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Short communication

Characterization of the α_1-adrenergic chronotropic response in neuropeptide Y-treated cardiomyocytes

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Abstract

The cardiac α_1-adrenergic chronotropic response changes from stimulatory to inhibitory post-natally. The mature inhibitory response is mediated by the α_{1B}-adrenoceptor and a pertussis toxin sensitive G protein. In vivo and in vitro studies identify sympathetic innervation as critical for the maturation of this inhibitory response. Additional experiments in a culture model indicate the effect of innervation is dependent on neurally released neuropeptide Y. The present study establishes that the individual signaling elements in the neuropeptide Y induced α_1-adrenergic cascade are the same as those appearing during normal in vivo development. In addition, the data demonstrate that the effect of neuropeptide Y does not result from activation of the putative cardiac Y_3 neuropeptide Y receptor subtype, since it is reproduced by the peptide fragment neuropeptide Y-(13–36) but not by [Leu^{31}, Pro^{34}]neuropeptide Y.

Keywords: Neuropeptide Y; α-Adrenergic; Innervation; Ventricle; Development; Chronotropy

1. Introduction

Heart rate and force are modulated through the opposing action of the sympathetic and parasympathetic limbs of the autonomic nervous system, with the former being largely excitatory (increased rate and force) and the latter inhibitory (decreased rate). However, the parasympathetic system develops earlier, resulting in the possibility of a transitory imbalance and excess inhibition. Mitigating this potential imbalance is the observation that at the post-synaptic level in cardiomyocytes many autonomic responses change developmentally from a vigorous excitation in the neonate to a more modest excitation, or even inhibition, in the adult (Robinson, 1996). For example, the newborn exhibits a non-M_2 positive chronotropic muscarinic response (Sun et al., 1994) which contrasts with the purely inhibitory M_2-response in the adult. β-Adrenoceptor sensitivity is enhanced in the newborn relative to the adult, and includes a prominent β_2-receptor component (Kuznetsov et al., 1995). Finally, the neonate demonstrates exclusively a positive α_1-adrenergic chronotropic response which, with maturation, changes to a predominantly negative chronotropic response (Drugge et al., 1985). While the tendency for greater excitatory responsiveness in the neonate protects against a transitory sympathetic–parasympathetic imbalance, it may lead to arrhythmias if these excitatory responses fail to become more inhibitory at the appropriate developmental milestone(s). Thus, it is important to elucidate the mechanisms controlling age-dependent changes in these signaling cascades.

While the mechanism(s) responsible for many of these age-dependent changes in autonomic responsiveness are unknown, numerous in vivo and in vitro studies have implicated cardiac sympathetic innervation in the developmental maturation of the α_1-adrenergic response (Drugge et al., 1985; Steinberg et al., 1985; Malfatto et al., 1990). Moreover, individual components in the α_1-adrenergic signaling cascades in neonatal and adult cardiomyocytes have been identified (Del Balzo et al., 1990; Steinberg et al., 1996). α_1-Adrenoceptors constitute a multi-gene family of three distinct receptor subtypes which can be distinguished pharmacologically on the basis of their sensitivity to subtype-selective antagonists (Hieble et al., 1995). While

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all three α_1-adrenoceptor subtypes are detectable in cardiomyocytes (Stewart et al., 1994), the inhibitory α_1-adrenergic chronotropic response is specifically mediated by the α_{1B}-adrenoceptor (which is identified by its sensitivity to irreversible alkylation by chloroethylclonidine) linked to a pertussis toxin sensitive G protein (Steinberg et al., 1985; Del Balzo et al., 1990). Initial studies established that in vitro innervation not only resulted in the appearance of an inhibitory α_1-adrenergic response, but also led to an increase in the level of pertussis toxin sensitive G protein α subunit(s) (Steinberg et al., 1985). More recent experiments suggest that this increase in G protein α subunit(s) may not be essential for the innervation-dependent interaction of the α_{1B}-adrenoceptor and pertussis toxin sensitive G protein(s) (Steinberg et al., 1996). Finally, in vitro studies indicate that neurally released neuropeptide Y plays a critical role in the innervation-dependent maturation of the α_1-adrenergic response (Sun et al., 1991). However, it has not been determined if the α_1-adrenoceptor subtype and signaling cascade leading to the inhibition of automaticity in neuropeptide Y treated cultures are the same as those expressed in the adult heart. Nor has it been determined if neuropeptide Y influences the level of pertussis toxin sensitive G protein α subunit(s), as does innervation. The current study addresses these issues. Further, this study provides the first characterization of the neuropeptide Y receptor subtype regulating maturation of cardiac α_1-adrenoceptor responsiveness.

