ELSEVIER

European Journal of Pharmacology 365 (1999) 91–102

ejp

Complex formation of skeletal muscle Ca^{2+}-regulatory membrane proteins by halothane

Gabriele R. Froemming, Derek J. Dillane, Kay Ohlendieck *

Department of Pharmacology, National University of Ireland, University College Dublin, Belfield, Dublin 4, Ireland

Received 17 August 1998; revised 10 November 1998; accepted 13 November 1998

Abstract

In skeletal muscle, halothane affects the functions of several Ca^{2+}-regulatory membrane proteins involved in the excitation–contraction–relaxation cycle. To investigate the mechanism by which this volatile anesthetic interferes with Ca^{2+}-homeostasis, we studied potential changes in protein–protein interactions by halothane. Using comparative immunoblotting of microsomal muscle proteins separated on native and denaturing gels, we show here that halothane induces oligomerization of the terminal cisternae Ca^{2+}-binding protein calsequestrin, the junctional ryanodine receptor Ca^{2+}-release channel and the transverse-tubular α_1-dihydropyridine receptor. This agrees with previous reports on the modulation of Ca^{2+}-release activity by halothane since interactions between the voltage-sensing α_1-dihydropyridine receptor, the ryanodine receptor and the luminal Ca^{2+}-reservoir might result in a rapid release of Ca^{2+}-ions. Furthermore, this study supports the idea that specific protein sites are involved in the action of inhalational anesthetics and that halothane might trigger abnormal Ca^{2+}-homeostasis in malignant hyperthermia via oligomerization of the mutated ryanodine receptor.

Keywords: Halothane; Calsequestrin; Ryanodine receptor; Dihydropyridine receptor; Oligomerization; Skeletal muscle

1. Introduction

Halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) is a widely employed inhalational anesthetic and is often used as a standard against which other potent anesthetic agents are measured (Koblin, 1994). The effect of halothane on regulatory muscle membrane proteins involved in Ca^{2+}-homeostasis has been intensively studied. At clinical concentrations, halothane influences the functions of the calmodulin-dependent Ca^{2+}-ATPase of the plasmalemma (Lopez and Kosk-Kosicka, 1995) and the sarcoplasmic reticulum Ca^{2+}-ATPases (Karon et al., 1995). Recently, Kosk-Kosicka et al. (1997) could show binding of halothane to discrete sites in the Ca^{2+}-ATPase demonstrating direct interaction between a volatile anesthetic and a muscle membrane protein. Vesicular preparations of the sarcoplasmic reticulum from skeletal and cardiac muscle exhibit a pH-dependent increase in Ca^{2+}-release in response to halothane (Louis et al., 1992; Beltran et al., 1996). Electrophysiological studies on the isolated ryanodine receptor Ca^{2+}-release channel from the sarcoplasmic reticulum showed increased duration of channel opening following incubation with halothane (Bull and Marengo, 1994; Connelly and Coronado, 1994). These findings are of clinical importance since the main cause of death due to anesthesia is the pharmacogenetic disorder malignant hyperthermia. In susceptible individuals, halothane may trigger an episode of this ion channel disease which is characterized by a rapid increase in body temperature, tachycardia, metabolic acidosis and muscle stiffness (Mickelson and Louis, 1996). Primary defects in the ryanodine receptor Ca^{2+}-release channel (MacLennan et al., 1990; Quane et al., 1994) or the α_1-subunit of the dihydropyridine receptor (Monnier et al., 1997) were described as responsible for malignant hyperthermia.

The dihydropyridine-sensitive L-type Ca^{2+}-channel of the transverse-tubular membrane system exists in an α_1–α_2/δ–β–γ-configuration whereby the α_1-subunit was shown to be the principal subunit with respect to antagonist binding, ion permeability and voltage sensing (Catterall, 1995). The auxiliary dihydropyridine receptor sub-

* Corresponding author. Tel.: +353-1-706-1557; Fax: +353-1-269-2749; E-mail: kay.ohlendieck@ucd.ie

0014-2999/99/$ - see front matter
PII: S0014-2999(98)00854-1

units were demonstrated to be important for modulation of channel activity and receptor targeting (Gurnett and Campbell, 1996). The ryanodine-sensitive Ca^{2+}-release channel contains four identical 565-kDa subunits (Franzini-Armstrong and Protasi, 1997) and a modulatory 12-kDa subunit which was found to be a binding-protein for immunosuppressive drugs (Brilliantes et al., 1994). Within the muscle membrane systems responsible for signal transduction and Ca^{2+}-release, the transverse-tubular dihydropyridine receptor and the ryanodine receptor Ca^{2+}-release channel of the junctional sarcoplasmic reticulum were clearly shown to form large complexes (Block et al., 1988; Marty et al., 1994), estimated to be of approximately 3000 kDa (Murray and Ohlendieck, 1997). In addition, the major luminal Ca^{2+}-reservoir protein calsequestrin (MacLennan and Wong, 1971; Yano and Zarain-Herzberg, 1994) also exists in oligomeric form (Maguire et al., 1997) and is closely associated with the triad receptor complex (Zhang et al., 1997; Murray and Ohlendieck, 1998). While calsequestrin of apparent 63 kDa is a medium-affinity, high-capacity Ca^{2+}-binding protein localized mainly to the terminal cisternae region (Jorgensen et al., 1983; Fliegel et al., 1987), sarcalumenin of approximately 160 kDa appears to be the luminal sarcoplasmic reticulum Ca^{2+}-binding component of the longitudinal tubules (Leberer et al., 1990). These findings support the currently held hypothesis of excitation–contraction coupling in mature skeletal muscle which suggests that the α_1-dihydropyridine receptor acts as a voltage sensor and that this receptor induces Ca^{2+}-release via direct physical interactions with the tetrameric ryanodine receptor complex (Meissner and Lu, 1995; Murray et al., 1998). Following skeletal muscle contraction, the highly efficient sequestration of Ca^{2+}-ions is due to the rapid pump action of the sarcoplasmic reticulum Ca^{2+}-ATPase (MacLennan et al., 1997). The physiological pump units were shown to be of oligomeric nature, probably representing tetrameric structures of 110-kDa Ca^{2+}-ATPase monomers (Martonosi, 1995; Maguire and Ohlendieck, 1996).

Since it is not known by what molecular mechanism(s) halothane interferes with skeletal muscle Ca^{2+}-homeostasis in normal and pathological tissues, we investigated the potential effects of this volatile anesthetic on the oligomerization of key membrane components of Ca^{2+}-regulation. In analogy to a study on the effect of halothane on the skeletal muscle Ca^{2+}-ATPase (Karon and Thomas, 1993) and considering clinical levels of halothane are estimated to be 0.03 to 0.05 mol of halothane per mol of lipid in the bilayer (Taraschi et al., 1991), the same halothane concentration range was investigated here with respect to its effect on muscle membrane proteins. Immunoblotting with highly specific antibodies was used to unequivocally identify the fate of calsequestrin, the α_1-dihydropyridine receptor and the ryanodine receptor following treatment of microsomal vesicles with halothane. A comparative approach was utilized in the present study using native, non-denaturing polyacrylamide gels and denaturing sodium dodecyl sulfate polyacrylamide gels in order to analyze complex formation following halothane treatment.

2. Materials and methods

2.1. Materials

Protease inhibitors, acrylamide and peroxidase-conjugated secondary antibodies were purchased from Boehringer Mannheim (Lewis, East Sussex, UK). Western blotting chemiluminescence substrates (Super Signal®) were obtained from Pierce & Warriner (Chester, Cheshire, UK). Immobilon-NC nitrocellulose was from Millipore (Bedford, MA). Halothane, digitonin and all other chemicals were of analytical grade and purchased from Sigma (Poole, Dorset, UK).

2.2. Antibodies

A polyclonal antibody to the ryanodine receptor was from Upstate Biotechnology (Lake Placid, NY), monoclonal antibodies 20A and XIIC4 against the α_2-dihydropyridine receptor and sarcalumenin, respectively, were purchased from Affinity Bioreagents (Golden, CO) and monoclonal antibody MY32 to the fast myosin heavy chain was obtained from Sigma. Production and characterization of monoclonal antibodies IIID5 to the α_1-dihydropyridine receptor and $VIIID1_2$ against calsequestrin, which were a generous gift from Dr. K.P. Campbell (Howard Hughes Medical Institute, University of Iowa, IA), were performed as previously described in detail (Ohlendieck et al., 1991).

