Characterization of sheep brain ryanodine receptor ATP binding site by photoaffinity labeling

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Abstract Two high M_r protein bands (440 and 420 kDa) in sheep brain microsomal membranes were labeled with the photoaffinity ATP analog, *O*-(4-benzoyl)benzoyl adenosine 5′-triphosphate (Bz_2ATP). The 420 kDa band is labeled by [α-^{32}P]-Bz_2ATP with about 1000-fold higher affinity than the 440 kDa band. The heavily labeled 420 kDa band is identified as dynein heavy chain based on its partial amino acid sequence, and cross-reactivity with anti-dynein antibodies. The 440 kDa protein is immunologically identified as the type-2 RyR. Bz_2ATP binding is obtained in the absence of divalent cations. Bz_2ATP and ATP increased the binding of ryanodine to its receptor up to 3-fold, and increased the binding affinity up to 6-fold. Other nucleotides stimulate ryanodine binding with decreasing effectiveness: $Bz_2ATP > ATP > ADP > AMP > AMP\text{-}PNP > GTP > cAMP$. With respect to nucleotide specificity, this binding site is similar to the skeletal muscle RyR (type 1). However, the brain RyR may have additional one or more sites with lower affinity with inhibitory effect on ryanodine binding. These results suggest that the major RyR isoform in sheep brain corresponds to the type-2 isoform, and that modulation of ryanodine binding by ATP involves its binding to the RyR protein. The association of dynein with brain microsomal membranes may reflect a linkage of RyR to the cytoskeleton.

Key words: Ryanodine receptor; Ca^{2+}-release channel; Endoplasmic reticulum; ATP binding site; Photoaffinity; Brain; Dynein

1. Introduction

The concentration of intracellular free Ca^{2+} plays an important role in the regulation of cell function in both contractile and non-contractile cells [1]. The endoplasmic reticulum (ER) contains two well characterized Ca^{2+}-release channels, the $InsP_3$ receptor and the ryanodine receptor (RyR) [2–7], and less well characterized channels including a voltage-sensitive cation channel that is also highly permeable to Ca^{2+} [8]. RyR proteins, originally purified from skeletal and cardiac muscle SR (as type-1 and type-2 receptors, respectively), comprise large homotetramers with an apparent subunit M_r of ~450 kDa on SDS-PAGE (reviewed in [9]). In practice, they show anomalously fast mobility in SDS-PAGE: the *ryr1* cDNA cloned from rabbit skeletal muscle encodes a RyR predicted to have a subunit molecular mass of 565 kDa [4,5], while the *ryr2* cDNA corresponding to the rabbit cardiac muscle type-2 RyR corresponds to a protein of 566 kDa [6,7]. The amino acid sequence of a 'type-3' RyR was first deduced from rabbit brain cDNA [13]. It has a predicted mass of 552 kDa, slightly lower than that of the skeletal and cardiac RyRs.

It is now clear that all three major isoforms are widely distributed in different mammalian tissues, so that while, quantitatively, the major mammalian brain isoform generally appears to be the type-2 RyR, other proteins are also present [9]. RyRs are also present in mammalian brain [10–13], liver [14] and smooth muscle [15,16].

The function of RyR proteins, including those in brain ER, is modulated by endogenous Ca^{2+}, Mg^{2+} and adenine nucleotides (reviewed in [9]). The RyR amino acid sequence contains at least twice the ATP binding consensus sequence [4]. However, their exact location is unknown. Using the photoreactive ATP analog, Bz_2ATP, the ATP binding site(s) of the skeletal muscle RyR were localized to 27 kDa and 13 kDa proteolytic fragments derived from the C-terminal of the molecule [17]. In brain microsomes, ATP was found to stimulate ryanodine binding at concentrations below 1 mM and to inhibit at higher concentrations [11,18], suggesting the involvement of two or more different binding sites. In this study, we demonstrate that the major sheep brain RyR is the type-2 isoform, and characterized its nucleotide binding sites. We also identify dynein heavy chain as a major ATP binding protein in ER microsomal membranes.

