Neuropeptide Y Is an Essential In Vivo Developmental Regulator of Cardiac $I_{\text{Ca,L}}$

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Abstract—Cell culture studies demonstrate an increase in cardiac L-type Ca$^{2+}$ current ($I_{\text{Ca,L}}$) density on sympathetic innervation in vitro and suggest the effect depends on neurally released neuropeptide Y (NPY). To determine if a similar mechanism contributes to the postnatal increase in $I_{\text{Ca,L}}$ in vivo, we prepared isolated ventricular myocytes from neonatal and adult mice with targeted deletion of the NPY gene (Npy$^{-/-}$) and matched controls (Npy$^{+/+}$). Whole-cell voltage clamp demonstrates $I_{\text{Ca,L}}$ density increases postnatally in Npy$^{+/+}$ (by 56%), but is unchanged in Npy$^{-/-}$. Both $I_{\text{Ca,L}}$ density and action potential duration are significantly greater in adult Npy$^{+/+}$ than Npy$^{-/-}$ myocytes, whereas $I_{\text{Ca,L}}$ density is equivalent in neonatal Npy$^{+/+}$ and Npy$^{-/-}$ myocytes. These data indicate NPY does not influence $I_{\text{Ca,L}}$, prenatally, but the postnatal increase in $I_{\text{Ca,L}}$ density is entirely NPY-dependent. In contrast, there is a similar postnatal negative voltage shift in the I-V relation in Npy$^{+/+}$ and Npy$^{-/-}$, indicating NPY does not influence the developmental change in $I_{\text{Ca,L}}$ voltage-dependence. Immunoblot analyses and measurements of maximally activated $I_{\text{Ca,L}}$ (in presence of forskolin or BayK 8644) show that the differences in current density between Npy$^{+/+}$ and Npy$^{-/-}$ cannot be attributed to altered Ca$^{2+}$ channel $\alpha_{1C}$ subunit protein expression. Rather, these results suggest that the in vivo NPY-dependent postnatal increase in $I_{\text{Ca,L}}$ density in cardiac myocytes results from regulation $I_{\text{Ca,L}}$ properties by NPY. (Circ Res. 2003;93:972-979.)

Key Words: neuropeptide Y • development • Ca$^{2+}$ channel • innervation • ventricle

The mammalian cardiac L-type Ca$^{2+}$ current ($I_{\text{Ca,L}}$) increases in density during postnatal development. Cell culture studies suggest sympathetic innervation may contribute, because in vitro innervation of neonatal rat ventricular myocytes by sympathetic neurons increases $I_{\text{Ca,L}}$ density. However, the nature of the in vitro neural signal has been debated, and the extent to which neural signaling contributes to normal maturation of $I_{\text{Ca,L}}$ during development in vivo is unknown.

Some data suggest the neural signal regulating $I_{\text{Ca,L}}$ maturation is the neurotransmitter norepinephrine (NE). Our own studies suggest the relevant neural signal is neuropeptide Y (NPY), a 36-amino acid peptide present in and released from sympathetic nerve terminals. NPY fully mimics the effect of innervation in cell culture, and sustained exposure of innervated neonatal myocytes to an NPY antagonist fully prevents the effect of innervation in vitro. However, evidence is lacking for the relevance of NPY to normal postnatal maturation of $I_{\text{Ca,L}}$. Therefore, we have taken advantage of a transgenic mouse containing a targeted deletion of the NPY gene to determine the effect of NPY deficiency during normal development on $I_{\text{Ca,L}}$ in ventricular myocytes. The results demonstrate the essential role of NPY as an in vivo developmental regulator of $I_{\text{Ca,L}}$ in the postnatal mammalian heart and provide insights into the mechanism of $I_{\text{Ca,L}}$ modulation by NPY during maturation.

Materials and Methods

Animals were maintained and experiments conducted in accordance with the US NIH Guide for the Care and Use of Laboratory Animals (publication No. 85-23). NPY-deficient (Npy$^{-/-}$) mice in a 129SvEv background were bred at Columbia University and strain-matched wild-type (Npy$^{+/+}$) mice were obtained from Taconic Laboratories (Germantown, NY).

Isolation of Cardiac Myocytes

Single ventricular cells were isolated from adult (8- to 10-week-old male mice) and newborn (3- to 4-day-old mice of either sex) hearts using a Langendorff perfusion of enzyme containing solution. A mixture of collagenase and trypsin (adult) or collagenase and protease (newborn) was used in the initial perfusion (see expanded Materials and Methods section in the online data supplement available at http://www.circresaha.org). Isolated cells were maintained in enzyme-free solution at 4°C and used within 8 hours of isolation. Single cells were prepared from 44 adult (21 Npy$^{+/+}$, 23 Npy$^{-/-}$) and 6 neonatal (3 of each type) animals.