2.3. Subcellular fractionation

All membrane samples used derived from homogenates of New Zealand white rabbit hind leg and back skeletal muscle, obtained from the Biomedical Facility, University College Dublin, Ireland. Crude microsomes, as well as vesicular fractionates enriched in heavy sarcoplasmic reticulum or triad junctions were prepared at 0–4°C by standard methodology (Rosemblatt et al., 1981; Sharp et al., 1987; Murray and Ohlendieck, 1997). To minimize proteolytic degradation of muscle membrane proteins, all buffers contained a protease inhibitor cocktail (0.2 mM pefabloc, 1.4 μM pepstatin, 0.15 μM aprotinin, 0.3 μM E-64, 1 μM leupeptin, 0.5 μM soybean trypsin inhibitor and 1 mM EDTA). Membrane pellets were resuspended at a protein concentration of 10 mg/ml and used immediately for halothane treatment or quick-frozen in liquid nitrogen and then stored at −70°C prior to further usage. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.4. Incubation of muscle membranes with halothane

Crude microsomes, resuspended in 10% (w/v) sucrose, 25 mM HEPES, pH 7.5, 5 mM EGTA, 3 mM $MgCl_2$, were treated on ice (Figs. 1–5) or at 25°C (Fig. 6) at a final protein concentration of 1 mg/ml with 0.25 to 10% (v/v) halothane. Halothane was added from an acetone-containing 50% (v/v) stock solution. Following incubation for 30 min, membrane samples were prepared for native or denaturing gel electrophoresis. Non-denaturing buffer contained 50 mM Tris–Cl, pH 6.8, 20% (w/v) sucrose, 20% (v/v) glycerol and 0.1% (w/v) digitonin (Chang and Hosey, 1990), while denaturing buffer consisted of 125 mM Tris–Cl, pH 6.8, 3% (w/v) sodium dodecyl sulfate, 20% (w/v) sucrose and 20% (v/v) glycerol (Dunn and Bradd, 1993). Both buffers also contained 0.002% (w/v) bromophenol blue as a dye marker.

2.5. Gel electrophoresis and immunoblot analysis

Modified polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) and Chang and Hosey (1990) using 5% (w/v) or 6% (w/v) resolving gels with a 4% (w/v) stacking gel. Electrophoretic separation was performed under non-reducing conditions for 360 or 440 Vh employing a Mini-Protean II electrophoresis system from Bio-Rad Laboratories (Hempel Hempstead, Herts., UK), whereby 5 to 25 μg protein was loaded per well. In non-denaturating gel electrophoresis, 0.1% (w/v) digitonin but no sodium dodecyl sulfate was present in the resolving and stacking gel, while the running buffer contained 0.015 (w/v) sodium dodecyl sulfate (Chang and Hosey, 1990). Two types of denaturing gel electrophoresis were performed. Samples were treated with no sodium dodecyl sulfate or 3% (w/v) sodium dodecyl sulfate and were then subsequently separated by standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970). For two-dimensional electrophoresis, samples were separated in the first dimension using native polyacrylamide rod gels and then incubated for 2 h using sodium dodecyl sulfate-containing sample buffer (Laemmli, 1970). Tube gels were mounted on top of the second dimension slab gel with the help of 1% (w/v) agar and subsequently electrophoresed in the presence of ionic detergent as described above. Myofibrils, prepared from rat skeletal muscle homogenates, served as a source of high-molecular mass protein markers (Murray and Ohlendieck, 1997). Proteins separated by non-denaturing or denaturing polyacrylamide gel electrophoresis were transferred for 75 min at 100 V onto nitrocellulose membranes by the method of Towbin et al. (1979). Immunoblotting was performed by standard methods and immunodecoration of nitrocellulose sheets was evaluated by the enhanced chemiluminescence technique (Bradd and Dunn, 1993). Densitometric scanning of enhanced chemiluminescence blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) with ImageQuant V3.0 software.

3. Results

3.1. Effect of halothane on the α_1-dihydropyridine receptor

Since halothane has a modulatory effect on the sarcoplasmic reticulum Ca^{2+}-release at clinical concentrations and because direct protein–protein interactions are believed to underlie excitation–contraction coupling in mature skeletal muscle, we investigated the effect of halothane on the oligomerization of Ca^{2+}-regulatory membrane proteins. Initial blotting experiments with halothane-treated rabbit skeletal muscle microsomes revealed that this inhalational anesthetic reduces the relative electrophoretic mobility of the transverse-tubular α_1-dihydropyridine receptor in non-denaturating gels. Incubation of native muscle membrane vesicles with 0.25 to 5% (v/v) halothane resulted in a shift from a 175-kDa monomer to a 3000-kDa complex in non-denaturing gels (Fig. 1). While the intensity of immunodecoration of the monomer decreased with higher concentrations of the drug, labeling of the high-molecular mass band intensified. Based on these initial findings, we performed a comparative immunoblot analysis of key components of excitation–contraction coupling and Ca^{2+}-homeostasis, i.e., the major Ca^{2+}-binding protein of the terminal cisternae region, calsequestrin, the junctional sarcoplasmic reticulum Ca^{2+}-release channel, the ryanodine receptor and the voltage-sensor of the transverse-tubules, the α_1-dihydropyridine receptor. Following treatment with halothane, microsomal proteins were analyzed using three different gel systems. Non-denaturing gels contained low concentrations of the non-ionic detergent digitonin, but no sodium dodecyl sulfate was added to the sample buffer and gel. The other two gel systems employed contained sodium dodecyl sulfate in the gel only or ionic detergent both in the incubation buffer and the gel, respectively.

3.2. Halothane-induced oligomerization of calsequestrin

As illustrated in Fig. 2a, the non-denatured calsequestrin monomer from rabbit skeletal muscle microsomes, triads and sarcoplasmic reticulum runs as a double protein band of approximately 60 to 70 kDa. In immunoblotting, monoclonal antibody $VIIID1_2$ also recognizes high-molecular mass bands between 170 and 210 kDa which were previously designated as calsequestrin-like proteins (Maguire et al., 1997; Murray and Ohlendieck, 1997; Murray and Ohlendieck, 1998). These luminal sarcoplasmic reticulum proteins, which might represent calsequestrin isoforms, are recognized as a band of apparent 180 kDa under non-denaturing conditions (Fig. 2a). To properly visualize very high-molecular mass complexes, higher protein con-

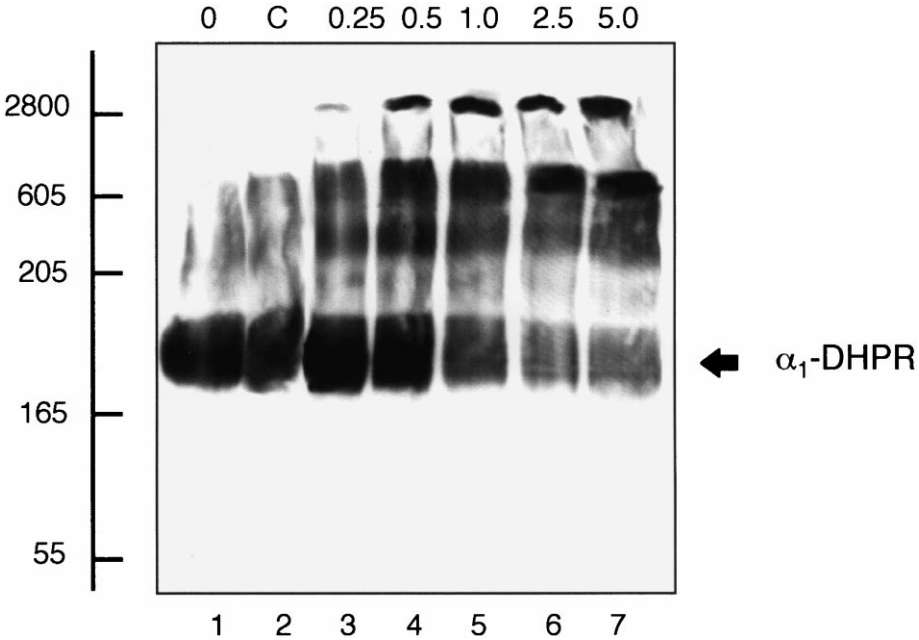

Fig. 1. Effect of halothane on the α_1-subunit of the dihydropyridine receptor. Shown is an immunoblot labeled with monoclonal antibody IIID5 to the α_1-subunit of the dihydropyridine receptor (α_1-DHPR). Lanes 1 to 7 represent rabbit skeletal muscle microsomes treated with 0, 0, 0.25, 0.5, 1.0, 2.5 and 5.0 (v/v) halothane, respectively. Lane 2 is a control (C) showing microsomes treated with 5% (v/v) acetone but no halothane. Membrane samples (15 μg protein/lane) were dissolved in non-denaturing buffer and electrophoretically separated on a 6% (w/v) gel for 360 Vh under non-reducing conditions. The position of the apparent monomer is marked by an arrow and the sizes of molecular mass standards (in kDa) are indicated on the left.

centrations were used in Fig. 2b–d resulting in broader calsequestrin bands under control conditions. While the carrier solvent acetone had no effect on the electrophoretic mobility of calsequestrin bands, incubation with halothane reduced the intensity of immunodecoration of the monomer band and the calsequestrin-like protein bands but caused the appearance of an approximately 3000-kDa complex (Fig. 2b). With the exception of the highest halothane concentration employed, the presence of sodium dodecyl sulfate in the gel system prevented the formation of the extremely high-molecular mass band (Fig. 2c). Pre-incubation of halothane-treated muscle membrane vesicles with sodium dodecyl sulfate completely abolished a shift in electrophoretic mobility (Fig. 2d).

