Chloroform extract of hog barn dust modulates skeletal muscle ryanodine receptor calcium-release channel (RyR1)

Chengju Tian,1 Chun Hong Shao,1 Danielle S. Fenster,1 Mark Mixan,1 Debra J. Romberger,2,3 Myron L. Toews,1 and Keshore R. Bidasee1,3,4

Departments of 1Pharmacology and Experimental Neuroscience, 2Internal Medicine, Pulmonary and Critical Care Medicine Section, and 3Environmental, Agricultural and Occupational Health, University of Nebraska Medical Center, Omaha; and 4Nebraska Redox Biology Center, Lincoln, Nebraska

Submitted 4 February 2010; accepted in final form 22 June 2010

Tian C, Shao CH, Fenster DS, Mixan M, Romberger DJ, Toews ML, Bidasee KR. Chloroform extract of hog barn dust modulates skeletal muscle ryanodine receptor calcium-release channel (RyR1). J Appl Physiol 109: 830–839, 2010. First published June 24, 2010; doi:10.1152/japplphysiol.00123.2010.—Skeletal muscle weakness is a reported ailment in individuals working in commercial hog confinement facilities. To date, specific mechanisms responsible for this symptom remain undefined. The purpose of this study was to assess whether hog barn dust (HBD) contains components that are capable of binding to and modulating the activity of type 1 ryanodine receptor Ca2+-release channel (RyR1), a key regulator of skeletal muscle function. HBD collected from confinement facilities in Nebraska were extracted with chloroform, filtered, and rotary evaporated to dryness. Residues were resuspended in hexane-chloroform (20:1) and precipitated, referred to as HBDorg, were air-dried and studied further. In competition assays, HBDorg dose-dependently displaced [3H]ryanodine from binding sites on RyR1 with an IC50 of 1.5 ± 0.1 μg/ml (Ki = 0.4 ± 0.0 μg/ml). In single-channel assays using RyR1 reconstituted into a lipid bilayer, HBDorg exhibited three distinct dose-dependent effects: first it increased the open probability of RyR1 by increasing its gating frequency and dwell time in the open state, then it induced a state of reduced conductance (55% of maximum) that was more likely to occur and persist at positive holding potentials, and finally it irreversibly closed RyR1. In differentiated C2Cl2 myotubes, addition of HBD triggered a rise in intracellular Ca2+ that was blocked by pretreatment with ryanodine. Since persistent activation and/or closure of RyR1 results in skeletal muscle weakness, these new data suggest that HBD is responsible, at least in part, for the muscle ailment reported by hog confinement workers.

PORK CONSUMPTION IS INCREASING WORLDWIDE, and to keep up with demand, farmers are continuously expanding the size of their operations (26, 36). In developed and middle-income countries, hogs are housed in large confinement facilities, where they are fed a diet rich in grains and beans in preparation for market. The dust from the feed mixes with feces, urine, dander, bacteria, molds, and pungent volatile substances emanating from nearby manure pits to produce what is referred to as hog barn dust (HBD) (9, 11, 38). A growing body of literature from nearby manure pits to produce what is referred to as hog barn dust (HBD) (9, 11, 38). A growing body of literature
Spectrum Laboratories (Rancho Dominguez, CA). All other reagents and solvents used were of the highest grade commercially available.

**Extraction of HBD**

HBD collected 1–2 m from the floor of hog confinement facilities in Nebraska were extracted three times with chloroform (CHCl₃, 5 g in 70 ml for 1 h × 3 extractions). Pooled extracts from each sample were gravity filtered (Whatman no. 1 paper) and rotary evaporated to dryness (HBDCHCl₃). HBDCHCl₃ were then redissolved in hexane-CHCl₃ (20:1, 40 ml), and the resultant precipitates were collected by gravity filtration, air-dried, and labeled HBDorg.

**Composition of HBDorg**

The composition of HBDorg was determined by spotting on silica gel thin layer plates and chromatographed using varying ratios of CHCl₃-methanol (MeOH) with either triethylamine (TEA) or acetic acid (HAc). Compounds were visualized under 365-nm wavelength UV light. Plates were sprayed with 1% sulphuric acid and heated to dryness (HBDCHCl₃). HBDCHCl₃ were then redissolved in hexane-CHCl₃ (20:1, 40 ml), and the resultant precipitates were collected by gravity filtration, air-dried, and labeled HBDorg.

