

Primary structure and distribution of a novel ryanodine receptor/calcium release channel from rabbit brain

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The complete amino acid sequence of a novel ryanodine receptor/calcium release channel from rabbit brain has been deduced by cloning and sequence analysis of the cDNA. This protein is composed of 4872 amino acids and shares characteristic structural features with the skeletal muscle and cardiac ryanodine receptors. RNA blot hybridization analysis shows that the brain ryanodine receptor is abundantly expressed in corpus striatum, thalamus and hippocampus, whereas the cardiac ryanodine receptor is more uniformly expressed in the brain. The brain ryanodine receptor gene is transcribed also in smooth muscle.

Ryanodine receptor; Calcium release channel; cDNA cloning; RNA blot hybridization; Smooth muscle; Rabbit brain

1. INTRODUCTION

Cytoplasmic free Ca^{2+} plays important roles in the regulation of various cellular functions, and Ca^{2+} release from intracellular stores is a major source of cytoplasmic Ca^{2+} . Ca^{2+} can be released from the endoplasmic reticulum (ER) via two types of calcium release channel: the inositol 1,4,5-trisphosphate (IP_3) receptor and the ryanodine receptor, the latter being activated by cytoplasmic Ca^{2+} (Ca^{2+} -induced Ca^{2+} release, caffeine and ryanodine [1–3]. The ryanodine receptor is abundantly present in skeletal muscle. The ryanodine receptor in skeletal muscle forms a homo-tetrameric complex with the 'foot' structure, which spans the gap between the transverse-tubule (T-tubule) and sarcoplasmic reticulum (SR) membranes [4]. The skeletal muscle ryanodine receptor releases Ca^{2+} from SR following depolarization of the T-tubule membrane, although the molecular mechanism of this coupling remains to be elucidated [1,3]. Cloning and sequence analysis of cDNAs have revealed the primary structure of the ryanodine receptor from skeletal muscle [5,6] as well as from heart [7,8]. Expression of the cDNAs in Chinese hamster ovary cells [9] or *Xenopus* oocytes [7] directs the formation of functional calcium release channels.

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Abbreviations: ER, endoplasmic reticulum; IP_3 , inositol 1,4,5-trisphosphate; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

Several lines of evidence suggest that the ryanodine receptor is involved in Ca^{2+} signalling also within neurons [10–12]. RNA blot hybridization analysis and cloning and sequence analysis of partial cDNAs have suggested that the cardiac ryanodine receptor is expressed in the brain [7], but existence of other types of ryanodine receptor was not known. We report here the complete amino acid sequence of a novel type of ryanodine receptor/calcium release channel of rabbit brain deduced from the cDNA sequence. The tissue distribution of the brain ryanodine receptor mRNA, together with that of the skeletal muscle and cardiac ryanodine receptor mRNAs, has been studied by blot hybridization analysis.

2. MATERIALS AND METHODS

2.1. Cloning and sequencing of cDNAs

An oligo(dT)-primed, size-selected ($> \sim 2.5$ kilobase pairs (kb)) cDNA library [13], constructed in phage λ ZAPII (Stratagene) using poly(A)⁺ RNA isolated from adult rabbit whole brain [14,15], was screened ($\sim 3.0 \times 10^5$ plaques) with the *Sma*I(13290)/*Sma*I(15181) fragment from a skeletal muscle ryanodine receptor cDNA clone pRR616 [5], to isolate λ BRR110; restriction endonuclease sites are identified by numbers (in parentheses) indicating the 5'-terminal nucleotide generated by cleavage; nucleotide residues are numbered in the 5'-to-3' direction, beginning with the first residue of the ATG triplet encoding the putative initiating methionine. Another oligo(dT)-primed, size-selected ($> \sim 2.5$ kb) cDNA library, constructed in phage λ gt10 as in [16], was screened ($\sim 4.0 \times 10^5$ plaques) with the 2.1-kb *Eco*RI(13354)/*Eco*RI(vector) fragment derived from λ BRR110 to yield λ BRR9. A randomly primed, size-selected ($> \sim 2.5$ kb) cDNA library, constructed in phage λ gt10, was screened ($\sim 4.0 \times 10^5$ plaques) seven times with different probes: the 0.7-kb *Eco*RI(vector)/*Eco*RI(13354) fragment from λ BRR9 yielded 2 clones including λ BRR61; the 1.0-kb *Eco*RI(vector)/*Rsa*I(12963) fragment from λ BRR61 yielded 3 clones including λ BRR71 and λ BRR74; the 0.9-kb *Eco*RI(vector)/

