Differential expression and regulation of ryanodine receptor and *myo*-inositol 1,4,5-trisphosphate receptor Ca^{2+} release channels in mammalian tissues and cell lines

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Ryanodine receptors (RyRs) and $\text{Ins}(1,4,5)\text{P}_3$ receptors ($\text{Ins}(1,4,5)\text{P}_3\text{Rs}$) represent two multigene families of channel proteins that mediate the release of Ca^{2+} ions from intracellular stores. In the present study, the expression patterns of these channel proteins in mammalian cell lines and tissues were investigated by using isoform-specific antibodies. All cell lines examined expressed two or more $\text{Ins}(1,4,5)\text{P}_3\text{R}$ isoforms, with the type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ being ubiquitous. RyR isoforms were detected in only six out of eight cell lines studied. Similarly, of the nine rabbit tissues examined, RyR protein expression was detected only in brain, heart, skeletal muscle and uterus. Specific [³H]ryanodine binding was found in a number of rabbit tissues, although it was not detected in mammalian cell lines. Subcellular fractionation of SH-SY5Y human neuroblastomas revealed that the type 2 RyR and type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ co-localize among the

fractions of a sucrose-cushion separation of crude microsomal membrane fractions. Manipulation of SH-SY5Y cells by chronic stimulation of muscarinic acetylcholine receptor (mAChR) results in a decrease in their type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ levels but not in the abundance of the type 2 RyR. Differentiation of these neuroblastomas by using retinoic acid did not detectably alter their expression of Ca^{2+} -release channel proteins. Finally, differentiation of BC₃H1 cells affects the expression of their Ca^{2+} -release channel proteins in an isoform-specific manner. In summary, this study demonstrates that mammalian cell lines display distinct patterns of Ca^{2+} -release channel protein expression. The abundance of these proteins is differentially regulated during phenotypic modifications of a cell, such as differentiation or chronic stimulation of mAChR.

INTRODUCTION

Two well-characterized classes of ion channel mediate the rapid mobilization of Ca^{2+} ions from intracellular stores: the inositol 1,4,5-trisphosphate receptors [$\text{Ins}(1,4,5)\text{P}_3\text{Rs}$] and the ryanodine receptors (RyRs). Both families form high-conductance, low-selectivity cation channels by the association of four high-molecular-mass protein monomers in combination with a number of accessory proteins such as calmodulin and FK506-binding proteins [1,2]. The $\text{Ins}(1,4,5)\text{P}_3\text{R}$ family consists of at least three distinct gene products, encoding monomers of molecular mass approx. 300 kDa, all of which bind their endogenous agonist $\text{Ins}(1,4,5)\text{P}_3$ and share approx. 70% amino acid identity with one another. The type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ isoform is expressed at greatest levels in cerebellar Purkinje cells, although in common with the other members of this channel family [3] it has very broad tissue and cell distribution. Three RyR isoforms have been identified in mammals, each of calculated molecular mass approx. 560 kDa. RyR isoforms share approx. 70% amino acid identity with one another and show limited similarity with the $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$, which is most extensive within the putative channel domain. Type 1 and type 2 RyRs were originally identified in tissues in which they are expressed at greatest abundance (skeletal muscle and heart respectively) by virtue of their interaction with the neutral plant alkaloid ryanodine. The third RyR isoform was originally identified in mink lung epithelial cells [4] and in rabbit brain [5], although the mRNA encoding this protein displays a

broad tissue distribution [6]. Similarly, mRNA species encoding the type 1 and type 2 RyRs have recently been detected in a range of mammalian tissues and cell lines [6–9].

RyRs and $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ might be co-expressed in mammalian cells. The possible reasons for the co-existence of multiple Ca^{2+} -release mechanisms within a single cell have been discussed extensively [3,10]. These include suggestions that $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$, RyRs and subtypes within each family are functionally distinct [11]; that their activity is differentially modulated by endogenous factors; that their expression is differentially regulated during differentiation and development; or that they are targeted to discrete subcompartments of intracellular Ca^{2+} stores. Both types of channel seem to be located predominantly in the sarcoplasmic/endoplasmic reticulum [12,13], although several reports indicate that they might also be present at the plasma membrane [14,15] or nuclear membrane [16]. Various studies indicate that RyRs have distinct but overlapping distributions compared with those of $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ within a cell. For example, both RyRs and $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ are expressed in the cell bodies and dendrites of chicken Purkinje neurons, but only the latter are present in dendritic spines [17]. Membrane subfractionation of bovine adrenal chromaffin cells reveals a close, although not complete, co-localization of the type 2 RyR, the type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ and sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) pumps [18]. Immunofluorescence studies suggest that different $\text{Ins}(1,4,5)\text{P}_3\text{R}$ isoforms might be expressed in distinct but over-

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3\text{R}$, $\text{Ins}(1,4,5)\text{P}_3$ receptor; mAb, monoclonal antibody; mAChR, muscarinic acetylcholine receptor; methacholine, acetyl- β -methylcholine chloride; pAb, polyclonal antibody; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; Sulfo-SMCC, sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

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lapping subcellular compartments in mammalian macrophage- and lymphocyte-derived cell lines [19].

