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Immunohistochemical study on the distribution of sarcoplasmic reticulum calcium ATPase in various human tissues using novel monoclonal antibodies

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Summary. Novel monoclonal antibodies were raised against sarcoplasmic reticulum calcium (Ca^{2+})-ATPase of human skeletal muscle. Immunohistochemical analysis demonstrated that these antibodies, designated 6F5 and 7F10, bind Ca^{2+}-ATPase of non-muscle tissue of the adult including parathyroid, islets of Langerhans, anterior lobe of the pituitary gland and photoreceptor cells of the retina as well as skeletal muscle. A positive reaction was also found for fetal tissues including skeletal muscle, heart, chondrocytes and peripheral nerves. Our results for distribution suggest that Ca^{2+}-ATPase is strongly expressed in the tissues and cells in which signal transduction is actively carried out by Ca^{2+} release from the cytoplasmic Ca^{2+} pool.

Key words: Calcium – Adenosine triphosphatase – Novel monoclonal antibody – Immunohistochemistry

Introduction

The calcium (Ca^{2+})-ATPase of mammalian skeletal muscle sarcoplasmic reticulum (SR) is an ion-transport enzyme, first isolated in 1970 (MacLennan 1970) and extensively investigated at the molecular level (MacLennan and Reithmeier 1982). cDNA cloning and sequencing have shown that the Ca^{2+} pumps of the mammalian sarco(endo)plasmic reticulum are products of at least two different but closely related genes (Campbell and MacLennan 1981; MacLennan et al. 1985; Brandl et al. 1986, 1987; de La Bastie et al. 1988; Gunteski-Hamblin et al. 1988; Korczak et al. 1988; Lytton and MacLennan 1988; Eggermont et al. 1989; Komuro et al. 1989; Lompre et al. 1989; Lytton et al. 1989). Gene 1 encodes a SR Ca^{2+} pump which is only expressed in fast-twitch skeletal muscle (Brandl et al. 1986), while expression of gene 2 is believed to predominate in other tissues (MacLennan et al. 1985; Brandl et al. 1987; de La Bastie et al. 1988; Eggermont et al. 1989; Gunteski-Hamblin et al. 1988; Komuro et al. 1989; Lompre et al. 1989; Lytton and MacLennan 1988; Lytton et al. 1989). Tissue-dependent alternative splicing of the primary transcript of gene 2 results in either transcript 2.1 or in transcript 2.2. The 2.1 transcript has been detected in cardiac muscle (Brandl et al. 1987), slow-twitch skeletal muscle (MacLennan et al. 1985; Brandl et al. 1987), smooth muscle (Eggermont et al. 1989; Lytton et al. 1989), and renal tissue (Lytton and MacLennan 1988). The alternatively spliced 2.2 transcript is found in non-muscle tissues (Gunteski-Hamblin et al. 1988; Lytton and MacLennan 1988). There are some indications of the existence of a third gene, but this question requires further attention (Gunteski-Hamblin et al. 1988; Lytton and MacLennan 1988). Available data show that a high homology of amino acid sequence has been maintained among these gene products.

Ca^{2+} is believed to play an important role in cytoplasmic signal transduction, not only in muscle cells but also in non-muscle cells. Although it has been postulated that the endoplasmic reticulum (ER) of non-muscle cells constitutes a cytoplasmic Ca^{2+} pool equivalent to the SR of muscle cells, little is known about the distribution of human SR type Ca^{2+}-ATPase in non-muscle tissues. We therefore raised two kinds of novel monoclonal antibodies (mAbs) reactive with the A1 fragment of SR Ca^{2+}-ATPase of human skeletal muscle, and found that they cross-reacted with Ca^{2+}-ATPase of human non-muscle cells. The present paper documents finding for distribution of this enzyme in non-muscle tissues as assessed immunohistochemically.

Materials and methods

SR membranes were purified from human skeletal muscle and rabbit fast-twitch muscle according to the method of Kawakita et al. (1980).

