

# Cryo-EM of the native structure of the calcium release channel/ryanodine receptor from sarcoplasmic reticulum

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**ABSTRACT** The native structure of the calcium release channel (ryanodine receptor) from rabbit skeletal muscle has been analyzed in two dimensions from electron micrographs of frozen hydrated specimens. Within a resolution of 3.0 nm there is excellent agreement between the structure as seen in vitreous water and in negative stained specimens. Features seen in the three-dimensional reconstruction of the negatively stained channel can be identified in the projection of the unstained receptor.

## INTRODUCTION

The calcium release channel is a major component of the triad junction that is located in the gap between the terminal cisternae region of the sarcoplasmic reticulum membrane and the transverse tubule, an invagination of the plasmalemma (reviewed by Fleischer and Inui, 1989). The calcium release channel is identical to the "foot" structure observed in electron micrographs of sarcoplasmic reticulum (Inui et al., 1987) and is believed to be the site where excitation-contraction coupling occurs, a process by which depolarization of the plasmalemma leads to opening of the calcium release channel and release of calcium into the cytoplasm.

The analysis presented here is a two-dimensional electron microscopical study of the isolated channel in frozen-hydrated preparation (Adrian et al., 1984) which is compared with the structural results obtained from specimens prepared in negative stain. Frozen-hydrated specimens are known to better preserve the native structure of the complex and offer the possibility of extending the resolution beyond the limitations imposed by the negative staining technique. The main difficulty in the electron microscopy of frozen hydrated specimens is their inherent low contrast and radiation sensitivity. To overcome these problems minimal dose electron microscopy and image-averaging techniques have been applied.

## MATERIALS AND METHODS

The calcium release channel from rabbit fast twitch skeletal muscle was purified (Inui et al., 1987a, b) from junctional terminal cisternae SR vesicles (Saito et al., 1984) with some modifications (Inui and Fleischer, 1988). The purified calcium release channel was stored frozen at  $-80^{\circ}\text{C}$  in a buffer containing 5 mM potassium phosphate, pH 7.4, 0.5M KCl, 2 mM dithiothreitol, 5 mg/ml CHAPS ([3-(3-

cholamidopropyl)-dimethylammonio]-1-propanesulfonate)), and 0.5  $\mu\text{g/ml}$  leupeptin.

For cryo-electron microscopy a solution of macromolecules was applied to a specimen grid (300 mesh copper coated with a thin carbon film supported by a thick holey carbon film), blotted to form a thin ( $\sim 0.1 \mu\text{m}$ ) film, and then frozen rapidly by plunging into liquid ethane, such that the water assumes a noncrystalline or vitreous state (Lepault et al. 1983; Milligan et al. 1984).

The results were compared with those obtained from negatively stained specimens. For these preparations the particles had been applied to grids using a sandwich technique (Stöffler-Meilicke and Stöffler, 1988). The negative stain was 0.5% uranyl acetate.

The specimen was loaded into the microscope using a Gatan 626 cryo-transfer system. Micrographs were recorded with minimal dose ( $10 \text{ el}/\text{\AA}^2$ ) on Kodak S0163 film (Eastman Kodak, Rochester, NY) and developed in full strength Kodak D19 developer for 12 min. The defocus value used was  $\sim 1.5 \mu\text{m}$ . For the underfocus value of  $1.5 \mu\text{m}$  the maximum frequency transfer occurs at  $\sim 3.5 \text{ nm}$  (100 kV,  $C_s = 2 \text{ mm}$ ) and the first zero transition is at a spatial frequency of 2.4 nm. Consequently, the micrograph of the frozen-hydrated specimen is expected to contain structural information to a limiting resolution of  $\sim 2.4 \text{ nm}$ .

In frozen hydrated preparations, as in the previously analyzed preparations in negative stain, the calcium release channel assumes a preferred orientation. Over 90% of all particles seen in the micrograph form a square shaped image in the plane of the specimen support. All particles show the same handedness, which indicates that only one side attaches to the supporting carbon. There are indications that the side attached to the carbon is the platform of the channel that in the sarcoplasmic reticulum is embedded into the membrane.

