Pestic. Sci. 1998, **54**, 345–352

Characterisation of Antibody Models of the Ryanodine Receptor for Use in High-Throughput Screening†

Andrew J. Dinsmore,[1] William Rees-Blanchard,[2] Philip Bentley,[2] Terence Lewis,[2] Steven D. Kahl,[3‡] Peter S. McPherson,[3§] Michael J. Mullinnix,[3] Kevin P. Campbell,[3] John D. Windass[2] and Fergus G. P. Earley[2]*

[1] Antibody Group, Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TF, UK
[2] Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET, UK
[3] Howard Hughes Medical Institute, Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA

(Received 3 July 1998; revised version received; accepted 12 August 1998)

Abstract: The syntheses of seven novel synthetic analogues of the naturally occurring insecticide ryanodine are described. These, and other synthetic and naturally occurring analogues, have been used to characterise the selectivity of a monoclonal antibody which has been produced by immunisation with 9-hydroxy-21-(4-azidobenzyloxy)-9-epiryanodine photo-conjugated to keyhole limpet haemocyanin. The antibody binds [^{3}H]ryanodine with a dissociation constant of 0·37 nM. The specificity of this antibody in terms of its ability to recognise 11 natural and synthetic analogues of ryanodine has been determined by [^{3}H]ryanodine displacement and shown to be similar (for a partially overlapping set of analogues) to that determined earlier for a rabbit polyclonal antibody (Kahl, S. D., *et al.*, *Anal. Biochem.*, **218** (1994) 55–62). The selectivity of the antibodies is shown to be related to that of the sarcoplasmic reticulum Ca^{2+} release channel from rabbit skeletal muscle, both for this set of ryanodine analogues and for three structurally dissimilar, low-molecular-weight compounds identified by high-throughput screening. The advantages of these antibody models of the Ca^{2+} release channel for screening are illustrated by their superior performance in a homogeneous binding assay. © 1998 Society of Chemical Industry

Pestic. Sci., **54**, 345–352 (1998)

Key words: ryanodine; antibodies; radio-immunoassay; ryanodine analogues; screening technology

* To whom correspondence should be addressed.
† One of a collection of papers on various aspects of agrochemicals research contributed by staff and collaborators of Zeneca Agrochemicals UK and Zeneca Ag Products USA. The papers were collected and collated by Dr B. C. Baldwin and Dr D. Tapolczay.
‡ Present address: Lilly Research Laboratories, Lilly Corporate Center, Drop Code 1543, Indianapolis, IN 46285, USA.
§ Present address: McGill University, Montreal Neurological Institute, Room 736, 3801 University Street, Montreal, Quebec H3A 2B4, Canada.

 Pestic. Sci. 0031–613X/98/$17.50. Printed in Great Britain

1 INTRODUCTION

Simple, low cost, very high throughput screening technologies are sometimes difficult to apply to targets of interest. An example is the receptor for the unusual alkaloid ryanodine, a plant natural product with potent insecticidal effects.[1] Ryanodine modifies the function of a family of intracellular calcium channels, which mediate muscle contraction and intracellular signalling, by binding with very high affinity to the open channel state.[2–4] The vertebrate channel is a homotetramer with monomer molecular weight of 500 kDa and there may be other associated proteins important for function. Studies in insect tissues indicate that the receptor has similar properties and size to its mammalian homologue.[5,6] These properties present considerable difficulties for cloning and functional expression, which would be required to enable high-throughput screening against receptors from insects.

Although there are some known differences in pharmacology,[7] the vertebrate receptor can be considered as a suitable model,[8] and vertebrate skeletal muscle provides a rich source of the receptor from which purified fractions can easily be prepared. These fractions can be used in screening for novel ligands by [^{3}H]ryanodine displacement using filtration methods, but are not suitable for the cheaper and more convenient homogeneous binding assay technologies such as those marketed by Amersham (scintillation proximity assay) or NEN™ Life Science Products (FlashPlate®). These formats are best suited to receptors which can be immobilised on the detecting surface at high density.

There have been several reports of antibodies that mimic receptors in their ligand-binding pharmacology,[9–11] and it follows that certain antibodies may be satisfactory substitutes for the natural receptor in screening for novel ligands. As part of an earlier study designed to characterise the ryanodine binding site, a polyclonal antibody was produced which was shown to have a related pharmacology to the receptor for a limited number of synthetic and natural analogues of ryanodine.[12] In this paper we report the further characterisation of the pharmacology of this antibody, examine the performance of antibodies in high-throughput homogeneous binding assays, and also report the generation and characterisation of high-affinity monoclonal antibodies to ryanodine.

2 METHODS

2.1 Chemical synthesis

Tetrahydrofuran (THF) was dried by distillation from sodium and benzophenone and stored over 4 Å 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^{-1} and detection was carried out at 268 nm. NMR spectra were recorded in deuteromethanol on a JEOL GSX 270 spectrometer. Mass spectra were determined by fast atom bombardment ionisation (FAB) using a JEOL JMX-DX 303 instrument.

