Sustained negative inotropism mediated by α -adrenoceptors in adult mouse myocardia: developmental conversion from positive response in the neonate

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1 Inotropic responses to α -adrenoceptor stimulation and the effects of antagonists were examined in isolated ventricular preparations from neonatal and adult mice.

2 Phenylephrine, in the presence of propranolol, produced positive inotropic responses in neonates up to 1 week after birth, while it produced negative inotropic responses in mice older than 3 weeks.

3 Both positive and negative responses to phenylephrine in neonates and adults, respectively, were antagonized by prazosin, WB4101 (2-([2,6-dimethoxyphenoxyethyl]aminomethyl)-1,4-benzodioxane) and 5-methylurapidil, but not by atropine, yohimbine or chlorethylclonidine.

4 Noradrenaline (NA) produced positive inotropic responses both in the neonate and adult; the responses were observed in a lower concentration-range in the neonate than in the adult. WB4101 produced a significant leftward shift of the concentration-response curve for noradrenaline in adult preparations while only a slight rightward shift was observed in the neonate.

5 Our results demonstrate the presence of α -adrenoceptor-mediated inotropic responses in the mouse ventricular myocardia. The response to phenylephrine changes from a positive to a negative effect during postnatal development. The responses are mediated by α_1 -adrenoceptors, and modulate the overall inotropic response to NA in the adult.

Keywords: Myocardium; inotropism; noradrenaline; α -adrenoceptor; development

Introduction

The sympathetic nervous system is a major regulator of myocardial function. Although myocardial effects of the neurotransmitter, noradrenaline (NA), are considered to be mediated through β -adrenoceptors, α -adrenoceptors are also present in myocardial tissue of various species, and their stimulation is known to affect cardiac rhythm and contractility (reviewed by Fedida, 1993; Terzic *et al.*, 1993). Although effects of α -adrenoceptor stimulation are considered to be mediated through mechanisms different from β -adrenoceptor stimulation, many questions on the mechanism and the species difference of α -adrenoceptor-mediated responses remained unsolved. Also, how α -adrenoceptors contribute to the overall inotropic action of NA or sympathetic stimulation is even less well understood.

Cardiac responsiveness to agents, including physiological neurotransmitters, is often different in developing animals when compared with adults (reviewed by Kojima *et al.*, 1990; Shigenobu, 1990). Many endogenous factors including autonomic innervation are known to affect cardiac responsiveness and in some cases abnormalities of the developing myocardium are considered to be involved in the pathogenesis of cardiovascular diseases (Tucker & Johnson, 1984; Tanaka *et al.*, 1988b). In our previous study (Tanaka *et al.*, 1994), we examined the positive inotropic responses to NA of isolated ventricular myocardia from neonatal and adult mice and demonstrated a developmental increase in sympathetic innervation and a tenfold decrease in the sensitivity to NA. In the present study, we examined whether α -adrenoceptor stimulation has any inotropic effect on mouse myocardia. We found that α -adrenoceptor-mediated inotropic responses in the mouse ventricular myocardia change from positive to negative during postnatal development and that this change contributes to the developmental decrease in the overall inotropic sensitivity to NA.

Methods

Right ventricular free wall strips were rapidly isolated from 1-day old neonatal and adult (5 to 6 weeks old) *ddy* strain mice. Preparations were placed horizontally in a 20 ml organ bath containing physiological salt solution of the following composition (mM concentration): NaCl 135, KCl 5, CaCl₂ 2, MgCl₂ 1, NaHCO₃ 15 and glucose 5.5 (pH 7.4). The nutrient solution was aerated with 95% O₂: 5% CO₂ and maintained at 36.5°C. The preparations were driven by a pair of platinum plate electrodes (field stimulation) with rectangular current pulses (1 Hz, 5 ms, 1.2 $\times$ threshold voltage) generated from an electronic stimulator (Dia Medical System, DPS-165B). Addition of propranolol (10⁻⁶M) had no effect on the basal contractile force indicating that endogenous catecholamines which might be released by electrical stimulation did not modify the basal contractile activities. The resting tension applied to each preparation was adjusted so that the muscle was stretched to the peak of its length-tension curve. Developed tension was recorded isometrically with a force-displacement transducer (Nihon Kohden TB-611T) connected to a minipolygraph (Nihon Kohden RM-6100).