For production of monoclonal antibodies against Ca^{2+}-ATPase, 6-week-old mice (Balb/c, SLC Japan, Shizuoka) were immunized intraperitoneally with 10 mg purified SR membranes of rab-

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Table 1. Immunohistochemical analysis of the human tissues in fetuses and adults

Tissue and cell	Reactivity[a]			
	6F5		7F10	
	Fetus	Adult	Fetus	Adult
Skin and skin appendages				
Epidermis				
Keratinocyte	−	−	−	−
Langerhans' cell		−		−
Dermis	−	−	−	−
Appendages				
Sweat gland		−		−
Hair follicle		−		−
Mammary gland		−		−
Endocrine				
Pituitary gland				
Anterior		+		−
Posterior		−		−
Thyroid gland	−	−	−	−
Parathyroid gland		+		+
Langerhans islet	−	+	−	+
Adrenal gland	−	−	−	−
Cardiovascular				
Heart	+[b]	−	+[b]	−
Artery and vein	−	−	−	−
Capillary	−	−	−	−
Lymphatic vessel	−	−	−	−
Haemopoietic				
Lymph node		−		−
Thymus	−	−	−	−
Spleen		−		−
Bone marrow		−		−
Respiratory				
Trachea	−	−	−	−
Lung	−	−	−	−
Digestive				
Salivary gland		−		−
Tongue	−	−	−	−
Oesophagus	−	−	−	−
Stomach	−	−	−	−
Small and large intestine	−	−	−	−
Liver	−	−	−	−
Pancreatic acinus	−	−	−	−
Locomotor				
Bone	−	−	−	−
Chondrocyte	+	−	+	−
Muscle	+	+	+	+
Urogenital				
Kidney				
Glomerulus	−	−	−	−
Tubules	−	−	−	−
Pelvis		−		−
Ureter		−		−
Bladder		−		−
Testis		−		−
Epididymis		−		−
Prostate		−		−
Ovary		−		−
Fallopian tube		−		−
Uterus		−		−
Neurological				
Cerebrum	−	−	−	−
Cerebellar Purkinje cell		−		−
Spinal cord	−	−	−	−
Peripheral nerve	+	−	+	−
Chorioid plexus		−		−
Eye				
Cornea	−	−	−	−
Sclera	−	−	−	−
Retina	−	+	−	−
Lens	−	−	−	−
Placenta				
Amnion sac		−		−
Decidua		−		−
Chorionic villi				
Syncytiotrophoblast		−		−
Cytotrophoblast				

[a] +, Positive reaction with monoclonal antibody; −, negative
[b] More strongly positive in the right ventricle than in the left

bit fast-twitch muscle, emulsified with incomplete Freund's adjuvant. Two additional intraperitoneal immunizations with the same dose were performed at 2 week intervals. Three days before performance of cell fusion, a final immunization at the same dose was executed intravenously. Spleen cells of an immunized mouse were fused with P3UI mouse myeloma cells uisng a published procedure (McKearn et al. 1980; Gaetani et al. 1983). Hybrid clones were selected in hypoxanthine-aminopterin-thymidine medium (Sigma, St. Louis, Mo., USA). Of the 695 hybrids generated, 2 showed exclusive reactivity with Ca^{2+}-ATPase of SR by enzyme-linked immunosorbent assay (Engvall and Perlmann 1971). The selected hybridomas were cloned by submitting them to limiting dilution 2 times and then transplanted to mice (Balb/c, SLC Japan, Shizuoka) that had been treated with pristane (Aldrich, Milwaukee, Wis., USA) 2 weeks in advance. After a period of 14–18 days, ascites fluid was collected and used for immunostaining and immunoblotting.

For mAb isotype determination, the immunoglobulin isotype of mAb was determined by a Serotec mouse monoclonal typing kit (Serotec, Oxford, UK).

