Developmental Difference in the Stimulation of Cardiac Myofibrillar Mg^{2+} -ATPase Activity by Calmidazolium

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ABSTRACT. We probed possible developmentally related changes in thin filament activity in rat hearts with the aid of calmidazolium (CDZ). CDZ is a calmodulin antagonist that also binds to troponin C and stimulates Ca^{2+} troponin C-dependent activation of cardiac myofibrillar contractile activity. In paired experiments, we compared the effects of 10, 30, 50, 70, and 100 μM CDZ on Mg^{2+} -dependent ATPase activity of myofibrillar preparations from adult and neonatal rat hearts. Over the dose-response curve, the ATPase activity of neonatal myofibrils was significantly less stimulated than was the ATPase activity of the adult preparations. To know whether this difference in response to CDZ was related to differences in the thin or thick filaments, we studied hybrid adult and neonatal myofibrillar preparations. These hybrid myofibrils had native thin filaments, but the thick filaments were displaced with rabbit skeletal myosin. The relative insensitivity of the neonatal preparations to the effect of CDZ was retained in the hybrid myofibrils. This suggested that developmental transitions in the population of thin filament proteins are responsible for the difference between adult and neonatal myofibrils in their response to CDZ. Recently, we and others have reported developmental switching of troponin I isoforms in the rat heart. Since troponin I reacts strongly with troponin C in a Ca^{2+} -dependent manner, we speculate that developmentally related changes in troponin I isoforms may contribute to the differential effect of CDZ in neonatal cardiac myofibrils. (*Pediatr Res* 28: 46–49, 1990)

Abbreviations

CDZ, calmidazolium
TnC, troponin C
TnI, troponin I
pCa, negative log of calcium concentration

Neonates of several species have been demonstrated to be relatively insensitive to inotropic agents, including digoxin (1–3), and β -adrenergic agonists (4, 5). With the development of newer inotropic agents with novel modes of action, it is important to know whether effects of these agents are also age related. This has been shown to be so for phosphodiesterase inhibitors (6) that

elevate cAMP levels by bypassing the β -receptor. However, to our knowledge there have been no studies of agents that interact directly with myofilament proteins and act as so-called Ca^{2+} -sensitizers. In this study, our aim was to determine whether the effects of CDZ on myofibrillar contractile activity were different in neonatal than in adult rat hearts.

CDZ is generally considered a calmodulin antagonist, yet this agent also acts directly on the regulatory proteins of the cardiac myofilaments. CDZ promotes Ca^{2+} -binding to TnC and thus increases submaximal force and Mg^{2+} -ATPase activity (7). This action of CDZ is similar to the action of a number of inotropic drugs, including sulmazole, that act as TnC agonists and calmodulin antagonists (8). We chose to study CDZ because it has been shown to act through the thin filament regulatory complex and because there is evidence for the existence of developmentally regulated populations of cardiac thin filament proteins. Our results indicate that, compared with adult, neonatal heart myofibrils are less sensitive to stimulation by CDZ, and this difference may be related to developmental switching in thin filament proteins. We hypothesize that a developmental switch of TnI isoforms may be responsible for this difference.

MATERIALS AND METHODS

Myofibrils were prepared by extracting all membranes with the aid of Triton X-100 according to the modification of previously described procedures (9). For each experiment, myofibrils were prepared from a single adult rat heart and from pooled hearts from a single litter (age 5–11 d). Hybrid myofibrils were prepared from hearts pooled from several litters. The hearts were rapidly removed from pentobarbital anesthetized rats, minced, and placed in cold standard buffer (60 mM KCl, 30 mM imidazole pH 7.0, 2 mM $MgCl_2$). The hearts were homogenized with a motor-driven pestle in a glass-Teflon homogenizer. The homogenates were centrifuged at $12\,000 \times g$ for 15 min. The pellets were resuspended in a solution of 10 mM EGTA, 8.2 mM $MgCl_2$, 14.4 mM KCl, 60 mM imidazole pH 7.0, 5.5 mM ATP, 12 mM creatine phosphate, 10 U/mL creatine phosphokinase and 1% Triton 100-X and left on ice for 30 min. After recentrifugation at $1100 \times g$ for 15 min, the pellets were washed twice in the standard buffer. The myofibrils were either used fresh or diluted 1:1 with glycerol and stored at 0°C. Frozen myofibrils were diluted in the standard buffer and washed before use. Myosin was prepared from rabbit longissimus dorsi by the procedure of Shiverick *et al.* (10). The myosin was shown by SDS-PAGE to be free of thin filament proteins. The myosin preparation was functionally accessed as described in the next section. CDZ (Boehringer Mannheim, Mannheim, West Germany) was prepared in 100% ethanol, then diluted in H_2O for the assay.

