Inositol 1,4,5-Trisphosphate Receptor in Heart: Evidence for its Concentration in Purkinje Myocytes of the Conduction System

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Abstract. Inositol 1,4,5-trisphosphate (IP_3) is one of the second messengers capable of releasing Ca^{2+} from sarcoplasmic reticulum/ER subcompartments. The mRNA encoding the intracellular IP_3 receptor (Ca^{2+} channel) has been detected in low amounts in the heart of various species by Northern blot analysis. The myocardium, however, is a heterogeneous tissue composed of working myocytes and conduction system cells, i.e., myocytes specialized for the beat generation and stimulus propagation. In the present study, the cellular distribution of the heart IP_3 receptor has been investigated. [^{3}H]IP_3 binding experiments, Western blot analysis and immunofluorescence, with anti-peptide antibodies specific for the IP_3 receptor, indicated that the majority of Purkinje myocytes (the ventricular conduction system) express much higher IP_3 receptor levels than atrial and ventricular myocardium. Heterogeneous distribution of IP_3 receptor immunoreactivity was detected both at the cellular and subcellular levels. In situ hybridization to a riboprobe generated from the brain type 1 IP_3 receptor cDNA, showed increased accumulation of IP_3 receptor mRNA in the heart conduction system. Evidence for IP_3-sensitive Ca^{2+} stores in Purkinje myocytes was obtained by double immunolabeling experiments for IP_3 receptor and cardiac calsequestrin, the sarcoplasmic reticulum intralumenal calcium binding protein. The present findings provide a molecular basis for the hypothesis that Ca^{2+} release from IP_3-sensitive Ca^{2+} stores evoked by α_1-adrenergic stimulation is responsible for the increase in automaticity of Purkinje myocytes (del Balzo, U., M. R. Rosen, G. Malfatto, L. M. Kaplan, and S. F. Steinberg. 1990. *Circ. Res.* 67:1535–1551), and open new perspectives in the hormonal modulation of chronotropism, and generation of arrhythmias.

ONE of the mechanisms of cell activation relies on receptor-stimulated hydrolysis of phosphatidyl inositol 4,5-bisphosphate ($PtInsP_2$[1]) via G protein-dependent phospholipase C (Berridge and Irvine, 1989). Hydrolysis of $PtInsP_2$ yields two intracellular second messengers, diacylglycerol and IP_3.

In cardiac muscle, several plasma membrane receptors (α_1-adrenergic, muscarinic, endothelin, angiotensin III) are coupled to $PtInsP_2$ turnover (Eckel et al., 1991; Renard and Poggioli, 1987; Yorikane et al., 1990; Kem et al., 1991; Brown et al., 1985; Kentish et al., 1990; Vites and Pappano, 1990), and different metabolic pathways lead to IP_3 production (Brown et al., 1985; Renard and Poggioli, 1987). The mRNA encoding the intracellular IP_3 receptor (Ca^{2+} channel) has been detected in low amounts in the heart of various species by Northern blot analysis (Furuichi et al., 1990; Mignery et al., 1990; Nakagawa et al., 1991). IP_3-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) of both atrial and ventricular myocytes (Fabiato, 1986; Kentish et al., 1990; Eckel et al., 1991; Vites and Pappano, 1991) has been demonstrated, and a positive inotropic effect has been postulated (Eckel et al., 1991; Vites and Pappano, 1991). Moreover, Borgatta et al. (1991) have recently shown the occurrence of a low-conducting Ca^{2+} release channel sensitive to IP_3 in canine heart SR vesicles. IP_3-gated Ca^{2+} channels were almost exclusively recorded from SR vesicles isolated from the ventricle septum (Borgatta et al., 1991), where cells of the conduction system are numerous (Sommer and Jennings, 1986).

The myocardium is a heterogeneous tissue composed of working (atrial and ventricular) myocytes and conduction system myocytes. The heart conduction system is composed of subpopulations of myocytes specialized for the beat generation and stimulus propagation, that display precise anatomical localization (Sommer and Jennings, 1986). Sino-atrial node myocytes represent the pacemaker, atrio-ventricular nodal myocytes control the ventricular excitation and coordinate it through the atrio-ventricular bundle, the right and

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1. *Abbreviations used in this paper*: CS, calsequestrin; IP_3, inositol 1,4,5-trisphosphate; $PtInsP_2$, phosphatidyl inositol 4,5-bisphosphate; SR, sarcoplasmic reticulum.

left bundle branches and the peripheral network of ventricular conduction system myocytes, i.e., the Purkinje myocytes.

In this paper, we have investigated the cellular distribution of the IP_3 receptor in cardiac muscle by biochemical, immunohistochemical and in situ hybridization methods. We found that the majority of Purkinje myocytes express much higher IP_3 receptor levels than atrial and ventricular myocytes, and other portions of the conduction system. Heterogeneous distribution of IP_3 receptor immunoreactivity was detected both at the cellular and subcellular levels. The biological relevance of these observations is discussed in relation to phenomena such as automaticity, chronotropism and arrhythmias.

Materials and Methods

Materials

[^{3}H]IP_3 and mouse anti-desmin mAbs were obtained from Amersham International (Buckinghamshire, UK), alkaline-phosphatase-conjugated anti-rabbit IgGs were obtained from Sigma Chemical Co. (St. Louis, MO) goat rhodamine-labeled anti-mouse IgGs from Cappel Laboratories (Malvern, PA) and swine fluorescein-labeled anti-rabbit IgGs from Dako Corporation (Carpinteria, CA).

Tissue Sources

Adult rats were killed by decapitation and their cerebella and hearts were quickly removed and stored at −80°C until used. Bovine and horse hearts were obtained from a local slaughterhouse. Samples of different cardiac regions corresponding to both working myocardium and conduction system were dissected out. Analogous samples were excised from mongrel dog and chicken hearts. Whole hearts were dissected out from bovine fetuses collected at 3, 4, and 5 mo of gestational age. All samples were immediately frozen in liquid nitrogen and stored at −80°C until used.