Averaging techniques were applied to obtain a virtually noise-free representative image of the calcium release channel. 320 particle images were extracted from the set of micrographs. The particles were translationally and rotationally aligned (Frank and Verschoor, 1981). The calcium release channel is known to be a homotetramer, which at least at low resolution exhibits a fourfold symmetry (Saito et al., 1988; Wagenknecht et al. 1989; Saito et al., 1989). Accordingly, the rotational cross-correlation function showed four peaks. Slight differences in the heights of the four peaks were attributed to noise, not to a significant deviation from the fourfold symmetry.

Few images were excluded visually because the particles shown were either obscured by a dust particle on the emulsion, did not show a complete particle, or visibly had not been aligned to the same orientation as the particle average. Correspondence analysis was

applied to the image set (Van Heel and Frank, 1981; Frank and van Heel, 1982), followed by classification, using a hybrid method (Lebart et al., 1984; Frank et al., 1987) that combines the K-means algorithm with hierarchical ascendant classification. If the channel would have lacked the postulated fourfold symmetry then the image set should be split by classification: namely into two classes if the symmetry was twofold, corresponding to two 90°-related images, or into four classes, corresponding to four 90°-related views, if no true symmetry was present. No such separation into different classes could be observed and it can be concluded that at least at the present level of resolution the calcium release channel has fourfold symmetry. The maps obtained from correspondence analysis were used to reduce the image set to a smaller set with less variability. Of the 320 images originally selected, 258 images remained for the calculation of the averages.

## RESULTS AND DISCUSSION

Close inspection of two micrographs (Fig. 1), one of a frozen hydrated preparation and one of a negatively stained specimen, shows that the calcium release channel images have similar morphologies for the two methods of specimen preparation, but the noise content and the variability among the images, especially for the low contrast frozen-hydrated specimen, severely limit the

amount of structural detail that can be interpreted. It should be noted that the contrast of the calcium release channel images is reversed for the frozen-hydrated relative to the negatively stained specimens. This reversal occurs because in the frozen-hydrated specimen the channel complexes are more dense than the surrounding ice, whereas for negative stain the enveloping stain is much more dense than the channel.

The resolution of the average image from the frozen hydrated preparation is limited to 2.4 nm by the transfer function of the microscope. The contrast transfer for specimens in vitreous water and specimens in negative stain differs at low spatial frequencies. The amount of amplitude contrast in frozen hydrated specimens is under discussion (Toyoshima and Unwin, 1988; Smith and Langmore, 1991, Quantitation of molecular densities by cryo-electron microscopy: determination of the radial density distribution of tobacco mosaic virus [submitted for publication]), and the contribution of inelastic scattering is much larger than for negatively stained specimens. However, because a study done earlier (Wagenknecht et al., 1988), where averages from negatively stained particles and particles in vitreous