Drugs were added after preparations had been allowed to equilibrate for more than 30 min, after which the developed tension of the preparations was well maintained. All drug solutions were prepared immediately before the start of the experiments by dissolving them in distilled water. Small aliquots of the solutions were added to the bath to give the desired final concentrations. The preparations were incubated with antagonists (except for chlorethylclonidine) for 15 min before exposure to phenylephrine or NA. In the case of chlorethylclonidine, the preparations were exposed to the drug for 40 min and the drug was not removed during exposure to phenylephrine. Concentration-response curves were obtained by cumulative addition of agonists. Each curve was obtained in a different preparation to avoid exhaustion of preparations. Significance of difference in pD₂ values were evaluated by Duncan's multiple range test, and a *P* value less than 0.05 was considered significant. The drugs used were

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(-)-noradrenaline hydrogen tetrates monohydrate (Wako), (-)-phenylephrine hydrochloride (Wako), propranolol hydrochloride (Sigma), atropine sulphate monohydrate (Tokyo Kasei), yohimbine hydrochloride (Tokyo Kasei), prazosin hydrochloride (Wako), chlorethylclonidine dihydrochloride (RBI), WB4101 hydrochloride (RBI) and 5-methylurapidil (RBI).

Results

Phenylephrine, in the presence of 10^{-6} M propranolol, produced a concentration-dependent positive inotropic effect in neonatal myocardia (Figure 1a,b). The maximum contractile force was about 200% of the basal contractile force and the pD_2 value was 5.23 ± 0.13 . In contrast, in the adult (five to six weeks after birth), phenylephrine, in the presence of 10^{-6} M propranolol, produced a concentration-dependent negative inotropic effect. The contractile force decreased to about 50% of the basal value and the pD_2 value was 5.98 ± 0.20 (Figure 1a,b). Application of 10^{-4} M phenylephrine, in the presence of 10^{-6} M propranolol, resulted in increases in contractile force up to 1 week after birth, slight

Figure 1 Inotropic responses of ventricular myocardia from neonatal and adult mice to phenylephrine. All experiments were performed in the presence of 10^{-6} M propranolol. (a) Traces of contractile force in neonatal (upper trace) and adult (lower trace) preparations; (▼) indicates the addition of 10^{-4} M phenylephrine. The horizontal bar indicates 1 min and the vertical bar indicates 100 mg. (b) Concentration-response curves for phenylephrine in neonatal (○) and adult (●) preparations. (c) Effect of 10^{-4} M phenylephrine on preparations from various ages. Each point in (b) and (c) represent the mean $\pm$ s.e. from five to ten experiments.

Figure 2 Effect of antagonists on the positive inotropic responses of neonatal myocardia to phenylephrine. All experiments were performed in the presence of 10^{-6} M propranolol. (a) Increases in contractile force by 10^{-4} M phenylephrine in the presence of antagonists were expressed as a percentage of the increase in the absence of antagonists in the same preparation. The concentration of the antagonists used were: atropine, 10^{-6} M; yohimbine, 10^{-6} M; prazosin, 10^{-6} M; chlorethylclonidine (CEC), 5×10^{-5} M and WB4101, 10^{-6} M. (b) Concentration-response curves for phenylephrine in the absence (●) and presence of 3×10^{-3} M (▲) and 10^{-8} M (■) 5-methylurapidil. Each column or point in (a) and (b) respectively, represent the mean $\pm$ s.e. from five experiments.

decreases or no effect were observed at 2 weeks after birth, and decreases were observed in myocardia from mice older than 3 weeks (Figure 1c).

