Neuropeptide Y contributes to innervation-dependent increase in $I_{Ca,L}$ via ventricular $Y_2$ receptors

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Protas, Lev, and Richard B. Robinson. Neuropeptide Y contributes to the innervation-dependent increase in $I_{Ca,L}$ via ventricular $Y_2$ receptors. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H940–H946, 1999.—The developmental increase in L-type Ca current ($I_{Ca,L}$) density in the rat ventricle is reproduced in vitro by culturing neonatal myocytes with sympathetic neurons. We tested whether this effect of sympathetic innervation results from a chronic or sustained action of neurally released neuropeptide Y (NPY). Ventricular myocytes from newborn rats were cultured in serum-free medium with or without sympathetic neurons, NPY, or NPY analogs. Ca currents were measured in single myocytes at room temperature using the perforated patch clamp. In all cell groups (control, innervated, or NPY treated), the current-voltage relation for $I_{Ca,L}$ was represented by a bell-shaped curve with maximal value near 0 mV. The current density at 0 mV normalized to that of corresponding mean control values was 1.63 ± 0.12 and 1.52 ± 0.16 for innervated and NPY-treated myocytes, respectively. Both groups differed significantly from control ($P < 0.05$). NPY analogs exhibited the following rank order of effectiveness: NPY ≥ NPY-(13–36) ≥ PYY >> [Leu$^{31}$Pro$^{34}$]NPY, suggesting that the NPY effect occurs via a $Y_2$-receptor subtype. In confirmation, chronic treatment of innervated cultures with a $Y_2$-selective NPY antagonist prevented the innervation-dependent increase in $I_{Ca,L}$. These results indicate that sympathetic innervation contributes to the developmental increase in $I_{Ca,L}$ via neurally released NPY acting at $Y_2$ receptors on the ventricular myocytes.

sympathetic innervation; neonatal cardiomyocytes; calcium current

THE CONTRACTILITY of the mammalian heart increases during development (4, 14, 20, 29). The improvement of contractile performance is associated with changes in a number of factors, including the maturation of excitation-contraction coupling (3, 6, 25, 37, 40), acquisition of adult pattern of contractile proteins (7), and the increased expression of L-type Ca channels, defined by both Ca current measurements and binding assays (15–17, 28, 40).

Studies using an in vitro model of innervation have suggested that the ontogeny of myocardial sympathetic innervation contributes to this developmental increase in cardiac contractility and Ca current density. In the rat ventricle, sympathetic innervation is largely postnatal (cf. Ref. 31). One can therefore directly test for a trophic effect of innervation by growing neonatal rat ventricular myocytes with or without sympathetic neurons or ganglia and comparing the characteristics of the noninnervated and innervated myocytes after several days in culture. Using this nerve-muscle coculture, Lloyd and Marvin (18) showed that 3- to 4-day innervated cultures contracted more strongly than noninnervated ones. More recently, Ogawa et al. (27) reported that sympathetic innervation of myocytes for 3–4 days markedly augmented the L-type Ca current ($I_{Ca,L}$) and increased the number of dihydropyridine (DHP) receptors. Similar effects (increase in $I_{Ca,L}$ and DHP receptors) and a transient increase in mRNA for DHP receptors could be seen when noninnervated cardiac myocytes were cultured for 24 h in media containing norepinephrine (19). Although these data are consistent with the idea that the trophic influence of sympathetic nerves on Ca channels in neonatal rat ventricular myocytes is mediated by the neurotransmitter norepinephrine, there are some inconsistencies and uncertainties with this interpretation. Most notably, the effect of in vitro innervation on contractility is global [i.e., it is mimicked by nerve-conditioned medium (18)], whereas the effect on $I_{Ca,L}$ density is localized [i.e., only physically innervated myocytes were reported to have enhanced current density, as opposed to nearby noninnervated cells in the same culture dish (27)]. Furthermore, the time course of the innervation and norepinephrine experiments was not comparable.