In the present investigation, the expression profiles of Ins(1,4,5) P_3 R and RyR proteins were determined in a variety of mammalian cell lines and tissues by using isoform-specific antisera. The subcellular distribution of these proteins in SH-SY5Y cells was examined by a membrane subfractionation technique and compared with that of the SERCA pumps. The effects of differentiation of BC₃H1 and SH-SY5Y cells, or of chronic stimulation of muscarinic acetylcholine receptor (mAChR) of these human neuroblastomas, on the expression of their Ca²⁺-release channel proteins were examined. The results presented indicate that RyRs and Ins(1,4,5) P_3 Rs display marked differences in their expression patterns, as well as differences in their subcellular distribution. Furthermore these proteins are differentially regulated by chronic stimulation of mAChR or by differentiation. This distinct regulation of protein expression occurs not only between RyRs and Ins(1,4,5) P_3 Rs but also between different isoforms of each channel family.

MATERIALS AND METHODS

Materials

Acetyl- β -methylcholine chloride (methacholine), atropine, 3,3'-diaminobenzamidine and horseradish peroxidase-conjugated secondary antibodies were from Sigma (Poole, Dorset, U.K.). [³H]Ryanodine (60 Ci/mmol) and enhanced chemiluminescence reagents were from Amersham (Little Chalfont, Bucks., U.K.). Sulphosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was from Pierce and Warriner (Chester, Cheshire, U.K.). A monoclonal antibody (mAb Y1/F4) against SERCA proteins was donated by Dr. F. Michelangeli (University of Birmingham, Birmingham, U.K.). Monoclonal antibodies against the type 2 (mAb KM1083) and type 3 (mAb KM1082) Ins(1,4,5) P_3 Rs were generously supplied by Dr. M. Hasegawa (Tokyo Research Laboratories, Tokyo, Japan). All other reagents used were of A.R. grade.

Preparation of antibodies

Mouse mAbs against type 2 Ins(1,4,5) P_3 R (mAb KM1083), type 3 Ins(1,4,5) P_3 R (mAb KM1082) and SERCA proteins (mAb Y1/F4) were generated and characterized as described previously [19,20]. Isoform-specific regions of type 1, type 2 or

type 3 RyRs of high predicted antigenicity were selected with MacVector® software (International Biotechnologies, New Haven, CT, U.S.A.). Peptide sequences corresponding to these regions were compared with all sequences in the GenBank database to minimize the possibility of cross-reaction with known polypeptides. Peptides corresponding to unique amino acid sequences in the rabbit type 1 RyR, rabbit type 2 RyR and mink type 3 RyR (Table 1) were synthesized on an Applied BioSystems Model 430A peptide synthesizer. Peptides were coupled to keyhole-limpet haemocyanin by using Sulfo-SMCC in accordance with the manufacturer's instructions; the resulting conjugates were used to immunize rabbits by standard protocols [21]. Crude antisera were screened for immunoreactivity against type 1 Ins(1,4,5) P_3 R and type 3 RyR in rabbit cerebellum, type 1 RyR in rabbit skeletal muscle and type 2 RyR in pig heart by immunoblot assay of microsomal proteins. High-titre antisera against type 1 Ins(1,4,5) P_3 R [polyclonal antibody (pAb) 40], type 1 RyR (pAb 405), type 2 RyR (pAb 10 and pAb 129) and type 3 RyR (pAb 1094) were obtained. The specificity of these antisera was examined by immunoblot assays, with antiserum [diluted in 5% (w/v) non-fat milk/PBS (m-PBS)] preincubated with the appropriate synthetic peptide at 10 μ g/ml for 1 h as a negative control.

Cell culture

All cells were grown at 37 °C in media containing 50 i.u./ml penicillin G, 50 μ g/ml streptomycin and 2.5 μ g/ml fungizone, in humidified air/CO₂ (19:1). SH-SY5Y human neuroblastomas, originally a gift from Dr. J. Beidler (Sloane-Kettering Institute for Cancer Research, Rye, NY, U.S.A.), were grown in minimal essential medium with Earle's Salts containing 10% (v/v) FCS, as described previously [20]. These cells were differentiated by culture in 10 μ M retinoic acid/1% (v/v) heat-inactivated FCS for 6 days [22]. BC₃H1 cells, a gift from Dr. C. W. Taylor (University of Cambridge, Cambridge, U.K.), were cultured in Dulbecco's modified Eagle's medium containing 20% (v/v) FCS and were differentiated in the same medium containing only 0.5% (v/v) FCS [23]. All other cell lines were originally obtained from and cultured by the protocols of the European Collection of Animal Cell Cultures.

Preparation of microsomes and membrane subfractionation

All steps were performed at 2–4 °C. Crude microsomal membranes were prepared on a small scale from 75 cm² cell mono-

Table 1 Summary of synthetic peptides used to generate isoform-selective antisera against RyRs

Synthetic peptides representing unique regions of RyR isoforms were selected using MacVector software. Amino acid sequence identity was calculated from aligned RyR sequences. Sequence similarity was calculated by taking conserved amino acid residue changes as similar. The type 3 RyR peptide was selected from a mink lung type 3 RyR partial sequence and so is not 100% identical with the rabbit form of this protein.