The chemical structures are given in Fig. 1. Compounds 5–8 and 10 were prepared as described previously .[12]

2.1.1 15-O-Benzoylryanodine (1)

Ryanodine (17 mg; 34·5 μmole) was dissolved in dry DMF (1 ml). To this solution was added sodium methoxide (3·7 mg; 69 μmole). The mixture was left to stir at 20°C for 2 h and then benzoyl chloride (9·5 mg; 69 μmoles) in dry THF (1 ml) was added. Stirring was continued at 20°C for a further 18 h. The reaction was quenched with water (1 ml) and methanol (1 ml). This solution was then evaporated under reduced pressure to give the crude product (29 mg), which was purified by reverse-phase HPLC using methanol + water (72 + 28 by volume) to give 5 mg (25%) of pure product (t_R = 20 min). [^{1}H]NMR (δ ppm): 7·70 (d, 2H, J = 7·5 Hz), 7·48 (t, 1H, J = 7·5 Hz), 7·34 (t, 2H, J = 7·5 Hz), 6·85 (m, 1H), 6·78 (m, 1H), 6·08 (m, 1H), 5·57 (s, 1H), 3·69 (d, 1H, J = 10 Hz), 3·20 (d, 1H, J = 14 Hz), 2·49 (d, 1H, J = 14 Hz), 2·33 (m, 1H), 2·21 (m, 1H), 1·70 (m, 1H), 1·47 (s, 3H), 1·36 (m, 2H), 1·19 (m, 1H), 1·10 (d, 3H, J = 6 Hz), 0·87 (d, 3H, J = 6 Hz), 0·81 (s, 3H), 0·65 (d, 3H, J = 6 Hz).

2.1.2 21-Phenyl-9,21-dehydroryanodine and 21-phenyl-8,9-dehydroryanodine

9,21-Dehydroryanodine (30 mg; 60 μmole) and iodobenzene (24 mg; 120 μmole) were dissolved in dry DMF (1 ml). To this was added tri-(*ortho*-tolyl)phosphine (19 mg; 60 μmole), palladium (II) acetate (6·8 mg; 30 μmole) and, finally, ethyl diisopropylamine (7·8 mg; 60 μmole) in dry DMF (0·5 ml). The solution was stirred at 120°C for 22 h in a nitrogen atmosphere. On cooling, the reaction was quenched with water (1 ml) and extracted with diethyl ether (× 8). The extracts were evaporated under reduced pressure to give 32 mg of the crude product which was purified by reverse-phase HPLC using methanol + water (63 + 35 by volume) to give 11·2 mg (32%) 21-phenyl-9,21-dehydroryanodine [t_R = 11·5 min; [^{1}H]NMR (δ ppm): 7·09 (m, 5H), 6·91 (m, 1H), 6·74 (m, 1H), 6·54 (m, 1H), 6·11 (m, 1H) 5·54 (s, 1H), 2·67 (m, 1H), 2·44 (d, 1H, J = 14 Hz), 2·17 (m, 2H), 1·92 (m, 1H), 1·79 (d, 1H, J = 14 Hz), 1·32 (s, 3H), 1·22 (m, 1H), 1·00 (d, 3H, J = 6 Hz), 0·80 (s, 3H), 0·63 (d, 3H, J = 6 Hz)] and 6·5 mg (18%) 21-phenyl-8,9-dehydroryanodine [t_R = 9·0 min; [^{1}H]NMR (δ ppm): 7·09 (m, 5H), 6·94 (m, 1H), 6·77 (m, 1H), 6·14 (m, 1H), 5·53 (s, 1H), 5·28 (m, 1H), 4·24 (m, 1H), 3·51 (m, 1H), 3·28 (m, 1H), 2·55 (m, 1H), 2·53 (d, 1H, J = 14 Hz), 2·18 (m, 1H), 1·89

