Identification of the cardiac ryanodine receptor channel in membrane blebs of sarcoplasmic reticulum

Thomas Böhle[a,*], Mathias C. Brandt[a], Nadine Henn[a], Annette Schmidt[b], Wilhelm Bloch[b], Dirk J. Beuckelmann[a]

[a] *Department of Medicine III, University of Cologne, Joseph-Stelzmann-Str. 9, D-50924 Cologne, Germany*
[b] *Institute of Anatomy I, University of Cologne, Joseph-Stelzmann-Str. 9, D-50924 Cologne, Germany*

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Abstract Blebs of the sarcoplasmic reticulum (SR) membrane of heart muscle cells were generated after saponin perforation of the plasma membrane followed by complete hypercontraction of the cell. Although characteristic proteins of the plasma membrane, namely the β1-adrenoreceptor and Gαi, were stained by monoclonal antibodies in the hypercontracted cells, these proteins could not be detected in the adjacent blebs. Monoclonal antibodies to the cardiac ryanodine receptor (RyR2), calsequestrin and SERCA2 bound at different amounts to surface components of the blebs and to components of the hypercontracted cells. From the immunofluorescence signals we conclude that the blebs are mainly constituted of corbular and junctional SR membrane, and only to a lesser extent of network SR membrane. Deconvolution microscopy revealed that the membrane location of RyR2, calsequestrin and SERCA2 in the bleb is comparable to native SR membrane. At the bleb membrane gigaohm seals could be obtained and patches could be excised in a way that single-channel currents could be measured, although these are not completely identified.

Key words: Sarcoplasmic reticulum membrane; Membrane bleb; Immunohistochemistry; Deconvolution microscopy; Human myocardial ryanodine receptor; In situ identification

1. Introduction

Contraction in heart muscle is triggered mainly by Ca^{2+}-induced Ca^{2+} release [1]. Ca^{2+} entering the cardiomyocyte through L-type Ca^{2+} channels, located both in the surface and the T-tubular membrane, triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptor channels (RyR2), representing the main isoform of this protein in heart muscle.

While for electrophysiological characterization the L-type Ca^{2+} channel is directly accessible to patch-clamp recordings, channel proteins as RyR2 located in intracellular membranes are not. Thus, lipid bilayers made of e.g. phosphatidylserine and phosphatidylethanolamine serve as artificial membranes, in which after purification RyR2 is incorporated by spontaneous reconstitution. With two purification methods several electrophysiological investigations have been performed on RyR2 [2–5]. In method 1 SR membrane vesicles containing RyR2 are biochemically purified in several steps. In method 2 the subfractions of the SR membrane are further purified and finally RyR2 is isolated. Since method 1 is closer to native conditions than method 2, electrophysiological results from SR membrane vesicles are assumed to be comparable with the in situ situation, an assumption that has not yet been verified.

Results on RyR2 are mainly from experiments with cardiac tissue obtained from various animals [4,6]. In experiments on RyR2 with diseased human heart tissue Holmberg et al. [7] used method 1 and found no differences in the channel characteristics compared to normal animal tissue. In both methods the purification process probably causes alteration of physiological and pathophysiological functions of RyR2 [8]. This may be due to changes of the native lipid environment, when incorporation into an artificial bilayer for electrophysiological experiments is necessary. Furthermore, regulatory and accessory proteins [9], e.g. FKBP12, sorcin, calmodulin, calsequestrin, junctin, and triadin, may be altered.

More than a decade ago Stein and Palade [10] developed a technique, which allows for measurements of the native skeletal muscle ryanodine receptor. Subsequently with this method various membrane proteins of skeletal muscle SR were characterized electrophysiologically [11–14]. In the present paper a new technique is described, in which generation of SR membrane blebs from cardiac tissue opens up the possibility for an in situ patch-clamping of RyR2.

2. Materials and methods

2.1. Cell isolation

Right atrial appendage specimens were obtained from patients mostly suffering from coronary heart disease and thus undergoing heart surgery. The procedure for isolation of cardiomyocytes from human atrial appendages has previously been described in detail by Brandt et al. [15]. Cells from guinea-pig cardiac ventricle were isolated by Langendorff perfusion similar to the method described by Isenberg and Klöckner [16].

2.2. Solutions and temperature

The cardioplegic solution for transport of the atrial tissue contained (mM): NaCl 15, KCl 9, $MgCl_2$ 4, histidine hydrochloride 18, histidine 180, tryptophan 2, mannitol 30, $CaCl_2$ 0.015, glutaric acid 1, KOH 2.0. The modified Tyrode's solution for cell isolation and storage contained (mM): NaCl 135, KCl 4, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES

*Corresponding author. Fax: (49)-221-478 6490.
E-mail address: thomas.boehle@medizin.uni-koeln.de (T. Böhle).