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Electrophysiology

Only clearly striated rod-shaped adult and spindle-shaped newborn cells were studied. Cells were superfused with 35°C Tyrode solution containing (in mmol/L) 140 NaCl, 5.4 KCl, 1.0 CaCl2, 1.0 MgCl2, 5.0 HEPES, and 10 dextrose (pH 7.4). Whole-cell patch clamp recordings used 2 to 3 MΩ borosilicate glass pipettes (Sutter Instrument), pCLAMP 7 software, DigiData 1200 interface, and Axopatch 1D or 200B amplifier (Axon Instruments). To record action potentials, pipettes contained (in mmol/L) 130 aspartic acid, 146 KOH, 10 NaCl, 2.0 CaCl2, 1.0 MgCl2, 5.0 EGTA, 10 HEPES, and 2.0 MgATP (pH 7.2). For I\textsubscript{Na\textsc{L}} recordings, pipettes contained (in mmol/L) 80 aspartic acid, 10 NaCl, 70 CsOH, 40 CsCl, 2.0 MgCl2, 10 EGTA, 10 HEPES, 2.0 ATP Na\textsubscript{2}, and 0.1 GTP Na\textsubscript{2} (pH 7.2).

I\textsubscript{Na\textsc{L}} was recorded in Cs-containing bath solution: (in mmol/L) 135 NaCl, 10 CsCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 HEPES, 10 dextrose, and 0.01 tetrodotoxin (pH 7.4). In control experiments, I\textsubscript{Na\textsc{L}} was recorded as a single voltage step every 4 seconds to assess rundown and eliminate possible changes in density and inactivation kinetics from facilitation (see Anderson\textsuperscript{4}). No significant difference in rundown rate was seen between adult Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} myocytes: after 4 minutes, peak amplitude was (as percent initial) 91 ± 2.8 versus 90 ± 2.8, respectively. In I-V experiments, current stability was usually checked for 2 minutes before beginning the protocol. In cells from newborn mice, rundown was faster (peak amplitude 95 ± 10% after 2 minutes in Npy\textsuperscript{−/−}, n = 3), and the I-V protocol completed within 1 minute of membrane rupture.

I-V curves were generated as nifedipine-sensitive (10 μmol/L) difference currents using 200-ms steps from −30 to +60 mV (holding potential −40 mV), and activation relations calculated as previously described.\textsuperscript{6} To insure that larger current amplitudes in adult myocytes did not cause loss of voltage control and error in the activation relation, we performed regression analysis of current protocols as described by Dun et al.,\textsuperscript{7} except the test voltage duration (in mmol/L) 80 aspartic acid, 10 NaCl, 70 CsOH, 40 CsCl, 2.0 MgCl\textsubscript{2}, 10 EGTA, 10 HEPES, 2.0 ATP Na\textsubscript{2}, and 0.1 GTP Na\textsubscript{2} (pH 7.2).

The two curves differ significantly by ANOVA. Action potentials were recorded from 17 adult Npy\textsuperscript{+/+} myocytes and 15 adult Npy\textsuperscript{−/−} myocytes in current-clamp mode, with cells stimulated by 3- to 5-ms pulses at a frequency of 1 Hz.

Because I\textsubscript{Na\textsc{L}} is a major repolarizing current in mouse ventricle, we asked if the reduced APD in Npy\textsuperscript{−/−} myocytes resulted from enhanced I\textsubscript{Na\textsc{L}}. Peak current at +60 mV was

Results

Basic Characterization of Npy\textsuperscript{−/−} and Npy\textsuperscript{+/+} Myocytes

As previously reported,\textsuperscript{4} Npy\textsuperscript{−/−} mice were of normal body weight, with no significant difference in weight of adult ventricles (107.6 ± 5.9 mg in Npy\textsuperscript{+/+} versus 95.7 ± 2.8 mg in Npy\textsuperscript{−/−}). Myocyte size did not differ for either adult [capacitance, 126 ± 3.9 pF (n = 74) for Npy\textsuperscript{+/+}; 136 ± 3.8 pF (n = 68) for Npy\textsuperscript{−/−}] or newborn [23.7 ± 4.1 pF (n = 8) and 24.7 ± 2.2 pF (n = 6), respectively] animals.