For electrophoresis and western blot analysis, the purified SR membranes (Ca^{2+}-ATPase molecules) of rabbit and human skeletal muscle were stored at a protein concentration of 15 mg/ml at −80° C. For SDS-PAGE, the purified SR membranes were mixed with 2× sample buffer and analysed according to the method of Laemmli (1970) and electrophoretically transferred to nitrocellulose filters. Autoclave pretreatment for filter papers immersed in distilled water was performed (hydrate autoclaving) before incubation with mAb (Shin et al. 1991). Filter papers were incubated in 5% dried milk in 150 mM sodium chloride and TRIS-HCl buffer (pH 7.5) containing 0.025% Tween 20 (TBS-T) at 4° C overnight to block non-specific binding to papers. Filter papers were then incubated for 1 h at room temperature with each of the mAbs diluted 1:100 in TBS-T and washed three times with the same solution for 10 min at each washing. Control incubations were performed with preimmune mouse sera. Finally the filter papers were incubated for 1 h with affinity-purified goat anti-mouse whole Ig conjugated with peroxidase, followed by several washes in TBS-T, and either developed with chloronaphthol or detected by enhanced chemoluminescence using ECL western blotting detection reagents (Amersham, Tokyo, Japan).

Tryptic digestion of rabbit SR membranes was carried out for western blot analysis according to the method of Saito et al. (1984) at 0° C.

For immunoperoxidase staining, human normal tissues of adults and of fetuses at a gestational age of 12 weeks were examined

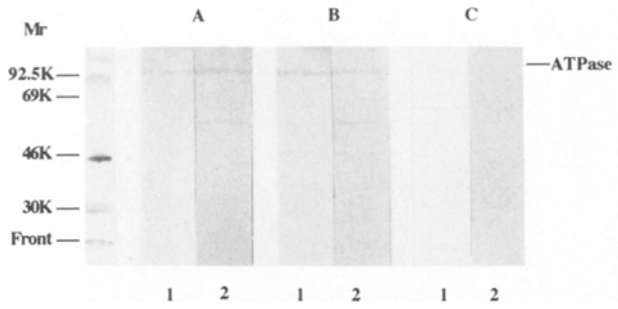

Fig. 1. Binding of antibodies with Ca^{2+}-ATPase from human skeletal muscle sarcoplasmic reticulum (SR). SR membranes of human skeletal muscle were prepared according to the method of Kawakita et al. (1980), separated on 10% SDS-PAGE, and transferred to a nitrocellulose membrane sheet. Autoclave pretreatment in distilled water (hydrate autoclaving) was performed for lane 2 but not lane 1 of each set before incubation with monoclonal antibody (Shin et al. 1991). Set A was incubated with antibody 6F5, set B with 7F10 and set C with normal mouse serum. 6F5 and 7F10 reacted with the Ca^{2+}-ATPase molecule of SR, showing the main band at 110 kDa. No reaction products were observed in the negative control study using normal mouse serum (set C). A 2 μg aliquot of SR membranes was applied to each lane

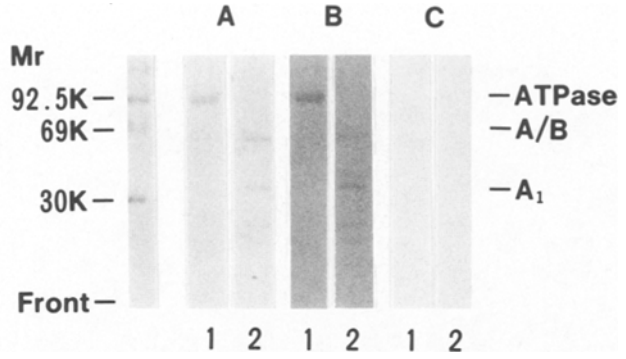

Fig. 2. Tryptic digestion fragments of the Ca^{2+}-ATPase from rabbit skeletal muscle SR and their reaction with antibodies. Rabbit SR protein (2 mg/ml) was digested at 0° C for twenty minutes in the presence of 5 mM calcium chloride glycerol at a trypsin/SR protein ratio of 1/100 (w/w) (Saito et al. 1984). The digestion was stopped with solution containing 4% SDS, 10% glycerol, 1.32 M mercaptoethanol, and 62.5 mM TRIS HCl buffer (pH 6.8) in 1.12 volume of reaction mixture, followed by Laemmli SDS/polyacrylamide-gel electrophoresis. Set A was incubated with antibody 6F5, set B with 7F10 and set C with normal mouse serum. Trypsin digestion was performed for lane 2 but not lane 1 before immunoblotting. 6F5 and 7F10 both recognized the A1 fragment showing a single band at 33 kDa (lane 2). No reaction products were observed in the negative control study using normal mouse serum (set C)