**Preparation of SR Vesicles**

The University of Nebraska Medical Center, Institutional Animal Care and Use Committee approved rabbits used for the study, and their short-term housing adhered to the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals (27). Crude SR membrane vesicles (SR vesicles) were prepared from back muscle (28) according to a protocol later by Lai and coworkers (20) with some modifications. SR vesicles were fractionated by placing on top a discontinuous sucrose gradient (26 ml each, 0.8, 1.0, 1.2, and 1.5 M) and centrifuging at 110,000 gav for 2 h (Ti45 rotor). The vesicles at the interface between 1.2 and 1.5 M sucrose were collected, resuspended in fresh isolation buffer, and harvested by centrifugation at 110,000 gav for 2 h. The resultant pellet enriched in junctional SR vesicles was then solubilized in buffer (19 ml) containing 1.0 M NaCl, 0.05 mM EGTA, 0.35 mM Ca²⁺, 5 mM AMP, 20 mM Na/PIPES, pH 7.4, 0.3 mM Pefabloc, 0.03 mM leupeptin, 0.9 mM DTT, 1.5% CHAPS, and 5 mg/ml phosphati-dylcholine for 10 min at room temperature (24°C), followed by 1 h incubation in ice. CHAPS-insoluble material was removed by centrifugation at 26,163 gav (Beckman Ultra Centrifuge, Ti75 rotor) for 30 min at 4°C, and the supernatant was further fractionated on a linear sucrose gradient (7–15% sucrose, 34 ml per tube, 6 tubes, 3 ml of solubilized protein per tube) by centrifugation at 89,454 gav (Beckman Ultra Centrifuge, SW28 rotor) for 17 h at 4°C. [³H]ryanodine (3.3 nM) was added to one of the tubes to serve as a guide for RyR1 purification. After centrifugation, 2.0-ml aliquots from the [³H]ryanodine-labeled tube were collected from the bottom of the tube upwards and counted to determine the location of [³H]ryanodine and RyR1. Twenty-microliter volumes from each aliquot were mixed with gel dissociation medium and electrophoresed on 4–15% linear gradient Tris-glycine polyacrylamide gel at 150 V for 180 min and then silver stained according to manufacturer’s protocol (BioRad, Burlingame, CA). RyR1-containing fractions from the five unlabeled tubes were pooled and dialyzed at 4°C for 44 h in buffer containing 0.5 M NaCl, 0.1 mM EGTA, 0.2 mM CaCl₂, 10 mM Na/PIPES, pH 7.4, 0.13 mM DTT, and 0.4 mM PMSF with three buffer changes at 2, 6, and 12 h.

**Preparation of RyR1 Reconstituted into Proteoliposomes**

Proteoliposomes containing RyR1 were prepared as described earlier by Lai and coworkers (20) with some modifications. SR vesicles were fractionated by placing on top a discontinuous sucrose gradient (26 ml each, 0.8, 1.0, 1.2, and 1.5 M) and centrifuging at 110,000 gav for 2 h (Ti45 rotor). The vesicles at the interface between 1.2 and 1.5 M sucrose were collected, resuspended in fresh isolation buffer, and harvested by centrifugation at 110,000 gav for 2 h. The resultant pellet enriched in junctional SR vesicles was then solubilized in buffer (19 ml) containing 1.0 M NaCl, 0.05 mM EGTA, 0.35 mM Ca²⁺, 5 mM AMP, 20 mM Na/PIPES, pH 7.4, 0.3 mM Pefabloc, 0.03 mM leupeptin, 0.9 mM DTT, 1.5% CHAPS, and 5 mg/ml phosphatidylycholine for 10 min at room temperature (24°C), followed by 1 h incubation in ice. CHAPS-insoluble material was removed by centrifugation at 26,163 gav (Beckman Ultra Centrifuge, Ti75 rotor) for 30 min at 4°C, and the supernatant was further fractionated on a linear sucrose gradient (7–15% sucrose, 34 ml per tube, 6 tubes, 3 ml of solubilized protein per tube) by centrifugation at 89,454 gav (Beckman Ultra Centrifuge, SW28 rotor) for 17 h at 4°C. [³H]ryanodine (3.3 nM) was added to one of the tubes to serve as a guide for RyR1 purification. After centrifugation, 2.0-ml aliquots from the [³H]ryanodine-labeled tube were collected from the bottom of the tube upwards and counted to determine the location of [³H]ryanodine and RyR1. Twenty-microliter volumes from each aliquot were mixed with gel dissociation medium and electrophoresed on 4–15% linear gradient Tris-glycine polyacrylamide gel at 150 V for 180 min and then silver stained according to manufacturer’s protocol (BioRad, Burlingame, CA). RyR1-containing fractions from the five unlabeled tubes were pooled and dialyzed at 4°C for 44 h in buffer containing 0.5 M NaCl, 0.1 mM EGTA, 0.2 mM CaCl₂, 10 mM Na/PIPES, pH 7.4, 0.13 mM DTT, and 0.4 mM PMSF with three buffer changes at 2, 6, and 15 h.

