Oxford, UK) using LipofectAMINE™ 2000 (Invitrogen Ltd., Paisley, UK). Transfection efficiencies of 70% were achieved routinely. Cells were maintained in minimal essential medium with Earle's Salts supplemented with 10% foetal calf serum and 5% L-glutamine under antibiotic selection (SUR2A, 727 μg/ml G418; Kir6.2/SUR2A, 727 μg/ml G418, 364 μg/ml zeocin).

Isolation of single ventricular myocytes

Rat isolated ventricular myocytes were prepared using the Langendorff technique with collagenase (type 1, Sigma) and protease (type XV, Sigma) as described previously[21,22] and resulted, typically, in a 70-90 % yield of quiescent, rod-shaped myocytes.

Immunocytochemistry

Coverslips (25 mm) were washed with a 1:1 mixture of chloroform and methanol, rinsed once with water, soaked in 0.01 % poly-L-lysine (Sigma-Aldrich, Poole, UK) and dried 12-16 hours prior to fixation of cells. HEK 293 cells stably expressing Kir6.2/SUR2A or rat isolated ventricular myocytes were adhered to the glass coverslips at 4^{o}C for 20 min and the cells fixed in 2 % paraformaldehyde at room temperature for 10 min. The fixative was removed and 100 mM glycine added to quench the paraformaldehyde for 10 min. Cells were treated with permeabilization solution (0.1 % Triton X-100 in PBS; 2.7 mM KCl, 15 mM potassium phosphate, 137 mM NaCl, 8 mM sodium phosphate) and this solution was replaced with solution containing glycine for 10 min. Finally the cells were washed 3 times in PBS for 5 min and stored at 4^{o}C for 12-16 h in PBS.

Cells were incubated for 30 min with antibody-diluting buffer (2 % goat serum, 1 % bovine serum albumin, 0.05 % Triton X-100 in SCC; 150 mM NaCl, 15 mM Na citrate). Antibody diluting buffer was replaced with buffer containing primary antibody at 1:250 or 1:500 dilutions and incubated for one hour at room temperature. Cells were washed for 10 min in 0.05 % Triton X-100 in SCC and then incubated with anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate (1:1000 dilution, Sigma-Aldrich, Poole, UK). After three 10 min washes anti K_{ATP} channel antibody-labeled cells were viewed with a confocal dual laser-scanning microscope at 488 nm. Mitochondria were localized in co-localization experiments by incubating antibody-labeled cells with 100 nM Mitofluor 589 (Molecular Probes, Poort Gebouwin, The Netherlands) PBS for 10

minutes. The fluorescent emission from labeled mitochondria was detected at 580 nm. Transverse tubules (t-tubules) were visualized using a monoclonal $IXE11_2$ antibody (1:250 dilution) directed to a 28 kDa T-tubule specific protein[23] (a gift from Dr. K. Campbell, University of Iowa, USA) and binding detected with anti-mouse IgG-rhodamine (1:1000 dilution, TRITC, Sigma-Aldrich, Poole, UK) conjugate at a wavelength of 525 nm.

Confocal Microscopy

Confocal microscopy was performed as described previously[24] with excitation wavelengths of 488 nm for the detection of anti-K_{ATP} channel subunit antisera, 568 nm for Mitofluor red 589 and the anti-transverse tubule (t-tubule) marker ($IXE11_2$ antibody). All images were analyzed using a background subtraction method off-line. The background was defined as a region of interest proximal to the cell and subtracted automatically from subsequent images.

Data acquisition, image processing

Confocal image processing was performed using the Ultra-view software (v4.0) (Perkin Elmer, Beaconsfield, UK). Data was presented as mean ± standard deviation and exported as an ASCII text file. As a device to represent distribution of fluorescent intensity throughout the cells, data was presented as a line created from one image drawn either horizontally or vertically through the confocal plane. The line graph

represents the colour coded fluorescent profile from the whole cell area defined off line from the confocal images.

Co-localization analysis

Co-localization analysis consisted of both the correlation and the co-localization between two images. The two images were mapped to remove the background signals and the images combined to a single color image. Two forms of results were generated. The co-localization correlation analysis then takes into account the intensity of a signal within a pixel as well as its x,y coordinates. The percentage co-localization is based only on whether there is any signal from each component in each pixel.
The correlation between the two signals was then calculated on intensity as follows:

$C = \frac{a \times b}{\sqrt{a^2 \times b^2}}$ where: C= correlation, a= Image 1-Image 2, b= Image 2- Image 1

Preparation of mitochondria

Mitochondria were prepared from rat ventricle according to the method of Santos *et al*.[25] and monitored by the enrichment of cytochrome c oxidase activity.[26] The microsomal membrane fraction was recovered in the supernatant after centrifugation of tissue homogenate at 9000 × g for 10 min.

Results

Anti-K_{ATP} channel subunit antisera

A panel of site-directed polyclonal antisera (anti-Kir6.1, Kir6.2, SUR2A and SUR2B) was prepared to permit Kir6.0 and SUR2 isoforms to be distinguished. In each case, antisera were shown to be of high titre measured against 20 ng immunizing peptide per well in ELISA, with a positive signal of > 0.15 absorbance units (A_{430}) under standard assay conditions with antisera diluted at least $1:10^4$ (not shown). Specificity was confirmed by the demonstration of an absence of direct cross reactivity with other short peptides including those derived from related isoforms and block of the ELISA signal by preincubation for 16 h of diluted antisera with the immunizing peptide (10 μg/ml) but not by any other short peptides, including those derived from related isoforms.

All antisera recognized their respective subunit polypeptides (Kir6.1, 45 kDa; Kir6.2, 40 kDa, SUR2A and SUR2B, 140 kDa) in Western blots of *in vitro* expressed subunit polypeptides (Fig. 1A and B). Moreover, all antibodies were shown to immunoprecipitate specifically [^{35}S]methionine-labelled *in vitro* translated subunit polypeptides (Fig. 1C and D), to bind to fixed and permeabilized HEK 293 cells only when transfected with cDNA encoding the target polypeptide (Fig. 2) and to produce unique staining patterns in isolated ventricular myocytes (see below, Fig. 8). In all the above assays, specificity was confirmed by the demonstration of an absence of direct cross-reactivity with other subunit polypeptides and/or block of immunoreaction by preincubation of diluted antisera with the immunizing peptide (10 μg/ml) for 16 h but not by any other short peptides including those derived from related isoforms.

Localization of Kir6.0 subunits in isolated ventricular myocytes

The localization of K_{ATP} channel subunits was investigated by immunocytochemistry and confocal microscopy in isolated adult rat ventricular myocytes. Anti-Kir6.1 C-terminal antiserum was used to localize Kir6.1 subunit distribution in fixed and permeabilized isolated ventricular myocytes. A strong intracellular signal was observed with longitudinal streaks of immunofluorescence, consistent with an association of this isoform with myofibril structures within the cells (Figure 3A). In some cells, punctate anti-Kir6.1-associated immunofluorescence was also localized either on or just under regions of the sarcolemma. The pixel profile taken across a stained myocyte demonstrated 3-fold higher anti-Kir6.1-associated fluorescence of internal structures than at the sarcolemma (Figure 3B). The specificity of anti-Kir6.1 antibody binding to these structures was confirmed by the abolition of fluorescence signal after preadsorption of the anti-Kir6.1 antiserum with 10 μg/ml immunizing peptide (Figure 3C and 3D) but not by non-specific peptides, e.g. the corresponding C-terminal peptide from Kir6.2 (not shown).

A predominantly mitochondrial localization for Kir6.1 subunits has been suggested previously from immunoblot analysis of subcellular fractions of skeletal muscle and liver and from immunogold labeling of mitochondrial structures.[9] To investigate whether Kir6.1 subunits were localized in mitochondria associated with myofibrils in ventricular myocytes, mitochondria were labeled with the fluorescent mitochondrial marker, MitoFluor red 589 and the co-localization of anti-Kir6.1-associated fluorescence was analyzed by comparing fluorescent emission on excitation at 568 and 488 nm, respectively (Fig. 4A-C). Analysis of the pixel profile (Fig. 4D) for the

distribution of the two fluorescent labels in six cells revealed that of all pixels positive for anti-Kir6.1, 86 ± 12 % were also positive for MitoFluor red. Conversely, of all pixels positive for MitoFluor red, 74 ± 17 % were also positive for anti-Kir6.1. The correlation coefficient for co-localization of anti-Kir6.1 and MitoFluor red labeling was 0.63 ± 0.05.