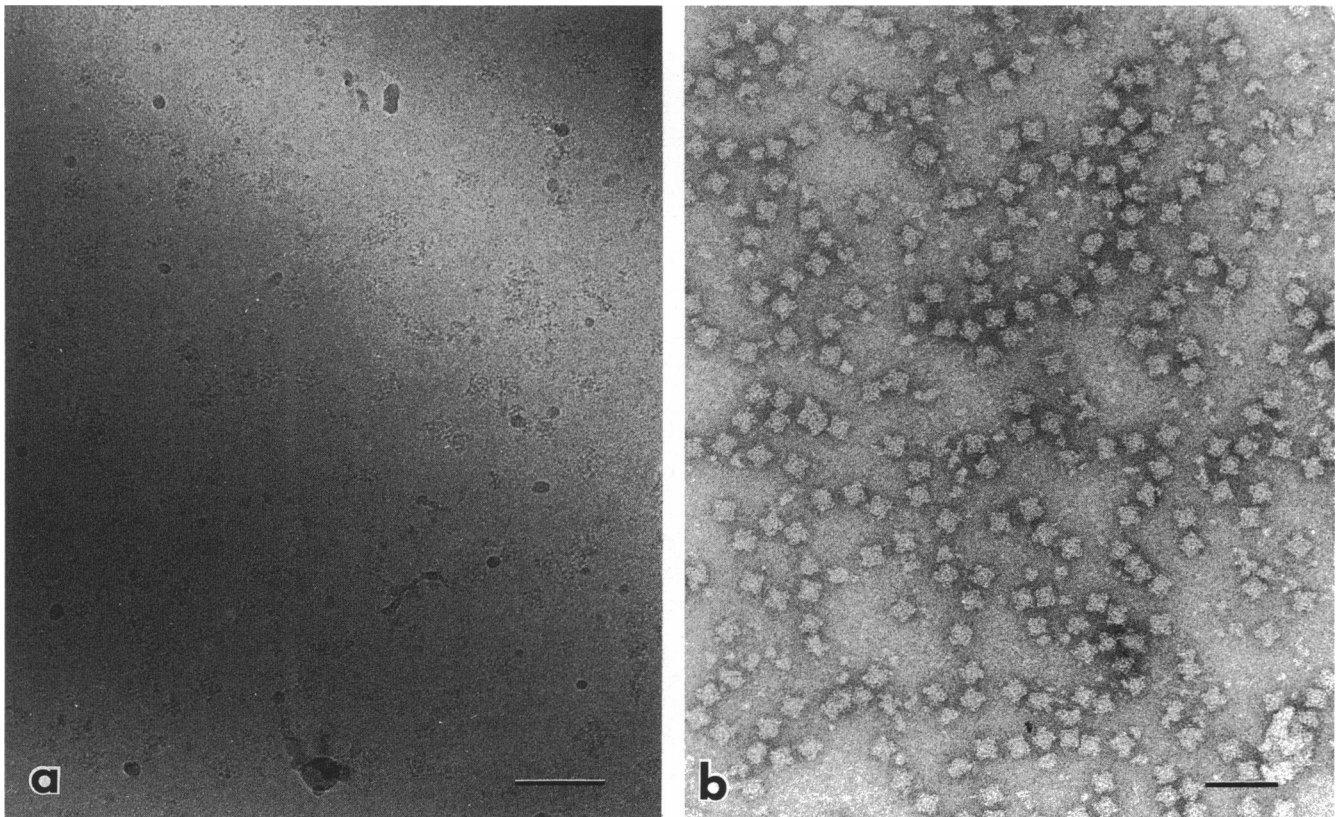


FIGURE 1 Portions of micrographs of the frozen-hydrated, non-stained (a), and negatively stained (b) isolated calcium release channels (scale bar, 100 nm).