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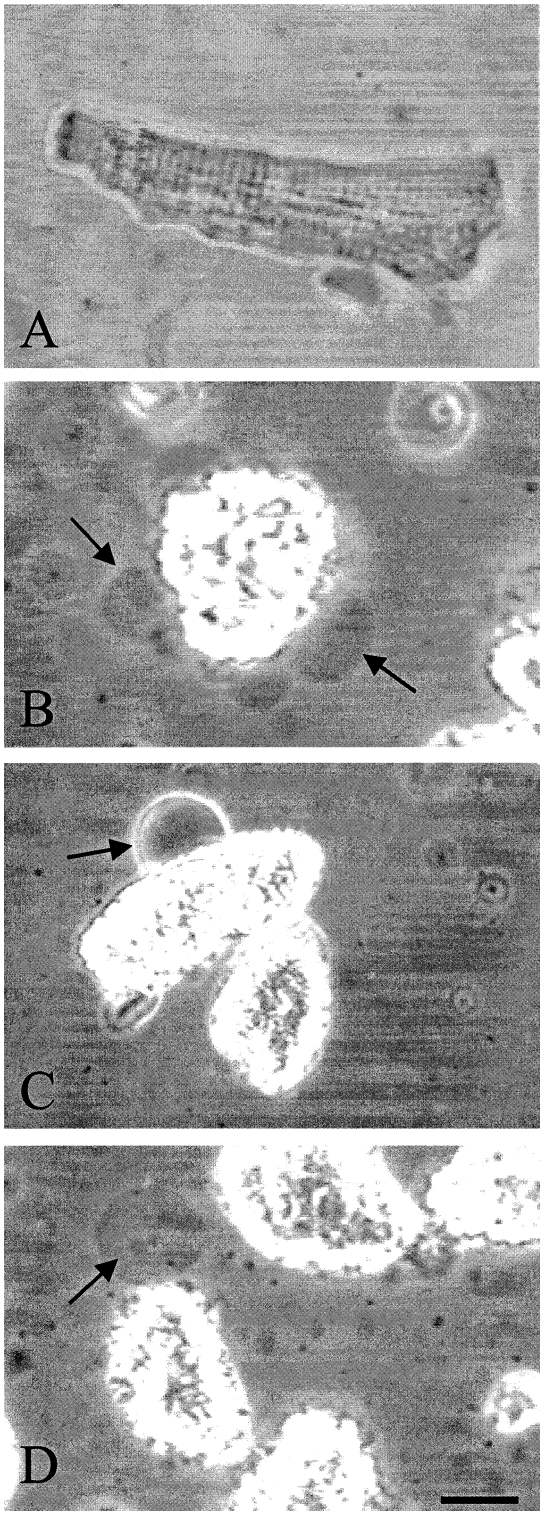

Fig. 1. Vital microscopical images of untreated (A) and hypercontracted guinea-pig cardiomyocytes with blebs after exposure to saponin (B–D). A: The untreated cardiomyocyte is brick-shaped and shows cross striation. B: Multiple blebs (arrows) of different sizes are formed around a hypercontracted cardiomyocyte. C: A hypercontracted cardiomyocyte with a single bleb (arrow). D: A bleb (arrow) losing contact with the hypercontracted cardiomyocyte. Bar = 10 μm.

10, glucose 10, pH 7.3. The physiological saline solution (PSS) for triggering generation of the SR membrane blebs contained (mM): NaCl 117, KCl 2.5, $CaCl_2$ 1.8, MOPS 5, pH 7.2. The solution (CRS) for patch-clamping excised patches of SR bleb membrane was symmetric and contained (mM): $Ca(gluconate)_2$ 50, $CaCl_2$ 2.5, sucrose 100, 10 μg/ml hydroxycholesterol, pH 7.3. All experiments were performed at room temperature (22–24°C).