From the similarity of the functional and pharmacological properties of K_{ATP} channels when Kir6.2 and SUR2A subunits are co-expressed in heterologous expression systems compared to native cardiac sarcolemmal channels, the cardiac sarcolemmal K_{ATP} channel is proposed to comprise this subunit combination.[27] Consistent with this proposal, anti-Kir6.2-associated fluorescence was localized over the sarcolemma of ventricular myocytes with weaker fluorescence associated with intracellular structures (Fig. 5A and 5B). This signal was blocked by preadsorption of the anti-Kir6.2 antiserum with 10 μg/ml immunizing peptide (Figure 5C and 5D) but not by non-specific peptides, e.g. the corresponding C-terminal peptide from Kir6.1 (not shown). Transverse pixel profiles taken across myocytes showed strong florescence intensity associated with the sarcolemmal membrane with intracellular fluorescence approximately half that at the sarcolemma (Fig. 5B and F).

In cells co-labeled with anti-Kir6.2 antiserum (1:250 dilution) and MitoFluor Red (Fig. 5E), analysis of the pixel profile (Fig. 5F) for the distribution of the two fluorescent labels in six cells revealed that of all pixels positive for anti-Kir6.2, 45 ± 3 % were also positive for MitoFluor red. Conversely, of all pixels positive for MitoFluor red, 32 ± 10 % were also positive for anti-Kir6.2. The correlation coefficient for co-localization of

anti-Kir6.1 and MitoFluor red labeling was 0.56 ± 0.05. In cells labeled with anti-Kir6.2 antiserum at higher dilution (1:500), labeling of Kir6.2 was observed associated with the sarcolemmal membrane only (not shown) further suggesting that the density of Kir6.2 subunit at the sarcolemma is higher than that in mitochondria. Together, these results suggested strong association of Kir6.2 subunits with the sarcolemma but also the presence of Kir6.2 subunits in mitochondrial membrane at a lower abundance.

Localization of SUR2 subunits in isolated ventricular myocytes

As predicted from the proposed involvement of the SUR2A subunit in the sarcolemmal K_{ATP} channel[27], anti-SUR2A-associated fluorescence was localized strongly over the sarcolemma (Fig 6A-C). As with Kir6.2-associated immunofluorescence, weaker anti-SUR2A-associated fluorescence was localized with intracellular structures. Preadsorption of the anti-SUR2A antiserum with 10 μg/ml immunizing peptide (Fig. 6A and 6B) but not non-immunizing peptides, e.g. the corresponding C-terminal peptide from SUR2B (not shown), confirmed that anti-SUR2A antibody binding to sarcolemmal and internal structures was specific to the SUR2A polypeptide. In co-localization experiments (Fig. 6C-E), analysis of the pixel profile (Fig. 6F) for the distribution of anti-SUR2A labeling and MitoFluor red in six cells revealed that of all pixels positive for anti-SUR2A-associated fluorescence, 43 ± 4 % were also positive for MitoFluor red. Conversely, of all pixels positive for MitoFluor red, 72 ± 5 % were also positive for anti-SUR2A. The correlation coefficient for co-localization of anti-SUR2A and MitoFluor red was 0.61 ± 0.06, suggestive of a probable mitochondrial, as well as, a sarcolemmal distribution of the SUR2A subunit.

Anti-SUR2B-associated fluorescence displayed a distinct regular transverse striated pattern of labeling (Fig. 7A), which was further illustrated on examination of the longitudinal pixel profile of the fluorescent emission with this antiserum (Fig. 7B). Preadsorption of the anti-SUR2B antiserum with 10 μg/ml immunizing peptide (Fig. 7C and D) but not non-immunizing peptides, e.g. the corresponding C-terminal peptide from SUR2A (not shown), confirmed that anti-SUR2B antibody associated immunofluorescence was specific to the SUR2B polypeptide. To examine whether this pattern of transverse striations corresponded to the localization of t-tubules, the co-localization of SUR2B labeling with that of a specific 28kDa t-tubule protein marker recognized by monoclonal $IXE11_2$ antibody[23] was investigated. Analysis of the pixel profile for the distribution of anti-SUR2B and $IXE11_2$ antibody labeling revealed that of all pixels positive for anti-SUR2B-associated fluorescence, 50 ± 5 % were positive for the t-tubule marker. Conversely, of all pixels positive for $IXE11_2$ binding, 77 ± 7 % were also positive for anti-SUR2B binding. The correlation coefficient for co-localization of anti-SUR2B and the $IXE11_2$ t-tubule marker was 0.81 ± 0.05, suggesting that SUR2B subunits are highly localized to the t-tubule system in ventricular myocytes.

No staining of myocytes was seen with an anti-SUR1 subunit antiserum, similar to that prepared by Aguilar-Bryan *et al.*[28] against the N-terminal domain of this subunit (not shown).

Western blotting of K_{ATP} channel subunits in isolated ventricular myocytes

The subunit content of K_{ATP} channels in isolated ventricular myocytes was investigated by Western Blotting (Fig. 8). A polypeptide of 45 kDa was detected with anti-Kir6.1 antiserum corresponding to the native Kir6.1 subunit (Fig. 8A). Preadsorption of the diluted antiserum with Kir6.1 C-terminal peptide blocked anti-Kir6.1 recognition of the 45 kDa species completely. Anti-Kir6.2 antiserum labeled a polypeptide of 40 kDa in the absence but not the presence of the Kir6.2 C-terminal peptide consistent with specific immunodetection of the native Kir6.2 subunit (Fig 8A).

Immunodetection of electrophoretically separated ventricular myocyte proteins with both anti-SUR2A and anti-SUR2B antisera most often resulted recognition of multiple species of apparent molecular weights between approximately 120 and 160 kDa (Fig. 8B). Moreover, additional polypeptides of lower molecular weights were also labeled (not shown). This contrasted to labeling of SUR2 subunits in Western blots of polypeptides transcribed and translated *in vitro* from cDNA, where distinct bands corresponding to polypeptides of the predicted molecular weight were always observed (Fig. 1B). In blots of myocyte proteins, most of the polypeptides recognized by anti-SUR2 subunit antisera were no longer labeled after preadsorption of the antiserum with the appropriate immunizing SUR2 C-terminal peptide (Fig. 8B).

K_{ATP} channel subunit distribution in subcellular fractionations of ventricular myocytes

Subcellular fractions corresponding to mitochondria and low buoyant density microsomes (containing sarcolemma) were prepared to allow the subcellular localization of K_{ATP} channel subunits to be investigated in Western blots. Cytochrome c

oxidase activity in the mitochondrial fraction (1089 μmol/min/mg protein) was enriched approximately 6-fold over both the microsomal fraction (196 μmol/min/mg protein) and cell homogenate (173 μmol/min/mg protein). Enrichment of mitochondria was also established by labeling with anti-cytochrome c antiserum of a polypeptide of 12 kDa in mitochondrial but not microsomal membranes (Fig. 8G). Conversely, concanavalin A-alkaline phosphatase conjugate bound to multiple glycoproteins in the microsomal fraction and homogenate, while no polypeptides were detected in the mitochondrial fraction (not shown). Comparison of the detection of a representative concanavalin A-labeled polypeptide of 104 kDa in microsomal and mitochondrial fractions is shown in Fig. 8H.

When blots of electrophoretically separated mitochondrial fraction proteins were probed with anti-Kir6.1 antiserum, a single band of 48 kDa was labeled in the absence but not the presence of competing Kir6.1 C-terminal peptide (10 μg/ml, Fig. 8C). Similarly, polypeptides of 40 (Fig. 8C) and 80 kDa (not shown) were labeled specifically in the mitochondrial fraction by the anti-Kir6.2 antiserum. Detection of these species was reduced in the presence of competing Kir6.2 C-terminal peptide (10 μg/ml, Fig. 8C). When blots were probed with anti-SUR2 antiserum, no labeling of polypeptides corresponding to the migration of native SUR2 subunits was observed (Fig.8D). Additional polypeptides of lower apparent molecular size were labeled but this labeling was considered to be non-specific, as antibody binding was not reduced by preadsorption with the appropriate immunizing peptide.