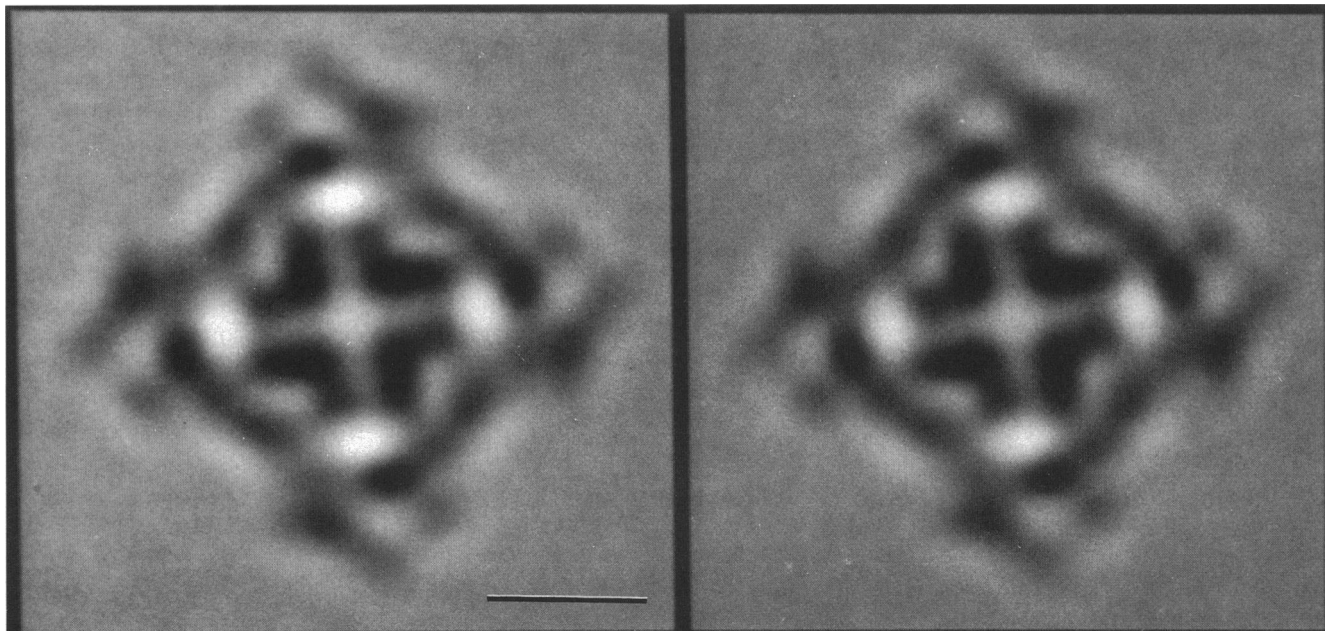


FIGURE 2 Two subaverages of the frozen hydrated calcium release channel calculated from 129 images each. Both averages are fourfold symmetrized (scale bar, 10 nm).

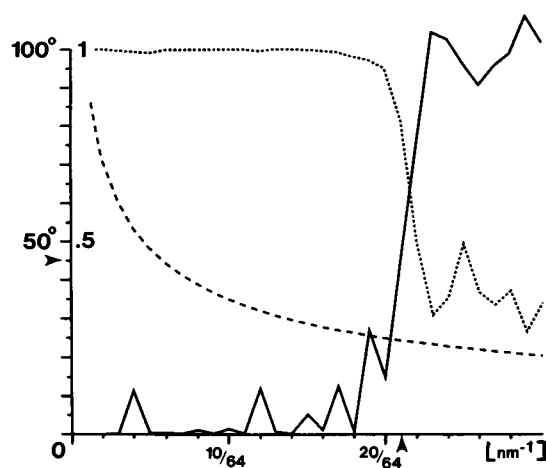


FIGURE 3 Curves showing the differential phase-residual (*solid line*) and Fourier ring-correlation (*small dashed line*) between the two averages shown in Fig. 2. (*Long dashed line*) Twice the value of the Fourier ring-correlation of two images containing pure noise. (y-axis) Phase-residual (from 0° to 100°), and Fourier ring-correlation (from 0 to 1). (x-axis) Radii of rings used for the comparison of the Fourier coefficients from both subaverages (Fig. 2) in reciprocal Fourier units of  $[\frac{1}{\text{nm}}]^{-1}$ . The resolution limit according to the 45° phase-residual criterion is found at 3.0 nm (arrows at 45° phase-residual value and  $\frac{2}{64} \text{ nm}^{-1}$  Fourier radius). The curve of the Fourier ring-correlation between the two subaverages does not intersect the curve of two times the noise ring-correlation within the Fourier radii shown here (maximum  $\frac{1}{2} \text{ nm}^{-1}$ ); thus, the resolution according to the Fourier ring-correlation criterion is better than 2 nm.

water were compared in a series of different band-pass filtrations, did not show significant differences in the appearance of the averages from the two different preparations, and because the average calculated here is in no contradiction to the average image of the calcium release channel prepared in negative stain, no correction for the transfer function at low spatial frequencies has been attempted.

Three averages were computed from the 258 images, two each from half the particle set and one from the complete set. The averages were fourfold symmetrized to increase the signal to noise ratio (Fig. 2). The two subaverages were used to determine the resolution by the 45° differential phase-residual criterion (Frank et al., 1981) and the Fourier ring-correlation criterion (Saxton and Baumeister, 1982; van Heel et al., 1982; Radermacher, 1988) (Fig. 3).<sup>1</sup> The resolution according to the