**A** HBDorg in basic mobile phases

<table>
<thead>
<tr>
<th>Chloroform: methanol: triethylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>97:3:0.05</td>
</tr>
<tr>
<td>93:7:0.05</td>
</tr>
<tr>
<td>85:15:0.05</td>
</tr>
</tbody>
</table>

**B** HBDorg in acidic mobile phases

<table>
<thead>
<tr>
<th>Chloroform: methanol: acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>93:7:0.05</td>
</tr>
<tr>
<td>85:15:0.05</td>
</tr>
</tbody>
</table>

**C** HBD and Ryanodine

<table>
<thead>
<tr>
<th>Chloroform: methanol: triethylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>88:12 0.05</td>
</tr>
</tbody>
</table>

Fig. 1. Chromatographic mobilities of hog barn dust (HBD) chloroform extract (HBDCHCl₃) precipitates (HBDorg). A and B: 5 µl from 5 mg/ml solutions of HBDorg spotted on a thin-layer silica gel plate and developed for 45 min in solvents containing mixtures of CHCl₃/MeOH with either triethylamine (A) or acetic acid (B). The plates were air-dried, visualized under 365-nm wavelength light, and photographed. Rₜ values were determined by measuring the distance the compound moved from the origin relative to the distance moved by the solvent. C: 10 µl from 5 mg/ml solution of HBDorg and 10 µl from 1 g/10 ml solution of HBD aqueous extract (HBDaq) spotted on a thin-layer silica gel plate alongside ryanodine (10 µl from 10 mg/ml).
Competition Binding Assays

Competition binding assays were used to assess the ability of HBDorg to bind to RyR1. This assay was also used to calculate IC50 and Ki of HBDorg, as well as general information on the location(s) of RyR1-selective ligand ryanodine was run concurrently in each assay. Displacement data were fitted using nonlinear regression to a one- and a two-site competition model given by equations as described by Motulsky and Christopoulos (25) respectively.

\[
Y = Y_{\text{min}} + \left( Y_{\text{max}} - Y_{\text{min}} \right) \frac{1}{1 + 10^{(X - \log EC_{50})/L}} \quad (1)
\]

\[
Y = Y_{\text{min}} + \left( Y_{\text{max}} - Y_{\text{min}} \right) \frac{1}{1 + 10^{(X - \log EC_{50})/L}} \left( \frac{1}{\text{fraction1}} \right) \left( 1 + 10^{(X - \log EC_{50})/L \text{fraction1}} \right) \quad (2)
\]

where Y is specific binding, X is the logarithm of the amount of the unlabeled ligand, and log IC50 is the competitor concentration that displaces one-half of the specifically bound [3H]ryanodine. Goodness of fit was evaluated by F-test.

Equilibrium inhibition constant (Ki) for HBDorg was calculated using the Cheng–Prusoff relationship (6) given by equation 3

\[
K_i = IC_{50}/\left(1 + (L/K_L)\right) \quad (3)
\]

where L is the concentration of [3H]ryanodine used (6.7 nM) and K_L is the equilibrium dissociation constant of [3H]ryanodine (2.4 nM for RyR1) (4).

Ca2+-dependency of HBDorg actions. Ca2+ is routinely used in the binding buffer to activate or open RyR1, and, under these conditions, the amount of [3H]ryanodine bound to RyR1 is substantially increased. In the absence of Ca2+ or in high Ca2+ (>900 μM), RyR1 becomes deactivated or closed, and the amount of [3H]ryanodine bound to RyR1 decreases precipitously (2). We, therefore, reasoned that competition assays employing a fixed amount of HBDorg in the presence of varying amounts of Ca2+ could provide insights into whether the actions of HBDorg are dependent on the open or closed conformation of RyR1. SR membrane vesicles (0.1 mg/ml) were incubated in binding buffer containing 2.0 μg/ml HBDorg and increasing amounts of Ca2+ (2–3,900 μM) for 2 h at 37°C. After incubation, vesicles were filtered and washed, and the amount of [3H]ryanodine bound was determined using liquid scintillation counting.

Single-channel Measurement and Analyses

Single-channel analyses were performed as described earlier (5). Briefly, phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in a ratio of 5:3:2 (35 mg/ml of lipid) in n-decane were painted across a 200-μm-diameter hole of the bilayer cup.