2. Materials and methods

2.1. Materials

ATP, EGTA, EDTA, Tris, MOPS, AMP, ADP, AMP-PNP, GTP, cAMP, Caps, were obtained from Sigma. Benzoyl-benzoyl-ATP (Bz_2ATP) and [α-^{32}P]-Bz_2ATP were synthesized and purified as described by Williams and Coleman [19] with some modifications to scale down. Briefly, ATP concentration was 0.55 mM, 1,1′-carbonyldiimidazole (CDI) and benzoyl-benzoic acid ratio was 2.2 and the acetone extraction step was omitted. [α-^{32}P]-ATP was obtained from Amersham, and [^{3}H]ryanodine (60 Ci/mmol) was purchased from New England Nuclear. Unlabeled ryanodine was obtained from Calbiochem. Monoclonal anti-RyR was kindly provided by Campbell [20], and a rabbit anti-cardiac RyR polyclonal antiserum was kindly provided by S. Fleischer. An anti-dynein antibody was obtained from Sigma, and horseradish peroxidase (HRP)-conjugated anti-mouse, and HRP-conjugated anti-rabbit antibodies were from Promega.

2.2. Membrane preparations

A crude synaptosomal fraction was prepared from whole sheep brain essentially as described previously [21]. Crude microsomes

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Abbreviations: EDTA, ethylene-diaminetetraacetate; EGTA, ethylene glycol bis(aminoethylether)tetraacetate; tricine, *N*-[2-hydroxy-1,1-bis(hydroxy-methyl)-ethyl]-glycine; MOPS, 3-(*N*-morpholino) propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; Bz_2ATP, 3′-*O*-(4-benzoyl)benzoyl adenosine triphosphate; AMP-PNP, 5-adenylyl imidodiphosphate; UV, ultraviolet; $InsP_3$, inositol 1,4,5-trisphosphate; RyR, ryanodine receptor

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were further purified by sucrose gradient centrifugation and the membranes were frozen in liquid nitrogen, and stored at −70°C. In most experiments the membranes at the 1.0/1.2 M sucrose interface were used. Junctional SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito et al. [22]. Sheep cardiac SR membranes were prepared by the method of Chamberlain et al. [23]. Protein concentrations were determined by the standard Lowry procedure [24].

2.3. [^{3}H]ryanodine binding

Unless otherwise specified, brain microsomal membranes were incubated for 1–2 h at 37°C in a standard binding solution containing 0.5 or 1 M NaCl, 20 mM MOPS (pH 7.4), 50 μM free [Ca^{2+}] and 5 to 20 nM [^{3}H]ryanodine. Unbound ryanodine was separated from protein bound ryanodine by sample filtration through Whatman GF/C filters, followed by three washes with 4 ml ice-cold buffer containing 0.2 M NaCl, 10 mM MOPS (pH 7.4) and 50 μM $CaCl_2$. The retained radioactivity in the dried filters was determined by liquid scintillation counting technique. Specific binding of [^{3}H]ryanodine is defined as the difference between the binding in the absence and the presence of 100 μM unlabeled ryanodine.

2.4. Photoaffinity labeling of ATP binding proteins by [α-^{32}P]-Bz_2ATP

Brain microsomal membranes (1 mg/ml) were irradiated with UV light for 3–4 min in the presence of 1 nM to 5 μM of [α-^{32}P]-Bz_2ATP (4×10^6 to 10^7 cpm/nmol) in 50 μl of 20 mM MOPS (pH 7.4) and 0.4 M NaCl, and other reagents as indicated in the figure and table legends. The irradiated membranes were immediately diluted 1:1 with a buffer containing 125 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS and 2% (v/v) 2-mercaptoethanol, and incubated for 3 min at 90°C. The samples were analyzed by SDS-PAGE as described below. Autoradiography of the dried gels was carried out using Kodak X-Omat film. Quantitative analysis of the labeled protein bands was determined by densitometric scanning using a Molecular Dynamics personal densitometer, using the Image Quant software provided by the manufacturer. Band densities were confirmed to be linearly related to film exposure times over the periods chosen for analysis.