For immunohistochemical studies performed with anti calsequestrin (CS) antibodies, tissues were fixed with 4% paraformaldehyde in PBS for 2 h at 4°C, cryoprotected with 18% sucrose and frozen in liquid nitrogen.

cRNA Probes and Abs

A rat IP_3 cDNA (clone pI2a; Mignery et al., 1990) containing a 2-kb EcoRI fragment of the 3′ coding and noncoding regions of the type 1 IP_3 receptor mRNA, was used for in situ hybridization analysis.

The 19-mer COOH-terminal peptide of the mouse/rat type 1 IP_3 receptor sequence (cf. Furuichi et al., 1989; Mignery et al., 1990) was synthesized, conjugated to keyhole limpet hemocyanine (protein carrier) by Multiple Peptide Systems (San Diego, CA), and used as immunogen in rabbits. Antibody specificity for the IP_3 receptor was ascertained by Western blot of cerebellum microsomal fractions (see Fig. 1 *a*).

The mouse polyclonal antibody specific for dog cardiac CS were obtained as described (Biral et al., 1992). The specificity of the antibody was assessed by Western blot (Biral et al., 1992).

Mouse mAbs specific for either the myosin heavy chain subunit (BA-D5) or the α myosin heavy chain (BA-G5) were obtained and characterized as described in Schiaffino et al. (1989) and Rudnicki et al. (1990), respectively.

Isolation of Microsomal Fractions and Biochemical Assays

Microsomes from rat cerebellum and different areas of bovine heart (atrium, ventricular wall, septum and false tendon) were prepared by differential centrifugation as described by Volpe et al. (1991). Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Microsomal fractions were analyzed by SDS PAGE (5-10% linear gradient), transferred to nitrocellulose and reacted with antibodies as described (Volpe et al., 1991).

[^{3}H]IP_3 binding was carried out essentially as described (Alderson and Volpe, 1989) in a medium containing 0.1 M KCl, 50 mM Tris-Cl, pH 8.3, 1 mM EDTA, 150 μg of microsomal protein, 50 nM [^{3}H]IP_3, in the absence and presence of 5 μM cold IP_3 for total and nonspecific binding, respectively. Filtration and rinsings were as described (Volpe et al., 1991).

Immunocytochemistry

Indirect immunofluorescence was performed as previously described (Sartore et al., 1978; Gorza et al., 1986). Paraformaldehyde-fixed cryosections were quenched before incubation with antibodies in 0.2 M Tris-glycine, pH 7.4, for 30 min at room temperature. 5- to 10-μm cryosections were incubated with adequate dilutions of anti-IP_3 receptor Abs for 20 min at 37°C. After several washes with PBS, sections were incubated with fluorescein-conjugated anti-rabbit IgGs, rinsed again in PBS, mounted with glycerol and observed with a Zeiss Axioplan microscope equipped with epifluorescence optics.

For double-labeling immunofluorescence, anti-IP_3 receptor antibodies were mixed with mouse anti-CS or anti-myosin heavy chain Abs in the first incubation. Anti-mouse and anti-rabbit secondary antibodies, labeled with rhodamine or fluoresceine, respectively, were sequentially applied, after absorption with heterologous Ig in order to eliminate interspecies cross-reactivity (cf. Vitadello et al., 1990).

In Situ Hybridization

Antisense and sense cRNA probes were transcribed from the 2-kb pI2a cDNA clone (Mignery et al., 1990) after linearization with Hind III and BamHI, respectively, of the Bluescript plasmid and labeled with ^{35}S as described (Ausoni et al., 1991; Gorza et al., 1993). Probes were digested to 50–100 nucleotides by mild alkaline hydrolysis. Cryosections were fixed with formaldehyde, digested with proteinase K (Gorza et al., 1993), and hybridized overnight at 52°C with 10 μl of probe at a concentration ranging between 2.5 and 20 × 10^4 cpm/μl, and washed at 65°C with 50% formamide, 2 × SSC and 0.1 M DTT. Sections were dehydrated, dipped in Kodak NTB-2 autoradiographic emulsion diluted 1:1 with water, and exposed for 7–21 d at 4°C. Slides were developed with Kodak D 19 for 3.5 min, fixed and examined with a Zeiss Axioplan microscope equipped with dark field optics. Serial sections of the rat heart were processed for routine hematoxylin-eosin histology.

Results

Biochemical Studies

The bulk of the experiments were carried out on bovine heart because the false tendon could be identified, dissected out and used also for biochemical analysis. The false tendon contains, within a coat of collagen fibers, bundles of Purkinje myocytes, sympathetic nerves and their terminals, some blood vessels and fibroblasts (Sommer and Jennings, 1986).

Microsomal fractions were isolated from different areas of bovine heart, and probed with polyclonal anti-peptide antibodies specific for the IP_3 receptor in Western blot experiments. Fig. 1 shows that false tendon microsomes (lane *b*) contained an immunologically reactive polypeptide having the same electrophoretic mobility of the rat cerebellum IP_3 receptor (lane *a*; about 260,000 D), whereas microsomes isolated from the ventricle wall or the conduction-system-free septum were not reactive (lanes *c* and *d*, respectively).

Specific [^{3}H]IP_3 binding sites were quantitated in microsomes of atrium, ventricle, free septum and false tendon. Table I shows that false tendon microsomes contained 640 fmol IP_3 bound/mg of protein, i.e., levels of IP_3 receptor about eightfold higher than any other heart region investigated. The apparent discrepancy between low level of [^{3}H]IP_3 binding and lack of immunoreactivity reactivity in working myocardium microsomes (Fig. 1), could be due to the lower detection sensitivity of blotting techniques.