Action potential characteristics were studied in adult myocytes (Figure 1). Neither action potential amplitude nor resting potential differed significantly between Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} myocytes (amplitude, 125.9 ± 2.8 versus 123.4 ± 2.8 mV; resting potential, −67.2 ± 1.1 versus −64.9 ± 0.9 mV) (P > 0.05; n = 17 and 15, respectively). Action potential duration (APD) was measured at times ranging from 30% to 90% repolarization. ANOVA indicated APD was significantly greater in Npy\textsuperscript{+/+} than Npy\textsuperscript{−/−} myocytes.

Because I\textsubscript{Na\textsc{L}} is a major repolarizing current in mouse ventricle, we asked if the reduced APD in Npy\textsuperscript{−/−} myocytes resulted from enhanced I\textsubscript{Na\textsc{L}}. However, I\textsubscript{Na\textsc{L}} magnitude was not significantly greater in Npy\textsuperscript{−/−}. Peak current at +60 mV was

Electrophoresis and Immunoblotting

Light sarcolemmal (LS) and heavy membrane (HM) fractions were isolated from ventricles of Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} hearts according to Anborg et al.\textsuperscript{8} with modification. Proteins were resolved by SDS-PAGE, and immunoblot analysis was performed with antibodies to the cardiac type α\textsubscript{c} Ca\textsuperscript{2+} channel, caveolin-3, the Na/K-ATPase α\textsubscript{1} subunit, and cardiac type V/VI adenylyl cyclase (AC). See online data supplement for details.

All samples were run at 2-fold different levels of protein loading, which were optimized in preliminary experiments to be well within the dynamic range of the ECL detection for each protein (to allow for differences in protein abundance and/or antibody sensitivity). ECL is not a linear detection system: under the conditions used in these experiments, a 2-fold difference in protein loading corresponded to ∼2- to 3-fold difference in immunoreactivity (necessitating a separate analysis of results obtained at different protein loadings).

Data Analysis

Activation and inactivation data were fit to a Boltzmann function and recovery from inactivation data fit to a biexponential function (Origin 6, Microcal). All data are presented as mean ± SE. Statistical significance was determined by ANOVA or Student’s t test as appropriate and defined as P < 0.05.
the linear portion of the positive limb of the $I-V$ relation as shown. Peak $I-V$ relations (Figure 3), with maximum at $P$ density. Capacitance transients have been cropped. B, Mean l-V curves; adult $Npy^{-/-}$ cells had smaller current density than $Npy^{+/+}$ cells (ANOVA, $P<0.001$); n=9 and 10, respectively.

26.6±4.5 and 23.3±3.8 pA/pF in $Npy^{+/+}$ and $Npy^{-/-}$, respectively (n=11 and 8; $P=0.596$).

$I_{Ca,L}$ Activation and Inactivation
In Figure 2, typical traces of the nifedipine-sensitive current and mean I-V curves for adult $Npy^{+/+}$ and $Npy^{-/-}$ cells are shown. Peak $I_{Ca,L}$ density in adult cells was maximal at 0 mV in both preparations; however, current density was significantly smaller in $Npy^{-/-}$ than $Npy^{+/+}$ cells (ANOVA, two curves differ significantly; $P<0.001$). At 0 mV $Npy^{-/-}$ current density was 36% smaller (9.64±0.77 pA/pF, n=9, versus 15.05±1.28 pA/pF, n=10). Unlike in adult, $I_{Ca,L}$ from $Npy^{-/-}$ and $Npy^{+/+}$ newborn cells had statistically equivalent I-V relations (Figure 3), with maximum at +10 mV. Maximal peak-current density was 9.19±1.26 pA/pF (n=8) and 9.91±1.04 pA/pF (n=6) for newborn $Npy^{-/-}$ and $Npy^{+/+}$ cells, respectively. Thus, there is no prenatal effect of NPY deficiency on cardiac $I_{Ca,L}$. Both peak current density and the I-V relation were equivalent in newborn ventricle cells from $Npy^{-/-}$ and $Npy^{+/+}$ animals. Further, in the absence of NPY exposure in vivo, there is no postnatal increase in $I_{Ca,L}$ density, as the adult peak current density in the $Npy^{-/-}$ animals is equivalent to that in either newborn animal (Figure 4A).