We have previously demonstrated that a norepinephrine-mediated effect on another ionic current is global [i.e., mimicked by nerve-conditioned medium (41)]. We also previously reported that a localized effect of innervation, to alter $\alpha$-adrenergic signaling, is mediated by neurally released neuropeptide Y (NPY) and prevented by chronic exposure of innervated cultures to an NPY antagonist (36). NPY, a 36-amino acid peptide, is stored in most sympathetic nerves as a cotransmitter with norepinephrine. Although its function in the heart is not fully understood (22), NPY has been shown to have some trophic effects in cardiac myocytes (24, 32, 35, 36) and vasculature (42).

The localized nature of the effect of innervation on $I_{Ca,L}$, in conjunction with our previous studies of norepinephrine and NPY actions, raised questions as to the actual trophic factor(s) involved in the innervation-dependent increase in Ca current. To test whether NPY participates in the trophic effect of innervation on Ca channel properties in cardiac myocytes, we have measured $I_{Ca,L}$ in neonatal ventricular myocytes cultured in NPY-containing medium and in innervated cultures grown in the presence of an NPY antagonist. The results indicate that activation of an NPY $Y_2$ receptor is essential for the trophic action of sympathetic neurons on Ca current density. A portion of these results has been presented as an abstract (30).
Methods

Isolation and culture of cardiac myocytes. Experiments were performed on cultured neonatal rat ventricular myocytes grown with or without sympathetic nerves. The ventricles of 1- to 2-day-old neonatal rats were quickly removed, cut into 1- to 2-mm² pieces, placed in Ca²⁺- and Mg²⁺-free saline solution (133 mM NaCl, 4.7 mM KCl, 20 mM HEPES, and 16.5 mM dextrose) containing 0.1% trypsin, and triturated for 15 min at 37°C. A series of 12 solution changes were completed, and the removed solution was kept on ice. At the end of the procedure, cells were collected from all samples by centrifugation, preplated to remove contaminating fibroblasts, and then plated onto fibronectin- or protamine sulfate-coated glass coverslips in petri dishes filled with MEM containing 10% FBS, at a final density of 1–2 x 10^5 cells/ml. This low plating density provided cultures with a large number of single cells suitable for voltage-clamp experiments. The dishes were incubated at 37°C in 5% CO₂. On the next day, the culture medium was exchanged for serum-free medium (34), and NPY or related peptides were added to some dishes in a final concentration of 0.1 µM, with the exception of the NPY antagonist. T₄₅[NPY-(33–36)]₄ (a template-assembled synthetic protein molecule), which was added at a final concentration of 350 nM to approximate its dissociation constant in binding assays (12). The next replacement of the medium was made 3 days later.

For nerve-muscle coculture, sympathetic chains were removed from 3- to 4-day-old rats, cleaned from surrounding fat, treated with trypsin (0.5% in above saline solution), dispersed mechanically by trituration, and collected by centrifugation as previously described (5). Neurons were then plated on fibronectin-coated coverslips and incubated for 2 h, and then freshly prepared neonatal cardiac myocytes were added as described above. Nerve growth factor, 20 ng/ml, was added to both nerve-muscle and control cultures. All cultures (myocytes treated with peptides, nerve-muscle and control) were used for electrophysiological experiments 4–6 days after initial cell isolation. Only single myocytes, not in contact with adjacent myocytes, were studied.

Current recording. Coverslips with cultured myocytes were placed on the glass bottom of a 0.25- to 0.5-ml experimental chamber mounted on the stage of an inverted microscope and superfused with Tyrode solution of the following composition (in mM): 130 aspartic acid, 146 NaCl, 1 MgCl₂, 1 CaCl₂, 5 EGTA, 10 HEPES, and 5 mM dextrose (pH 7.4). The NPY analogs used for chronic conditionments (33–36)₄, were obtained from the Foundation for Cardiovascular Research and Hypertension (Lausanne, Switzerland); NPY and all other peptides as well as nifedipine were obtained from Sigma.

