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Acquisition by Innervated Cardiac Myocytes of a Pertussis Toxin–Specific Regulatory Protein Linked to the α_1-Receptor

Abstract. During development, the chronotropic response of rat ventricular myocardium to α_1-adrenergic stimulation changes from positive to negative. The α_1-agonist phenylephrine increases the rate of contraction of neonatal rat myocytes cultured alone but decreases the rate of contraction when the myocytes are cultured with functional sympathetic neurons. The developmental induction of the inhibitory myocardial response to α_1-adrenergic stimulation in intact ventricle and in cultured myocytes was shown to coincide with the functional acquisition of a substrate for pertussis toxin. A 41-kilodalton protein from myocytes cultured with sympathetic neurons and from adult rat myocardium showed, respectively, 2.2- and 16-fold increases in pertussis toxin–associated ADP-ribosylation (ADP, adenosine diphosphate) as compared to controls. In nerve-muscle cultures, inhibition of the actions of this protein by pertussis toxin–specific ADP-ribosylation reversed the mature inhibitory α_1-adrenergic response to an immature stimulatory pattern. The results suggest that innervation is associated with the appearance of a functional pertussis toxin substrate by which the α_1-adrenergic response becomes linked to a decrease in automaticity.

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The effects of α_1-adrenergic catecholamines on cardiac automaticity change as a function of development. Purkinje fibers from dogs less than 1 day old often exhibit an increase in automaticity after exposure to the α-agonist phenylephrine. In contrast, adult canine Purkinje fibers show predominantly a decrease in rate in response to α-adrenergic input (1). Similarly, the neonatal rat heart responds to α-adrenergic stimulation by an increase in rate, whereas the adult rat ventricle responds by a decrease in rate (2). In both species, the developmental change in the α-adrenergic response from an increase to a decrease in rate parallels the maturation of functional sympathetic innervation in the heart. This observation prompted the suggestion that the cardiac sympathetic nerves are responsible for the change in cardiac responsiveness to the α-adrenergic catecholamines (3).

This hypothesis was tested in primary cultures of neonatal rat ventricular cells grown alone or cocultured with nerves from sympathetic ganglion cells (2). The neonatal rat ventricular cells showed an exclusive, positive chronotropic response to phenylephrine when cultured alone. However, when cultured with functional (4) sympathetic neurons, the myocytes showed a predominantly negative chronotropic response to the α-agonist (2).

The developmental induction of an inhibitory chronotropic response to α-adrenergic catecholamines provides an opportunity to examine potential changes in the biochemical regulators of the response. One specific candidate is the inhibitory guanine nucleotide binding protein, designated N_i, which mediates effects linked to the inhibition of adenylate cyclase (5). This inhibitory protein is also important in the function of effector systems not mediated by adenosine 3′,5′-monophosphate (cyclic AMP) (6). The latter observation is pertinent to a consideration of α_1-adrenergic catecholamines that are not believed to be regulated by cyclic AMP under most circumstances (7). N_i can be monitored directly with pertussis toxin, which catalyzes the transfer of ADP-ribose from NAD (ADP, adenosine diphosphate; NAD, nicotinamide-adenine dinucleotide) to the α subunit of N_i (8). The ADP-ribosylation of N_i inactivates it and permits unopposed utilization of stimulatory pathways (9). Thus, pertussis toxin can be used in pharmacological experiments to assess the functional relevance of N_i.

We present data supporting the hypothesis that the developmental conversion of the α-adrenergic chronotropic response in the heart from positive to negative is due to an increase in the functional inhibitory guanine nucleotide binding protein, presumably N_i. We used two experimental systems: (i) neonatal and adult rat ventricles and (ii) neonatal rat myocardial cells cultured alone and in the presence of sympathetic neurons.

The effect of pertussis toxin on the α_1-adrenergic response in muscle cultures and in nerve-muscle cocultures is shown in Fig. 1. Pertussis toxin did not alter significantly the basal autonomic rate of noninnervated neonatal rat myocytes. Similarly the concentration-response relation to phenylephrine was not significantly changed by pertussis toxin (Fig. 1A). The positive chronotropic response to phenylephrine, in the presence or absence of pertussis toxin, was inhibited by the α_1-adrenergic antagonist prazosin (data not shown). Thus, in neonatal muscle cells cultured alone, pertussis toxin had no significant action. In muscle cells cultured with sympathetic nerves (Fig. 1B), pertussis toxin, which did not change basal automaticity, altered the response to phenylephrine so that all concentrations were associated with a positive chronotropic response. The responses of both the control and the pertussis toxin–treated nerve-muscle cultures to phenylephrine were inhibited by prazosin.