In neonatal preparations (1 day after birth), positive inotropic responses to 10^{-4} M phenylephrine, in the presence of 10^{-6} M propranolol, were inhibited by 10^{-6} M prazosin and 10^{-6} M WB4101, but not by 10^{-6} M atropine, 10^{-6} M yohimbine or 5×10^{-5} M chlorethylclonidine (Figure 2a). 5-Methylurapidil concentration-dependently shifted to the right the concentration-response curves for the positive inotropic effect of phenylephrine, in the presence of 10^{-6} M propranolol (Figure 2b); the slope of the Schild plot was close to unity (0.99 ± 0.11) and the pA_2 value was 8.59 ± 0.07 .

In adult preparations, negative inotropic responses to 10^{-4} M phenylephrine, in the presence of 10^{-6} M propranolol, were inhibited by 10^{-6} M prazosin and 10^{-6} M WB4101, but not by 10^{-6} M atropine, 10^{-6} M yohimbine or 5×10^{-5} M chlorethylclonidine (Figure 3a). 5-Methylurapidil concentration-dependently shifted to the right the concentration-response curves for the negative inotropic effect of phenylephrine, in the presence of 10^{-6} M propranolol; the slope of the Schild plot was close to unity (0.91 ± 0.04) and the pA_2 value was 8.73 ± 0.11 (Figure 3b).

Noradrenaline produced positive inotropic responses both in the neonate and adult (Figure 4a). The response was observed with a lower concentration-range in the neonate than in the adult, pD_2 values for neonates and adults being 7.05 ± 0.01 and 6.09 ± 0.04 , respectively. WB4101 (10^{-8} M) produced a significant leftward shift of the concentration-response curve for noradrenaline in adult preparations while only a slight rightward shift was observed in the neonate. pD_2 values for NA in the presence of 10^{-6} M WB4101 in

Figure 3 Effect of antagonists on the negative inotropic responses of adult myocardia to phenylephrine. All experiments were performed in the presence of 10^{-6} M propranolol. (a) Decreases in contractile force by 10^{-4} M phenylephrine in the presence of antagonists were expressed as a percentage of the decrease in the absence of antagonists. The concentration of the antagonists used were: atropine, 10^{-6} M; yohimbine, 10^{-6} M; prazosin, 10^{-6} M; chlorethyl-clonidine (CEC), 5×10^{-5} M and WB4101, 10^{-6} M. (b) Concentration-response curves for phenylephrine in the absence (●) and presence of 3×10^{-8} M (▲), 10^{-8} M (■) and 3×10^{-8} M (◆) 5-methylurapidil. Each column or point in (a) and (b) respectively, represent the mean $\pm$ s.e. from five experiments.

neonates and adults were 6.87 ± 0.09 and 6.79 ± 0.06 , respectively. In the adult, 10^{-7} to 10^{-5} M NA, in the presence of 10^{-6} M propranolol, produced negative inotropic responses, while it produced positive responses at concentrations higher than 10^{-4} M (Figure 4b).

Discussion

We have previously reported a tenfold decrease in the sensitivity to NA during postnatal development of the mouse myocardium (Tanaka *et al.*, 1994). As sympathetic innervation of the mouse myocardium increases postnatally (Yamada *et al.*, 1980; Tanaka *et al.*, 1994), increased NA uptake into sympathetic nerve terminals may contribute to the decrease in sensitivity to NA; this was pharmacologically confirmed to be the case in our previous paper (Tanaka *et al.*, 1994). As the developmental decrease in sensitivity was also observed with isoprenaline, which is not taken up into sympathetic nerve endings, and with NA in the presence of desipramine, a neuronal uptake inhibitor, it was suggested that a decrease in the sensitivity of myocardial β -adrenoceptor-mediated mechanisms also takes place during development (Tanaka *et al.*, 1994). In addition to these two mechanisms, other mechanisms may be involved, i.e., the existence of an α -adrenoceptor-mediated inotropic response in the mouse ventricular myocardia and its change from positive to negative during postnatal development, as was revealed in the present study (Figure 1). In neonatal myocardium, the concentration-response curve for NA was only slightly affected by WB4101, indicating the small contribution of the α -adrenoceptor-mediated positive effect to the overall inotropic response to

