Biochimica et Biophysica Acta, 1064 (1991) 89–102

ADONIS 000527369100177T

BBAMEM 75203

Functional characterisation of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum

Allan R.G. Lindsay and Alan J. Williams

Department of Cardiac Medicine, National Heart and Lung Institute, University of London, London (U.K.)

(Received 10 August 1990)

Key words: Sarcoplasmic reticulum; Ryanodine receptor; Calcium-release channel; (Heart)

Sheep cardiac muscle sarcoplasmic reticulum ryanodine receptors have been isolated by density-gradient centrifugation following solubilisation with the zwitterionic detergent, CHAPS. The functional state of the receptor complex has been assessed by quantification of [^{3}H]ryanodine binding and by characterisation of single-channel conductance and gating properties following reconstitution into unilamellar proteo-liposomes and incorporation into planar phospholipid bilayers. A method of solubilisation is described which yields a receptor displaying high-affinity [^{3}H]ryanodine binding (K_d 2.8 nM, B_{max} 352 pmol/mg protein) and which functions as a cation-selective, ligand-regulated channel under voltage clamp conditions. Previous reports of channel activity of purified rabbit skeletal and canine cardiac muscle ryanodine receptors describe a range of sub- or variable-conductance events. In contrast, the sheep cardiac ryanodine receptor-channels isolated using the optimal conditions described in this report consistently display a single open state conductance with either Ca^{2+} or K^+ as the charge carrying species.

Introduction

Contraction of muscle cells is initiated by an elevation of the cytosolic Ca^{2+} concentration. In striated muscle, the bulk of this Ca^{2+} is released from an internal membrane store, the sarcoplasmic reticulum (SR), in response to the depolarisation of the cell membrane or sarcolemma. The mechanisms linking excitation to Ca^{2+} release are believed to be somewhat different in cardiac and skeletal muscle [1–4]. However, in both muscle types Ca^{2+} release from the SR is mediated by a specific membrane channel protein located in specialised junctional regions of the SR membrane network [5]. The properties of the Ca^{2+}-release channel have been investigated at a macroscopic level by following uni-directional $^{45}Ca^{2+}$ efflux from isolated SR membrane vesicles [6–11] and at the single-channel level, under voltage clamp conditions, following the incorporation of isolated SR vesicles into planar phospholipid bilayers [12–18]. The Ca^{2+}-release channels of both cardiac and skeletal muscle display properties consistent with them playing a rôle in excitation-contraction coupling. Channel open probability (P_o) is increased by physiological and pharmacological activators of Ca^{2+} release (Ca^{2+} [12,13,18,19], ATP [12,13,17], anthraquinones [20–22] and caffeine and related compounds [15,23–25]). Whilst P_o is reduced by substances known to inhibit Ca^{2+} release from the SR (Mg^{2+} [13,18], ruthenium red [12,17] and calmodulin [26]). SR Ca^{2+} release [10,27] and single-channel properties [16,28] are also modified by the plant alkaloid ryanodine which disrupts excitation-contraction coupling in both forms of striated muscle [29,30]. Ryanodine binds specifically, and with high affinity, to a receptor in the junctional regions of the SR membrane network [27,31–35] and the availability of [^{3}H]ryanodine has made possible the purification of the receptor proteins from both skeletal and cardiac muscle SR. The ryanodine receptor protein has been isolated from rabbit skeletal muscle SR by a number of groups using a range of solubilisation and separation techniques [32,36–40]. Similar procedures have also been employed for the isolation of the ryanodine receptor of canine cardiac muscle SR membranes [36,41,42].

Purification of the receptor proteins has permitted detailed structural [37,39,43–46] and biochemical [47]

Correspondence: A. Williams, Department of Cardiac Medicine, National Heart and Lung Institute, University of London, Dovehouse Street, London SW3 6LY, U.K.

investigations of the proteins and it is now established that the high-affinity ryanodine-binding complex consists of a 30 S homotetramer and is equivalent to the feet structures which span the sarcolemmal-SR junction in intact muscle [37,44,46]. cDNAs encoding the rabbit [48–50] and human [50] forms of the skeletal muscle SR ryanodine receptor have been cloned and sequenced, and functional expression of ryanodine-binding [48] and cation conductance [51] have been obtained in a hamster ovary cell line. Less information is available concerning the primary structure of the cardiac ryanodine receptor. However, it is clear that differences do exist between the receptor proteins found in the two forms of striated muscle. Although there is extensive sequence homology, the genes encoding the two forms are located on different chromosomes and expression is tissue specific [52]. Immunological investigations demonstrate no cross reactivity between monoclonal antibodies raised to the canine cardiac ryanodine receptor protein and skeletal SR membranes [53].

Despite the burgeoning structural details of the ryanodine receptor, only limited information is available concerning the functional aspects of the complex as a ligand-regulated ion channel. The studies that have been undertaken reveal considerable differences between the conductance properties of the various purified proteins. Three groups have demonstrated cation-selective, ligand-regulated channel activity following incorporation of rabbit skeletal muscle ryanodine-binding proteins into planar phospholipid bilayers [38–40,54–57]. All three groups have reported the common occurrence of sub- or variable-conductance states, with one group obtaining unit conductance values of between 4 and > 120 pS [54]. Similar multiple-conductance levels have been reported for purified canine cardiac ryanodine receptor proteins [45,58].

Isolation of the ryanodine receptor-channel from the SR membrane and hence from accompanying K^+ and Cl^- channel proteins, provides considerable advantages for the investigation of ion discrimination. Our initial attempts to apply the method devised by Lai et al. [41] for the purification of the canine cardiac receptor, to sheep cardiac SR proved unsuccessful; yielding poorly-selective, multiple-conductance level channels on reconstitution. In this report we describe a minor modification of the CHAPS solubilisation and density-gradient centrifugation procedures described by Lai et al. [41] which, when applied to sheep cardiac SR membranes, yields high-affinity [^{3}H]ryanodine binding proteins which function as ligand-regulated, cation-selective channels displaying a single open conductance level. We propose that this corresponds to the unit conductance of the receptor-channel complex and that previously reported multiple-conductance states reflect modification of the complex during solubilisation and reconstitution.

Experimental Procedures

Materials. [^{3}H]Ryanodine was purchased from New England Nuclear Ltd. Unlabelled ryanodine was purchased from Progressive Agri Systems, U.S.A. Phosphatidylethanolamine was purchased from Avanti Polar Lipids, U.S.A. Phosphatidylcholine (commercial grade) was purchased from Sigma Ltd. Aqueous counting scintillant was purchased from Amersham International. All other chemicals were of AnalaR or best available grade from Aldrich Ltd, BDH Ltd., or Sigma Ltd.

Preparation of junctional/heavy sarcoplasmic reticulum membrane vesicles (HSR). Sheep hearts were obtained from a local abattoir and transported to the laboratory in ice-cold cardioplegic solution [59]. HSR membrane vesicles were isolated from septal and left ventricular free wall tissue as previously described [24]. A mixed membrane fraction obtained following homogenisation and differential centrifugation was further fractionated by discontinuous sucrose density centrifugation. HSR membrane vesicles were collected from the 30/40% (w/v) interface, suspended in 0.4 M KCl and sedimented by centrifugation at 36 000 rpm (100 000 × g_{av}) for 60 min in a Sorvall A641 rotor. The resulting pellet was resuspended in a solution containing 0.4 M sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) titrated to pH 7.2 with tris(hydroxymethyl)methylamine (Tris), snap frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined using assays described by either Markwell et al. [60] or a Coomassie brilliant blue binding assay (Bio-Rad, Herts., U.K.) with bovine serum albumin as standard.

[^{3}H]Ryanodine binding. [^{3}H]Ryanodine binding to solubilised HSR membranes and isolated receptor was performed using a modification of the method for [^{3}H]ryanodine binding to intact HSR vesicles described by Holmberg and Williams [35]. Solubilised HSR membrane vesicles or isolated receptors were incubated with various concentrations of [^{3}H]ryanodine, in a solution containing 1 M KCl, 100 μM $CaCl_2$, 5 μM phenylmethylsulphonyl fluoride (PMSF), 25 mM piperazine-*N*,*N'*-bis(2-ethanesulphonic acid) (Pipes)-KOH (pH 7.4), in 1 ml total volume. After 60 min incubation at 37°C the medium was diluted with 5 ml ice-cold incubation solution and filtered through Whatman GF-B filters which had been pre-soaked in 5% polyethylimine and washed with 3 × 5 ml ice-cold deionised H_2O. The tube was then rinsed with a further 2 × 5 ml of ice-cold incubation solution and its contents passed through the filter. Following transfer to scintillation vials, 10 ml of scintillant was added to the filters which were allowed to stand overnight before counting. Non-specific binding was determined by carrying out binding assays in the presence of excess unlabelled ryanodine (2.5 μM). All incubations were performed in triplicate. Specific

binding to the isolated receptor was characterised in the presence of between 0.5 and 20 nM ^{3}[H]ryanodine. Values of K_d and B_{max} were obtained using a non-linear regression fitting procedure (Enzfitter, Biosoft, Cambridge, U.K.).