Antiserum designation	Peptide sequence	Predicted specificity	Sequence identity/similarity (%)		
			RyR1	RyR2	RyR3
pAb 405	EPPKKAPPSPPAKEEAG RyR1 4515-4534	Type 1 RyR	100/100 (rabbit)	5.5/33.3 (rabbit)	22.2/38.8 (rabbit)
pAb 129	KAALDFSDAREKKPKKDSSLSAV RyR 2 4675-4697	Type 2 RyR	20.8/37.5 (rabbit)	100/100 (rabbit)	33.3/45.8 (rabbit)
pAb 10	EDKGKQKLRQLMTMYGEPE RyR 2 4459-4478	Type 2 RyR	10.5/26.3 (rabbit)	100/100 (rabbit)	10.5/31.6 (rabbit)
pAb 1094	WADVTKKKRRRGQKVEKPE RyR 3 4367-4387	Type 3 RyR	5/25 (rabbit)	5/25 (rabbit)	100/100 (mink) 95/100 (rabbit)

layers, with a modification of a technique described previously [20]. In brief, monolayers were washed twice with 0.02% EDTA/0.9% NaCl/10 mM Hepes (pH 7.2) and were harvested in 5 ml of the same buffer by agitation. Cells were collected by centrifugation at $500 g_{\text{max}}$ for 2 min and the resulting pellet was homogenized in 500 μl of buffer I [25 mM Tris/Hepes (pH 7.4)/0.5 mM MgCl_2] by 50 strokes of a hand-held glass-Teflon homogenizer. An equal volume of buffer II [25 mM Tris/Hepes (pH 7.4)/0.5 M sucrose/0.3 M KCl] was added and the suspension was subjected to another 25 strokes of the homogenizer. The homogenate was centrifuged at $500 g_{\text{max}}$ for 2 min (Biofuge 28RS; Heraeus Sepatech, Heraeus Ltd., Brentwood, Essex, U.K.). The supernatant was centrifuged at $30000 g_{\text{max}}$ for 60 min and the resulting microsomal pellet was resuspended in 100 μl of buffer II by ten passes through a 25-gauge needle. All buffers contained protease inhibitors (2 mM iodoacetamide/1 mM benzamidine/1 $\mu\text{g}/\text{ml}$ leupeptin/1 $\mu\text{g}/\text{ml}$ pepstatin A/1 $\mu\text{g}/\text{ml}$ aprotinin/0.5 mM PMSF). Crude cell microsomes were subfractionated on a sucrose/CsCl cushion as described previously [20].

Crude microsomal membranes from various adult rabbit tissues were prepared by differential centrifugation. Fresh tissues were trimmed free of excess fat, major nerves and blood vessels, and were then cut into approx. 1 cm^3 pieces. Tissues were homogenized with three 30 s bursts of an Ultra-Turrax homogeniser (T-25 probe, maximum speed) in 10 vol. (per wet weight) of ice-cold buffer A consisting of 0.25 M sucrose, 10 mM Mops, pH 7.1, and protease inhibitors. Homogenates were centrifuged at $1100 g_{\text{max}}$ for 10 min (3000 rev./min, Beckman JA-20.1 rotor, J2-21 centrifuge) and resulting supernatants were then centrifuged at $7700 g_{\text{max}}$ for 20 min (8000 rev./min, Beckman JA-20.1 rotor). Crude microsomal membranes were collected from this supernatant at $105000 g_{\text{max}}$ for 60 min (30000 rev./min, Beckman Ti50.2 rotor). Membranes were resuspended in buffer A to a protein concentration of 10–20 mg/ml by ten up-and-down strokes of a loose-fitting glass-Teflon homogenizer, then were used immediately or stored at -70°C until required.

SDS/PAGE and immunoblotting

Membrane proteins were resolved by SDS/PAGE [5% (w/v) gel] or on minigels under denaturing conditions, then transferred to nitrocellulose by using semi-dry blotting apparatus (Atto, Tokyo, Japan). The transfer buffer consisted of 150 mM glycine/20 mM Tris/0.037% SDS; RyRs were transferred at 4 mA/ cm^2 constant current for 4 h; InsP_3Rs /SERCA proteins were blotted at 2 mA/ cm^2 for 2 h. Blots were blocked overnight in m-PBS, then incubated with antibodies, diluted as described in figure legends, for 2 h. Nitrocellulose membranes were washed three times in PBS for 30 min, incubated with a 1:500 dilution of horseradish peroxidase-conjugated anti-(mouse IgG) or a 1:2000 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG) in m-PBS for 1 h. Blots were washed as described above, then immunoreactive proteins were detected with enhanced chemiluminescence reagents or by staining with 3,3'-diaminobenzamide, in accordance with the manufacturers' instructions.