Fig. 2. Screening HBD for type 1 ryanodine receptor Ca2+-release channel (RyR1) activity. [3H]ryanodine competition assays were conducted with various amounts of HBDCHCl3, HBDorg, HBDaq, and solvent controls. For this, sarcoplasmic reticulum (SR) vesicles (0.1 mg/ml) were incubated in binding buffer consisting of 500 mM KCl, 20 mM Tris-HCl, 0.3 mM CaCl2, 0.1 mM EGTA, pH 7.4, 6.7 nM [3H]ryanodine with HBDorg (220, 110, and 55 μg/ml), and the solvent [CHCl3/MeOH/dimethylformamide (DMF); 2:1:1] for 2 h at 37°C. At the end of the incubation, samples were rapidly filtered through GF/C filters using a cell harvester (Brandel, Gaithersburg, MD) and washed three times with 3 ml of ice-cold binding buffer, and the amount of [3H]ryanodine bound to the filters was determined by liquid scintillation counting. Data for each compound represent means ± SE from four experiments. aDose response for chloroform extract of HBD. bDose response for organic extract of HBD. cSignificantly different from control (P < 0.05). dSignificantly less than that of 2 and 0.2 μg/ml HBDorg (P < 0.05).

After dialysis, proteoliposomes containing RyR1 were diluted 1:1 in buffer containing 0.1 mM EGTA, 0.2 mM CaCl2, 10 mM Na/PIPES, pH 7.4, 0.1 mM EDTA, and 0.4 mM PMSF and centrifuged at 163,520 g (Ti75 rotor) for 2 h. The pellet was then resuspended in 0.1 M NaCl, 10 mM Na/PIPES, pH 7.4, and 0.3 M sucrose; aliquoted (100 μl); quick-frozen in liquid nitrogen; and stored frozen in the vapor phase of liquid nitrogen until use.

Fig. 3. Determining the affinity of HBDorg for RyR1. Displacement [3H]ryanodine binding assays were used to determine the affinity of HBDorg for RyR1. For this, SR vesicles (0.1 mg/ml) were incubated in binding buffer consisting of 500 mM KCl, 20 mM Tris-HCl, 0.3 mM CaCl2, 0.1 mM EGTA, pH 7.4, and 6.7 nM [3H]ryanodine with 12 concentrations of HBDorg (0.007–15.75 μg/ml) for 2 h at 37°C. At the end of the incubation, samples were rapidly filtered through GF/C filters using a cell harvester (Brandel) and washed three times with 3 ml of ice-cold binding buffer, and the amount of [3H]ryanodine bound to the filters was determined by liquid scintillation counting. Data for each compound represent means ± SE from four experiments. For comparison, ryanodine was also used in this assay.
HOG DUST MODULATES SKELETAL MUSCLE RYANODINE RECEPTOR (RyR1)

Fig. 4. Determining the Ca\(^{2+}\) dependency of HBD\(_{org}\) effect on RyR1. For this, SR vesicles (0.1 mg/ml) were incubated in binding buffer consisting of 500 mM KCl, 20 mM Tris·HCl, 0.1 mM EGTA, pH 7.4, 6.7 nM [\(^3\)H]ryanodine, and increasing amounts of Ca\(^{2+}\) (2–3,900 \(\mu\)M) for 2 h at 37°C. At the end of the incubation, samples were rapidly filtered through GF/C filters using a cell harvester (Brandel) and washed three times with 3 ml of ice-cold binding buffer, and the amount of [\(^3\)H]ryanodine bound to the filters was determined by liquid scintillation counting. Data for each compound represent means \pm SE from four experiments. *Significantly different from [\(^3\)H]ryanodine bound in absence of HBD\(_{org}\) (P < 0.05).

Purified RyR1 in proteoliposomes was then fused to the bilayer. The side of the bilayer to which the proteoliposomes were added was designated the cis side. The trans side was defined as ground. Single channels were recorded in symmetric KCl buffer solution (0.25 mM KCl, 20 mM K/HEPES, pH 7.4) with 3.3 \(\mu\)M Ca\(^{2+}\) or as specified. In this study, free calcium concentrations were titrated against EGTA. HBD\(_{org}\) was made up in CHCl3/MeOH/DMF (2:1:1) at concentrations this study, free calcium concentrations were titrated against EGTA.