Figure 4 Effect of WB4101 and propranolol on the positive inotropic response to noradrenaline (NA) in ventricular muscles from neonatal (open symbols) and adult (closed symbols) mice. (a) Concentration-response curves for NA were obtained in the absence (circles) and presence (squares) of 10^{-8} M WB4101. (b) Concentration-response curves for NA of adult preparations in the presence of 10^{-6} M propranolol. Each point represents the mean $\pm$ s.e. from five experiments.

NA. This may be due to the greater sensitivity of the myocardium to the β -adrenoceptor-mediated positive effects of NA in the neonate (Tanaka *et al.*, 1994). In contrast, in adult myocardium, a significant leftward shift of the concentration-response curve for NA by WB4101 was observed (Figure 4a), indicating that the α -adrenoceptor-mediated negative effect significantly contributes to the overall inotropic response to NA. It was also confirmed that not only phenylephrine but also NA can produce negative inotropic responses during β -adrenoceptor blockade (Figure 4b). Thus, the developmental conversion of α -adrenoceptor-mediated inotropic response from positive to negative contributes to the postnatal decrease in the overall inotropic sensitivity to NA.

The factors triggering the developmental changes remain to be clarified. In the case of the ventricular myocardium of rats (reviewed by Shigenobu, 1990), a tenfold decrease in the sensitivity to NA and isoprenaline occurs in the early postnatal period concurrently with the increase in sympathetic innervation density (Shigenobu *et al.*, 1988; Tanaka & Shigenobu, 1990a). Sympathetic denervation studies (Ishii *et al.*, 1982; 1985; Goto *et al.*, 1985) and organ culture experiments (Tanaka *et al.*, 1988a; Tanaka & Shigenobu, 1990b) revealed that sympathetic innervation is not only responsible for the pre-junctional component of the decreased sensitivity, but also exerts a long-term influence on myocardial properties to maintain the sensitivity to agonists at normal level. Whether the developmental decrease in sensitivity of the β -adrenoceptor-mediated effects (Tanaka *et al.*, 1994) and conversion of α -adrenoceptor-mediated effects from positive to negative (Figure 1) in mouse myocardia have causal relationship with the concurrent increase in its

sympathetic innervation is a matter of interest. α -Adrenoceptor agonists are reported to increase automaticity in non-innervated ventricular myocytes or neonatal ventricles but decrease automaticity in innervated ventricular myocytes or adult ventricles (Malfatto *et al.*, 1990). Alternatively, it is also possible that developmental conversion of α -adrenoceptor-mediated responses is triggered by other *in vivo* factors. Myocardial α -adrenoceptor-mediated responses or sarcolemmal α -adrenoceptor density are reported to be altered by abnormal thyroid function (reviewed by Osnes *et al.*, 1985), in hypoxia (Heathers *et al.*, 1988), hypertension (Limas & Limas, 1987) and experimentally induced diabetes mellitus (Heijnis & van Zwieten, 1992).