2.3. Formation of SR membrane blebs

After cells had been isolated they were kept in PSS for 5 min. Then the cells were superfused with PSS with addition of 0.2 mg/ml saponin. This was the four-fold concentration used by Endo and Iino [17] in skeletal muscle fibers to preserve SR function while perforating the surface membrane. At this stage of the experiment the cells were carefully observed on a monitor by means of a camera, which was connected to the side tube of an inverted microscope. Presumably reflecting perforation by saponin, first the thickness of the plasma membrane seemed to diminish. Then the regular cross striation of the cell began to dissolve, probably as a consequence of local increase of internal Ca^{2+}. During the time interval of 10–30 min following the addition of saponin a first contraction of the cell was observed. In order to preserve the SR membrane a prompt switching to saponin-free PSS was essential at this moment. During the following 10 min local contractions occurred, subsequently becoming more frequent and synchronous, then spreading over the whole cell in a wave-like fashion, and finally resulting in a hypercontraction of the myocyte. Just at the moment of maximum hypercontraction about 3–10 SR membrane blebs originated through the perforated surface membrane.

2.4. Immunohistochemistry

After induction of membrane blebs cardiomyocytes were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline for 30 min. Then cells were washed several times with 0.05 M Tris-buffered saline (TBS) and subjected to 0.25% Triton-X 100 and 0.5 M NH_4Cl in 0.05 M TBS. The probes were blocked with 5% bovine serum albumin (BSA) in TBS (1 h, room temperature). The primary antibodies were incubated overnight (4°C) in 0.8% BSA: mouse anti-SERCA2 (1:500; ABR, Inc., Golden, CO, USA), mouse anti-calsequestrin (1:300; ABR), rabbit anti-β1-adrenoreceptor (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-RyR2 (1:100, ABR) as well as rabbit anti-Gαi (1:500, Santa Cruz). The secondary antibodies, goat-anti rabbit-conjugated biotin and goat-anti mouse-conjugated biotin (1:400; Dako A/S, Glostrup, Denmark) were incubated for 1 h at room temperature. For detection Extravidin CY3 was used (1:1000, Sigma Chemical Co., St. Louis, MO, USA).

2.5. Deconvolution

After immunohistochemistry cells were employed for deconvolution microscopy. For this purpose an inverted microscope (Zeiss Axiovert 100 M) with an oil-immersion objective (Zeiss 63 Planapochromat), as well as the commercial software package KS 400 from Zeiss were used. A band-pass filter CY3 (AHF) without overlap in the fluorescence emission spectrum was utilized. Each specimen was acquired as an image stack with 64 planes. The distances of the image planes were set to 100 nm to assure proper oversampling of the optical resolution for the subsequent iterative deconvolution process. Both theoretical