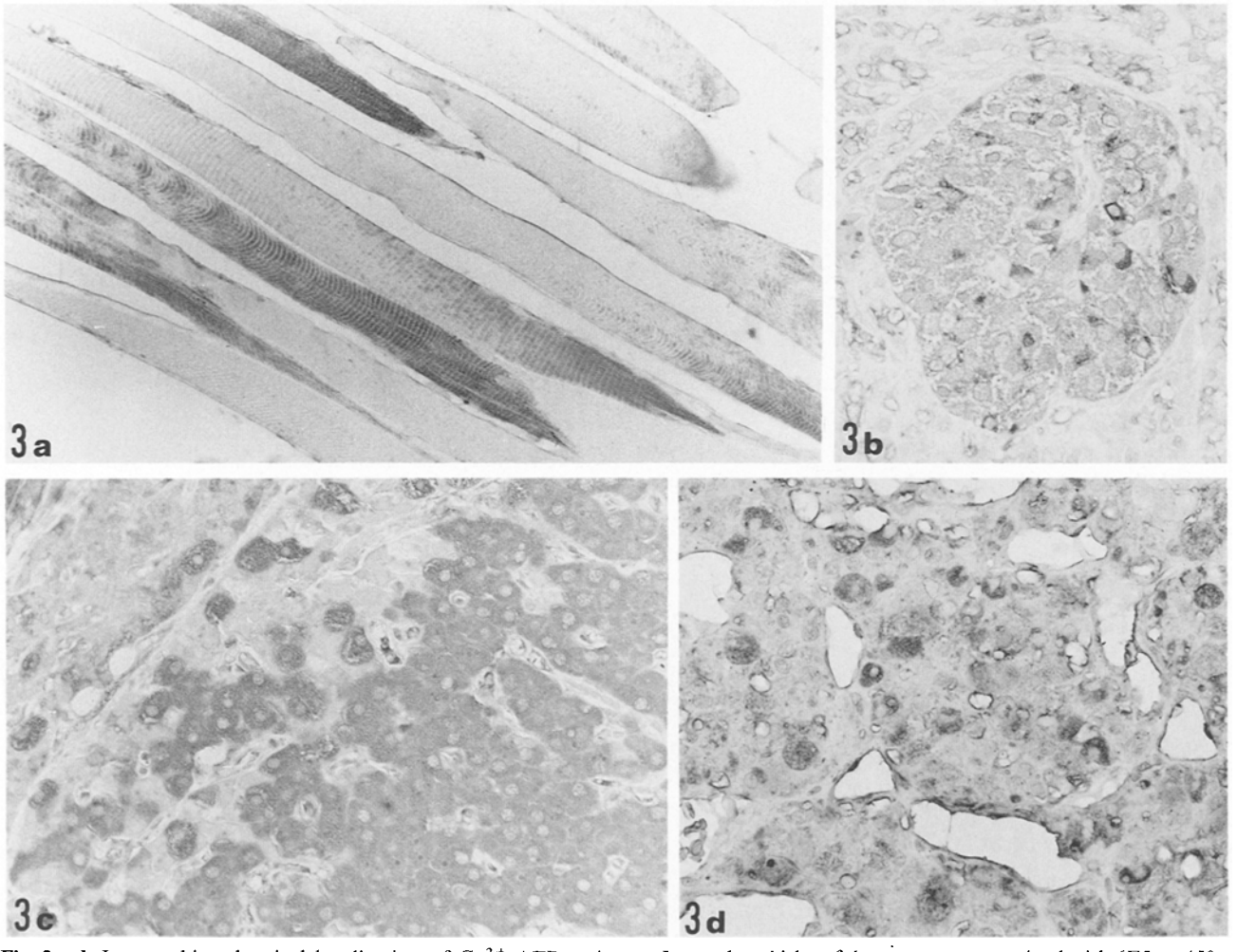

Fig. 3a–d. Immunohistochemical localization of Ca^{2+}-ATPase in human adult tissues using monoclonal antibodies 6F5 and 7F10. **a** Skeletal muscle exhibits positive immunostaining with 7F10 mainly localized at the cross-striations. ×340. **b** Some cells in a Langerhans' islet of the pancreas are stained with 6F5. ×450. **c** Oxyphilic cells and some chief cells in the parathyroid gland are positive for 7F10. ×450. **d** In the anterior lobe of the pituitary gland, scattered cells are observed to bind 6F5. ×450