The results of both Western blot and [^{3}H]IP_3 binding experiments per se do not warrant the conclusion that the IP_3

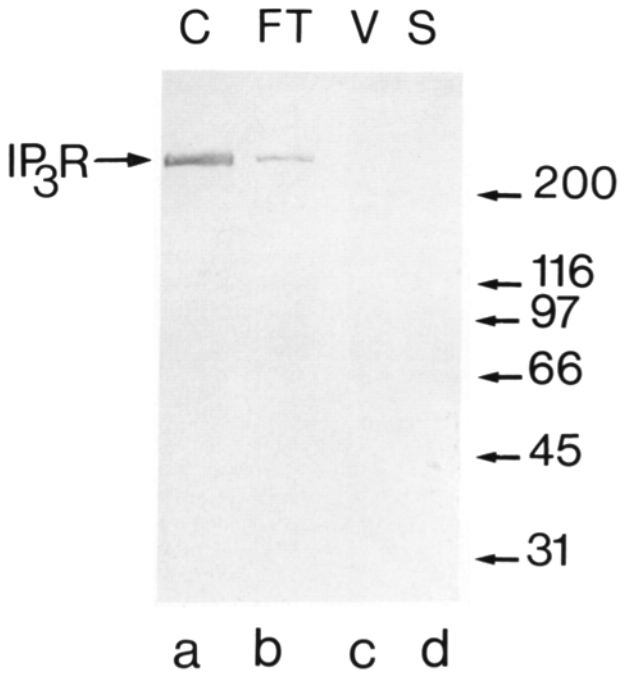

Figure 1. Western blotting of microsomal fractions obtained from different areas of bovine heart with anti-IP_3 receptor antibodies. SDS PAGE (5–10% linear gradient), electrophoretic transfer to nitrocellulose and immunoblot were carried out as described in Materials and Methods. Microsomes (100 μg of protein) derived from rat cerebellum (lane *a*), bovine heart false tendon (lane *b*, *FT*), ventricle wall (lane *c*, *V*) and free septum (lane *d*, *S*) were probed with anti-IP_3 receptor Abs. Bio-Rad (Bio-Rad Laboratories) molecular weight standards are indicated by arrows. IP_3-R, IP_3 receptor.

receptor is either localized or restricted to Purkinje myocytes, the main, yet not exclusive, constituents of the false tendon (see above). This question was further addressed by immunofluorescence and in situ hybridization experiments.

Immunocytochemistry

Cryosections of bovine heart were stained by immunofluorescence with antibodies specific for type 1 IP_3 receptor (Fig. 2, *b*, *c*, *e–g*). Myocytes of the working atrial (not shown) and ventricular (Fig. 2, *c*, *e–g*) myocardium were uniformly weakly labeled. In contrast, the distribution of IP_3 receptor immunoreactivity varied among the different regions of the conduction system (diagram in 2 *a*). Sino-atrial node myocytes were weakly reactive (not shown), atrio-ventricular node myocytes were only in part strongly labeled (*arrows*, *b*), whereas Purkinje myocytes were, in general, strongly reactive. Fig. 2 *c* shows bundles of large immunoreactive cells that were identified as myocytes on the account of their reactivity with anti-myosin antibodies, as judged by double labeling experiments (Fig. 2 *d*). Fig. 2 *e* shows peripheral Purkinje strands, at the most distal portions of the conduction system, where IP_3 receptor-labeled specialized myocytes intermingle with and can not be distinguished morphologically from ordinary myocytes.

Heterogeneity in staining intensity was also observed within and between Purkinje myocytes. Fig. 2 *c* shows that areas close to cell to cell junctions displayed a lower level of labeling with anti-IP_3 antibodies than central areas. Weakly-labeled Purkinje myocytes (*arrowheads*, Fig. 2 *f*) were detected either grouped in the same bundle or packed together with strongly positive myocytes (*arrow*). At the level of peripheral Purkinje strands (Fig. 2 *g*), homogeneously weakly labeled bundles (*arrowhead*, Fig. 2 *g*) were seen adjacent to strongly positive bundles (*arrow*, Fig. 2 *g*). In Fig. 2 *h* (serial section to Fig. 2 *g*), Purkinje myocytes were identified on the account of their immunoreactivity for anti-desmin antibodies (Thornell et al., 1985). Although subpopulations of conduction system myocytes can be distinguished on the basis of immunoreactivity to distinct myosin heavy chain isoforms, such as nodal myosin in sino-atrial and atrio-ventricular nodes and α-myosin heavy chain in the Purkinje myocytes (Gorza et al., 1986; Sartore et al., 1980), IP_3 receptor labeling was not obviously related to a specific myosin heavy chain composition (not shown).

IP_3 receptor immunoreactivity was also observed in the ventricular conduction system of the other mammalian species investigated and in the chicken heart (not shown).

The cellular compartmentalization of IP_3 receptor immunoreactivity in Purkinje myocytes of the bovine heart appears to occur very early during prenatal development. A population of myocytes of the ventricular septum of a 3-mo-old bovine fetus, corresponding to the right and left bundle branches, reacted strongly with anti-IP_3 receptor antibodies (*RB* in Fig. 3 *a*), at variance with the weak reactivity displayed by ventricular myocytes (*V* in Fig. 3 *a*). Higher magnification images of serial sections (Fig. 3 *b* and *c*) clearly show that labeling for the IP_3 receptor (*b*) was restricted to myocytes also reactive for anti-myosin antibodies (*c*). Comparable results were obtained in later stages of development (4- and 5-mo old fetuses): bundles of large myocytes were strongly reactive with anti-IP3 receptor antibodies (Fig. 3 *d*). In serial section (*e*), Purkinje myocytes were unambiguously identified on the account of their reactivity with antibodies specific for α myosin heavy chain, a marker of Purkinje myocytes in developing bovine heart (Thornell et al., 1984). Other conduction system myocytes e.g., nodal myocytes, were as weakly labeled as working myocytes (not shown).