3.3. Halothane-induced oligomerization of the α_1-dihydropyridine receptor

The α_1-subunit of the dihydropyridine receptor runs as a single protein band representing a monomer of apparent 175 kDa in non-denaturing gels of crude microsomes and membranes enriched in the sarcoplasmic reticulum or triads (Fig. 3a). Acetone did not induce a marked change in the electrophoretic mobility of this transverse-tubular protein. However, halothane clearly caused a shift of the α_1-dihydropyridine receptor to complexes of approximately 700 and 3000 kDa in native gels (Fig. 3b). The presence of sodium dodecyl sulfate in the gel matrix did result in a clearer monomer band but had no significant effect on the halothane-induced complex formation (Fig. 3c). In stark contrast, pre-incubation of halothane-treated membranes with sodium dodecyl sulfate completely abolished high-molecular mass bands representing the α_1-subunit of the dihydropyridine receptor. Even at a halothane concentration of 10% (v/v), only an approximately 175-kDa protein band was visualized by monoclonal antibody IIID5 (Fig. 3d).

3.4. Halothane-induced oliogomerization of the ryanodine receptor

To investigate the potential effect of halothane on the sarcoplasmic reticulum Ca^{2+}-release channel, the above described gel systems were also employed for the analysis of ryanodine receptor oligomerization. While the ryanodine receptor runs in native gels of crude microsomes, the sarcoplasmic reticulum and junctional couplings as an apparent monomer (Fig. 4a), incubation with halothane reduced the abundance of this 565-kDa band and concomitantly induced the appearance of a very high-molecular mass band (Fig. 4b). While sodium dodecyl sulfate in the gel matrix did not reduce the electrophoretic mobility of the apparent 3000-kDa band, incubation of halothane-treated membranes with the ionic detergent caused most of the ryanodine receptor to run further into the separating gel. However, in contrast to calsequestrin and the α_1-dihydropyridine receptor, the sodium dodecyl sulfate-treated ryanodine receptor is represented by a much broader band and at high halothane concentrations some of the 3000-kDa complexes are still immunodecorated (Fig. 4d).

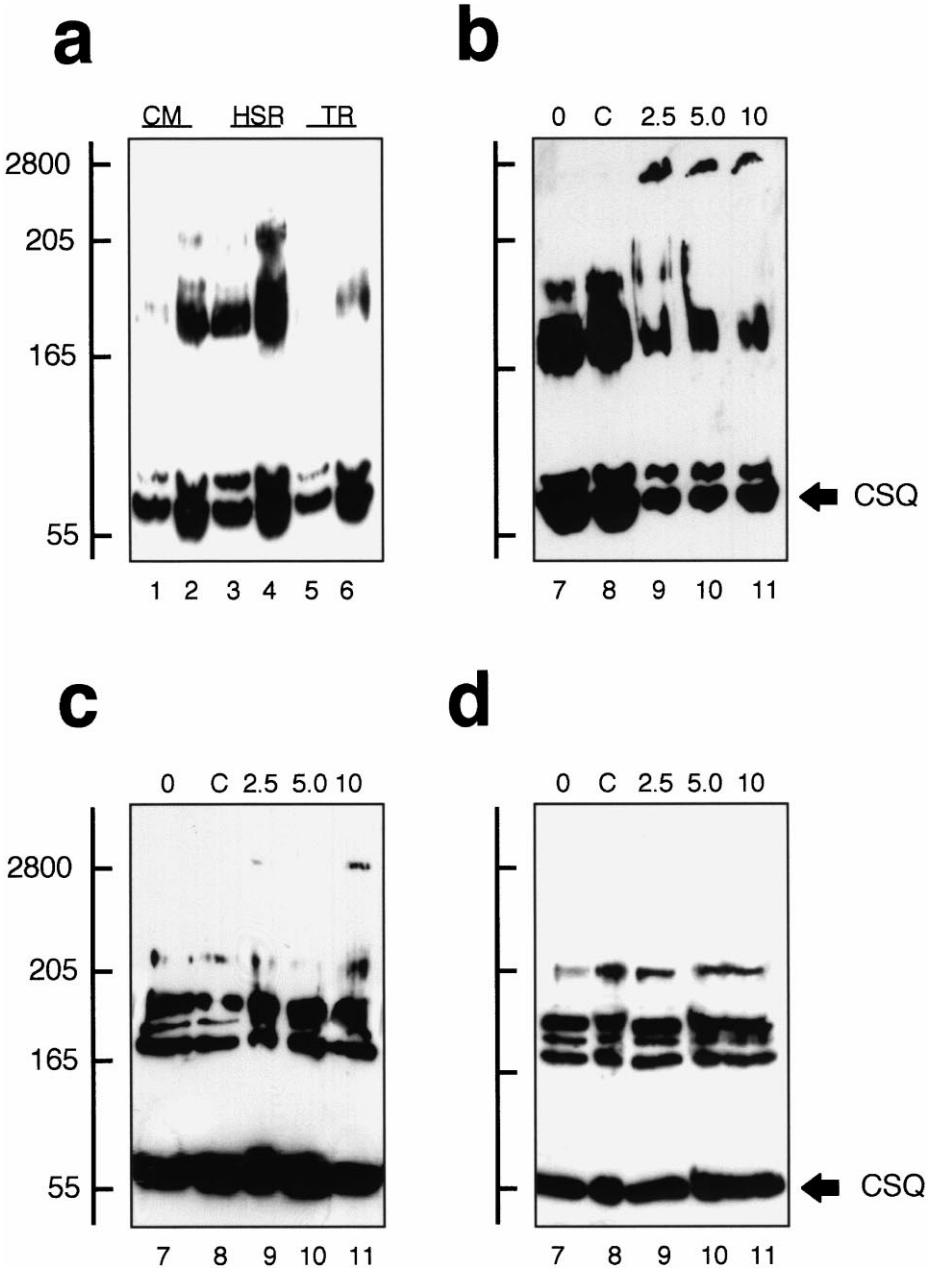

Fig. 2. Halothane-induced oligomerization of the Ca^{2+}-binding protein calsequestrin. Shown are immunoblots transferred from 6% (w/v) gels and immunodecorated with monoclonal antibody $VIIID1_2$ to calsequestrin (CSQ). Lanes 1 to 6 represent 5 and 10 μg protein/lane extracted from crude microsomes (CM), heavy sarcoplasmic reticulum (HSR) and triads (TR), respectively (a). Lanes 7 to 11 represent rabbit skeletal muscle microsomes (15 μg protein/lane) treated with 0, 0, 2.5, 5.0 and 10.0 (v/v) halothane, respectively (b–d). Lane 8 is a control (C) showing microsomes treated with 10% (v/v) acetone but no halothane. Protein samples were dissolved in non-denaturing buffer (a–c) or sodium dodecyl sulfate-containing sample buffer (d) and were then electrophoretically separated for 360 Vh under non-reducing conditions employing a gel system which contained no ionic detergent (a and b) or sodium dodecyl sulfate (c and d). The position of the apparent calsequestrin monomer is marked by an arrow and the sizes of molecular mass standards (in kDa) are indicated on the left.