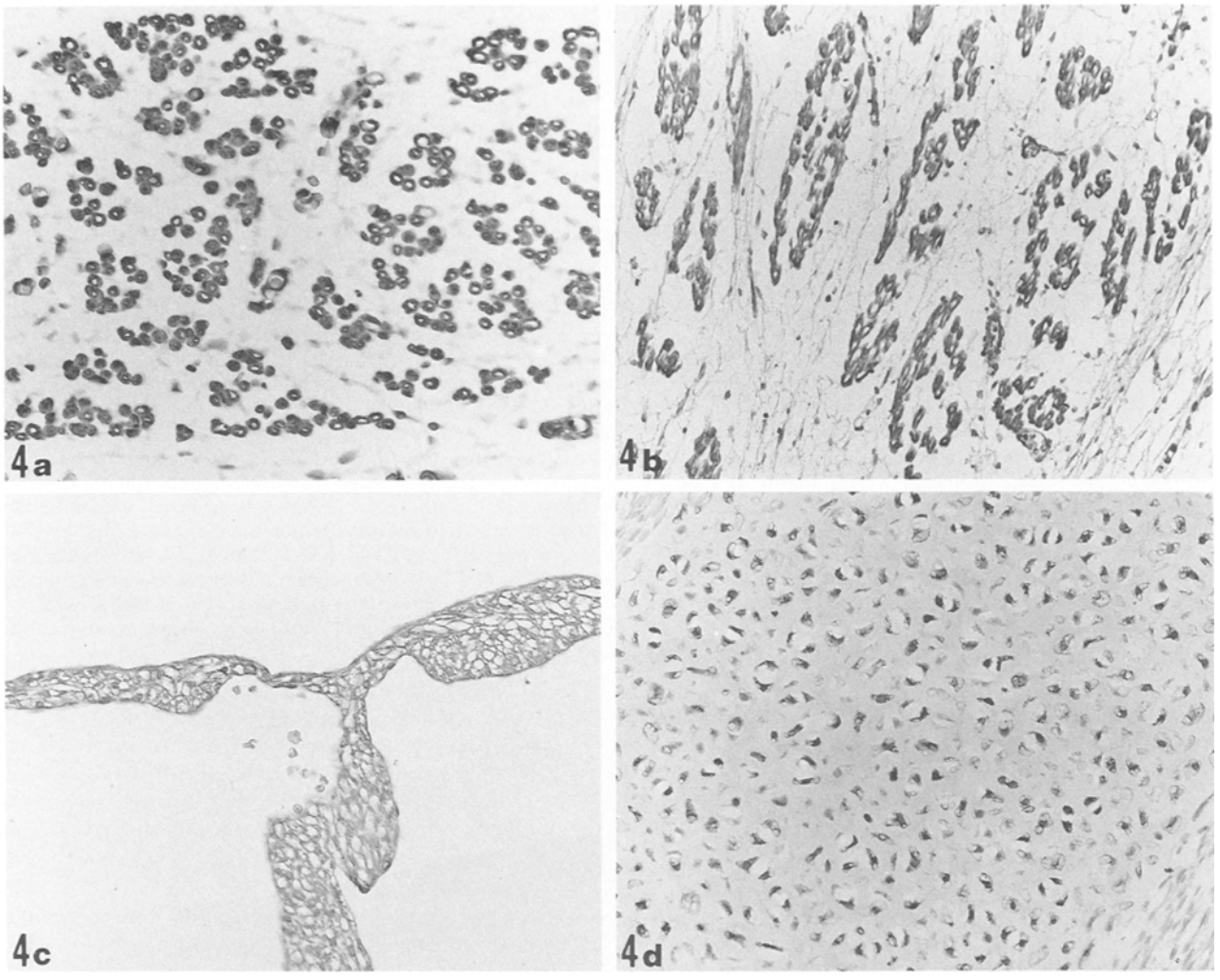

Fig. 4a–d. Immunohistochemical localization of Ca^{2+}-ATPase in human fetal tissues using monoclonal antibodies 6F5 and 7F10. **a, b** Positive reaction for 6F5 is shown in skeletal muscle by immunostaining. ×340. **c** Strong positive staining is observed in the right ventricle of the heart with 7F10. ×230. **d** Chondrocytes exhibit positive staining for 6F5. ×230