The cellular mechanisms involved in the developmental conversion of α -adrenoceptor-mediated responses also remain to be investigated. Receptor-binding studies of mouse myocardia indicate that, the density of β -adrenoceptors is about 60% higher in the neonate than in the adult, while that of α -adrenoceptors is not different between early postnatal and adult periods (Yamada *et al.*, 1980). In the present study, both the positive and negative inotropic responses to phenylephrine were antagonized by prazosin but not by yohimbine indicating the involvement of α_1 -receptors. This was further confirmed with the observation that the concentration-response curves for phenylephrine were shifted to the right by 5-methylurapidil (Figures 2b, 3b). The positive inotropic responses following α -adrenoceptor stimulation in several other species are reported to be mediated by α_1 -adrenoceptors (reviewed by Endoh, 1991). α_1 -Adrenoceptors can be further subdivided into at least two pharmacologically distinct subtypes, α_{1A} and α_{1B} (reviewed by Minneman, 1988). The α_{1A} -subtype has a higher affinity than the α_{1B} -subtype for the antagonist WB4101, 5-methylurapidil and (+)-niguldipine, while the α_{1B} -subtype is irreversibly alkylated by chlorethylclonidine. The subtype responsible for the α_1 -adrenoceptor mediated inotropic responses varies among species. For example, it is reported to be mainly the α_{1A} -subtype in rat myocardium (Rokosh & Sulakhe, 1991), while the α_{1B} -subtype mediates responses of the rabbit myocardium (Takanashi *et al.*, 1991). In the present study, both the positive and negative responses to phenylephrine in neonatal and adult mouse myocardia, respectively, were sensitive to WB4101 and 5-methylurapidil (Figures 2, 3), indicating that the receptors mediating both effects are of the α_{1A} -subtype. The pA_2 values for 5-methylurapidil against phenylephrine in neonatal and adult myocardium is close to those for α_1 -adrenoceptors in the rabbit aorta (Suzuki *et al.*, 1990) and bronchus (Takayanagi & Moriya, 1991). As for

the subclassification of receptor types, recent papers suggest the existence of additional types of α -adrenoceptors (Noguchi *et al.*, 1993) and the possibility remains that the inotropic responses of mouse myocardia are mediated by some of the novel types of receptors. In any case, no evidence for developmental changes in α -adrenoceptor subtypes mediating the inotropic effects was obtained in the present study. Thus it is likely that the developmental conversion of α -adrenoceptor-mediated response from positive to negative is due to developmental changes in the signal transduction pathways and/or excitation-contraction mechanisms of the mouse myocardium rather than to changes in α -adrenoceptor subtypes.

The contractile responses following α -adrenoceptor stimulation are reported to vary among species (reviewed by Fedida, 1993; Terzic *et al.*, 1993). In the myocardium of the rat (Tohse *et al.*, 1987), cat (Hartmann *et al.*, 1988) and rabbit (Skomedal *et al.*, 1990), α -adrenoceptor stimulation results in increases in contractile force, while in that of the dog (Endoh *et al.*, 1978), guinea-pig (Hescheler *et al.*, 1988) and ferret (Endoh *et al.*, 1989), inotropic effects are small or do not exist. Varying results have been reported with human myocardium (Jakob *et al.*, 1988; Osnes *et al.*, 1989). Many questions on the mechanisms of α -adrenoceptor-mediated inotropic responses remain unsolved. One reason for this may be the presence of both positive and negative effects. For example, in the rat ventricular myocardia, stimulation of α_1 -adrenoceptors results in a triphasic inotropic response (Tohse *et al.*, 1987). An initial increase in contractile force is followed by a transient decline and finally reaches a maximum level which persists for more than 20 min. Also, in other species, it is possible that the positive response to α -adrenoceptor stimulation is obscured by a simultaneous unrecognized negative component. The adult mouse myocardium is unique in that α -adrenoceptor stimulation results in a sustained negative inotropic response (Figure 1a), and may be useful in studies of α -adrenoceptor-mediated inotropism.

In summary, our results demonstrated that the α -adrenoceptor-mediated inotropic response in the mouse ventricular myocardia changes from positive to negative during post-natal development. The effects are most likely mediated through α_{1A} receptors, and contribute to the overall inotropic response to NA. Thus, the mouse myocardium provides an interesting model for investigation of the factors regulating and the cellular mechanisms of α -adrenoceptor-mediated inotropism.

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(Received May 10, 1994

Revised August 16, 1994

Accepted September 28, 1994).