Cell Culture and Ca\(^{2+}\) Transients

Undifferentiated C\(_2\)C\(_1\)2 cells (mouse myoblast cell line), obtained as a gift from Dr. Gregory S. Taylor (Department of Biochemistry, UNMC), were grown in Dulbecco’s modified Eagle’s medium containing 1.8 mM CaCl\(_2\) (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and antibiotics (100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 100 \(\mu\)g/ml gentamicin, pH 7.3) to 70–80% confluency. Cells were then subcultured and plated onto laminin-coated glass-bottom chambers and placed in an incubator for 24 h with 5% CO\(_2\)/95% air at 37°C. After this time, the media was changed, fetal bovine serum was reduced to 2%, and cells were allowed to differentiate for 96 h into myotubes. Differentiated myotubes in DMEM (containing 1.8 mM Ca\(^{2+}\)) were then loaded with fluo 3-AM (5 \(\mu\)M) for 30 min at 37°C, washed, and placed on the stage of a laser confocal microscope (Zeiss Confocal LSM 410 confocal microscope equipped with an Argon-Krypton Laser, 25 mW argon laser, 2% intensity, Thornwood, NJ). HBD\(_{org}\) in dimethylsulfoxide was then manually added to a corner of the glass chamber and allowed to diffuse into the cell medium, and Ca\(^{2+}\) transients (\(\Delta F\)) were recorded. The final dimethylsulfoxide in the chamber was \(<5\%\). In some experiments, after loading fluo 3 and washing, 500 mM EGTA were added to DMEM media to chelate extracellular Ca\(^{2+}\). In other experiments, differentiated C\(_2\)C\(_1\)2 cells were incubated with 50 \(\mu\)M ryanodine 20 min at 37°C before loading.
with fluo 3-AM. Cells were then challenged with HBDorg, and Ca2+/H11001 transients were recorded. Fluo 3 was excited by light at 488 nm, and fluorescence was measured at wavelengths of 515 nm. Data were analyzed using Microsoft Excel (Microsoft, Seattle, WA) and Sigma Plot (Systat, Chicago, IL).

Statistical Analyses

Data shown are means ± SE. Differences among concentrations were analyzed using analysis of variance, followed by the Bonferroni (post hoc) test using Prism 4 (GraphPad Software, San Diego, CA).

RESULTS

Preparation of Chloroform Extract of HBD

Chloroform extraction of HBD (HBDCHCL3) afforded a pale yellow paste (~5% yield) that exhibited greenish-blue fluorescence under 365-nm wavelength light. At 305 nm, the greenish-blue fluorescence was less intense. When sprayed with 1% sulfuric acid and heated, the major compounds overlaid with the UV-active compounds. Sulfuric acid also revealed low levels of other non-UV active compounds. When redissolved in hexane-CHCl3 (20:1), a brownish precipitate (HBDorg, 40% yield) was obtained that fluoresced under UV light. The discarded hexane-CHCl3 (20:1) fraction contained very weak UV-active spots that did not react with sulfuric acid.

Chromatographic Characterization of HBDorg

Basic (TEA) and acidic (HAc) mobile phases of increasing polarities were employed to gain insights into the composition of HBD. When spotted on a thin-layer chromatographic plate and developed using a mobile phase consisting of CHCl3/MeOH/TEA (97:3:0.05), all four HBDorg extracts contained three major compounds that migrated with Rf 0.65, (#1), 0.52 (#2), 0.52 (#3; Fig. 1A, left, arrows). Compounds 2 and 3 were better resolved in sample 1. In this solvent system, all four HBDorg samples exhibited streaking near the origin of the plate. Increasing the polarity of the basic mobile phase to 7% methanol resulted in the appearance of two additional compounds with Rf values of 0.2 (#4) and 0.15 (#5). Increasing the polarity of the mobile phase to 15% methanol did not reveal any additional compounds. However, the resolution of compounds 4 and 5 was enhanced. In all three basic mobile phases of increasing polarities, bright streaks were seen at the origin, suggesting that HBDorg also contains very polar, nonbasic compounds.

Fig. 6. Effects of HBDorg on single RyR1. Single-channel currents were recorded at +40 mV (left, upward deflections) and −40 mV (right, downward deflections) in symmetric KCl buffer solution (0.25 mM KCl, 20 mM K/HEPES, pH 7.4), as described in the text. The top right and left panels show representative recordings of RyR1 in the presence of 3.3 μM cytosolic Ca2+/H11001 before addition of HBDorg to the cis chamber. The panels below thereafter represent the effect of varying concentrations of HBDorg. The second and third panels from the top show increases in open probability (Po), the fourth panel shows the induction of a subconductance state, and the fifth and sixth panels show induction of the closed state of the channel. Data shown are representative of six separate channels. HP, holding potential; O, open; C, closed; M, modified.

Fig. 7. Effects of HBDorg on the Po of RyR1. The currents of single RyR1 incorporated into lipid bilayers were recorded at +40 mV and −40 mV (in symmetric KCl buffer), and Po of the channel (transition from closed to full open state) in the presence of varying amounts of HBDorg in the cis chamber were plotted. Data shown are means ± SE from six channels.
Replacing TEA with HAc in the mobile phase also revealed the presence of five major compounds in HBDorg (Fig. 1B). Bright streaks were also seen at origin in acidic mobile phases. At this time, it is not clear whether the compounds that separated with the basic mobile phases are the same as those that separated with the acidic mobile phases.