[^3H]Ryanodine binding

[^3H]Ryanodine binding assays were performed essentially as described previously for cardiac and skeletal muscle RyRs [24]. Microsomal protein (1 mg/ml) prepared from various cell types or from rabbit cardiac muscle was incubated at 37°C in 1 M NaCl/150 μM CaCl_2 /100 μM EGTA/10 mM AMP/20 mM Na-Pipes (pH 7.1)/200 μM PMSF, containing 10 nM [^3H]ryanodine. After 120 min, aliquots of each reaction were filtered, on a vacuum filtration manifold, through Whatman GF/C filters,

which were then washed three times with 5 ml of ice-cold deionized water or placed in a scintillation vial to determine the total amounts of radioactivity added. Radioactivity remaining on filters and in the totals was determined by liquid-scintillation counting. Non-specific binding was determined in duplicate reactions incubated in the presence of 100 μM unlabelled ryanodine.

Miscellaneous

Protein concentrations were determined by the method of Bradford [25], with BSA as a standard. Scanning densitometry was used to determine the band densities of immunoreactive proteins on immunoblots as detailed in the Results section (Bio-Rad model GS-670 scanning densitometer). Densitometric data were compared with Student's *t* test for paired observations. The relationship between band density and protein loading was examined with the following positive controls: rabbit whole-brain microsomes for pAbs 40 and 1094 (which recognize $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ and RyR3 respectively); rabbit heart microsomes for pAbs 10 and 129 (RyR2); rabbit skeletal muscle for pAb 405 (RyR1); or J774.2 monocyte macrophage microsomes for mAbs KM1083 and KM1082 [$\text{Ins}(1,4,5)\text{P}_3\text{R2}$ and $\text{Ins}(1,4,5)\text{P}_3\text{R3}$]. A note has been made in the Results section where proteins were detected below the linear band density-protein concentration relationship for each of these antibodies.

RESULTS

Specificity of anti-RyR antisera and distribution of immunoreactive proteins in mammalian tissues

The distribution of different RyR subtypes among rabbit tissues was analysed on immunoblots with isoform-specific antisera (Figure 1). Antiserum pAb 405, generated against a type 1 RyR-specific synthetic peptide, recognized a protein of very high apparent molecular mass in skeletal muscle (RyR1, lane 6) and at lower levels in uterus microsomes (lane 9). Diaphragm also expresses type 1 RyR, as assessed with pAb 405, at levels greater than those in uterus. Proteins of identical electrophoretic mobility were also detected with pAb G715 [26], an antiserum raised against purified skeletal muscle RyR (results not shown). Anti-(type 2 RyR) antiserum pAb 129 detected a protein at greatest abundance in heart (RyR2, lane 2) and present at lower levels in whole brain (lane 1), which was of slightly greater electrophoretic mobility than that recognized by pAb 405 in skeletal muscle. A similar expression profile of the type 2 RyR in rabbit tissues was revealed with a second isoform-specific anti-synthetic peptide antiserum, pAb 10 (data not shown). Antiserum pAb 1094, raised against a type 3 RyR-derived peptide, specifically recognized a protein of electrophoretic mobility indistinguishable from that of the type 2 RyR, which was consistently detected only in whole brain (RyR3, lane 1).

Analysis of Ca^{2+} -release channel expression in mammalian cell lines

The presence of distinct Ca^{2+} -release channel proteins in a range of mammalian cell lines was investigated by immunoblotting. All cell lines co-expressed at least two distinct isoforms of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ (Table 2). The type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ protein was detectable in every cell line examined, whereas the type 2 isoform displayed a more limited distribution. Type 3 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ was also detectable in all cell lines examined except SH-SY5Y neuroblastomas. The two neuroblastoma types investigated showed distinct $\text{Ins}(1,4,5)\text{P}_3\text{R}$ isoform profiles, with high levels of type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ in SH-SY5Y cells, compared with high

Figure 1 Distribution of RyR isoforms in rabbit tissues

Microsomal protein (100 μ g per lane) was resolved by SDS/PAGE [5% (w/v) gel] then either stained with Coomassie R250 or blotted on nitrocellulose membranes. Blots were probed with 1:500 dilutions of pAb 405 [anti-(type 1 RyR), RYR1], pAb 129 [anti-(type 2 RyR), RYR2], or pAb 1094 [anti-(type 3 RyR), RYR3], then immunoreactive proteins were stained by using horseradish peroxidase-conjugated anti-(rabbit IgG), with 3,3'-diaminobenzidine as a substrate. Microsomes were prepared from rabbit whole brain (lane 1), heart (lane 2), kidney (lane 3), liver (lane 4), spleen (lane 5), skeletal muscle (lane 6), stomach (lane 7), urinary bladder (lane 8) and uterus (lane 9). Numbers to the left of the Coomassie R250-stained gel indicate the apparent molecular masses (in kDa) of prestained standards.

Table 2 Distribution of Ca^{2+} -release channel isoforms among mammalian cell lines

The expression of Ca^{2+} -release channel proteins in mammalian cell lines was assessed by immunoblot assays. Symbols: —, lack of detectable immunoreactivity; +/—, inconsistent, weak signals; +, weak immunoreactivity; ++, intermediate immunoreactivity; +++, strong immunoreactivity. Each experiment was performed at least twice; for type 2 RyR, expression of this protein was verified with antisera directed against two different sites (pAb 10 and pAb 129; see Table 1).