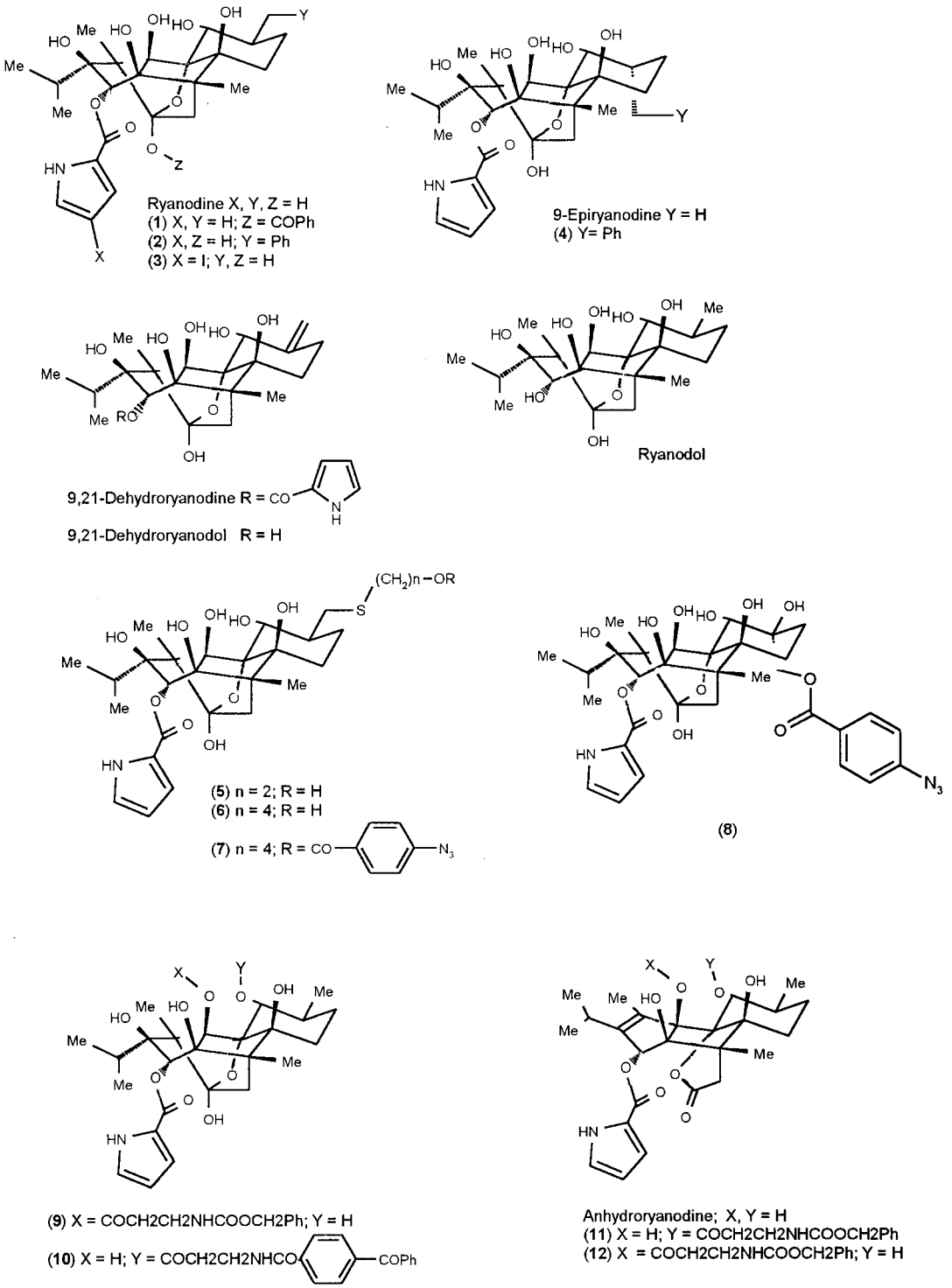

Fig. 1. Structures of ryanodine and derivatives used in this study.

(d, 1H, J = 14 Hz), 1·71 (m, 1H), 1·29 (s, 3H), 1·00 (d, 3H, J = 6 Hz) 0·80 (s, 3H), 0·65 (d, 3H, J = 6 Hz).

2.1.3 21-Phenylryanodine (2)

21-Phenyl-8,9-dehydroryanodine (10·2 mg; 18 μmole) was dissolved in methanol (20 ml). To this was added 5% palladium on charcoal catalyst (10 mg). The mixture was hydrogenated for 3 h at 3 bar pressure and 20°C. The catalyst was filtered off and the filtrate evaporated under reduced pressure to give the crude product (10 mg). This material was purified by reverse-phase HPLC using methanol + water (58 + 42 by volume) to give 7·5 mg (73%) of (**2**) (t_R = 23·5 min). [^{1}H]NMR (δ ppm): 7·07 (m, 5H), 6·93 (m, 1H), 6·77 (m, 1H), 6·13 (m, 1H), 5·54 (s, 1H), 3·88 (d, 1H, J = 10 Hz), 3·08 (m, 2H), 2·45 (d, 1H, J = 14 Hz), 2·15 (m, 2H), 1·89 (m, 1H),

1·81 (d, 1H, J = 14 Hz), 1·34 (s, 3H), 1·16 (m, 3H), 1·03 (d, 3H, J = 6 Hz), 0·77 (s, 3H), 0·65 (d, 3H, J = 6 Hz).

2.1.4 25-Iodoryanodine (3)
Ryanodine (10 mg; 20 μmole) was dissolved in acetonitrile (1 ml). To this solution was added sodium iodide (3 mg; 20 μmole) followed by chloramine-T (5 mg; 20 μmole). The reaction was stirred at 20°C for 3 h and the solvent was then removed under reduced pressure to give the crude product (18 mg). Purification was by reverse phase HPLC using methanol + water (70 + 30 by volume) to give 10 mg (80%) of the pure product (**3**) (t_R = 10·5 min). [^{1}H]NMR δ ppm): 7·00 (m, 1H), 6·80 (m, 1H), 5·55 (s, 1H), 3·70 (d, 1H, J = 10 Hz), 2·42 (d, 1H, J = 14 Hz), 2·14 (m, 1H), 2·01 (m, 1H), 1·85 (d, 1H, J = 14 Hz), 1·74 (m, 1H), 1·37 (m, 2H), 1·30 (s, 3H), 1·03 (d, 3H, J = 6 Hz), 0·93 (d, 3H, J = 6 Hz), 0·80 (s, 3H), 0·64 (d, 3H, J = 6 Hz). FAB MS m/e 619 M^+.