2. Materials and methods

Cultures were prepared from neonatal rat ventricular myocytes using a standard trypsin dissociation procedure, as described previously (Drugge et al., 1985). Fibroblast contamination was minimized by preplating, and the remaining myocytes cultured in Minimal Essential Medium (MEM) with 10% fetal calf serum, 0.6 μg/ml hypoxanthine and 20 μg/ml gentamicin sulfate. Medium was changed on day 1 (24 h after initial culture) and again on day 3 or 4. Cells were used for experimentation on days 4–6. For studying the effect of chronic exposure to neuropeptide Y or related peptides, the peptide at a final concentration of 10^{-7} M was added at the day 1 feeding and replaced with fresh drug at the day 3–4 feeding.

Automaticity experiments were conducted in the culture dish in the presence of culture medium as described previously (Sun et al., 1991). Drugs were added as 20 μl aliquots at 100 $\times$ final concentration into the 2 ml volume of medium and dispersed by gentle pipetting. If antagonist was employed it was added first, and then a series of increasing concentrations of agonist were added to generate a cumulative dose–response relation. Spontaneous rate was measured opto-electrically 2 min after addition of each antagonist or agonist aliquot, to allow time for steady-state to be achieved. A temperature controller and feedback circuit maintained the culture dish and solution at 37°C, while a constant flow of humidified 5% CO_2–95% O_2 maintained a constant pH. Dose–response curves were compared by nested analysis of variance (ANOVA). Individual groups were compared by ANOVA or Student's *t*-test, as appropriate. $P < 0.05$ was taken as significant. All data are expressed as mean $\pm$ S.E.M.

Detailed description of the methods for membrane preparation, pertussis toxin catalyzed ADP-ribosylation reactions, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for measurements of pertussis toxin sensitive G protein α subunits are published (Del Balzo et al., 1990).

3. Results

Initial studies compared pertussis toxin sensitive G protein α subunit levels in control and in neuropeptide Y treated cultures to determine if chronic neuropeptide Y treatment mimics this aspect of the effect of in vitro innervation. Fig. 1A demonstrates that the level of this protein in control (58 ± 4 fmol/mg) and neuropeptide Y treated cultures (54 ± 2 fmol/mg) does not differ ($n = 3$, NS). Thus, although neuropeptide Y treatment results in the appearance of an inhibitory α-adrenergic response (Sun et al., 1991), this is not associated with increased pertussis toxin sensitive G protein α subunit expression. Accordingly, we next determined if the inhibitory α-adrenergic response of the neuropeptide Y treated cultures was mediated by a pertussis toxin sensitive pathway (as is the case with innervated myocytes) or a distinct mechanism. Since many actions of neuropeptide Y are themselves mediated by a pertussis toxin sensitive cascade, we first identified conditions that would acutely disrupt the α-adrenergic response without interfering with a chronic trophic neuropeptide Y pathway. We found that a 6-h treatment with 100 ng/ml pertussis toxin was sufficient to fully ADP-ribosylate and inactivate all susceptible G protein α subunits in the myocyte culture (data not shown). However, removal of neuropeptide Y for the final 6 h of culture did not result in a loss of the α_1-inhibitory chronotropic response. Therefore, for the next experiments, cultures were treated with pertussis toxin (100 ng/ml) for 6 h in neuropeptide Y-free medium immediately prior to measurements of chronotropic responses. This would abolish an inhibitory α_1-adrenergic response mediated by a pertussis toxin sensitive G protein, but would not reverse the trophic actions of neuropeptide Y. Fig. 1B demonstrates that neuropeptide Y treated cultures exhibit the expected negative chronotropic response to the α-adrenergic agonist phenylephrine. However, pretreatment of these cultures with pertussis toxin abolishes this response, leaving only a positive chronotropic response characteristic of control (i.e., not neuropeptide Y treated) neonatal myocytes. These results demonstrate that the inhibitory α_1-adrenergic