Assessing the Ability of HBDorg to Bind to RyR1

HBDorg dose-dependently displaced [3H]ryanodine from RyR1 and, as expected, was ~10 times more potent than HBDCHiCl3 (Fig. 2). Ryanodine (1 µM) displaced >90% of [3H]ryanodine from RyR1. CHCl3/MEOH/TEA (2:1:1) used to dissolved HBDCHiCl3 and HBDorg (4%) had minimal effect on the binding of [3H]ryanodine to RyR1. HBDorg did not significantly affect the binding of [3H]ryanodine RyR1 at the concentrations used (20 and 40 µl from a 1 g/10 ml extract in 400 µl of binding buffer).

The affinity of HBDorg for RyR1 was then determined using a wide concentration range of HBDorg in displacement assays. As shown in Fig. 3, HBDorg displaced [3H]ryanodine from RyR1 in a concentration-dependent manner. The experimental data fitted well to a one-site binding model (r2 = 0.95, F = 0.92), affording an IC50 value of 1.5 ± 0.10 µg/ml. The data also fitted well to a two-site model (r2 = 0.95, F = 0.92). Since displacement occurred over two log units, the less complicated single-site model was used for fitting data (25). For comparison, the displacement isotherm of ryanodine is shown in the solid circles, exhibiting an IC50 of 3.8 ± 0.2 ng/ml (IC50 = 7.7 ± 0.4 nM, given molecular mass of 493 Da). Using Eq. 3 with L = 13.59 ng/ml (6.7 nM [3H]ryanodine) and Kf of ryanodine of 4.8 ng/ml (2.4 nM), the Kf for HBDorg binding to RyR1 was calculated to be 0.4 µg/ml. The binding isotherms of RyR1 and HBDorg were also parallel, suggesting that both ryanodine and the active compound(s) of HBDorg are binding to the same site(s) on RyR1.

To gain insight into whether the effect of HBDorg is dependent on the open or closed conformation of RyR1, displacement assays were conducted with varying Ca2+ concentrations. As shown in Fig. 4, in the absence of Ca2+ or at high Ca2+ (≥900 µM), HBDorg was to unable displace [3H]ryanodine from RyR1. However, HBDorg competed with [3H]ryanodine at all concentrations of Ca2+ that activated or opened RyR1 (2–300 µM).

Effects of HBDorg on Single RyR1 Channels

Figure 5 shows the elution profile and a silver-stained gel of aliquots collected following solubilization and centrifugation of junctional SR membranes. Fractions 3, 4, and 5 were collected, dialyzed, and used in single-channel assays, to gain additional insight into mechanisms by which HBDorg alters the activity of RyR1. Three distinct effects were observed when HBDorg was added to the cis chamber of the bilayer cup. At a concentration of 3.5 µg/ml, HBDorg increased the probability of RyR1 opening (Po) from 0.08 ± 0.01 to 0.37 ± 0.05 at +40 mV, and from 0.02 ± 0.01 to 0.35 ± 0.08 at −40 mV within 3 min after addition (Fig. 6, left, 1st and 2nd panels). This increase in P0 resulted from increases in both the frequency of transitions from the closed to the fully open state (number of events increased from 356 ± 48 to 841 ± 154/s, P < 0.05) and dwell time in the open state (from 0.39 ± 0.11 to 0.73 ± 0.13 ms, P < 0.05). Dwell time in the closed state also decreased from 4.2 ± 0.8 to 1.2 ± 0.5 ms (P < 0.05). The increase in Po was seen at both +40 mV and −40 mV holding potentials. The solvent used to dissolve HBDorg had no significant effect on the activity of RyR1 (data not shown).

Tripling the cis concentration of HBDorg to 10.5 µg/ml further increased the Po of RyR1 at both positive and negative holding potentials. This increase in Po also resulted from increases both in its gating frequency of RyR1 (number of events increased to 748 ± 201, P < 0.05) and its dwell time in the open state (1.8 ± 0.9 ms). Dwell time in the closed state also decreased to 0.6 ± 0.2 ms. Increasing the amount of HBDorg in the cis chamber of the bilayer bath to 17.5 µg/ml induced a state of reduced conductance (subconductance) with current amplitude of 55 ± 4% of the fully open state (391 ± 45 vs. 710 ± 90 pS, P < 0.05). Induction of this subconduc-
tance state (with Po = 1) was more likely to occur and persisted for longer times at +40 than at −40 mV. In fact, the time to modification and the probability of occurrence of the subconduc-
tance state were about five times more likely at positive holding potentials than at negative holding potentials. At positive holding potentials and with 17.5 µg/ml HBDorg, no transitions were observed from the subconductance state to either the full open or closed states (Fig. 6, left, 4th panel). At −40 mV, the subconductance state was noticeably noisier than at positive holding potentials, and the channel transitioned to the fully open state with high Po. Increasing the concentration of HBDorg even further to 21.5 µg/ml resulted in the channel predominantly residing in a subconductance state at both positive and negative holding potentials, with occasional transitions to the closed state. Higher concentrations (≥35.0 µg/ml) of HBDorg resulted in irreversible channel closure. Figure 7 shows the pooled data of the effects of HBDorg on the Po of RyR1.