2.1.5 21-Phenyl-9-epiryanodine (4)
21-Phenyl-9,21-dehydroryanodine (11·2 mg; 20 μmole) was dissolved in methanol (20 ml). To this was added 5% palladium on charcoal catalyst (10 mg) and the mixture was hydrogenated under 3 bar pressure of hydrogen for 3 h at 20°C. The catalyst was filtered off and the filtrate evaporated under reduced pressure to leave the crude product (11·0 mg). This material was purified by reverse-phase HPLC using methanol + water (58 + 42 by volume) to give 10·8 mg (96%) of pure product (4) (t_R = 27·5 min). [^{1}H]NMR δ ppm): 7·04 (m, 5H), 6·93 (m, 1H), 6·88 (m, 1H), 6·13 (m, 1H), 5·52 (s, 1H), 4·34 (d, 1H, J = 6 Hz), 3·03 (m, 1H), 2·64 (m, 1H), 2·50 (d, 1H, J = 14 Hz), 2·12 (m, 3H), 1·88 (d, 1H, J = 14 Hz), 1·58 (m, 1H), 1·36 (m, 1H), 1·33 (s, 3H), 1·06 (m, 1H), 1·02 (d, 3H, J = 6 Hz), 0·80 (s, 3H), 0·67 (d, 3H, J = 6 Hz).

*2.1.6 12-*O*-(3-benzoyloxycarbamoylpropionyl)ryanodine (9), 10-*O*-(3-benzoyloxycarbamoylpropionyl) anhydroryanodine (11) and 12-*O*-(3-benzoyloxycarbamoylpropionyl)anhydroryanodine (12)*
Ryanodine (540 mg, 1·1 mmole) was dissolved in dry THF (20 ml) followed by 3-benzyloxycarbamoylpropionic acid (260 mg, 1·17 mmole), dicyclohexylcarbodiimide (DCC; 1 g, 4·85 mmole) and a catalytic amount of 4-dimethylaminopyridine (DMAP) (20 mg). After stirring for 18 h, more DCC (500 mg) and 3-benzyloxycarbamoylpropionic acid (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 water + methanol (50 + 50 by volume; 30 ml), filtered and the filtrate was evaporated under reduced pressure. The crude product was resuspended in the aqueous methanol (10 ml), and an oil separated (280 mg), which was chromatographed on silica (methanol + chloroform, 5 + 95 by volume, as the mobile phase) and then twice on reverse-phase HPLC, in methanol + water (35 + 65 by volume) to give 10-*O*-(3-benzyloxycarbamoylpropionyl)-ryanodine[1] 53 mg (t_R = 16·5 min, 7%), together with (**9**) 11 mg (t_R = 25 min, 1·4%), (**11**) 12 mg (t_R = 29 min, 1·6%) and (**12**) 3·2 mg (t_R = 24 min, 0·42%). [^{1}H]NMR δ ppm):

(**9**) 7·26 (m, 5H), 6·95 (m, 1H), 6·89 (m, 1H), 6·14 (m, 1H), 5·58 (s, 1H), 5·03 (m, 2H), 3·73 (d, 1H, J = 10 Hz), 3·34 (m, 2H), 2·98 (d, 1H, J = 14 Hz), 2·46 (m, 2H), 2·42 (d, 1H, J = 14 Hz), 2·26 (m, 1H), 1·76 (m, 1H), 1·48–1·33 (m, 2H), 1·37 (s, 3H), 1·28–1·15 (m, 2H), 1·07 (d, 3H, J = 6 Hz), 0·94 (d, 3H, J = 6 Hz), 0·82 (s, 3H), 0·68 (d, 3H, J = 6 Hz).

(**11**) 7·23 (m, 5H), 6·97 (m, 1H), 6·77 (m, 1H), 6·16 (m, 1H), 6·05 (m, 1H), 5·49 (d, 1H, J = 10 Hz), 4·97 (s, 1H), 3·32 (m, 4H), 2·65 (m, 1H), 2·53 (m, 2H), 1·90 (m, 1H), 1·69 (m, 2H), 1·53 (m, 2H), 1·05 (d, 3H, J = 6 Hz), 1·00 (d, 3H, J = 6 Hz), 0·88 (s, 3H), 0·86 (d, 3H, J = 6 Hz), 0·78 (d, 3H, J = 6 Hz).

(**12**) 7·23 (m, 5H), 6·97 (m, 1H), 6·77 (m, 1H), 6·23 (m, 1H), 6·15 (m, 1H), 4·98 (s, 1H), 3·93 (d, 1H, J = 10 Hz), 3·33 (m, 4H), 2·67 (m, 1H), 2·51 (m, 2H), 1·89 (m, 1H), 1·65 (m, 2H), 1·50 (m, 2H), 1·05 (d, 3H, J = 6 Hz), 1·00 (d, 3H, J = 6 Hz), 0·89 (s, 3H), 0·87 (d, 3H, J = 6 Hz), 0·78 (d, 3H, J = 6 Hz).