Cell line (cell type)	Ins(1,4,5) P_3 R1	Ins(1,4,5) P_3 R2	Ins(1,4,5) P_3 R3	RyR1	RyR2	RyR3
BC ₃ H1 (mouse brain tumour)	+	+++	++	+	+	++
DDT ₁ MF-2 (hamster leiomyosarcoma)	+	—	++	+	++	+ / —
HeLa (human cervix carcinoma)	+	+	+++	—	+ / —	—
J774.2 (mouse tumour monocyte macrophage)	++	+++	+++	—	—	—
N2A (mouse neuroblastoma)	+	++	+	—	—	—
PC-12 (rat adrenal pheochromocytoma)	++	+ / —	++	—	+	—
SH-SY5Y (human neuroblastoma)	++	+	—	—	+	—
Y1 (mouse adrenal cortex tumour)	+	—	++	—	+	—

levels of the type 2 isoform in N2A cells, relative to the other cell types examined. RyR expression was less widespread than that of the Ins(1,4,5) P_3 Rs. Type 2 RyR was the most commonly detected isoform, being present in six of the eight cell lines investigated. Detectable levels of type 1 and type 3 RyR proteins were present only in 'muscle-like' cells, BC₃H1 and DDT₁MF-2. As with Ins(1,4,5) P_3 Rs, these cell lines co-express multiple RyR isoforms (Table 2).

[³H]Ryanodine binding in rabbit tissues and mammalian cell line microsomes

High-affinity [³H]ryanodine-binding sites were detected in microsomes prepared from a number of rabbit tissues, with highest levels being present in striated muscles (Table 3). Unexpectedly, some smooth-muscle-enriched tissues (stomach and vas deferens) displayed high levels of [³H]ryanodine binding, despite the lack of detectable immunoreactive proteins to anti-RyR antisera (Figure 1). Furthermore RyRs could not be detected with these

antisera even when equivalent amounts of [³H]ryanodine binding, rather than total protein, were immunoblotted (results not shown). Under identical assay conditions, specific [³H]ryanodine binding could not be detected in microsomes prepared from the mammalian cell lines examined in the present study, even though several of these cell lines expressed RyR proteins as assessed by immunoblotting (Table 2). In the cell lines investigated, a major technical problem with the [³H]ryanodine binding assays employed was a high level of non-specific binding. Alteration of the assay parameters (such as incubation time, ionic composition, temperature, filter type and concentration of unlabelled ryanodine to define non-specific binding) did not satisfactorily resolve this difficulty. It should be noted that the levels of [³H]ryanodine binding might not accurately reflect the levels of RyR in tissues and cell lines because only a single concentration of this radioligand was employed. However, the small amounts of protein obtained in all cell lines and some tissue membrane preparations prevented the determination of full radioligand-binding isotherms.

Table 3 [3 H]Ryanodine binding in mammalian tissues and cell lines

[3 H]Ryanodine binding assays were performed as described in the Materials and methods section. All tissue microsomes were prepared from rabbit tissues. Results are means $\pm$ S.E.M. for the number of experiments shown in parentheses, performed in duplicate. Abbreviation: n.d., that no specific [3 H]ryanodine binding was detected.

Tissue/cell line	Specific [3 H]ryanodine binding (fmol/mg of protein) (<i>n</i>)
Brain	302 $\pm$ 19 (8)
Diaphragm	1413 $\pm$ 91 (4)
Heart	1285 $\pm$ 67 (8)
Skeletal muscle (back and hindlimb)	4474 $\pm$ 349 (8)
Stomach	65 $\pm$ 11 (4)
Urinary bladder	n.d. (4)
Uterus	139 $\pm$ 20 (8)
Vas deferens	220 $\pm$ 4 (4)
BC $_3$ H1 (undifferentiated)	n.d. (6)
DDT $_1$ MF-2	n.d. (6)
PC-12	n.d. (6)
SH-SY5Y	n.d. (6)
Y1	n.d. (6)

Figure 2 Subcellular fractionation of Ca^{2+} -release channel proteins and SERCA pumps in SH-SY5Y human neuroblastomas

Crude microsomal membranes were prepared from SH-SY5Y cells and subfractionated as described in the Materials and methods section. Equal quantities of protein from each fraction were resolved on SDS/PAGE minigels [5% (w/v) gel], transferred to nitrocellulose and immunostained with: 1:2000 pAb 10 (RyR2), 1:2000 pAb 40 (IP3R1), 1:200 mAb KM1083 (IP3R2) or 1:200 mAb Y1/F4 (SERCA). Lane 1 was loaded with 0.5 μ g of rabbit heart microsomal protein; all other lanes were loaded with 30 μ g of SH-SY5Y membrane fractions, namely crude cell homogenate (lane 2), low-speed fraction (lane 3), high-speed ('crude microsomal') fraction (lane 4), 0.3 M sucrose subfraction (lane 5), interphase subfraction (lane 6), 1.3 M sucrose subfraction (lane 7) and pellet subfraction (lane 8).