Solubilisation of the ryanodine receptor. The method of solubilisation of the sheep cardiac muscle HSR ryanodine receptor, described in this report, is based upon the method described by Lai et al. [41] for the purification of the canine cardiac muscle ryanodine receptor. We undertook a series of experiments designed to produce optimal conditions for solubilisation of the sheep cardiac ryanodine receptor with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate (CHAPS); that is, maximal solubilisation of the receptor with maximal retention of binding activity. Frozen HSR membrane vesicles were stored at −80°C overnight before suspension in buffer A (1 M NaCl, 0.1 mM ethyleneglycol bis(β-aminoethyl ether)tetraacetic acid (EGTA), 0.15 mM $CaCl_2$, 25 mM Pipes-NaOH (pH 7.4)) at a final concentration of between 0.3 and 1.5 mg protein/ml. Solubilisation was initiated by the addition of various volumes of a stock solution containing 10% CHAPS and 50 mg/ml L-α-phosphatidylcholine (PC) to give final CHAPS concentrations of between 0.5% and 2.0%. The vesicles were then incubated on ice for 1 hour; at the end of this period unsolubilised material was sedimented by centrifugation for 45 min at 36000 rpm (100000 × g_{av}) in a Sorvall T-875 rotor. [^{3}H]Ryanodine binding to aliquots of the solubilised material was determined as described above at a concentration of 5 nM.

Separation of the ryanodine receptor. The sheep cardiac HSR ryanodine binding protein was separated from other solubilised membrane components by sedimentation on continuous sucrose density gradients, essentially as described by Lai and colleagues [41]. Solubilised material was placed at the top of a 5–25% (w/v) linear sucrose gradient in buffer A supplemented with 0.5% CHAPS and 2.5 mg/ml PC, formed above a 40% sucrose cushion. Material was sedimented overnight at 28000 rpm (100000 × g_{av}) in a Sorvall AH-629 rotor. Gradient fractions were collected in 2-ml. volumes drawn from the base of the tube. Fractions containing the receptor were identified by comparison with an identical gradient which contained material incubated in the presence of 5 nM [^{3}H]ryanodine during the solubilisation period. Peak fractions (100 μl aliquots) were subjected to SDS-polyacrylamide gel electrophoresis on 5–12% gels prior to silver staining.

Reconstitution of receptor into liposomes. For single-channel conduction and gating studies, the solubilised receptor was reconstituted into unilamellar liposomes by dialysis overnight with 4 volumes of buffer A containing 0.5% CHAPS and 2.5 mg/ml PC, against 1 litre of 0.1 M NaCl, 0.1 mM EGTA, 0.15 mM $CaCl_2$, 25 mM Pipes-NaOH (pH 7.4) with four changes of solution. At the end of dialysis, an equal volume of 0.4 M sucrose was added to the vesicles before snap freezing in liquid nitrogen and storage at −80°C. The experiments reported here were carried out using proteo-liposomes derived from six solubilisations taken from five HSR membrane preparations.

Planar lipid bilayer methods. Planar lipid bilayers were formed from suspensions of phosphatidylethanolamine (PE) in decane (35 mg/ml). Bilayers were painted across a 200 μm diameter hole in a polystyrene partition separating two fluid filled compartments, referred to as the *cis* chamber (volume 0.5 ml) and the *trans* chamber (1.0 ml). The *trans* chamber was held at virtual ground and the *cis* chamber could be clamped at various holding potentials relative to ground. Current flow through the bilayer was measured using an operational amplifier as a current-voltage converter as described by Miller [61]. During bilayer formation both the *cis* and *trans* chambers contained 210 mM KCl, 20 mM Hepes-KOH (pH 7.4). An osmotic gradient was established by the addition of 100 μl of 3 M KCl to the *cis* chamber. Proteo-liposomes were added to the *cis* chamber and stirred, whereupon a second aliquot of 3 M KCl was added to the *cis* chamber to induce fusion of vesicles with the bilayer. On incorporation of a channel(s) further incorporation was prevented by perfusion of the *cis* chamber with 210 mM KCl, 20 mM Hepes-KOH (pH 7.4). For experiments in which Ca^{2+} was used as the permeant ion, purified receptors were incorporated as described above, before perfusion of the *trans* chamber with a solution containing 250 mM glutamic acid titrated to pH 7.4 with $CaOH_2$ (free Ca^{2+}, 63 mM) and the *cis* chamber with a solution containing 250 mM Hepes, 125 mM Tris (pH 7.4) which contained a free Ca^{2+} concentration of 10 μM. Native sheep HSR Ca^{2+} channel activity was monitored in these solutions following the incorporation of HSR vesicles as described previously [18,62]. Free Ca^{2+} concentrations of all solutions were determined using a Ca^{2+} selective electrode (Orion 93-20). All experiments were performed at room temperature (23 ± 1°C).

Single-channel data acquisition and analysis. Single-channel current fluctuations were displayed on an oscilloscope and stored on either FM or video recording tape. For analysis, data were replayed, low pass-filtered using an 8-pole Bessel filter (Frequency Devices 902) at 1.0–4.0 kHz and digitised at 2.0–8.0 kHz using either an AT-based system (Intracel, Cambridge, U.K.) or a PDP 11/73 based system (Indec, Sunnyvale, CA, U.S.A.). Single-channel current amplitudes were obtained from digitised data. Open probabilities (P_o) and open and closed lifetimes were determined by 50% amplitude threshold analysis. Lifetimes accumulated over 3-min periods, under steady-state conditions, were

stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to probability density functions using the method of maximum likelihood [63]. With Ca^{2+} as the permeant ion, events with durations < 1 ms were not fully resolved and were therefore excluded from the fitting procedure. A missed events correction was employed [63] and a likelihood ratio test was used to compare fits to multiple exponentials [64].

Results

Receptor purification

Influence of CHAPS and protein concentrations on [^{3}H]ryanodine binding and receptor solubilisation. All previous reports on the purification of the ryanodine receptor from cardiac muscle have used canine heart as starting material [36,41,42,45]. Consequently, we carried out a preliminary investigation aimed at determining the optimal conditions for [^{3}H]ryanodine binding and solubilisation of the receptor from sheep cardiac muscle. Specific [^{3}H]ryanodine binding, following incubation with CHAPS, was influenced by the concentration of membrane protein incubated with the detergent (data not shown). By using a final protein concentration of 1.5 mg/ml, we observed [^{3}H]ryanodine binding levels of approx. 90% of control values determined in the absence of added CHAPS, at detergent/lipid concentrations ranging from (%CHAPS/mg PC per ml) 0.5%/2.5 mg per ml to 2.0%/10 mg per ml (Fig. 1). The degree of receptor solubilisation was determined at CHAPS concentrations ranging from 0.5 to 2.0% with a final protein concentration of 1.5 mg/ml. Following incubation with CHAPS, unsolubilised membrane com-

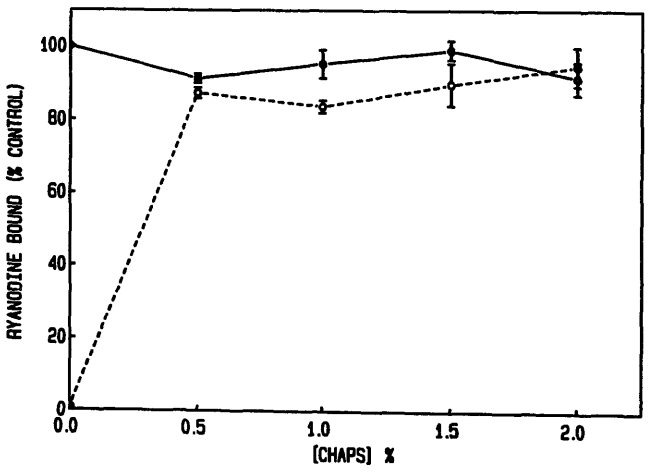

Fig. 1. Influence of CHAPS on ^{3}[H]ryanodine binding to HSR. Binding is expressed as % of control values obtained with intact HSR in the absence of CHAPS. ●, Binding to HSR in the presence of increasing concentrations of CHAPS. ○, Binding to solubilised fraction of HSR following incubation with increasing concentrations of CHAPS. Experimental details are given in Methods. Points are means of nine experiments ± S.E.