In microsomal fractions, anti-Kir6.1 and 6.2 antisera detected polypeptides of 48 and 40 kDa, respectively, and both anti-SUR2A and SUR2B antisera detected polypeptides of 140 kDa. Taken together, Western blotting indicates the presence of Kir6.1, Kir6.2 only in mitochondria, while Kir6.1, Kir6.2, SUR2A and SUR2B are all present in the low buoyant density microsome fraction.

Discussion

Immunological methods have been applied to provide direct evidence for the cellular localization of K_{ATP} channel subunit isoforms in rat isolated ventricular myocytes. Four subunit specific antisera were prepared against C-terminal domains in each subunit and characterized against synthetic peptides in ELISA, and in Western blots and immunoprecipitation experiments using *in vitro* translated subunit polypeptides. Antisera were further tested against HEK 293 cells transfected either stably or transiently with specific K_{ATP} channel subunits to further establish specificity. This combination of screens established that all four antisera recognized the immunizing antigen only and provided isoform specific recognition for the subunit localization experiments.

Kir6.1 was shown to be associated predominantly with the mitochondria of ventricular myocytes. Confocal microscopy using the anti-Kir6.1 antiserum revealed an intracellular distribution of the Kir6.1 subunit associated with myofibril structures. Anti-Kir6.1 immunofluorescence was significantly co-localized with the mitochondrial marker MitoFluor red suggesting that Kir6.1 is the predominant mitoK_{ATP} channel pore

forming subunit in ventricular myocytes. This result was corroborated by the strong labeling by anti-Kir6.1 antiserum of a polypeptide of 48 kDa in Western blots of mitochondrial fractions. A mitochondrial localization for Kir6.1 has been suggested previously from immunogold histochemistry in rat skeletal muscle using an anti-Kir6.1 antibody against a different epitope (amino acids 375-386).[9] It is noteworthy that a putative mitochondrial targeting sequence is found in Kir6.1, consistent with a mitochondrial localization for this isoform (PSORT, UK Human Genome Mapping Project Resource Centre, http://menu.hgmp.mrc.ac.uk/cgi-bin/psort). Whether Kir6.1 is a component of the mitoK_{ATP} channel has been questioned from the inability of a dominant negative form of Kir6.1 (Kir6.1 AFA) to ablate the mitochondrial redox response of increased flavoprotein fluorescence to diazoxide[29] and from the unchanged diazoxide-stimulated changes in flavoprotein fluorescence in Kir6.1$^{-/-}$ knockout mice compared to wild type.[30] It is possible that an alternative Kir subunit contributes to the mitoK_{ATP} channel but several studies have suggested that K_{ATP} channel modulators can elicit effects on mitochondrial metabolism independent of K_{ATP} channel activity[12-14] and thus it is possible that flavoprotein fluorescence may be an inappropriate assay of mitoK_{ATP} channel activity.

It has been suggested from Western blot analysis that Kir6.1 subunits are present in the plasma membrane of rabbit liver[9] and murine cardiomyocytes[31] but only limited evidence for a plasma membrane localization for this subunit was observed in this study. Detection of Kir6.1 was predominantly intracellular in this study but the presence of Kir6.1 in cardiac sarcolemma was supported by the observation of occasional

punctate anti-Kir6.1 antiserum-associated immunofluorescence at the cell boundary of some cells.

Kir6.2 subunits are widely held to comprise the pore of the sarcK_{ATP} channel from the resemblance of the properties of co-expressed Kir6.2/SUR2A expressed in heterologous systems with those of the native sarcK_{ATP}[27] and from the reduction in functional sarcolemmal K_{ATP} channels in Kir6.2 knockout mice.[8] Anti-Kir6.2 subunit-associated immunofluorescence at high antiserum dilution (1:500) was localized almost exclusively on the cell boundary. To our knowledge, this is the first direct evidence in support of the contention that Kir6.2 subunits are present in the sarcK_{ATP} channel. When the anti-Kir6.2 antiserum dilution was reduced (1:250), significant anti-Kir6.2 subunit-associated immunofluorescence was also observed on intracellular structures that co-localized significantly with the distribution of the mitochondrial marker, MitoFluor red. The presence of Kir6.2 subunits in mitochondria was confirmed by specific recognition of a polypeptide of 40 kDa by anti-Kir6.2 antiserum in Western blots of isolated mitochondrial fraction. Together, our results suggest that Kir6.2 is the predominant Kir6.0 isoform in the sarcolemma and that it is also present in mitochondria.

It has been shown that, in principle, Kir6.1 and Kir6.2 can co-assemble into functional mixed hetero-multimers.[20] Five distinct channel populations with differing single-channel amplitudes were observed on co-expression of Kir6.1, Kir6.2 and SUR2B in HEK 293 cells and co-expressed Kir6.1 and Kir6.2 subunits were co-immunoprecipitated. In addition, Kir6.2/SUR2B currents were suppressed by co-expression of Kir6.1 dominant negative subunits and Kir6.1/SUR2B currents, by co-

expression of Kir6.2 dominant negative subunits, respectively.[20] The strong labeling of Kir6.2 and the occasional punctate staining of Kir6.1 only at the sarcolemma in this study suggest that Kir6.2 homotetramers are likely to predominate in sarcK_{ATP}. This conclusion is consistent with the absence of sarcK_{ATP} channel currents in cardiomyocytes of Kir6.2$^{-/-}$ knockout mice[8] and the presence of unmodified sarcK_{ATP} channels in cardiomyocytes of Kir6.1$^{-/-}$ knockout mice.[30] The inference that Kir6.2 is the predominant K_{ATP} pore-forming subunit in the sarcolemma and that significant hetero-multimerization of Kir6.1 and Kir6.2 does not occur in cardiac tissue was also drawn from a study in rabbit ventricular myocytes in which gene transfer of dominant negative Kir6.2 subunit was reported to suppress pinacidil-induced sarcK_{ATP} channel currents, whereas dominant negative Kir6.1 subunits did not.[32] Why dominant negative Kir6.1 subunit was ineffective against Kir6.2/SUR2A containing channels in HEK A549 cells and cardiomyocytes in the study Seharaseyon *et al*.[32] but disrupted Kir6.2/SUR2B channels in the study of Cui *et al*.[20] is not clear.

Consistent with the contention that SUR2A subunits comprise the sulphonylurea accessory subunit of the sarcK_{ATP} channel, from comparisons of functional properties of heterologously expressed Kir6.2/SUR2A channels and native channels,[27] distinct localization of anti-SUR2A-associated immunofluorescence to the cell boundary was observed in confocal microscopy of ventricular myocytes. Anti-SUR2A-associated immunofluorescence was also localized to intracellular structures and there was a significant correlation with the distribution of the mitochondrial marker MitoFluor red indicative of a mitochondrial localization of this subunit. The low sensitivity to diazoxide conferred on K_{ATP} channels by the SUR2A subunit argues against a primary

role for this subunit in mitoK_{ATP} channels.[6] Moreover, the insensitivity of Kir6.1/SUR2A channels to either diazoxide or 5HD argues against this combination in mitoK_{ATP} channel structure.[6] In view of some reservations over the validity of the association of potassium channel modulator-induced changes in flavoprotein fluorescence with mitoK_{ATP} channel activity,[12-14] care is required in attributing a pharmacological profile to the mitoK_{ATP} channel and, therefore, all three of the K_{ATP} channel subunits identified in mitochondria herein may contribute to its structure.