2.2 Preparation of conjugates for antibody production

A photo-activatable derivative of ryanodine, 9-hydroxy-21-(4-azidobenzoyloxy)-9-epiryanodine (azido-ryanodine, **8**) was covalently linked to keyhole limpet haemocyanin (KLH) and bovine serum albumin (BSA), essentially as described previously.[12] Azido-ryanodine (1 mg) was dissolved in water + ethanol (50 + 50 by volume; 200 μl) and then KLH (1·66 mg) dissolved in phosphate buffered saline (PBS; 800 μl) was added. Similarly, azido-ryanodine (1 mg) in ethanol + water (50 + 50 by volume; 200 μl) was mixed with BSA (1 mg) dissolved in PBS (800 μl). Both mixtures, in plastic cuvettes, were irradiated with UV light at 365 nm for 30 min. The resulting conjugates were split into aliquot portions and stored at −20°C.

2.2.1 Generation of antibodies
The rabbit polyclonal antibody was identical to that used in a previous study.[12] For preparation of monoclonal antibodies Balb/c mice were immunised with the ryanodine–KLH conjugate. They were dosed at 14-day intervals with 30 μg of conjugate, delivered subcutaneously in an emulsion with Freund's complete adjuvant for the first dose and Freund's incomplete adjuvant for the second and third doses. At least six weeks after the third dose, mice were dosed with 30 μg of conjugate delivered intravenously in PBS. Four days after the fourth dose, spleens were removed aseptically. Monoclonal antibodies were generated by fusing 10^7 NS0 myeloma cells with 10^8 splenocytes using polyethylene

glycol 1500. Fusion, cloning and antibody production were performed by standard methods.[13]

2.2.2 Analysis of monoclonal supernatants by enzyme linked immunosorbant assay (ELISA)

Initial selections of hybridomas secreting suitable antibodies were made using an ELISA assay with ryanodine–BSA conjugate. Ninety-six-well polystyrene plates were coated with conjugate diluted to 1 μg ml^{-1} in carbonate/bicarbonate buffer (pH 9·6) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0·5 g $litre^{-1}$ Tween 20 (PBS/T). Antibody supernatants were added to the wells and incubated for 2 h at room temperature. The plates were washed with PBS/T and horseradish-peroxidase-labelled anti-mouse IgG second antibody diluted 1 : 4000 in PBS/T was added to the wells and incubated for 1 h at room temperature. The plates were washed with PBS/T and 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) substrate (1 mg ml^{-1}) TMB in citrate phosphate buffer pH 5·0, 1 μl ml^{-1} of 30% hydrogen peroxide) was added and incubated for 30 min at room temperature in the dark. Sulfuric acid (3 M) was added to the wells and optical densities at 450 nm were measured.

2.3 Radioimmunoassay (RIA)

The ability of the selected antibodies to bind [^{3}H]ryanodine (New England Nuclear) was determined by RIA. Serial dilutions of antibodies were prepared in duplicate in PBS containing 2% bovine serum. A final concentration of 1 nM [^{3}H]ryanodine was added to each tube. The tubes were incubated for 2 h at room temperature. Chilled saturated ammonium sulfate was added to each tube, the tubes were mixed and incubated for 1 h at 4°C, then centrifuged at 13 000 rev min^{-1} to pellet precipitated antibody. The supernatants were removed and the pellets were washed with chilled 50% saturated ammonium sulfate. The tubes were recentrifuged and the antibody pellets were redissolved in PBS. [^{3}H]ryanodine binding was measured by counting the redissolved antibody samples in a liquid scintillation counter. Tubes without antibody and tubes containing [^{3}H]ryanodine alone were included.

2.4 Affinity and specificity of monoclonal antibodies

The affinity of ryanodine analogues and other chemicals for binding to the antibody was determined by competition for [^{3}H]ryanodine using the RIA described above or by a simpler microplate assay which used 96-well polystyrene plates coated with mab 823 by incubating at a dilution of 1 : 1600 (with respect to tissue culture supernatant) for 1 h at room temperature. The antibody solution was then replaced with a solution of bovine serum albumin (100 g $litre^{-1}$) and further incubated at room temperature for 30 min. After washing, solutions containing [^{3}H]ryanodine (1 nM final concentration) and various concentrations of competing ligands between 10^{-12} and 10^{-7} M were added and incubated at room temperature for 1 h. The plates were rapidly washed three times by flooding with ice-cold water and the radioactivity in the wells determined by scintillation counting. Non-specific binding was determined by inclusion of excess unlabelled ryanodine (1 μM) and was less than 10% of total binding [^{3}H]ryanodine binding was plotted as a function of the concentration of competing ligand, and the concentration giving 50% displacement of specific binding (IC_{50}) was determined by fitting the data to a simple hyperbola using the programme MicroCal Origin™. The mean of at least four experiments was used for analysis.

2.5 Receptor binding assays

The affinity of ryanodine analogues and other chemicals for membranes prepared from rabbit skeletal muscle was determined by competition for [^{3}H]ryanodine in a filtration binding assay using the method previously described.[12] The concentration of competing ligand giving 50% displacement of maximal [^{3}H]ryanodine binding (IC_{50}) was calculated as described for the radioimmunoassay.