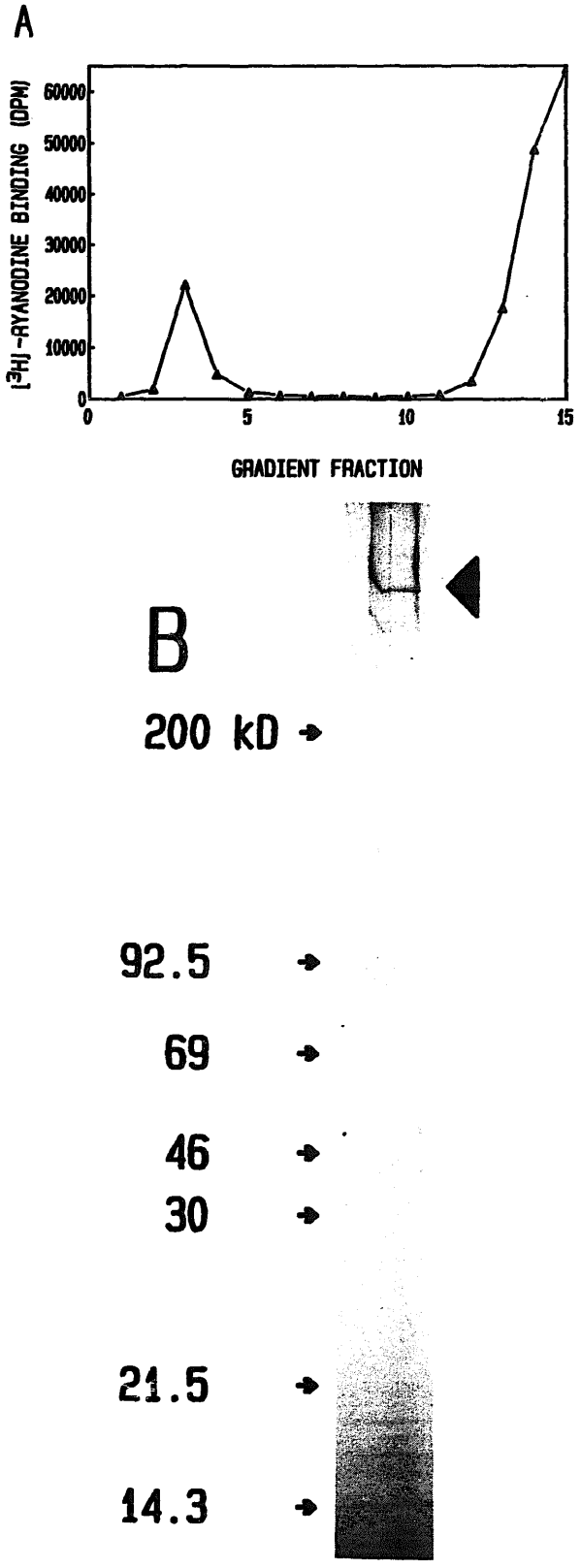

Fig. 2. (A) Solubilisation profile of ^{3}[H]ryanodine binding protein on a continuous 5–25% sucrose density gradient following centrifugation at 100000 × g_{av} for 16 h. A single peak was obtained at approx. 22% sucrose. Unbound ^{3}[H]ryanodine remained at the top of the gradient (fractions 12–15). (B) Silver stained SDS-polyacrylamide gel of a ^{3}[H]ryanodine binding fraction. A major component with an estimated molecular mass of approx. 350000 daltons is indicated. Positions of molecular mass markers are shown to the left of the gel.

ponents were sedimented by centrifugation and [^{3}H]ryanodine binding to the soluble fraction was determined as described in Methods. The data presented in Fig. 1 demonstrate that essentially complete solubilisation of the receptor was achieved in the presence of 0.5% CHAPS. Based on the data presented in Fig. 1, we used a protein concentration of 1.5 mg/ml and a CHAPS concentration of 0.5% for all subsequent solubilisation and purification procedures.

Purification of the ryanodine receptor by sucrose density-gradient centrifugation

The sheep cardiac mu cle ryanodine receptor was separated from other solubilised HSR membrane components by sedimentation on continuous sucrose density-gradients as described by Lai et al. for the canine cardiac muscle receptor [41]. Sedimentation of [^{3}H]ryanodine-labelled solubilised membrane fractions produced a single peak at approx. 22% sucrose (Fig. 2A), yielding approx. 20 μg protein from an original 3 mg HSR protein. Polyacrylamide gel electrophoresis profiles of the peak fractions are shown in Fig. 2B. Fractions contain a major protein component with an apparent molecular weight of approx. 350000.

Functional assessment

[^{3}H]Ryanodine binding to the purified receptor. [^{3}H]Ryanodine binding to the unlabelled purified receptor fraction was investigated at ryanodine concentrations ranging from 1 to 20 nM (Fig. 3). A Scatchard plot of the data (inset to Fig. 3) provides a K_d of 2.8 nM and a B_{max} of 352 pmol/mg protein. Comparison of these values to those determined in an earlier study for binding of [^{3}H]ryanodine to native sheep cardiac muscle HSR vesicles (K_d 1.4 nM, B_{max} 9.7 pmol/mg protein) [35] suggests that the binding activity of the receptor is well preserved during solubilisation and that the receptor is significantly enriched following sucrose density gradient centrifugation.

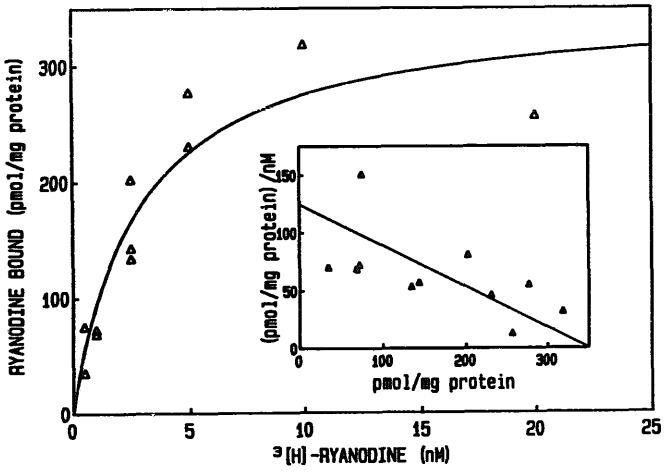

Fig. 3. Specific binding of ^{3}[H]ryanodine to the isolated sheep cardiac HSR receptor. The data were obtained using binding to 0.6–1 μg of receptor protein taken from four different solubilisation and isolation procedures. The inset shows a Scatchard plot of the data. In both plots the solid lines are drawn with a K_d of 2.8 nM and B_{max} of 352 pmol/mg protein. These values were obtained from non-linear regression analysis of the data (see Methods for details).

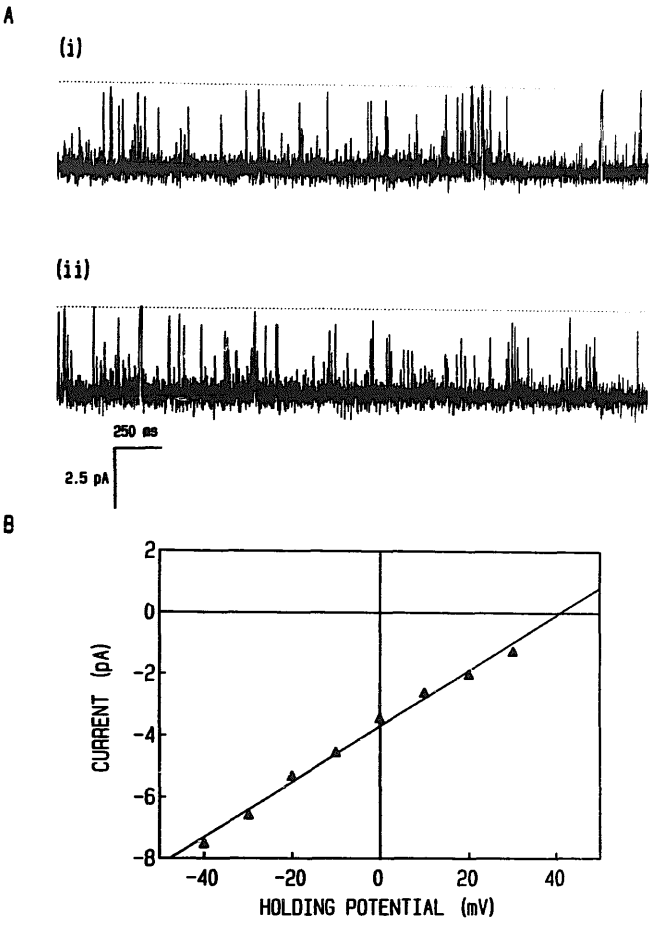

Fig. 4. (A) Single-channel current fluctuations of the reconstituted, isolated sheep cardiac HSR ryanodine receptor with Ca^{2+} as the permeant ion (details of the solutions are given in the text). Traces i and ii show a continuous recording at a holding potential of 0 mV. The open level of the channel is indicated by a dotted line. (B) Single-channel current-voltage relationship for an isolated sheep cardiac HSR receptor-channel with Ca^{2+} as the permeant ion. The slope conductance obtained from the plot is 90.4 pS and the reversal potential is 40.4 mV.

Channel conductance and gating. The purified ryanodine receptor proteins of skeletal and canine cardiac muscle HSR have been shown to function as cation-selective channels following their reconstitution into planar phospholipid bilayers [39,40,55,56]. Similarly, the purified sheep cardiac muscle HSR ryanodine receptor displays characteristic conductance and gating properties consistent with its rôle as the pathway for Ca^{2+} release from the SR membrane network during excitation-contraction coupling. Fig. 4 shows typical single-channel current fluctuations of the purified sheep cardiac muscle ryanodine receptor incorporated into a PE planar bilayer following reconstitution into PC liposomes. In this experiment Ca^{2+} is the permeant ion from the *trans* side of the bilayer and the channel is

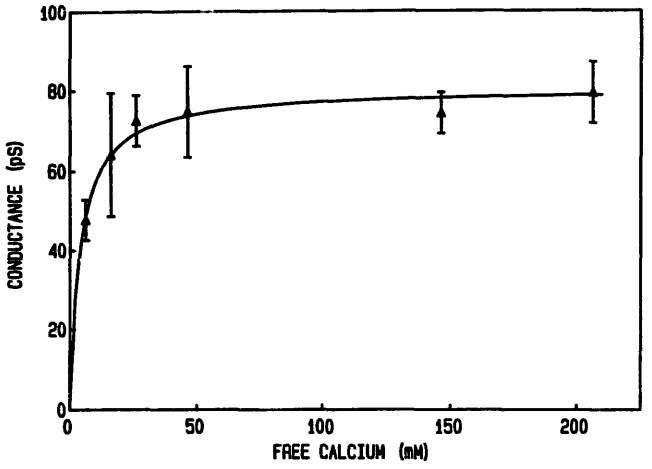

Fig. 5. Saturation of single-channel conductance with increasing free Ca^{2+} concentration. Slope conductance was monitored at *trans* free Ca^{2+} concentrations ranging from 6.3 to 206.3 mM. The data were fitted to a Michaelis-Menten scheme using non-linear regression (Enzfitter). The solid line is drawn with values obtained from this analysis: maximal conductance (80.3 pS) with half-maximal conductance achieved at a free Ca^{2+} concentration of 4.11 mM.

activated by 10 μM Ca^{2+} at the *cis* side of the membrane (details of ionic conditions are given in Methods); the holding potential is 0 mV. The conditions used in this experiment are identical to those which we have used previously to investigate conduction and gating behaviour of the native sheep cardiac SR calcium-release channel [18,24,25,62]. An inspection of the single-channel current-voltage relationship of the channel shown in Fig. 4A provides information on the slope conductance of the channel and the relative permeability of the channel to Ca^{2+} and the only other permeant ion in the system; $Tris^+$. The slope conductance obtained from the plot in Fig. 4B is 90.4 pS and the reversal potential is 40.4 mV which corresponds to a $Ca^{2+}/Tris^+$ permeability ratio [12] of 15.3. Both these values are in good agreement with those obtained by us for the native sheep cardiac SR Ca^{2+}-release channel [24,25].