Immunoblot analysis of myocyte proteins with both anti-SUR2 subunit antisera resulted in the specific detection of multiple polypeptides. Since the two anti-SUR2 antisera were shown not to recognize their highly related family member in Western blots of *in vitro* translated subunits, i.e. anti-SUR2A did not recognize SUR2B and vice versa, and specific antibody binding was competed in each case by the appropriate immunizing peptide, it is likely that multiple detection of bands in tissue blots was not a product of low antibody specificity but rather the presence of a number of related antigenic polypeptides. Those polypeptides with apparent molecular weights near that predicted for the SUR2 subunits may have been multiple glycosylation products of the target subunit, proteolytic products of the native subunit or, possibly, the result of expression of different splice variants.[33] Labeled polypeptides with apparent molecular weights significantly lower than the predicted size are likely to have been derived proteolytically from the native subunit. Inclusion of protease inhibitor cocktails in all steps following myocyte dissociation did not reduce the number of polypeptides detected suggesting that the SUR2A subunit is relatively labile on disruption of cardiac cells.

Although the presence of SUR2A in mitochondria was suggested from immunocytochemical experiments, when the presence of SUR2A in mitochondria was examined directly in immunoblots of mitochondrial proteins, anti-SUR2A antiserum did not label any polypeptide specifically. This inability to detect the presence of SUR2A may have been due to the proteolytic lability of the SUR2A subunit.

The distribution of anti-SUR2B-associated immunofluorescence in isolated ventricular myocytes was distinctly different to that observed with any of the other three anti-K_{ATP} channel subunit antisera. In this case, a distinct regular transverse striated pattern of labelling was strongly associated with t-tubular structures ($IXE11_2$ antibody localisation). In the absence of a similar pattern of localisation for either Kir6.1 or Kir6.2, the identity of the pore-forming partner(s) for this subunit remains to be determined. To date, the SUR2B subunit has been associated primarily with K_{ATP} channels in the sarcolemma of smooth muscle.[1] Specific localization of SUR2B to the t-tubules of cardiomyocytes suggests a possible novel function in this location. The inward rectifiers Kir2.1 and Kir2.2 have both been implicated in contributing to a slow inward tail current (I_{tail}) in t-tubules of ventricular myocytes.[34,35] In addition, the presence of an important K_{ATP} channel in t-tubules is suggested from the parallel loss in cultured rabbit ventricular myocytes of ATP-sensitive potassium current ($I_{K\text{-}ATP}$) and membrane capacitance,[36] which has been attributed in turn to the disappearance of t-tubules.[37,38] The presence of SUR2B localized specifically in t-tubules provides direct evidence for a K_{ATP} channel in these structures.

In summary, this study provides direct immunological evidence from confocal microscopy and subcellular fractionation followed by Western blotting for distinct subcellular localization of K_{ATP} channel subunits in ventricular myocytes. Kir6.2 and SUR2A were universally distributed over the sarcolemma. Since Kir6.1 was largely localized intracellularly, it can be inferred that the main sarcK_{ATP} channel oligomer is Kir6.2/SUR2A. Within the t-tubule system SUR2B was the predominant SUR. Although neither Kir6.0 subunit displayed a similar discrete transverse striated distribution, we hypothesize that the t-tubule form of the sarcK_{ATP} channel is likely to be Kir6.2/SUR2B, since Kir6.2 was by far the predominant sarcolemmal Kir6.0 subunit and only relatively small amounts of Kir6.1 appeared to be present in punctate locations in the surface membrane.

SarcK_{ATP} channels containing Kir6.2 subunits are clearly important in maintaining cardiac cellular homeostasis, as well as responding to metabolic challenges.[39,40] Cardiomyocytes from Kir6.2$^{-/-}$ knockout mice lack the appearance of electrocardiographic ST-segment elevation in response to ischemia seen in wild type cells.[39] In addition, the ability to adjust membrane excitability and calcium handling in response to sympathetic stimulation is compromised in Kir6.2$^{-/-}$ knockout mice, suggesting that important mechanisms responsible for adaptation to stress involving Kir6.2-containing sarcK_{ATP} channels remain to be elucidated.[40] Experiments on Kir6.2$^{-/-}$ knockout mice have also shown that Kir6.2-containing sarcK_{ATP} channels are important for the depression of cardiac excitability and that they are likely to be important in mediating IPC in cardiac tissue.[11]

In mitochondria, both Kir6.1 and Kir6.2 subunits were shown to be present. Whether homo-oligomers containing Kir6.1 and/or Kir6.2 or hetero-oligomers of Kir6.1/Kir6.2 constitute the mitoK_{ATP} channel requires further study. Although there was no evidence from Western blotting of mitochondrial fractions, immunocytochemistry also suggested the presence of SUR2A in mitochondria. This study implicates Kir6.1, Kir6.2 and SUR2A as potential components of the mitoK_{ATP} channel but the precise composition of the mitoK_{ATP} remains to be elucidated. Experiments to investigate the co-immunoprecipitation of K_{ATP} channel subunits from different subcellular compartments are ongoing in our laboratory and may resolve this issue. Interestingly, no apparent changes in cardiac sarcK_{ATP} or mitoK_{ATP} channels were evident in Kir6.1$^{-/-}$ knockout mice.[30] The major effect of Kir6.1 knockout in these mice was an increased susceptibility to spontaneous coronary spasm leading to a lethal atrioventricular block, resembling a Prinzmetal angina in humans. This condition was associated with defects in vascular sarcolemmal K_{ATP} channels, rather than their cardiac counterparts.[30] In view of these results and reservations concerning the use of flavoprotein fluorescence to monitor mitoK_{ATP} channel activity[12-14], the confirmation of the presence and potential rôle of a cardiac mitoK_{ATP} channel remains elusive.

Acknowledgements

We thank for their generous gifts: Professor S. Seino for Kir6.1, SUR2A and SUR2B clones, Professor Y. Karachi for a Kir6.2 clone, Dr. A. Tinker for the Kir6.2/SUR2A expressing HEK 293 cell lines and Professor K.P. Campbell for the IXE11$_2$ antibody. We thank Mrs C. Orme for help with cell culture. This work was supported by a British

Heart Foundation Project Grant (98011) and Medical Research Council Studentships to HS and RR.

References

1. Yokoshiki H, Sunagawa M, Seke T, Sperelakis N. ATP-sensitive K^+ channels in pancreatic, cardiac and vascular smooth muscle cells. Am J Physiol 1998; 274: C25-C37.
2. Cohen MV, Baines CP, Downey JM. Ischaemic preconditioning: from adenosine receptor to K_{ATP} channel. Annu Rev Physiol 2000; 62: 79-109.
3. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. Circ Res 1999; 84: 973-979.
4. Noma, A. ATP-regulated K^+ channels in cardiac muscle. Nature 1983; 305: 147-148.
5. Inoui I, Nagase H, Kishi K, Higuti T. ATP-sensitive K^+ channel in the mitochondrial inner membrane. Nature 1991; 352: 244-247.
6. Liu Y, Ren G, O'Rourke B, Marban E, Seharaseyon J. Pharmacological comparison of native mitochondrial K_{ATP} channels with molecularly defined surface K_{ATP} channels. Mol Pharmacol 2001; 59: 225-230.
7. Clement IV JP, Kunjilwar K, Gonzalez G, Schwanstecher M, Panten U, Aguilar-Bryan L, Bryan J. Association and stoichiometry of K_{ATP} channel subunits. Neuron 1997; 18: 827-838.

8. Suzuki M, Li RA, Miki T, Uemura H, Naoya S, Ohmoto-Sekine Y, Tamagawa M, Ogure T, Seino S, Marban E, Nakaya H. Functional roles of cardiac and vascular ATP-sensitive potassium channels clarified by Kir6.2 knockout mice. Circ Res 2001; 88: 570-577.

9. Suzuki M, Kotake K, Fujikura K, Inagaki N, Suzuki T, Gonoi T, Seino S, Takata K. Kir6.1: a possible subunit of ATP-sensitive K^+ channels in mitochondria. Biochem Biophys Res Commun 1997; 241: 693-697.

10. Murry CR, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136.

11. Suzuki M, Sasaki N, Miki T, Sakamoto N, Ohmoto-Seine Y, Tamagawa M, Seino S, Marban E, Nakaya H. Role of sarcolemmal K_{ATP} channels in cardioprotection against ischaemia/reperfusion injury in mice. J Clin Invest 2002; 109: 509-516.