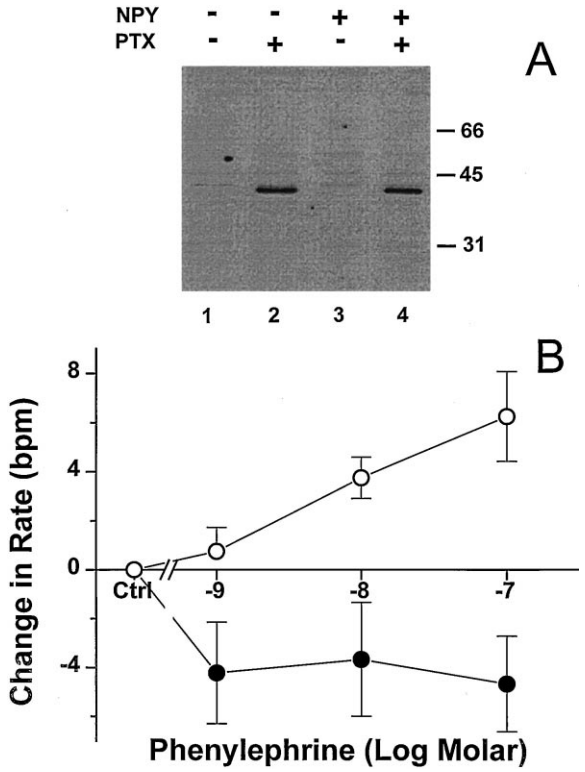

Fig. 1. Pertussis toxin dependent [^{32}P]ADP-ribosylation of membranes from control and neuropeptide Y (NPY) treated cultures and the pertussis toxin sensitivity of the NPY induced inhibitory α_1-adrenergic chronotropic response. (A) Control cultures (lanes 1 and 2) and NPY-treated cultures (lanes 3 and 4) were subjected to the ADP-ribosylation reaction in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of pertussis toxin (PTX). Results are from a single representative experiment. (B) Cultures chronically exposed to neuropeptide Y were treated with 100 ng/ml PTX or vehicle for 6 h immediately preceding the measurements of the chronotropic response to phenylephrine. Initial rates were 91.1 ± 3.6 bpm ($n = 9$) in the absence of PTX treatment (filled symbols) and 83.5 ± 4.0 bpm ($n = 8$) in the presence of PTX (unfilled symbols). There was no significant difference between the two initial rates. Data are plotted relative to these initial rates (CTRL). The two curves differ by nested ANOVA.

chronotropic response in neuropeptide Y treated cultures is mediated by a pertussis toxin sensitive pathway.

The inhibitory chronotropic response in the adult heart is mediated by the α_{1B}-adrenoceptor. This receptor subtype is detectable in the neonatal heart, but its linkage to an inhibitory chronotropic response is confined to the adult heart (Del Balzo et al., 1990). The α_1-adrenoceptor that mediates the inhibitory response in innervated and neuropeptide Y treated cultures has not been identified. To test whether the post-neuropeptide Y inhibitory chronotropic response in neuropeptide Y treated cultures also is mediated by the α_{1B}-adrenoceptor, neuropeptide Y treated cultures were exposed to 10^{-7} M chloroethylclonidine prior to measuring phenylephrine-dependent changes in automaticity. For comparison, matched neuropeptide Y treated cultures were exposed to 10^{-7} M WB4101 (an α_{1A}/α_{1D}-adrenoceptor antagonist (Hieble et al., 1995)) which has previously been shown to block the positive chronotropic response in the neonatal heart. The chloroethylclonidine exposed cultures exhibited exclusively a positive chronotropic response, while cultures exposed to WB4101 exhibited a negative α-adrenergic chronotropic response typical of cultures not exposed to any antagonist (Fig. 2A). Collectively, these results indicate that neuropeptide Y treatment mimics the normal maturational induction of an inhibitory α_1-adrenergic response that is mediated by an α_{1B}-adrenoceptor linked to a pertussis toxin sensitive G protein(s). However, the mechanism does not require increased pertussis toxin sensitive G protein expression, as