using both frozen and paraffin-embedded sections. For cryostat sections, the excised tissues were embedded in Tissue Tek OCT compound (Miles, Naperville, Ill., USA), and were snap-frozen in acetone cooled to −80° C. Acetone-fixed 4-μm sections were treated with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity prior to staining with mAbs. For paraffin-sections, the tissues were fixed with 10% buffered formalin, dehydrated and embedded in paraffin. Autoclave pretreatment of 3-μm sections immersed in distilled water was performed (hydrate autoclaving) (Shin et al. 1991). All sections were incubated with one of the two mAbs, diluted 1:100 in 20% fetal calf serum in phosphate-buffered saline. Control incubations were performed with preimmune mouse sera. The streptavidin-biotin-peroxidase complex method (Histofine, Nichirei, Tokyo, Japan) (Zuo-Rong et al. 1988) was applied for the immunoperoxidase staining.

Results

Two mAbs, designated 6F5 and 7F10, were raised, the immunoglobulin subclass of 7F10 being IgM, and that of 6F5 being IgG_{2b}. Western blotting revealed that both 6F5 and 7F10 reacted with the SR Ca^{2+}-ATPase from human and rabbit skeletal muscle showing a main band at 110 kDa (Fig. 1). SR membranes from rabbit skeletal muscle was divided into the three main fragments, namely $SR(A_1)$, $SR(A_2)$ and SR(B), by treatment with trypsin for 20 min. Western blotting using these fragments as antigens demonstrated 6F5 and 7F10 to recognize the A1 fragment showing a single band at 35 kDa (Fig. 2).

Immunohistochemical binding of mAbs 7F10 and 6F5 could be demonstrated in various tissues of human fetuses and adults as summarized in Table 1. Only the skeletal muscles were examined using both frozen and paraffin sections, while the other tissues were examined using paraffin sections. With regard to adult tissues, positive immunoreactivity for both antibodies was found in the skeletal muscle, Langerhans' islets of the pancreas and parathyroid gland. 6F5 also reacted with the anterior lobe of the pituitary gland and photoreceptor cells of the retina. In the skeletal muscle, both frozen and paraffin sections showed similar immunoreaction patterns for 6F5 and 7F10, the staining being restricted to the cytoplasm at cross-striations (Fig. 3a). In Langerhans' islets of the pancreas, cells positive for 6F5 and 7F10 were scattered, as illustrated in Fig. 3b. In the

parathyroid gland, most of the oxyphilic cells were stained with 6F5 and 7F10 (Fig. 3c). Positive cells for 6F5 were also found scattered in the anterior lobe of the pituitary (Fig. 3d). In fetuses, a positive staining with both antibodies was observed in the skeletal muscle, heart muscle, chondrocytes and peripheral nerves (Fig. 4a–d). The reaction was most pronounced in the right ventricle of the heart for both 6F5 and 7F10. No reaction products were observed in negative control tissues reacted with normal mouse serum.

Discussion

Intracellular organelles play major roles in the regulation of cytosolic Ca^{2+} concentration due to their ability to sequester Ca^{2+} and to release it following cell activation. In muscle cells, release of Ca^{2+} from SR occurs in response to either plasma membrane depolarization or Ca^{2+}-induced Ca^{2+} release. In both cases the Ca^{2+}-release channel known as the ryanodine receptor channel is involved (Fill and Coronado 1988). In non-muscle cells, Ca^{2+} release from intracytoplasmic Ca^{2+} stores can occur in response to inositol-1,4,5-triphosphate[Ins-(1,4,5)P_3] generated by phospholipase C following receptor activation (Berridge and Irvine 1989). Many non-muscle cells also possess intracellular Ca^{2+} stores which, in some cases, show similarities to the muscle SR case in being sensitive to both ryanodine and caffeine. The Ins(1,4,5)P_3 and ryanodine receptors have been characterized and sequenced by molecular cloning and described to be distinct but related proteins (Furuichi et al. 1989; Takashima et al. 1989). Active transport of Ca^{2+} across the membranes of ER or SR is carried out by Ca^{2+}-transport ATPase (Ca^{2+} pump) in both muscle and non-muscle tissues and the Ca^{2+} pump in both ER and SR has a homologous structure (Gunteski-Hamblin et al. 1988; Lytton and MacLennan 1988; Whytack et al. 1989).