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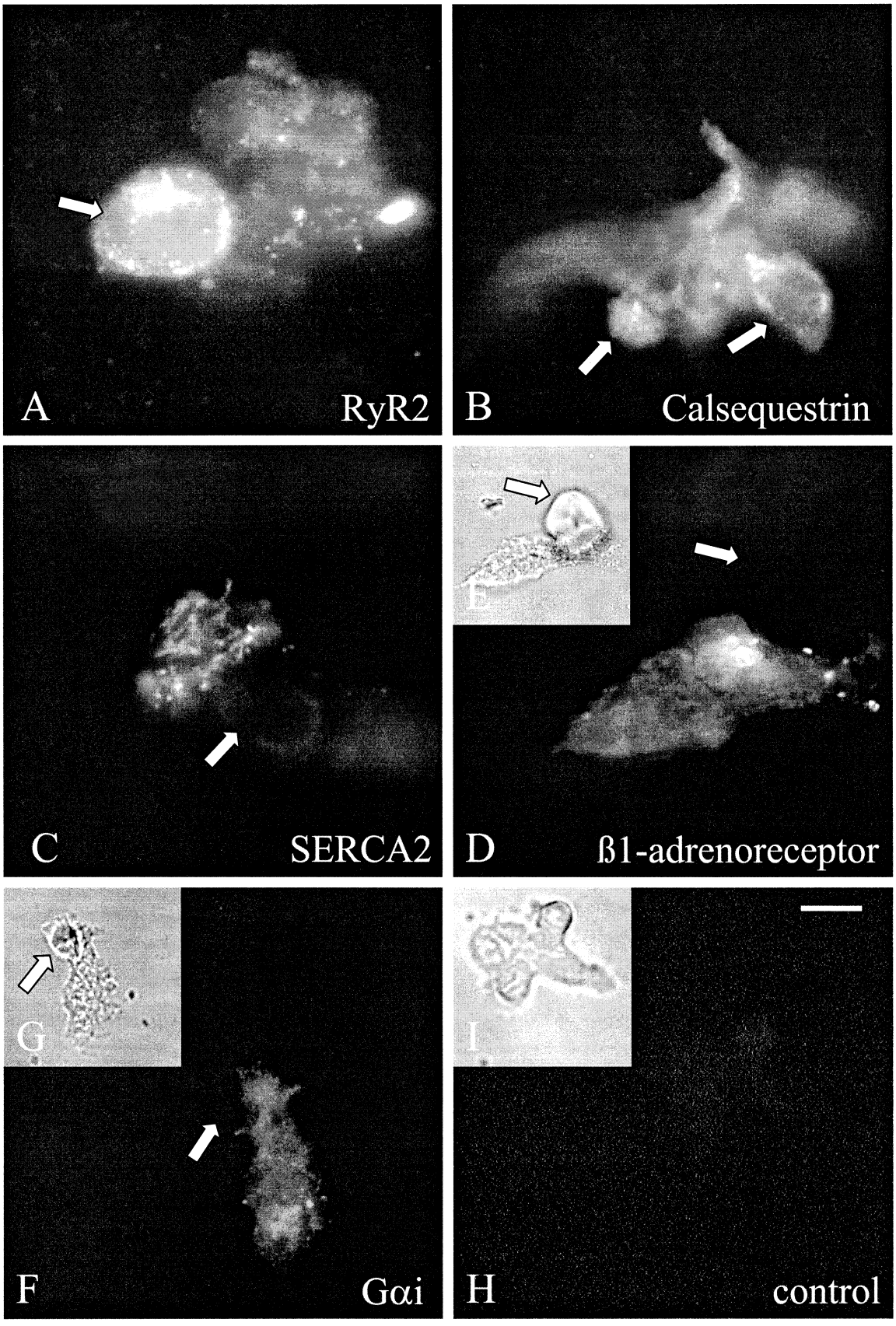
Fig. 2. Immunohistochemical detection of proteins located in the SR or in the plasma membrane of guinea-pig cardiomyocytes. A: RyR2 is highly expressed on the bleb (arrow), while the hypercontracted cardiomyocyte reveals a lower immunohistochemical staining. B: Intense calsequestrin staining is apparent on the hypercontracted cardiomyocyte as well as on the blebs (arrows). C: Staining for SERCA2 is higher on the hypercontracted cardiomyocyte than on the bleb (arrow). D: Immunohistochemical detection of the β1-adrenoreceptor results in staining of the hypercontracted cardiomyocyte only, no signal is visible on the bleb (arrow). E: Same as D, but without fluorescence excitation reveals position of the bleb (arrow). F: Gαi is highly expressed in the hypercontracted cardiomyocyte, while no staining can be found on the bleb (arrow). G: Same as F, but without fluorescence excitation reveals position of the bleb (arrow). H: Without the primary antibody no staining of the hypercontracted cardiomyocyte can be seen, also shown in I (transmitted light). Bar = 10 μm.


A
RyR2
B
Calsequestrin
C
SERCA2
E
D
ß1-adrenoreceptor
G
F
Gαi
I
H
control

point-spread functions (PSF), based on microscope and specimen data, and measured PSF, calculated from fluorescent beads with a diameter of 1 μm (Molecular Probes, T-14792), were used. The conjugated gradient-maximum likelihood deconvolution algorithm with 60 iterations was performed on the image stacks. Output intensities were scaled between gray values 0 and 255. Theoretical and measured PSF generated comparable results with slightly higher resolution in the resulting images by using the measured PSF.

2.6. Patch-clamping

Five minutes were allowed for the myocytes to form adhesions to the bottom of the recording chamber. After triggering generation of the SR membrane bleb in PSS this solution was replaced by CRS and patch-clamping was started after at least 10 min. Ultra-thick-walled patch pipettes (outer and inner diameters of the raw material 2.0 mm and 0.25 mm, respectively) were used, which were shortened to about 5 mm and inserted into a silver pipette holder of small dimensions [18]. Pipette tips were generated only seconds before starting of giga-ohm seal formation by gently breaking off the final tip region at the glass bottom of the bath chamber [19]. Thus, an ultra-clean glass surface was generated, at which giga-ohm seals developed by touching the bleb membrane and application of slight suction.

2.7. Current data acquisition and analysis

Unitary currents were recorded at a sampling rate of 3 kHz in the excised patch configuration with an Axopatch-200B amplifier (Axon Instruments, Inc., Foster City, CA, USA). Analog filtering was performed with an intrinsic filter at a band width of 1 kHz. The holding potential was 0 mV and 500 ms pulses ranging from −100 mV to +100 mV were applied at a rate of 1.5 Hz. Capacitive transients were compensated for carefully via compensation circuits containing two exponentials. Leakage and remaining capacitive currents were removed by subtracting parts of traces, in which the channels were closed. Recording was performed on a PC-80486 with the pCLAMP software. Analysis was performed on a PC-Pentium with the ISO2 software (MFK-Computer, Niedernhausen, Germany) after first converting the data with the PCV software (MFK-Computer, Niedernhausen, Germany).