Subcellular distribution of RyRs and Ins(1,4,5) P_3 Rs in SH-SY5Y cells

SH-SY5Y neuroblastoma whole-cell homogenates contain the type 1 Ins(1,4,5) P_3 R at high levels, a low density of the type 2 Ins(1,4,5) P_3 R and of the type 2 RyR (Figure 2, lane 2). Although the levels of the type 2 RyR could not be quantified, owing to the high non-specific binding in the radioligand binding assays, SH-

Figure 3 Effects of chronic stimulation of mAChR on the abundance of Ca^{2+} -release channel proteins in SH-SY5Y human neuroblastomas

SH-SY5Y neuroblastomas were grown for 24 h in the presence of vehicle (lane 1), 100 μ M methacholine (lane 2) or 100 μ M methacholine plus 10 μ M atropine (lane 3). Microsomal membranes (30 μ g protein/lane) prepared from these cells were resolved on SDS/PAGE minigels [5% (w/v) gel], transferred to nitrocellulose and immunostained with a 1:2000 dilution of pAb 40 (IP3R1) or pAb 10 (RyR2). Numbers to the right of each blot represent the apparent molecular mass (in kDa) of prestained markers.

SY5Y cells expressed low levels of this channel because the pAb 10 immunoreactivity in a 30 μ g sample of crude homogenate protein from these neuroblastomas (Figure 2, RyR2, lane 2) was less intense than that in only 0.5 μ g of rabbit heart microsomes (lane 1). This observation is not a consequence of a difference in the ability of pAb 10 to recognize type 2 RyR from various species, because the amino acid sequence used to generate this antiserum was 100% identical between rabbit and human forms of this protein [8]; furthermore similar results were obtained with pAb 129, raised against a distinct peptide sequence of this protein. Type 2 RyR was most enriched in a crude SH-SY5Y microsomal fraction (Figure 2, RyR2, lane 4) in the differential centrifugation protocol employed, whereas the type 1 Ins(1,4,5) P_3 R was at greatest abundance in a low-speed pellet (Ins(1,4,5) P_3 R1, lane 3), in which the SERCA pumps were also present at the highest levels. On fractionation of crude microsomal membranes, type 2 RyR, type 1 Ins(1,4,5) P_3 R, type 2 Ins(1,4,5) P_3 R and SERCA proteins co-segregated to the three most dense fractions (Figure 2, lanes 6, 7 and 8). However, this co-segregation was not absolute because a low level of type 1 Ins(1,4,5) P_3 R was also present in the least dense fraction (lane 5), in which the other three proteins were undetectable.

Regulation of Ca^{2+} -release channel proteins in SH-SY5Y cells during chronic stimulation of mAChR

Chronic stimulation of SH-SY5Y cells of mAChR leads to a loss of their Ins(1,4,5) P_3 R proteins [27]. These observations were confirmed in the present study, whereby 24 h of stimulation of these neuroblastomas with the mAChR agonist methacholine resulted in an $83 \pm 9\%$ ($n = 4$; $P < 0.01$ compared with untreated control) decrease in anti-[type 1 Ins(1,4,5) P_3 R] immunoreactivity compared with untreated cells (Figure 3, IP3R1, lane 2 compared with lane 1). Atropine (10 μ M), a muscarinic antagonist, blocked ($100 \pm 3\%$; $\bar{x} \pm$ S.E.M. $n = 4$) this decrease in type 1 Ins(1,4,5) P_3 R protein (lane 3). In methacholine-treated neuroblastomas there was a trend towards an increase in type 2 RyR protein compared with control (Figure 3, RyR2, lane 2) [$133 \pm 16\%$ compared with control (100%); $n = 4$] that was not observed in the presence of atropine (Figure 3, RyR2, lane 3) ($103 \pm 8\%$; $n = 4$). However, the methacholine-evoked increase in RyR2 was not significant ($P > 0.05$), the variability in these data reflecting the difficulty in quantifying low levels of RyR proteins expressed in cell lines.

Figure 4 Effects of retinoic acid-induced differentiation on Ca^{2+} -release channel protein levels in SH-SY5Y human neuroblastomas

SH-SY5Y cells were cultured in normal medium (lane 1), differentiated with $10 \mu\text{M}$ retinoic acid (lane 2), or were grown in differentiation medium alone (lane 3) for 6 days. Microsomal membranes ($30 \mu\text{g}$ of protein per lane) prepared from these neuroblastomas were then immunostained for the presence of $\text{Ins}(1,4,5)\text{P}_3\text{R1}$ (IP3R1) or RyR2, as described in the legend to Figure 3.

Regulation of RyRs and $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$ during differentiation of SH-SY5Y neuroblastomas

SH-SY5Y cells might be differentiated towards a phenotype displaying 'neuronal-like' characteristics by culture for 6 days in low-serum medium containing $10 \mu\text{M}$ retinoic acid [22,28]. Despite morphological alterations, the levels of type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ protein in SH-SY5Y cells were unaltered in neuroblastomas treated with retinoic acid (Figure 4, IP3R1, lane 2; $101 \pm 4\%$; $n = 4$) or differentiation medium only (lane 3; $95 \pm 5\%$; $n = 4$) compared with untreated cells. Similarly, the levels of type 2 RyR protein were not significantly altered on differentiation with retinoic acid (Figure 4, RyR2, lane 2; $109 \pm 7\%$, $n = 4$). However, the levels of type 2 RyR were

increased significantly in SH-SY5Y cells treated with differentiation medium alone (lane 3; $190 \pm 20\%$; $n = 4$; $P < 0.01$).