<sup>1</sup>For the determination of the reproducible resolution two subaverages formed from two mutual exclusive subsets of one data set are compared. The equation for the phase-residual is:

$$\Delta\theta = \left[ \frac{\sum (|F_1| + |F_2|)(\delta\theta)^2}{\sum (|F_1| + |F_2|)} \right]$$

where  $F_1$  and  $F_2$  are the Fourier coefficients in the Fourier transforms of image 1 and image 2, respectively, and  $\delta\theta$  is the phase difference between each two coefficients. The summation is carried out over all coefficients on rings at increasing Fourier radii. The limit of the resolution is determined by the Fourier radius at which the phase

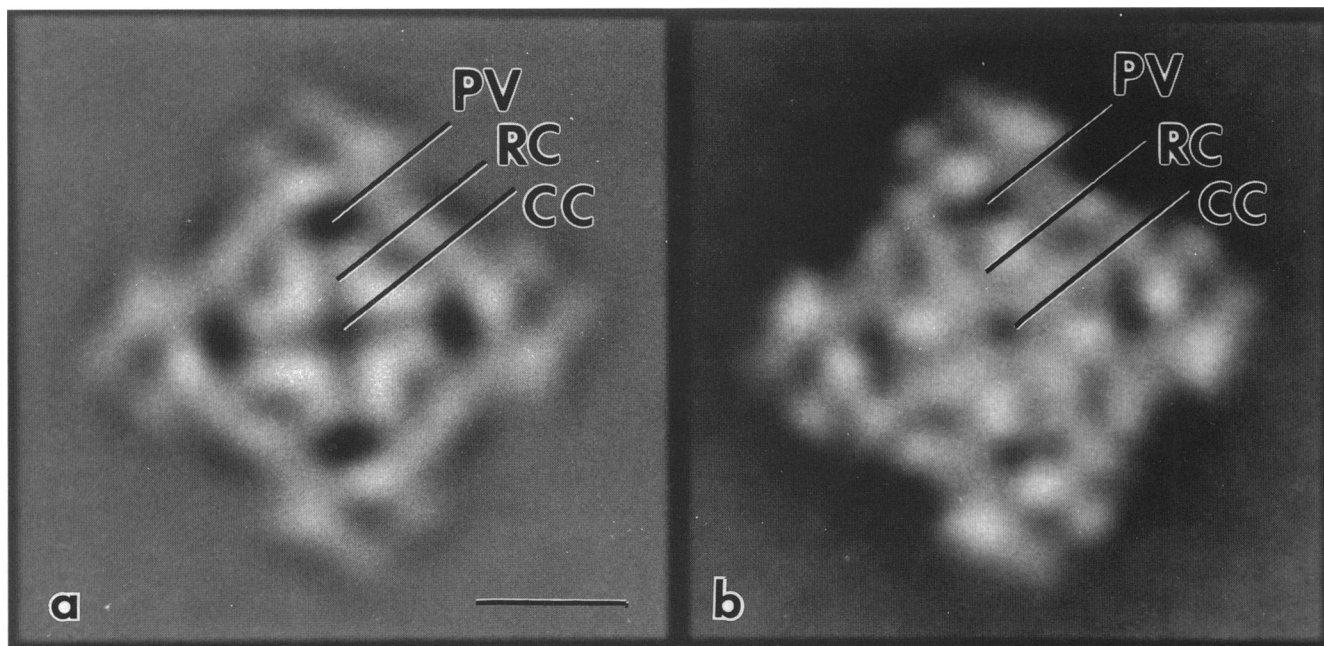


FIGURE 4 Results of averaging images of (a) frozen-hydrated, and (b) negatively stained calcium release channels. The average in *a* is calculated from all 258 images and fourfold symmetrized. The average in *b* is calculated from 280 images. (Resolutions) (a) 3 nm, (b) 2.5 nm. The contrast of the average of the frozen hydrated specimen has been reversed to match the contrast of the negative stain average. Labeled are some of the features that can be clearly recognized in the projection of frozen hydrated calcium release channels and in negative stain preparation. CC, central cavity; RC, radial canals; PV, peripheral vestibules (see also Fig. 5) (scale bars, 10 nm).

phase-residual criterion was 3.0 nm (better than 2 nm according to the Fourier ring-correlation criterion, given only for comparison) and is lower than that of the negative stain average (2.5 nm) obtained earlier (Saito et al., 1988) (Fig. 4). This result was not unexpected because of the degree of underfocus used to record the micrograph of the frozen-hydrated calcium release channels. However, the averages from frozen-hydrated specimens and those obtained from negatively stained particles agree well with each other.