Results

Description of the Ca current in control experiments. Single, spontaneously beating neonatal myocytes were chosen for data recording. Mean capacitance of all control cells employed in this study was 14.6 ± 0.7 pF (n = 42). An increase in the peak value of the Ca current (run-up) was seen when a single pulse from −40 to 0 mV (200 ms in duration) was repeatedly imposed at the beginning of the experiment. These changes, most probably connected to the transition from resting state to steady-state conditions (2), were prominent during the first one to two pulses. The I-V relation protocol was initiated after the seventh to eighth pulse when a stabilization of the peak current occurred. Once this stabilization was achieved, rundown of the inward current was small through the experiment. In the case of n = 3 cells, the current at the start of recording and 15 min later was 129 ± 39 and 108 ± 23 pA, respectively. This average decrease of 0.8%/min did not significantly influence measurement of I-V curves in the present study, which were typically complete within 5 min.

Inward currents (measured as peak minus steady state) exhibited a typical bell-shaped I-V relation with activation threshold between −30 and −20 mV and a maximum at 0 mV (Fig. 1). The holding potential of −40 mV prevented contamination by other inward currents (fast Na current, T-type Ca current); CdCl₂ (200 µM) completely eliminated the current (not shown).
**NPY AND DEVELOPMENTAL INCREASE IN I_{Ca,L}**

**Acute effect of NPY on I_{Ca,L}.** NPY acutely added to the bath solution during patch-clamp experiments can change $I_{Ca,L}$ in ventricular myocytes isolated from some adult mammals, including rats (22). An "acute" but long-lasting effect of NPY might interfere with or mimic the trophic effect of chronically applied NPY in our experiments. To determine the acute effect of NPY on $I_{Ca,L}$ in neonatal cardiac myocytes, we measured $I_{Ca,L}$ produced by repetitive 200-ms pulses to 0 mV from a holding potential of −40 mV every 5 s while exposing a cell to 100 nM NPY. An example of such an experiment is depicted in Fig. 3. NPY, 100 nM, was added after 4 min of control recording. No change in peak $I_{Ca,L}$ occurred during superfusion of the cell with NPY-containing solution or after NPY was washed out. In five experiments, the current at the end of exposure to NPY was 97.5 ± 3.5% of control values, and the difference was not statistically significant. This experiment also illustrates the absence of significant rundown.

**Chronic effect of NPY on I_{Ca,L}.** No significant change in Ca current was seen in cardiac myocytes chronically treated with 1 nM NPY (data not shown, peak $I_{Ca,L}$ was $-8.3 \pm 2.3 \text{ pA/pF}$, n = 7, and $-8.0 \pm 1.8 \text{ pA/pF}$, n = 5, in control and NPY-treated cells, respectively; P = 0.91). However, chronic exposure of cardiac myocytes to 100 nM NPY produced an increase in current density (Fig. 4, A and B). As in the case with innervation, neither the

**Effect of innervation on I_{Ca,L}.** Single, spontaneously beating myocytes in apparent contact with two or more branches of axons were chosen for recording. Comparison with noninnervated myocytes was based on separate culture dishes prepared from the same litters but grown without nerves. The capacitance of innervated and control (noninnervated) myocytes was 17.1 ± 1.7 pF (n = 12) and 15.8 ± 1.3 pF (n = 12), respectively. Representative families of current traces obtained from a noninnervated and an innervated cell are shown in Fig. 2A. Mean I-V relations from a series of cells are compared in Fig. 2B. Innervation did not change the shape of the I-V relation but proportionally increased current densities at all voltages. The current density at 0 mV in innervated myocytes was 1.63 ± 0.12 times that in control cells (−10.6 ± 0.8 pA/pF, n = 12, and −6.5 ± 0.5 pA/pF, n = 13, respectively; P < 0.001).