Ca^{2+} -release channel isoforms display distinct patterns of expression during differentiation of $\text{BC}_3\text{H1}$ cells

Undifferentiated $\text{BC}_3\text{H1}$ cells expressed all three $\text{Ins}(1,4,5)\text{P}_3\text{R}$ and all three RyR isoforms investigated in the present study (Table 2 and Figure 5). On differentiation by serum starvation, there were marked changes in the abundance of some of the proteins involved in Ca^{2+} signalling in these cells. The SERCA pump was up-regulated on differentiation (Figure 5, SERCA), as has been reported previously [29]. Although the levels of type 1 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ protein were decreased in differentiated $\text{BC}_3\text{H1}$ cells (Figure 5, IP3R1, lane 2 compared with lane 1) to $12 \pm 2\%$ of those in untreated cells ($n = 4$; $P < 0.001$), the levels of the other two $\text{Ins}(1,4,5)\text{P}_3\text{R}$ isoforms were not significantly altered (IP3R2, lane 2 compared with lane 1; $95 \pm 5\%$, $n = 4$; IP3R3, lane 2 compared with lane 1; $140 \pm 30\%$, $n = 4$). In contrast, all RyR isoforms displayed marked alterations on differentiation of $\text{BC}_3\text{H1}$ cells. Type 1 RyR was up-regulated (Figure 5, RyR1, lane 2 compared with lane 1) by $840 \pm 90\%$ ($n = 4$; $P < 0.005$), whereas type 2 (Figure 5, RyR2, detected lane 1 compared with not detected lane 2) and type 3 (RyR3, lane 1 compared with lane 2; to $7 \pm 5\%$ of undifferentiated control; $n = 4$; $P < 0.001$) RyRs were markedly down-regulated on differentiation. Type 3 RyR was undetectable in microsomes prepared from adult rabbit skeletal muscle (Figure 5, RyR3, lane 3).

DISCUSSION

Reports on the distribution of Ca^{2+} -release channel proteins among established mammalian cell lines have so far been limited. Examination of the abundance of proteins, rather than the

Figure 5 $\text{Ins}(1,4,5)\text{P}_3\text{R}$ and RyR proteins show distinct changes in abundance during differentiation of $\text{BC}_3\text{H1}$ muscle cells

$\text{BC}_3\text{H1}$ cells were grown in the presence of 20% (v/v) FCS (lane 1) or were differentiated by growth in 0.5% (v/v) FCS for 12 days (lane 2). Microsomal membranes from these cells ($30 \mu\text{g}$ of protein per lane) were immunoblotted as described in the legend to Figure 3, with 1:500 pAb 405 (RyR1), 1:500 pAb 129 (RyR2), 1:500 pAb 1094 (RyR3), 1:2000 pAb 40 (IP3R1), 1:200 mAb KM1083 (IP3R2), 1:200 mAb KM1082 (IP3R3) or 1:200 Y1/F4 (SERCA). Control samples were $1 \mu\text{g}$ of rabbit skeletal muscle microsomal protein (lane 3), $5 \mu\text{g}$ of rabbit brain microsomal protein (lane 4) or $30 \mu\text{g}$ of J774.2 monocyte macrophage microsomal protein (lane 5).

mRNA species encoding them, is important because mRNA levels do not necessarily reflect the density of a protein within a cell. For example, mRNA encoding the type 3 RyR is reported to display a widespread tissue distribution [4,5], although the corresponding protein could be detected only in brain [6]. Down-regulation of SH-SY5Y type 1 Ins(1,4,5) P_3 R protein by chronic stimulation of mAChR is not mirrored by changes in the mRNA encoding it [27]. Furthermore, in many cases where the expression of Ca^{2+} -release channels has been investigated at the level of protein abundance, the antibodies used were not isoform-specific [3,17,30–32]. The present report describes the development of RyR isoform-specific antisera, which were used in combination with previously developed subtype-specific anti-RyR [15] and anti-Ins(1,4,5) P_3 R [19] antibodies to assess the distribution and regulation of these proteins in a number of mammalian tissues and cell lines.

Reports on Ca^{2+} -release channel mRNA distribution among pig [33] or murine [6] tissues suggest that type 3 RyR mRNA is present in uterus. In the present study, type 1 RyR protein, but not type 3 RyR, was detected in uterine microsomes. Such discrepancies could arise from differences in the species investigated or from differences in the detection methods employed. The type 1 RyR protein in rabbit uterus, like that from skeletal muscle [26], sediments as a characteristic approx. 30 S high-affinity [3H]ryanodine-binding complex on solubilization and sucrose density gradient centrifugation (J. J. Mackrill and F. A. Lai, unpublished work). The immunoblot analyses of type 2 and type 3 RyRs in rabbit tissues in the present study are consistent with other reports on the expression of these proteins, although not of the mRNA species encoding them, in mammalian tissues.