12. Ovide-Bordeaux S, Ventura-Clapier R, Veksler V. Do modulators of the mitochondrial K_{ATP} channel change the function of mitochondria *in situ*? J Biol Chem 2000; 275: 37291-37295.

13. Lawrence CL, Billups B, Rodrigo GC, Standen NB, The K_{ATP} channel opener diazoxide protects cardiac myocytes during metabolic inhibition without causing

mitochondrial depolarisation or flavoprotein oxidation. Br J Pharmacol 2001; 134: 535-542.

14. Hanley PJ, Mickel M, Löffler M, Brandt U, Daut, J. K_{ATP} channel-independent targets of diazoxide and 5-hydroxydecanoate in the heart. J Physiol 2002; 542: 735-741.

15. Atherton E, Sheppard RC. Solid-phase peptide synthesis using N-alpha-fluorenylmethoxycarnonylamino acid pentafluorophenyl esters. J Chem Soc 1985: 3: 165-166.

16. LaRochelle WJ, Wray BE, Sealock R, Froehner SC. Immunochemical demonstration that amino acids 360-377 of the acetylcholine receptor γ-subunit are cytoplasmic. J Cell Biol 1985; 100: 684-691.

17. Stonehouse AH, Pringle JH, Norman RI, Stanfield PR, Conley EC, Brammar WJ. Characterisation of Kir2.0 proteins in the rat cerebellum and hippocampus by polyclonal antibodies. Histochem Cell Biol 1999; 112: 457-465.

18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 690-685.

19. Towbin H, Gordon J. Immunoblotting and dot immunobinding – current status and outlook. J Immunol Methods 1984; 72: 313-340.

20. Cui Y, Giblin JP, Clapp LH, Tinker A. A mechanism for ATP-sensitive potassium channel diversity: Functional coassesmbly of two pore-forming subunits. Proc Natl Acad Sci USA 2001; 98: 729-734.

21. Mitra, R. & Morad, M. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. Am J Physiol 1985; 249: H1056-H1060.

22. Lawrence CL, Rodrigo GC. A Na^+-activated K^+ current ($I_{k,Na}$) is present in guinea-pig but not rat ventricular myocytes. Pflugers Arch 1999; 437: 831–838.

23. Jorgensen AO, Arnold W, Shen AC-Y, Yuan S, Gaver M, Campbell KP. Identification of novel proteins unique to either transverse tubules (TS28) or the sarcolemma (SL50) in rabbit skeletal muscle. J Cell Biol 1990; 110: 1173-1185.

24. Hudman D, Rainbow RD, Lawrence CL, Standen NB. The origin of calcium overload in rat cardiac myocytes following metabolic inhibition with 2,4-dinitrophenol. J Mol Cell Cardiol 2002; 34: 859-871.

25. Santos AS, Nathanael L, Maurice G and Guido K. Purification of mitochondria for apoptosis assays. Methods in Enzymology 2000; 322: 205-208.

26. Cooperstein SJ, Lazarow A. A microspectrophotometric method for the determination of cytochrome oxidase. J Biol Chem 1950; 186: 129-139.

27. Inagaki N, Gonoi T, Clement IV JP, Wang C-Z, Aguilar-Bryan L, Bryan J, Seino S. A family of sulphonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. Neuron 1996; 16: 1011-1017.

28. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JP, Boyd III AE, González G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA. Cloning of the β cell high affinity sulphonylurea receptor: a regulator of insulin secretion. Science 1995; 268: 423-426.

29. Seharaseyon J, Ohler A, Sasaki N, Fraser H, Sato T, Johns DC, Rourke BO, Marbán, E. Molecular composition of mitochondrial ATP-sensitive potassium channels probed by viral Kir gene transfer. J Mol Cell Cardiol 2000; 32: 1923-1930.

30. Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, Yamaguchi K, Koseki H, Iwanaga T, Nakaya H, Seino S. Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. Nature Med. 2002; 8: 466-472

31. Rosner E, Lopez G, Porter LM, Pountney DJ, Nakamura TY, Coetzee WA. Kir6.1 K^+ channel subunits are expressed in mouse ventricular myocytes and associate with the actin cytoskeleton (Abstract). Biophys J 2002; 82: 589a.

32. Seharaseyon J, Sasaki N, Ohler A, Sato T, Fraser H, Johns DC, O'Rourke B, Marbán E. Evidence against functional heteromultimerization of the K_{ATP} channel subunit Kir6.1 and Kir6.2. J Biol Chem 2000; 275: 17561-17565.

33. Chutkow WA, Makielski JC, Nelson DJ, Burant CF, Fan Z. Alternative splicing of *sur2* Exon 17 regulates nucleotide sensitivity of the ATP-sensitive potassium channel. J Biol Chem 1999; 274: 13656-13665.

34. Clark RB, Tremblay A, Melnyk P, Allen BG, Giles WR, Fiset C. T-tubule localization of the inward-rectifier K^+ channel in mouse ventricular myocytes: a role in K^+ accumulation. J Physiol 2001; 537: 979-992 .

35. Leonoudakis D, Mailliard W, Wingerd K, Clegg D, Vandenberg C. Inward rectifier potassium channel Kir2.2 is associated with synapse-associated protein SAP97. J Cell Sci 2001; 114: 987-998.

36. Christé, G. Localization of K^+ channels in the t-tubules of cardiomyocytes as suggested by the parallel decay of membrane capacitance, IK_1 and IK_{ATP} during culture and by delayed IK_1 response to barium. J Mol Cell Cardiol 1999; 31: 2207-2213.

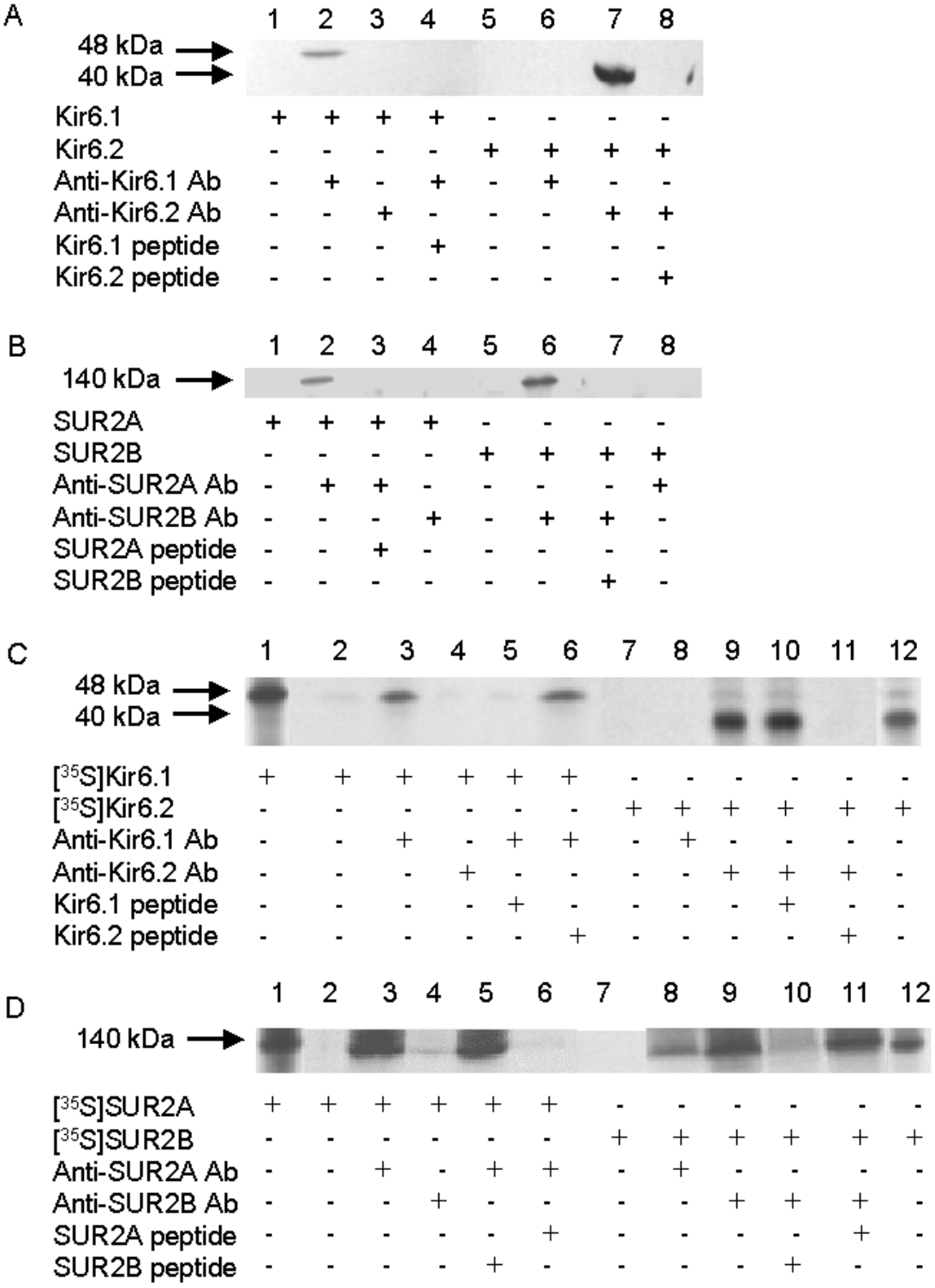
37. Lipp P, Hüsher J, Pott L, Niggli E. Spatially non-uniform Ca^{2+} signals induced by the reduction of transverse tubules in citrate-loaded guinea-pig ventricular myocytes in culture. J Physiol 1996; 497: 589-597.