3. Results

Isolated guinea-pig cardiomyocytes, which were nearly completely relaxed (Fig. 1A), were treated with saponin and brought to hypercontraction. Under vital microscopical observation the cells then showed structural alterations as loss of the typical cross striation and rounding.

In addition to these changes multiple small blebs of membrane were detected on the surface of the cardiomyocyte (Fig. 1B), which were able to fuse, often resulting in a larger single bleb (Fig. 1C). In some cases a bleb lost its connection to the cardiomyocyte (Fig. 1D). Each bleb was covered by a thin membrane, and the diameter of individual blebs ranged from about 2 to 30 μm.

Vital microscopy of the cardiomyocytes does not give information about the origin of the blebs. Therefore, we performed immunohistochemical stainings for different plasma membrane and SR membrane proteins. Microscopical investigation of the immunofluorescence-stained cardiomyocytes gives evidence of an SR origin of the blebs (Fig. 2). Especially for RyR2 (Fig. 2A) and calsequestrin (Fig. 2B) intense labeling of the blebs was visible, which was often stronger compared to the staining seen in the adjacent and fully contracted cardiomyocytes. Only a faint staining of the blebs in compar-

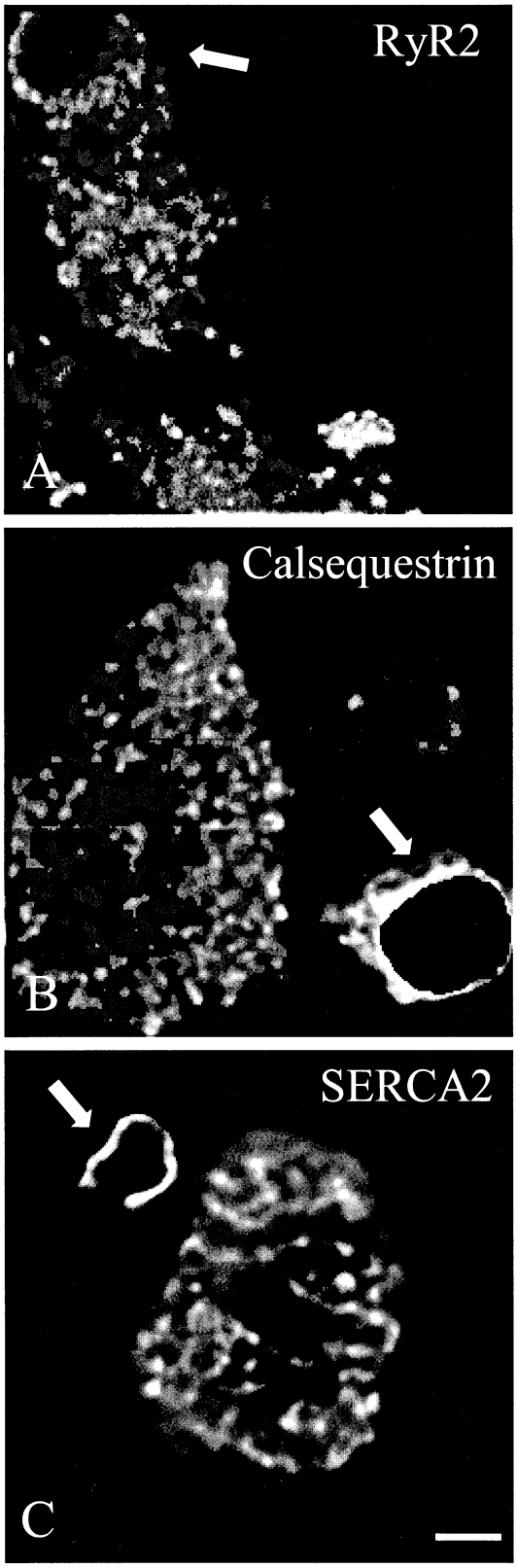


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Fig. 3. Deconvolution microscopy images of immunohistochemically stained cardiomyocytes with blebs (arrows). Each figure shows a single digital slice of 100 nm vertical expansion. A hypercontracted cardiomyocyte and the adjacent bleb is shown for RyR2 (A), calsequestrin (B), and SERCA2 staining (C), respectively. The immunohistochemical signals of RyR2, calsequestrin, and SERCA2 are restricted to the surface of the blebs, while the hypercontracted cardiomyocytes express the three types of proteins throughout the cell, but not on their surfaces. Bar = 5 μm.