The reversal potential, determined from extrapolation of the linear portion of the positive limb of the I-V curves, did not differ between groups and was used to construct activation relations (Figure 4B). The two newborn curves were the most positive and essentially superimposable, with midpoints (V_{1/2}) of −2.0±2.0 mV (n=8) and −2.9±1.5 mV (n=6) in $Npy^{-/-}$ and $Npy^{+/+}$, respectively. The adult activation curves were significantly more negative (V_{1/2}: −11.1±1.9 mV, n=10, for adult $Npy^{+/+}$, $P=0.005$ relative to newborn $Npy^{+/+}$; −9.2±1.0 mV, n=9, for adult $Npy^{-/-}$, $P=0.004$ relative to newborn $Npy^{-/-}$). The adult V_{1/2} values did not differ from each other. Slope factors of the activation curves for adult $Npy^{+/+}$ and $Npy^{-/-}$ cells also did not differ significantly (4.9±0.2 and 5.1±0.2 mV, respectively), but were significantly smaller than values for newborn cells (6.2±0.2 and 6.5±0.4 mV for $Npy^{+/+}$ and $Npy^{-/-}$, respectively; $P<0.005$ for both adult versus newborn comparisons), suggesting an age-dependent steepening of the activation relation independent of postnatal NPY exposure.

The reduced current density in adult $Npy^{-/-}$ could result from a difference in voltage dependence of inactivation or recovery from inactivation, such that a smaller fraction of channels were available during the voltage protocol in $Npy^{-/-}$ than $Npy^{+/+}$ cells. Therefore, we determined the steady-state inactivation relation. We found no significant difference in $I_{Ca,L}$ availability between adult $Npy^{+/+}$ and $Npy^{-/-}$ cells (Figure 5). Threshold for inactivation was about −40 mV for both groups. V_{1/2} values were −25.3±0.8 mV (n=8) and −26.7±1.3 mV (n=6), respectively; slope factors were 4.7±0.2 and 5.2±0.5 mV, respectively. Thus, the reduced peak current in $Npy^{-/-}$ cells does not result from a shift in inactivation relation.

The reduced peak current in $Npy^{-/-}$ cells also could arise from slower recovery from inactivation. Figure 6 illustrates this is not the case, and that recovery from inactivation is faster in $Npy^{-/-}$. Mean time dependence of recovery of $I_{Ca,L}$.
from inactivation is illustrated, with the initial portion expanded in the inset. The time course of recovery is best described by a biexponential function. Although both the fast and slow time constants (τ) are statistically equivalent in both groups (fast τ, 47.9 ± 9.2 ms, n = 10, and 49.4 ± 5.2 ms, n = 8 for Npy+/− and Npy−/−, respectively; slow τ, 470.3 ± 144.3 and 355.2 ± 66.2 ms, respectively), the fraction of current ascribed to the fast component was significantly greater in Npy+/− (0.85 ± 0.03 versus 0.68 ± 0.07, P = 0.033). In some cases, at interpulse intervals of 1.0 to 1.6 seconds, ICa,L produced by the second (test) pulse was greater than control current, which may be a result of facilitation. This was seen in 1 of 8 Npy+/− and 3 of 10 Npy−/− cells. We further explored this phenomenon in conditions (see Materials and Methods) similar to those in the recovery experiments. In both groups the amplitude of the current induced by 0.5 Hz stimulation after a 60-second period of rest increased, and reached a maximum at the 4th test pulse (not shown); the increase was modest for both preparations but significantly (P = 0.011) greater in Npy+/− cells (8.5 ± 1.4%, n = 7) than Npy−/− (3.5 ± 1.1%, n = 8). Both the faster recovery from inactivation and greater facilitation in Npy−/− myocytes, if also present at physiological resting potentials, would tend to mitigate the difference in peak current density at the rapid heart rates typical of mouse.