From this and other studies [4,6,7,17,19], it is apparent that mammalian cell lines might express multiple Ca^{2+} -release channel subtypes, of both the Ins(1,4,5) P_3 R and RyR families. The findings presented concentrate on the investigation of the expression of these proteins in populations of cells, because a number of the antibodies employed proved unsuitable for the immunolocalization of these channels within single cells. Previous investigations indicate that multiple Ca^{2+} -release channel proteins might be expressed within a single cell and that these display discrete subcellular localizations [17–19]. Ins(1,4,5) P_3 Rs and RyRs in SH-SY5Y cells are enriched in distinct crude differential centrifugation fractions, although they display similar distributions between subfractions of microsomal membranes. The Ca^{2+} -release channel proteins in SH-SY5Y membrane subfractions might not represent mature, functional channels but could be present in membrane systems involved in their processing. However, several lines of evidence argue against this. First, the type 2 RyR was recognized by antisera raised against two distinct sites on the protein; secondly, both the type 1 Ins(1,4,5) P_3 R [27] and type 2 RyR have very long biochemical half-lives. Although the turnover of type 2 RyR in SH-SY5Y cells could not be accurately determined with [^{35}S]methionine pulse-chase studies, owing to its low abundance, its half-life was at least as great (approx. 8 h) as that of the type 1 Ins(1,4,5) P_3 R ([27], and J. J. Mackrill, R. A. J. Challiss and S. R. Nahorski, unpublished work). Finally, preliminary $^{45}Ca^{2+}$ mobilization experiments demonstrate that permeabilized SH-SY5Y cells contain a caffeine-releasable, ryanodine-sensitive Ca^{2+} -pool, indicating that they contain functional RyR channel complexes. SK-N-BE, another human neuroblastoma cell line, also contains a ryanodine-sensitive Ca^{2+} -pool, which is coupled to δ -opioid receptors at the plasma membrane [34].

The densities of Ins(1,4,5) P_3 Rs and RyRs within a cell also seem to be regulated in distinct ways, not only between these two

classes but also between isoforms within each family. For example, the type 1 Ins(1,4,5) P_3 R is down-regulated by chronic stimulation of SH-SY5Y neuroblastomas with methacholine, whereas the type 2 RyR is unaffected. Similarly, different Ins(1,4,5) P_3 R isoforms display distinct rates of down-regulation [35]. This is paradoxical because the down-regulation of type 1 Ins(1,4,5) P_3 Rs by this treatment is mediated by a Ca^{2+} -dependent cysteine protease, probably a member of the calpain family [36], for which the RyRs are also targets [37]. Reasons for this selective digestion of the Ins(1,4,5) P_3 Rs include that the RyR calpain cleavage sites might be inaccessible to the protease; that the calpain-like protease is physically associated with the Ins(1,4,5) P_3 Rs; or that this is a novel enzyme, with selectivity for the Ins(1,4,5) P_3 Rs. Although there is little experimental evidence to support any of these possibilities, the concept that this protease is intimately associated with the Ins(1,4,5) P_3 Rs is attractive because down-regulation of these proteins seems to be dependent on activation of their channels, and several accessory proteins are known to interact with these channel complexes.

Differentiation of SH-SY5Y neuroblastomas with retinoic acid has no detectable effect on the expression of Ca^{2+} -release channel proteins. However, this treatment leads to a number of phenotypic changes in these cells, including increasing neurite length and number, and elevation of neuron-specific enolase [22]. The effects of differentiation on Ins(1,4,5) P_3 R and RyR function in SH-SY5Y neuroblastomas are as yet undetermined. In contrast, differentiation of BC₃H1 cells from a fibroblastic to a muscle-like phenotype results in a marked alteration in the expression of Ca^{2+} -release channel proteins. Differentiation of these cells leads to an increase in the size of the caffeine-sensitive Ca^{2+} pool and a decrease in the Ins(1,4,5) P_3 -sensitive Ca^{2+} store [23]. Although an elevation of type 1 RyR mRNA [38] and protein [30] resulting from differentiation of these cells has been reported previously, the isoform specificity of the antibodies employed was not assessed. The use of subtype-specific antibodies in the present study demonstrates that Ca^{2+} -release channel expression is regulated in an isoform-dependent manner. This isoform-dependent regulation of the expression of these channels is likely to increase the flexibility of cellular Ca^{2+} signalling. The use of such systems might prove useful in the assessment of the functional properties of poorly characterized Ca^{2+} -release channel proteins, such as the type 3 RyR and the type 2 and type 3 Ins(1,4,5) P_3 Rs.

In summary, the presence of various Ca^{2+} -release channel proteins in mammalian tissues and cell lines has been demonstrated by using subtype-specific antisera. Different tissues and cell lines display distinct patterns of expression of these proteins, which might have a bearing on their roles. Different Ins(1,4,5) P_3 R and RyR isoforms display discrete distributions in subcellular fractionation studies, as well as distinct regulation by cellular differentiation or chronic muscarinic stimulation. This suggests that Ca^{2+} -release channel proteins might often play key roles in the adaptation of cellular responses to extracellular stimuli.

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