Single-channel conductance of the purified sheep cardiac SR Ca^{2+}-release channel saturates as the free Ca^{2+} concentration on the *trans* side of the membrane is increased (Fig. 5, each point is the mean $\pm$ S.D. $n \geq 5$). In these experiments, single-channel slope conductance was determined with 10 μM Ca^{2+}, 250 mM Hepes, 125 mM Tris (pH 7.4) on the *cis* side of the membrane and 300 mM mannitol, 10 mM Hepes titrated with $CaOH_2$ to give a pH of 7.4, plus various amounts of $CaCl_2$ to give free Ca^{2+} concentrations of between 6.3 and 206.3 mM on the *trans* side of the membrane. The line drawn through the points in Fig. 5 is a Michaelis-Menten saturation curve fitted by non-linear regression, with a maximal conductance of 80.3 pS and a half-maximal conductance achieved at a free Ca^{2+} concentration of 4.11 mM.

The purified rabbit skeletal and canine cardiac muscle ryanodine receptors have been shown to function as

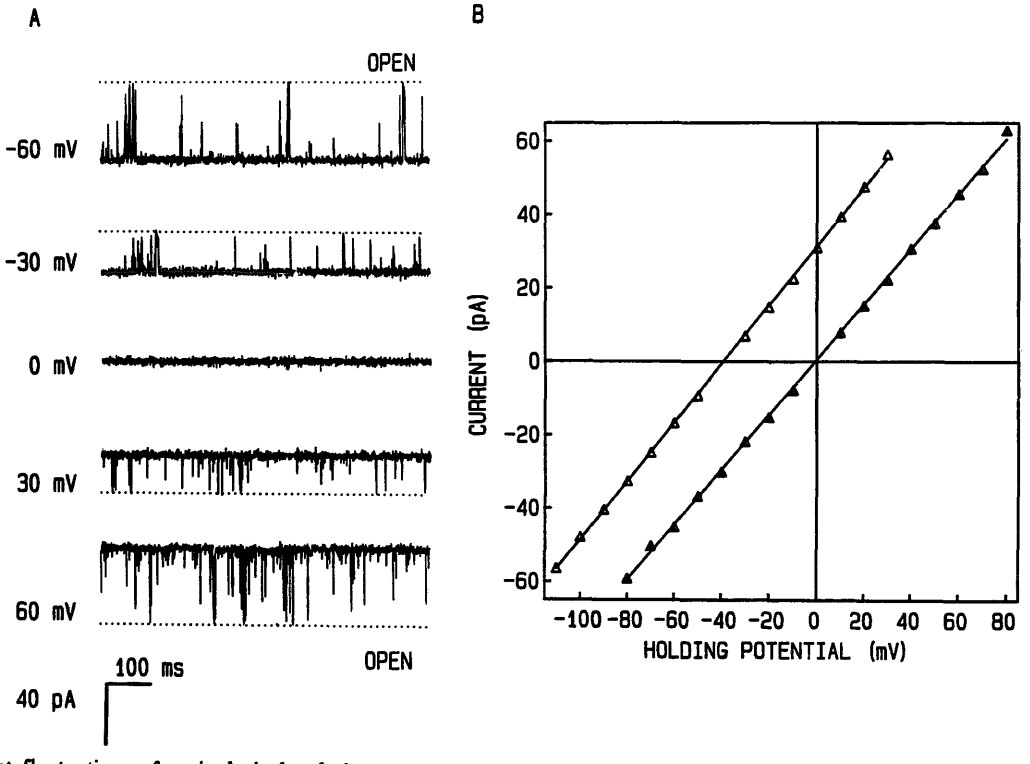

Fig. 6. (A) Current fluctuations of a single isolated sheep cardiac HSR receptor-channel in symmetrical 210 mM K^+ at a range of holding potentials. (B) Single-channel current-voltage relationships for a single isolated receptor-channel in symmetric (210 mM, ▲) and asymmetric (840 mM *cis*, 210 mM *trans*, △) KCl. Slope conductance and reversal potential values are given in the text.

variable-conductance channels when monovalent cations are used as the permeant species [39,40,45,55]. Fig. 6A shows typical single-channel current fluctuations of a purified sheep cardiac HSR ryanodine receptor at a range of holding potentials with 210 mM K^+ (200 mM KCl, 10 mM Hepes, 10 mM KOH, pH 7.4) on both sides of the bilayer. Under these, and all other conditions examined, the sheep cardiac receptor-channel consistently displays a single open-conductance state. Open events not attaining the full open level in this illustration represent brief events, the amplitudes of which are attenuated by the bandwidth limitations of the recording equipment. The single-channel current-voltage relationship for this channel is shown in Fig. 6B, together with a current-voltage relationship obtained with a 4:1 KCl gradient imposed across the bilayer (*cis*: 840 mM, *trans*: 210 mM). In both cases a linear relationship is observed, with a slope conductance of 730 pS in the symmetric solutions and 800 pS with the asymmetric solutions. The reversal potential of 39 mV seen with a 4:1 KCl gradient is indistinguishable from that predicted from the Nernst equation for an ideally K^+ selective system under these conditions, indicating that the channel is totally impermeant to Cl^-.

The relative permeability of the purified sheep cardiac HSR ryanodine receptor-channel to monovalent and divalent cations can be assessed by monitoring conductance with K^+ as the permeant ion on the *cis* side of the membrane and Ca^{2+} as the permeant ion on the *trans* side of the membrane. The single-channel current-voltage plot shown in Fig. 7 was obtained with 210 mM K^+ (200 mM KCl, 20 mM Hepes, 10 mM KOH, pH 7.4) *cis* and 210 mM Ca^{2+} (200 mM $CaCl_2$, 40 mM Hepes, 10 mM $CaOH_2$, pH 7.4) *trans*. Under these conditions, a reversal potential of +40 mV is obtained, yielding a Ca^{2+}/K^+ permeability ratio of 7.4 [12,13].

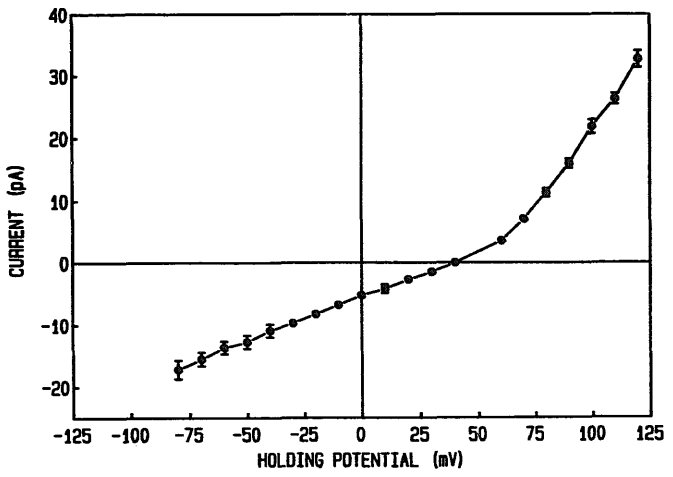

Fig. 7. Single-channel current-voltage relationship for single isolated receptor-channels with 210 mM K^+ *cis* and 210 mM Ca^{2+} *trans*. Points represent mean values ± S.D. for six experiments. Currents fluctuations reversed at 40 mV yielding a Ca^{2+}/K^+ permeability ratio of 7.4 (see text for details).

A number of physiological and pharmacological agents have been shown to influence the permeability of isolated skeletal and cardiac muscle HSR membrane vesicles to Ca^{2+} [7–11,19,27]. Consistent with these observations, the open probabilities of Ca^{2+}-release channels of HSR of both species of muscle are similarly regulated, and recent reports suggest that the modulatory effects of a range of these compounds are preserved in the purified ryanodine receptor proteins of skeletal [39,40,54,55] and canine cardiac muscle [42,45,58].

Our earlier studies of the gating behaviour of the native sheep cardiac HSR membrane Ca^{2+}-release channel have established that, with Ca^{2+} as the permeant ion, channel opening occurs as bursts of very brief events [18,24,25]. Analysis of open and closed lifetime durations suggests that for the native channel at room temperature, best fits to open lifetimes are obtained with two exponential components, with more than 90% of events occurring to the shorter distribution; whilst best fits to closed lifetimes require three exponential components [18,24,25]. An example of lifetime analysis of a typical native sheep cardiac HSR Ca^{2+}-release channel is presented in Fig. 8. Lifetimes are displayed as non-cumulative histograms together with probability density functions (pdfs) obtained from maximum likelihood fitting to the individual lifetimes. Details of the pdfs are given in the figure legend. Similar lifetime analysis of a typical purified sheep cardiac HSR ryanodine receptor-channel, under identical conditions, is also shown in Fig. 8. A comparison of the channel traces and lifetime analyses indicates that a virtually identical pattern of channel gating has been retained following solubilisation, purification and reconstitution of the ryanodine receptor-channel.