2.5. Gel electrophoresis and immunoblot analysis

Analysis of protein profiles by SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system of Laemmli [25] in 1.5 mm thick slab gels containing a gradient of 3.5–6% (w/v) acrylamide. Gels were stained with Coomassie blue. The molecular mass standards were: myosin (200 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); bovine serum albumin (66.2 kDa) and ovalbumin (42.7 kDa), (Bio-Rad), and cross-linked phosphorylase b (97 to 873 kDa, monomer to octamer, Sigma). The separated proteins were electrophoretically transferred onto nitrocellulose membranes using either Tris/glycine (pH 8.3) [26] or 20 mM Caps buffer containing 0.01% (w/v) SDS (pH 10.5). The membranes were blocked with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 in 10 mM Tris-HCl, pH 7.8 and 0.15 M NaCl. The membranes were then incubated with anti-skeletal muscle RyR, anti-cardiac RyR or anti-dynein antibodies, and then with horseradish peroxidase (HRP)-conjugated anti-mouse or HRP-conjugated anti-rabbit antibodies. Antibody detection was carried out using enhanced chemiluminescence.

2.6. Internal amino acid sequence analysis

Briefly, brain microsomes were separated by SDS-PAGE (3–6% acrylamide) and the 420 kDa protein bands were cut out of the gel, digested by endoproteinase Lys C. The resulting peptides were separated by reverse phase HPLC on a C18 column, and were subjected to amino acid sequencing (in the Biology Center, Technion, Israel).

3. Results

3.1. Effects of nucleotides on [^{3}H]ryanodine binding to brain microsomes

Fig. 1 shows that ATP is an activator of ryanodine binding to sheep brain ryanodine receptors. Half-maximal stimulation was obtained at about 0.3 mM, and maximal stimulation (of about 2-fold) at 1 mM ATP. The degree of stimulation was decreased at higher ATP concentrations (see also Table 1). The frank inhibition of ryanodine binding by ATP concentrations above 1 mM that has been seen in some other studies [11,18] could be due to differences in assay conditions, such as the presence of 1 mM Mg^{2+} in the assay medium, or to species differences in relative RyR isoform distributions. The ability of ATP to stimulate ryanodine binding decreases as the binding comes near its maximal level. Increasing the concentration of NaCl enhances ryanodine binding, and markedly decreases the extent of stimulation by ATP (Table 1). As a result, 0.5 M NaCl was used throughout these studies to optimize measurements in the relevant experiments.

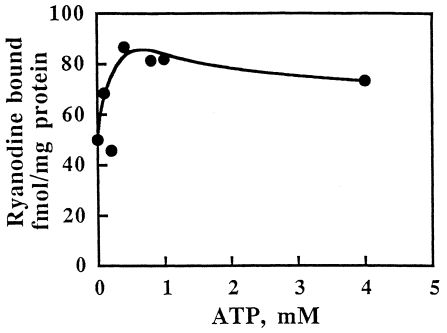

Fig. 1. Stimulation of ryanodine binding by ATP. Ryanodine binding to brain microsomes (2.5 mg/ml) was assayed as described in Section 2 in the presence of 0.5 M NaCl, 10 nM ryanodine, 50 μM free Ca^{2+} (calculated as in [23]) and the indicated concentrations of ATP.

Fig. 2 shows the binding of [^{3}H]ryanodine as a function of its concentration (in 0.5 M NaCl), and in the absence and the presence of 0.5 mM ATP. ATP increased the ryanodine binding affinity, decreasing the K_D about 3-fold, from 54 ± 6 nM (n = 3) to 19 ± 5 nM (n = 2, means ± S.D.). Thus, the increase in ryanodine binding produced by ATP appears to be attributable to increased affinity of the receptor for ryanodine. Table 2 shows that in the dark the photoreactive ATP analog, 3′-*O*-(4-benzoyl)benzoyl adenosine triphosphate (Bz_2ATP), acts like ATP to increase the binding of ryanodine to brain microsomes. On photoactivation, very low (μM) concentrations of Bz_2ATP stimulated ryanodine binding, suggesting that photoactivated Bz_2ATP binds irreversibly to one or more ATP binding sites. The effect of various nucleotides on ryanodine binding is presented in Table 3. The relative stimulation of ryanodine binding decreases in the following order of effectiveness: $Bz_2ATP > ATP > ADP > AMP >$ AMP-PNP≥GTP, while cAMP had no effect. This nucleotide specificity is similar to that of the skeletal muscle RyR ATP binding site [17].