3.5. Complex formation between excitation–contraction coupling components by halothane

Since Figs. 2–4 clearly demonstrate that halothane triggers oligomerization in triad components involved in excitation–contraction coupling and Ca^{2+}-homeostasis, we were interested in whether the induced high-molecular mass bands exhibited a potential overlap. To address this question, native gels were run under identical conditions and immunoblotted for the ryanodine receptor, the α_1-dihydropyridine receptor and calsequestrin. Fig. 5a–c illustrate that halothane-induced complexes of all three triad proteins are of approximately 3000 kDa. In control experiments it was shown that an abundant marker of the contractile apparatus and a junctional triad component, as well as a major Ca^{2+}-binding protein of the longitudinal sarco-

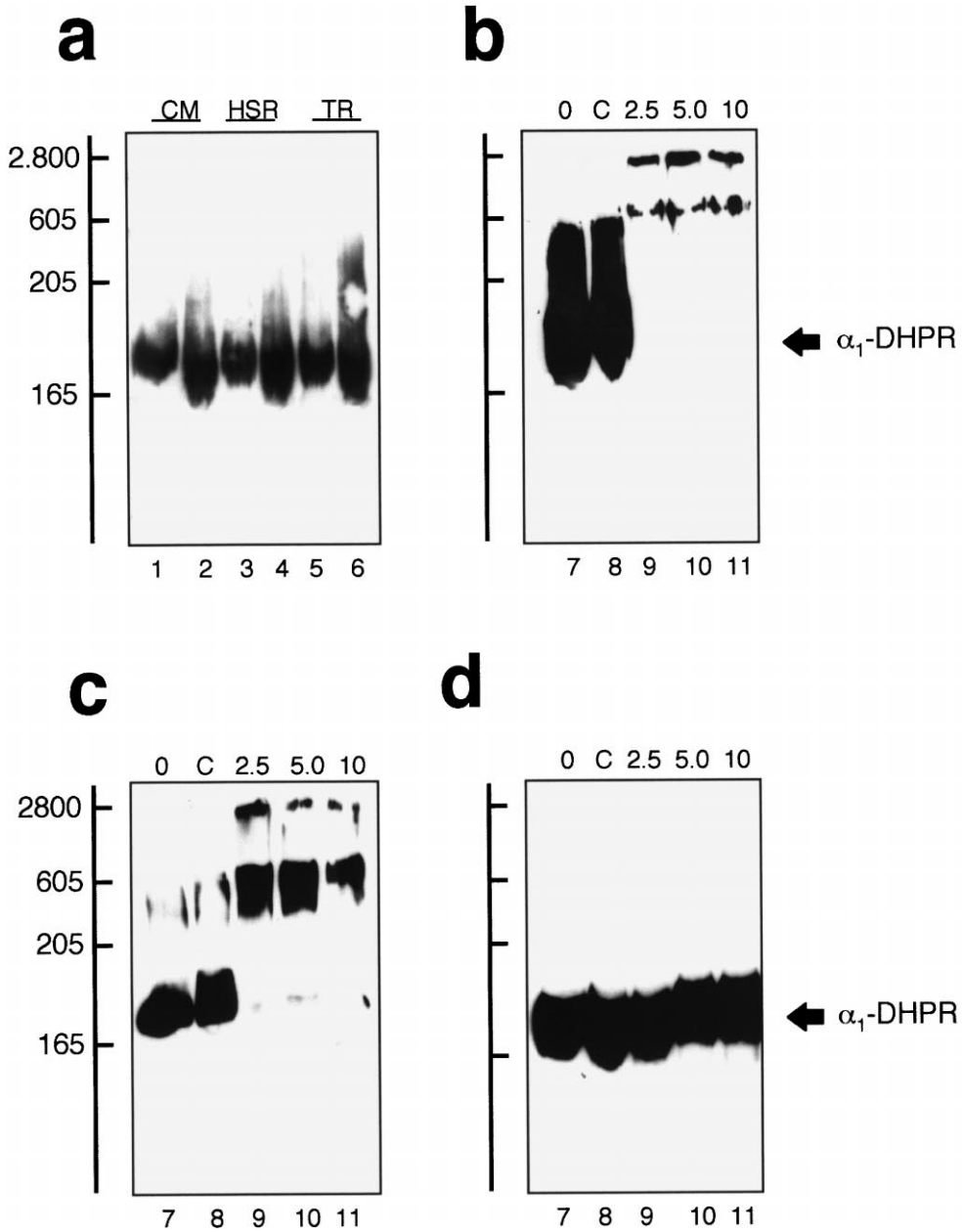

Fig. 3. Halothane-induced oligomerization of the α_1-dihydropyridine receptor. Shown are immunoblots transferred from 6% (w/v) gels and immunodecorated with monoclonal antibody IIID5 to the α_1-subunit of the dihydropyridine receptor (α_1-DHPR). Lanes 1 to 6 represent 5 and 10 μg protein/lane extracted from crude microsomes (CM), heavy sarcoplasmic reticulum (HSR) and triads (TR), respectively (a). Lanes 7 to 11 represent rabbit skeletal muscle microsomes (15 μg protein/lane) treated with 0, 0, 2.5, 5.0 and 10.0 (v/v) halothane, respectively (b–d). Lane 8 is a control (C) showing microsomes treated with 10% (v/v) acetone but no halothane. Protein samples were dissolved in non-denaturing buffer (a–c) or sodium dodecyl sulfate-containing sample buffer (d) and were then electrophoretically separated for 360 Vh under non-reducing conditions employing a gel system which contained no ionic detergent (a and b) or sodium dodecyl sulfate (c and d). The position of the apparent α_1-dihydropyridine receptor monomer is marked by an arrow and the sizes of molecular mass standards (in kDa) are indicated on the left.

plasmic reticulum are not affected by a halothane concentration which clearly caused complex formation of the other junctional components studied. As can be seen in Fig. 5d and e, the dimer of the fast myosin heavy chain and the apparent 170-kDa α_2-subunit of the dihydropyridine receptor do not shift to higher molecular mass complexes following treatment with the inhalational anesthetic. Thus, halothane-induced triad protein clustering does not generally affect rabbit skeletal muscle proteins but appears to be specific for calsequestrin, the α_1-dihydropyridine receptor and the ryanodine receptor at the concentrations used in this study. Labeling with antibodies to sarcoplasmic reticulum Ca^{2+}-ATPases and the junctional component triadin did not reveal enough specific immunodecoration for proper analysis (not shown). We could thus not investigate the effect of halothane on