Table I. [^{3}H]IP_3 Binding to Microsomal Fractions Isolated from Bovine Atrium, Ventricle, Free Septum, False Tendon and Cerebellum

	fmol/mg of protein
Atrium	85.8 ± 9.4 (4)
Ventricle	88.0 ± 7.8 (5)
Free septum	83.6 ± 13.8 (5)
False tendon	640.4 ± 55.3 (5)
Cerebellum	8860.3 ± 310.6 (3)

Experiments were carried out as described in Materials and Methods. Data are given as mean ± SD for the number of different preparations shown in parenthesis. Scatchard plot analysis of [^{3}H]IP_3 binding to cerebellum and false tendon microsomes yielded K_d values of 12.3 and 14.1 nM, respectively, to a single class of binding sites.

In Situ Hybridization

Analysis at the mRNA level was performed by in situ hybrid-

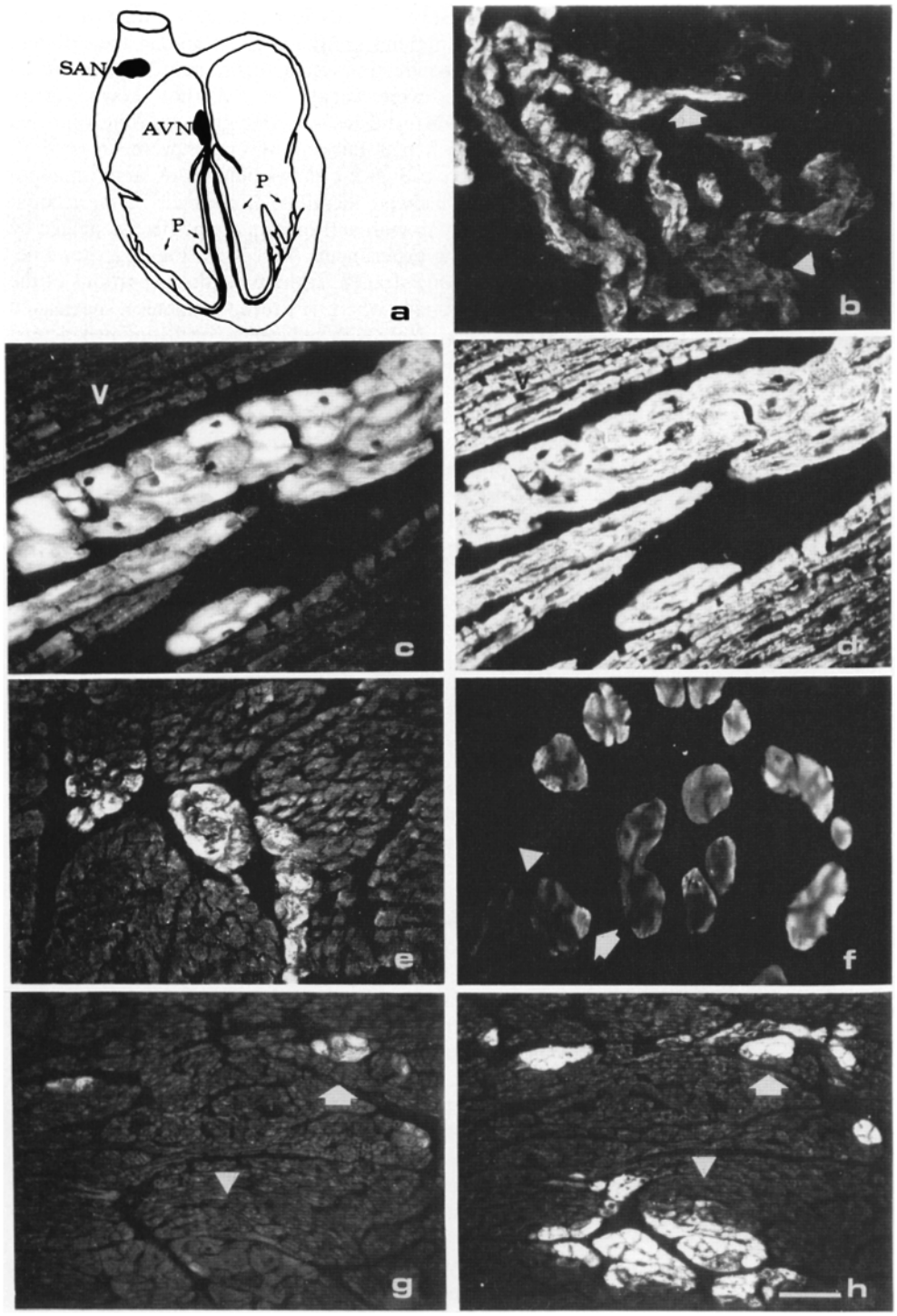

Figure 2. Immunofluorescence staining of bovine heart cryosections with anti-IP_3 receptor (*b*, *c*, *e–g*), anti-myosin heavy chain (*d*) and anti-desmin (*h*) antibodies. The diagram in *a* outlines the composition and anatomical localization of the heart conduction system: sino-atrial node (*SAN*) myocytes, the pacemaker, atrio-ventricular nodal (*AVN*) myocytes, the atrio-ventricular His bundle, the right and left bundle branches and the peripheral network of ventricular conduction system myocytes (*P*), i.e., the Purkinje myocytes. (*b*) Bundles of the *AVN* myocytes; arrows and arrowheads point to strongly and weakly reactive myocytes, respectively. (*c* and *d*) Section through bundles of the right bundle branch, double labeled with either anti-IP_3 receptor (*c*) or anti-myosin (*d*) antibodies: *c*, note weakly-labeled areas close to cell-to-cell junctions. (*e*) Peripheral Purkinje strands. (*f*) IP_3 receptor-labeled bundles of the right bundle branch; arrows and arrowheads point to strongly and weakly reactive Purkinje myocytes, respectively. (*g* and *h*) Serial sections labeled with anti-IP_3 receptor (*g*) and anti-desmin (*h*) antibodies: among Purkinje myocytes, only a few are strongly labeled by anti-IP_3 receptor antibodies (*arrow*, *g*), the majority being weakly reactive (*arrowhead*, *g*). In *h*, arrow and arrowhead point to Purkinje myocytes identified on the account of their reactivity with anti-desmin antibodies. Abbreviation: *V*, ventricle. Bar, *b–f*, 60 μm; *g* and *h*, 160 μm.

ization to a riboprobe transcribed from a 2-kb cDNA corresponding to the 3′ end of rat brain type 1 IP_3 receptor mRNA (Mignery et al., 1990; Sudhof et al., 1991).

Antisense cRNA hybridized strongly to rat cerebellar Purkinje neurons (Fig. 4 *a*), as expected, given the high density of IP_3 receptor in these cells (Worley et al., 1987; Furuichi et al., 1989; Satoh et al., 1990; Villa et al., 1991; Takei et al., 1992). Fig. 4 *b* represents a hematoxylin-stained section serial to those of Fig. 4, *c* and *d*. Hybridization signals varied among rat cardiac myocytes: working ventricular myocytes were weakly reactive, whereas conduction system myocytes of either the atrioventricular node (*AVN*) and the His bundle (*H*), as shown in Fig. 4 *c*, or subendocardial myocytes, probably corresponding to Purkinje bundles (*P*), as shown in Fig. 4 *d*, hybridized strongly to IP_3 cRNA. Hybridization to sense cRNA was negative (not shown). In the rat heart, strong hybridization to IP_3 receptor mRNA was detected in smooth muscle myocytes of coronary vessels (inset in Fig. 4 *d*; cf. Furuichi et al., 1990).

Only a very weak signal was observed in canine Purkinje

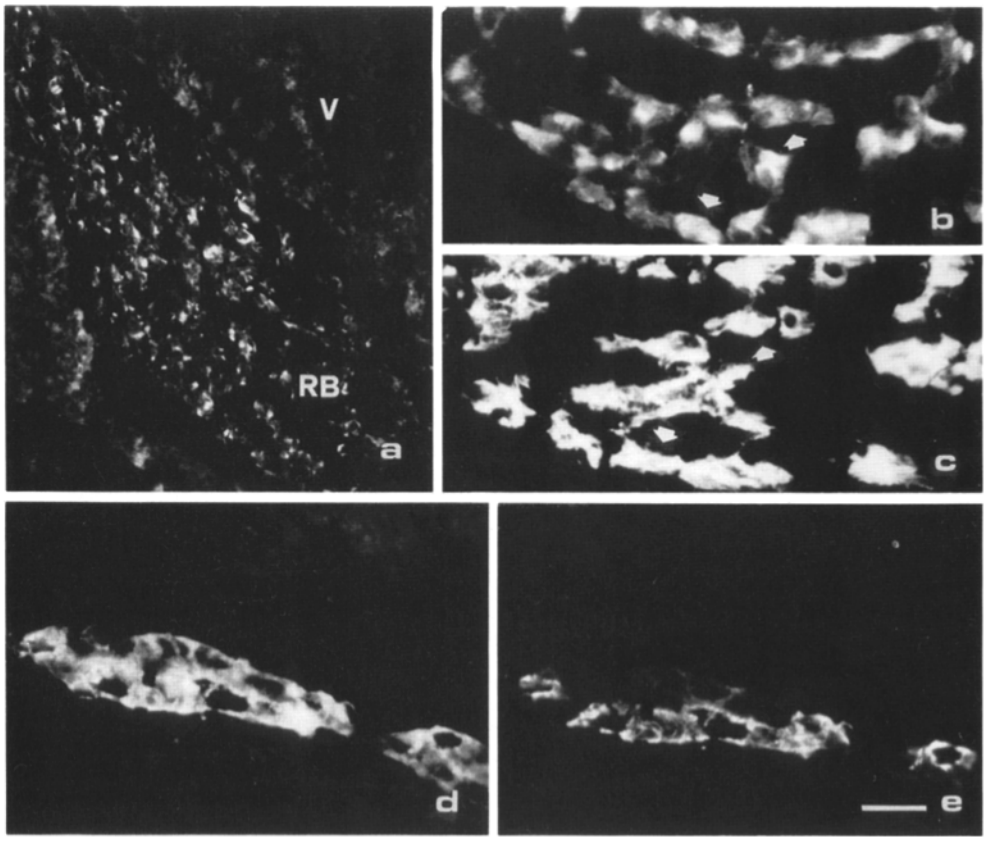

Figure 3. Immunofluorescence staining of bovine embryonic heart cryosections with anti-IP_3 receptor (*a*, *b* and *d*), anti-myosin heavy chain (*c*), and anti-α myosin heavy chain (*e*) antibodies. Serial sections of the ventricular septum of a 3-mo old fetus were labeled with anti-IP_3 receptor (*b*) and anti-myosin heavy chain (*c*) antibodies; labeling for IP_3 receptor is detectable in myosin-positive myocytes of the right bundle branch (arrows, *b* and *c*). Serial sections of the ventricular myocardium of a 5-mo-old fetus were labeled with anti-IP_3 receptor (*d*) and anti-α myosin heavy chain (*e*) antibodies: IP_3 receptor-positive bundles of large conduction myocytes were detected. Abbreviations: *V*, ventricle, *RB*, right bundle branch. Bar, *a*, 60 μm; *b–e*, 30 μm.

neurons with rat IP_3 receptor cRNA and no signal at all in both bovine and canine myocytes (not shown).

Subcellular Localization and Heterogeneous Distribution of the IP_3 Receptor and Calsequestrin

The cellular and subcellular distribution of both IP_3 receptor and CS, the SR intralumenal low-affinity, high-capacity Ca^{2+} binding protein (MacLennan and Wong, 1971) was investigated by immunofluorescent labeling in bovine Purkinje myocytes.

Double labeling revealed uneven distribution of the two proteins in IP_3 receptor-positive Purkinje myocytes. The IP_3 receptor labeling was widespread throughout the cytoplasm, showing regional staining differences (Fig. 5, *a* and *b*), i.e., the staining was more intense in the central cytoplasmic region (see also Fig. 2 *c*) that is filled with intermediate filament proteins in bovine Purkinje myocytes (Thornell et al., 1985). On the other hand, the punctate staining of CS (cf. Jorgensen et al., 1984) was more evident in, but not limited to, subsarcolemmal areas (Fig. 5, *c* and *d*) where myofibrils are concentrated. In all panels, large arrows point to myocytes showing strong reactivity for the IP_3 receptor in central areas apparently devoid of CS; small arrows point to subsarcolemmal regions reactive for both the IP_3 receptor and CS.

Discussion

Heterogeneous Distribution of the Cardiac Type 1 IP_3 Receptor and Its Concentration in Purkinje Myocytes

In this paper we demonstrate, for the first time, that the type 1 IP_3 receptor is highly concentrated in the majority of Purkinje myocytes, the distal elements of the heart conduction system, and some fascicles of the atrio-ventricular node. We also show much lower amounts of IP_3 receptor in the working myocardium and other portions of the conduction system. The differential distribution of the IP_3 receptor between conduction system myocytes and working myocytes is also observed during embryonic development, i.e., at the earliest stage examined, conduction myocytes are found to express a higher level of IP_3 receptor.

There are, thus, two levels of heterogeneity whose functional relevance remains to be investigated: one among the different subpopulations of the heart conduction system and the working myocytes, the other between and within the Purkinje myocytes themselves. In the latter cases, the heterogeneity of Purkinje myocytes pertains both to the subcellular and cellular level, as judged by serial section analysis: there are, in fact, areas close to cell–cell junctions which are weakly stained (Fig. 2 *c*) as compared to central cytoplasmic areas; moreover, either individual myocytes within a bundle (Fig. 2 *f*) or entire bundles of conduction system myocytes (cf. Fig. 2, *f–h*) are weakly labeled as compared to either adjacent myocytes or bundles.

A rat type 2 IP_3 receptor isoform, the product of a distinct gene (Sudhof et al., 1991), has been recently described. Ligand binding studies on recombinant IP_3 receptor proteins, have shown that type 2 IP_3 receptor displays slightly higher affinity for IP_3 than type 1 IP_3 receptor. Analysis of the deduced amino acid sequence indicates divergence in the COOH-terminal 19-mer peptide, chosen to raise the antibodies specific for type 1 IP_3 receptor and used in this study. Low degree of homology is also observed in the 3′