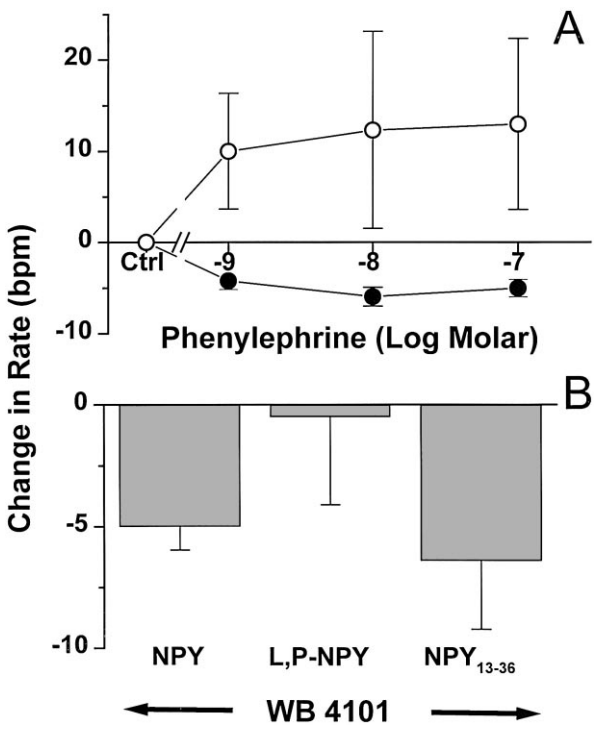

Fig. 2. Subtype selectivity of α_1-adrenergic response and the action of neuropeptide Y (NPY). (A) Cultures chronically exposed to neuropeptide Y were first exposed to either 10^{-7} M chloroethylclonidine (unfilled symbols) or 10^{-7} M WB4101 (filled symbols) for 2 min, and then exposed to progressively higher concentrations of phenylephrine in the continued presence of antagonist, as described in Section 2. The initial rates of the two groups did not differ from each other and did not differ before and after antagonist (chloroethylclonidine group: initial rate 74.2 ± 7.1 bpm, rate in chloroethylclonidine 75.4 ± 5.3; WB4101 group: initial rate 78.4 ± 3.4, rate in WB4101 81.8 ± 2.9). Data are plotted relative to rate in antagonist but prior to addition of any agonist (labeled Ctrl). The two curves differ from each other by nested ANOVA. $n = 6$ for the chloroethylclonidine group; $n = 23$ for WB4101 group. (B) Subtype-selective action of NPY in modifying α-adrenergic responsiveness. Cultures were chronically exposed to either NPY, [Leu^{31}, Pro^{34}]NPY (L,P-NPY) or neuropeptide Y-(13–36) (NPY_{13-36}), all at a concentration of 10^{-7} M. They were then exposed to 10^{-7} M phenylephrine in the presence of 10^{-7} M WB4101 and the change in rate relative to control was measured. The [L,P]NPY ($n = 8$) and NPY_{13-36} ($n = 10$) data are from a matched culture, while the NPY data ($n = 15$) are a subset of (A). Both neuropeptide Y and neuropeptide Y-(13–36) treatment resulted in a significant negative chronotropic response to 10^{-7} M phenylephrine, while [Leu^{31}, Pro^{34}]neuropeptide Y did not.

chronic neuropeptide Y exposure does not elevate pertussis toxin sensitive G protein α subunit(s) levels.

The next studies examined the neuropeptide Y receptor subtype that influences maturation of α_1-adrenergic responsiveness. Based on pharmacological and molecular studies, as many as seven neuropeptide Y receptor subtypes have been suggested (cf. Gehlert, 1994; Balasubramaniam, 1997), of which the neuropeptide Y Y_3 receptor subtype is reported to be present in adult cardiac tissue (Balasubramaniam, 1997). The neuropeptide Y Y_1 and Y_2 receptor subtypes are the best characterized, and can be distinguished on the basis of their sensitivity to the peptide agonists [Leu^{31}, Pro^{34}]neuropeptide Y and neuropeptide Y-(13–36), respectively. [Leu^{31}, Pro^{34}]neuropeptide Y is approximately 100 × more potent at neuropeptide Y Y_1 than Y_2 receptors, while neuropeptide Y-(13–36) is at least 300 × more potent at neuropeptide Y Y_2 than Y_1 receptors. At the neuropeptide Y Y_3 receptor, the two agonists are reported to be either equipotent, or [Leu^{31}, Pro^{34}]neuropeptide Y is somewhat more potent than neuropeptide Y-(13–36). Particularly relevant is the observation that, other than the neuropeptide Y Y_2 subtype, the so-called 'peptide YY preferring' subtype is the only one exhibiting greater potency for neuropeptide Y-(13–36) than for [Leu^{31}, Pro^{34}]neuropeptide Y. Fig. 2B demonstrates that cultures grown in the sustained presence of [Leu^{31}, Pro^{34}]neuropeptide Y fail to express an inhibitory response to 10^{-7} M phenylephrine. In contrast, cultures grown in the sustained presence of an identical concentration of neuropeptide Y-(13–36) exhibit an inhibitory α-adrenergic response equivalent to neuropeptide Y treated cultures. To eliminate interference from the opposing positive chronotropic α-adrenergic response, these experiments were conducted in the presence of WB4101. Taken together, these data suggest maturation of α_1-adrenoceptor responsiveness is influenced by a neuropeptide Y Y_2 or peptide YY preferring receptor, rather than neuropeptide Y Y_1, Y_3 or other receptor subtypes.