The effects of HBDorg on gating and conductance of RyR1 are reminiscent of that seen with the plant alkaloid ryanodine. Accordingly, we assessed whether ryanodine might be present in HBDorg. When spotted on a silica gel thin-layer chromatographic plate and developed using a mobile phase consisting of CHCl3/ MeOH/TEA (88:12:0.05), neither HBDorg nor HBDaq contained any compound with a chromatographic mobility (Rf) similar to that of ryanodine (Fig. 1C, arrow, Rf for ryanodine = 0.39).
ryanodine for 20 min to block intracellular RyR1 blunted the increase in cytoplasmic Ca\(^{2+}\) induced by HBD\(_\text{org}\) (Fig. 8, \(A_i\) and \(B\)). Using EGTA to chelate extracellular Ca\(^{2+}\) minimally impact the ability of HBD\(_\text{org}\) to trigger Ca\(^{2+}\) release from C\(_2\)C\(_{12}\) cells, indicating that HBD\(_\text{org}\) is inducing intracellular Ca\(^{2+}\) release (data not shown).

**DISCUSSION**

Hog production is a major agricultural industry in the United States, Europe, and several middle-income countries (36). While the economic impact is clear, environmental and health-related issues resulting from this industry are experiencing an avalanche of public discussion. Leading the debate is the issue of the effect of confinement dust on the health and well-being of workers and individuals living in communities surrounding these facilities. It is becoming clearer that inhalation of HBD contributes to the increased incidence of respiratory ailments, including shortness of breath, wheezing, coughing, and chest tightness, among confinement facility workers (10, 14, 34, 37, 39, 40). However, what is not clear whether HBD is also contributing to skeletal muscle weakness and fatigue, and, if it does, what are the mechanism(s) (8, 17)?

The principal finding of the present study is that chloroform extracts of HBD collected from confinement facilities across Nebraska bind to and modulate the activity of RyR1. Using displacement assays, we found that all four samples of HBD\(_\text{org}\) exhibited high affinities for RyR1 (\(K_i = 0.39 \mu\text{g/mL}\)) and that the binding of HBD\(_\text{org}\) to RyR1 is preferred when the channel is in an activated or opened state. The latter is very important, as it suggests that active or working muscles are more prone to the effects of HBD than nonactive muscles. Using black lipid bilayers, we also found that HBD\(_\text{org}\) exhibited three distinct, dose-dependent effects on binding to RyR1. At low microgram amounts, HBD\(_\text{org}\) increased the \(P_0\) of RyR1 by mechanisms that involved increasing both the gating of RyR1 and its dwell time in the open state. At higher concentrations of HBD\(_\text{org}\), RyR1 transitioned into a state of reduced conductance (55% of full open) with a \(P_0\) of 1. In the presence of even more HBD\(_\text{org}\), RyR1 irreversibly closed. Irreversible closure of RyR1 could be a result of slow dissociation of HBD\(_\text{org}\) from RyR1. Thus HBD\(_\text{org}\) is a potent modulator of RyR1.

Parallel findings were also observed in skeletal muscle myotubes. When added to the culture chamber, HBD\(_\text{org}\) increased Ca\(^{2+}\) in differentiated C\(_2\)C\(_{12}\) myotubes in a dose-dependent manner. The rise in intracellular Ca\(^{2+}\) persisted for \(~1\) min and then decayed to near or even below basal. Removal of Ca\(^{2+}\) from the medium minimally impacted the ability of HBD\(_\text{org}\) to increase intracellular Ca\(^{2+}\), indicating that the Ca\(^{2+}\) rise is originating from inside and not from outside the cell. Pretreating differentiated C\(_2\)C\(_{12}\) cells with ryanodine to close intracellular RyR1 blunted the ability of HBD\(_\text{org}\), suggesting that the initial intracellular Ca\(^{2+}\) rise occurs via activation of RyR1. The inability of caffeine to trigger Ca\(^{2+}\) release post-HBD\(_\text{org}\) treatment is also consistent with the initial intracellular Ca\(^{2+}\) rise occurring via activation of RyR1. From single-channel studies, the rise in intracellular Ca\(^{2+}\) in C\(_2\)C\(_{12}\) cells is likely the result of HBD\(_\text{org}\) increasing the gating frequency and inducing a state of reduced conductance of RyR1. The lower basal cytoplasmic Ca\(^{2+}\) post-HBD\(_\text{org}\) treatment and the inability of caffeine to trigger Ca\(^{2+}\) release post-HBD\(_\text{org}\) suggest that HBD\(_\text{org}\) is shutting RyR1, consistent with single-channel data.