It is possible that pertussis toxin enhances either basal cyclic AMP levels or cyclic AMP accumulation in response to stimulatory agents. Such elevations of myocyte intracellular cyclic AMP concentration are associated with an increase in automaticity (10). Thus, the effect of pertussis toxin could simply have been due to an indirect action to raise cyclic AMP levels in the nerve-muscle cultures. Several results, however, make this suggestion unlikely. First, the experiments were performed in the presence of both the β-adrenergic antagonist propranolol (0.1 μM) and the muscarinic inhibitor atropine (0.1 μM), negating a cyclic AMP response through a β-adrenergic mechanism or through reversal of a muscarinic receptor–dependent effect. Second, cyclic AMP levels in muscle and nerve-muscle cultures were measured by a sensitive radioimmunoassay and found not to be changed under any of our experimental conditions. Muscle and nerve-muscle cultures had similar basal cyclic AMP levels that did not change after addition of phenylephrine. Furthermore, there was no significant increase in cellular cyclic AMP concentration in innervated or noninnervated myocytes after pertussis toxin ex-

posure (innervated, 5.8 ± 0.5 versus 4.5 ± 0.5 pmol of cyclic AMP per 2×10^5 cells; noninnervated, 5.0 ± 0.3 versus 5.9 ± 0.6 pmol of cyclic AMP per 2×10^5 cells; $n = 5$) (11). Finally, phenylephrine's effect of increasing automaticity in nerve-muscle cultures previously exposed to pertussis toxin was not associated with any changes in cyclic AMP levels (4.5 ± 0.5 versus 5.1 ± 0.5 pmol of cyclic AMP per 2×10^5 cells; $n = 5$). This result, however, does not
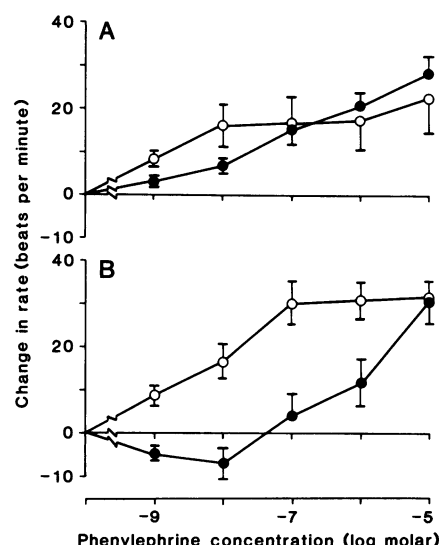

Fig. 1. The effect of pertussis toxin on the chronotropic response to phenylephrine of noninnervated and innervated myocardial cultures. Muscle and nerve cells were prepared from the ventricle and paravertebral sympathetic chain of neonatal rat (2, 18). For the pertussis toxin–treated cultures (O), the medium was replaced 16 to 24 hours before the pharmacological experiment with fresh medium containing 0.25 or 0.5 μg of pertussis toxin per milliliter. Control cultures (●) received fresh medium without pertussis toxin at the same time. Concentration-response curves for phenylephrine in the presence of propranolol (0.1 μM) and atropine (0.1 μM) were obtained according to the method of Drugge et al. (2). (A) Myocytes cultured alone. All cells respond to phenylephrine with an increase in spontaneous rate. The two curves are not statistically different by nested analysis of variance ($P > 0.05$). For both control and pertussis toxin–treated cultures, $n = 10$. (B) Myocytes cultured with nerves. Seventy percent of the control myocardial cells responded to phenylephrine with a decrease in spontaneous rate. The average response of all cells (both positive and negative responding) is shown. The subsequent increase in rate at concentrations of phenylephrine greater than 1 μM is probably the result of a crossover β-adrenergic effect (2). None of the pertussis toxin–treated cells responded to phenylephrine with a decrease in spontaneous rate. The two curves differ significantly, as tested by nested analysis of variance ($P < 0.05$). For controls, $n = 10$; for pertussis toxin–treated cells, $n = 18$. All data are shown as means ± standard error of the mean. The samples studied came from three separately cultured litters.

rule out the unlikely possibility that a small subpopulation of cells experiences a significant change in cyclic AMP levels.

The data suggest that the appearance of a negative α-adrenergic chronotropic response to phenylephrine in nerve-muscle cultures correlates with the functional acquisition of a substrate for the action of pertussis toxin. The only known substrates are the guanine nucleotide regulatory proteins, N_i, transducin, and N_o (8, 12), all of which can be ADP-ribosylated by pertussis toxin. We, therefore, assessed the amount of pertussis toxin substrate in noninnervated and innervated myocytes. In pure muscle cell cultures, which do not respond to pertussis toxin, there is only a very small amount of pertussis toxin–specific incorporation of ADP-ribose into molecular weight regions of pertussis toxin substrates (Fig. 2A, lanes 3 and 4). In contrast, nerve-muscle cultures, which respond dramatically to pertussis toxin, show more than twice as much specific incorporation of ADP-ribose into a band of the same molecular weight (Fig. 2, lanes 5 and 6). The amount of pertussis toxin–specific ADP-ribosylation is 152 ± 90 (standard error of the mean) fmol per milligram of protein in the muscle cell cultures and increases to 339 ± 151 fmol/mg in the cocultures ($P = 0.01$; $n = 5$ separate cultures; paired Student's t test). A similar experiment was performed in membranes prepared from innervated myocytes already activated by pertussis toxin under the conditions used for the pharmacological experiments. The labeled band at 41 kD is now not observed, indicating that conversion of the negative to the positive chronotropic response by pertussis toxin is associated with complete ADP-ribosylation of the pertussis toxin substrate by endogenous NAD (Fig. 2a, lanes 7 and 8; similar experiments in muscle cultures are shown in lanes 1 and 2). The small amount of pertussis toxin substrate that we have detected in isolated sympathetic ganglia does not account for the 2.2-fold increase in pertussis toxin substrate observed in the cocultures.