In the present study we raised two novel mAbs, 6F5 and 7F10, and demonstrated their reaction with SR Ca^{2+}-ATPase from human and rabbit skeletal muscle on western blotting, and the A1 fragment in particular after trypsin digestion. Adapting an immunohistochemical approach we further showed the mAbs to react with various non-muscle tissues as well as skeletal muscle of both adults and fetuses. The staining pattern of the skeletal muscle showing accentuation at the cross-striations confirms the high specificity of these antibodies for SR Ca^{2+}-ATPase. Positive staining of Langerhans' islets of the pancreas, parathyroid and pituitary clearly indicates that their cytoplasmic Ca^{2+}-ATPase maintains a close homology with SR Ca^{2+}-ATPase. The localization of Ca^{2+}-ATPase to such endocrine tissues implies that the cytoplasmic Ca^{2+} pool in these cells is well developed and that therefore Ca^{2+} might play an important role in their signal transduction. In the parathyroid gland, the fact that positive cells were only observed scattered through the tissue suggests that they are functionally activated, while those demonstrating no binding are relatively inactive. Concerning the Langerhans' islets of the pancreas, some recent reports have indicated that inositolphospholipid turnover participates in the stimulation of insulin secretion (Prentki and Matschinsky 1987; Wollheim and Regazzi 1990; Zawalich and Rasmusen 1990). The present evidence for the presence of a Ca^{2+} pump in the Langerhans' islets of pancreas raises the question of whether Ca^{2+} release from the cytoplasmic pool may be related to hormone secretion from the Langerhans' islets.

While Walton et al. (1991) showed the co-existence of Ins(1,4,5)P_3 and ryanodine receptors in avian Purkinje cells and Villa et al. (1991) have described immunohistochemical localization of Ca^{2+}-ATPase in chicken Purkinje neurons, no immunoreactivity in human Purkinje cells was found in the present study. The reason for this discrepancy is presumably due to their use of mAbs to cardiac slow twitch SR Ca^{2+}-ATPase since we know that a material cross-reactive with a mAb to the A2 fragment of SR Ca^{2+}-ATPase (1E5, Ono and Kawakita, unpublished observation) is strongly expressed in Purkinje cells. There is a possibility that the Ca^{2+} pump of Purkinje cells may have a highly homologous structure to the A2 fragment of SR Ca^{2+}-ATPase, but not to the A1 fragment of SR Ca^{2+}-ATPase, which is recognized by our mAbs.

The observed differences in expression between fetus and adult are interesting, especially with regard to the endocrine system. Two possibilities require consideration. One is that the Ca^{2+}-ATPase of the fetus in non-muscle tissues is a different form from that found in adults. The other is that the amount of Ca^{2+}-ATPase in the fetal endocrine system is smaller than in the adult, possibly due to developmental immaturity.

It was shown in this study that the heart muscle was positive for Ca^{2+}-ATPase in fetuses but not in adults, suggesting that Ca^{2+}-ATPase of the fetal heart muscle has homologous structure to the A2 fragment of SR Ca^{2+}-ATPase.

Intracytoplasmic signal transduction, in which Ca^{2+} release from cytoplasmic Ca^{2+} pool participates, is thought to be ubiquitous to various cells. Our results show that, in addition to the amount of Ca^{2+}-ATPase differing in different tissues, distribution in a given tissue can also vary with cell type. Thus in some endocrine tissues, some cells scattered in the tissues showed a strong reaction, while the rest of them did not. Ca^{2+}-ATPase may be expressed preferentially in some cells in which signal transduction carried out by Ca^{2+} release from cytoplasmic pool is in an active state in tissues with strong expression.

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