RsaI(10596) fragment from λ BRR74 yielded 2 clones including λ BRR92; the 1.0-kb *EcoRI*(vector)/*PvuII*(9188) fragment from λ BRR92 yielded 3 clones including λ BRR105 and λ BRR121; the 2.5-kb *EcoRI*(vector)/*PvuII*(8101) fragment from λ BRR105 yielded 4 clones including λ BRR133; the 0.4-kb *EcoRI*(vector)/*PstI* (4590) fragment from λ BRR133 yielded λ BRR142; the 0.6-kb *EcoRI*(vector)/*PstI*(4590) fragment from λ BRR142 yielded 2 clones including λ BRR151. A synthetic primer complementary to nucleotide residues 2492–2521 was elongated by the procedures described previously [5] and the resulting clones were selected with the 0.4-kb *EcoRI*(vector)/*HindIII*(2538) fragment from λ BRR151 to yield 5 clones including λ BRR331 and λ BRR341. All cDNA inserts were subcloned into the *EcoRI* site of pBluescript SK(–) (Stratagene). Nested deletions were made and DNA sequencing was carried out on both strands [17]. The cDNA clones used for nucleotide sequence analysis were as follows: λ BRR331 (carrying nucleotides –50 to 2426), λ BRR341 (–69 to 131; residues downstream not sequenced), λ BRR151 (2184 to 4339), λ BRR133 (4205 to 5972), λ BRR142 (4012 to 5304; residues 4212 to 5104 not sequenced), λ BRR121 (5958 to 8289), λ BRR105 (5578 to 8934; residues 6395 to 7299 not sequenced), λ BRR92 (8241 to 10,024), λ BRR74 (9721 to 12,184), λ BRR71 (11,834 to 13,353), λ BRR61 (12,000 to 13,353), λ BRR9 (12,692 to 14,673) and λ BRR110 (13,354 to 15,472).

An oligo(dT)-primed, size-selected (>0.3 kb) cDNA library was constructed in λ gt10 using poly(A)⁺ RNA from adult rabbit aorta (TimeSaver, Pharmacia). The library was screened (~1.5 × 10⁵ plaques) with the *EcoRI*(11834)/*EcoRI*(13354) fragment from λ BRR71 to yield λ ARR1, λ ARR2 and λ ARR3. Both ends of the cDNA inserts of these clones (λ ARR1: residues 11,146 to 11,257 and 15,370 to 15,472, λ ARR2: 11,074 to 11,193 and 15,300 to 15,472, λ ARR3: 8558 to 8695 and 15,434 to 15,472) were sequenced by the dsDNA cycle sequencing system (Bethesda Research Laboratories). The cDNA inserts were subcloned into the *NotI* site of pBluescript SK(–) and analysed with restriction endonucleases *EcoRI*, *PstI* and *HincII*.

2.2. RNA blot hybridization analysis

Total RNA was extracted from rabbit tissues by the LiCl–urea method [18]. In brief, tissues were homogenized in a solution containing 3 M LiCl and 6 M urea with a blender on ice and RNA was precipitated. The procedures used for RNA blot hybridization were essentially the same as described previously [19], except that 1.2% agarose gels were used and that the filters were washed at 50°C with 0.3 × SSC containing 0.1% SDS. Because probes derived from partial cDNAs of the brain ryanodine receptor gave only faint signals, pNRR42 carrying the entire protein coding sequence of the brain ryanodine receptor cDNA was constructed, which contains the following fragments: 2.5-kb *KpnI*(on pBluescript SK(–))/*DraI*(2395) derived from λ BRR331, *DraI*(2395)/*PmaCI*(4299) from λ BRR151, *PmaCI*(4299)/*ApaI*(5917) from λ BRR133, *ApaI*(5917)/*KpnI*(6249) from λ BRR105, *KpnI*(6249)/*HindIII*(7523) from λ BRR121, *HindIII*(7523)/*XbaI*(8405) from λ BRR105, *XbaI*(8405)/*PstI*(9788) from λ BRR92, *PstI*(9788)/*EcoRI* (11834) from λ BRR74, *EcoRI*(11834)/*EcoRI*(13354) from λ BRR71, *EcoRI*(13354)/*HincII* (14656) from λ BRR110, 25-base-pair *EcoRV*/*XhoI* from pBluescript SK(–) and 2.9-kb *XhoI*/*KpnI* from pBluescript SK(–). The probes used for RNA blot hybridization analysis were the 14.8-kb *XhoI*(vector)/*XhoI*(vector) fragment of pNRR42 for the brain ryan-

odine receptor mRNA, an equimolar mixture of the 3.8-kb *EcoRI*(vector)/*EcoRI*(vector) fragment from pHRR160 and the 3.2-kb *EcoRI*(vector)/*EcoRI*(10073) and the 2.4-kb *EcoRI*(10073)/*EcoRI*(vector) fragment from pHRR60 for the cardiac ryanodine receptor mRNA [7], and an equimolar mixture of the 0.9-kb *EcoRI*(vector)/*EcoRI*(2296) and the 2.1-kb *EcoRI*(2296)/*EcoRI*(vector) fragment from pRR229, the 2.3-kb fragment *SacI*(vector)/*XhoI*(6467) from pRR72 and the 1.0-kb *EcoRI*(vector)/*EcoRI*(11636) and the 0.8-kb *EcoRI*(11636)/*EcoRI*(vector) fragment from pRR451 for the skeletal muscle ryanodine receptor mRNA [5]. The probes were labelled by the random primer method with [α -³²P]dCTP [20]. The presence of equivalent amounts of rRNAs (18 S and 28 S) on each lane was confirmed by ethidium bromide staining of the gel. Dehybridization and rehybridization experiments with different probes were carried out for each blot in order to verify the uniformity of the three blots.