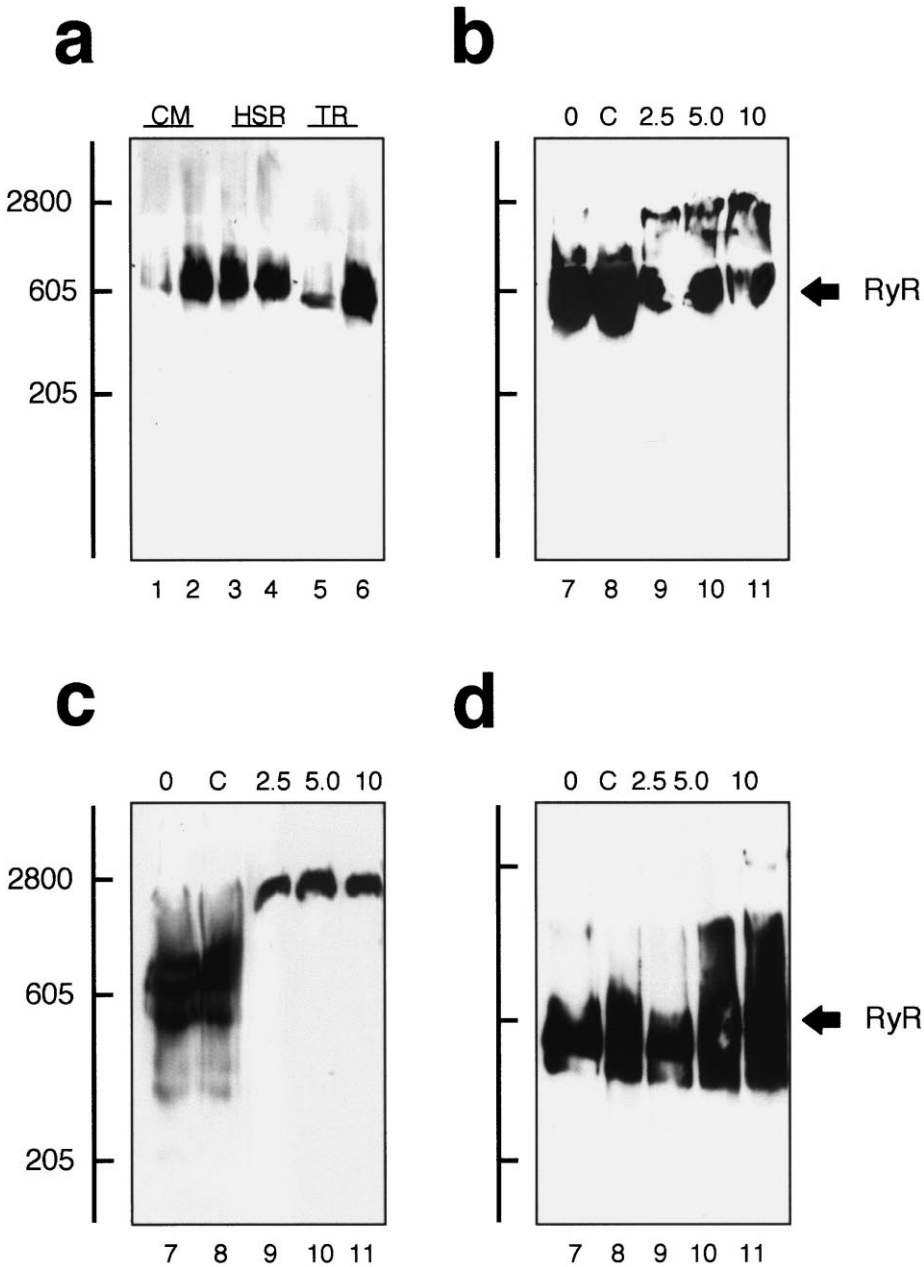

Fig. 4. Halothane-induced oligomerization of the ryanodine receptor Ca^{2+}-release channel. Shown are immunoblots transferred from 5% (w/v) gels and immunodecorated with a polyclonal antibody to the ryanodine receptor (RyR) Ca^{2+}-release channel. Lanes 1 to 6 represent 5 and 10 μg protein/lane extracted from crude microsomes (CM), heavy sarcoplasmic reticulum (HSR) and triads (TR), respectively (a). Lanes 7 to 11 represent rabbit skeletal muscle microsomes (25 μg protein/lane) treated with 0, 0, 2.5, 5.0 and 10.0 (v/v) halothane, respectively (b–d). Lane 8 is a control (C) showing microsomes treated with 10% (v/v) acetone but no halothane. Protein samples were dissolved in non-denaturing buffer (a–c) or sodium dodecyl sulfate-containing sample buffer (d) and were then electrophoretically separated for 440 Vh under non-reducing conditions employing a gel system which contained no ionic detergent (a, b) or sodium dodecyl sulfate (c, d). The position of the apparent ryanodine receptor monomer is marked by an arrow and the sizes of molecular mass standards (in kDa) are indicated on the left.

oligomerization of the abundant sarcoplasmic reticulum Ca^{2+}-pumps and triadin clustering. Immunoblotting with monoclonal antibody XIIC4, which recognizes both sarcalumenin of apparent 160-kDa and the 53-kDa sarcoplasmic reticulum-glycoprotein, revealed no labeling of the above described 3000-kDa molecular mass region. Although the monomers of both sarcalumenin and the 53-kDa sarcoplasmic reticulum-glycoprotein were still recognized at the highest halothane concentration used in this comparative study, a smeared immunodecorative background was evident at 2.5% (v/v) halothane (Fig. 5f).

Fig. 6a–c illustrate halothane-induced oligomerization of excitation–contraction coupling components at 25°C, as compared to 0–4°C shown in Figs. 1–5. A clear difference

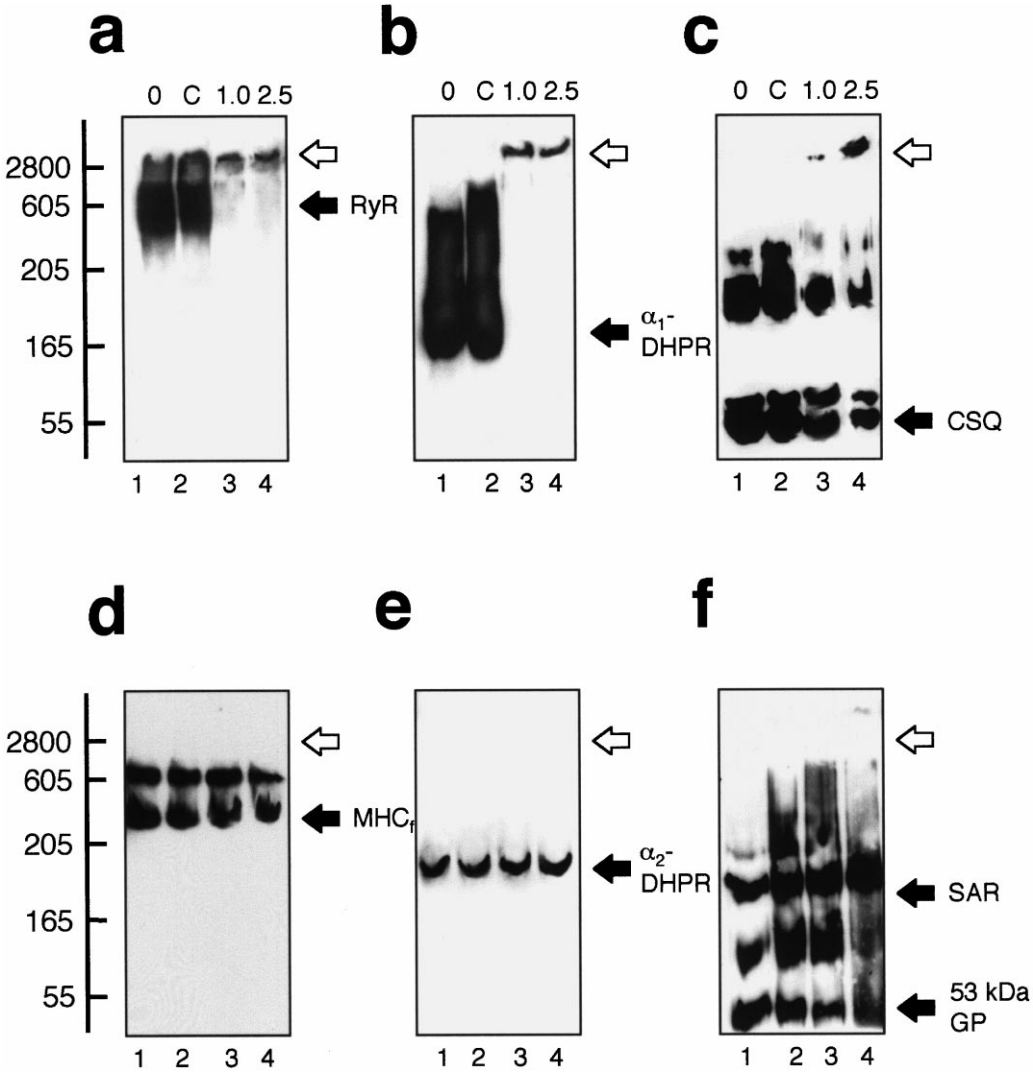

Fig. 5. Complex formation between excitation–contraction coupling components by halothane. Shown are identical immunoblots transferred from 6% (w/v) gels and immunodecorated with a polyclonal antibody to the ryanodine receptor (RyR) (a), monoclonal antibody IIID5 against the α_1-subunit of the dihydropyridine receptor (α_1-DHPR) (b), monoclonal antibody VIIID1$_2$ to calsequestrin (CSQ) (c), monoclonal antibody MY32 against the fast heavy chain of myosin (MHC$_f$) (d), monoclonal antibody 20A to α_2-subunit of the dihydropyridine receptor (α_2-DHPR) (e) and monoclonal antibody XIIC4 to 160 kDa sarcalumenin (SAR) and the 53-kDa sarcoplasmic reticulum-glycoprotein (GP) of the sarcoplasmic reticulum (f). Lanes 1 to 4 represent rabbit skeletal muscle microsomes (15 μg protein/lane) treated with 0, 0, 1.0 and 2.5% (v/v) halothane, respectively. Lane 2 is a control (C) showing microsomes treated with 2.5% (v/v) acetone but no halothane. Protein samples were dissolved in non-denaturing buffer and were then electrophoretically separated for 360 Vh under non-reducing conditions employing a gel system containing no ionic detergent. Apparent monomers are indicated by solid arrows and the relative position of triad complexes is marked by open arrows. The sizes of molecular mass standards (in kDa) are indicated on the left.

in the relative electrophoretic mobility is apparent when one compares the protein species, representing the ryanodine receptor (Fig. 6a), the α_1-dihydropyridine receptor (Fig. 6b) and calsequestrin (Fig. 6c), run under native conditions as compared to samples treated with ionic detergent. On the other hand, immunoblotting of muscle membrane proteins which had been incubated with halothane at 37°C did not reveal strong enough signals for a proper comparative analysis (not shown). Possibly, higher temperatures trigger accelerated rates of proteolytic degradation and thereby negatively interfere with the immunoblot analysis of anesthetic-treated proteins.