Modulation of channel open probability by physiological and pharmacological agents is preserved following solubilisation, purification and reconstitution of the sheep cardiac muscle HSR ryanodine receptor-channel. Channel open probability is elevated by increasing concentrations of Ca^{2+} added to the cis face of the bilayer (Fig. 9). In this example, with K^+ as the permeant ion, P_o was markedly increased by raising the activating Ca^{2+} from 10 ($P_o = 0.04$) to 110 μM ($P_o = 0.14$). Addition of 1.3 mM ATP (producing a final free Ca^{2+} concentration of 10 μM and a final free ATP concentration of 1 mM) further increases P_o (0.20) (Fig. 9). Mg^{2+} (Fig. 10) and ruthenium red (Fig. 11) decrease P_o of native HSR Ca^{2+}-release channels [12,13,17,18] and, when added to the *cis* chamber, these agents decrease P_o of the purified sheep cardiac HSR ryanodine receptor-channel.

The plant alkaloid ryanodine is a specific marker for the Ca^{2+}-release channel of the HSR. When added in

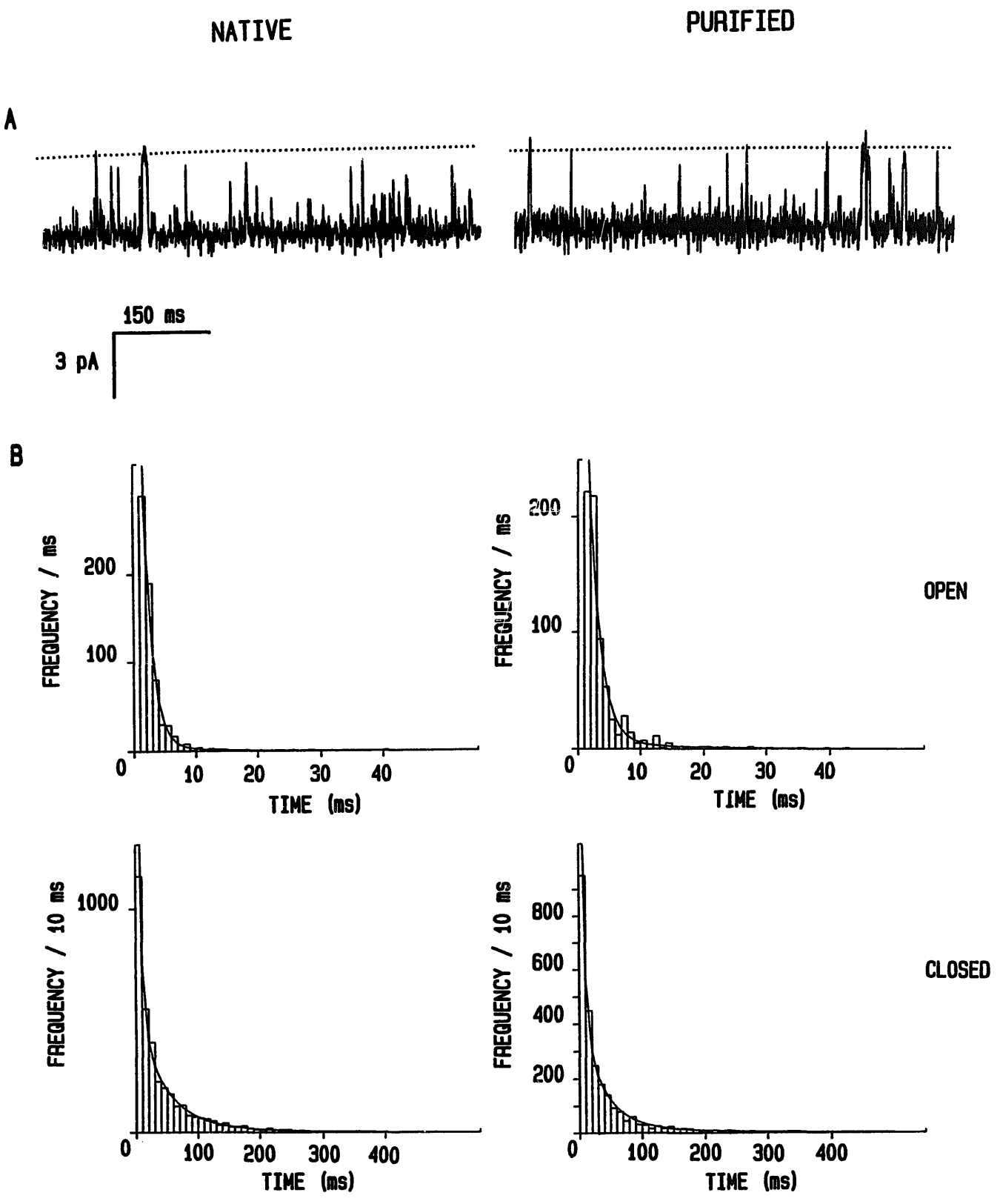

Fig. 8. Gating kinetics of native and purified sheep HSR ryanodine receptor-channels with Ca^{2+} as permeant ion. (A) Single-channel current fluctations of native and purified receptor-channels. In both cases channel open level is indicated by a dotted line and the holding potential was 0 mV. (B) Non-cumulative lifetime histograms together with probability density functions (solid lines) obtained by the method of maximum likelihood. Open and closed lifetime distributions for a native channel are shown on the left of the diagram. Individual lifetimes were fitted to pdfs such that $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots a_n(1/\tau_n)\exp(-t/\tau_n)$ [63]. For both the native and purified channels, open lifetimes are best fitted to double exponentials described by the following pdfs. Native: $f(t) = 0.96(1/1.4)\ \exp(-t/1.4) + 0.04(1/9.5)\ \exp(-t/9.5)$. Purified: $f(t) = 0.93(1/1.8)\ \exp(-t/1.8) + 0.07(1/13.9)\ \exp(-t/13.9)$. Closed lifetimes of both native and purified channels are best described by triple exponentials with the following pdfs. Native: $f(t) = 0.29(1/5.3)\ \exp(-t/5.3) + 0.56(1/42.5)\ \exp(-t/42.5) + 0.15(1/129.7)\ \exp(-t/129.7)$. Purified: $f(t) = 0.29(1/4.9)\ \exp(-t/4.9) + 0.56(1/35.1)\ \exp(-t/35.1) + 0.15(1/226.5)\ \exp(-t/226.5)$.

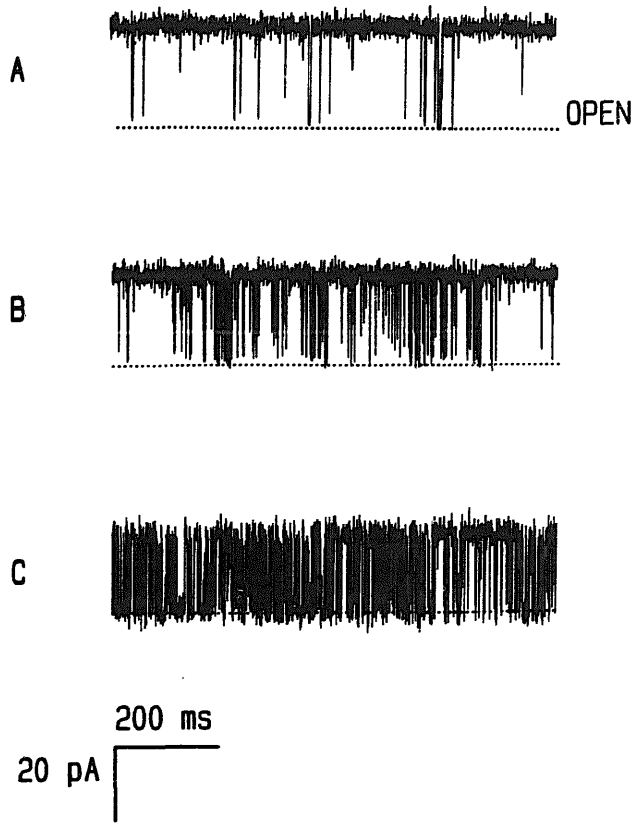

Fig. 9. Modulation of purified receptor-channel gating by Ca^{2+} and ATP. Traces were obtained at a holding potential of 40 mV with symmetric 210 mM K^+. (A) Channel activated by 10 μM Ca^{2+} *cis*, $P_0 = 0.04$. (B) Channel activated by 110 μM Ca^{2+} *cis*, $P_0 = 0.14$. (C) Channel activated by 10 μM Ca^{2+} + 1 mM ATP *cis* (see text for details), $P_0 = 0.20$.