3. RESULTS

3.1. cDNA cloning and protein structure

Fig. 1 shows the amino acid sequence of the rabbit brain ryanodine receptor as predicted from the cloned cDNA (for cloning procedure, see Materials and Methods). The primary structure of this protein was deduced by using the open reading frame corresponding to the amino acid sequences of the rabbit skeletal muscle and cardiac ryanodine receptors [5,7]. The nucleotide sequence GAGCCATGG surrounding the translational initiation codon agrees reasonably well with the consensus initiation sequence CCA(G)CCATGG [21]. The 3'-noncoding region of the cDNA is 856 nucleotides long (excluding the poly(dA) tract); the polyadenylation signal AATAAA [22] is found 13 nucleotides upstream from the poly(dA) tract. The rabbit brain ryanodine receptor is composed of 4872 amino acid residues, its calculated *M_r* (including the initiating methionine) being 551,901. Amino acid sequence comparison of the brain, cardiac and skeletal muscle ryanodine receptors reveals 70%, 67% and 67% identities between the brain/cardiac, brain/skeletal muscle and skeletal muscle/cardiac pairs, respectively. The hydropathicity profile of the brain ryanodine receptor is similar to those of the skeletal muscle and cardiac counterparts in that there are four highly hydrophobic segments (referred to as M1, M2, M3 and M4) in the carboxy-terminal tenth of the molecule, that the remaining region is largely hydrophilic and that there is no hydrophobic amino-terminal sequence indicative of the signal sequence. The carboxy-

Fig. 1. Amino acid sequence (in one-letter code) of the rabbit brain ryanodine receptor (top), deduced from the cDNA sequence, and its alignment with those of the rabbit cardiac [7] (middle) and the rabbit skeletal muscle [5] (bottom) ryanodine receptor. The sets of three identical residues at one position are enclosed with solid lines, and sets of three identical or conservative residues with broken lines [39]. Gaps (–) have been inserted to achieve maximum homology and a continuous stretch of gaps was counted as one substitution regardless of its length. Amino acid residues are numbered from the initiating methionine. The putative transmembrane segments M1–M4 are indicated; the termini of each segment are tentatively assigned by comparison with the rabbit skeletal muscle and cardiac ryanodine receptors. Four repeated sequences occurring in two tandem pairs are bounded by arrows. Two stretches of amino acid residues which are highly conserved between the ryanodine receptor and the IP₃ receptor are underlined.