It is known that L-type current inactivation consists of both a voltage-dependent and Ca-dependent component (2). To determine if innervation altered the time course of current decay, we fit each trace to a double-exponential equation and plotted the result as a function of voltage (Fig. 2C). No significant differences were found in the values of either the fast or slow time constant between the noninnervated and innervated myocytes.

![Fig. 1. Effect of nifedipine on L-type Ca current ($I_{Ca,L}$). Peak current-voltage (I-V) relation curves from a holding potential of −40 mV; for waveform configuration, see Figs. 2 or 4. □. Control currents; ○, currents in the presence of nifedipine, 10 µM; n = 10 myocytes for both curves; values are means ± SE.](http://ajpheart.physiology.org/)
NPY AND DEVELOPMENTAL INCREASE IN \( I_{\text{Ca,L}} \)

**Fig. 3.** Absence of effect of acute exposure to neuropeptide Y (NPY) on \( I_{\text{Ca,L}} \). Peak current values produced by voltage steps from \(-40 \text{ mV} \) to \( 0 \text{ mV} \) (holding potential \(-40 \text{ mV}\)). Currents were recorded with interval of 5 s (every third value is presented here). Horizontal bar, time of superfusion of NPY-containing solution in the bath. Top inset: voltage step used to produce \( I_{\text{Ca,L}} \) and a sample of the current recorded. One of 5 similar experiments.

The shape of the \( I-V \) relation nor the current decay kinetics (Fig. 4C) were altered by NPY treatment. The peak current was recorded at 0 mV and was 1.52 ± 0.16 times greater in NPY than in control cells (\(-15.8 ± 1.7 \text{ pA/pF}, n = 7, \) and \(-10.4 ± 1.5 \text{ pA/pF}, n = 10, \) respectively; \( P = 0.03 \)). Nifedipine, 10 µM, decreased the current by 89.11 ± 1.15% (\( n = 4 \)) and by 89.7 ± 1.7% (\( n = 4 \)) in NPY-treated and control cells, respectively (data not shown), confirming that the increase in current produced by NPY treatment is due to augmentation of \( I_{\text{Ca,L}} \).

Chronic effects of other NPY agonists. In this same set of experiments, the chronic effect on \( I_{\text{Ca,L}} \) of another natural peptide from the PP family, PYY, was studied under the same conditions. PYY was used because the natural peptide from the PP family, PYY, was studied in the heart under the same conditions. PYY was used because the natural peptide from the PP family, PYY, was studied in the heart under the same conditions. PYY is comparable with that of NPY (91 and 79%, respectively). The effectiveness of NPY-(13—36) and PYY, and ineffectiveness of [Leu31Pro34]NPY, is consistent with an action via a Y\(_2\) NPY receptor subtype. Chronic exposure of innervated cultures to a Y\(_2\)-selective antagonist. If NPY participates in the mechanism by which innervation produces the increase in \( I_{\text{Ca,L}} \), a chronic blockade of NPY receptors on cardiomyocytes should attenuate or diminish the effect of innervation. Therefore, innervated cultures next were grown in the presence or absence of the Y\(_2\)-selective NPY antagonist T\(_4\)(NPY-(33—36))\(_4\) (350 nM). As can be seen in Fig.

![Graph](image)