3.2. Identification of ryanodine receptor isoforms and dynein

Fig. 3A shows the protein profiles of skeletal muscle SR, cardiac SR and of brain microsomal preparation. Three high MW protein bands are present, all near the expected position of RyR proteins (these are labeled 1, 2 and 3). The major band (band 3) does not correspond to the type-1 RyR of skeletal muscle SR, which has an apparent mass on SDS-PAGE of 460 kDa, nor does it correspond to the skeletal muscle RyR degradation product of 375 kDa. Band 2 corresponds to the cardiac type-2 RyR (440 kDa on SDS-PAGE), and its identity is confirmed by its cross-reactivity with anti-

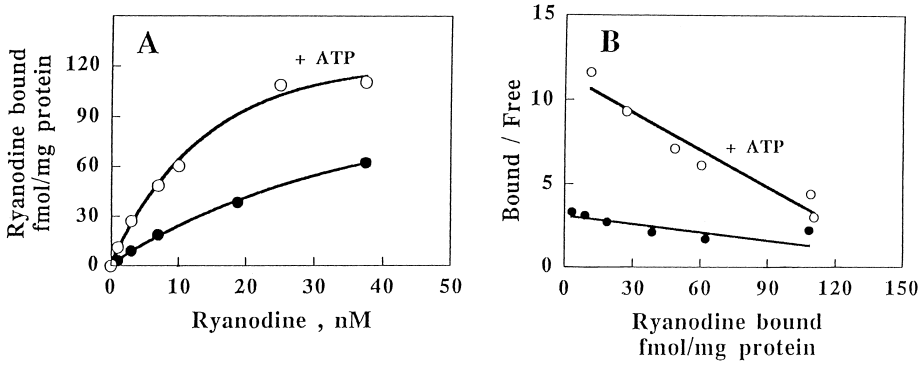

Fig. 2. The influence of ATP on the affinity of the ryanodine binding site. A: The binding of [^{3}H]ryanodine was assayed as described in Section 2 in the presence of 0.5 M NaCl and 50 μM free Ca^{2+} with (○) or without (●) 0.5 mM ATP. B: Scatchard plot analysis (K_D = 57 and 13 nM, B_{max} = 170 and 150 fmol/mg protein) in the absence and the presence of ATP, respectively.

type-2 RyR antibodies (Fig. 3). Band 1 does not cross-react with either of the anti-RyR antibodies, and it has no ATP binding site (Fig. 3A and B). To determine whether band 3 (420 kDa) represented an additional RyR isoform (e.g. the type-3 protein), or was a distinct protein we used different antibodies. Anti-RyR-3 antibodies are not yet commercially available. An anti-RyR antibody kindly supplied by V. Sorrentino failed to react specifically with RyR. To identify this protein band, brain microsomes were subjected to SDS-PAGE and band 3 was cut of the gel and subjected to amino acid sequencing. After digestion by endoproteinase Lys C, peptides were separated by reverse phase HPLC and a selected peptide was sequenced. We obtained a sequence of 18 amino acids with 100% identity to the sequence of the following fragment of rat brain cytosolic dynein heavy chain [27], part of the mechanochemical ATPase that powers the traffic of organelles along microtubules [28]:

Residue no.:	4397																4414
	K	R	T	V	E	N	I	K	D	P	L	F	R	F	F	E	R	E

3.3. Bz_2ATP labeling of brain microsomal membrane proteins

We used the photoreactive ATP analogue, Bz_2ATP, to label the ATP binding sites in brain microsomes. Fig. 3B shows that irradiation of brain microsomes with [α-^{32}P]-Bz_2ATP resulted in covalent binding of the label to several membrane proteins, including two high MW proteins corresponding to band 2 and band 3 (440 and 420 kDa, respectively). The labeling of both protein bands by [α-^{32}P]-Bz_2ATP was prevented in the presence of excess of ATP or ADP (data not shown). The 440 kDa polypeptide which was labeled with [α-^{32}P]-Bz_2ATP cross-reacts with both monoclonal anti-skeletal muscle and anti-cardiac RyR antibodies (Fig. 3C). However, the 420 kDa protein band is labeled much more intensively with [α-^{32}P]-Bz_2ATP, and it cross-reacts with anti-dynein antibodies. The same anti-dynein antibodies also cross-react with a 420 kDa protein band in cardiac but not in skeletal muscle SR. In skeletal muscle SR, there was weak cross-reactivity of anti-dynein antibodies with two bands corresponding to the type-1 RyR and its proteolytic fragment. It should be noted that we found that dynein was retained in several rats and sheep brain microsomal preparations, even after purification by sucrose density gradient centrifugation.

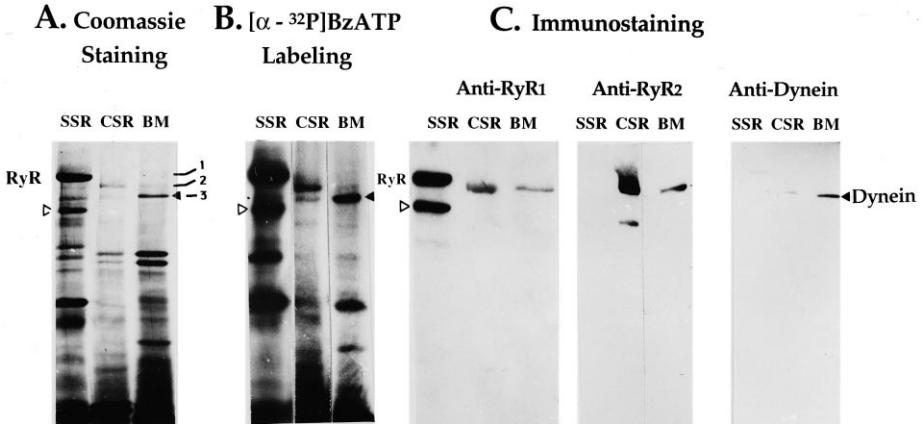

Fig. 3. Electrophoretic profile, immunoreactivity and [α-^{32}P]-Bz_2ATP labeling of skeletal muscle SR, cardiac SR and brain microsomes. Brain microsomes (BM), skeletal SR (SSR) and cardiac SR (CSR) membranes were labeled with 1 μM [α-^{32}P]-Bz_2ATP and subjected to SDS-PAGE in 3–6% (w/v) acrylamide followed by Coomassie blue staining (A), autoradiography (B) or immunoblot analysis (C). Western blotting was carried out as described in Section 2 using anti-skeletal muscle RyR (1:500), anti-dynein (1:100) or anti-cardiac muscle RyR (1:1000) antibodies, followed by HRP-conjugated anti-mouse or anti-rabbit antibodies as appropriate, and ECL detection. The skeletal muscle type-1 RyR protein band is labeled in the Coomassie-stained gel (A) and in the anti-RyR-1 immunoblot (C). Note labeling of this band in SSR lane in (B). The open arrow indicates the RyR-1 proteolytic fragment that is also labeled by [α-^{32}P]-Bz_2ATP. In the BM lane in (A), three high MW Coomassie-stained bands are labeled (1–3), and in the BM lane in (B) the filled arrow indicates dynein heavy chains (labeled as dynein in C).