B	MAECCGEG	EDFIOFLRTD	EVYLOC	ATVHKEDOR	CLAAEG	GNRCLFLEPT	SEAKFI	PPOLC	MFVLEOSL	SVRALQERLANT	CEHOC	93
SK	MADGEG	EDFIOFLRTD	EVYLOC	ATVHKEDOR	CLAAEG	GNRCLFLEPT	SEAKFI	PPOLC	MFVLEOSL	SVRALQERLANT	CEHOC	98
	MADGEG	EDFIOFLRTD	EVYLOC	ATVHKEDOR	CLAAEG	GNRCLFLEPT	SEAKFI	PPOLC	MFVLEOSL	SVRALQERLANT	CEHOC	91
B	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	188
SK	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	198
	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	186
B	GNITVD	DAFTMT	LNHNP	HTCS	SSIE	ETGLIG	GVYRL	FGH	DECTI	STQ	MDGHR	287
SK	GNITVD	DAFTMT	LNHNP	HTCS	SSIE	ETGLIG	GVYRL	FGH	DECTI	STQ	MDGHR	298
	GNITVD	DAFTMT	LNHNP	HTCS	SSIE	ETGLIG	GVYRL	FGH	DECTI	STQ	MDGHR	283
B	NVTG	HTL	LA	TE	UO	GL	LO	DR	CKA	D	T	387
SK	NVTG	HTL	LA	TE	UO	GL	LO	DR	CKA	D	T	395
	NVTG	HTL	LA	TE	UO	GL	LO	DR	CKA	D	T	380
B	EGHMD	UGT	LO	RC	REES	OA	AR	IT	NT	TA	LS	478
SK	EGHMD	UGT	LO	RC	REES	OA	AR	IT	NT	TA	LS	491
	EGHMD	UGT	LO	RC	REES	OA	AR	IT	NT	TA	LS	480
B	EGM	LA	LY	AC	TD	NR	HT	MS	AR	FA	IG	578
SK	EGM	LA	LY	AC	TD	NR	HT	MS	AR	FA	IG	591
	EGM	LA	LY	AC	TD	NR	HT	MS	AR	FA	IG	580
B	GHKS	IS	LL	DK	GR	NR	HY	LV	LS	CL	CV	678
SK	GHKS	IS	LL	DK	GR	NR	HY	LV	LS	CL	CV	691
	GHKS	IS	LL	DK	GR	NR	HY	LV	LS	CL	CV	680
B	HLRV	GWA	S	CT	PT	PG	GG	W	CG	NG	V	778
SK	HLRV	GWA	S	CT	PT	PG	GG	W	CG	NG	V	791
	HLRV	GWA	S	CT	PT	PG	GG	W	CG	NG	V	760
B	VS	FA	Q	Y	VR	FL	GG	RR	GE	FL	PP	878
SK	VS	FA	Q	Y	VR	FL	GG	RR	GE	FL	PP	891
	VS	FA	Q	Y	VR	FL	GG	RR	GE	FL	PP	880
B	LW	VR	NR	HT	LA	TE	UO	GL	LO	DR	CKA	978
SK	LW	VR	NR	HT	LA	TE	UO	GL	LO	DR	CKA	991
	LW	VR	NR	HT	LA	TE	UO	GL	LO	DR	CKA	980
B	DE	IL	VD	KA	EA	NA	HN	VA	RD	IR	Q	1076
SK	DE	IL	VD	KA	EA	NA	HN	VA	RD	IR	Q	1090
	DE	IL	VD	KA	EA	NA	HN	VA	RD	IR	Q	1077
B	ES	TI	AV	RS	IR	WY	FE	FA	VT	GS	RR	1176
SK	ES	TI	AV	RS	IR	WY	FE	FA	VT	GS	RR	1190
	ES	TI	AV	RS	IR	WY	FE	FA	VT	GS	RR	1177
B	AF	AD	IE	TE	NA	GV	FI	CS	CL	DS	LS	1276
SK	AF	AD	IE	TE	NA	GV	FI	CS	CL	DS	LS	1280
	AF	AD	IE	TE	NA	GV	FI	CS	CL	DS	LS	1277
B	GS	NS	TD	IM	FE	LS	MS	PI	CE	AS	FS	1301
SK	GS	NS	TD	IM	FE	LS	MS	PI	CE	AS	FS	1374
	GS	NS	TD	IM	FE	LS	MS	PI	CE	AS	FS	1377
B	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	1368
SK	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	1466
	ADGGG	HRTLLYGHAI	LLRHSA	FSGMYTIC	LSST	OTOKLAFOWG	LOCH	HA	TEACMMW	HPASKORSEGEKVR	1473
B	VT	LD	GE	RC	REES	OA	AR	IT	NT	TA	LS	1468
SK	VT	LD	GE	RC	REES	OA	AR	IT	NT	TA	LS	1564
	VT	LD	GE	RC	REES	OA	AR	IT	NT	TA	LS	1573
B	PL	SA	AT	KT	KE	RT	VP	CP	PR	LD	U	1568
SK	PL	SA	AT	KT	KE	RT	VP	CP	PR	LD	U	1664
	PL	SA	AT	KT	KE	RT	VP	CP	PR	LD	U	1673
B	CAL	GS	NR	HY	LV	LS	CL	CV	W	CG	NG	1662
SK	CAL	GS	NR	HY	LV	LS	CL	CV	W	CG	NG	1758
	CAL	GS	NR	HY	LV	LS	CL	CV	W	CG	NG	1772
B	PG	FK	ST	PT	CG	EV	1756
SK	PG	FK	ST	PT	CG	EV	1852
	PG	FK	ST	PT	CG	EV	1872
B	EH	SA	1841
SK	EH	SA	1936
	EH	SA	1969
B	QAN	OR	RY	NA	LM	SA	AL	TAR	K	IE	FR	1936
SK	QAN	OR	RY	NA	LM	SA	AL	TAR	K	IE	FR	2034
	QAN	OR	RY	NA	LM	SA	AL	TAR	K	IE	FR	2068
B	KIT	GP	PE	KE	PT	TE	2033
SK	KIT	GP	PE	KE	PT	TE	2131
	KIT	GP	PE	KE	PT	TE	2167
B	VIA	CR	EE	KL	MI	NR	GL	DM	NR	HY	LV	2132
SK	VIA	CR	EE	KL	MI	NR	GL	DM	NR	HY	LV	2231
	VIA	CR	EE	KL	MI	NR	GL	DM	NR	HY	LV	2266
B	PS	MR	ST	PL	DA	AA	SY	ON	NE	LA	LO	2232
SK	PS	MR	ST	PL	DA	AA	SY	ON	NE	LA	LO	2364
	PS	MR	ST	PL	DA	AA	SY	ON	NE	LA	LO	
B	GP	AL	RG	EG	GL	LA	AM	GO	AT	RT	ST	2330
SK	GP	AL	RG	EG	GL	LA	AM	GO	AT	RT	ST	2464
	GP	AL	RG	EG	GL	LA	AM	GO	AT	RT	ST	
B	DLY	GI	TS	IS	PI	KT	PS	IN	KD	VS	EP	2430
SK	DLY	GI	TS	IS	PI	KT	PS	IN	KD	VS	EP	2530
	DLY	GI	TS	IS	PI	KT	PS	IN	KD	VS	EP	2564

