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Radioimmunoassay for the Calcium Release Channel Agonist Ryanodine

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A novel photo-activatable derivative of ryanodine, 9-hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine, has been synthesized and conjugated to keyhole limpet hemocyanin for the production of antibodies with high affinity and specificity to ryanodine. The anti-ryanodine antibodies reacted specifically on immunoblots with the azido-ryanodine compound covalently conjugated to bovine serum albumin. A radioimmunoassay specific for ryanodine was developed using the anti-ryanodine antibodies, and a dissociation constant for ryanodine of 1 nM was determined. Half-maximal inhibition constants (IC_{50}) for various ryanodine derivatives were found to range between 3.2 and 200 nM. These IC_{50} values correlated very well with the IC_{50} values obtained for the compounds binding to the skeletal muscle membrane receptor. These antibodies should be useful for the characterization of the ryanodine binding site on the sarcoplasmic reticulum Ca^{2+} release channel.

Intracellular levels of calcium are regulated by movement of calcium through channels in cellular membranes. Calcium release through an ion channel present in the terminal cisternae of the sarcoplasmic reticulum initiates contraction in skeletal muscle (1). This Ca^{2+} release channel (reviewed in Ref. 2) has been shown to bind the neutral plant alkaloid ryanodine with high affinity (3–7) and can be regulated by ryanodine, Ca^{2+}, ATP, KCl, Mg^{2+} ruthenium red, caffeine, and calmodulin (8–11).

The use of radiolabeled ryanodine as a probe has led to the identification, purification, and biochemical characterization of the Ca^{2+} release channel/ryanodine receptor from skeletal, cardiac, and neuronal tissue (12–16). The predicted primary structure of the Ca^{2+} release channel/ryanodine receptor has been determined in several species and tissues (17–19), but the specific site modulated by ryanodine is unknown.

Ryanodine has different effects on the channel conductance and gating properties depending on the type of tissue and concentration of ryanodine. In skeletal and cardiac muscle for instance, micromolar concentrations of ryanodine inhibit Ca^{2+} release by closing the Ca^{2+} release channel (4,5,20,21), while nanomolar concentrations of ryanodine stimulate Ca^{2+} release by locking this channel in the open state (4,5). In neuronal tissue, micromolar concentrations of ryanodine completely block the channel; however, lower concentrations of ryanodine do not appear to affect the brain Ca^{2+} release channel (16).

Knowledge about the site of action for ryanodine could provide additional information about receptor structure and function. Since immunologic probes have been widely used for the characterization of membrane proteins and ion channels (22,23), one approach toward characterization of the ryanodine binding site would be the production of high-affinity antibodies to ryanodine which mimic the binding characteristics of the skeletal muscle membrane receptor.

Preparation of antibodies to peptides or small molecules like ryanodine (MW 493) is usually accomplished by conjugation to a carrier protein for optimal antigenicity (24–26). Derivatization of ryanodine would allow coupling to carrier proteins. Selection of the site for coupling is obviously a very important determinant of

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the properties of the resulting antibodies. Other groups have prepared ryanodine derivatives and examined their pharmacological, toxicological, and biochemical properties (27–30), but not their ability to be linked to proteins. We have synthesized a novel photo-activatable azido-ryanodine[2] derivative and covalently conjugated this compound to KLH for production of antibodies. The antibodies generated were used in the development of a competitive radioimmunoassay for ryanodine and ryanodine derivatives.

MATERIALS AND METHODS

Preparation of Compounds

Tetrahydrofuran (THF) was dried by distillation from sodium and benzophenone and stored over 4A molecular sieves under nitrogen. HPLC separations were carried out using a Gilson 303 system and a Spherisorb S5 ODS2 column. The flow rate was 10 ml/min and detection was carried out at 268 nm. NMR spectra were recorded on a JEOL GSX 270 spectrometer. Mass spectra were determined by fast atom bombardment ionization (FAB) using a JEOL JMX-DX 303 instrument.

*9,21-Dihydroxy-9-epiryanodine (**1**).* Dehydroryanodine (50 mg, 102 μM) was added to a solution of osmium trichloride (4 mg, 24 μM) and *N*-methylmorpholine (30 mg, 240 μM) in 50% aqueous THF (2 ml). The mixture was stirred at room temperature for 24 h. The reaction was quenched by adding saturated aqueous solutions of $NaHSO_3$ (2 ml) and $NaHCO_3$ (2 ml) and then evaporated to dryness. The residue was extracted with a mixture of methanol and chloroform (1:3, 10 ml) and, after filtration, the solution was passed through a short silica column which was then eluted with more solvent. Evaporation of the resulting solution gave the crude product (95 mg). The product was purified on reverse-phase HPLC using aqueous methanol (55:45) as the mobile phase (t_R = 12.5 min) to give 51 mg (95%) of pure material, identical with that reported previously (27), FAB MS *m/e:* 526 (MH^+).

*9-Hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine (**2**).* Compound **1** (70 mg, 133 μM) was dissolved in dry THF and to this solution at room temperature was added 4-azidobenzoyl chloride (27 mg, 150 μM) and dry pyridine (12 mg). The mixture was stirred in subdued light for 5 days and then evaporated under reduced pressure. The resulting residue was taken up in aqueous methanol (3:7, 2 ml) and passed down a short reverse-phase column, which was further eluted with the same solvent. The eluent was evaporated under reduced pressure to give the crude product (105 mg). The product was purified by twice passing it through a reverse-phase HPLC column in aqueous methanol (3:7) to give 35 mg (39%) of **2** (t_R = 13.5 min). ir (90% $CHCl_3$, 10% MeOH) 2118 cm^{-1} (N_3). ^1H NMR (CD_3OD, δ ppm): 8.03 (d, 2H, J = 7 Hz), 7.08 (d, 2H, J = 7 Hz), 6.96 (m, 1H), 6.79 (m, 1H), 6.16 (m, 1H), 5.56 (s, 1H), 4.95 (d, 1H, J = 12 Hz), 4.49 (d, 1H, J = 12 Hz), 4.36 (s, 1H), 2.51 (d, 1H, J = 14 Hz), 2.19 (sept, 1H, J = 6 Hz), 2.06 (dt, 1H, J = 6, 14 Hz), 1.96 (m, 1H), 1.85 (d, 1H, J = 14 Hz), 1.82 (m, 1H), 1.36 (s, 3H), 1.25 (m, 1H), 1.04 (d, 3H, J = 6 Hz), 0.84 (s, 3H), 0.67 (d, 3H, J = 6 Hz). FAB MS *m/e:* 671 (MH^+).

*21-(2-Hydroxyethylmercapto)-ryanodine (**3**).* Dehydroryanodine (100 mg, 204 μM) and 2-mercaptoethanol (100 mg, 1280 μM) were dissolved in dry THF (5 ml), together with catalytic amounts of AIBN (10 mg) and tributyltin oxide (14 mg). The mixture was stirred at room temperature for 7 days and then evaporated to dryness under reduced pressure. The residue was dissolved in aqueous methanol (55:45, 2 ml) and filtered through a short column of silica, which was then washed with aqueous methanol (10 ml). The total solution was evaporated to dryness to give the crude product (115 mg). This material was then purified by reverse-phase HPLC using aqueous methanol (55:45) to give 85 mg (73%) of pure product **3** (t_R = 25 min). ^1H NMR (CD_3OD, δ ppm): 6.96 (m, 1H), 6.79 (m, 1H), 6.15 (m, 1H), 5.56 (s, 1H), 3.95 (d, 1H, J = 10 Hz), 3.60 (t, 2H, J = 7 Hz), 2.94 (m, 1H), 2.58 (dt, 2H, J = 2.7 Hz), 2.50 (d, 1H, J = 14 Hz), 2.36 (m, 1H), 2.21 (sept, 1H, J = 6 Hz), 2.01 (m, 1H), 1.89 (m, 1H), 1.87 (d, 1H, J = 14 Hz), 1.76 (m, 1H), 1.45 (m, 1H), 1.32 (s, 3H), 1.24 (m, 1H), 1.05 (d, 3H, J = 6 Hz), 0.83 (s, 3H), 0.67 (d, 3H, J = 6 Hz). FAB MS *m/e:* 570 (MH^+).

*21-(4-Hydroxybutylmercapto)-ryanodine (**4**).* This compound was made in 19% yield by a route similar to that for **3**, from dehydroryanodine (50 mg) and 4-mercaptobutanol (50 mg). On reverse-phase HPLC (40:60 aqueous methanol) t_R = 10.5 min. ^1H NMR (CD_3OD, δ ppm): 6.96 (m, 1H), 6.79 (m, 1H), 6.16 (m, 1H), 5.56 (s, 1H), 3.90 (d, 1H, J = 10 Hz), 3.50 (t, 2H, J = 7 Hz), 2.93 (m, 1H), 2.49 (d, 1H, J = 14 Hz), 2.47 (m, 2H), 2.31 (m, 1H), 2.19 (sept, 1H, J = 6 Hz), 2.0 (m, 1H), 1.93 (m, 1H), 1.85 (d, 1H, J = 14 Hz), 1.75 (m, 1H), 1.56 (m, 4H), 1.44 (m, 1H), 1.31 (s, 1H), 1.23 (m, 1H), 1.03 (d, 3H, J = 6 Hz), 0.81 (s, 3H), 0.67 (d, 3H, J = 6 Hz).

*21-(2-[4-Azidobenzoyloxy]-ethylmercapto)-ryanodine (**5**).* Compound **3** (8 mg, 14 μM) was dissolved in dry THF (1 ml). To this was added 4-azidobenzoic acid (4.5 mg, 28 μM) and dicyclohexylcarbodiimide (DCC; 6 mg, 28 μM) and DMAP (1 mg). The mixture was then stirred in subdued light at room temperature for 4 days. The

[2] Abbreviations used: BSA, bovine serum albumin; FAB, fast atom bombardment ionization; KLH, keyhole limpet hemocyanin; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BLOTTO, bovine lacto transfer technique optimizer; TSG, tris/saline/gelatin; IC_{50}, inhibition constant at 50% binding; azido-ryanodine, 9-hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine; THF, tetrahydrofuran; DCC, dicyclohexylcarbodiimide; and DMAP, 4-dimethylaminopyridine.

reaction mixture was evaporated under reduced pressure and the residue dissolved in aqueous methanol (3:7) and passed down a short reverse-phase column. The eluent was evaporated to leave the crude product (22 mg). This material was purified twice by reverse-phase HPLC, with aqueous methanol (32:68) as the mobile phase (t_R = 26 min). The pure product, **5**, 7.5 mg (75%), was then obtained. ^{1}H NMR (CD_3OD, δ ppm): 7.98 (d, 2H, J = 7 Hz), 7.10 (d, 2H, J = 7 Hz), 6.96 (m, 1H), 6.79 (m, 1H), 6.15 (m, 1H), 5.56 (s, 1H), 4.36 (t, 2H, J = 7 Hz), 3.99 (d, 1H, J = 10 Hz), 3.03 (m, 1H), 2.83 (dt, 2H, J = 2, 7 Hz), 2.50 (d, 1H, J = 14 Hz), 2.43 (m, 1H), 2.19 (sept, 1H, J = 6 Hz), 2.01 (m, 1H), 1.89 (m, 1H), 1.87 (d, 1H, J = 14 Hz), 1.76 (m, 1H), 1.46 (m, 1H), 1.32 (s, 3H), 1.24 (m, 1H), 1.05 (d, 3H, J = 6 Hz), 0.83 (s, 3H), 0.67 (d, 3H, J = 6 Hz).

*21-(2-[4-Azidobenzoyloxy]-butylmercapto)-ryanodine (**6**).* By a route analogous to that above, **6** was made in 40% yield from compound **4** (8 mg, 14 μM). It was purified by reverse-phase HPLC (aqueous methanol 25:75, t_R = 17 min). ^{1}H NMR (CD_3OD, δ ppm): 7.97 (d, 2H, J = 8 Hz), 7.10 (d, 2H, J = 8 Hz), 6.95 (m, 1H), 6.79 (m, 1H), 6.17 (m, 1H), 5.56 (s, 1H), 4.25 (t, 2H, J = 7 Hz), 3.92 (d, 1H, J = 10 Hz), 2.91 (m, 1H), 2.52 (m, 2H), 2.48 (d, 1H, J = 14 Hz), 2.33 (m, 1H), 2.19 (sept, 1H, J = 16 Hz), 1.99 (m, 1H), 1.92 (m, 1H), 1.85 (d, 1H, J = 14 Hz), 1.84–1.6, m, 5H), 1.44 (m, 1H), 1.32 (s, 3H), 1.22 (m, 1H), 1.03 (d, 3H, J = 6 Hz), 0.83 (s, 3H), 0.67 (d, 3H, J = 6 Hz).

*21-(2-[3,3,3-Trifluoro-2-diazopropionyloxy]-ethylmercapto)-ryanodine (**7**).* Compound **3** (20 mg, 40 μM) was dissolved in dry THF (1.5 ml). To this was added pyridine (8 mg, 100 μM) and 3,3,3-trifluoro-2-diazopropionyl chloride (14 mg, 80 μM) and the mixture was stirred in subdued light for 7 days at room temperature. The solution was then evaporated under reduced pressure to give the crude product (41 mg) which was purified twice by reverse-phase HPLC (t_R = 11 min) using aqueous methanol 28:72 as the mobile phase, which had been preadjusted to pH 7.6 with triethylamine to limit decomposition of the product. The product (4 mg, 16%) showed the following spectral characteristics: ^{19}F (CD_3OD, δ_{FCCL_3}) −57.5 ppm. ^{1}H NMR (CD_3OD, δ ppm): 7.96 (m, 1H), 6.81 (m, 1H), 6.16 (m, 1H), 5.56 (s, 1H), 4.33 (t, 2H, J = 7 Hz), 3.93 (d, 1H, J = 10 Hz), 3.00 (m, 1H), 2.75 (dt, 2H, J = 2, 7 Hz), 2.50 (d, 1H, J = 14 Hz), 2.39 (m, 1H), 2.19 (sept, 1H, J = 6 Hz), 2.02 (m, 1H), 1.92 (m, 1H), 1.86 (d, 1H, J = 14 Hz), 1.72 (m, 1H), 1.43 (m, 1H), 1.31 (s, 3H), 1.22 (m, 1H), 1.03 (d, 3H, J = 6 Hz), 0.81 (s, 3H), 0.69 (d, 3H, J = 6 Hz). FAB MS *m/e*: 728 (MNa^+).

*10-O-(3-Benzyloxycarbamoyl-propionyl)-ryanodine (**8**).* Ryanodine (540 mg, 1.1 mM) was dissolved in dry THF (20 ml) followed by 3-benzyloxycarbamoyl-propionic acid (260 mg, 1.17 mM), DCC (1 g, 4.85 mM), and a catalytic amount of DMAP (20mg). After stirring for 18 h further amounts of DCC (500 mg) and the propionic acid derivative (130 mg) were introduced and stirring was continued for a further 24 h. The solution was then evaporated to dryness and the remainder was suspended in 50% aqueous methanol (30 ml) and filtered and the filtrate was evaporated under reduced pressure. The crude product was then resuspended in a smaller volume of 50% aqueous methanol (10 ml) from which an oil separated (280 mg). The oil was chromatographed on silica (5% methanol in chloroform as the mobile phase) and then twice on reverse-phase HPLC, in aqueous methanol (35:65, t_R = 16.5 min) to give **8** (53 mg, 7%). ^{1}H NMR (CD_3OD, δ ppm): 7.33–7.18 (m, 5H), 6.97 (m, 1H), 6.80 (m, 1H), 6.17 (m, 1H), 5.51 (s, 1H), 5.25 (d, 1H, J = 10 Hz), 5.04 (d, 1H, J = 12 Hz), 4.97 (d, 1H, J = 12 Hz), 3.37 (t, 2H, J = 6 Hz), 2.53 (t, 2H, J = 6 Hz), 2.51 (d, 1H, J = 14 Hz), 2.19 (sept, 1H, J = 6 Hz), 2.15–1.90 (m, 2H), 1.88 (d, 1H, J = 14 Hz), 1.56–1.40 (m, 2H), 1.31 (s, 3H), 1.24 (m, 1H), 1.03 (d, 3H, J = 6 Hz), 0.83 (s, 3H), 0.76 (d, 3H, J = 6 Hz), 0.68 (d, 3H, J = 6 Hz).

*10-O-(3-Aminopropionyl)-ryanodine (**9**).* Compound **8** (23 mg, 33 μM) was dissolved in methanol, a palladium catalyst (5% Pd on charcoal; 2 mg) was added and the mixture was hydrogenated under a 2-bar pressure of hydrogen for 1.5 h. The reaction mixture was filtered through hi-flo and the filtrate evaporated under reduced pressure to give the product (19.5 mg). This material showed a single spot on TLC (R_f = 0.1 on SiO_2 using $CHCl_3$:MeOH:40% aq.$MeNH_2$ = 85:15:2) and was not purified further. ^{1}H NMR (CD_3OD, δ ppm): 6.96 (m, 1H), 6.79 (m, 1H), 6.16 (m, 1H), 5.50 (s, 1H), 5.24 (m, 1H), 2.85 (t, 2H, J = 6 Hz), 2.49 (d, 1H, J = 14 Hz), 2.47 (t, 2H, J = 6 Hz), 2.18 (sept, 1H, J = 6 Hz), 2.15–1.90 (m, 2H), 1.87 (d, 1H, J = 14 Hz), 1.56–1.40 (m, 2H), 1.33 (s, 3H), 1.23 (m, 1H), 1.03 (d, 3H, J = 6 Hz), 0.82 (s, 3H), 0.77 (d, 3H, J = 6 Hz), 0.67 (d, 3H, J = 6 Hz).

*10-O-(3-[4-Azidobenzamido]-propionyl)-ryanodine (**10**).* Compound **9** (7 mg, 12.4 μM) in THF (1 ml) was reacted at 20°C with 4-azidobenzoic acid (4.5 mg, 27 μM) and a solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (18 mg, 94 μM) in dichloromethane (1 ml). After stirring in subdued light for 1 h the mixture was evaporated to dryness and the residue was purified by chromatography first on a silica column (in 85% $CHCl_3$ 15% MeOH) and then on a reverse-phase HPLC column using aqueous methanol (35:65, t_R = 19 min) to give **10** (5.5 mg, 63%). ^{1}H NMR (CD_3OD, δ ppm): 7.78 (d, 2H, J = 8 Hz), 7.06 (d, 2H, J = 8 Hz), 6.95 (m, 1H), 6.78 (m, 1H), 6.15 (m, 1H), 5.48 (s, 1H), 5.25 (d, 1H, J = 10 Hz), 3.60 (m, 2H), 2.64 (t, 2H, J = 6 Hz), 2.50 (d, 1H, J = 14 Hz), 2.18 (sept, 1H, J = 6 Hz), 2.13–1.89 (m, 2H), 1.87 (d, 1H, J = 14 Hz), 1.54–1.40 (m, 3H), 1.31 (s, 3H), 1.24 (m, 1H), 1.02 (d, 3H, J = 6 Hz), 0.83 (s, 3H), 0.76 (d, 3H, J = 6 Hz), 0.64 (d, 3H, J = 6 Hz).

*10-O-(3-[2-Nitro-5-azidobenzamido]-propionyl)-ryanodine (**11**).* This compound was made by a method similar to that for **10,** using 2-nitro-5-azidobenzoic acid. The product (5 mg, 53%) on HPLC had a $t_R = 12$ min. ^{1}H NMR (CD_3OD, δ ppm): 8.09 (d, 1H, $J = 9$ Hz), 7.24 (dd, 1H, $J = 3$, 9 Hz), 7.11 (d, 1H, $J = 3$ Hz), 6.96 (m, 1H), 6.78 (m, 1H), 6.15 (m, 1H), 5.50 (s, 1H), 5.23 (d, 1H, $J =$ 10 Hz), 3.59 (t, 2H, $J = 6$ Hz), 2.67 (t, 2H, $J = 6$ Hz), 2.49 (d, 1H, $J = 14$ Hz), 2.15 (sept, 1H, $J = 6$ Hz), 2.14–1.78 (m, 2H), 1.86 (d, 1H, $J = 14$ Hz), 1.53–1.4 (m, 2H), 1.32 (s, 3H), 1.21 (m, 1H), 1.03 (d, 3H, $J = 6$ Hz), 0.81 (s, 3H), 0.77 (d, 3H, $J = 6$ Hz), 0.65 (d, 3H, $J = 6$ Hz).

*10-O-(3-[4-Benzoylbenzamido]-propionyl)-ryanodine (**12**).* Using 4-benzoylbenzoic acid and a method similar to that for **10,** the product **12** (4 mg, 42%) was obtained. On HPLC it had a $t_R = 18$ min. ^{1}H NMR (CD_3OD, δ ppm): 7.89 (d, 2H, $J = 8$ Hz), 7.73 (m, 4H), 7.57 (m, 1H), 7.47 (m, 2H), 6.96 (m, 1H), 6.79 (m, 1H), 6.16 (m, 1H), 5.47 (s, 1H), 5.26 (d, 1H, $J = 10$ Hz), 3.65 (m, 2H), 2.69 (m, 2H), 2.49 (d, 1H, $J = 14$ Hz), 2.15 (sept, 1H, $J = 6$ Hz), 2.14–1.86 (m, 2H), 1.85 (d, 1H, $J = 14$ Hz), 1.57–1.39 (m, 2H), 1.29 (s, 3H), 1.23 (m, 1H), 1.00 (d, 3H, $J = 6$ Hz), 0.82 (s, 3H), 0.75 (d, 3H, $J = 6$ Hz), 0.63 (d, 3H, $J = 6$ Hz).

Preparation of Azido-Ryanodine Conjugates

Azido-ryanodine (compound **2**) was covalently coupled to the carrier proteins KLH and BSA for antibody production and analysis of antiserum. Lyophilized carrier protein (5 mg) was dissolved in 0.5 ml sterile filtered H_2O and 6 mg azido-ryanodine was dissolved in 0.5 ml 50% ethanol. The carrier protein was added to 2 ml PBS, pH 7.4, mixed, and combined with the dissolved azido-ryanodine in a 30-mm-diameter plastic disposable petri dish. The mixture was irradiated in the petri dish for 30 min at 4°C using a Spectroline Model ENF-280C handheld ultraviolet lamp at 365 nm. Following irradiation, the carrier protein–ryanodine conjugates were aliquoted and stored at −20°C. All steps, prior to uv irradiation, in the preparation of the carrier protein–ryanodine conjugate were performed in dim or indirect lighting.

Immunization and Production of Anti-Ryanodine Antibodies

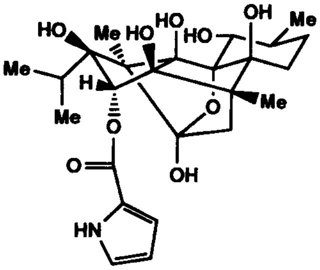
Female New Zealand white rabbits were bled on Day 0 prior to injection to obtain preimmune sera. The rabbits received 0.9 mg of KLH–ryanodine conjugate (based on KLH concentration in the mixture) emulsified in Freund's complete adjuvant at multiple subcutaneous and intramuscular sites. Subsequent injections of 0.1 to 0.5 mg of KLH–ryanodine conjugate were emulsified in Freund's incomplete adjuvant and administered at 2- to 3-week intervals. Rabbits were bled 1 week after the second injection and again 1 week following each boost. Blood was collected from the outer marginal ear vein, allowed to clot at 25°C for 15 min, and stored at 4°C for 1–2 h. Serum was removed following centrifugation for 20 min at 10,000 rpm in a Beckman JA-17 rotor and stored frozen at −20°C.

Immunoblot Analysis of Anti-Ryanodine Antibodies

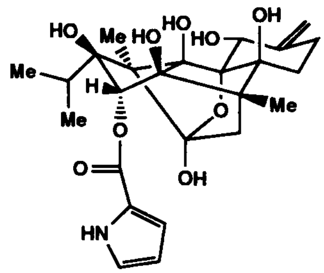
BSA or BSA–ryanodine conjugate (1 μg each) was separated by 3–12% SDS–PAGE and transferred to nitrocellulose as previously described (31). Immunoblots were blocked with PBS–BLOTTO (PBS–5% non-fat dried milk) for 1 h and incubated overnight on a rocker at room temperature with anti-ryanodine antibodies at a 1:1000 dilution. The nitrocellulose membranes were washed twice for 10 min and then incubated for 1 h with a 1:1000 dilution of horseradish peroxidase-linked goat anti-rabbit secondary antibody. The membranes were then washed twice for 10 min and developed using 4-chloro-1-naphthol as substrate. All washes and incubations were performed using PBS–BLOTTO.

[^{3}H]Ryanodine Binding—Dextran-Coated Charcoal Assay

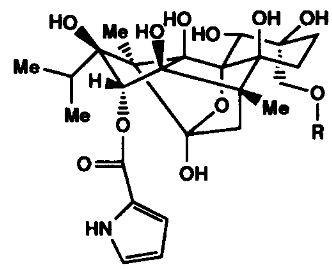
[^{3}H]Ryanodine binding characteristics of the isolated anti-ryanodine antibodies were determined using a dextran-coated charcoal assay similar to an assay developed for the 1,4-dihydropyridine calcium channel blockers (32,33). Binding was performed in triplicate in 1.5-ml polypropylene tubes. Each assay tube contained 1 ml of TSG (Tris/saline/gelatin consisting of 150 mM NaCl, 10 mM Tris, pH 7.2, and 0.1% gelatin) and a final concentration of 1 nM [^{3}H]ryanodine. Following the addition of serial dilutions of antibody, the tubes were shaken and incubated for 1 h at room temperature. The tubes were placed on ice for 10 min and then 0.2 ml of stirred, ice-cold dextran-coated charcoal (0.0625% Dextran T-70, 0.625% Norit A charcoal in TSG) was added to each tube. The tubes were shaken and incubated for an additional 10 min on ice. The charcoal was removed by centrifugation (850*g*) for 15 min in a Beckman TJ-6R centrifuge and [^{3}H]ryanodine binding was measured by counting 0.5 ml of the supernatant in a liquid scintillation counter. Controls without antiserum were included to determine the amount of radiolabel that could not be precipitated by the charcoal. These values were subtracted from values obtained for the antiserum to determine the total specific [^{3}H]ryanodine bound to the antibody. Greater than 98% of the [^{3}H]ryanodine was removed from the supernatant by the dextran-coated charcoal in the absence of antibody. The antibody dilution required to bind 50% of the [^{3}H]ryanodine in the absence of unlabeled ryanodine (titer) was used for the competitive binding assays.



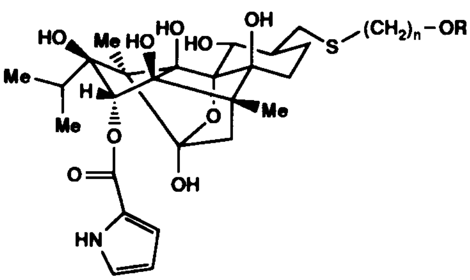
RYANODINE



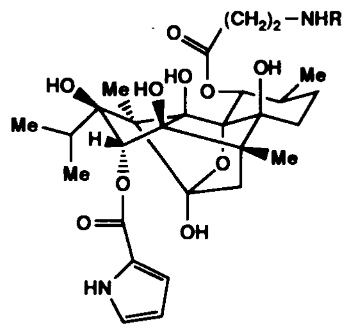
DEHYDRORYANODINE



(1) R = H
(2) R = 4-N_3-BENZOYL



(3) R = H, n = 2
(4) R = H, n = 4
(5) R = 4-N_3-BENZOYL, n = 2
(6) R = 4-N_3-BENZOYL, n = 4
(7) R = $CF_3C(N_2)CO$-, n = 2



(8) R = CO_2Bn
(9) R = H
(10) R = 4-N_3-BENZOYL
(11) R = 2-NO_2-5-N_3-BENZOYL
(12) R = 4-BENZOYLBENZOYL

FIG. 1. Derivatives of ryanodine. Shown are structures for ryanodine, dehydroryanodine, and ryanodine derivatives prepared as described under Materials and Methods.

Affinity and Specificity of Anti-Ryanodine Antibodies

Inhibition of [^{3}H]ryanodine binding was performed using the dextran-coated charcoal assay described above in the presence of unlabeled ryanodine or ryanodine derivatives (10^{-12} to 10^{-5} M). Ryanodine derivatives were prepared by serial dilution in TSG. The percentage of [^{3}H]ryanodine bound was plotted as a function of the concentration of unlabeled ryanodine compound. The inhibition of [^{3}H]ryanodine at 50% of maximal binding (IC_{50}) was determined from the plot.

[^{3}H]Ryanodine Binding to Rabbit Skeletal Membranes

Rabbit skeletal muscle triads were prepared as previously described (34). [^{3}H]Ryanodine binding to triad membranes was performed as described (12) using a glass fiber filter assay. Ryanodine and ryanodine derivatives were used at concentrations varying from 10^{-12} to 10^{-5} M in competition assays. The inhibition of [^{3}H]-ryanodine at 50% of maximal binding (IC_{50}) was determined from a plot of bound [^{3}H]ryanodine versus concentration of competitor.

Materials

[^{3}H]Ryanodine (61.5 Ci/mmol) was obtained from New England Nuclear. Ryanodine used in biochemical studies was obtained from Penick (Lyndhurst, NJ). Ryanodine and dehydroryanodine used in synthesis were obtained as an approximately 1:1 mixture from Agra Systems International (Windgap, PA) and separated by reverse-phase HPLC. Dextran T-70 was obtained from Sigma and Norit A was obtained from Fisher Scientific. Bovine serum albumin and keyhole limpet hemocyanin were from Pierce Chemical Co. Gelatin was obtained from Bio-Rad and electrophoresis reagents and secondary antibodies were from Boehringer

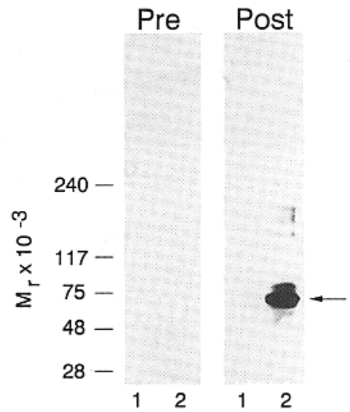


FIG. 2. Antibodies to ryanodine. One microgram of BSA (lane 1) or BSA–ryanodine conjugate (lane 2) was separated by SDS–PAGE on a 3–12% gradient gel, transferred electrophoretically to nitrocellulose, and analyzed using indirect immunoperoxidase staining methods. The left column (Pre) was stained with a 1:1000 dilution of rabbit serum obtained on Day 0 prior to immunization and the right column (Post) was stained with a 1:1000 dilution of postimmune serum obtained on Day 68. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the left and the arrow indicates the position of the BSA–ryanodine conjugate.

Mannheim. All other reagents were of reagent grade quality.

RESULTS

Preparation of Azido-Ryanodine Derivative

Dehydroryanodine (Fig. 1) was dihydroxylated to form 9,21-dihydroxy-9-epiryanodine (compound **1,** Fig. 1). This compound was subsequently acylated with 4-azidobenzoyl chloride to form 9-hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine (compound **2,** Fig. 1). This provided an analog of ryanodine with an added azido group which could be activated by uv light for attachment to carrier proteins (such as KLH or BSA) via available amines. Other compounds were prepared from substituted ryanodine or intermediates to test their specificity and selectivity on the Ca^{2+} release channel/ryanodine receptor (Fig. 1).

Production of Anti-Ryanodine Antibodies

Figure 2 shows the positive reaction of the postimmune serum with BSA–ryanodine conjugate (right column, lane 2, indicated by arrow) but not with BSA alone (right column, lane 1). There was no reaction on an identical blot when stained with preimmune serum (Fig. 2, left column). Furthermore, when a similar blot was incubated with postimmune serum in the presence of 1 μM unlabeled ryanodine, the reaction with the immobilized BSA–ryanodine conjugate was abolished (data not shown).

[^{3}H]Ryanodine Binding to the Anti-Ryanodine Antibodies

[^{3}H]Ryanodine binding to the anti-ryanodine antibodies was tested using a dextran-coated charcoal radioimmunoassay. This assay was used because the dextran-coated charcoal is able to precipitate free [^{3}H]ryanodine but not [^{3}H]ryanodine that is bound to the anti-ryanodine antibodies. In a typical assay, the anti-ryanodine antibodies bound greater than 95% of the added [^{3}H]-ryanodine and were specifically inhibited by 1 μM unlabeled ryanodine (data not shown). In addition, the precipitation of [^{3}H]ryanodine in the absence of added antibody or in the presence of preimmune antiserum were only at background levels (less than 2% of total binding, data not shown). Similar assays were performed using anti-ryanodine antibodies immobilized on protein A-Sepharose with identical binding results (data not shown). These assays demonstrate the specific interaction of the anti-ryanodine antibodies with [^{3}H]ryanodine.

In order to use the anti-ryanodine antibodies to determine inhibition constants for ryanodine and ryanodine derivatives, it was necessary to determine an appropriate dilution of antibody to use for the competitive radioimmunoassay. A titer test, using serial dilutions of the anti-ryanodine antibodies, was performed to calculate the concentration of antibody required for 50% of the added [^{3}H]ryanodine to be bound in the absence of unlabeled ryanodine. This dilution of antibody was chosen since it has been shown to be appropriate for accu-

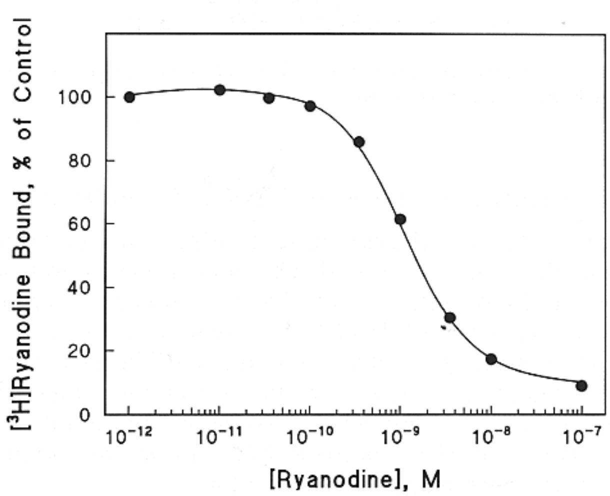


FIG. 3. Displacement of specifically bound [^{3}H]ryanodine by unlabeled ryanodine. A competitive radioimmunoassay for [^{3}H]ryanodine was performed using the dextran-coated charcoal assay as described under Materials and Methods in the presence of 1 nM [^{3}H]ryanodine, a 1:8000 dilution of anti-ryanodine antibody, and various concentrations of unlabeled ryanodine (10^{-12} to 10^{-7} M). Percentage [^{3}H]-ryanodine bound was plotted as a function of unlabeled ryanodine concentration. The data shown are from a representative experiment of triplicates with the SEM <2%. The half-maximal inhibition (IC_{50}) of [^{3}H]ryanodine binding determined from the plot is 1.6 nM.

TABLE 1

Inhibition of [^{3}H]Ryanodine-Antibody Binding by Ryanodine and Ryanodine Derivatives

Compound	IC_{50} (nM)
Ryanodine	1.6
Ryanodine derivatives	
21-(2-[3,3,3-Trifluoro-2-diazopropionyloxy]-ethylmercapto)-ryanodine (**7**)	4
21-(4-Hydroxybutylmercapto)-ryanodine (**4**)	25
21-(2-[4-Azidobenzoyloxy]-butylmercapto)-ryanodine (**6**)	198
10-*O*-(3-[4-Azidobenzamido]-propionyl)-ryanodine (**10**)	3.2
10-*O*-(3-[2-Nitro-5-azidobenzamido]-propionyl)-ryanodine (**11**)	3.2
10-*O*-(3-[4-Benzoylbenzamido]-propionyl)-ryanodine (**12**)	6

Note. Numbers in parentheses correspond to the compound numbers in Fig. 1.

rate calculation of the average antibody affinity constant (35). The titer for the specific antibody used for the competitive radioimmunoassays in this study was determined to be 1:8000 or 0.125 μl/ml (data not shown).