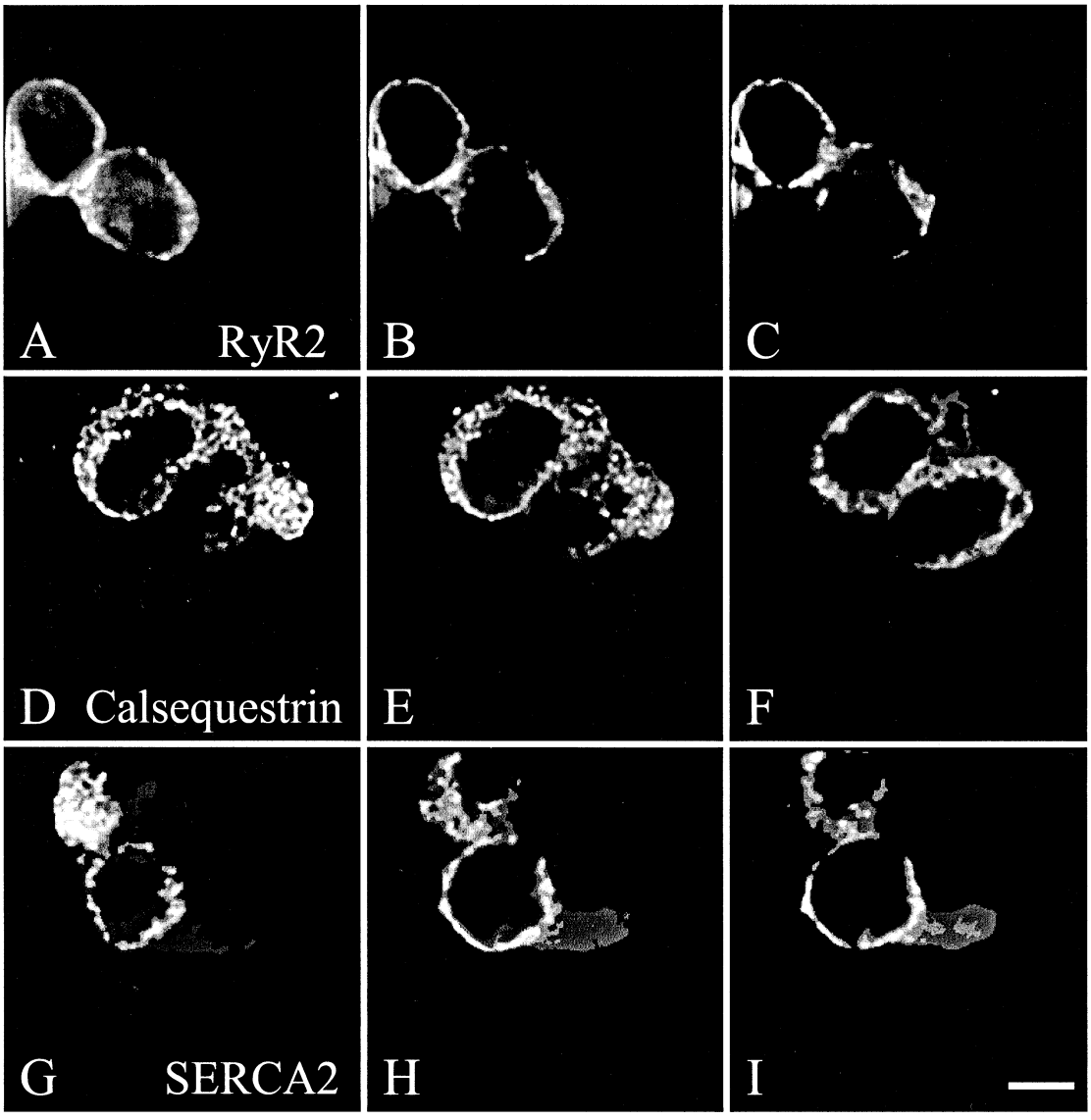

Fig. 4. Digital slices at different planes of immunohistochemically stained blebs. Each row shows one type of staining in two coherent blebs. A–C: RyR2 is located in the membrane of the bleb. D–F: Calsequestrin is detected in a larger border area beneath the bleb membrane. G–I: SERCA2 is located in the membrane of the bleb. No immunostaining for any of the three SR-derived proteins could be found inside of the blebs. Bar = 5 μm.

ison to the cardiomyocytes was visible for SERCA2 (Fig. 2C). No evidence of characteristic plasma membrane proteins could be found in the blebs. While the hypercontracted cardiomyocytes were immunohistochemically stained with antibodies for β1-adrenoreceptor (Fig. 2D,E) and Gαi (Fig. 2F,G), no immunofluorescence signals were detected in the blebs themselves. The possibility of non-specific labeling was excluded in control experiments without the primary antibody (Fig. 2H,I).