B	CAPLFAQTEH	YISLIDSL	ITITLSTK	ASLTAKAORD	TEECCLAT	INHLRPSM	QOLLRRLVFDV	QULWQYCKMPLKLLTNH	FSQCKKTYTCLH	GWGQST	2530	
B	CAPLFAQTEH	YISLIDSL	ITITLSTK	ASLTAKAORD	TEECCLAT	INHLRPSM	QOLLRRLVFDV	QULWQYCKMPLKLLTNH	FSQCKKTYTCLH	GWGQST	2530	
SK	CAPLFAQTEH	YISLIDSL	ITITLSTK	ASLTAKAORD	TEECCLAT	INHLRPSM	QOLLRRLVFDV	QULWQYCKMPLKLLTNH	FSQCKKTYTCLH	GWGQST	2530	
B	GLAVFEELH	TEELFWGTF	LSHKKYD	HTFMSL	PCSAI	AGALPPD	YOTIRIT	ATLQKQVSV	DAQNT	OPAPIT	MTIF	2633
B	GLAVFEELH	TEELFWGTF	LSHKKYD	HTFMSL	PCSAI	AGALPPD	YOTIRIT	ATLQKQVSV	DAQNT	OPAPIT	MTIF	2633
SK	GLAVFEELH	TEELFWGTF	LSHKKYD	HTFMSL	PCSAI	AGALPPD	YOTIRIT	ATLQKQVSV	DAQNT	OPAPIT	MTIF	2633
B	KWALCEIS	SGSMKVC	ISLDEHY	KITHTP	LAPIT	RTYKKE	KEITRWPI	ARESLK	ITMLAY	GMIT	WEATAE	2725
B	KWALCEIS	SGSMKVC	ISLDEHY	KITHTP	LAPIT	RTYKKE	KEITRWPI	ARESLK	ITMLAY	GMIT	WEATAE	2725
SK	KWALCEIS	SGSMKVC	ISLDEHY	KITHTP	LAPIT	RTYKKE	KEITRWPI	ARESLK	ITMLAY	GMIT	WEATAE	2725
B	ISNVYLS	RELOQ	AVIEV	AEYTH	WAKKK	LELES	SGG	HP	LLPYD	TLTAK	ER	2825
B	ISNVYLS	RELOQ	AVIEV	AEYTH	WAKKK	LELES	SGG	HP	LLPYD	TLTAK	ER	2825
SK	ISNVYLS	RELOQ	AVIEV	AEYTH	WAKKK	LELES	SGG	HP	LLPYD	TLTAK	ER	2825
B	KLIRYVD	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	2925
B	KLIRYVD	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	2925
SK	KLIRYVD	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	AEH	2925
B	ITNVYLS	CHT	LAO	LOIT	RTYK	SG	SE	LYK	AEH	FE	SA	3025
B	ITNVYLS	CHT	LAO	LOIT	RTYK	SG	SE	LYK	AEH	FE	SA	3025
SK	ITNVYLS	CHT	LAO	LOIT	RTYK	SG	SE	LYK	AEH	FE	SA	3025
B	QVSCRT	IT	SLY	LG	TH	IT	VEH	OR	AL	GE	CL	3125
B	QVSCRT	IT	SLY	LG	TH	IT	VEH	OR	AL	GE	CL	3125
SK	QVSCRT	IT	SLY	LG	TH	IT	VEH	OR	AL	GE	CL	3125
B	RTTTPH	VEY	IT	CP	ML	NY	LS	Y	W	WE	RG	3221
B	RTTTPH	VEY	IT	CP	ML	NY	LS	Y	W	WE	RG	3221
SK	RTTTPH	VEY	IT	CP	ML	NY	LS	Y	W	WE	RG	3221
B	CEKLRKA	AV	KV	TO	CE	TO	CE	TO	CE	TO	CE	3321
B	CEKLRKA	AV	KV	TO	CE	TO	CE	TO	CE	TO	CE	3321
SK	CEKLRKA	AV	KV	TO	CE	TO	CE	TO	CE	TO	CE	3321
B	REINNA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3421
B	REINNA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3421
SK	REINNA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3421
B	RSLO	OPA	AV	W	OL	IL	TS	LE	TS	LE	TS	3507
B	RSLO	OPA	AV	W	OL	IL	TS	LE	TS	LE	TS	3507
SK	RSLO	OPA	AV	W	OL	IL	TS	LE	TS	LE	TS	3507
B	YVLF	IG	Y	KS	W	IE	TS	LE	TS	LE	TS	3602
B	YVLF	IG	Y	KS	W	IE	TS	LE	TS	LE	TS	3602
SK	YVLF	IG	Y	KS	W	IE	TS	LE	TS	LE	TS	3602
B	EN	FE	KE	ME	KO	RT	LY	QO	AR	HE	GA	3702
B	EN	FE	KE	ME	KO	RT	LY	QO	AR	HE	GA	3702
SK	EN	FE	KE	ME	KO	RT	LY	QO	AR	HE	GA	3702
B	QKKA	EG	LG	W	IE	TS	LE	TS	LE	TS	LE	3802
B	QKKA	EG	LG	W	IE	TS	LE	TS	LE	TS	LE	3802
SK	QKKA	EG	LG	W	IE	TS	LE	TS	LE	TS	LE	3802
B	NFSKA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3902
B	NFSKA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3902
SK	NFSKA	LA	FT	IG	SS	SK	SK	SK	SK	SK	SK	3902
B	ESS	TV	EM	L	K	F	D	M	F	K	L	4002
B	ESS	TV	EM	L	K	F	D	M	F	K	L	4002
SK	ESS	TV	EM	L	K	F	D	M	F	K	L	4002
B	LSCH	PH	ND	SL	KL	LD	PA	ES	VL	NT	FE	4102
B	LSCH	PH	ND	SL	KL	LD	PA	ES	VL	NT	FE	4102
SK	LSCH	PH	ND	SL	KL	LD	PA	ES	VL	NT	FE	4102
B	QISE	SD	LA	DR	FE	IE	GE	ES	SV	Y	LD	4201
B	QISE	SD	LA	DR	FE	IE	GE	ES	SV	Y	LD	4201
SK	QISE	SD	LA	DR	FE	IE	GE	ES	SV	Y	LD	4201
B	FTY	Y	GL	F	OT	LS	TS	Y	FG	GL	VE	4293
B	FTY	Y	GL	F	OT	LS	TS	Y	FG	GL	VE	4293
SK	FTY	Y	GL	F	OT	LS	TS	Y	FG	GL	VE	4293
B	YK	HE	VE	GL	LD	SE	IG	DE	PT	LE	ST	4300
B	YK	HE	VE	GL	LD	SE	IG	DE	PT	LE	ST	4300
SK	YK	HE	VE	GL	LD	SE	IG	DE	PT	LE	ST	4300
B	FMAN	F	K	GT	IE	Y	OT	R	L	N	Y	4471
B	FMAN	F	K	GT	IE	Y	OT	R	L	N	Y	4471
SK	FMAN	F	K	GT	IE	Y	OT	R	L	N	Y	4471
B	GYMA	HT	LA	LA	Y	HT	IS	LV	CY	Y	CL	4571
B	GYMA	HT	LA	LA	Y	HT	IS	LV	CY	Y	CL	4571
SK	GYMA	HT	LA	LA	Y	HT	IS	LV	CY	Y	CL	4571
B	RT	EL	LO	LA	LA	Y	HT	IS	LV	CY	Y	4669
B	RT	EL	LO	LA	LA	Y	HT	IS	LV	CY	Y	4669
SK	RT	EL	LO	LA	LA	Y	HT	IS	LV	CY	Y	4669
B	KOLV	Y	GL	LA	V	Y	Y	Y	Y	Y	Y	4769
B	KOLV	Y	GL	LA	V	Y	Y	Y	Y	Y	Y	4769
SK	KOLV	Y	GL	LA	V	Y	Y	Y	Y	Y	Y	4769
B	LI	DA	FE	LD	OO	Q	VE	ME	TK	FC	IG	4869
B	LI	DA	FE	LD	OO	Q	VE	ME	TK	FC	IG	4869
SK	LI	DA	FE	LD	OO	Q	VE	ME	TK	FC	IG	4869
B	Q	GL										4872
B	Q	GL										4872
SK	Q	GL										4872