38. Mitcheson JS, Hancox JC, Levi AJ. Action potentials, ion channel currents and transverse tubule density in adult rabbit ventricular myocytes maintained for 6 days in cell cultures. Pflügers Archiv 1996; 431: 814-827.

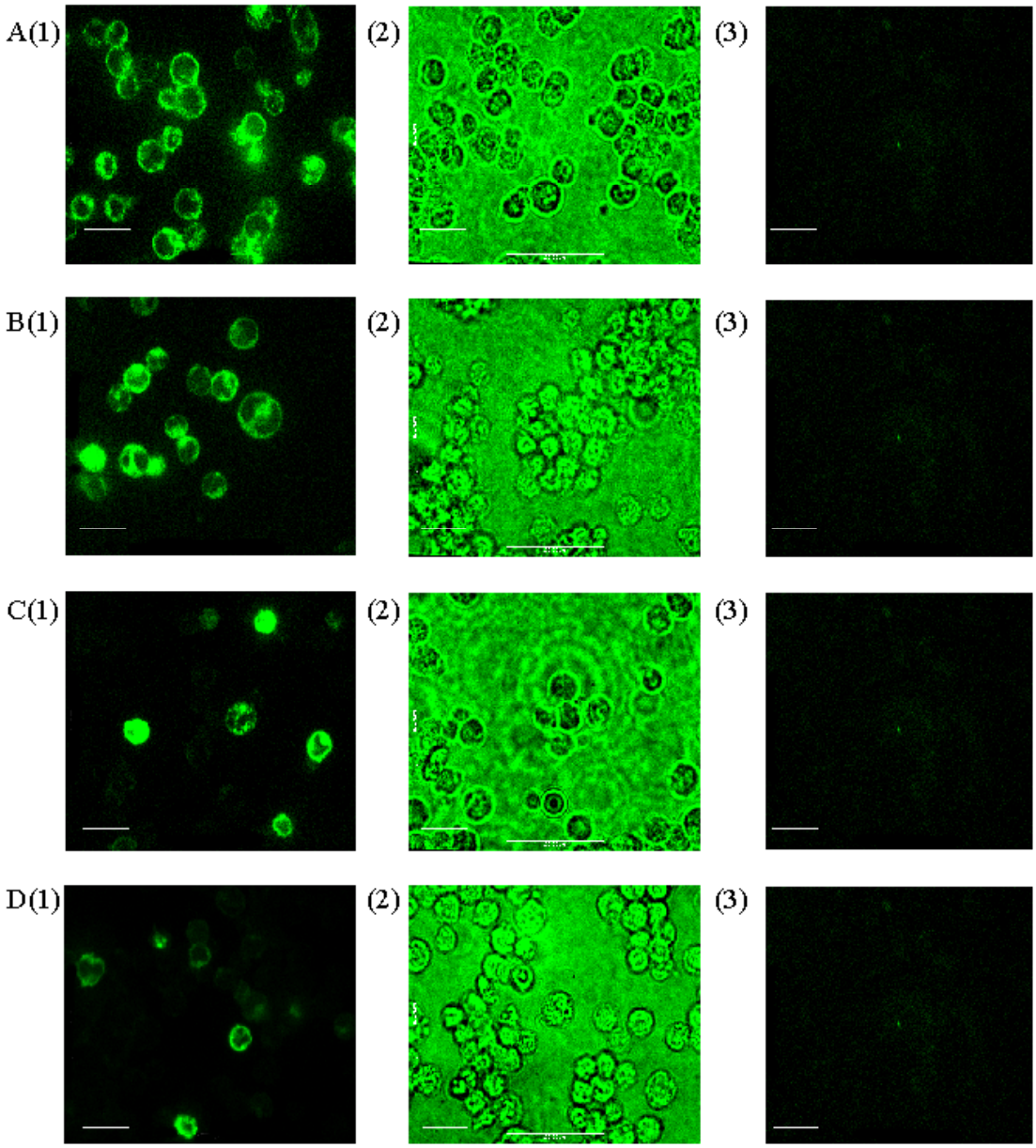
39. Li RA, Leppo M, Miki T, Seino S, Marbán E. Molecular basis of electrocardiographic ST-segment elevation. Circ Res 2000; 87: 837-839.

40. Zingman LV, Hodgson DM, Bast PH, Kane GC, Perez-Terzic C, Gumina RJ, Pucar D, Bienengraeber M, Dzeja PP, Miki T, Seino S, Alekseev AE, Terzic A. Kir6.2 is required for adaptation to stress. Proc Natl Acad Sci USA 2002; 99: 13278-13283.

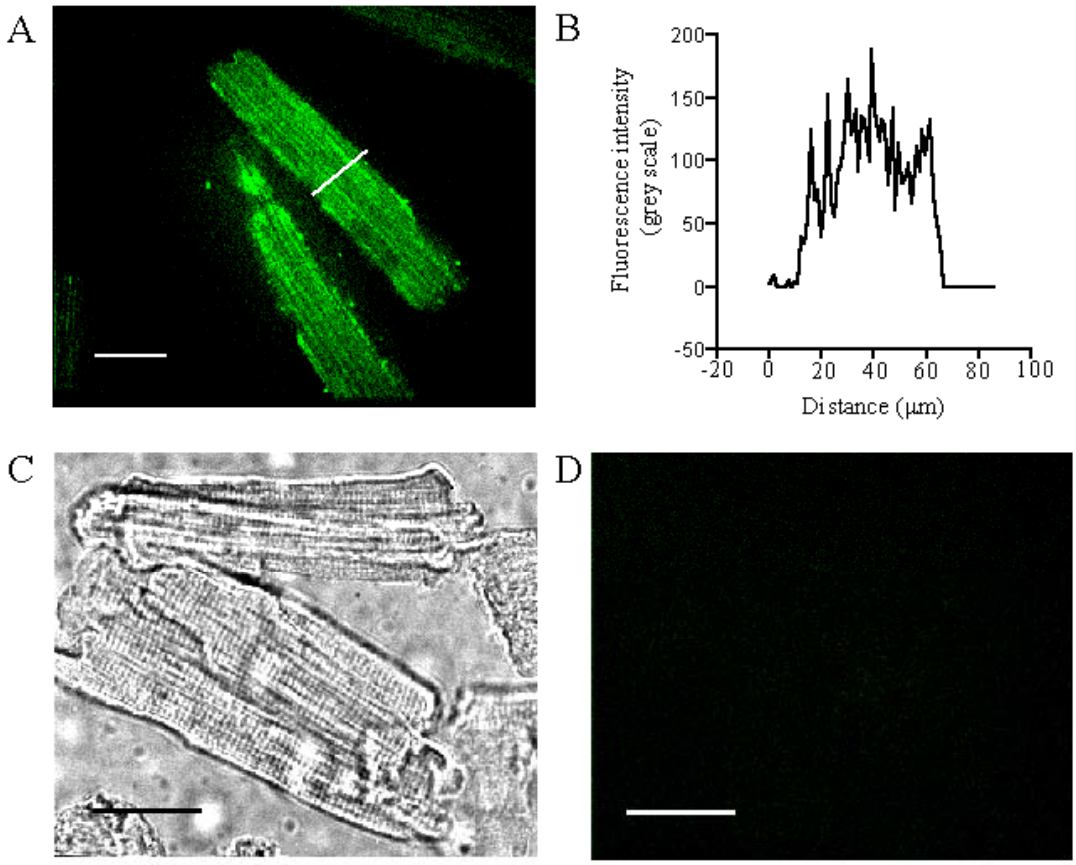
Singh et al., 2002 Figure 1. K_{ATP} channel subunit localisation in cardiac myocytes