2.6 Homogeneous binding assays

The rabbit polyclonal antiserum or membranes enriched in ryanodine receptor from rabbit skeletal muscle were incubated in FlashPlates® (NEN™ Life Science Products) for 1 h at room temperature. Dilutions were in PBS. Any remaining binding sites were then blocked by addition of an equal volume of a solution of BSA (10 mg ml^{-1} in PBS) and incubation for a further 30 min. The plates were washed with PBS and [^{3}H]ryanodine (2·6 nM, 2·9 TBq $mmole^{-1}$) added to all wells. Unlabelled ryanodine (1 μM) was added to some wells for determination of non-specific binding. After 1 h the plates were counted in the Packard TopCount scintillation counter.

3 RESULTS AND DISCUSSION

3.1 The relationship between the pharmacology of the polyclonal antibody and that of the rabbit muscle receptor extends beyond structural analogues of ryanodine

For a limited number of ryanodine analogues, displacement of [^{3}H]ryanodine from a rabbit polyclonal anti-

body has previously been shown to correlate very well with displacement from the rabbit muscle receptor.[12] However, to have confidence in the antibody as a model for the receptor binding site, it is important to demonstrate that the correlation extends to molecules structurally dissimilar to the hapten. To test this, three compounds which were detected in a high-throughput screening exercise as being able to displace [^{3}H]ryanodine from its receptor (Fig. 2A) were assessed for their ability to displace [^{3}H]ryanodine from the rabbit polyclonal antibody. All three compounds gave greater than 90% displacement of [^{3}H]ryanodine in the receptor binding assay when tested at 10 μg ml^{-1} (results not shown) and, at the same concentration, all three also gave significant displacement of [^{3}H]ryanodine from the polyclonal antibody (Fig. 2B). The ability of the antibody to recognise these small molecules is striking because they are so different, both structurally and in physical chemical terms, from ryanodine. It strongly suggests conservation of the structural aspects of ligand–protein interaction between the antibody and the receptor. The ability of a number of structurally unrelated basic molecules to interact with the receptor is well known.[14]

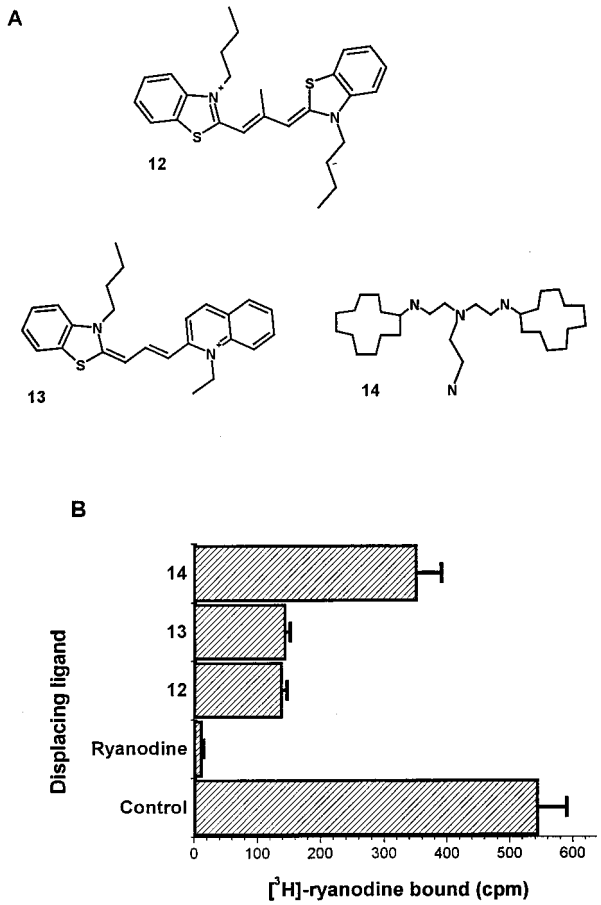

Fig. 2. Displacement of [^{3}H]ryanodine binding from rabbit polyclonal antibody by three compounds, **12–14**, found through high-throughput screening. A. structures; B. binding results.

3.2 The polyclonal antibody is suitable for screening using SPA format

The suitability of the rabbit polyclonal antiserum for determination of [^{3}H]ryanodine displacement in a format where free and bound radioactivity are distinguished by proximity to a solid scintillant was assessed using NEN™ Life Science's FlashPlate® technology. This format has the advantage of removing the need for hazardous and time-consuming wash steps required in traditional RIA or filtration binding assays.[15] A good signal was obtained when the plates were coated with the antibody up to the highest dilution tested (1 : 1600) (Fig. 3A). In contrast, no specific binding could be seen when the plates were coated with the rabbit skeletal muscle receptor preparation at up to 200 μg per well (Fig. 3B). This was due to a failure of sufficient receptor to bind to the plate, since solubilising the contents for traditional scintillation counting also failed to detect a signal. An attempt to increase receptor binding by pretreatment of the plates with polyethylenimine had no effect. However, this treatment decreased total binding and increased non-specific binding in antibody-coated plates (results not shown).

3.3 A high affinity monoclonal antibody with properties similar to that of the polyclonal has been generated

Selected monoclonal antibodies were titrated with [^{3}H]ryanodine to determine the concentration of each antibody required for 50% binding. This dilution of an antibody has been shown to be appropriate for accurate

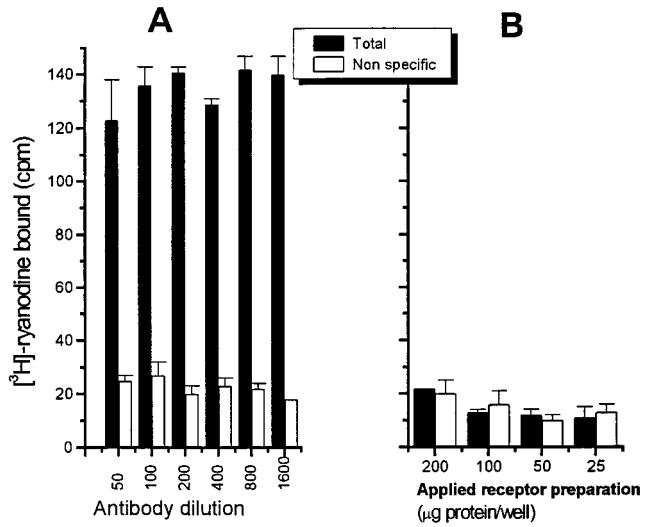

Fig. 3. Binding of [^{3}H]ryanodine to; (A) rabbit polyclonal antiserum, and (B) rabbit skeletal muscle receptor preparation, tested using FlashPlate® technology (details of the determination of total and non-specific binding are described in Section 2.6).

calculation of antibody affinity constants.[16] One monoclonal antibody (mab 823) had a 50% titre of 1 : 1600. The apparent dissociation constant K_D calculated according to Muller[16] was 0·37 nM. This compared well with a dissociation constant of 1 nM calculated for the rabbit polyclonal antibody.[12]

The specificity of mab 823 was determined using various ryanodine derivatives at concentrations between 10^{-12} and 10^{-14} M in competition. Results are expressed as IC_{50} (molar concentrations of competitor required to give half-maximal inhibition of [^{3}H]ryanodine binding) and are presented in Table 1 together with the IC_{50} determinations for displacement of [^{3}H]ryanodine from the receptor preparation from rabbit skeletal muscle. The correlation between the two sets of data is presented graphically in Fig. 4.

This set of analogues expands on those tested in the earlier study using the rabbit polyclonal antiserum.[12] For those common to both studies, compounds **6**, **7** and **10** (**4**, **6** and **12** respectively, in Ref. 12) the relationship between affinity at the receptor and affinity at the antibody is conserved, with compound **7** having lowest and compound **10** having highest affinity for both. This not only confirms the relationship in structural specificity between antibody and receptor but also reveals a close relationship in specificity between the two antibodies. In general, the monoclonal antibody has between 10- and 100-fold higher affinity than the rabbit muscle receptor preparation for the broader range of analogues tested in this study, which is consistent with its increased affinity for [^{3}H]ryanodine (27 ×). It is less sensitive to substitutions at the 9 and 10 positions (compounds **2**, **4**, **5**, **6**, **9** and **10**). It is, perhaps, not surprising that receptor binding is more sensitive to substitution at these positions, as compound **8** was used to generate the hapten. Ryanodol and dehydroryanodol are exceptions, having comparatively lower affinity for the antibody, indicating that the pyrrole moiety is relatively more important for antibody recognition.

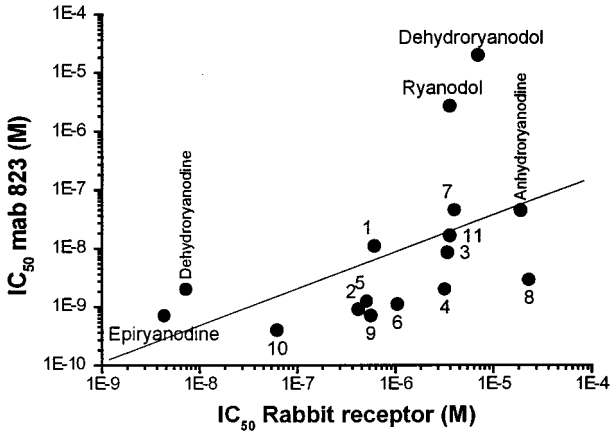

Fig. 4. Correlation between binding of chemicals to rabbit skeletal muscle receptor and monoclonal antibody mab 823. The line is the result of linear regression analysis for all points displayed (R (correlation coefficient) = 0·52, P (probability that R is 0) = 0·04, SD (standard deviation of the fit) = 1·17).

The intercellular calcium release channel that mediates muscle contraction (also known as the ryanodine receptor) is a target of interest for insecticide development, principally because of the insecticidal properties of ryanodine and related alkaloids; however the complexity of ryanodine does not make it a good starting

TABLE 1
Comparison of the Ability of Ryanodine Derivatives to Displace [^{3}H]ryanodine Binding from Rabbit Skeletal Muscle Receptor and Monoclonal Antibody mab 823

Compound	*IC_{50} (M) Rabbit skeletal muscle receptor*[a]	*IC_{50} (M) Monoclonal antibody mab 823*[a]
9,21-Dehydroryanodine	7·20E-09	2·00E-09
9-Epiryanodine	4·31E-09	7·00E-10
Ryanodol	3·60E-06	2·70E-06
9,21-Dehydroryanodol	7·01E-06	2·00E-05
Anhydroryanodine	1·90E-05	4·40E-08
1	6·10E-07	1·10E-08
2	4·20E-07	9·00E-10
3	3·42E-06	8·40E-09
4	3·20E-06	2·00E-09
5	5·12E-07	1·25E-09
6	1·05E-06	1·10E-09
7	4·03E-06	4·50E-08
8	2·30E-05	2·90E-09
9	5·60E-07	7·07E-10
10	6·23E-08	4·00E-10
11	3·60E-06	1·64E-08

[a] IC_{50} values were determined as described in Section 2.4.

point for a purely synthetic approach. An alternative approach to generating novel chemical effectors of this target is through high-throughput screening of large chemical libraries. This approach is limited by both cost and the amount of chemical available for screening. Antibody models of the natural binding site can offer several advantages over the receptor itself for screening, since they are easy to produce in large quantity and are adaptable to less labour-intensive and more easily miniaturised methods.

In this and a previous study,[12] antibodies have been generated that bind a variety of natural and synthetic ryanodine analogues in a way that is related to their binding to the receptor. What is particularly encouraging is that the relationship extends to simple low-molecular-weight compounds that are unrelated in structure to ryanodine. These antibodies can therefore be used with some degree of confidence to detect, or even purify, ryanodine-like molecules and other ligands with potential to interact with the receptor.

ACKNOWLEDGEMENTS

We thank the laboratory of Professor A. G. Lee, School of Biological Sciences, University of Southampton, UK, for the provision of ryanodine receptor preparations from rabbit skeletal muscle.

REFERENCES

1. Jefferies, P. R., Toia, R. F., Brannigan, B., Pessah, I. & Casida, J. E., Ryania insecticide analysis and biological activity of 10 natural ryanoids. *J. Agric. Food Chem.*, **40** (1992) 142–6.
2. Sorrentino, V. & Volpe, P., Ryanodine receptors: how many, where and why? *Trends Pharmacol. Sci.*, **14** (1993) 98–103.
3. Sutko, J. L. & Airey, J. A., Ryanodine receptor Ca^{2+} release channels: does diversity in form equal diversity in function? *Physiol. Rev.*, **76** (1996) 1027–71.
4. Wagenknecht, T. & Radermacher, M., Ryanodine receptors: structure and macromolecular interactions. *Curr. Opin. Struct. Biol.*, **7** (1997) 258–65.
5. Schmitt, M., Turberg, A., Londershausen, M. & Dorn, A., Binding sites for Ca^{2+}-channel effectors and ryanodine in *Periplaneta americana*: Possible targets for new insecticides. *Pestic Sci.*, **48** (1996) 375–88.
6. Takeshima, H., Nishi, M., Iwabe, N., Miyata, T., Hosoya, T., Masai, I. & Hotta, Y., Isolation and characterization of a gene for a ryanodine receptor-calcium release channel in *Drosophila melanogaster*. *FEBS Lett.*, **337** (1994) 81–7.
7. Lehmberg, E. & Casida, J. E., Similarity of insect and mammalian ryanodine binding sites. *Pestic. Biochem. Physiol.*, **48** (1994) 145–52.
8. Jefferies, P. R., Yu, P. & Casida, J. E., Structural modifications increase the insecticidal activity of ryanodine. *Pestic Sci.*, **51** (1997) 33–8.
9. Couraud, P. O. & Strosberg, A. D., Anti-idiotypic antibodies against hormone and neurotransmitter receptors. *Trans. Biochem. Soc.*, **19** (1991) 147–51.
10. Ducancel, F., Merienne, K., Fromen-Romano, C., Tremeau, O., Pillet, L., Drevet, P., Zinn-Justin, S., Boulain, J. C. & Menez, A., Mimicry between receptors and antibodies. Identification of snake toxin determinants recognized by the acetylcholine receptor and an acetylcholine receptor-mimicking monoclonal antibody. *J. Biol. Chem.*, **271** (1996) 31345–53.
11. Linthicum, D. S., Bolger, M. B., Kussie, P. H., Albright, G. M., Linton, T. A., Combs, S. & Marchetti, D., Analysis of idiotypic and anti-idiotypic antibodies as models of receptor and ligand. *Clin. Chem.*, **34** (1988) 1676–80.
12. Kahl, S. D., McPherson, P. S., Lewis, T., Bentley, P., Mullinnix, M. J., Windass, J. & Campbell, K. P., Radioimmunoassay for the calcium release channel agonist ryanodine. *Anal. Biochem.*, **218** (1994) 55–62.
13. Galfre, G. & Milstein, C., Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.*, **73** (1981) 3–46.
14. Palade, P., Drug-induced Ca^{2+} release from isolated sarcoplasmic reticulum. I. Use of pyrophosphate to study caffeine-induced Ca^{2+} release. *J. Biol. Chem.*, **262** (1987) 6135–41.
15. Holland, J. D., Singh, P., Brennand, J. C. & Garman, A. J., A non-separation microplate receptor binding assay. *Anal. Biochem.*, **222** (1994) 516–18.
16. Muller, R., Determination of affinity of specificity of anti-hapten antibodies by competitive radioimmunoassay. *Methods Enzymol.*, **92** (1983) 589–601.