Fig. 1. continued.

terminal region that encompasses the M3 and M4 segments is highly conserved in all ryanodine receptors. On the other hand, there are rather divergent regions, for example, the region around the amino acid residue 1300 where some hundred residues are deleted in the brain ryanodine receptor and the region immediately preceding the M1 segment. The brain ryanodine receptor contains four repeated sequences occurring in two tandem pairs (amino acid residues 840–953, 954–1069, 2602–2713 and 2714–2823), as described for the skeletal muscle [6] and cardiac [7] counterparts.

Potential ligand binding sites were analysed using the proposed consensus amino acid sequences. A sequence that bears some resemblance to the EF-hand [23] is found (residues 3934–3945), and this region is relatively well conserved in the skeletal muscle and cardiac ryanodine receptors. One potential calmodulin-binding site (residues 3472–3495), made up from an amphipathic helix with two clusters of positive charges separated by a hydrophobic region [24], is found, being particularly well conserved in both the skeletal muscle and cardiac counterparts. There are four copies of the nucleotide-binding consensus sequence GXGXXG [25] (residues 697–702, 699–704, 2238–2243 and 2526–2531), one of which (2238–2243) is conserved in both the skeletal muscle and cardiac counterparts. Using the consensus sequence RXXS/T [26], there are twenty-one potential phosphorylation sites for Ca^{2+} /calmodulin-dependent protein kinase, four of which (serine residue 2711 and threonine residues 130, 290 and 4163) are conserved in the skeletal muscle and cardiac counterparts. There are two potential cyclic AMP-dependent phosphorylation sites (threonine residues 1243 and 4156), defined as KRXXS/T or RRXS/T [26], but they are not conserved in the skeletal muscle or cardiac counterparts. All of these potential ligand binding sites described are located on the putative cytoplasmic side according to our transmembrane model [5]. Arginine residue 613, substitution of cysteine for whose equivalent residue in the skeletal muscle ryanodine receptor has been found to be associated with malignant hyperthermia of swine [27] and human [28], is conserved in three types of ryanodine receptor.