**Fig. 4.** Chronic effect of NPY and PYY on \( I_{\text{Ca,L}} \). A: original traces of currents recorded from a control myocyte cultured without NPY (top traces) and from a myocyte cultured 4 days in media containing 100 nM NPY (bottom traces). From a holding potential of \(-40 \text{ mV}, \) voltage steps from \(-30 \text{ to } 50 \text{ mV} \) were applied (see waveform configuration shown above traces). B: peak \( I-V \) relation for \( I_{\text{Ca,L}} \) in control myocytes and in myocytes cultured for 3–5 days with NPY, 100 nM, or another natural peptide from the PP family, PYY, 100 nM. C: voltage dependence of time constant values for fast (bottom points) and slow (top points) components of \( I_{\text{Ca,L}} \) decay; \( n \) is >7 for each value. Values in B and C are means ± SE.
6, the antagonist-treated cultures exhibit a peak current at 0 mV 42% smaller ($P < 0.04$) than that of the untreated innervated cultures ($26.26 \pm 6.10$ pA/pF, $n = 6$, and $210.69 \pm 1.71$ pA/pF, $n = 7$, respectively). This difference is very close to that between innervated and noninnervated myocytes (39%, see Effect of innervation on $I_{\text{Ca,L}}$), indicating that the antagonist fully prevented the effect of innervation. In separate control experiments, sustained exposure to the antagonist did not inhibit the current in noninnervated cultures; peak current density at 0 mV in myocytes treated with $T_4\text{-[NPY-(33—36)]}_4$ was $-11.35 \pm 1.78$ pA/pF ($n = 4$) versus $-8.92 \pm 1.87$ pA/pF ($n = 5$) in matching control noninnervated cultures, and this difference was not significant ($P = 0.4$).

**DISCUSSION**

The present study provides several lines of evidence that NPY, which can be detected in cardiac nerves in developing rat ventricle as early as norepinephrine (26), is a trophic factor that contributes significantly to the modulation of $I_{\text{Ca,L}}$ density by sympathetic innervation. First, sustained NPY exposure, over a time course of several days (equivalent to the duration of the innervation experiments), mimics the effect of in vitro innervation. Second, the use of subtype-selective NPY agonists indicates that this action of NPY is via the $Y_2$ receptor on cardiac myocytes. This is in accordance with the observation that other trophic effects of NPY are mediated through $Y_2$ receptors (see below). Third, the effect of in vitro innervation is prevented by sustained exposure of innervated myocytes to a $Y_2$-selective antagonist $T_4\text{-[NPY-(33—36)]}_4$. This antagonist had no effect on $I_{\text{Ca,L}}$ in noninnervated cultures.

Effect of innervation and NPY on $I_{\text{Ca,L}}$. We did not attempt to directly measure the NPY degradation kinetics in our cell culture conditions. However, others have reported that the first step of degradation in vitro in cardiac membranes is cleavage of the N-terminal first two amino acids, yielding NPY-(3—36) (23), which is a naturally occurring $Y_2$-selective agonist, as is PYY-(3—36) (9, 10). This is consistent with our results with subtype-selective agonists and antagonists, suggesting that the relevant NPY receptor subtype is $Y_2$. Although early binding studies on adult heart suggested the presence of $Y_3$ receptors (defined as PYY insensitive; see Ref. 1), a more recent study concluded that $Y_1$ and $Y_2$ receptors were present in the heart (21). In addition, we recently reported that the ability of chronic NPY exposure to mimic the effect of sympathetic innervation on $\alpha$-adrenergic signaling appears to involve the $Y_2$ receptor subtype, based on the relative effectiveness of NPY-(13—36) and $[\text{Leu}^{31}\text{Pro}^{34}]\text{NPY}$ (35). Thus the NPY $Y_2$-receptor subtype appears to play a significant role in the trophic actions of sympathetic innervation during cardiac development. Interestingly, the $Y_2$ receptor also is considered to be the main NPY receptor responsible for the angiogenic effect of NPY (42). It also should be noted that there are additional cardiac trophic effects of NPY that have yet to be ascribed to a specific NPY receptor subtype. For ex-

![Fig. 5. Effect of selective NPY agonists on $I_{\text{Ca,L}}$. A: peak current at 0 mV in myocytes cultured for 3–5 days with NPY and PYY. Values are given as percentage of current recorded in control myocytes (cultured without agonists). Full $I$-$V$ curves from experiments represented in this panel are depicted in Fig. 4B. B: results from a separate set of experiments measuring the effect of NPY, NPY-(13—36) (an agonist selective for $Y_2$ receptors), and $[\text{Leu}^{31}\text{Pro}^{34}]\text{NPY}$ (an agonist selective for $Y_1$ receptors). A and B represent two sets of experiments, each with its own control values. All agonists were given at a concentration of 100 nM; $n = 8$ for each bar; values are means $\pm SE$. *Significant difference from control. C: comparison of increase in peak current produced by agonists at 0 mV; the increase produced by NPY is taken as 100%.](http://ajpheart.physiology.org/)