Assay. The Mg^{2+} -ATPase activity of the myofibrillar prepara-

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tions was measured as described previously (11, 12). Incubation conditions were established by varying the total concentration of metals, salts, and ligands as calculated using a computer-program after specification of the desired pH, free Ca^{2+} , free Mg^{2+} , MgATP^{2-} , and ionic strengths using stability constants compiled by Fabiato (13). ATPase activity was expressed in nmol of inorganic phosphate/mg of protein/min. For comparison of adult to neonatal animals, a free calcium level that produced submaximal ATPase activity was chosen. Mg^{2+} -ATPase activity was determined under basal conditions and with addition of CDZ 10–100 μM . Ethanol was added to a final concentration of 1%. All assays were performed in triplicate. The activity of the skeletal myosin preparation was evaluated by determining the Ca^{2+} - and K^{+} -EDTA-activated ATPase activity in the absence of thin filament or actin. These activities (227 and 1117 nmol/mg/min, respectively) were as expected for rabbit skeletal myosin (14). Filament displacement experiments were performed by preparing hybrid myofilaments generated by the addition of a 10-fold molar excess of skeletal myosin to the neonatal and adult myofibrils as previously described (15). Protein concentration was determined by the Lowry assay using BSA as the standard (16).

Statistics. Two-way analysis of variance was used to determine the statistical significance of age-related difference in sensitivity to CDZ (17). Paired two-tailed *t* test was used to determine the statistical significance of the findings in the displacement experiments. A *p* value of less than 0.05 was considered significant.

RESULTS

Differential response to CDZ. Figure 1 illustrates the typical response to CDZ over a range of free calcium concentration. The increase in activity is at basal and submaximal levels of calcium. This is consistent with previous results on cardiac tissue of other species (7). A qualitatively similar response to 50 μM CDZ was seen with a preparation of neonatal myofibrils. At basal free calcium concentrations (pCa 7.5–6.25), the neonatal myofibrils had a mean 58% increase over control activity when the data points from pCa 7.5 to 6.25 were combined. This compares to a 127% increase for the mean of those data points illustrated in Figure 1 for the adult preparation. At submaximal calcium concentration (pCa 6.125–5.875), the neonatal preparation demonstrated a mean 48% increase versus a 78% increase

Fig. 1. Effect of CDZ on the relation between pCa and relative Mg^{2+} -ATPase activity of adult rat heart myofibrils. Measurements were made on a preparation from one rat heart at pH 7.0, 2 mM MgATP^{2-} , 2 mM Mg^{2+} , ionic strength 0.12 M, 30°C, with (♦) and without (■) 50 μM CDZ. Free Ca^{2+} was varied by varying the total CaCl_2 and EGTA to obtain constant 1 mM CaEGTA . The maximal ATPase velocity of the control myofibrils was 287 nmol/mg/min. The concentration of the ethanol vehicle was the same in all experiments.

in activity for the adult preparation. There was essentially no response to 50 μM CDZ at maximal calcium concentrations for either preparation. The remainder of the experiments were performed at pCa 5.875, a level of free Ca^{2+} that corresponds approximately to the half-maximal activity for both adult and 7-d neonatal rat myofibrils (18).

Results depicted in Figures 2 and 3 compare the dose response curves in the neonatal and adult myofibrillar preparations. Control ATPase activities were greater in adult preparations, as seen in Figure 2, which illustrates the absolute data. Two-way analysis of variance of the absolute data indicated that there was a significant age-related difference in the effect of CDZ for the overall curve as well as at concentrations of 50, 70, and 100 μM . In Figure 3, this data is illustrated as percent increase from control. At each data point, there was a greater percent increase in activity for the adult preparations.

These experiments indicate a greater relative response to CDZ in the adult myofibrils. The absolute ATPase activities were

Fig. 2. CDZ dose dependence of stimulation of adult (♦) and neonatal (■) myofibrillar ATPase activity. Measurements were made at pCa 5.875 under conditions described in the legend to Figure 1. Results are presented as means $\pm$ SEM for six preparations and were obtained in paired experiments. Two-way analysis of variance was performed for the overall curve and for each drug level with comparison to the control activity. This indicated a significant age-related difference in drug effect at doses of 50 μM ($p = 0.006$), 70 μM ($p = 0.036$), and 100 μM ($p = 0.018$) as indicated by the asterisks, as well as for the overall curve ($p = 0.009$).

Fig. 3. CDZ dose dependence as in Figure 2, but with the data represented as percent increase over control. The asterisks indicate data points with a significant age-related difference in drug effect as determined using analysis of variance of the absolute data.