Following incubation with halothane, detergent-solubilized complexes were treated with immobilized antibodies to triad receptors in order to achieve immunoprecipitation of anesthetic-induced oligomeric structures (not shown). This approach failed, possibly due to protein–protein and/or halothane–protein interactions that interfered with proper antibody binding to the respective antigen binding sites. However, two-dimensional gel electrophoresis using native gels in the first dimension and standard sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension clearly demonstrated complex formation between the triad receptors and the Ca^{2+}-binding protein

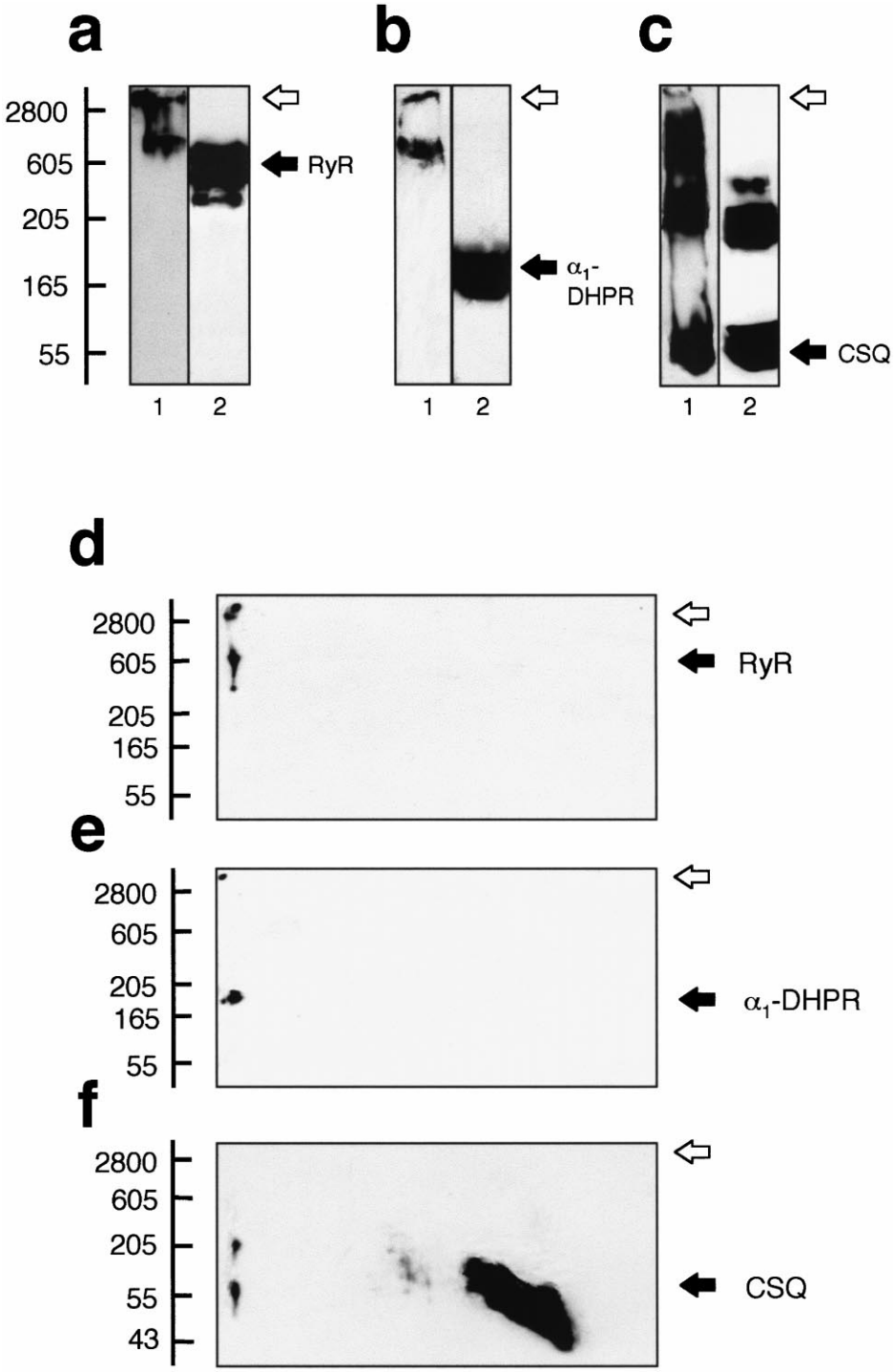

Fig. 6. Two-dimensional immunoblot analysis of halothane-induced complexes between excitation–contraction coupling components. Shown are immunoblots transferred from native gels (a–c, lane 1), sodium dodecyl sulfate-containing gels (a–c, lane 2) and two-dimensional gels (d–f). All samples (15 μg protein/lane) had been incubated with 2.5% (v/v) halothane prior to electrophoretic separation. Two-dimensional gel electrophoresis was performed with 6% (w/v) native gels in the first dimension and 5% (w/v) (d, e) or 6% (w/v) (f) sodium dodecyl sulfate-containing polycarylamide gels in the second dimension. Proteins were separated in the first dimensions for 360 Vh (a–f) and 440 Vh in the second dimension (d–f). Immunodecoration was performed with a polyclonal antibody to the ryanodine receptor (RyR) (a, d), monoclonal antibody IIID5 against the α_1-subunit of the dihydropyridine receptor (α_1-DHPR) (b, e) and monoclonal antibody VIIID1$_2$ to calsequestrin (CSQ) (c and f). Apparent monomers are indicated by solid arrows and the relative position of triad complexes is marked by open arrows. The sizes of molecular mass standards (in kDa) are indicated on the left.

studied. Fig. 6d and e show that most of the ryanodine receptor and the α_1-dihydropyridine receptor form extremely high-molecular mass complexes following halothane treatment. On the other hand, the anesthetic appears to induce oligomerization of only a small percentage of the overall calsequestrin population (Fig. 6f). Im-

portantly, on the second dimension slab gel, separation of the halothane-induced protein complexes caused the three different protein species to move again at a higher relative electrophoretic mobility, mostly representing the monomeric state. Hence, under native conditions halothane induces a specific aggregation of these excitation–contraction coupling components.

4. Discussion

Since a diverse group of simple substances produce narcotic effects and because a close correlation exists between anesthetic potency and lipid solubility, inhalation anesthetics have long been regarded as non-specific inhibitors of the central nervous system. It has been proposed that interference with the electrical excitability and impulse propagation in neurons is due to the uptake of anesthetics into the hydrophobic interior of the neuronal plasmalemma (Koblin, 1994). This view has changed dramatically in recent years and the major action of anesthetics is no longer believed to be based on perturbation of lipid bilayers, but rather is likely to be due to direct interactions with neuronal proteins (Franks and Lieb, 1994; Eckenhoff and Johansson, 1997). Although the pharmacology of anesthetics may vary between different types of neurons, their major mechanism of action appears to be the modification of ion channel activity in chemical synapses. Neurotransmitter-gated receptor channels shown to be sensitive to anesthetics include the nicotinic acetylcholine receptor (Dickinson et al., 1995; Raines and McClure, 1997), the 5-hydroxytryptamine receptor (Jenkins et al., 1996), as well as the γ-aminobutyric acid and glycine receptors (Mihic et al., 1997). In analogy to these findings and since halothane has an effect on the function of Ca^{2+}-regulatory membrane proteins of the sarcoplasmic reticulum (Louis et al., 1992; Bull and Marengo, 1994; Connelly and Coronado, 1994; Beltran et al., 1996; Kosk-Kosicka et al., 1997), we studied the effect of halothane on the complex formation of important skeletal muscle membrane components involved in voltage-sensing, Ca^{2+}-release and Ca^{2+}-sequestration.

The immunoblot analysis presented in this report reveals that halothane induces oligomerization of the ryanodine receptor Ca^{2+}-release channel, the α_1-subunit of the dihydropyridine receptor and calsequestrin. In native gels, the ryanodine receptor, the α_1-dihydropyridine receptor and calsequestrin exhibited not only a greatly reduced electrophoretic mobility in response to halothane, but distinct overlapping immunodecoration was observed between the high-molecular mass bands of the two triad receptors and the Ca^{2+}-binding protein. Thus, the volatile anesthetic investigated appears to have a direct effect on protein–protein interactions between Ca^{2+}-regulatory proteins within skeletal muscle triads. Since junctional couplings of skeletal muscle membranes are formed by contact zones between a central transverse-tubule and two surrounding terminal cisternae of the sarcoplasmic reticulum (Franzini-Armstrong and Jorgensen, 1994), protein complex formation was induced between receptors existing in two different membrane systems and clusters of a peripheral protein, calsequestrin, which is present in the terminal cisternae lumen. Incubation with the ionic detergent sodium dodecyl sulfate resulted in the disintegration of the halothane-induced complex. Hence, the induction of covalent bonds between triad receptors via halothane or halothane-derived metabolites is unlikely. Possibly, halothane-mediated hydrophobic interactions between Ca^{2+}-regulatory membrane proteins account for the biological cross-linking observed. This was previously shown to be the case in interactions between serum albumin and halothane (Eckenhoff, 1996). However, the non-covalent bonding must be relatively strong since in sodium dodecyl sulfate polyacrylamide gel electrophoresis lacking ionic detergent in the sample buffer, similar shifts for the ryanodine receptor Ca^{2+}-release channel and the α_1-subunit of the dihydropyridine receptor were produced as had been observed in native gels.