Calcium Channel α1C Subunit Protein Expression and Maximal Responsiveness

Given that the reduced peak current density of adult Npy−/− myocytes cannot be explained by a difference in steady-state inactivation or recovery from inactivation, we used immunoblot analysis to determine whether L-type Ca2+ channel α1C subunit protein expression differs between the two preparations. To optimize immunodetection of the membrane-
associated Ca\textsuperscript{2+} channel protein, we used a differential centrifugation protocol to separate LS membranes from other cellular membranes. The LS fraction contains only a minor amount of total membrane protein and is highly enriched in Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and caveolin-3 (plasma membrane and lipid raft markers), relative to the HM fraction (Figure 7A); unexpectedly, protein recovery in the LS from Npy\textsuperscript{+/+} is significantly higher than from Npy\textsuperscript{−/−}. Protein recovery in the HM from Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} does not differ. Figure 7B shows that Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit immunoreactivity is detected as a \(\approx 200\)-kDa band, which corresponds to mobility of the full-length protein,\textsuperscript{9} in both LS and HM fractions. An additional minor \(\approx 160\)-kDa immunoreactive species (which is consistent with a C-terminal truncation of the pore-forming Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit) was detected with the anti-\(\alpha_{1C}\) subunit antibody (directed against the N-terminus) only in HM. Both \(\approx 200\)-and \(\approx 160\)-kDa bands are epitope specific and are blocked by competing antigen peptide. Figures 7C and 7D show that \(\alpha_{1C}\) immunoreactivity (expressed per mg protein) is similar in LS membranes from Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−}; \(\alpha_{1C}\) immunoreactivity in HM fractions (which contain the bulk of membrane protein) is actually \(\approx 30\%\) higher in Npy\textsuperscript{−/−} ventricles, compared with the Npy\textsuperscript{+/+}. These results indicate that the smaller current density in Npy\textsuperscript{−/−} hearts does not result from a gross decrease in total L-type Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit expression. Rather, the reduced yield of total LS protein, in the context of higher levels of HM fraction \(\alpha_{1C}\) subunit and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, the abundance of AC V/VI and caveolin-3 in the HM fraction is too low for accurate quantification. Western blot analyses in C and the quantification in D illustrate results obtained at a single level of protein loading; results with 2-fold greater amounts of protein loading were in each case identical and (for economy of space) are not included.

To further investigate whether there is a difference in total available functional channel density, we conducted experiments to compare maximally activated current density in myocytes from Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} animals. We reasoned that if there is a difference in basal channel activity rather than density of functional channel protein, then a protocol designed to maximally stimulate protein kinase A (PKA)–dependent phosphorylation might eliminate the difference between Npy\textsuperscript{−/−} and Npy\textsuperscript{+/+} cells. Forskolin is a direct activator of AC (which is expressed at equivalent levels in Npy\textsuperscript{−/−} and Npy\textsuperscript{+/+} ventricles; Figure 7) and has been used successfully in previous developmental studies of \(I_{CaL}\) in rabbit myocytes.\textsuperscript{10} Therefore, we exposed adult cells to

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Figure 7. Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit expression in light sarcolemma (LS) and heavy membranes (HM) from Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} mouse ventricles. A, LS and HM (100 μg each) from Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} hearts were immunoblotted for plasma membrane (Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and cardiac type V/VI AC) and caveolae (CAV-3) markers. B, LS and HM (100 μg each) from Npy\textsuperscript{+/+} mouse hearts were subjected to SDS-PAGE and immunoblot analysis with anti-cardiac \(\alpha_{1C}\) Ca\textsuperscript{2+} channel antibody without or with antibody preblocking with antigen (+Antigen). C, Immunoblot analysis of LS and HM (35 μg per lane each) from three Npy\textsuperscript{+/+} and Npy\textsuperscript{−/−} ventricle preparations for Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit protein expression. D, Quantification of protein yield, Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit, AC V/VI, Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and caveolin-3 expression in Npy\textsuperscript{+/+} (open bars) and Npy\textsuperscript{−/−} (filled bars) ventricles (n=4, *P<0.05). At the levels of protein loading used for the detection of Ca\textsuperscript{2+} channel \(\alpha_{1C}\) subunit and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, the abundance of AC V/VI and caveolin-3 in the HM fraction is too low for accurate quantification. Western blot analyses in C and the quantification in D illustrate results obtained at a single level of protein loading; results with 2-fold greater amounts of protein loading were in each case identical and (for economy of space) are not included.
forskolin (10 μmol/L) to maximally stimulate AC during repetitive imposition of a voltage step to 0 mV, and determined the full I-V relation before and 2 to 3 minutes after start of forskolin exposure. For these experiments $I_{\text{Ca,L}}$ was defined as peak minus steady-state current, rather than the nifedipine-sensitive current. There is a strong and reversible response to forskolin (Figure 8) and the magnitude of that response is significantly greater in Npy$^{-/-}$ than Npy$^{+/+}$ myocytes ($P=0.025$). In the absence of forskolin, the measured $I_{\text{Ca,L}}$ density statistically differs between Npy$^{-/-}$ and Npy$^{+/+}$ cells ($P=0.015$), but after exposure to forskolin, current density no longer statistically differs between the two groups.

A similar result was obtained using the L-type channel agonist BayK 8644 (Figure 8C).