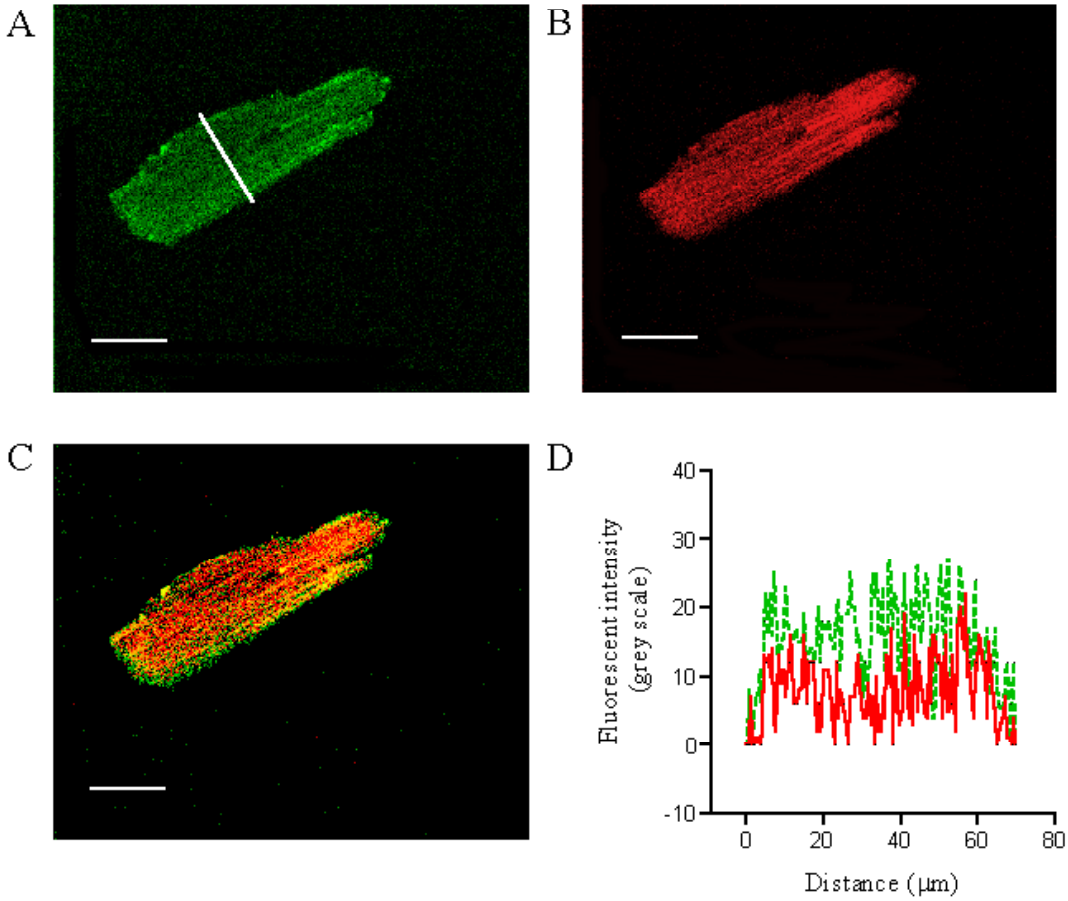
Singh et al., 2002 Figure 2. K_{ATP} channel subunit localisation in cardiac myocytes



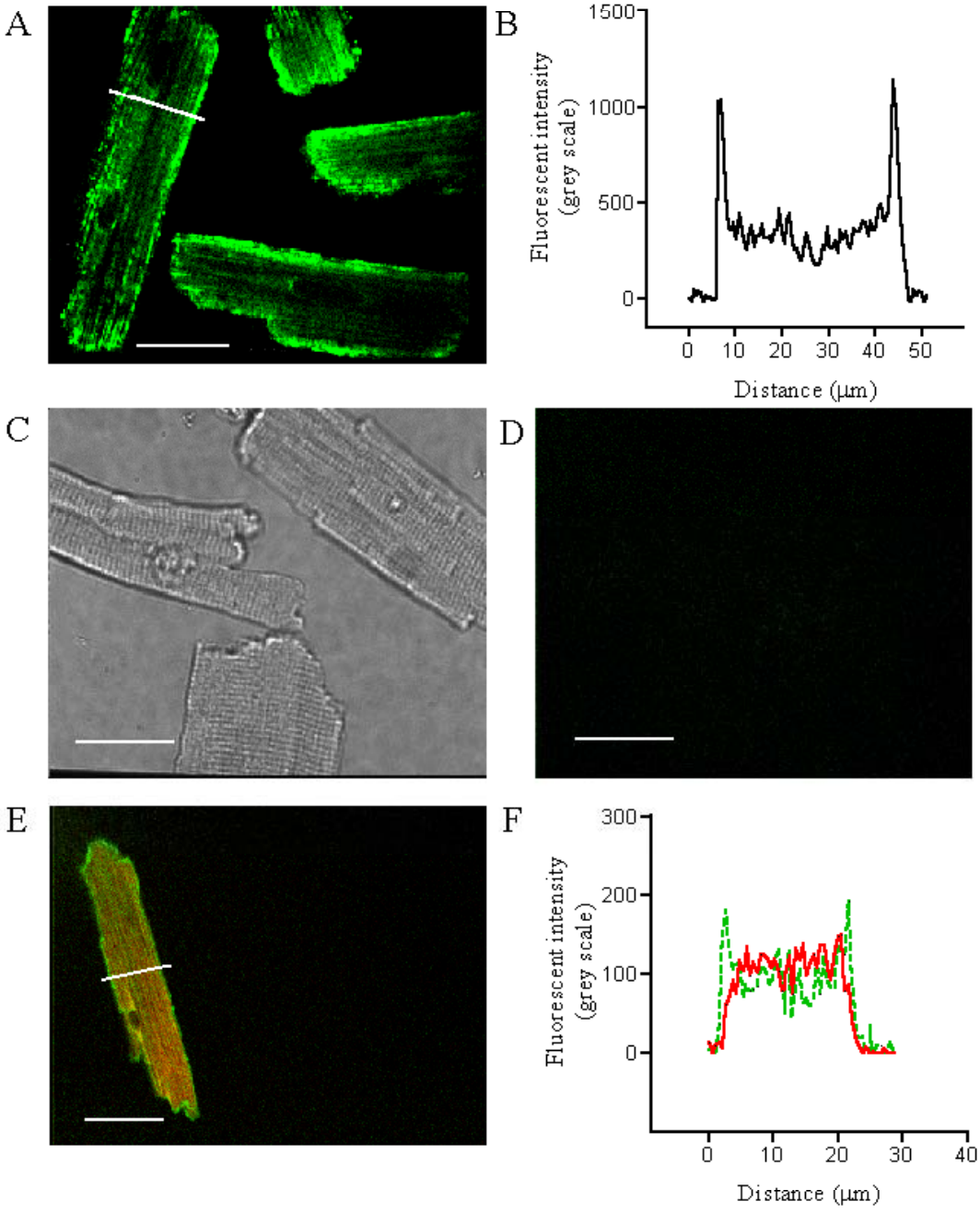
Singh et al., 2002 Figure 3. K_{ATP} channel subunit localisation in cardiac myocytes