The effect of halothane on muscle protein oligomerization described in this study agrees with the more recently established protein theory of anesthetic action (Franks and Lieb, 1997) and might at least partially explain the response of muscle Ca^{2+}-release units to halothane. On the molecular level, anesthetic agents seem to directly modulate receptor activity (Mihic et al., 1997), so halothane-induced complex formation between triad receptors and calsequestrin is not implausible. Direct physical interactions between the voltage-sensing α_1-subunit of the dihydropyridine receptor and the ryanodine receptor Ca^{2+}-release channel are believed to be the underlying mechanism of excitation–contraction coupling in adult skeletal muscle fibers (Fleischer and Inui, 1989; Rios et al., 1991; Melzer et al., 1995). Both Ca^{2+}-channels are pharmacologically distinct receptors. The ryanodine receptor function can be modulated by an enormous amount of exogenous substances as reviewed by Zucchi and Ronca-Testoni (1997). The sarcoplasmic reticulum Ca^{2+}-channel, however, is insensitive to the action of blocking agents of the transverse-tubular L-type Ca^{2+}-channel, such as verapamil, diltiazem and nifedipine. In contrast, halothane appears to have a profound effect on both triad receptors and the anesthetic-induced biological cross-linking could trigger Ca^{2+}-release by direct physical means. Complex formation between the α_1-subunit of the dihydropyridine receptor and the ryanodine receptor might trigger transient opening of the Ca^{2+}-release channel. This supports the increased Ca^{2+}-release from microsomal vesicles from muscle in response to volatile anesthetics (Bull and Marengo, 1994; Connelly and Coronado, 1994; Louis et al., 1992; Beltran et al., 1996; Zucchi and Ronca-Testoni, 1997). Since the medium-affinity, high-capacity Ca^{2+}-binding protein calse-

questrin might actually be an endogenous regulator of the ryanodine receptor (Donoso et al., 1995; Hidalgo and Donoso, 1995), halothane-induced complex formation between calsequestrin and the Ca^{2+}-release channel also agrees with the above described physiological changes by halothane. Possibly changes in the protein–protein interactions between the Ca^{2+}-binding protein and the ryanodine receptor trigger the rapid release of Ca^{2+}-ions from calsequestrin and thus provide a large amount of this second messenger molecule for release via the junctional channel pore.

The response of native muscle membrane proteins essential for signal transduction and Ca^{2+}-homeostasis to incubation with halothane also has potential implications for explaining the response of malignant hyperthermia-susceptible skeletal muscle fibers to volatile anesthetics. If clinical concentrations of halothane induce complex formation between the α_1-dihydropyridine receptor, the ryanodine receptor and calsequestrin, which then triggers opening of the Ca^{2+}-release channel, this might result only in a negligible disturbance of Ca^{2+}-homeostasis in healthy skeletal muscle. In normal skeletal muscle, the ryanodine receptor should be only transiently open and might cause only a slight change in the cytosolic Ca^{2+}-concentration. In stark contrast, the mutated RyR1 isoform found in individuals susceptible to malignant hyperthermia, might stay open much longer and thus results in a pathophysiological increase in cytosolic Ca^{2+}-levels (Mickelson and Louis, 1996). Future experiments could address potential differences of halothane effects on protein–protein interactions in normal skeletal muscle vs. muscle fibers from patients susceptible to malignant hyperthermia. However, an essential prerequisite for such a study is the availability of highly specific antibodies to all key Ca^{2+}-regulatory membrane proteins which cross-react with human muscle tissues. In addition, a sufficient amount of human muscle tissue has to be accessible for the preparation of microsomal preparations. In conclusion, we could show here that halothane has a direct effect on muscle membrane proteins involved in excitation–contraction coupling. This is not only potentially important for understanding the pathophysiology of malignant hyperthermia, but is a further demonstration that inhalational anesthetics can directly interact with proteins thus confirming the protein hypothesis of the effect of volatile substances (Franks and Lieb, 1997).

Acknowledgements

This work was supported by the Training and Mobility of Researchers Network Grant FMRX-CT960032 from the European Community. We thank Dr. Kevin P. Campbell (Howard Hughes Medical Institute, Iowa City, IA, USA) for the generous gift of monoclonal antibodies to muscle membrane proteins.

References

Beltran, M., Bull, R., Donoso, P., Hidalgo, C., 1996. Ca^{2+}- and pH-dependent halothane stimulation of Ca^{2+} release in sarcoplasmic reticulum from frog muscle. Am. J. Physiol. 271, C540–C546.

Block, B.A., Imagawa, T., Campbell, K.P., Franzini-Armstrong, C., 1988. Structural evidence for direct interaction between the molecular components of the transverse-tubules/sarcoplasmic reticulum junction in skeletal muscle. J. Cell Biol. 107, 2587–2600.

Bradd, S.J., Dunn, M.J., 1993. Analysis of membrane proteins by Western blotting and enhanced chemiluminescence. Methods Mol. Biol. 19, 211–218.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brilliantes, A.B., Ondrias, K., Scott, A., Kobrinsky, E., Ondriasova, E., Moschella, M., Jayaraman, T., Landers, M., Ehrlich, B., Marks, A.M., 1994. Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein. Cell 77, 513–523.

Bull, R., Marengo, J.J., 1994. Calcium-dependent halothane activation of sarcoplasmic reticulum calcium channels from frog skeletal muscle. Am. J. Physiol. 266, C391–C396.

Catterall, W.A., 1995. Structure and function of voltage-gated ion channels. Annu. Rev. Biochem. 64, 493–532.

Chang, C.F., Hosey, M.M., 1990. A combined non-denaturating and denaturating gel electrophoretic analysis of the subunit composition of a membrane protein: the skeletal muscle L-type calcium channel. Biochem. Biophys. Res. Commun. 172, 751–758.

Connelly, T.J., Coronado, R., 1994. Activation of the Ca^{2+} release channel of cardiac sarcoplasmic reticulum by volatile anesthetics. Anesthesiology 81, 459–469.

Dickinson, R., Lieb, W.R., Franks, N.P., 1995. The effects of temperature on the interactions between volatile general anesthetics and a neuronal nicotinic acetylcholine receptor. Br. J. Pharmacol. 116, 2949–2956.

Donoso, P., Prieto, H., Hidalgo, C., 1995. Luminal calcium regulates calcium release in triads isolated from frog and rabbit skeletal muscle. Biophys. J. 68, 507–515.

Dunn, M.J., Bradd, S.J., 1993. Separation and analysis of membrane proteins by SDS–polyacrylamide gel electrophoresis. Methods Mol. Biol. 19, 203–210.

Eckenhoff, R.G., 1996. Amino acid resolution of halothane binding sites in serum albumin. J. Biol. Chem. 271, 15521–15526.

Eckenhoff, R.G., Johansson, J.S., 1997. Molecular interactions between inhaled anethetics and proteins. Pharmacol. Rev. 49, 343–367.

Fleischer, S., Inui, M., 1989. Biochemistry and biophysics of excitation–contraction coupling. Annu. Rev. Biophys. Biophys. Chem. 18, 333–364.

Fliegel, L., Ohnishi, M., Carpenter, M.R., Khanna, V.K., Reithmeier, R.A.F., MacLennan, D.H., 1987. Amino acid sequence of rabbit fast-twitch skeletal muscle calsequestrin deduced from cDNA and peptide sequencing. Proc. Natl. Acad. Sci. USA 84, 1167–1171.

Franks, N.P., Lieb, W.R., 1994. Molecular and cellular mechanisms of general anesthesia. Nature 367, 607–613.

Franks, N.P., Lieb, W.R., 1997. Anesthetics set their sites on ion channels. Nature 389, 334–335.

Franzini-Armstrong, C., Jorgensen, A.O., 1994. Structure and development of E–C coupling units in skeletal muscle. Annu. Rev. Physiol. 56, 509–534.

Franzini-Armstrong, C., Protasi, F., 1997. Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. Physiol. Rev. 77, 699–729.

Gurnett, C.A., Campbell, K.P., 1996. Transmembrane auxiliary subunits of voltage-dependent ion channels. J. Biol. Chem. 271, 27975–27978.

Hidalgo, C., Donoso, P., 1995. Luminal calcium regulation of calcium release from sarcoplasmic reticulum. Biosci. Rep. 15, 387–397.

Jenkins, A., Franks, N.P., Lieb, W.R., 1996. Actions of general anesthetics on 5-HT_3 receptors in N1E-115 neuroblastoma cells. Br. J. Pharmacol. 117, 1507–1515.

Jorgensen, A.O., Shen, A.C.Y., Campbell, K.P., MacLennan, D.H., 1983. Ultrastructural localization of calsequestrin in rat skeletal muscle by immunoferritin labeling of ultrathin frozen sections. J. Cell Biol. 97, 1573–1581.

Karon, B.S., Thomas, D.D., 1993. Molecular mechanisms of Ca-ATPase activation by halothane in sarcoplasmic reticulum. Biochemistry 32, 7503–7511.

Karon, B.S., Geddis, L.M., Kutchai, H., Thomas, D.D., 1995. Anesthetics alter the physical and functional properties of the Ca-ATPase in cardiac sarcoplasmic reticulum. Biophys. J. 68, 936–945.

Koblin, D.D., 1994. Mechanisms of action. In: Miller, R.D. (Ed.), Anesthesia, 4th edn. Livingstone, New York, pp. 67–100.

Kosk-Kosicka, D., Fomitcheva, I., Lopez, M.M., Eckenhoff, R.G., 1997. Heterogeneous halothane binding in the SR Ca^{2+}-ATPase. FEBS Lett. 402, 189–192.

Laemmli, U.K., 1970. Cleavage of bacteriophage T7 early RNAs and proteins on slab gels. Nature 227, 680–685.

Leberer, E., Timms, B.G., Campbell, K.P., MacLennan, D.H., 1990. Purification, calcium binding properties and ultrastructural localization of the 53,000- and 160,000 (sarcalumenin)-Da glycoproteins of the sarcoplasmic reticulum. J. Biol. Chem. 265, 10118–10124.

Lopez, M.M., Kosk-Kosicka, D., 1995. How do volatile anesthetics inhibit Ca^{2+}-ATPases?. J. Biol. Chem. 270, 28239–28245.

Louis, C.F., Zualkernan, K., Roghair, T., Mickelson, J.R., 1992. The effects of volatile anesthetics on calcium regulation by malignant hyperthermia-susceptible sarcoplasmic reticulum. Anesthesiology 77, 114–125.

MacLennan, D.H., Wong, P.T.S., 1971. Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc. Natl. Acad. Sci. USA 68, 1231–1235.

MacLennan, D.H., Duff, C., Zorzato, F., Fujii, J., Philipps, M., Korneluk, R.G., Frochis, W., Britt, B.A., Worton, R.G., 1990. Ryanodine receptor gene is a candidate for predisposition to malignant hyperthermia. Nature 343, 559–561.

MacLennan, D.H., Rice, W.J., Green, N.M., 1997. The mechanism of Ca^{2+} transport by sarco(endo)plasmic reticulum Ca^{2+}-ATPases. J. Biol. Chem. 272, 28815–28818.

Maguire, P.B., Ohlendieck, K., 1996. Oligomerization of sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle. FEBS Lett. 396, 115–118.

Maguire, P.B., Briggs, F.N., Lennon, N.J., Ohlendieck, K., 1997. Oligomerization is an intrinsic property of calsequestrin in normal and transformed skeletal muscle. Biochem. Biophys. Res. Commun. 240, 721–727.

Martonosi, A.N., 1995. The structure and interactions of Ca^{2+}-ATPase. Biosci. Rep. 15, 263–282.

Marty, I., Robert, M., Villaz, M., DeJongh, K., Lai, L., Catterall, W.A., Ronjat, M., 1994. Biochemical evidence for a complex involving dihydropyridine receptor and ryanodine receptor in triad junctions of skeletal muscle. Proc. Natl. Acad. Sci. USA 91, 2270–2274.

Meissner, G., Lu, X., 1995. Dihydropyridine receptor–ryanodine receptor interactions in skeletal muscle excitation–contraction coupling. Biosci. Rep. 15, 399–408.

Melzer, W., Herrmann-Frank, A., Luettgau, H.C., 1995. The role of Ca^{2+} ions in excitation–contraction coupling of skeletal muscle fibres. Biochim. Biophys. Acta 1241, 59–116.

Mickelson, J.R., Louis, C.F., 1996. Malignant hyperthermia: excitation–contraction coupling, Ca^{2+} release channel and cell Ca^{2+} regulation defects. Physiol. Rev. 76, 537–592.

Mihic, S.J., Ye, Q., Wick, M.J., Koltchine, V.V., Krasowski, M.D., Finn, S.E., Mascia, M.P., Valenzuela, C.F., Hanson, K.K., Greenblatt, E.P., Harris, R.A., Harrison, N.L., 1997. Sites of alcohol and volatile anesthetic action on $GABA_A$ and glycine receptors. Nature 389, 385–389.

Monnier, N., Procaccio, V., Stieglitz, P., Lunardi, J., 1997. Malignant-hyperthermia susceptibility is associated with a mutation of the alpha 1-subunit of the human dihydropyridine-sensitive L-type voltage-dependent calcium-channel receptor in skeletal muscle. Am. J. Hum. Genet. 60, 1316–1325.

Murray, B.E., Ohlendieck, K., 1997. Cross-linking analysis of the ryanodine receptor and α_1-dihydropyridine receptor in rabbit skeletal muscle. Biochem. J. 324, 689–696.

Murray, B.E., Ohlendieck, K., 1998. Complex formation between calsequestrin and the ryanodine receptor in fast- and slow-twitch rabbit skeletal muscle. FEBS Lett. 429, 317–322.

Murray, B.E., Froemming, G.R., Maguire, P.B., Ohlendieck, K., 1998. Excitation–contraction–relaxation cycle: role of the Ca^{2+}-regulatory membrane proteins in normal, stimulated and pathological skeletal muscle: review. Int. J. Mol. Med. 1, 677–687.

Ohlendieck, K., Ervasti, J.M., Snook, J.B., Campbell, K.P., 1991. Dystrophin–glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. J. Cell Biol. 112, 135–148.

Quane, K.A., Keating, K.E., Manning, B.M., Healy, J.M.S., Monsieurs, K., Heffron, J.J.A., Lehane, M., Heytens, L., Krivosic-Horber, R., Adnet, P., Ellis, F.R., Monnier, N., Lunardi, J., McCarthy, T.V., 1994. Detection of a novel common mutation in the ryanodine receptor gene in malignant hyperthermia: implications for diagnosis and heterogeneity studies. Hum. Mol. Genet. 3, 471–476.

Raines, D.E., McClure, K.B., 1997. Halothane interactions with nicotinic acetylcholine receptor membranes. Anesthesiology 86, 476–486.

Rios, E., Ma, J., Gonzalez, A., 1991. The mechanical hypothesis of excitation–contraction (EC) coupling in skeletal muscle. J. Muscle Res. Cell Motil. 12, 127–135.

Rosemblatt, M., Hidalgo, C., Vergara, C., Ikemoto, N., 1981. Immunological and biochemical properties of the transverse-tubule membranes isolated from rabbit skeletal muscle. J. Biol. Chem. 256, 8140–8148.

Sharp, A.H., Imagawa, T., Leung, A.T., Campbell, K.P., 1987. Identification and characterization of the dihydropyridine-binding subunit of the skeletal muscle dihydropyridine receptor. J. Biol. Chem. 262, 12309–12315.

Taraschi, T.F., Lee, Y.C., Janes, N., Rubin, E., 1991. Anesthetic potency and conformational stability in membranes. Ann. New York Acad. Sci. 625, 698–706.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

Yano, K., Zarain-Herzberg, A., 1994. Sarcoplasmic reticulum calsequestrins: structural and functional properties. Mol. Cell Biochem. 135, 61–70.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y.M., Jones, L.R., 1997. Complex formation between junctin, triadin, calsequestrin and the ryanodine receptor: proteins of the cardiac junctional sarcoplasmic reticulum membrane. J. Biol. Chem. 272, 23389–23397.

Zucchi, R., Ronca-Testoni, S., 1997. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol. Rev. 49, 1–51.