**Discussion**

This is the first study to provide direct evidence that NPY exerts an effect on maturation of any cardiac ion channel during postnatal development in vivo. Our main finding is that $I_{\text{Ca,L}}$ recorded in ventricular myocytes from NPY-deficient mice has a reduced density compared with the current from Npy$^{+/+}$ animals. Consistent with this finding, APD was significantly shorter in NPY$^{-/-}$ myocytes, and this was not due to an increase in $I_{\text{Ca,L}}$ These results demonstrate that NPY is an obligatory developmental factor controlling the properties of L-type Ca$^{2+}$ channels in the heart and confirms our previous results using an in vitro cell culture system.2

Additional information on the role of NPY in developmental regulation of L-type Ca$^{2+}$ current was provided in the newborn animal experiments. The existence of $I_{\text{Ca,L}}$ in cultured ventricular myocytes was previously demonstrated for fetal or postnatal stages of mouse development,11–13 but this study provides the first data comparing $I_{\text{Ca,L}}$ in freshly isolated postnatal newborn and adult mouse cardiac myocytes. In agreement with data from other mammalian species,14–16 the results with Npy$^{+/+}$ cells show a developmental increase in $I_{\text{Ca,L}}$ density. However, Npy$^{-/-}$ and Npy$^{+/+}$ newborn myocytes had $I_{\text{Ca,L}}$ of identical density and voltage-dependence, indicating the divergence of $I_{\text{Ca,L}}$ in Npy$^{-/-}$ and Npy$^{+/+}$ ventricles is established postnatally, along with maturation of sympathetic innervation of the heart. Further, it is striking that in the absence of NPY, there is no postnatal increase in current density, suggesting that NPY is the sole contributor to this developmental change in the mouse. It also is striking that the difference in current density, with adult Npy$^{-/-}$ being 36% reduced from that of Npy$^{+/+}$, is remarkably similar to the difference observed in neonatal rat ventricle cultures as a result of sympathetic innervation (39%) or NPY (34%).5

Not all developmental changes in $I_{\text{Ca,L}}$ are NPY-dependent. NPY-independent factors appear responsible for the age-dependent shift in voltage-dependence of $I_{\text{Ca,L}}$ activation (seen in both the voltage corresponding to the peak of the I-V relation and in the calculated activation relations), because an equivalent negative developmental shift occurred in both Npy$^{-/-}$ and Npy$^{+/+}$ cells. This shift was not parallel: the steepness of the activation curves constructed in both adult cell preparations was significantly greater than that of either newborn cell preparation. For reasons detailed in the Materials and Methods section, we do not believe this negative shift in activation results from loss of voltage control when clamping the larger adult cells with their correspondingly greater $I_{\text{Ca,L}}$. Further, the shift is equivalent in Npy$^{-/-}$ and Npy$^{+/+}$ cells despite current amplitude being significantly greater in Npy$^{+/+}$ myocytes. However, because these are calculated activation relations, the results should be confirmed by direct determination of the activation relation from tail current measurements.

Although the present results argue that the postnatal increase in L-type current density is entirely NPY-dependent, others have suggested a contribution of adrenergic agonists.3 Cell culture studies using rat heart cells confirm that exposure to NE exerts transcriptional effects on the $\alpha_{1C}$ subunit of the channel, increases message and protein levels, and increases current density.3 Additional experiments in vivo demonstrate that sustained exposure to elevated NE levels in the adult animal also affects these parameters.17 However, it has not been demonstrated that exposure of innervated myocytes in culture to adrenergic receptor blockers prevents the effect of sympathetic innervation. Further, the effect of innervation in
vitro is localized, affecting only physically innervated myocytes and not adjacent ones. We previously demonstrated that effects of innervation in vitro on the Na⁺ channel, which are mediated by NE, are global and can be replicated by nerve-conditioned medium as well as by physical innervation. In contrast, other effects of innervation in vitro that are mediated by NPY are not replicated by nerve-conditioned medium, confirming the localized nature of this signaling pathway in cell culture. Thus, although NE clearly exerts transcriptional effects on the L-type Ca²⁺ channel, there is no direct evidence that this contributes to the increased I_{Ca,L} density resulting from either sympathetic innervation in vitro or normal development. In addition, given the known function of NPY as a negative feedback inhibitor of NE release, it would be expected that sympathetic activity might be greater in the Npy⁻/⁻ animals. Thus, if NE exerts an important developmental influence on I_{Ca,L}, one might predict current density would have actually been greater in the Npy⁻/⁻ myocytes.