Affinity and Specificity of the Anti-Ryanodine Antibodies

Figure 3 shows the displacement of specifically bound [^{3}H]ryanodine by the addition of increasing concentrations of unlabeled ryanodine. The average antibody apparent dissociation constant (K_d) for the [^{3}H]-ryanodine–antibody complex, determined according to the method of Müller (35), was calculated to be 1 nM. This value is similar to the K_d values reported for the ryanodine receptor in skeletal, cardiac, and brain membranes (12,36,37).

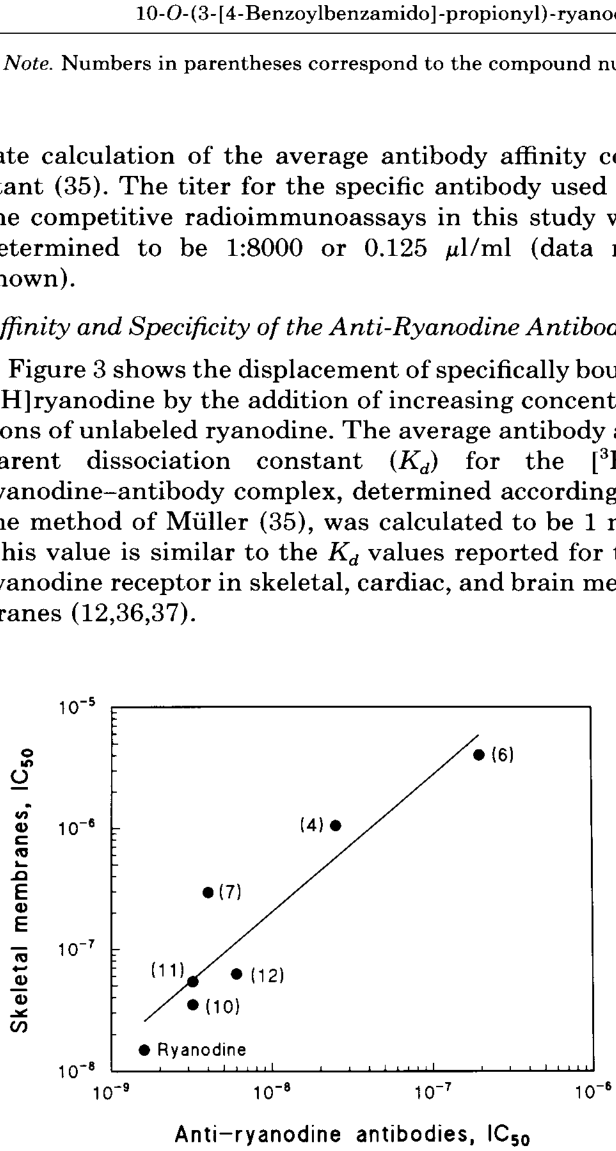


FIG. 4. Comparison of [^{3}H]ryanodine inhibition to skeletal membranes and anti-ryanodine antibodies. [^{3}H]Ryanodine binding to anti-ryanodine antibodies and rabbit skeletal membranes was performed in the presence of various concentrations of unlabeled ryanodine derivatives as described under Materials and Methods. Shown is a linear regression comparison of the half-maximal inhibition (IC_{50}) values for rabbit skeletal membranes and anti-ryanodine antibodies. Numbers next to the data points correspond to the compound numbers from Fig. 1.

The specificity of the anti-ryanodine antibodies was tested using various ryanodine derivatives from 10^{-12} to 10^{-5} M as competitors. All ryanodine derivatives were able to compete with [^{3}H]ryanodine for the antibody binding sites with differences in affinity resulting from the substitutions to the basic ryanodine structure as shown in Table 1. Derivatization of the C_{10} hydroxyl of ryanodine gave compounds with IC_{50} values similar to ryanodine, even when the substituents were large (compounds **10, 11,** and **12**). Comparitively, small S-linked substituents at C_{21} can be tolerated with little effect on the IC_{50} (compound **7**). However, more sterically demanding groups reduce binding substantially, notably the mercaptobutyl-linked compounds **4** and **6,** which bind about 10 and 100 times less tightly than ryanodine.

Comparison of Antibody and Skeletal Muscle Membrane Binding Characteristics

[^{3}H]Ryanodine competition binding was also performed on rabbit skeletal muscle membranes with ryanodine and ryanodine derivatives using a glass fiber filter assay. Concentrations at 50% inhibition were calculated from plots of percentage bound versus concentration of inhibitor (data not shown). Figure 4 shows a comparison of IC_{50} values plotted for [^{3}H]ryanodine competition binding to skeletal membranes and anti-ryanodine antibodies. This figure indicates the similarity in the trend of [^{3}H]ryanodine binding for skeletal membranes and anti-ryanodine antibodies. For example, ryanodine has the highest affinity for membranes and antibodies but compound **6** has the lowest affinity for both.

DISCUSSION

We have demonstrated that by use of a derivative of ryanodine (9-hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine) which can be photocoupled to carrier proteins, it is possible to prepare antibodies with high affinity and specificity for ryanodine. The binding characteristics of the anti-ryanodine antibodies produced compare favorably with those of the skeletal muscle membrane receptor for ryanodine and ryanodine derivatives.

These antibodies may be useful for the production of anti-idiotypic antibodies (directed against the ryanodine binding site) which may affect receptor function. Another potential use for these antibodies and the radioimmunoassay is screening for endogenous ryanodine-like compounds in skeletal, cardiac, and brain preparations. Detection of such endogenous ryanodine-like compounds would further the understanding of calcium release and excitation–contraction coupling.