Alignment of the amino acid sequences reveals a significant similarity between the ryanodine receptor and the IP_3 receptor [29–31]. Especially, the carboxy-terminal region of the ryanodine receptor, including the M3 and M4 segments, shows a remarkable amino acid sequence similarity to the corresponding region of the IP_3 receptor. Furthermore, two stretches of highly conserved amino acid residues are found between the ryanodine receptor and the IP_3 receptor; RFLQLLCEGHN (residues 3739–3749; glycine is replaced by asparagine in the IP_3 receptor) and LTEYIQGPC (residues 3818–3826; isoleucine is replaced by cysteine in the IP_3 receptor). Interestingly, two regions which encompass the four repeats occurring in two tandem pairs of the ryan-

odine receptor (627–1583 and 2346–3615; see above) are missing in the IP_3 receptor.

3.2. RNA blot hybridization analysis

Total RNA was extracted from rabbit brain and other rabbit tissues and was subjected to blot hybridization analysis with cDNA probes of the rabbit brain, cardiac and skeletal muscle ryanodine receptors. A RNA species hybridizable with a brain ryanodine receptor cDNA probe is found abundantly in restricted areas of the brain (corpus striatum, thalamus and hippocampus), and at a much lower level in midbrain (superior and inferior colliculi), pons and medulla oblongata (Fig. 2A). Its estimated size is ~16 kb. On the other hand, no hybridization signal is observed with RNA preparations from cerebral cortex and cerebellum, although faint signals are detected in these lanes by Image Analyzer (Fuji Film Inc.) (data not shown). A RNA species hybridizable with cardiac ryanodine receptor cDNA probes is distributed throughout the brain except pituitary (Fig. 2B). Interestingly, the cardiac ryanodine receptor mRNA is relatively abundant in cerebellum, where the level of the brain ryanodine receptor mRNA is low, and only small amounts of the cardiac ryanodine receptor mRNA are detected in corpus striatum and thalamus, where the brain ryanodine receptor mRNA is abundant. The skeletal muscle ryanodine receptor mRNA is detected only in skeletal muscle (Fig. 2C), as was reported in [8].

Outside of the brain, a RNA species hybridizable with a brain ryanodine receptor cDNA probe is detected in tissues containing smooth muscle, such as aorta, esophagus, taenia coli, urinary bladder, ureter and uterus (Fig. 2A,D). The size of the RNA species in smooth muscle is ~16 kb, same as in the brain. Faint signals in skeletal muscle and heart may suggest existence of the brain ryanodine receptor mRNA (Fig. 2D), but they may result from cross-hybridization with the skeletal muscle and cardiac ryanodine receptor mRNAs because these mRNAs are present in a huge amount in skeletal muscle and heart (Fig. 2B,C).

3.3. Cloning of partial cDNAs from aorta

To identify the hybridizable RNA species present in smooth muscle, we screened a cDNA library derived from poly(A)⁺ RNA of rabbit aorta using a brain ryanodine receptor cDNA probe. Sequence analysis of three cDNA clones showed that they carry the partial sequences of the brain ryanodine receptor cDNA. Restriction endonuclease analysis showed that all these clones yield fragments common to those derived from the brain ryanodine receptor cDNA. These results indicate that the brain ryanodine receptor gene is transcribed in aorta, although the aortic mRNA may not be identical with the brain ryanodine receptor mRNA because transcription may be initiated at different promoters and/or because the transcription product may be subject to