μM concentrations to native cardiac and skeletal HSR channels incorporated into planar phospholipid bilayers, ryanodine characteristically modifies both conduction and gating properties, 'locking' the channel into

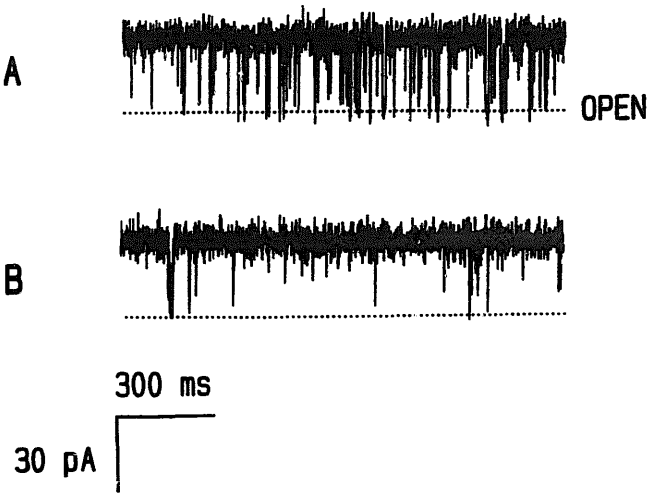

Fig. 10. Reduced channel opening in the presence of Mg^{2+}. (A) Single purified receptor-channel in symmetric 210 mM K^+ at 40 mV. Channel opening was activated by a combination of 10 μM Ca^{2+} and 1 mM ATP *cis*. (B) The same channel following the addition of 1 mM $MgCl_2$ to the solution in the *cis* chamber.

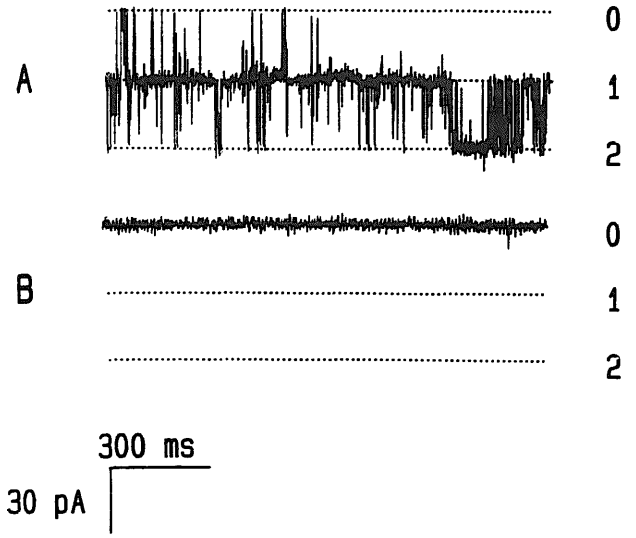

Fig. 11. Reduced channel opening in the presence of ruthenium red. (A) Receptor-channel current fluctuations from a bilayer containing at least two channels with symmetric 210 mM K^+ at 40 mV. Channels were activated with 110 μM Ca^{2+} *cis*. The numbers to the right of the trace indicate the number of receptor-channels in the open state. (B) Addition of 1 μM ruthenium red to the solution on the *cis* side of the membrane immediately abolishes channel opening.

a permanently open, reduced conductance state [16,18,28,35]. A similar pattern is observed following the addition of ryanodine to the *cis* solutions bathing purified, reconstituted, sheep cardiac HSR Ca^{2+}-release channels (Fig. 12). This diagram shows conduction and gating modification with both Ca^{2+} and K^+ as the charge carrying species. In both cases, following a variable period in the presence of ryanodine (A: 5 μM, B: 1 μM) the channel enters a characteristic reduced conductance state. As with the native channel, when Ca^{2+} is used as the permeant ion the modification invariably occurs from the fully open state of the channel. With K^+ as the permeant ion, it is common to observe the ryanodine modification occurring from the closed state. Following the interaction of ryanodine with its receptor, the channel remains in the reduced-conductance open state with only occasional, brief events, occurring to the normal non-conducting current level. Addition of ruthenium red to the solution in the *cis* chamber bathing a ryanodine-modified channel induces closing or blocking events to either the normal non-conducting level or variable low conductance levels. The frequency and duration of these events increases with increasing ruthenium red concentration (Fig. 12).

Discussion

Our initial attempts to purify functional sheep cardiac SR Ca^{2+}-release channels by duplicating the method developed by Lai et al. [41] for the purification of rabbit skeletal and canine cardiac muscle SR ryanodine receptors proved unsuccessful. This procedure yielded varia-

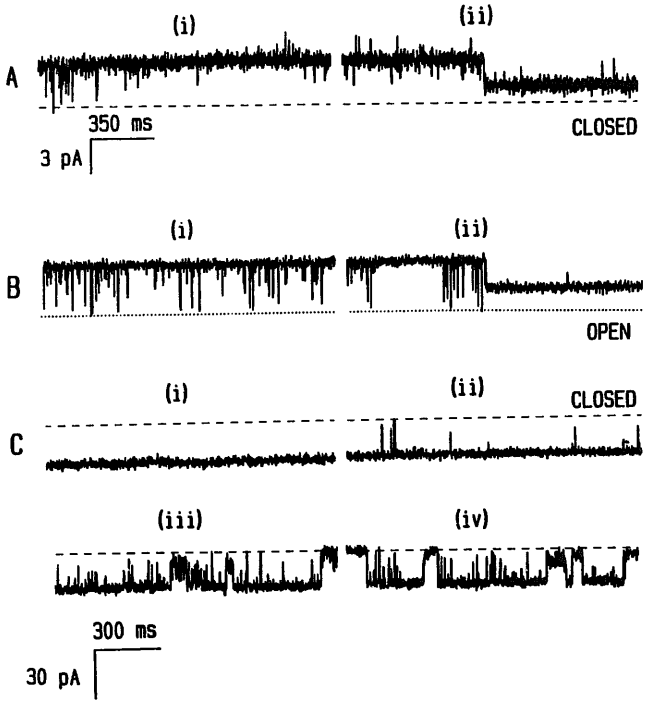

Fig. 12. Modification of purified receptor-channel gating and conductance by ryanodine with Ca^{2+} or K^+ as the permeant ion. (A) Bilayer containing at least two purified receptor-channels activated by 110 μM Ca^{2+} *cis* and with Ca^{2+} as the permeant ion (see Methods for details of solutions). Holding potential was 0 mV. The zero-current level is indicated by the dashed line. (B) Current fluctuations for a bilayer containing a single receptor-channel in symmetric 210 mM K^+ held at 40 mV. In this case, channel opening is indicated by a downward deflection and the open level is indicated by a dotted line. In both cases trace (i) represents activity prior to ryanodine induced modification. Examples of characteristic ryanodine modification are shown in trace (ii) following the addition of ryanodine to the solutions in the *cis* chambers. C (i) Single purified receptor-channel in symmetric 800 mM K^+ at a holding potential of 40 mV following modification by 1 μM ryanodine. Traces (ii), (iii) and (iv) are examples of the effects of the addition of a total of 2, 4 and 6 μM ruthenium red to the solution on the *cis* side of the bilayer. The dashed line represents the normal zero-current level.

ble [^{3}H]ryanodine binding and, on reconstitution, a range of poorly selective channels displaying multiple conductance states (data not shown). We therefore investigated modifications of the methods of Lai et al. in an attempt to find a method suitable for the purification of functional Ca^{2+}-release channels from sheep cardiac SR. Our aim was to define conditions which involved minimal exposure of the receptor-channel to detergent, whilst allowing for significant solubilisation of the receptor. A further modification was to carry out solubilisation on ice rather than at room temperature. [^{3}H]Ryanodine binding was used as the primary assessment of the functional state of the receptor. However, it is clear that high levels of ligand binding following isolation do not always reflect functional integrity of channel proteins. An example of this phenomenon is provided by the nicotinic acetylcholine receptor-channel of *Torpedo californica* electroplax, which was initially isolated as an α-bungarotoxin binding protein but did not function as a ligand-regulated ion channel following reconstitution into proteo-liposomes. Function was attained by modification of the solubilisation and reconstitution protocol [65]. Indeed, evidence is available to suggest that solubilisation of the skeletal muscle HSR ryanodine receptor can be achieved under conditions which produce significant [^{3}H]ryanodine binding but abnormal channel function. Such a situation was reported by Imagawa et al. following solubilisation of HSR with digitonin [38]. For the HSR ryanodine receptor-channel, functional assessment should include, in addition to [^{3}H]ryanodine binding, preservation of cation conductance and selectivity, together with regulation of gating by a range of physiological and pharmacological agents. This has been achieved, to a greater or lesser extent with the receptor-channels of rabbit skeletal and canine cardiac muscle. In all cases, following solubilisation with CHAPS, purified preparations have been produced which act as ligand-regulated cation channels. The major area of diversity between reports concerns the unit conductance of the purified functional receptor-channel. Hymel et al. have noted conductance levels ranging from 4 to greater than 120 pS following the reconstitution of rabbit skeletal [54] and canine cardiac [58] ryanodine receptor-channels. Of the two groups to carry out reasonably detailed examinations of receptor-channel conductance and selectivity, one report a range of different unit conductances; for example Ma et al. describe channel events of either 800, 400 or 200 pS in 250 mM K^+ solutions (approx. 80% of events were of the 400 pS form) for the rabbit skeletal receptor [56]. Whilst Meissner and colleagues describe channels from rabbit skeletal receptors with maximal conductance of 600 pS in 500 mM Na^+, displaying sub-conductance states at 450, 300 and 150 pS and a second population of receptor-channels with a maximum conductance of 300 pS, displaying sub-conductance levels at 225, 150 and 75 pS. The most frequently observed sub-conductance states were those which occurred to 50% of the maximal conductance [55]. The same group report similar sub-conductances with receptors isolated from canine cardiac muscle [45]. These authors have also noted that channels displaying multiple conductance states do not usually retain their properties of regulation by physiological and pharmacological agents [39].