ADP-ribosylation assays were also conducted in membranes obtained from neonatal and adult rat ventricles that show the same change in α-adrenergic responsiveness from positive (neonatal) to negative (adult) chronotropy as demonstrated in muscle and nerve-muscle culture (2). Although a small amount of pertussis toxin–specific ADP-ribosylation could be demonstrated in neonatal membranes, much more intensely labeled substrate migrating at the same

position in the polyacrylamide gel is observed in membranes obtained from the adult ventricle. The amount of pertussis toxin–dependent ADP-ribose incorporation is 16 times greater in adult than in neonatal membranes [690 ± 140 versus 43 ± 9 fmol/mg; $P < 0.005$; $n = 5$ separate preparations; Welch test (13)].

These results indicate that the inhibitory guanine nucleotide regulatory protein N_i—or a related protein—may mediate the innervation-induced acquisition of negative chronotropy to the α_1-adrenergic catecholamines in the developing heart. Although the acquisition of a functional membrane-associated regulatory protein has been suggested by recent studies of the developing muscarinic receptor complex in chicken ventricles (14), our data now document this possibility. However, the identity of the pertussis toxin substrate is not yet known. Three closely related guanosine triphosphate binding proteins have been identified as substrates for pertussis toxin–specific ADP-ribosylation (8, 12). These three complexes, designated N_i, N_o, and transducin, have similar β and γ subunits and ADP-ribosylatable α subunits of molecular weights that are within the range of the 41-kD protein identified in this study.

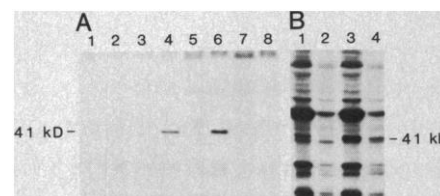This family of N_i-related proteins is

Fig. 2. Pertussis toxin–dependent ADP-ribosylation of myocardial preparations from myocyte and nerve-muscle cultures and from neonatal and adult rat ventricles. Membranes from pure myocyte cultures or nerve-muscle cultures (A) or from neonatal or adult rat ventricle (B) were prepared (19), and utilized for ADP-ribosylation assays, according to the method of Kaslow et al. with modifications (20). (A) Noninnervated myocytes were cultured for 16 to 24 hours alone (lanes 3 and 4) or in the presence of pertussis toxin (lanes 1 and 2). Innervated myocytes were similarly cultured alone (lanes 5 and 6) or in the presence of pertussis toxin (lanes 7 and 8). The ADP-ribosylation assay was subsequently performed in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of pertussis toxin. (B) Adult ventricular myocardial membranes were incubated with [^{32}P]NAD in the absence (lane 1) and presence (lane 3) of pertussis toxin. Neonatal ventricular myocardial membranes were incubated in the absence (lane 2) or presence (lane 4) of pertussis toxin. Quantitation of the amount of pertussis toxin substrate was accomplished by determining the amount of ^{32}P in the 41-kD band only and relating that to the protein concentration of the sample.

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thought to have a role in several other hormonal systems that are independent of cyclic AMP (6). Our data confirm that cyclic AMP is an unlikely candidate to mediate the α_1-adrenergic response. The α_1-adrenergic receptor complex appears to influence free cytosolic calcium through an inositol 1,4,5-triphosphate–dependent mechanism (15). Recent observations that binding of α_1-adrenergic agonists may be modulated by guanine nucleotides (16) and that treatment with pertussis toxin can inhibit α-adrenergic mediated phospholipid turnover (6) extend the role of N_i—or an N_i-related protein—to the α_1-adrenergic receptor system. These results also indicate that the acquisition of an N_i-like protein has an important role to play in the ontogeny of the developing heart.

The importance of innervation in the acquisition and maintenance of a negative modulatory effect of the α_1-adrenergic catecholamines has potential applications in clinical cardiology. In several pathological conditions, notably acute myocardial infarction and cardiac transplantation, normal patterns of innervation are altered or lost. If maintenance of the normal regulatory control afforded by the α-adrenergic catecholamines depends on continued neuronal input, the loss or alteration of neuronal input could lead to a change in physiological effects. In support of this possibility, enhanced excitatory α-adrenergic tone develops after acute myocardial infarction is induced in the cat (17). Such a reversion by the α-adrenergic catecholamines to an excitatory response has seminal implications for our understanding of the regulatory dynamics of catecholamine effects in the acutely damaged or jeopardized heart.

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