An Intrinsic Adrenergic System in Mammalian Heart

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Abstract

We have identified a previously undescribed intrinsic cardiac adrenergic (ICA) cell type in rodent and human heart. Northern and Western blot analyses demonstrated that ICA cell isolates contain mRNA and protein of enzymes involved in catecholamine biosynthesis. Radioenzymatic catecholamine assays also revealed that the catecholamine profile of adult rat ICA cell isolates differed from that of sympathetic neurons. Unlike sympathetic neuronal cells, isolated ICA cells have abundant clear vesicles on electron microscopy. Endogenous norepinephrine and epinephrine constitutively released by ICA cells in vitro affect the spontaneous beating rate of neonatal rat cardiac myocytes in culture. Finally, ICA cells could be identified in human fetal hearts at a developmental stage before sympathetic innervation of the heart has been documented to occur. These findings support the concept that these cells constitute an ICA signaling system capable of participating in cardiac regulation that appears to be independent of sympathetic innervation. (*J. Clin. Invest.* 1996. 98:1298–1303.) Key words: catecholamines • culture • heart • paracrine • tyrosine hydroxylase

Introduction

The importance of endogenous norepinephrine and epinephrine in augmenting cardiac function is well established. The cardiovascular augmentation during the classic "fight or flight" response is thought to be due primarily to catecholamine release from intracardiac sympathetic nerve endings and from the adrenal medulla. However, a cardiac response to exercise is not abolished in dogs that have undergone cardiac denervation and adrenalectomy (1). After cardiac denervation in dogs, norepinephrine levels fall (< 5% of that in normal heart), reflecting the lack of sympathetic innervation, while a large fraction (~ 50% of that in normal heart) of tissue epinephrine content remains in the heart (2). Patients with transplanted hearts also maintain adequate cardiac function in the absence of sympathetic reinnervation (3, 4). Furthermore, the obligatory role of catecholamines in fetal cardiac development has been underscored by the finding that mice with targeted disruption of catecholamine-synthesizing genes die in utero due to cardiac failure at a developmental stage before cardiac sympathetic innervation is known to occur (5, 6). Finally, recent evidence suggests that abnormal cardiac epinephrine production in heart failure patients may be derived in part from sources other than adrenal chromaffin cells or sympathetic nerves, because enhanced cardiac epinephrine spillover into the coronary circulation is unrelated to stress-induced cardiac sympathoadrenal activation (7). All these observations suggest that an adrenergic mechanism distinct from the classic sympathoadrenal axis may be involved in cardiovascular regulation. This hypothesis prompted us to determine if an intrinsic adrenergic system exists in the mammalian myocardium.

Methods

Morphologic studies

In situ and in vitro immunohistochemistry. Immunoperoxidase labeling (8) was performed with a monoclonal antibody (IgG) against tyrosine hydroxylase (TH)[1] (1:500, Incstar) on 3-μm paraffin sections of 4% paraformaldehyde fixed rat and human hearts (two adult, two neonatal, and two fetal human hearts). Nonspecific IgG served as a control. Antibodies (IgG) against neuron-specific enolase (NSE) and protein gene product 9.5 were also used at 1:5,000 dilution. Isolated intrinsic cardiac adrenergic (ICA) cells (see below) from adult rat hearts were stained using an alkaline phosphatase immunohistochemical method (9). Antibodies against TH and NSE were used at 1:50 and 1:100 dilution, respectively. Isolated myocytes served as a negative control for anti-TH and anti-NSE in ICA cells. The use of human discarded tissue was approved by Human Research Committee of Brigham and Women's Hospital.

Electron microscopic analysis. Transmission electron microscopic analysis was performed on primary isolates of adult rat and human ICA cells. Human ICA cells were isolated from specimens obtained at surgery (two hearts). Ventricular tissues were enzymatically dissociated, and human ICA cells were isolated in the same manner as for the rat. Isolated ICA cells were fixed in 1.5–4% glutaraldehyde for electron microscopy using routine methods.

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1. *Abbreviations used in this paper:* DBH, dopamine β-hydroxylase; ICA, intrinsic cardiac adrenergic; NSE, neuron-specific enolase; PNMT, phenylethanolamine *N*-methyltransferase; TH, tyrosine hydroxylase.

Cardiac myocyte and ICA cell isolation

Enzymatic dissociation of ventricular myocytes was performed as described previously (10). The isolation of ICA cells was made possible by the observation that ICA cells associated with magnetic beads after enzymatic dissociation of cardiac muscle tissue. Dissociated muscle cells were resuspended in 4 ml of phosphate-buffered saline containing 50 μl of IgG-free magnetic beads (8-4100B; Advanced Magnetics, Inc., Cambridge, MA) and incubated for 20 min with intermittent mixing on ice. ICA cells associated with the beads were recovered by magnetic separation (Dynal, Inc., Great Neck, NY). Isolated ICA cells were used for immunohistochemical staining as described above and for further biochemical assays.

Catecholamine assay

Cellular content. Measurements of norepinephrine, epinephrine, and dopamine content were performed in isolated ICA cells, sympathetic

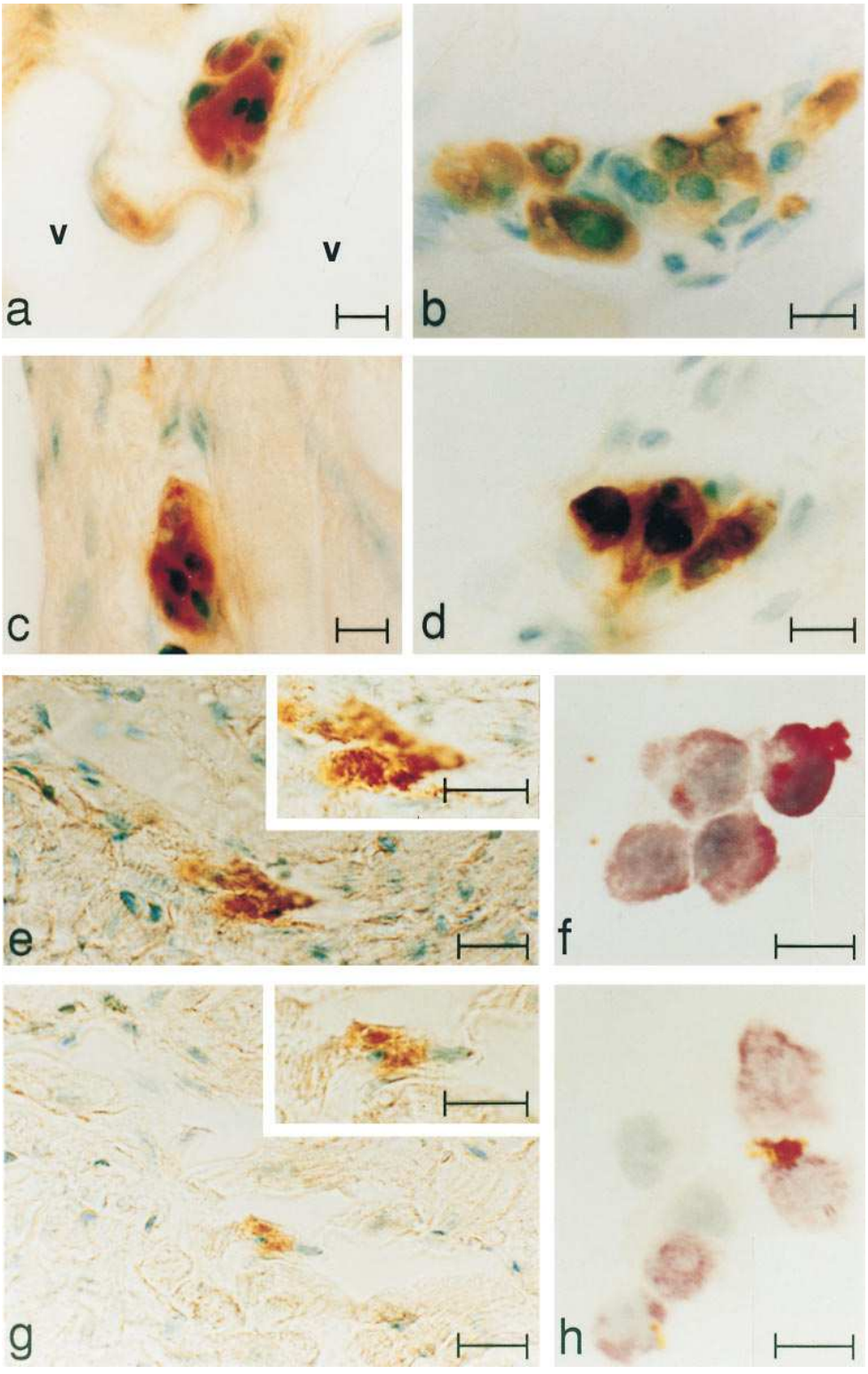

Figure 1. Immunoperoxidase labeling of TH and NSE in ICA cells in the adult rat heart. (*a*) Clusters of ICA cells were frequently observed near venules (*v*). (*b*) Epicardial localization of an ICA cell cluster. (*c*) ICA cells are also located at interfaces between arteriolar smooth muscle cells (*left side*) and cardiac myocytes (*right side*). (*d*) Location of ICA cell cluster in connective tissue. (*e* and *g*) Serial sections of adult rat ventricle. Immunoperoxidase labeling for TH (*e*) and NSE (*g*) were observed in ICA cells in serial sections of rat ventricular muscle. The insets illustrate higher magnification images of *e* and *g*, respectively. (*f* and *h*) Isolated ICA cells from adult rat heart. By alkaline phosphatase immunohistochemistry, 70–80% (n = 3 samples) of the isolated ICA cells stained positively with anti-TH (*f*) and anti-NSE (*h*) antibodies. Isolated myocytes were stained negatively with either anti-TH or anti-NSE (data not shown). Bars in *a–h*, 10 μm.

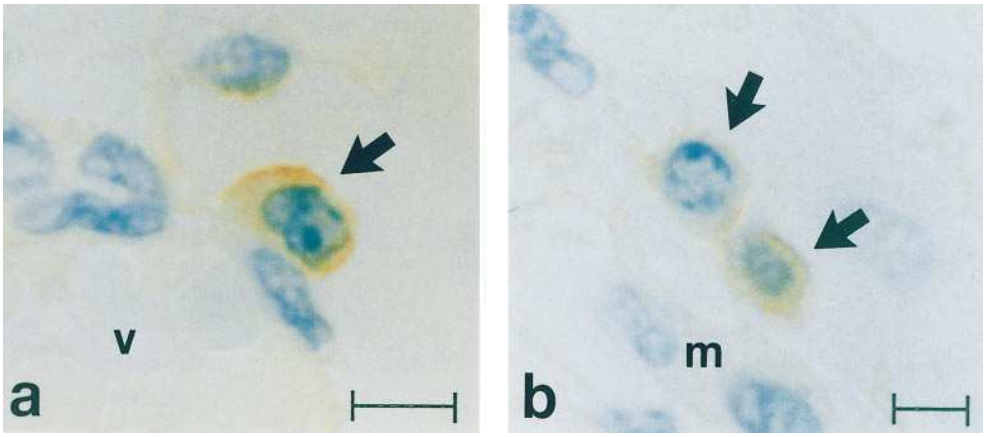

Figure 2. Immunoperoxidase labeling of TH in ICA cells in paraffin sections of two 20-wk-old fetal human hearts. *a* shows a perivascular binucleated ICA cell in ventricular muscle, and *b* shows ICA cells associated with ventricular myocytes (*m*). Arrows depict ICA cells labeled with anti-TH IgG. *v*, vessel. Bars, 10 μm.

neurons, and ventricular myocytes depleted of ICA cells in adult rats. Catecholamine content was quantitated with a catechol-*O*-methyltransferase–based radioenzymatic method (11). Rats were anesthetized and stellate ganglia were isolated. All cells were homogenized in a lysis buffer containing 1% Triton, 5 M NaCl, 1 M Tris, 0.5 M EDTA, 0.25 M EGTA, 20 mM phenylmethyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, and 0.5% NP-40. Cell lysates were centrifuged for 20 min at 5,800 *g* and the supernatants were used for catecholamine assays. For whole heart catecholamine assays, isolated hearts were perfused with Tyrode's solution (37°C) for 20 min to eliminate catecholamines in blood. Hearts were homogenized by a tissue homogenizer in 5 ml of lysis buffer, and the supernatants were collected.

Catecholamine release in vitro. Freshly isolated rat ICA cells were plated in a P-60 culture dish containing 3 ml of serum-free culture medium (DMEM; GIBCO-BRL, Gaithersburg, MD) with 1 mM Ca^{2+} incubated for 1 h (37°C, 5% CO_2), and conditioned media were taken for radioenzymatic catecholamine assay. ICA cells from each dish were then lysed for protein quantification.

ICA cell catecholamine release and neonatal myocyte beating rate in vitro

Neonatal rat ventricular cardiac cell cultures were prepared using established methods (12). ICA cells which are present in neonatal rat ventricular myocardium were not selectively removed from neonatal rat ventricular myocyte primary isolates using the magnetic cell isolation method described above. This allowed the observation of ICA cell–derived catecholamines on neonatal rat myocyte contractile function in vitro. The beating rate of myocytes plated on a coverslip was determined on a temperature-controlled (37°C) chamber on the stage of a microscope connected to a videomotion analyzer (10). The spontaneous beating of myocyte clusters converted to an analogue signal was recorded continuously by a pen recorder. The culture was allowed to equilibrate in Tyrode perfusate (2 ml/min) for 20 min before the β-adrenergic antagonist timolol (1 μM) was administered ($n = 5$ preparations). All data are expressed as mean±SEM. ANOVA and a Student's *t* test were used for statistical analyses.

Northern and Western blot analyses

Total RNA isolated from each cell type was electrophoretically resolved on 1% formaldehyde-agarose gels (13). Blots were hybridized overnight with a randomly primed 2.3-kb human genomic TH probe (American Type Culture Collection, Rockville, MD) at 42°C. The blot was washed for 30 min at 65°C in 2× SSC, 0.5% SDS followed by two additional 15-min washes in 0.1× SSC before autoradiography. For Western blots, cell lysates were prepared in a buffer as described above. Lysates were separated by 4–20% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes (14). The primary antiserum dilutions for anti-TH, anti–dopamine β-hydroxylase (DBH), and anti–phenylethanolamine *N*-methyltransferase (PNMT) (Incstar) were 1:6,000, 1:6,000, and 1:10,000, respectively. Sympathetic neurons taken from adult rat stellate ganglia were studied after 3 d in culture using a method described previously (15).

Results

Histological identification of ICA cells. Immunoreactivity for the rate-limiting catecholamine-forming enzyme TH was noted in a class of cardiac cells not associated with neurites or efferent sympathetic axons in adult and neonatal rat hearts. These cells, which we have termed ICA cells, formed clusters and were diffusely distributed throughout the heart including both atria and ventricles. ICA cells were closely associated with the coronary microvasculature (arterioles, venules, and capillaries) and were also in intimate contact with atrial and ventricular myocytes in adult rat heart (Fig. 1). TH colocalized with a common neuroendocrine/neuronal marker, NSE (16), in adult rat hearts (Fig. 1), as well as another neuroendocrine maker, protein gene product 9.5 (17) (data not shown). Importantly, ICA cells were also observed in early gestational human fetal hearts, primarily in a perivascular and intramyocardial distribution (Fig. 2). Although sympathetic axons, which were stained positively with anti-TH, were readily observed in adult and neonatal rat heart sections, such structures were not visualized in sections of human fetal hearts at 20 wk of gestation, a stage of development that precedes innervation of the heart by the sympathetic nervous system (18).

Isolated ICA cells in vitro retained their characteristic in situ adrenergic immunohistochemical profiles (Fig. 1, *e–h*). Electron microscopic examination of primary isolates of ICA cells revealed a single cell population with distinct ultrastructural features that were similar in rat and human hearts (Fig. 3). Importantly, neither cells nor cellular remnants containing dense core granules suggestive of chromaffin cells or sympathetic nerve endings were observed in ICA cell isolates (Fig. 3).

ICA cell catecholamine content and release in vitro. The content of norepinephrine and epinephrine in ICA cells was comparable with that of rat sympathetic neurons isolated from stellate ganglia after normalization for total cell protein. However, the dopamine content was lower in ICA cells than in sympathetic neurons (Table I). Adult rat ventricular myocyte primary isolates, from which ICA cells had been selectively removed, did not exhibit detectable dopamine and epinephrine, although a small amount of norepinephrine (∼ 1% of that

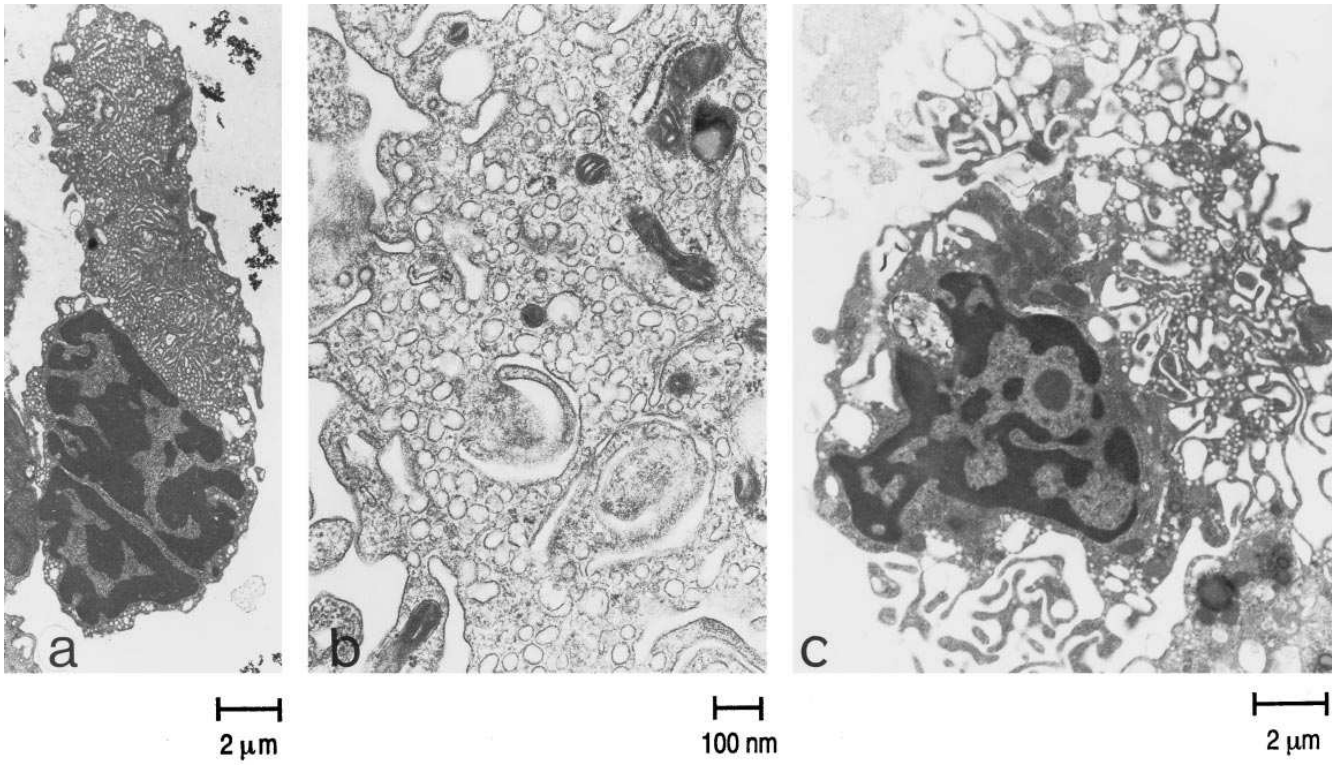

Figure 3. Transmission electron micrographs of primary isolates of adult rat (*a* and *b*) and adult human (*c*) ICA cells. *b* is a higher magnification view showing abundant clear cytoplasmic vesicles in a different rat ICA cell. The extensive vacuolation shown here (*c*) is presumably formed by extruded vesicles. The rat ICA cells in each preparation were samples from cell isolates prepared for catecholamine assay or immunocytochemical staining (Table I and Fig. 1 *f*).

present in ICA cells) could be detected, presumably due to residual contamination by ICA cells. Based on the catecholamine content of ICA cell isolates isolated from whole adult rat hearts, we estimate that catecholamines derived from ICA cells constituted at least 18, 13, and 16%, respectively (Table I), of total rat heart content of epinephrine, norepinephrine, and dopamine. Release of epinephrine, norepinephrine, and dopamine from adult ICA cell primary isolates in vitro was 0.8±0.3, 6.0±1.1, and 1.3±0.3 pmol/h/mg cell protein, respectively (Fig. 4 *A*).

Table I. Radioenzymatic Assays of Endogenous Catecholamine Content in Primary Isolates of ICA Cells from Adult Rat Hearts

	Epinephrine	Norepinephrine	Dopamine
		pg/mg protein	
Catecholamine content*			
ICA cells	446 (±56)	7,226 (±1370)	165 (±45)‡
Sympathetic neurons	544 (±136)	12,803 (±2262)	2,251 (±434)
Myocyte primary isolates depleted of ICA cells	Undetectable	91 (±61)	Undetectable
		ng/heart	
Ratio of catecholamine content of total ICA cells/heart to catecholamine content of whole adult rat heart			
Total ICA cells/heart	1.8 (±0.3)	21.4 (±7.0)	0.7 (±0.4)
Whole cardiac tissue	9.9 (±2.6)	162.8 (±44)	4.4 (±2.6)
Content ratio	18.2%	13.1%	16%

*In isolates of ICA cells, rats stellate ganglion sympathetic neurons, and cardiac myocyte primary isolates partially depleted of ICA cells from adult rat ventricular muscle. ‡$P < 0.01$ vs sympathetic neurons. $n = 4$ hearts from weight-matched animals.

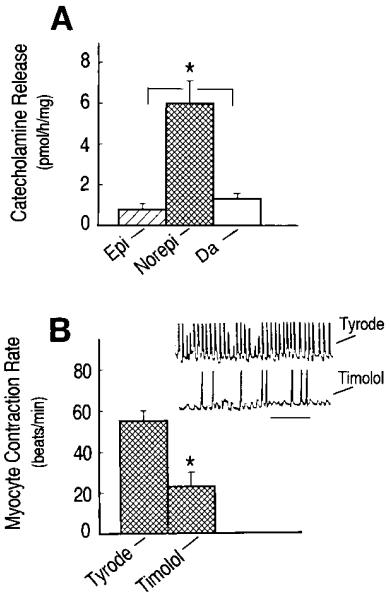

Figure 4. (*A*) In vitro determination of spontaneous release of catecholamines from primary isolates of ICA cells from adult rat hearts. $*P < 0.01$ compared with epinephrine or dopamine using ANOVA. Data were obtained from ICA cell isolates from pooled hearts in four separate preparations. (*B*) The addition of timolol to cultures of beating neonatal ventricular myocytes, from which ICA cells had not been depleted as described in Methods, resulted in a 58% reduction in the myocyte spontaneous beating rate. Scale in inset = 10 s.

To determine whether the basal beating rate of neonatal rat ventricular myocytes in culture was dependent upon catecholamine release by neonatal ICA cells in myocyte culture, the β-adrenoreceptor antagonist timolol (1 μM) was used. The spontaneous beating rate of neonatal rat ventricular myocytes was reduced by 58±8% by timolol in five culture preparations that contained both neonatal myocytes and ICA cells.

Catecholamine-synthesizing mRNA and proteins in ICA cells. Messenger RNA and protein for TH (19, 20) were detected in primary isolates of ICA cells (Fig. 5 *B*). TH protein was identified by Western blot in ICA cells isolated from adult rat hearts (Fig. 5 *C*) and an adult human heart (data not shown). In addition to TH, DBH (Fig. 5 *D*) and PNMT proteins, which catalyze the formation of norepinephrine and epinephrine, were identified as well in ICA cell primary isolates (Fig. 5 *E*).

Discussion

This study identified a previously undescribed type of cardiac cell capable of adrenergic paracrine signaling in mammalian hearts. These cells, which do not have neuronal or chromaffin cell ultrastructural morphology on electron microscopic analysis, we have termed ICA cells. Isolated ICA cells in vitro retained their characteristic in situ adrenergic immunohistochemical profiles (Fig. 1, *e–h*). ICA cells exhibited numerous clear vesicles and an extensive exocytotic profile suggestive of a secretory phenotype (Fig. 3). The presence of clear cytoplasmic vesicles distinguishes ICA cells from cardiac chromaffin cells, which contain electron-dense core granules and are largely confined to atrial parasympathetic ganglia (18, 21, 22).

ICA cells were also identified in human fetal myocardium. That no sympathetic axons were visualized in fetal hearts of 20 wk of gestation is consistent with a report of their absence determined by ultrastructural analyses of early gestational age human hearts (18). These data suggest that ICA cells are present within the developing myocardium before the initiation of sympathetic innervation. The obligatory role of catecholamines in fetal development has been underscored by the finding that in mice with targeted disruption of catecholamine-synthesizing genes, these animals succumb in utero due to heart failure, before the developmental stage at which cardiac sympathetic innervation occurs (5, 6). The identification of an adrenergic cell system outside of the sympathetic nervous system is compatible with phylogenetic studies suggesting that catecholamine-secreting cells that are not part of the sympathetic nervous system are diffusely distributed in vertebrate and mammalian species and are analogous to those of the adrenal medulla (23).

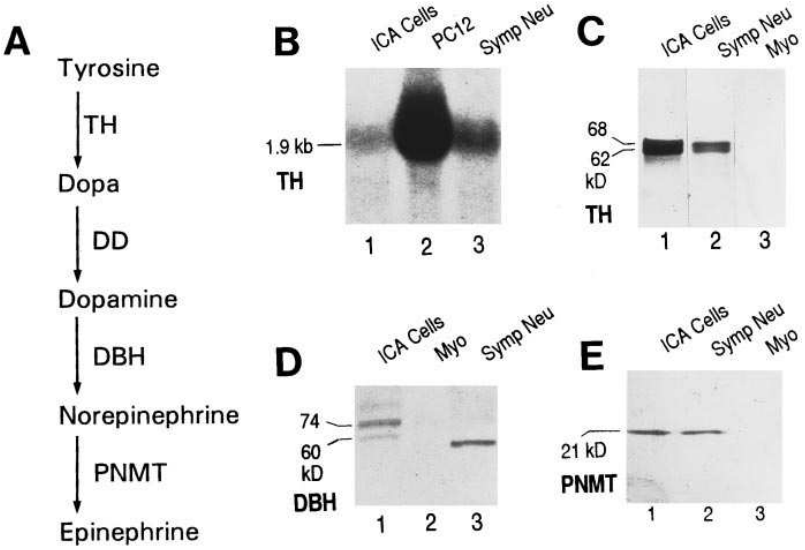

Figure 5. Catecholamine synthesizing enzymes in ICA cell lysates. (*A*) Steps in the enzymatic synthesis of catecholamines are illustrated. *DD*, dopa decarboxylase. (*B*) Northern blot analysis of TH mRNA from total RNA (20 μg RNA/lane). Lane *1*, rat ICA cells; lane *2*, rat pheochromocytoma (*PC12*) cells; lane *3*, isolated rat sympathetic neurons (*Symp Neu*). A 1.9-kb TH transcript was identified in three types of adrenergic cells. TH mRNA was barely detectable from rat ventricular myocytes depleted of ICA cells. (*C–E*) Western blot analyses of TH, DBH, and PNMT, respectively. (*C*) TH protein subunits (68 and 62 kD) were identified in ICA cells (lane *1*) and cultured sympathetic neurons (lane *2*) from rat stellate ganglia, but not in ventricular myocytes (*Myo*; lane *3*) (30, 20, and 30 mg protein loading for lanes *1*, *2*, and *3*, respectively). (*D*) Two major DBH isoforms were identified in ICA cells (lane *1*). No DBH was detected in cardiac myocytes depleted of ICA cells (lane *2*; 30 μg protein/lane). A single DBH isoform (smaller than 60 kD) was detected in cultured rat sympathetic neurons (lane *3*). (*E*) PNMT protein was detected in ICA cells and sympathetic neurons ($n = 2$ assays), but not in myocytes depleted of ICA cells (20 μg protein/lane).

Adult rat ICA cells spontaneously released catecholamines in vitro with high norepinephrine/epinephrine ratio (7.5/1) (Fig. 4 *A*). The addition of the β-adrenoreceptor antagonist timolol to primary cultures of spontaneously beating neonatal ventricular myocytes, from which ICA cells had not been systemically depleted, resulted in a 58% reduction in beating rate of myocytes (Fig. 4 *B*). This is consistent with the constitutive release of catecholamines by ICA cells and their positively chronotropic effect on spontaneously beating neonatal myocyte culture. The release ratio of norepinephrine/epinephrine (7.5/1) was not proportional to the ratio of their cellular content (16/1) (Table I), suggesting that specific packaging and secreting processes may occur for individual catecholamines. The catecholamines identified in ICA cell primary isolates could not have been due to contamination from sympathetic nerve endings because neither immunohistochemical nor electron microscopic analysis detected any sympathetic nerve fibers or axonal structures in primary ICA cell isolates.

The identification of mRNA and enzymes involved in catecholamine biosynthesis provides evidence for the existence of the cellular machinery responsible for catecholamine production in ICA cells. This, along with epinephrine synthesis and release in lysates of ICA cell isolates and in media from cultures of these cells (Table I and Fig. 4 *A*), is consistent with the observation that sympathetically denervated rat cardiac tissue retains adrenergic enzymatic activity and is capable of epinephrine production (24). Our data indicate that the mammalian heart possesses intrinsic adrenergic cells with a unique morphology and catecholamine biosynthetic profile, and that norepinephrine and epinephrine are constitutively released by ICA cells in vitro. The presence of this distinct adrenergic cell population may be essential during cardiac ontogeny and may have important implications for the regulation of postnatal cardiac function.

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