Receptor solubilisation and isolation. The sheep cardiac SR ryanodine receptor can be essentially completely solubilised by as little as 0.5% CHAPS in the presence of 2.5 mg/ml PC. The receptor, solubilised under these conditions, retains a high affinity for ryanodine and is significantly enriched in comparison with the native HSR membrane. SDS-polyacrylamide gel electrophoresis confirms this observation; the [^{3}H]ryanodine binding peak obtained following sucrose density gradient

centrifugation displays a major band at approximately 350 kDa. These findings are in good agreement with previous reports that the ryanodine receptor of both rabbit skeletal and canine cardiac HSR membranes consists of a 30 S homotetramer comprising monomers with molecular mass of approximately 400 kDa.

Reconstitution of the sheep cardiac ryanodine receptor into unilamellar liposomes by dialysis and subsequent incorporation of these liposomes into planar phospholipid bilayers, demonstrates that the isolated receptor functions as a ligand-operated, cation-selective channel, sharing a range of properties with both the native sheep HSR Ca^{2+}-release channel [18,24,25,62] and the purified ryanodine receptor-channels of rabbit skeletal and canine cardiac muscle [39,40,42,45,54,55,58].

Conduction. Measurement of ion conduction through the native HSR Ca^{2+}-release channel requires the use of very specific ionic conditions [12]. The channel is activated by a suitable ligand from the cytosolic side of the membrane and provided with a permeant ion at the luminal face of the channel. Current flow must be monitored in the absence of small monovalent cations and anions which have high conductance through monovalent cation [66,67] and anion [17,68] channels of the HSR membrane, which are incorporated into the planar bilayer along with the Ca^{2+}-release channel. Using these conditions, the native Ca^{2+}-release channels from both muscle types have been shown to display selectivity for cations over anions, a relatively high degree of selectivity for divalent cations over large monovalent cations such as $Tris^+$, and a high divalent cation conductance [12,13,19,24,25]. When investigated under the same ionic conditions, the purified sheep cardiac HSR ryanodine receptor displays selectivity and conductance properties identical to those of the native channel. Single-channel conductance saturates as the calcium concentration in the *trans* chamber is increased. The data can be fitted to a simple Michaelis-Menten scheme which would be consistent with single-ion channel behaviour [69], however, it does not conclusively exclude multi-ion occupancy of the conduction pathway. It is highly probable that the maximal conductance determined in these experiments is an underestimate of the true value and almost certainly reflects inhibition of Ca^{2+} conductance by mannitol (used in the solutions to maintain osmotic pressure). Indeed, higher Ca^{2+} conductance was observed in the experiments shown in Figs. 4 and 7. The putative conductance block observed in the presence of mannitol requires further investigation.

The isolation of the ryanodine receptor from the HSR membrane, and hence its separation from the SR monovalent cation and anion channels, permits a more detailed examination of the mechanisms involved in ion conduction and selectivity. As has been reported by others for the rabbit skeletal [39,40,55,56] and canine cardiac [45] ryanodine receptor-channels, the isolated sheep cardiac channel displays high single channel conductance and ideal selectivity for cations over anions when monovalent cations are used as the charge carrying species. The major difference between the data presented in this communication and that reported previously concerns the conductance level of the purified functional unit of the ryanodine receptor-channel. All other reports stress the common occurrence of either deviations from full conductance to sub-conductance states [39,45,54,55,58], or the occurrence of channels with different conductance levels [55,56]. Sub-conductance or reduced-conductance states were seen very rarely in the experiments reported here. We estimate that of the channels observed during this study (> 500), with a wide range of ionic conditions and at various holding potentials, under 5% showed any form of reduced conductance; the remainder displayed a single open-conductance level comparable to the maximum open levels reported by Meissner and his co-workers [39,45,55] and the largest conductance species, reported for a minority of channels, by Coronado's group [40,56].

What then is the conductance of the functional unit of the HSR ryanodine receptor-channel? A large body of ultrastructural [37,39,43–46] evidence suggests that both the native, *in situ*, channel and the purified 30 S ryanodine receptor are homotetramers, and it has been proposed that the various sub- and reduced-conductance levels seen with purified preparations of the receptor-channel reflect the sub-unit composition of the 30 S oligomer [40]. The variable conductance states described in earlier reports may result from modifications occurring during solubilisation and purification of the receptor-channel complex [55]. In support of this contention is the finding that fully functional native HSR Ca^{2+}-release channels do not display obvious sub-conductance states when Ca^{2+} is used as the charge carrying species; whilst purified receptor-channels which show a range of conductance levels with monovalent cations as the permeant species also show multiple conductance levels with Ca^{2+} [40,55]. High-affinity [^{3}H]ryanodine binding occurs with a stoichiometry of one binding site per homotetramer and there is evidence indicating that ryanodine binding to the 30 S complex may be influenced by co-operative interactions of the four 400 kDa sub-units [47]. The demonstration of variable conductance states following reconstitution of purified receptor-channels raises the possibility that the unit conductance of the functional receptor-channel may depend upon the co-ordinated gating of several conductance pathways (possibly one or two for each 400 kDa sub-unit) [55]. Loss of co-ordinated behaviour as the result of minor modifications occurring during solubilisation and reconstitution may give rise to the appearance of sub- or reduced-conductance levels. The 'milder' solubilisation conditions reported here may lead

to enhanced preservation of the functional unit and hence the very low frequency of sub- or reduced-conductance states.

Despite apparent differences in the unit conductance, the sheep cardiac HSR ryanodine receptor-channel described here has a Ca^{2+}/K^+ permeability ratio similar to that reported for the lower conductance rabbit skeletal receptor-channel [40]; suggesting analogies in the mechanisms governing ion discrimination.

Gating. Our previous investigations of the gating mechanisms of the native sheep cardiac HSR Ca^{2+}-release channel have revealed that with Ca^{2+} as the sole activating ligand and Ca^{2+}as the charge carrying ion, gating is characterised by bursts of brief opening events. Maximum likelihood fitting to individual lifetimes requires at least two exponential components for the open times, with the vast majority (> 90%) occurring to the shorter distribution, with a time constant only slightly in excess of the minimum fully resolvable duration. Three exponentials are required for maximum likelihood fits to the closed times. This basic pattern of gating is extremely well preserved following solubilisation, isolation and reconstitution of the receptor-channel.

The improved resolution produced by the use of K^+ as the charge carrying species reveals a basic Ca^{2+}-activated gating pattern not dissimilar to that observed with the native channel or the purified receptor-channel with Ca^{2+} as the permeant ion. Channel gating is characterised by a series of very brief opening events. A similar pattern of very brief opening events is apparent for the maximum conductance level (800 pS) reported for the rabbit skeletal muscle ryanodine receptor-channel [56].

The sheep cardiac receptor-channel also responds to physiological and pharmacological agents in a manner consistent with the preservation of the integrity of a range of sites of ligand interaction. Increasing free Ca^{2+} concentrations on the *cis* side of the bilayer increase P_0; the mechanisms involved in this activation have not yet been fully characterised, but preliminary observations appear to be consistent with our demonstration for the native channel, that Ca^{2+}-activated increases in P_0 result from an increased frequency of channel opening [18]. The purified receptor-channel is further activated by ATP added to the *cis* chamber and P_0 is reduced by Mg^{2+} and ruthenium red, again from the *cis* side of the bilayer.

Conductance and gating of the isolated receptor-channel are characteristically modified by ryanodine with either K^+ or Ca^{2+} as the permeant ion. In our experience, when ryanodine interacts with the native cardiac channel, the modification of gating invariably occurs from an open channel conformation [17,18,24,25,35]. The demonstration of an apparent interaction of ryanodine with closed states of the isolated receptor-channel in K^+ solutions is in agreement with previous observations [39,45,55] and may reflect improved resolution under these conditions; that is, resolution of ryanodine interaction 'during' an opening event. Earlier evidence provided by [^{3}H]ryanodine binding and single-channel studies with native sheep cardiac HSR membrane vesicles has been interpreted as suggesting that the ryanodine binding site is considerably more accessible in open- than in closed-channel conformations [32,35].

The concentration-dependent closing, or blocking, events induced by the addition of ruthenium red to ryanodine-modified channels is similar to that reported by Ma et al. [56] for the lower conductance rabbit skeletal muscle receptor-channel. The reduced open probabilities seen here are consistent with previous reports of ruthenium red-induced reductions in ryanodine-activated Ca^{2+} current through native-reconstituted skeletal SR Ca^{2+}-release channels [16] and the recent demonstration of the inhibition of ryanodine-induced Ca^{2+} efflux from the SR network of digitonin-permeabilised cardiac myocytes by ruthenium red [70]. The complex nature of the interaction of ruthenium red with the ryanodine-modified channel deserves fuller investigation; of particular interest is the possibility that the low conductance states induced by ruthenium red correspond to different conductance pathways of the receptor-channel complex.

Orientation of the receptor following reconstitution. A range of activating and inhibitory agents interact with the purified sheep cardiac HSR ryanodine receptor-channel when added to the *cis* chamber. It is well established that these agents interact with sites on the cytosolic face of the native channel protein [12,13,17–19] and therefore it is implied that the receptor-channels investigated here have been reconstituted into unilamellar vesicles, and hence incorporated into planar phospholipid bilayers, with a fixed orientation. Although we have seen occasional channels in reverse orientation, we believe that the vast majority do reconstitute with a fixed orientation so that the outside of the proteo-liposomes and hence the *cis* face of the bilayer, is equivalent to the cytosolic face of the native HSR membrane. We observed the same preferred orientation following solubilisation of rabbit skeletal muscle SR vesicles with cholate and reconstitution of K^+ channel proteins into liposomes for patch clamp studies [71].