Increasing the basal activity of RyR1, as is the case when its \(P_0\) is increased or a subconductance state is induced, would result in leakage of Ca\(^{2+}\) from the SR. As a result, the amount of Ca\(^{2+}\) available for contraction following depolarization would be compromised. Inactivating or closing RyR1, as would be the case with higher amounts of HBD\(_\text{org}\), would result in excitation-contraction uncoupling, as little or no Ca\(^{2+}\) would be released from the SR via an inactivated RyR1. Since effective skeletal muscle contraction requires the coordinated release of sufficient amounts of Ca\(^{2+}\) from the SR, these data provide, for the first time, a physiological rationale for the muscle weakness reported by hog confinement facility workers (8, 17). Since RyR1 is also a key Ca\(^{2+}\) release channel involved in diaphragm contraction (12, 19), the data from the present study also suggest that HBD could be compromising breathing, by another mechanism, i.e., impairment in diaphragm function. However, more work is needed to establish this. It would also be of interest to assess whether HBD\(_\text{org}\) is capable of modulating the activity of other cells that expresses RyR1 (13, 15).

HBD\(_\text{org}\) could enter into the circulation and access RyR1 via two major routes: absorption though the skin, and inhalation through the lung. While most companies require individuals working in concentrated animal feeding operations to wear protective coverings over hair and clothing and to use a respirator (24), it is conceivable that necks and faces are less likely to be protected, and these areas could serve as absorption sites for HBD components. Sweat produced during the course of work could also serve to increase the amount of HBD accumulated in these areas. The acidity of sweat is not likely to affect the components of HBD, as we did not observe any degradation in our chromatographic studies (Fig. 1). To what extent workers comply with the clothing policy is not clear. However, it is conceivable that thick or low porosity clothing worn during hot summer days could be a hindrance for workers.

Individuals working in concentrated animal feeding operations are also required to wear respirators. However, confinement facility workers find respirators to be a nuisance as they can become easily clogged (24). If these masks are not per-
sonalized, as is likely the case, leakage will occur, and inhalation could also serve to increase circulating concentrations of HBD components. However, the tolerance to inhalation is likely to be less than that which can be attached to the skin.

A surprising finding of the present study is that HBD\textsubscript{org} exhibits actions on RyR1 similar to those seen with the plant alkaloid ryanodine (5, 13, 20). Specifically, at low concentrations, it activated or increased the P\textsubscript{o} of RyR1, and at higher concentrations it closed RyR1. However, none of the HBD samples analyzed to date contained any compounds that migrated with a chromatographic mobility similar to that of ryanodine (or its congener 9,21-didehydroryanodine) (Fig. 1C). These data are exciting, as they indicate another source for ligands that can modulate the activity of RyR1. Which component of HBD\textsubscript{org} produces ryanodine-like actions is not known at this time. Because of this unexpected finding, it would be of great interest to assess whether HBD\textsubscript{org} can also modulate the activities of the other isoforms of RyR, namely type 2 ryanodine receptor (RyR2, predominant in heart) and type 3 ryanodine receptor (RyR3, predominant in diaphragm and brain).

It should be mentioned that, during preliminary fractionation of HBD\textsubscript{org}, a white crystalline solid was isolated and identified as lauric acid by \textsuperscript{1}H- and \textsuperscript{13}C-nuclear magnetic resonance spectroscopy (data not shown). In between batches of animals, confinement facilities are hosed and cleaned with commercial detergents, and it is likely that residual detergent molecules are serving as nuclei to attract HBD particles. In preliminary studies, up to 10 \mu g/ml lauric acid had no effect on the activity of \textsuperscript{3}H\textsubscript{-}ryanodine to bind to RyR1 (data not shown).

In conclusion, we have shown that RyR1 is important for the skeletal muscle weakness reported by hog confinement facility workers. They also raise several intriguing questions. Since RyR1 is present in many other cell types (the diaphragm, monocytes, B-cells, T-cells, dendritic cells, neurons, etc.), is modulation of RyR1 central to the reported effects of HBD? Also, since the actions of HBD on RyR1 are similar to that of the plant alkaloid ryanodine, could HBD also interact with and alter the activities of the other ryanodine receptor isoforms, i.e., RyR2 and RyR3? If the latter holds, then HBD could be the underlying cause for additional ailments. Identify the components of HBD that bind to and modulate the activity of RyR1 is, therefore, of utmost importance.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health (HL085061 to K. R. Bidasee and OH008539 to D. J. Romberger).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES