Neuroendocrine properties of intrinsic cardiac adrenergic cells in fetal rat heart

M.-H. Huang, J. J. Bahl, Y. Wu, F. Hu, D. F. Larson, W. R. Roeske, and G. A. Ewy. Neuroendocrine properties of intrinsic cardiac adrenergic (ICA) cells in developing heart constitute a novel adrenergic signaling system involved in cardiac regulation. Regulatory mechanisms of ICA cells remain to be defined. Immunohistochemical study of fetal rat hearts demonstrated ICA cells with catecholamine biosynthetic enzyme tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT). The mRNA of TH and PNMP was also detected in fetal rat hearts before sympathetic innervation. Immunoreactivity of norepinephrine transporter (NET) was localized to ICA cells in heart tissue and primary cell culture. For the functional study, the activity of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transients was quantified by a ratio fluorescent spectrometer in cultured ICA cells and myocytes. ICA cells generated spontaneous [Ca\(^{2+}\)]\(_i\) transients that were eliminated by tetrodotoxin or Ca\(^{2+}\)-free solutions and showed greatly reduced amplitude with the addition of L-type Ca\(^{2+}\) channel blocker nifedipine. \(^{[3]}\)Hnorepinephrine studies demonstrate release and uptake of norepinephrine. Functional interaction between catecholamines produced by the ICA cells and cocultured myocytes was evident by the effect of the \(\beta\)-adrenergic blocker atenolol eliciting a dose-dependent reduction in the amplitude and frequency of [Ca\(^{2+}\)]\(_i\), transients of beating myocytes. Hypoxia inhibited [Ca\(^{2+}\)]\(_i\) transients of ICA cells, which subsequently produced a reoxygenation-mediated rebound augmentation of [Ca\(^{2+}\)]\(_i\), transients. We conclude that ICA cells are capable of catecholamine synthesis, release, and uptake. They generate spontaneous [Ca\(^{2+}\)]\(_i\), transient activity that can be regulated by oxygen tension. ICA cells may provide an alternative adrenergic supply to maintain cardiac contractile and pacemaker function at rest and during stress in the absence of sympathetic innervation.

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The IMPORTANCE OF CATECHOLAMINES in augmenting heart function is well established. Catecholamines are obligatory for heart development. Animals with targeted disruption of catecholamine synthetic genes die in utero because of heart failure at an embryonic stage before sympathetic innervation (27). Although it has been proposed that the intrinsic cardiac nervous system contains adrenergic neurons involved in adult cardiac regulation (2), their role in fetal heart development is unknown. Huang et al. (14) identified, and it has been confirmed (8), that specialized nonneuronal cardiocytes, designated as intrinsic cardiac adrenergic (ICA) cells, possess catecholamine biosynthetic enzymes. ICA cells have been linked to fetal development, cardiac pacemaking and conduction, and blood pressure regulation (1, 8, 14, 23, 25). Recently, our laboratory (13) proposed that ICA cells might be responsible for the adrenergic activity in transplanted human hearts that lack sympathetic innervation. In the present study, we hypothesized that ICA cells, like neuroendocrine cells, would generate regulated intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transients necessary for neurotransmitter release (21). The objectives of this study included 1) molecular and immunohistochemical identification of ICA cells expressing catecholamine biosynthetic enzymes in fetal rat hearts; 2) demonstration of spontaneous [Ca\(^{2+}\)]\(_i\) transients generated by ICA cells in cell culture and the determination of Ca\(^{2+}\) channels involved in [Ca\(^{2+}\)]\(_i\) transient genesis; 3) examination of whether [Ca\(^{2+}\)]\(_i\) transients of ICA cells are regulated by hypoxia/reoxygenation, an important biological stressor associated with adrenergic stimulation; 4) demonstration of norepinephrine transporters (NET) in ICA cells that constitutively take up and release norepinephrine; and 5) determination of whether catecholamines derived from ICA cells are essential for maintaining optimal pacemaking and myocyte contractile function. This study advances our understanding of cardiac neurohormonal regulation in normal and diseased states.

MATERIALS AND METHODS

Adrenergic gene expression in the fetal heart. The mRNA from fetal rat hearts at embryonic day 16 (E16) and from maternal adrenal glands was extracted following the TRIzol reagent manufacturer’s instructions. The cDNA was reverse transcribed with primers AACTTCCACGGTTACTGTTT (sense) and GCATTGCCCT-GAGCTTCTCCT (antisense) for tyrosine hydroxylase (TH) and ACTGGGATGTTGACAGC (sense) and ACACGGAGAC-CACAGATAAGCCT (antisense) for phenylethanolamine N-methyltransferase (PNMT) (Integrated DNA Technologies).

Immunohistochemical studies. To identify ICA cells in myocardial tissue, immunohistochemical labeling was performed on 3-μm paraaffin sections of 4% formaldehyde-fixed fetal hearts (E16). The immunohistochemical methods for identifying ICA cells were described previously (14). The primary antibodies against TH and PNMT do not cross-react with other enzymes involved in the catecholamine biosynthetic cascade (14). The cell membranes were permeabilized with 0.1% Nonidet P-40 for 15 min. Mouse anti-TH antibody (1:500) (ImmunoStar, Hudson, WI) was used for labeling ICA cells. The secondary antibody was goat anti-mouse IgG conjugated to Texas red.
(Calbiochem, La Jolla, CA) and diluted 1:100. Antibody against PNMT (rabbit polyclonal, 1:1,000; ImmunoStar) was also used to identify ICA cells. The secondary antibody was donkey anti-rabbit IgG conjugated to fluorescein (Pierce, Rockford, IL) and diluted 1:100. For nuclear staining, sections were incubated with 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml, Sigma) for 5 min and then rinsed twice. For identification of ICA cells in culture, the cell coverslips were fixed for 15 min in 3% paraformaldehyde. After two rinses, the cells were made permeable with 0.2% Triton X-100 followed by rinses. The coverslips were incubated for 1 h with the primary antibodies, followed by incubation with the secondary antibodies for 1 h. For double staining of TH and NET in cultured ICA cells, the coverslips were incubated with a rabbit anti-NET antibody (1:1,000, Chemicon) for 1 h, followed by incubation with a secondary antibody (donkey anti-rabbit IgG conjugated to fluorescein and diluted 1:100) for 1 h. The double staining was completed by incubating slides with a mouse anti-TH antibody (1:500) overnight at 4°C, followed by incubation with a secondary antibody (goat anti-mouse IgG conjugated to Texas red and diluted 1:100) for 1 h at room temperature. The specificity of anti-TH, -PNMT, and -NET antibodies was tested by omission of the primary antibodies or by substituting rabbit anti-PNMT and -NET antibodies with normal rabbit serum diluted at 1:1,000.

Preparation of cardiac cell culture. Myocyte-ICA cell cultures (E16) were prepared as described previously (10). The dissociated cardiocytes were preplated in medium containing bovine serum albumin. This allows fibroblasts and endothelial cells to attach to the plate before the cell suspension for subsequent culture is poured off to remove fibroblasts and endothelial cells, enriching the primary population of myocytes and ICA cells in subsequent culture.

[^H]norepinephrine uptake and release assay. [^H]norepinephrine uptake and release assays (22) were performed in ICA cell-myocyte cocultures on a 24-well plate with a cell density of 2.5 ×10^5/well after 24 h in culture. The cells were incubated with 50 nmol/l [^H]norepinephrine (50 Ci/mmol, Amersham International) supplemented with ascorbic acid at 0.2 mmol/l in Tyrode solution for 2 h. Excess [^H]norepinephrine was removed by six washes. Cellular [^H]norepinephrine was extracted with perchloric acid (0.4 mol/l). The amount of [^H]norepinephrine uptake was expressed as picograms per milligram of protein per 2 h. To determine whether the [^H]norepinephrine uptake can be inhibited by norepinephrine and NET inhibitor, the uptake studies were performed in the presence of norepinephrine (1 μM) and the NET inhibitor nisoxetine (1 μM, Sigma) for 2 h, respectively. [^H]norepinephrine release was determined at 15, 30, and 60 min after 2 h of its uptake. This was done by quantifying the radioactivity released into a 0.5-ml Tyrode bathing solution of ICA cell-myocytes at 15, 30, and 60 min in three separate experiments (6 replicates for each). After collection of the bathing solution, the unreleased cytosolic [^H]norepinephrine was extracted accordingly. [^H]norepinephrine uptake was defined as the sum of released and unreleased radioactivity. The magnitude of [^H]norepinephrine release was expressed as percent release from its total uptake.