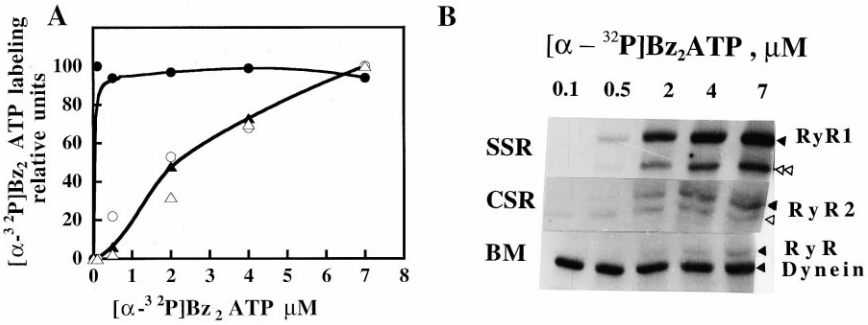

Fig. 4. Concentration dependence of [α-^{32}P]-Bz_2ATP labeling of RyR and dynein. Skeletal (○) and cardiac muscle (▲) SR membranes, and brain microsomes (△, ●) (50 μg), were labeled with the indicated concentrations of [α-^{32}P]-Bz_2ATP and subjected to SDS-PAGE, autoradiography and quantitative analysis of the autoradiograms as described in Section 2. The labeling intensity (relative to the maximal labeling of each protein) of the RyR monomers of skeletal (○), cardiac (▲) and brain (△), and of brain dynein heavy chains (●), is presented in A as a function of [α-^{32}P]-Bz_2ATP concentration. In B, the autoradiographs of the high MW protein bands (460, 440 and 420 kDa bands) obtained from skeletal, cardiac and brain microsomal membranes, respectively, and used for the quantitative analysis, are shown. The double open-headed arrow indicates RyR-1 proteolytic fragment and the single open-headed arrow indicates dynein heavy chain in cardiac SR, which has high affinity for [α-^{32}P]-Bz_2ATP and cross-reacts with anti-dynein antibody (Fig. 3C). This experiment is representative of four similar experiments.

The concentration dependence of [α-^{32}P]-Bz_2ATP binding to skeletal and cardiac muscle RyRs, and to the brain 440 kDa (RyR-2) and 420 kDa (dynein heavy chain) protein bands, is presented in Fig. 4. The results illustrate how the brain 420 kDa protein band, even with 10-fold lower Bz_2ATP concentrations, was much more intensively labeled than the skeletal, cardiac or brain RyRs. This protein band was again recognized by anti-dynein antibody, confirming that it represents dynein heavy chain (Fig. 3C). The labeling of each protein band relative to the amount of protein was: 8.7 ± 1.6 ($n = 6$), 2.0 ± 0.6 ($n = 3$), 2.1 ± 0.82 ($n = 6$) and 1.0 ($n = 6$) for dynein, and the brain microsomal membrane RyR, cardiac SR RyR and skeletal muscle SR RyR, respectively (means ± S.D., n = no. of experiments, all normalized to skeletal muscle). Our attempt to locate the RyR ATP binding site(s) more precisely in the protein primary sequence by limited proteolysis and microsequencing was unsuccessful because the very limited amount of RyR protein in sheep brain. It should be noted that a potential ATP binding site(s) has not yet mapped in the primary sequence of the more abundant skeletal or cardiac RyR.

4. Discussion

4.1. Identification of a sheep brain RyR and brain dynein

We used photoaffinity labeling with [α-^{32}P]-Bz_2ATP as a tool to identify and characterize the ATP regulatory site of sheep brain RyR(s)/Ca^{2+}-release channel(s). However, a dominant ATP binding protein band of 420 kDa is also present in the brain microsomal fraction, and depending on the SDS-PAGE conditions it co-migrates with a 440 kDa RyR. This 420 kDa protein band was identified as the heavy chain of dynein based on: (a) interaction with specific anti-dynein antibody (Fig. 3), (b) amino acid sequence of an 18 amino acid peptide showing complete identity to a sequence of rat brain dynein [27], and (c) over 1000-fold higher affinity for ATP in comparison to cardiac and skeletal muscle RyRs, as demonstrated by its labeling with the photoreactive ATP analogue [α-^{32}P]-Bz_2ATP (Figs. 3 and 4). It is thus important to be aware of the presence of dynein in brain microsomal fractions. An attempt to remove dynein by several approaches, including extraction with ATP [29] and UV irradiation in the presence of Mg^{2+}, ATP and vanadate [30], was ineffective in completely separating cytosolic dynein and brain microsomes. One sensitive approach that did clearly distinguish between RyR monomer and dynein heavy chain was the use of the photoreactive ATP analog, Bz_2ATP. Dynein was found to be highly labeled at 1000-fold lower [α-^{32}P]-Bz_2ATP concentrations (sub-micromolar concentrations, Fig. 4) in comparison to brain, cardiac or skeletal muscle RyRs. Amino acid sequence of both cytoplasmic and axonemal dynein heavy chains contains four ATP binding consensus sequences [31,32]. These sites, however, have not been characterized in detail. Biochemical analysis of Tetrahymena cilliary dynein has suggested that only one site participates directly in ATP hydrolysis [33]. A Michael's constant (K_m) for ATP hydrolysis (about 1 μM) indicates on high-affinity ATP binding site. The higher affinity determined in this study is expected because of the covalent binding nature of the photoreactive Bz_2ATP.

Following optimal separation by SDS-PAGE of the high MW protein bands in the range of 420–460 kDa, and the use of specific anti-dynein, anti-cardiac RyR and anti-skeletal muscle RyR antibodies, it is however clear that the 440 kDa protein is a RyR monomer (Fig. 3), and the cross-reactivity of this protein band with anti-RyR-2 antibody suggests that it is

Table 1
Effect of NaCl concentration on the stimulation of ryanodine binding by ATP

ATP concentration (mM)	Ryanodine bound % of control	
	0.5 M NaCl	1 M NaCl
None	100	100
1	190 ± 21	90 ± 10
2	170 ± 21	97 ± 8*
3	140 ± 17	76 ± 15

Ryanodine binding to brain microsomes (2.5 mg/ml) was assayed as in Fig. 1 in 0.5 or 1.0 M NaCl, 20 mM MOPS, pH 7.4, 50 μM free Ca^{2+}, in the absence and in the presence of the indicated ATP concentration. Control activity (100%) = 38 and 83 fmol/mg protein for ryanodine binding assayed in 0.5 and 1 M NaCl, respectively.
Results are shown ± S.D. (apart from *, which is the mean ± range of two determinations).

a type-2 RyR. Our monoclonal anti-skeletal muscle antibody does also recognize the 440 kDa protein band in both brain microsomes and cardiac SR (Fig. 3), but the proteins are highly homologous [9]. It has previously been shown that a monoclonal antibody to skeletal RyR detected several RyR fragments in rat brain microsomes after proteolysis [34]. We can not rule out the presence of another RyR isoform that is not recognized by anti-RyR-1 and anti-RyR-2 antibodies, for example a sheep type-3 isoform. However, given the non-availability of a specific and highly reactive anti-RyR-3 antibody, we cannot at this stage demonstrate the existence of RyR-3 in our brain microsomal preparations.

4.2. Characterization of the sheep brain RyR ATP binding site

The stimulatory effect of ATP on single Ca^{2+} channel activity and ryanodine binding appears to be due to a direct interaction of ATP with brain RyRs [10–12,18], but the precise mechanism by which nucleotides modulate the brain RyR/Ca^{2+}-release channel is unknown. It does not necessarily involve protein phosphorylation, as non-hydrolyzable ATP derivatives are effective. One possible mechanism is that ATP (or other nucleotides) bind to the RyR and stabilize a protein conformation, which has a higher binding affinity for ryanodine (Fig. 2). This may be related to an increased probability of the channel being open [12] and, in the simplest of models, increased access for the ligand to its binding site. Bz_2ATP enhanced the binding of ryanodine by up to 3-fold more than other nucleotides (see Table 3). This may suggest that the nucleotide binding sites have a higher affinity for Bz_2ATP than ATP. It has been previously shown that Bz_2ATP is more effective than ATP in activating the ATP-gated cation channel (purinergic receptors) [35–37]. This may suggest the nucleotide binding site binds better the more hydrophobic nucleotide. The observations that the aromatically substituted analog 2′,3′-trinitrophenyl-adenosine 5′-triphosphate binds better than ATP to (Na^++K^+) ATPase [38] and to the SR Ca^{2+}-ATPase [39] support the above suggestion. Alternatively, ATP may bind to two or more nucleotide binding sites with different affinities, where binding to the high-affinity site stimulates, and binding to the low-affinity site inhibits ryanodine binding (Fig. 1, Table 1 and [11,18]). The greater stimulation of ryanodine binding by Bz_2ATP may be due to its interaction with a high-affinity 'stimulatory', but not with the low-affinity 'inhibitory' nucleotide binding site, so that overall no inhibition of ryanodine binding is obtained. The biphasic effect of ATP on ryanodine binding – stimulation followed by inhibition with increased ATP concentrations (Fig. 1 and [11,17]) – supports the suggestion that there are two or more nucleotide binding sites in the RyR with different affinities and the produced effects.