![Fig. 6. Effect of a $Y_2$ antagonist, $T_4\text{-[NPY-(33—36)]}_4$, on $I_{\text{Ca,L}}$ in innervated cardiomyocytes. Peak $I$-$V$ relation for $I_{\text{Ca,L}}$ in innervated untreated myocytes (○) and innervated myocytes treated with $T_4\text{-[NPY-(33—36)]}_4$, 350 nM, for 3–5 days (■). For waveform configuration, see Figs. 2 or 4. Values are means $\pm SE$.](http://ajpheart.physiology.org/)
ample, chronic exposure of cultured adult rat ventricular myocytes to NPY produces hypertrophy of these myocytes (24), whereas prolonged incubation of neonatal rat ventricular myocytes with NPY (2–48 h) increases density of β-adrenergic receptors (32).

Our finding that innervated cardiomyocytes have a greater $I_{\text{Ca,L}}$ density than noninnervated ones confirms earlier studies by others on cultured rat ventricular myocytes (19, 27). These studies suggested that the effect of innervation on $I_{\text{Ca,L}}$ in culture was mediated by β-adrenergic catecholamines. They clearly show that β-adrenergic agonists transiently increase DHP message and result in elevated binding after 24 h. However, these results do not definitively demonstrate that innervation acts via the β-adrenergic cascade. For one thing, although β-adrenergic agonists clearly increased DHP message, the effect was transient and opposed to some extent by a slower-onset but sustained decrease in DHP message caused by α-adrenergic agonists. Thus the net effect over long times of exposure to the mixed agonist norepinephrine released from sympathetic nerves in the culture is not obvious. Furthermore, studies have not been reported in which innervated cultures were grown in the sustained presence of α- and/or β-adrenergic antagonists to determine if these agents are capable of preventing the trophic action of sympathetic neurons on DHP binding (or Ca current density). Even if innervation increases DHP binding and/or mRNA level via neurally released norepinephrine, this need not be the mechanism by which current density increases. Current density changes may occur independent of a change in protein level. The data indicate that the effect of innervation on current density is restricted to physically innervated cells, which may not be consistent with an action of norepinephrine (41). In addition, the chronic effect of norepinephrine on Ca current density was only reported at the 24-h time point, whereas studies of in vitro innervation typically involve 3–5 days of exposure.

In considering adrenergic versus neuropeptide mechanisms, it should be remembered that NPY has been shown to reduce norepinephrine release from sympathetic nerves in the human and guinea pig heart (8, 33, 39). In human atria, this effect is mediated by Y2 receptors (33). The release of NPY (and norepinephrine), in turn, can be attenuated by stimulation of presynaptic α2-adrenoceptors (13). Although it is not possible to estimate the balance between presynaptic effects of norepinephrine and NPY in our nerve-muscle cultures, an action of $T_{\alpha}[\text{NPY-(33–36)}]_4$ on postsynaptic Y2 receptors is consistent with the effectiveness of the Y2-selective agonist NPY-(13–36) in noninnervated cultures. If in fact this antagonist affected neural release of norepinephrine or NPY (i.e., prevented the negative feedback regulation by neurally released NPY), we would have seen a further increase in Ca current in innervated myocytes incubated in media with $T_{\alpha}[\text{NPY-(33–36)}]_4$ rather than a decrease to values characteristic for noninnervated myocytes as was seen in our experiments. However, this study does not rule out additional effects of norepinephrine on Ca current density, nor does it rule out the possibility of other nonneural factors that may contribute to the developmental increase in current density in vivo. It does, however, indicate that neurally released NPY is at least one of the factors regulating $I_{\text{Ca,L}}$ density during development.

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