Recording of ICA cell [Ca^{2+}]i, transients. Coverslips of ICA cell-myocyte cocultures were incubated with 1 mM fura-2 in Tyrode solution for 30 min. The coverslip was mounted on a temperature-controlled (37°C) closed chamber (0.8-cm² area, 0.5-mm depth, FCS2; Bioptechs, Butler, PA) on the stage of an inverted microscope equipped with a ratio fluorescent spectrometer (PTI, South Brunswick, NJ). Cell imaging used a ×100 oil-immersion lens. The ICA cell-myocyte coculture was perfused with oxygen-saturated Tyrode solution at a flow rate of 200 μl/min. ICA cells were characteristically small (<10 μm), round, and nonbeating (Fig. 1) in contrast to the enormously large, irregular, flat, and rhythmically beating myocytes cocultured in the same coverslip (Fig. 2). The putative ICA cells were defined as small, round cells (5–10 μm in diameter) expressing the immunoreactivity of TH and PNMT. It was estimated that ~85% of small (<10 μm) and round cells in fetal cardiocyte culture expressed TH and PNMT immunoreactivity. Thus, in at least 85% of instances, the spontaneous [Ca^{2+}]i transients recorded must be generated from putative ICA cells. In contrast to large-sized (~30 μm) ICA neurons that outgrow axons and dendrites in situ (20, 26) or neurites in culture (12), ICA cells lack neurite outgrowth. No ICA neurons were identified in fetal (E16) heart sections or cardiocyte cultures. Therefore, potential contamination of cardiac adrenergic neurons is unlikely to occur in fetal cardiocyte cultures. Myocytes that stained positive for sarcomeric myosin heavy chain (Fig. 2) demonstrated no TH or PNMT immunoreactivity. To study the [Ca^{2+}]i transients of the putative ICA cell, single, isolated, small ICA cells were selected. Caution was exercised to only study those ICA cells not in contact with underlying or adjacent beating myocytes so that the recorded [Ca^{2+}]i transients were exclusively from ICA cells. The fura-2-loaded ICA cells emit intense fluorescent light after being excited by UV light. The ICA cell was excited at 340/380 nm alternately. The 510-nm emission from the ICA cell was recorded as a fluorescence ratio (340/380 nm) representing cytosolic Ca^{2+} changes. To further ensure that the [Ca^{2+}]i transients recorded from small putative ICA cells were not from contaminated myocytes, the [Ca^{2+}]i transients of ICA cells were studied in those isolated from myocytes using a magnetic bead purification method (14).

Ionic properties and regulatory mechanisms of ICA cells. To determine whether the genesis of spontaneous [Ca^{2+}]i transients was triggered by Na+ influx, a selective voltage-sensitive Na+ channel blocker, tetrodotoxin (10 μM), was applied to ICA cells. To test whether the [Ca^{2+}]i transients generated by ICA cells were dependent on Ca^{2+} influx, Ca^{2+}-free solution was applied to ICA cells. The role of L-type Ca^{2+} channel and β-adrenergic receptors in the generation of [Ca^{2+}]i transients of ICA cells was determined by exposing ICA cells to nifedipine (1 and 10 μM) or atenolol (1 μM). The role of N- and P-type Ca^{2+} channels in the genesis of [Ca^{2+}]i transients of ICA cells was determined by administration of respective blockers ω-conotoxin (30 μM) and α-agatoxin IVA (30 μM) to the cells. To test whether [Ca^{2+}]i transients of ICA cells could be regulated by hypoxia/reoxygenation, ICA cells were exposed to hypoxia for 3–10 min, followed by reoxygenation. Hypoxia was produced by bubbling perfluorocarbons with N2-O2 gas (95.5:vol/vol; O2 tension ~44 mmHg).

Effects of ICA cells on myocyte [Ca^{2+}]i, transients. Myocytes characteristically flattened and generated rhythmic beating after 24 h in culture. To record myocyte [Ca^{2+}]i transients, a small cytoplasmic patch was selected without ICA cell contamination (Fig. 2). To assess the functional effect of endogenous catecholamines derived from ICA cells on the contractile function of adjacent myocytes, the beat-to-beat [Ca^{2+}]i transients generated by myocytes cocultured with ICA cells were studied. A cumulative atenolol dose-response experiment was performed with the concentration increased every 5 min (1, 10, 100, and 1,000 nM). Atenolol, a specific β1-blocker (11), has no direct effect on the contractile function of ventricular myocytes (15).