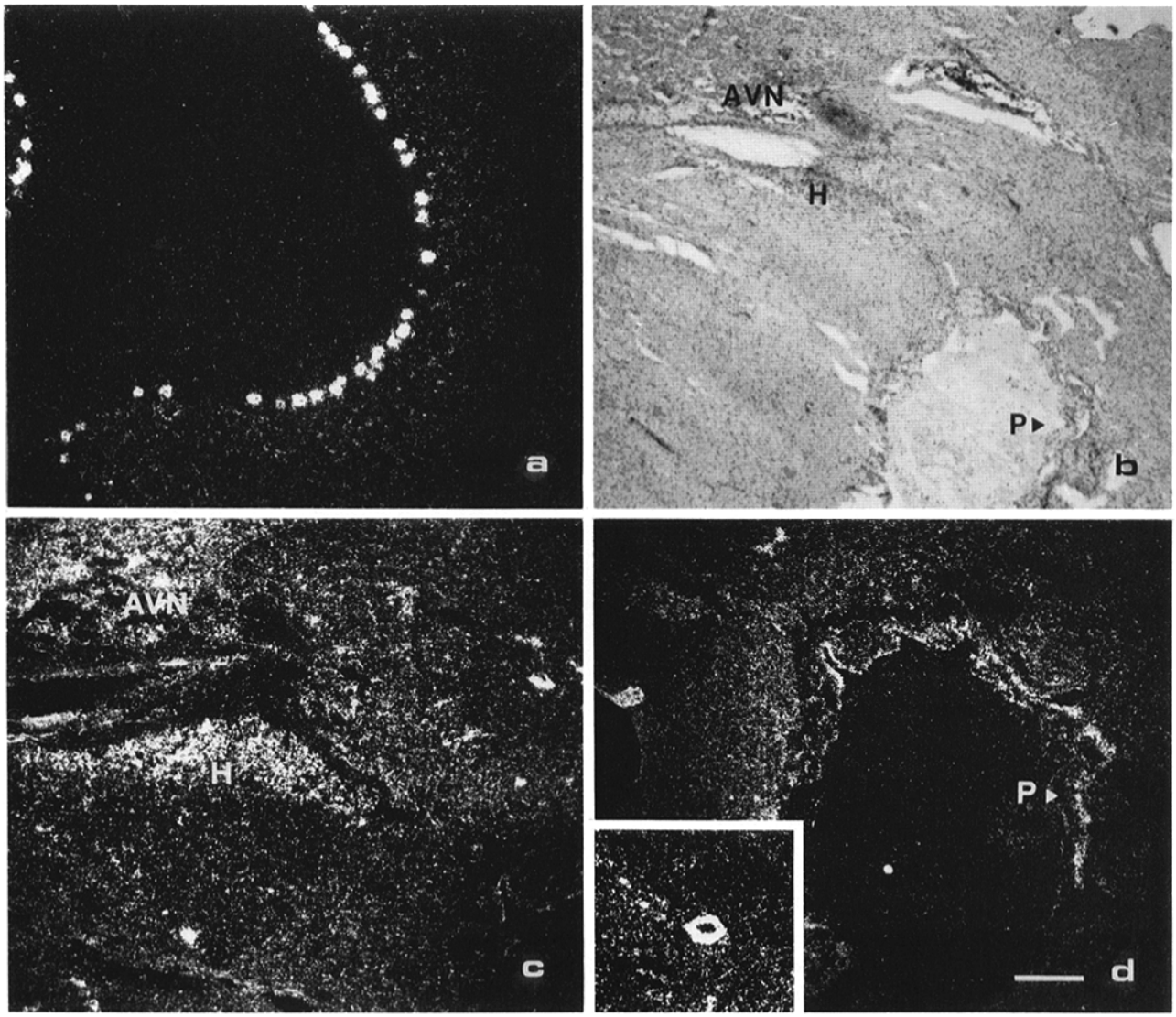

Figure 4. In situ hybridization. Dark fields micrographs of IP_3 receptor cRNA hybridized to rat cerebellum (*a*) and heart (*b* and *c*). Antisense probe hybridization was detected in the soma and dendrites of cerebellum Purkinje neurons (*a*), in coronary smooth muscle myocytes (*inset*, *d*), in subendocardial Purkinje myocytes (*d*, *P*, *arrowhead*), and in conduction myocytes of the AV node (*AVN*) and the His bundle (*H*: *c*); myocytes of the ventricular free wall (*V*) and septum (*S*) were weakly reactive (*c* and *d*). *b*, which represents a section serial to those of *c* and *d*, was stained with hematoxylin and eosin. Bar, *a*, *c*, *d*, and *inset*, 240 μm; *b*, 400 μm.

coding and noncoding regions of type 1 and 2 IP_3 receptor mRNAs (Sudhof et al., 1991). Although we cannot rule out with certainty that other IP_3 receptor isoforms are expressed (up to four distinct IP_3 receptor cDNAs have been described in the mouse; Ross et al., 1992), the specificity of the antibodies and cRNA probes used along with our $[^3H]IP_3$ binding data, strongly indicate that the type 1 IP_3 receptor represents a major isoform in Purkinje myocytes.

IP_3-sensitive Ca^{2+} Stores Purkinje Myocytes

In Purkinje myocytes, the SR is composed of three continuous membrane domains, the free SR, corbular SR and junctional SR (Sommer and Jennings, 1986) whose relative development shows regional variations. The corbular and junctional SR share ultrastructural features and contain intralumenal granules largely made up of CS (Jorgensen and Campbell, 1984; Jorgensen et al., 1988). Junctional SR profiles are observed exclusively in the subsarcolemmal rim all around myofibrils. Free and corbular SR, as well as ER cisternae are also observed in myofilament-free areas of central cytoplasm (Sommer and Jennings, 1986).

The IP_3 receptor is a ligand-gated Ca^{2+} channel (Maeda et al., 1991), found to be concentrated in the limiting membrane of smooth-surfaced ER subcompartments in both chicken and mammalian cerebellum Purkinje neurons (Satoh et al., 1990; Volpe et al., 1991; Takei et al., 1992). CS, an intralumenal low-affinity, high-capacity Ca^{2+} binding protein (MacLennan and Wong, 1971), is localized in striated muscle SR subcompartments, i.e., terminal cisternae (Jorgensen et al., 1984), and ER moderately dense-cored vacuoles in chicken cerebellum Purkinje neurons (Villa et al., 1991;

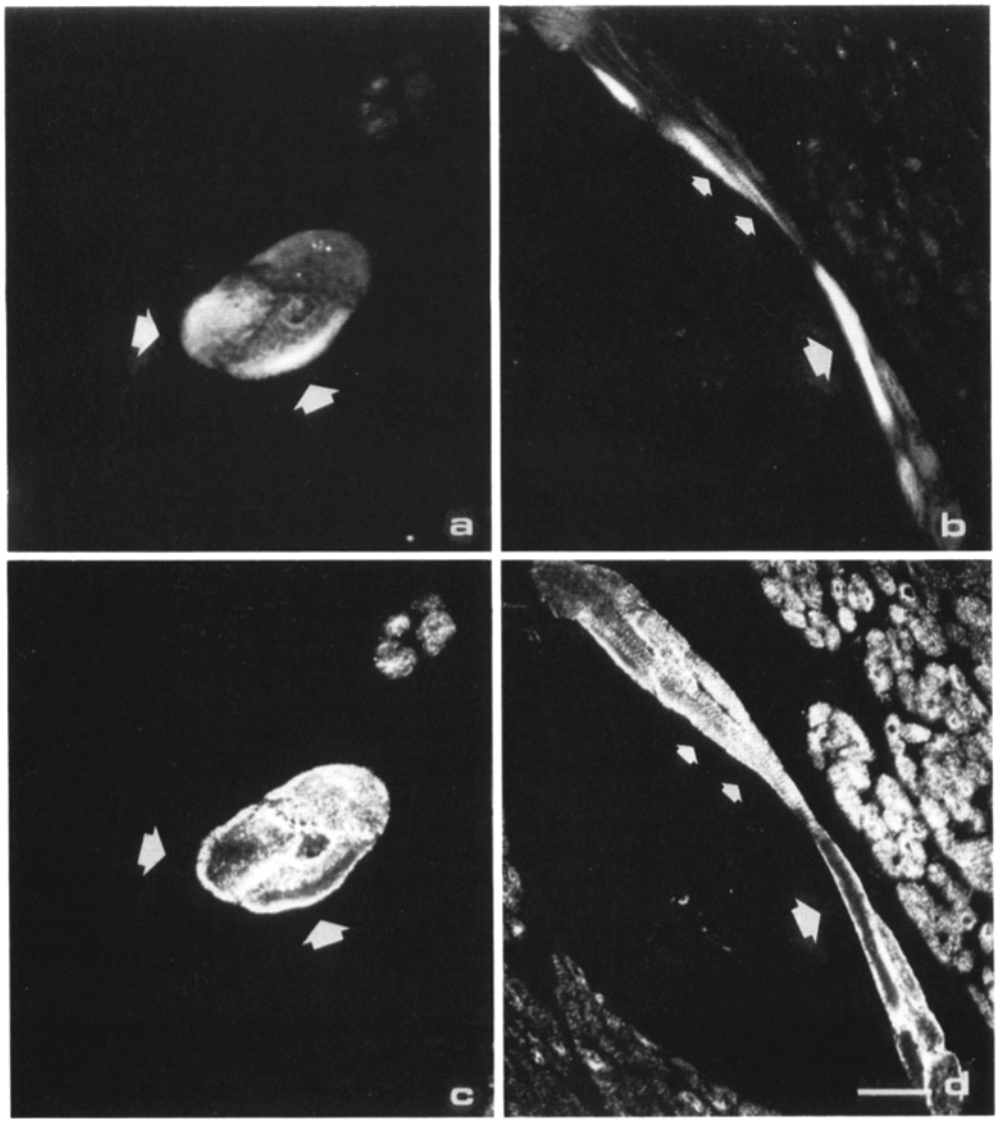

Figure 5. Double immunofluorescence staining of bovine heart cryosections with anti-IP_3 receptor (*a* and *b*) and anti-cardiac CS (*c* and *d*) Abs. Large arrows point to myocytes showing strong reactivity for the IP_3 receptor in central areas apparently devoid of CS; small arrows point to subsarcolemmal regions reactive for both the IP_3 receptor and CS. Bar, *a* and *c*, 40 μm; *b* and *d*, 60 μm.

Volpe et al., 1991). The occurrence of both CS and IP_3 receptor on the same subcellular compartments is taken as evidence of functional IP_3-sensitive Ca^{2+} stores (Volpe et al., 1991; Takei et al., 1992; reviewed in Meldolesi et al., 1992).

In the present study, Purkinje myocytes are labeled by antibodies for both the IP_3 receptor and CS (Fig. 5), thus indicating the existence of IP_3-sensitive Ca^{2+} stores. The labeling pattern indicates that the IP_3 receptor is not restricted to subsarcolemmal SR but possibly present in corbular SR (or parts of it) and ER subcompartments, scattered throughout the cell (Sommer and Jennings, 1986), which might be in lumenal continuity with junctional SR (cf. Volpe et al., 1992). The finding that central areas of a number of Purkinje myocytes are strongly reactive for the IP_3 receptor and poorly reactive for CS (Fig. 5), points to subcellular heterogeneity of Ca^{2+} stores in conduction system cells.

The relevance of the present observations and the detailed ultrastructural features of Ca^{2+} stores in Purkinje myocytes will be fully understood after completion of EM immunogold labeling experiments, which are in progress. Molecular heterogeneity of intracellular Ca^{2+} stores has been already demonstrated in the Purkinje neuron of the chicken, where different subcellular membrane compartments were found, by EM immunogold labeling, to be variably endowed with either CS or IP_3 receptor (Villa et al., 1991; Volpe et al., 1991; Takei et al., 1992).

Functional Significance of the IP_3 Receptor in Purkinje Myocytes

After plasma membrane depolarization, transient increases of $[Ca^{2+}]_i$ appear to be caused by other entry of extracellular Ca^{2+} and release of Ca^{2+} from the SR (Wier, 1980; Fabiato, 1985) of Purkinje myocytes. However, the physiological role of Ca^{2+} release has remained elusive thus far. In Purkinje myocytes, IP_3-sensitive Ca^{2+} stores may be involved in the increase of automaticity after α_1-adrenergic stimulation. del Balzo et al. (1990) have recently shown that WB 4101, a competitive inhibitor of α_{1A}-adrenergic receptor in canine Purkinje myocytes, antagonizes the α_1-adrenergic

positive chronotropic response, and prevents norepinephrine-dependent inositol phosphate accumulation. del Balzo et al. (1990), thus, suggested that the products of $PtInsP_2$ hydrolysis, either diacylglycerol, IP_3 or both, are "mechanistically involved in the α_1-adrenergic positive chronotropic response." Moreover, automatic arrhythmias induced by α_1-adrenergic stimulation in experimental models of "ischemic" and "reperfused" canine Purkinje myocytes (Anyukhowsky and Rosen, 1991; Molina-Viamonte et al., 1991), were abolished by pretreatment with WB 4101. IP_3-induced transient elevation of $[Ca^{2+}]_i$ may bring about decrease of membrane potential and, in turn, increased automaticity via activation of a nonspecific cation conductance (Colquoun et al., 1981; Kass and Tsien, 1982; Molina-Viamonte et al., 1990). Thus, IP_3-induced Ca^{2+} release appears to be causally related to both increase in automaticity and generation of some arrhythmias. The demonstration of the high level of IP_3 receptor in Purkinje myocytes provides a molecular basis for this interpretation.

In Purkinje myocytes, the heterogeneous distribution of the IP_3 receptor might be related to that of phospholipase C-coupled pertussis toxin-insensitive G proteins, which appear to be functionally present only in 50% of Purkinje myocytes (see del Balzo et al., 1990). The transduction pathway activated by α_{1A}-adrenergic agonists and controlled by a pertussis-insensitive G protein appears to be developmentally regulated. At birth almost all Purkinje myocytes respond to α_1-adrenergic agonists with an increase in automaticity (see del Balzo et al., 1990, and references therein) whereas only 50% of adult Purkinje myocytes do so. The IP_3 receptor immunolabeling pattern observed in adult and embryonic Purkinje myocytes is formally consistent with these changes, insofar as Purkinje myocytes are far less heterogeneous as compared to adult ones. However, other mechanisms might be responsible for the negative chronotropism induced in about 50% of adult Purkinje bundles upon stimulation with α_1-adrenergic agonists. A pertussis toxin-sensitive G protein has been shown to be expressed after birth and to couple activation of α_{1B}-adrenergic receptor subtype to a Na^+-K^+ pump, whose operation would determine the decrease in automaticity (Zaza et al., 1990). Interestingly, specific $\alpha 2$ and $\alpha 3$ isoforms of the Na^+-K^+ pump are expressed in conduction system myocytes (Zahler et al., 1992). It remains to be ascertained whether both transduction pathways are functional in adult Purkinje myocytes or are segregated to distinct Purkinje myocytes subpopulations.

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