To further study the subcellular distribution of the membrane proteins of the SR, we used high-resolution deconvolution microscopy. With this technique it was possible to perform digital slices with a thickness of only 100 nm from blebs and also from adjacent hypercontracted cardiomyocytes. Hence binding to membrane structures in the blebs could be discriminated from other binding. The deconvolution images reveal differences in the distribution of RyR2, calsequestrin and SERCA2 in the cardiomyocytes versus their adjacent blebs. While the immunostaining of RyR2, calsequestrin and SERCA2 is distributed throughout the whole hypercontracted cardiomyocytes but not on their surfaces, the fluorescence signal is only found on the surface of the blebs (Fig. 3A–C).

Serial digital sections of the blebs show a thin immunostained line on the surface of the blebs only after staining of RyR2 and SERCA2 (Fig. 4A–C,G,H). In contrast, calsequestrin immunofluorescence labeled blebs reveal a thicker fluorescent line (Fig. 4D–F). Due to the method of embedding the blebs are getting rather flattened, which results in nearly identical diameters of the three different digital bleb slices stained for the respective protein.

In order to look for membrane currents patch-clamp experiments were performed. Single-channel openings at different potentials are illustrated in Fig. 5. These current recordings are from an excised patch of an SR membrane bleb. The atrial cell originates from a patient suffering from coronary heart disease. The arrows indicate the beginning and the end of test

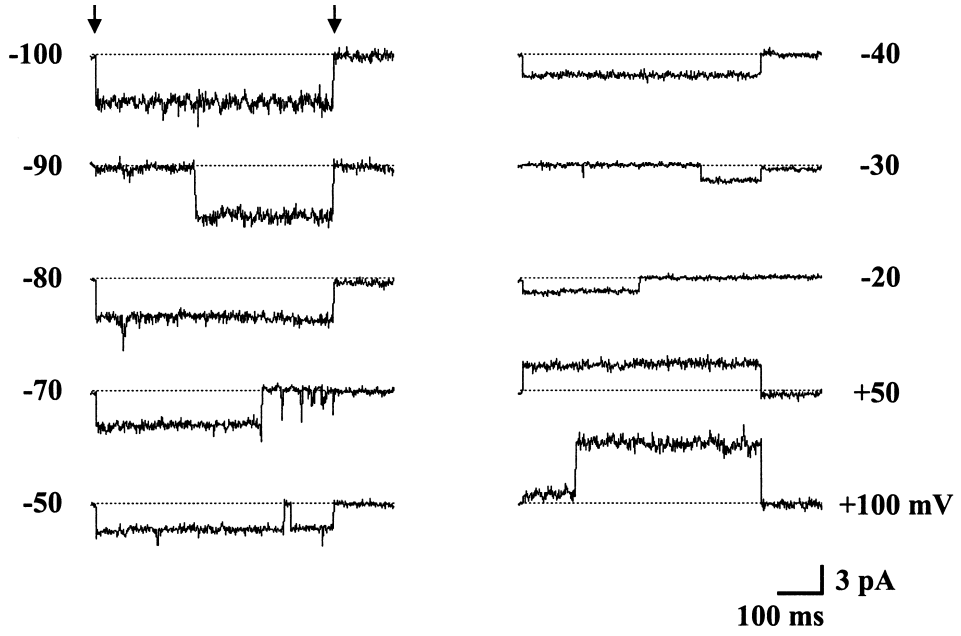

Fig. 5. Single-channel openings at different potentials. These current recordings are from an excised patch of an SR membrane bleb. The myocyte was isolated from the atrial appendage of a patient suffering from coronary heart disease. The holding potential is 0 mV, and the respective pulse potentials are indicated. The arrows mark the beginning and the end of test pulses.

pulses. The holding potential is 0 mV, and the respective pulse potentials are indicated.

4. Discussion

In order to study RyR2 in heart muscle cells from guinea-pig ventricle and human atrium, we have developed a method in which blebs of the SR membrane were generated after saponin perforation of the plasma membrane followed by complete hypercontraction of the myocyte. The blebs were studied by using vital, immunofluorescence and high-resolution deconvolution microscopy, as well as single-channel patch-clamping.

While the β1-adrenoreceptor and Gαi could not be detected in the blebs by monoclonal antibodies, RyR2, calsequestrin, and SERCA2 were present at different amounts. In the native cell RyR2 and calsequestrin are found in the junctional and the corbular SR, while SERCA2 is found in the network SR. Thus, the immunohistochemical stainings reveal that (1) proteins of the plasma membrane are not found in the blebs, (2) proteins of the SR membrane are found in the blebs, and (3) in the bleb membrane the portion of junctional and corbular SR is higher than of network SR.

The fact that the blebs consist mainly of membranes of the junctional and the corbular SR seems plausible. Being in close proximity of the perforated plasma membrane, these portions of SR are presumably pressed out more easily upon hypercontraction of the cell than the network SR being twisted between the myofilaments.

Possible differences in SR of human atrial cells and guinea-pig ventricular cells have to be discussed. In both types, the SR is a heterogeneous intracellular organelle, which is composed of junctional, network and corbular SR. Since atrial cells are devoid of T-tubules, junctional SR in these is connected only to the surface membrane. While it is assumed that mechanically skinned cardiac muscle cells [20] have no junctional SR [21], no information is available about junctional SR in our two types of saponin-perforated cardiomyocytes. Since both the time course of SR membrane bleb formation and the bleb appearance were indistinguishable between human atrial and guinea-pig ventricular cells, we conclude that blebs are of the same origin in both types of cells.

Deconvolution microscopy reveals that the antibodies against SR membrane proteins bound to surface components but not to the inside of the blebs. The serial sections suggest that RyR2 and SERCA2 are found in the bleb membrane and calsequestrin is found beneath the bleb membrane, like in native SR. In summary it is concluded that (1) blebs are surrounded by a single membrane, and (2) the location of the three SR proteins in this bleb membrane is similar to their in situ positions.

Point 1 is in contrast to results of Lewis et al. [22] about SR membrane blebs of skeletal muscle cells. These investigators detected a foam-like structure inside of such kind of blebs, which were obtained after mechanical skinning of the cells. One striking difference between cells from skeletal muscle tissue and cardiac tissue is that no corbular SR is found in the former [21]. Nevertheless, this is unlikely the underlying reason for the obvious difference in the constitution of the blebs. The method of mechanical skinning is more likely to destroy connections between surface membrane, T-tubules, and junctional SR, resulting in numerous membrane fragments, which might be found inside of the blebs after membrane fusion as the ultra-structural correlate of these foam-like structures.

Point 2 leads to the hopeful assumption that in cardiac SR membrane blebs accessory proteins, e.g. FKBP12, sorcin, and calmodulin on the cytosolic side such as calsequestrin, junctin, and triadin on the lumenal side, which presumably all regulate RyR2 are not disturbed in their physiological function and cooperation with RyR2 [9]. This is especially important when considering that pathophysiological defects, e.g. in heart

failure, might be triggered by incomplete functional cooperation of RyR2 with accessory proteins [23].

The patch-clamp recordings reveal that at the bleb membrane, formation of giga-ohm seals, excision of the patch, and single-channel recording are possible. We used excised patches, because the concentration of different ions inside of the bleb is unknown. At the Ca^{2+} concentration (symmetrical 52.5 mM) used in the present study from bilayer experiments the single-channel conductance of RyR2 is expected to be around 100–120 pS with the channel being mostly closed due to Ca^{2+}-dependent inactivation. From our present recordings it is not possible to determine whether the single-channel currents are being carried by Ca^{2+}, gluconate$^-$ or Cl^-. For this purpose experiments need to be performed in asymmetric ionic solutions to determine the current's reversal potential and to obtain the P_{Ca}/P_{anion} ratio.

Both short openings, the amplitude of which was cut off by the filter (1 kHz), and long openings, lasting for the whole pulse duration of 500 ms, were observed. This is similar to results from ryanodine receptors in excised patches of blebs of skeletal muscle SR [10], which were recorded in a solution of identical ion composition. Different to those results is the main conductance level of about 65 pS in our recordings, which is near a major single-channel subconductance level (78 pS) of the measurements of Stein and Palade [10]. If the channel in our measurements can be confirmed to be RyR2, a possible reason for a subconductance level might be the type of tissue we used, namely atrial cells of patients suffering from coronary heart disease. We are going to investigate this rather interesting result in further experiments.

Our method should enable other groups, which are interested in various cardiac SR membrane proteins, to perform experiments on real native cardiac SR membrane.

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