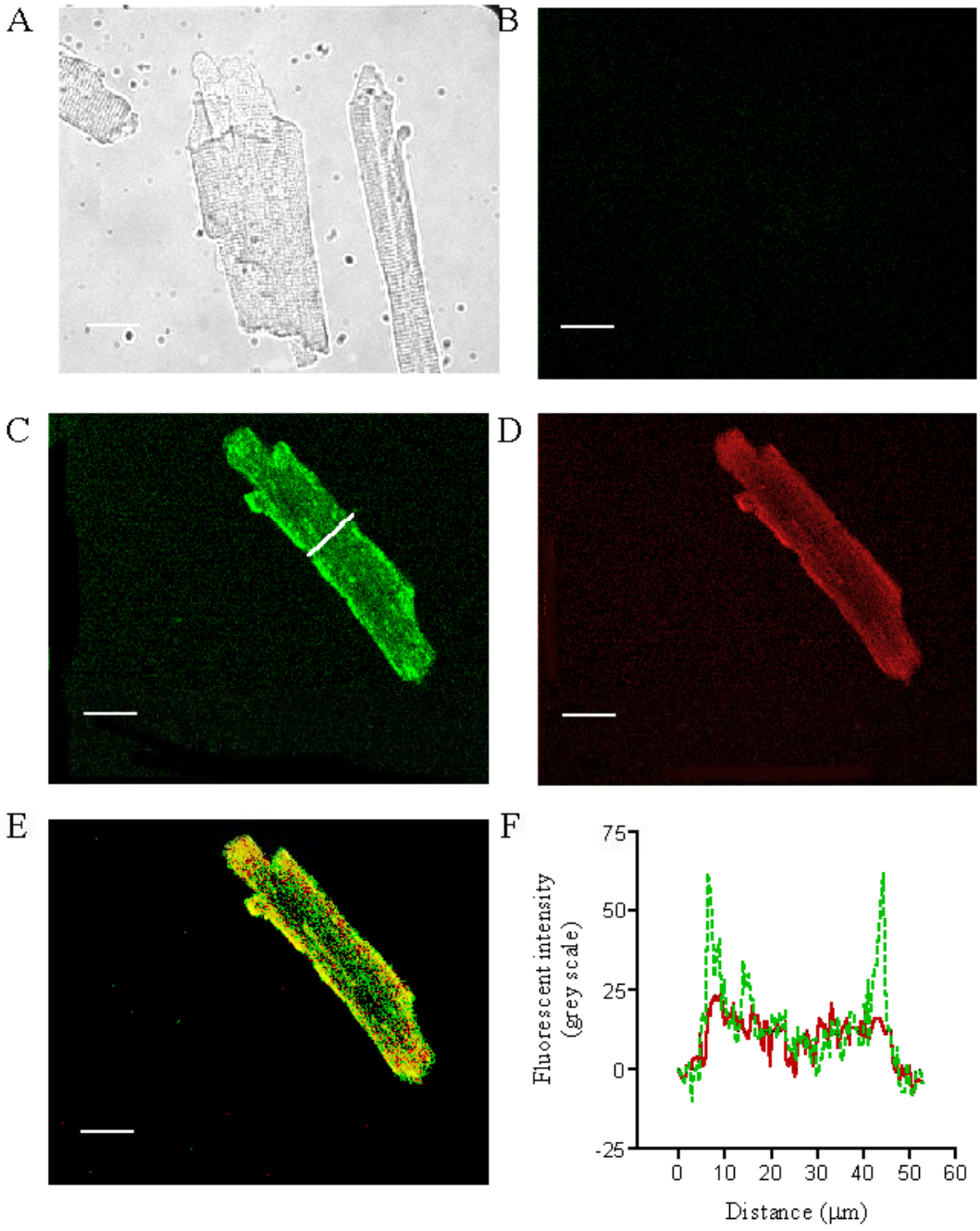
Singh et al., 2002 Figure 4. K_{ATP} channel subunit localisation in cardiac myocytes



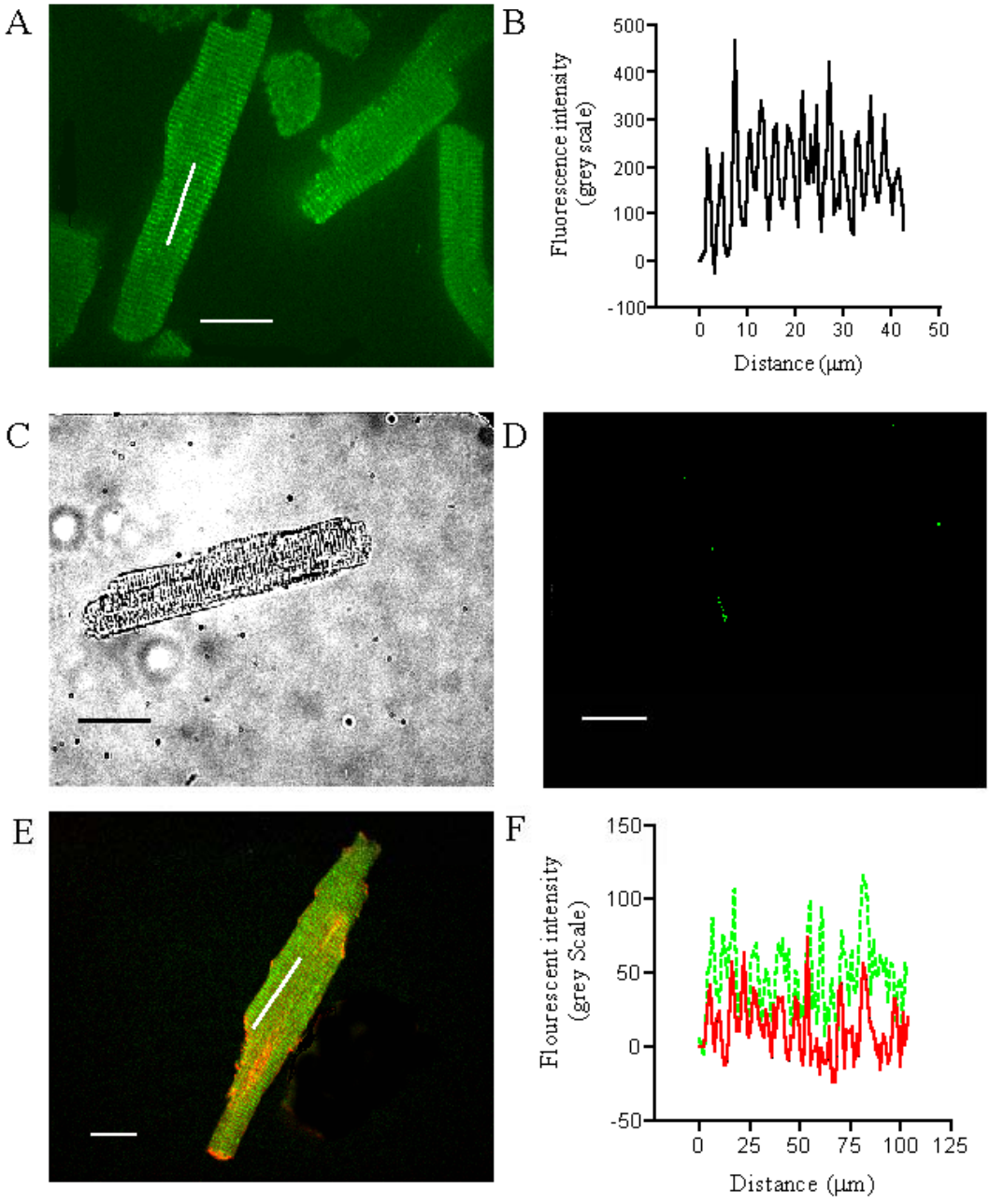
Singh et al., 2002 Figure 5. K_{ATP} channel subunit localisation in cardiac myocytes



Singh et al., 2002 Figure 6. K_{ATP} channel subunit localisation in cardiac myocytes



Singh et al., 2002 Figure 7. K_{ATP} channel subunit localisation in cardiac myocytes



Singh et al., 2002 Figure 8. K_{ATP} channel subunit localisation in cardiac myocytes