We have also demonstrated the ability of the anti-ryanodine antibodies to recognize protein–ryanodine covalent conjugates. Therefore, these antibodies may be a useful tool for the identification of the specific ryanodine binding site obtained from peptide fragments of the ryanodine receptor that have been covalently labeled with photoaffinity labeling derivatives of ryanodine. The identification of such a site could provide information on how ryanodine and other regulators of the Ca^{2+} release channel interact with the ryanodine receptor and affect channel function. In addition, knowing the specific ryanodine binding site would allow for the development of new ryanodine analogs. For example, new ryanodine-like compounds could be engineered which have lower toxicity levels and which may be useful for treatment of skeletal or cardiac disorders involving the Ca^{2+} release channel. Finally, knowing this specific site may help to answer the questions of whether the ryanodine binding site is located in the predicted channel region of the ryanodine receptor and whether the ryanodine binding site is conserved among the different genes of the ryanodine receptor (2).

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REFERENCES

1. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108.
2. McPherson, P. M., and Campbell, K. P. (1993) *J. Biol. Chem.* **268,** 13765–13766.
3. Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., and Fleer, E. A. M. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7256–7259.
4. Meissner, G. (1986) *J. Biol. Chem.* **261,** 6300–6306.
5. Lattanzio, F. A., Jr., Schlatterer, R. G., Nicar, M., Campbell, K. P., and Sutko, J. L. (1987) *J. Biol. Chem.* **262,** 2711–2718.
6. Pessah, I. N., Waterhouse, A. L., and Casida, J. E. (1985) *Biochem. Biophys. Res. Commun.* **128,** 449–456.
7. Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1986) *J. Biol. Chem.* **261,** 8643–8648.
8. Rousseau, E., Smith, J. S., Henderson, J. S., and Meissner, G. (1986) *Biophys. J.* **50,** 1009–1014.
9. Meissner, G., and Henderson, J. S. (1987) *J. Biol. Chem.* **262,** 3065–3073.
10. Smith, J. S., Rousseau, E., and Meissner, G. (1989) *Circ. Res.* **64,** 352–359.
11. Rousseau, E., LaDine, J., Liu, Q-Y., and Meissner, G. (1988) *Arch. Biochem. Biophys.* **267,** 75–86.
12. Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 16636–16643.
13. Inui, M., Saito, A., and Fleischer, S. (1987) *J. Biol. Chem.* **262,** 1740–1747.
14. Lai, A. F., Erickson, H. P., Rousseau, E., Liu, Q.-Y., and Meissner, G. (1988) *Nature* **331,** 315–319.
15. Inui, M., Saito, A., and Fleischer, S. (1987) *J. Biol. Chem.* **262,** 15637–15642.
16. McPherson, P. S., Kim, Y.-K., Valdivia, H., Knudson, C. M., Takekura, H., Franzini-Armstrong, C., Coronado, R., and Campbell, K. P. (1991) *Neuron* **7,** 17–25.
17. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. I., and Numa, S. (1989) *Nature* **339,** 439–445.
18. Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G., and MacLennan, D. H. (1990) *J. Biol. Chem.* **265,** 2244–2256.
19. Fujii, J., Otsu, K., Zorzato, F., DeLean, S., Khanna, V. K., Weiler, J. E., O'Brien, P. J., and MacLennan, D. H. (1991) *Science* **253,** 448–451.
20. Seiler, S., Wegner, A. A., Whang, D. D., Hathaway, D. R., and Jones, L. R. (1984) *J. Biol. Chem.* **259,** 8550–8557.
21. Chamberlain, B. K., Volpe, P., and Fleischer, S. (1984) *J. Biol. Chem.* **259,** 7547–7553.
22. Costa, M. R. C., and Catterall, W. A. (1984) *J. Biol. Chem.* **259,** 8210–8218.
23. Venter, J. C., Berzofsky, J. A., Lindstrom, J., Jacobs, S., Fraser, C. M., Kohn, L. D., Schneider, W. J., Green, G. L., Strosber, A. D., and Erlanger, B. F. (1984) *Fed. Proc. Fed. Soc. Exp. Biol.* **43,** 2352–2539.
24. DiMarchi, R., Booke, G., Gale, C., Cracknell, V., Doel, K. T., and Mowat, N. (1986) *Science* **232,** 639–641.
25. Patarroyo, M. E., Romero, P., Torres, M. L., Clavijo, P. I., Moreno, A., Martinez, A., Rodriguez, R., Guzman, F., and Cabezas, E. (1987) *Nature* **328,** 629–632.
26. Sutcliffe, J. G., Shinnick, T. M., Green, N., and Lerner, R. A. (1983) *Science* **219,** 660–666.
27. Waterhouse, A. L., Pessah, I. N., Francini, A. O., and Casida, J. E. (1987) *J. Med. Chem.* **30,** 710.
28. Gerzon, K., Humerickhouse, R. A., Besch, H. R., Bidasee, K. R., Emmick, J. T., Roeske, R. W., Tian, Z., Ruest, L., and Sutko, J. L. (1993) *J. Med. Chem.* **36,** 1319–1323.
29. Mais, E. E., Bowling, N., and Watanabe, A. M. (1992) *Biochem. Biophys. Res. Commun.* **183,** 462–467.
30. Jefferies, P. R., Lehmberg, E., Lam, W.-W., and Casida, J. E. (1993) *J. Med. Chem.* **36,** 1128–1135.
31. Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J., and Campbell, K. P. (1991) *Neuron* **7,** 499–508.
32. Campbell, K. P., Sharp, A., Strom, M., and Kahl, S. D. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2792–2796.
33. Campbell, K. P., Sharp, A. H., and Kahl, S. D. (1987) *J. Cardiovasc. Pharmacol.* **9(Suppl. 4),** S113–S121.
34. Sharp, A. H., Imagawa, T., Leung, A. T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 12309–12315.
35. Müller, R. (1983) *in* Methods in Enzymology (Langone, J. J., and Van Vunakis, H., Eds.), Vol. 92, pp. 589–601, Academic Press, New York.
36. Anderson, K., Lai, F. A., Liu, Q.-Y., Rousseau, E., Erickson, H. P., and Meissner, G. (1989) *J. Biol. Chem.* **264,** 1329–1335.
37. McPherson, P. S., and Campbell, K. P. (1990) *J. Biol. Chem.* **265,** 18454–18460.