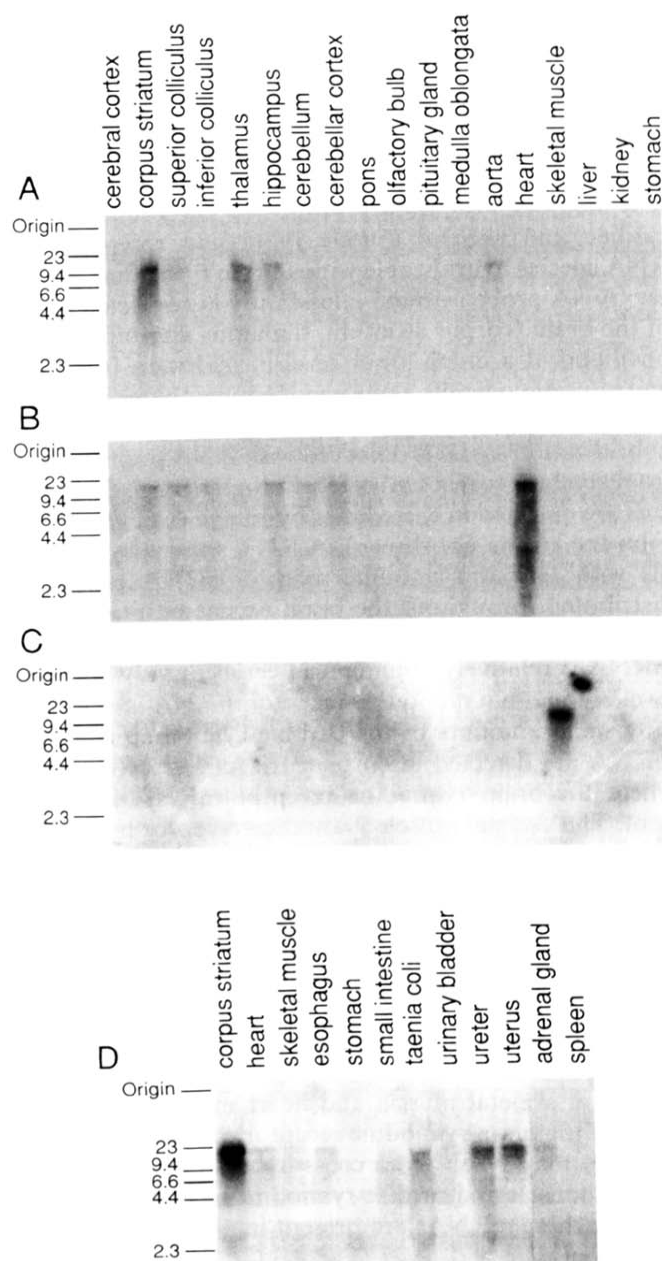


Fig. 2. Autoradiograms of blot hybridization analysis of RNA from different regions of brain and different tissues of adult rabbit with cDNA probes for the brain (A,D), cardiac (B) and skeletal muscle (C) ryanodine receptor mRNAs. 30 mg (A-C) or 60 mg (D) of total RNA was applied to each lane. Autoradiography was performed at -70°C for 4 days (A-C) and for 6 days (D) with an intensifying screen. The size markers used were the *Hind*III cleavage products of phage λ DNA (sizes in kilobases).

alternative splicing. The same may hold for the other putative smooth muscle ryanodine receptor mRNAs.

4. DISCUSSION

We have isolated and sequenced cDNA clones encoding the brain ryanodine receptor. The structural similarity suggests that the brain ryanodine receptor, like the

skeletal muscle and cardiac counterparts, consists of two main parts, the carboxy-terminal channel region containing four putative transmembrane segments (M1-M4) and the large cytoplasmic region, which corresponds to the foot structure. The ryanodine receptor shows amino acid sequence similarity to the IP_3 receptor [29-31], another type of calcium release channel on ER. The sequence similarity is remarkable in the carboxy-terminal region, which presumably contributes to forming the channel pore. The ryanodine receptor and the IP_3 receptor also share two stretches of highly homologous sequence (3739-3749 and 3818-3826). Since these sequences are located close to the putative transmembrane segments, they may contribute to the Ca^{2+} release mechanism common to both receptors. Furthermore, the regions encompassing the four repeats in two tandem pairs found in the ryanodine receptor are missing in the IP_3 receptor. This observation suggests that insertion of a pair of tandem repeats into the IP_3 receptor followed by duplication of the repeats contributed to evolutionary divergence between the ryanodine receptor and the IP_3 receptor.

Recently, evidence has been accumulated to suggest the involvement of the ryanodine receptor in the Ca^{2+} signalling in neurons [10-12]. Rat brain microsome membranes were found to contain high-affinity binding sites for ryanodine [32], and the purified ryanodine receptor protein has been demonstrated to function as a caffeine-sensitive calcium release channel when it is incorporated into planar bilayers [33]. Analysis with antibodies [33], RNA blot hybridization analysis and cloning and partial sequence of cDNAs [7] have suggested that the major species of the ryanodine receptor in brain is the product of the cardiac ryanodine receptor gene. In the present study, we show that while the cardiac ryanodine receptor mRNA is rather uniformly distributed, the brain ryanodine receptor is expressed abundantly in the specific regions of the brain, such as corpus striatum, thalamus and hippocampus. This restricted distribution may indicate that in these regions, Ca^{2+} regulation is different from that in other part of the brain. Furthermore, since these regions roughly correspond to the areas where 'delayed neuronal death' occurs after hypoxia in the brain [34], this type of ryanodine receptor may be important in the pathological state. Another possibility is that the brain ryanodine receptor is coupled more directly with neuronal calcium channels, as observed in skeletal muscle, being responsible for a rapid Ca^{2+} release following membrane depolarization [3].

Our present study has also provided evidence to indicate that the brain ryanodine receptor is expressed in smooth muscle. This result is in line with the recent observation that the ryanodine receptor isolated from aorta is distinct from the skeletal muscle and cardiac ryanodine receptors in single-channel conductance for Ca^{2+} and K^{+} [35].

In neurons and smooth muscle cells, cytoplasmic Ca^{2+} is regulated in various manners, some cells showing Ca^{2+} oscillation and others long-term Ca^{2+} signalling [36,37]. Since the voltage-dependent calcium channel, the IP_3 receptor and the ryanodine receptor are coexpressed in these cells and cooperatively play a major role in the regulation of cytoplasmic Ca^{2+} , multiple types of these components likely contribute to the molecular basis of heterogeneity of Ca^{2+} signalling systems. Functional characterization and morphological investigation of cellular and subcellular localization of three types of ryanodine receptor will help us to understand the Ca^{2+} regulation of brain and smooth muscle in normal and pathological states.

While preparing this manuscript, a partial sequence of a new type of ryanodine receptor from mink lung epithelial cells was reported [38], which corresponds to the carboxy-terminal one eighth of the rabbit brain ryanodine receptor and shows 94% amino acid identity.

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