Data analysis. The frequency of spontaneous [Ca^{2+}]i transients, transients of ICA cells and beat-to-beat frequency and amplitude of myocyte [Ca^{2+}]i, transients were analyzed. Data are expressed as means ± SE. Paired Student’s t-test and ANOVA were used for statistical analyses. A P value <0.05 was considered significant.

RESULTS

Adrenergic gene expression in fetal heart. The expression of mRNA of TH and PNMT was detected in the fetal heart at E16 when no sympathetic innervation was detected (see below). The PCR products of fetal heart mRNA for TH and PNMT matched the maternal adrenal gland products included as a positive control (Fig. 1).

Histological identification of ICA cells. Immunoreactivity of TH and PNMT identified ICA cells in heart tissue sections...
ICA cells were diffusely distributed throughout fetal rat heart, with the greatest density in the left atrium. The small ICA cells often formed clusters. No TH-positive nerve endings were identified in fetal rat hearts. In ICA cell-myocyte cocultures, ICA cells displayed colocalizing immunoreactivity of TH and NET (see Fig. 5), but myocytes contained no such immunoreactivity. No cellular immunoreactivity was detected in cardiac tissue sections or cell cultures when the primary antibodies were omitted or substituted with normal serum of the same species as primary antibodies (data not shown). No TH- or PNMT-positive neurons possessing neurites were identified in heart tissue sections or cardiocyte cultures. This may be due to the lack of development of cardiac adrenergic neurons in the early embryonic stage.

**[Ca^{2+}]_{i}** transients in ICA cells. ICA cells in ICA cell-myocyte coculture preparations generated spontaneous [Ca^{2+}]_{i} transients with markedly irregular rhythm. The spike frequency of [Ca^{2+}]_{i} transients recorded from a total of 42 cells varied, with a mean rate of 5 ± 4 spikes/min. The morphology of [Ca^{2+}]_{i} transients was characterized by a rapid upstroke with
Nifedipine markedly decreased the amplitude of the \([\text{Ca}^{2+}]_{\text{o}}\) transient of ICA cells. 340/380, Excitation at 340/380 nm. \([\text{Ca}^{2+}]_{\text{i}}\) transients after being perfused with \(\text{Ca}^{2+}\)-free solution (5 ± 2 to 0 spikes/min, \(n = 5\)) or tetrodotoxin at 10 \(\mu\text{M}\) (11 ± 7 to 0 spikes/min, \(n = 6\)). Nifedipine (1 and 10 \(\mu\text{M}\)) reduced the amplitude of \([\text{Ca}^{2+}]_{\text{i}}\) transients of ICA cells by 54 ± 8 and 82 ± 3% (\(P < 0.01\), \(n = 8\)), respectively (Fig. 3). Atenolol at 1,000 nM did not significantly increase the frequency of \([\text{Ca}^{2+}]_{\text{i}}\); transients of ICA cells [6 ± 3 vs. 11 ± 5 spikes/min, \(n = 5\), \(P = \text{not significant (NS)}\)]. \(\omega\)-Conotoxin and \(\omega\)-agatoxin IVA (both 30 \(\mu\text{M}\)) did not affect the \([\text{Ca}^{2+}]_{\text{i}}\) transients of ICA cells (data not shown). Acute hypoxia markedly inhibited \([\text{Ca}^{2+}]_{\text{i}}\) transients with a rapid onset. Reoxygenation immediately exerted a potent stimulatory effect on ICA cells with a maximum sixfold increase in \([\text{Ca}^{2+}]_{\text{i}}\) transient frequency. The stimulatory effect was rapid and sustained (up to 20 min) (Fig. 4). Burst activity producing temporal summation of \([\text{Ca}^{2+}]_{\text{i}}\) transients represents a distinct ICA cell response to reoxygenation (Fig. 4). ICA cells isolated from myocytes using the magnetic bead purification method (12) displayed the same immunohistological characteristics and generated a similar pattern of spontaneous \([\text{Ca}^{2+}]_{\text{i}}\) transients as those ICA cells cocultured with myocytes, as described above (data not shown).