4. Discussion

The present study has resulted in the following conclusions: (1) The inhibitory α_1-adrenergic response induced by neuropeptide Y is mediated by the α_{1B}-adrenoceptor subtype, similar to that which occurs in the adult heart following normal development; (2) neuropeptide Y does not mimic the effect of innervation to increase the level of pertussis toxin sensitive G protein α subunit(s), but appears to allow or facilitate α_{1B}-adrenoceptor and pertussis toxin sensitive G protein interactions; (3) innervation regulates α_1-adrenoceptor responsiveness via a neuropeptide Y Y_2 or 'peptide YY preferring' subtype of receptor. The putative cardiac-specific neuropeptide Y Y_3 receptor is not involved in this process.

Previous studies provide several lines of evidence to support the conclusion that the expression of the inhibitory α_1-adrenergic response is dependent upon sympathetic innervation of the heart. The developmental acquisition of an inhibitory chronotropic response can be induced by in vitro innervation of cardiomyocytes in culture (Drugge et al., 1985), and this innervation-dependent inhibitory response is pertussis toxin sensitive (Steinberg et al., 1985), as is that in the adult (Steinberg et al., 1996). Further, in vivo studies treating newborn rats with nerve growth factor or its antibody to accelerate or retard sympathetic nerve growth respectively, also speeds or slows the appearance of the inhibitory α_1-adrenergic response (Del Balzo et al., 1990). Additional studies using the cell culture model indicate that neuropeptide Y plays a critical role as a mediator of the innervation-dependent change in α_1-adrenoceptor-dependent chronotropic responsiveness (Sun et al., 1991).

The current study demonstrates that the neuropeptide Y induced α_1-adrenergic inhibitory chronotropic response is similar to that appearing during normal development, in that it is dependent on an interaction between the α_{1B}-adrenoceptor subtype and a pertussis toxin sensitive G protein(s). However, this study also reveals that neuropeptide Y treatment results in expression of a pertussis toxin sensitive α-adrenergic response without increasing the level of pertussis toxin sensitive G protein α subunit(s). This is consistent with the growing evidence that our traditional concepts of receptor signaling are oversimplifications. Productive signaling events are not likely to result from the random collision and interaction of individual components of the receptor complex (the receptor itself, the G protein, and the effector mechanism) which are uniformly distributed on the plasma membrane. Rather, there is new evidence that molecular heterogeneity of components of the receptor complex (including the G protein, where the identity of the γ subunit can influence the interactions of $\alpha\beta\gamma$ heterotrimers with receptors and effector mechanisms) as well as compartmentalization of receptors and their down-stream effectors may critically influence signaling events (Steinberg et al., 1998). These novel concepts should provide directions for future studies of the mechanism(s) underlying maturational changes in autonomic responsiveness. Further, this result argues for the existence of an additional neural factor, other than neuropeptide Y, that can regulate G protein expression.

In addition to providing further support for the previous conclusion that neuropeptide Y plays a critical role as a mediator of the innervation-dependent change in α_1-adrenergic responsiveness during normal development, the present study also provides new insights into the mechanism of action of neuropeptide Y. The effect of neuropeptide Y was reproduced by the peptide fragment neuropeptide Y-(13–36), but not by [Leu^{31}, Pro^{34}]neuropeptide Y. This is intriguing because neuropeptide Y-(13–36) is a neuropeptide Y Y_2 agonist that would be expected to be relatively

ineffective at the neuropeptide Y Y_3 receptor, which is the receptor subtype reported to be present in the adult heart (Balasubramaniam, 1997). In contrast, [Leu^{31}, Pro^{34}]neuropeptide Y is a neuropeptide Y Y_1 agonist with little Y_2 activity, but with a relatively high affinity for the neuropeptide Y Y_3 and Y_4 receptor subtypes. Thus, the ineffectiveness of this analog confirms the results with neuropeptide Y-(13–36) and strongly suggests that neurally-released neuropeptide Y acts to modulate α_1-adrenoceptor responsiveness in neonatal cardiomyocytes via an action at a non-Y_3 receptor subtype, presumably either the neuropeptide Y Y_2 or the peptide YY preferring (Gehlert, 1994) receptor. Interestingly, preliminary data indicate that another developmental action of neurally released neuropeptide Y (increase of L-type Ca current) has a similar pharmacologic profile (Protas and Robinson, 1998), suggesting that this non-Y_3 neuropeptide Y receptor subtype may trigger multiple developmental milestones in cardiac myocytes. In this regard, it should be noted that the characterization of the heart neuropeptide Y receptor as Y_3 is based on binding data in adult tissue. The identity of the neuropeptide Y receptor-subtype(s) expressed by neonatal cardiomyocytes has not previously been examined. This and the nature of the more distal elements of the signaling cascade initiated by neuropeptide Y in the neonatal heart remain to be determined.

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