Liu et al. reported an approximate 2-fold increase in α_{1C} subunit protein levels in rat heart homogenates between birth and adult, whereas Haase et al. reported a decline in α_{1C} protein levels postnatally in rat heart. We are not aware of comparable immunoblot studies in mouse heart. Although we did not conduct immunoblot studies in neonatal hearts from Npy⁻/⁻ or Npy⁻/⁺, our immunoblot analyses of adult tissue failed to identify a gross difference in α_{1C} subunit expression that could account for the NPY-dependent increase in calcium current density in mouse heart. However, the bulk of the α_{1C} subunit immunoreactivity (including the truncated form of the α_{1C} subunit) was recovered in a HM fraction that contained relatively low levels of surface membrane protein markers, and which exhibited elevated (rather than reduced) levels of α_{1C} subunit immunoreactivity in the Npy⁻/⁻ hearts. This raises the possibility that α_{1C} subunits in the HM might be intracellular channels, and that a functionally important pool of α_{1C} subunits in the LS membrane fraction is reduced in Npy⁻/⁻ hearts (with lower protein recovery in the LS fraction, relative to Npy⁺/⁺). However, we cannot rule out several alternative interpretations of the results. (1) It is possible that an NPY-induced change in cellular cytoarchitecture alters the efficiency of LS membrane recovery (i.e., that the difference in LS protein recovery is not due to a bone fide difference in the total amount of cell surface LS membrane in the tissue). (2) Because any biochemical approach that compares L-type calcium channel expression (or the expression of any other low abundance channel/signaling protein) must be performed on some type of enriched membrane fraction, some level of uncertainty related to the efficiency of channel protein recovery from the starting tissue sample is unavoidable. (3) Finally, NPY-dependent changes in I_{Ca,L} might be due to an alteration in the expression of other Ca²⁺ channel isoforms or subunits. In this regard, studies in other systems predict that an increase in α_{1D} expression could contribute to the negative shift in activation observed developmentally, whereas an increase in β₃-subunit expression could contribute to increased I_{Ca,L} density.

The functional studies with forskolin and BayK 8644, in conjunction with the immunoblotting results, strongly suggest that the difference in current density between Npy⁻/+ and Npy⁻/⁻ myocytes might be attributable to differences in posttranslational modifications or cellular factors that regulate channel activity, rather than differences in actual expression of channel protein. Consistent with this hypothesis, in the presence of maximal stimulation by forskolin or BayK 8644, current density no longer differs between the two preparations. A similar differential effect of forskolin in ventricle myocytes was observed in a developmental study of I_{Ca,L} in rabbit. The present results suggest that functionally relevant absolute protein levels are similar in the two preparations, but that under basal conditions the L-type Ca²⁺ channels of Npy⁻/+ and Npy⁻/⁻ myocytes possess distinct properties. Whereas the forskolin data are compatible with a difference in basal activation by the adenylyl cyclase pathway, the results with BayK 8644 argue that the difference in current density more likely arises from differences in the biophysical properties of the channel. Additional experiments, including single channel analysis, are needed to identify the precise biophysical characteristics influenced by NPY. Further, elucidation of the relevant signaling cascade that NPY influences will require studies with selective activators or inhibitors. In this regard, it may be relevant that exposure of neonatal mouse cardiac myocytes to NPY results in activation of a pathway that includes the Y₂, NPY receptor subtype, a pertussis toxin sensitive guanine nucleotide regulatory protein and activation of mitogen-activated protein kinase. Whether the same cascade is involved in long-term regulation of I_{Ca,L} density by NPY remains to be determined.

The effects of NPY in this preparation are not necessarily restricted to direct action on myocardial cell NPY receptors, as there may be indirect and compensatory actions in the developing animal in vivo. Because the magnitude of the NPY-dependent increase in I_{Ca,L} density observed in this study is comparable to that reported during incubation of neonatal myocytes with NPY in cell culture, such secondary NPY actions may not be relevant to regulation of cardiac L-type Ca²⁺ channels. However, secondary and/or compensatory effects, as well as additional direct effects not studied, could affect the physiological impact of reduced Ca²⁺ current density. For example, NPY has been reported to exert additional long-term effects on myocardial cells, including increased β-adrenergic receptor density and transient outward current density, and Npy⁻/+ animals also would be expected to have reduced presynaptic feedback inhibition of autonomic activity. At least some of these effects could act to reduce the physiological impact of reduced I_{Ca,L} density on Ca²⁺ influx during an action potential in vivo. Although we did not observe a significant difference in I_{Ca,L} in this study, we only measured peak 4-AP sensitive current. There are known to be multiple components of transient outward current in mouse ventricle with complex kinetics, and a more careful analysis of the individual components is required to fully resolve this question. In addition, the faster recovery from inactivation in Npy⁻/⁻ myocytes also might mitigate any difference in I_{Ca,L} density, although the extent of this difference at a normal resting potential remains to be determined. Similarly, the difference in APD and therefore its influence on repolarization time course and activation of other currents is likely to be highly dependent on intrinsic heart rate. Thus,
the extent of the differences in APD and $I_{Ca,L}$ in the intact animal, whether there are additional differences in other aspects of Ca$^{2+}$ homeostasis and the effect of any such differences on cardiac output, remain to be determined.

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MATERIALS AND METHODS

Isolation of cardiac myocytes

Adult (8- to 10-wk old) male mice were anesthetized with a mixture of ketamine (10 mg) and xylazine (0.7 mg), the heart removed, canulated through the aorta and perfused with 10 ml of Ca\(^{2+}\) free “basal” solution (in mmol/L): 112 NaCl, 5.4 KCl, 1.7 NaH\(_2\)PO\(_4\), 1.6 MgCl\(_2\), 4.2 NaHCO\(_3\), 20 HEPES, 10 taurine, 5.4 dextrose (pH 7.2 by NaOH). MEM vitamins (Gibco) 10 ml/L and MEM amino acids (Gibco) 20 ml/L also were included. Then the heart was mounted on a Langendorff apparatus and perfused for 8-10 min at 35°C with oxygenated “basal” solution containing collagenase type 2 (Worthington, 308 U/mg, 0.225 mg/ml) and trypsin (Gibco, 0.04 mg/ml). The ventricle was removed, minced and placed in a 35°C shaker water bath with “basal” solution containing 2x the original enzyme concentrations, 4.8 mg/ml bovine albumin and 0.75 mmol/L CaCl\(_2\). After 10 min the solution was removed and centrifuged to obtain a fraction of isolated cardiac myocytes, while the undigested tissue was placed into fresh enzyme solution for a total of up to 3 enzyme incubations and cell collections. Isolated cells were maintained in enzyme free solution of the same ionic composition at 4°C until used in electrophysiological experiments, within 8 hours of isolation.

The newborn mouse heart (3- to 4-day old, of either sex) was perfused in the Langendorff apparatus for 3-5 min with 35°C oxygenated “basal” solution in which the concentration of taurine was increased to 30 mmol/L and pH adjusted to 6.9. Then the heart was perfused with the same solution containing 0.7 mg/ml collagenase and 0.07 mg/ml protease (Sigma, 4.9 U/mg). After 5 min, the ventricle was removed, placed into KB solution (in mmol/L: 70 L-glutamic acid, 80 KOH, 20 KCl, 10 β-OH-butyric acid, 10 KH\(_2\)PO\(_4\), 10 HEPES-KOH, 10 taurine, 1 mg/ml
albumin, pH 7.4) and agitated to dissociate myocytes. Cells were maintained in this solution at 4°C until used, within 2 hours of isolation.

**Electrophoresis and immunoblotting**

Tissue (each preparation consisted of 0.6 g, from 6 ventricles; total of 24 Npy+/+ and 24 Npy−/− hearts) was thawed, minced on ice and disrupted by homogenization with a Polytron tissue grinder (Brinkmann Instruments, Inc.; generator PTA7) in 8 ml of homogenization buffer (HB: 30 mmol/L histidine, pH 7.6; 1 mmol/L EDTA; 0.1 mmol/L PMSF; 5 µmol/L pepstatin A). The homogenate was centrifuged at 500xg for 10 min. The supernatant was layered on top of 6 ml of 35% sucrose in HB and centrifuged at 38,000rpm for 90 min (SW40 rotor, Beckman Industry). Light membranes located at the interface of the supernatant and the 35% sucrose pad were designated light sarcolemma (LS) and the heavy pellet at the bottom of the ultracentrifuge tube was designated heavy membranes (HM).

Immunoblotting was with antibodies to the intracellular N-terminus of cardiac αICa²⁺ channel (Alomone Labs, cat.#ACC-013), caveolin-3 (mAb 26; BD Transduction Laboratories), the Na/K-ATPase α₁ subunit (Upstate Biotechnology, cat.#06-520) and cardiac types V/VI adenylyl cyclase (AC, with an antibody that distinguishes cardiac type V/VI AC from other AC isoforms, but not from each other; Santa Cruz, cat.#sc-590). Primary antibodies were diluted in 50 mmol/L Tris, pH 7.5, 0.2 mol/L NaCl containing 1% BSA (αIC; final dilution 1:200), 1% non-fat dry milk and 0.5% Tween 20 (caveolin-3; final dilution 1:1,000), or 3% non-fat dry milk and 0.1% Tween 20 (Na/K-ATPase final dilution 1:5,000; AC final dilution 1:100). Bound primary antibodies were visualized with ECL according to manufacturer’s instructions.