In conclusion, we have described modifications of previously reported methods for the isolation of ryanodine receptor-channels. The modifications were designed to subject the sheep cardiac HSR ryanodine receptor-channel oligomer to the minimum detergent exposure required for solubilisation. This procedure yields [^{3}H]ryanodine binding proteins which function as ligand-operated receptor-channels. Unlike previously reported preparations, the vast majority of these chan-

nels display a single open state conductance level which we believe represents the unit conductance of the functional receptor-channel complex.

Acknowledgements

We thank the British Heart Foundation for financial support and Drs. Di Manning and Rebecca Sitsapesan for useful comments on the manuscript. We are particularly grateful to Dr. Debbie Cumming for running the SDS-PAGE.

References

1 Fabiato, A. (1989) Mol. Cell. Biochem. 89, 135–140.
2 Nabauer, M., Callewaert, G., Cleemann, L. and Morad, M. (1989) Science 244, 800–803.
3 Melzer, W., Schneider, M.F., Simon, B.J. and Szucs, G. (1986) J. Physiol. 373, 481–511.
4 Rios, E. and Brum, G. (1987) Nature 325, 717–720.
5 Lai, F.A. and Meissner, G. (1989) J. Bioenerg. Biomembr. 21, 227–246.
6 Miyamoto, H. and Racker, E. (1982) J. Membr. Biol. 66, 193–201.
7 Meissner, G. (1984) J. Biol. Chem. 259, 2365–2374.
8 Meissner, G., Darling, E. and Eveleth, J. (1986) Biochemistry 25, 236–244.
9 Meissner, G. (1986) Biochemistry 25, 244–251.
10 Meissner, G. (1986) J. Biol. Chem. 261, 6300–6306.
11 Meissner, G. and Henderson, J.S. (1987) J. Biol. Chem. 262, 3065–3073.
12 Smith, J.S., Coronado, R. and Meissner, G. (1985) Nature 316, 446–449.
13 Smith, J.S., Coronado, R. and Meissner, G. (1986) J. Gen. Physiol. 88, 573–588.
14 Smith, J.S., Coronado, R. and Meissner, G. (1986) Biophys. J. 50, 921–928.
15 Rousseau, E. and Meissner, G. (1989) Am. J. Physiol. 256, 328–333.
16 Nagasaki, K. and Fleischer, S. (1988) Cell Calcium 9, 1–7.
17 Holmberg, S.R.M. and Williams, A.J. (1989) Circ. Res. 65, 1445–1449.
18 Ashley, R.H. and Williams, A.J. (1990) J. Gen. Physiol. 950, 981–1005.
19 Rousseau, E., Smith, J.S., Henderson, J.S. and Meissner, G. (1986) Biophys. J. 50, 1009–1014.
20 Nagasaki, K. and Fleischer, S. (1989) Cell Calcium 10, 63–70.
21 Holmberg, S.R.M. and Williams, A.J. (1989) J. Physiol. 418, 202. (Abstr.).
22 Holmberg, S.R.M. and Williams, A.J. (1990) Circ. Res. 670, 272–283.
23 Rousseau, E., LaDine, J., Liu, Q.Y. and Meissner, G. (1988) Arch. Biochem. Biophys. 267, 75–86.
24 Sitsapesan, R. and Williams, A.J. (1990) J. Physiol. 423, 425–439.
25 Williams, A.J. and Holmberg, S.R.M. (1990) J. Membr. Biol. 115, 167–178.
26 Smith, J.S., Rousseau, E. and Meissner, G. (1989) Circ. Res. 64, 352–359.
27 Lattanzio, F.A., Schlatterer, R.G., Nicar, M., Campbell, K.P. and Sutko, J.L. (1987) J. Biol. Chem. 262, 2711–2718.
28 Rousseau, E., Smith, J.S. and Meissner, G. (1987) Am. J. Physiol. 253, C364–368.
29 Penefsky, Z.J. and Kahn, M. (1969) Am. J. Physiol. 218, 1682–1686.
30 Sutko, J.L., Ito, K. and Kenyon, J.L. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. 44, 2984–2988.
31 Pessah, I.N., Waterhouse, A.L. and Casida, J.E. (1985) Biochem. Biophys. Res. Commun. 128, 449–456.
32 Pessah, I.N., Francini, A.O., Scales, D.J., Waterhouse, A.L. and Casida, J.E. (1986) J. Biol. Chem. 261, 8643–8648.
33 Pessah, I.N., Stambuk, R.A. and Casida, J.E. (1987) Mol. Pharmacol. 31, 232–238.
34 Fleischer, S., Ogunbunmi, E.M., Dixon, M.C. and Fleer, E.A.M. (1985) Proc. Natl. Acad. Sci. USA 82, 7256–7259.
35 Holmberg, S.R.M. and Williams, A.J. (1990) Biochim. Biophys. Acta 1022, 187–193.
36 Inui, M., Saito, A. and Fleischer, S. (1987) J. Biol. Chem. 262, 15637–15642.
37 Inui, M., Saito, A. and Fleischer, S. (1987) J. Biol. Chem. 262, 1740–1747.
38 Imagawa, T., Smith, J.S., Coronado, R. and Campbell, K.P. (1987) J. Biol. Chem. 262, 16636–16643.
39 Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y. and Meissner, G. (1988) Nature 331, 315–319.
40 Smith, J.S., Imagawa, T., Ma, J., Fill, M., Campbell, K.P. and Coronado, R. (1988) J. Gen. Physiol. 92, 1–26.
41 Lai, F.A., Erickson, H.P., Rousseau, E., Liu, Q-Y. and Meissner, G. (1988) Biochem. Biophys. Res. Commun. 151, 441–449.
42 Rardon, D.P., Cefali, D.C., Mitchell, R.D., Seiler, S.M. and Jones, L.R. (1989) Circ. Res. 64, 779–789.
43 Saito, A., Inui, M., Radermacher, M., Frank, J. and Fleischer, S. (1988) J. Cell Biol. 107, 211–219.
44 Block, B.A., Imagawa, T., Campbell, K.P. and Franzini-Armstrong, C. (1988) J. Cell Biol. 107, 2587–2600.
45 Anderson, K., Lai, F.A., Liu, Q.-Y., Rousseau, E., Erickson, H.P. and Meissner, G. (1989) J. Biol. Chem. 264, 1329–1335.
46 Wagenknecht, T., Grassucci, R., Frank, J., Saito, A., Inui, M. and Fleischer, S. (1989) Nature 338, 167–170.
47 Lai, F.A., Misra, M., Xu, L., Smith, H.A. and Meissner, G. (1989) J. Biol. Chem. 264, 16776–16785.
48 Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) Nature 339, 439–445.
49 Marks, A.R., Tempst, P., Hwang, K.S., Taubman, M.B., Inui, M., Chadwick, C.C., Fleischer, S. and Nadal-Ginard, B. (1989) Proc. Natl. Acad. Sci. USA 86, 8683–8687.
50 Zorzato, F., Fujii, J., Otsu, K., Green, N.M., Lai, F.A., Meissner, G. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 2244–2256.
51 Penner, R., Neher, E., Takeshima, H., Nishimura, S. and Numa, S. (1989) FEBS. Lett. 259, 217–221.
52 Otsu, K., Willard, H.F. and MacLennan, D.H. (1990) Biophys. J. 57, 503. (Abstract)
53 Imagawa, T., Takasago, T. and Shigekawa, M. (1989) J. Biochem. Tokyo. 106, 342–348.
54 Hymel, L., Inui, M., Fleischer, S. and Schindler, H. (1988) Proc. Natl. Acad. Sci. USA 85, 441–445.
55 Liu, Q.-Y., Lai, F.A., Rousseau, E., Jones, R.V. and Meissner, G. (1989) Biophys. J. 55, 415–424.
56 Ma, J., Fill, M., Knudson, M., Campbell, K.P. and Coronado, R. (1988) Science 242, 99–102.
57 Fill, M., Ma, J.J., Knudson, C.M., Imagawa, T., Campbell,, K.P. and Coronado, R. (1989) Ann. N.Y. Acad. Sci. 560, 155–162.
58 Hymel, L., Schindler, H., Inui, M. and Fleischer, S. (1988) Biochem. Biophys. Res. Commun. 152, 308–314.
59 Tomlins, B., Harding, S.E., Kirby, M.S., Poole-Wilson, P.A. and Williams, A.J. (1986) Biochim. Biophys. Acta 856, 137–143.
60 Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
61 Miller, C. (1982) Phil. Trans. Roy. Soc. Lond. B. 299, 401–411.

62 Williams, A.J. and Ashley, R.H. (1989) Ann. N.Y. Acad. Sci. 560, 163–173.
63 Colquhoun, D. and Sigworth, F.J. (1983) Single-channel recording (Sakmann, B. and Neher, E., eds.), pp. 191–263, Plenum Press, New York.
64 Blatz, A.L. and Magleby, K.L. (1986) J. Physiol. 378, 141–174.
65 Huganir, R.L. and Racker, E. (1982) J. Biol. Chem. 257, 9372–9378.
66 Miller, C. and Rosenberg, R.L. (1979) Biochemistry 18, 1138–1145.
67 Tomlins, B., Williams, A.J. and Montgomery, R.A.P. (1984) J. Membr. Biol. 80, 191–199.
68 Rousseau, E., Roberson, M. and Meissner, G. (1988) Eur. Biophys. J. 16, 143–151.
69 Läuger, P. (1973) Biochim. Biophys. Acta 311, 423–441.
70 Wimsatt, D.K., Hohl, C.M., Brierley, G.P. and Altschuld, R.A. (1990) J. Biol. Chem. 265, 14849–14857.
71 Tomlins, B. and Williams, A.J. (1986) Pflügers Arch. 407, 341–347.