\(^{[3]}\text{H}\)norepinephrine uptake and release. \(^{[3]}\text{H}\)norepinephrine uptake reached 73 ± 3 pg·mg protein\(^{-1}\)·h\(^{-1}\) in ICA cell-myocyte co-cultures. \(^{[3]}\text{H}\)norepinephrine uptake was reduced by 20 ± 5% (\(P < 0.05\)) and 36 ± 4% (\(P < 0.01\)) in the presence of added norepinephrine (1 \(\mu\text{M}\)) and nisoxetine (1 \(\mu\text{M}\)), respectively (Fig. 5). ICA cells spontaneously release \(^{[3]}\text{H}\)norepinephrine after the uptake, reaching 19 ± 2, 28 ± 2, 23 ± 1, and 20 ± 1 spikes/min, respectively (Fig. 6). The stimulated increase in frequency of \([\text{Ca}^{2+}]_{\text{i}}\) transients generated by two cells that subsequently give a rebound increase (2 ± 1 to 13 ± 4 spikes/min, **\(P < 0.001\), \(n = 10\) in \([\text{Ca}^{2+}]_{\text{i}}\)) transient spikes after exposure of ICA cells to hypoxia and reoxygenation, respectively. High-speed illustration of bursting \([\text{Ca}^{2+}]_{\text{i}}\) transients indicated in A (arrow) is shown in D.

Fig. 2. Myocytes in culture display immunoreactivity of myosin heavy-chain striations representing cytoplasmic myofilaments (A). Myocytes are characteristically flattened out after 24 h in culture. A patch of myocyte cytoplasm was microscopically selected (B) with the assistance of vertical and horizontal shutters. Caution was exercised to avoid the contamination of ICA cells in the field. This selected myocyte cytoplasm was used for intracellular \([\text{Ca}^{2+}]_{\text{i}}\) transient study. Calibration bar = 20 \(\mu\text{M}\).

Fig. 3. Effects of extracellular \(\text{Ca}^{2+}\) depletion (A), tetrodotoxin (TTX; B), and nifedipine (C) on \([\text{Ca}^{2+}]_{\text{i}}\) transients of ICA cells. ICA cells failed to generate \([\text{Ca}^{2+}]_{\text{i}}\) transients after being perfused with \(\text{Ca}^{2+}\)-free Tyrode solution or TTX. Nifedipine markedly decreased the amplitude of the \([\text{Ca}^{2+}]_{\text{i}}\) transients of an ICA cell. 340/380, Excitation at 340/380 nm.

Fig. 4. Hypoxia/reoxygenation regulates \([\text{Ca}^{2+}]_{\text{i}}\); transient activity of ICA cells. Exposure of ICA cells to hypoxia for 3 min (A) and 10 min (B) inhibits \([\text{Ca}^{2+}]_{\text{i}}\); transients generated by two cells that subsequently give a rebound increase in activity after reoxygenation (Re-O₂). Cytosolic \(\text{Ca}^{2+}\) levels are elevated through a process of temporal summation of rapid \([\text{Ca}^{2+}]_{\text{i}}\); transient spiking. **\(P < 0.001\) and \(0.001 < \text{other}< 0.01\); mean reduction (2 ± 1 to 0 spikes/min, **\(P < 0.001\), \(n = 10\)) in \([\text{Ca}^{2+}]_{\text{i}}\); transient spikes after exposure of ICA cells to hypoxia and reoxygenation, respectively. High-speed illustration of bursting \([\text{Ca}^{2+}]_{\text{i}}\) transients indicated in A (arrow) is shown in D.
and 52 ± 4% of the total uptake at 15, 30, and 60 min, respectively (Fig. 5).

Effect of β₁-adrenergic blockade on myocyte [Ca²⁺]i transients. At baseline, myocytes cocultured with ICA cells generated rhythmic beat-to-beat [Ca²⁺]i transients (60 ± 8 spikes/min) (Fig. 6). These fast and rhythmic [Ca²⁺]i transients generated by myocytes are distinctly different from those of ICA cells, with a [Ca²⁺]i spike frequency of only 5 ± 4 spikes/min in the same culture. The influence of catecholamines derived from ICA cells on [Ca²⁺]i transients of myocytes was assessed by the administration of atenolol to ICA cell-myocyte cocultures. Atenolol at 1, 10, 100, and 1,000 nM reduced the amplitude of myocyte [Ca²⁺]i transients by 19 ± 9, 49 ± 8, and 75 ± 10%, with a concurrent decrease in beating rate by 16 ± 7, 26 ± 8, 57 ± 11, and 62 ± 13%, respectively (Fig. 6).