Averages represent the structure as a projection in the

residual reaches 45°. The formula for the Fourier ring-correlation is:

$$\text{FRC} = \frac{\sum F_1 \cdot F_2^*}{\sum (|F_1|^2 \cdot |F_2|^2)^{1/2}}$$

As in the calculation of the phase-residual the sums are carried out over Fourier coefficients on rings at increasing radii. The resolution limit is determined to be at the radius where the Fourier ring correlation falls below twice the value that would be expected from a pair of images containing pure noise, which function is  $2/\sqrt{N}$ , where  $N$  is the number of points on the ring. The phase-residual criterion leads to a resolution value that shows details in the image where signal and noise have approximately the same strength. The resolution according to the Fourier ring-correlation normally is more optimistic, and details visible at that resolution may contain a much larger portion of noise.

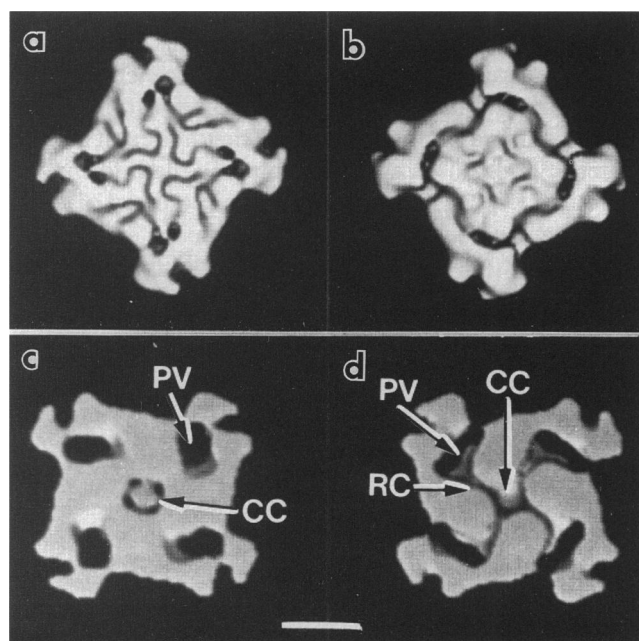


FIGURE 5 Three-dimensional reconstruction of the calcium release channel obtained from negatively stained specimens (Wagenknecht et al., 1989). Labeled are the central cavity (CC), the radial canal (RC), and the peripheral vestibules (PV). (a, b) Top and bottom faces; (c, d) reconstruction sliced open to reveal internal structure.

direction normal to the plane of the image. Many of the structural details present in the negatively stained average have been correlated with structural features observed in the three-dimensional reconstruction (Wagenknecht et al., 1989) (Fig. 5). Thus, in Fig. 4 we have labeled in the averaged images those features that correspond to the central cavity (CC), radial channels (RC), and peripheral vestibules (PV). Note that these same features are also clearly present in the frozen-hydrated average.

The agreement between the averages also indicates that the three-dimensional reconstruction calculated earlier from a negative stain preparation is most probably a faithful representation of the native structure of the calcium release channel within the resolution limit used here (3 nm).

## CONCLUSION

Cryo-electron microscopy of nonstained, frozen-hydrated calcium release channel and image analysis (two-dimensional) show an overall agreement with results obtained by the negative stain technique. In ice, as in negative stain, the calcium release channel exhibits the same preferred orientation. No conformational differences could be observed between the two preparations. Thus, the three-dimensional reconstruction computed from negatively stained specimens is probably representative of the native calcium release channel. In order to obtain higher resolution in the future, we plan to record micrographs of each specimen area at several values of underfocus and then combine the information from such a focal series. Because the preferred orientation is preserved, it also should now be feasible to compute a three-dimensional reconstruction from electron micrographs of nonstained frozen-hydrated calcium release channel.

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