Table 2
Effect of photoactivation of Bz_2ATP on ryanodine binding

Bz_2ATP concentration (μM)	[^{3}H]ryanodine bound % of control
0 (dark or UV)	100
10	100
10+UV	180
50	100
50+UV	190
100	150
100+UV	270
400	250
400+UV	380

Brain microsomes (1 mg/ml) were incubated in 0.4 M NaCl, 0.5 mM EDTA, 50 mM MOPS (pH 7.4) and the indicated concentration of Bz_2ATP for 3 min in the dark or exposed to ultraviolet light (UV). The samples were then diluted 2-fold with a solution to bring about final concentrations of 25 mM MOPS, pH 7.4, 0.5 M NaCl, 50 μM free Ca^{2+} and 10 nM ryanodine, and assayed (in the dark) for ryanodine binding as described in Table 1. The data are representatives of two similar experiments.

Table 3
Effect of different nucleotides on ryanodine binding by brain microsomes

Nucleotides	Concentration (mM)	[^{3}H]ryanodine bound, % of control
None	–	100
AMP	0.5	130 ± 14
	2.0	160 ± 16
cAMP	0.5	86 ± 9
	2.0	95 ± 7
ADP	0.5	140 ± 14
	2.0	120 ± 9
ATP	0.5	150 ± 15
	1.0	150 ± 3
AMP-PNP	0.5	110 ± 2
	1.0	150 ± 11
Bz_2ATP	0.5	230 ± 21
	1.0	280 ± 29
GTP	0.5	99 ± 1
	2.0	140 ± 17

[^{3}H]ryanodine binding was assayed in 0.5 M NaCl using 10 nM ryanodine in the absence and in the presence of the indicated nucleotide as described in Table 1. The free Ca^{2+} concentration was 50 μM in each case. Control activity for [^{3}H]ryanodine binding (100%) was between 38 to 100 fmol/mg protein. The results are averages of two to five experiments ± S.D. n.d., not determined.

4.3. Are brain microsomal membranes associated with the cytoskeleton via cytosolic dynein?

Brain dynein is a microtubule-activated ATPase that functions as a molecular motor to transport membrane organelles retrogradely in the axon [28]. The observation that dynein is associated with a brain microsomal membrane fraction suggests there may be a specific dynein-mediated interaction between microsomal ER membranes and the cytoskeleton. In this respect, the finding that RyR-1 and RyR-3 contain a cytoskeleton binding motif that could confer a structural and functional association with voltage-dependent Ca^{2+} channels in skeletal muscle is of considerable interest [40]. Neuronal L-type voltage-dependent Ca^{2+} channels and RyRs are clearly functionally coupled [41], and it is possible that an underlying molecular coupling may be partly mediated by microtubules and/or their associated motor protein dynein. An interaction between the membrane-associated cytoskeletal protein ankyrin and the mouse T-lymphoma cell RyR has been demonstrated [42]. Furthermore, the binding of kinesin and cytoplasmic dynein to brain microsomal fraction has been demonstrated and characterized [43]. Both motors exhibit saturation binding to the vesicles (1–6 binding sites per vesicle) and the binding is abolished by proteolysis of vesicle membrane proteins [40]. These findings may have broad significance in neuronal cell function, and suggest that the possible direct or indirect association of brain RyRs with cytoskeleton proteins should be further investigated.

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