DISCUSSION

We have demonstrated that ICA cells are capable of catecholamine synthesis, release, and uptake. They generate spontaneous [Ca²⁺]i transients that can be regulated by oxygen tension. ICA cells may provide an alternative adrenergic neuroendocrine supply to maintain contractile and pacemaker function at rest and during stress in the absence of cardiac sympathetic innervation.
Importance of ICA cells in maintaining cardiac adrenergic supply. Adrenergic gene expression is obligatory for fetal survival before cardiac sympathetic innervation (27). The detection of mRNA of TH and PNMT in the heart and localization of their respective enzyme proteins in ICA cells in the absence of TH-positive nerve endings (Fig. 1) demonstrate that ICA cells possess a well-developed catecholamine synthetic system in the heart before sympathetic innervation. These findings support the contention that ICA cells provide an obligatory adrenergic supply to maintain cardiac function in early fetal development. Furthermore, the presence of ICA cells in adult rat and human hearts (14) supports a concept that mammalian hearts possess an ICA system throughout adult life. The magnitude of adrenergic influence exerted by ICA cells on cocultured myocytes was revealed by the β-adrenergic blocking effect with atenolol (Fig. 6). Human and rodent myocardial β-adrenergic receptors are fully expressed at the embryonic stage before sympathetic innervation (6, 7). The findings of functional myocardial β-receptors and norepinephrine release from ICA cells and its regulatory effect on [Ca\(^{2+}\)]\(_i\) transients of fetal myocytes provide compelling evidence of a highly effective ICA signaling pathway that is critically important in early development.

ICA cells and Ca\(^{2+}\) influx. Ca\(^{2+}\) influx is the fundamental mechanism required for neurotransmitter release from neuromodulatory cells (21). The Ca\(^{2+}\) influx-mediated [Ca\(^{2+}\)]\(_i\) transients generated by ICA cells provide a physiological basis for catecholamine release. It appears that the activation of membrane voltage-sensitive Na\(^+\) channels is necessary for generating ICA cell [Ca\(^{2+}\)]\(_i\) transients, since they fail to generate [Ca\(^{2+}\)]\(_i\) transients in the presence of tetrodotoxin (Fig. 3), which specifically blocks voltage-sensitive Na\(^+\) channels and action potentials in excitable cells (5). The L-type calcium channels appear to be the primary mechanism responsible for the formation of [Ca\(^{2+}\)]\(_i\) transients, since the L-type calcium channel blocker nifedipine effectively reduced the amplitude of [Ca\(^{2+}\)]\(_i\) transients (Fig. 3). The unique pattern of [Ca\(^{2+}\)]\(_i\) transients generated by putative ICA cells rules out the possibility that such [Ca\(^{2+}\)]\(_i\) transients were recorded from contaminated myocytes in the cocultures. This argument is supported by the following evidence: 1) basal frequency of [Ca\(^{2+}\)]\(_i\) transients of ICA cells is 10 times slower than that of myocytes; 2) the rhythm of [Ca\(^{2+}\)]\(_i\) transients of ICA cells is rather irregular, in contrast to the regular beating rhythm generated by myocytes; 3) ICA cells but not myocytes display unique bursting activity (temporal summation) of [Ca\(^{2+}\)]\(_i\) transients; 4) the [Ca\(^{2+}\)]\(_i\) transients of ICA cells are not significantly affected (with a trend of increase) in the presence of the β-adrenergic blocker that greatly reduced the beating frequency of myocytes; and 5) ICA cells isolated from myocytes displayed characteristics similar to those of ICA cells recorded from ICA cell-myocyte cocultures.

Effects of acute hypoxia and reoxygenation. Acute hypoxia induces bradycardia. This oxygen-conserving mechanism is exemplified by the diving response in which the heart rate rapidly decelerates in water (9). The hypoxic bradycardia has been attributed to reflex-mediated parasympathetic activation (4). However, recent studies indicate that bradycardia during hypoxia is independent of reflex-mediated parasympathetic activation, since the bradycardia is not attenuated in rats and mice that have received vagotomy and/or atropine infusion (16, 24). Acute hypoxia markedly inhibited [Ca\(^{2+}\)]\(_i\) transients of ICA cells (Fig. 4). The mechanism underlying this inhibition is beyond the scope of this study. Nonetheless, this finding indicates that hypoxia-mediated ICA cell inactivation may have an important role in hypoxic bradycardia, presumably as a result of diminished catecholamine release. This contention is further supported by the histological evidence that ICA cells are closely associated with cardiac pacemaking and conduction tissue (8). Inhibition of ICA cells may act in coordination with autonomic reflex mechanisms to reduce myocardial oxygen consumption during acute hypoxia through the reduction of cardiac catecholamine release. The inhibitory response of ICA cells to hypoxia distinguishes them from adrenal chromaffin cells, which exhibit an excitatory response to hypoxia resulting in enhanced catecholamine release (17, 19). Such different responses to hypoxia may represent tissue-specific differences between sympathoadrenal and ICA cells.

Reoxygenation after hypoxia is a crucial process when the heart must quickly maximize cardiac output to restore adequate oxygen tension for the organs and peripheral tissues. Reoxygenation, compared with hypoxia, is a much more potent stimulant of myocardial catecholamine surge (18). Myocardial interstitial norepinephrine and epinephrine levels increase by severalfold during ventricular fibrillation-induced hypoxia but peak to >160 times baseline levels at the early reperfusion phase in anesthetized pigs (18). The origin of this powerful catecholamine surge induced by reperfusion cannot be fully explained by sympathetic activation in anesthetized pigs, since electrical stimulation of cardiac sympathetic trunks only induces a severalfold increase in catecholamine levels, a magnitude similar to that induced by hypoxia (18). Reoxygenation rapidly elicited a sixfold increase in the [Ca\(^{2+}\)]\(_i\) transient frequency of ICA cells. The burst activity of [Ca\(^{2+}\)]\(_i\) transients is a unique feature of ICA cells in response to reoxygenation. It occurs when [Ca\(^{2+}\)]\(_i\) transient spikes arrive in quick succession, so that each adds to the preceding one, producing sustained Ca\(^{2+}\) influx (Fig. 4). This responsive pattern may represent a highly effective mechanism for Ca\(^{2+}\) influx-dependent ICA cell activation during reoxygenation.

Norepinephrine uptake by ICA cells. ICA cells express immunoreactivity for the NET (Fig. 5). NET-mediated norepinephrine uptake was demonstrated by exogenous norepinephrine that competitively inhibited \(^{3}H\)norepinephrine uptake by 20%. A NET inhibitor, nisoxetine, reduced \(^{3}H\)norepinephrine uptake by 36% (Fig. 5). The fact that nisoxetine only partially blocked \(^{3}H\)norepinephrine uptake by ICA cells suggests that NET expressed by ICA cells may not have the same structural and/or functional properties as that located in sympathetic nerve endings. Believing that cardiac norepinephrine uptake occurs only at the sympathetic nerve endings, Bengel et al. (3) attributed norepinephrine uptake demonstrated by positron emission tomography using a radioactive norepinephrine analog in transplanted human hearts to sympathetic reinnervation. The demonstration of norepinephrine uptake and release in ICA cells provides an alternative catecholamine uptake and release mechanism in transplanted human hearts (13). This study has identified a novel adrenergic neuroendocrine system that possesses an active norepinephrine uptake mechanism in the heart independent of the sympathetic innervation.

Limitations of the study. The tetrodotoxin-sensitive spontaneous [Ca\(^{2+}\)]\(_i\) transients generated by ICA cells suggest mem-
brane excitability mediated by Na\(^+\) channel-dependent action potentials. However, the measurement of membrane action potentials was not attempted. Although \(^{3}H\)norepinephrine assays may provide qualitative information of catecholamine uptake and release, they cannot quantify the endogenous catecholamine release. Because of this limitation, norepinephrine release from ICA cells in response to hypoxia/reoxygenation was not determined in the current study. It would be necessary to perform HPLC for full determination of catecholamine content and identity in future studies.

We conclude that ICA cells are constitutively active and regulated neuroendocrine cells capable of catecholamine biosynthesis, release, and reuptake. ICA cells generate spontaneous \([\text{Ca}^{2+}]\) transients through \(\text{Ca}^{2+}\) influx, a mechanism necessarily required for a neuroendocrine system. The genesis of \([\text{Ca}^{2+}]\) transients by ICA cells depends on the specific membrane Na\(^+\) and \(\text{Ca}^{2+}\) channel activity and is tightly regulated by the change in oxygen tension. ICA cells may exert an important adrenergic influence to maintain optimal contractile and pacemaking function of the mammalian heart during early development when the sympathetic innervation is not fully established.

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