Differential Effects of pH on Calcium Activation of Myofilaments of Adult and Perinatal Dog Hearts
Evidence for Developmental Differences in Thin Filament Regulation

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SUMMARY. Our results show that calcium activation of myofilament preparations of dog heart in the perinatal period is unaffected by a reduction in pH from 7.0 to 6.5, which, in adult heart myofilaments, induces a 0.4 pCa unit (-log molar free calcium concentration) rightward shift in the relation between pCa and myofibrillar adenosine triphosphatase activity. Acidic pH also had no effect on calcium binding to myofibrillar troponin C of perinatal hearts. The stoichiometry of troponin C bound calcium at full myofilament activation (about 3 mol calcium/mol troponin C) was the same for adult and perinatal heart myofibrils, as was their myofibrillar troponin C content. Moreover, there were no differences in isoelectric pH of troponin C from adult and perinatal hearts. We tested whether variants of myofilament proteins other than troponin C could account for the differential effects of acidic pH. In adult and perinatal dog heart preparations, myosin heavy chain isoenzymes appeared the same as measured, using native pyrophosphate gel electrophoresis. No evidence for thick filament-related calcium regulation in the perinatal heart myofilaments was obtained, when tested in studies in which native thin filaments were displaced with a 10-fold molar excess of pure actin. In preparations in which native thick filaments were displaced with a 10-fold molar excess of pure skeletal muscle myosin, the effects of acidic pH on calcium activation were the same as in native adult and perinatal preparations. Our major conclusion from these results is that the perinatal heart myofilaments are likely to possess variations in thin filament activity and structure. Although not conclusive, preliminary investigations of this question by means of polyacrylamide gel electrophoresis of the adult and perinatal myofilaments in the presence of sodium dodecyl sulfate or urea at basic pH and acidic pH provide evidence that there may be variants of troponin I in the thin filaments of the perinatal hearts. It is also possible that variants of troponin T or unidentified low molecular weight polypeptides associated with the thin filaments may also be important in the relative insensitivity of calcium activation of perinatal heart myofilaments to acidic pH. (Circ Res 58: 721–729, 1986)

FETAL and neonatal hearts function under conditions of environment and load that are different from those of adult hearts. It is therefore not surprising that there are differences between mechanical properties of adult and perinatal hearts (Friedman, 1973). In general, when compared to the adult, preparations of heart from perinatal hearts show differences in rates of tension development, levels of peak tension, and sensitivity to inotropic interventions such as paired pulse stimulation, postextrasystolic potentiation and β-adrenergic stimulation (Park et al., 1980; Nishioka et al., 1981; Anderson et al., 1984). Moreover, immature hearts show a remarkable resistance to hypoxic stress. Studies (Jarmakani et al., 1978a, 1978b) with both dog and rabbit hearts have shown that the decrease in peak developed tension and rate of tension development, associated with hypoxic stress in the adult heart, is essentially absent in the newborn heart.

These differences in the activity of adult hearts and hearts in the perinatal period undoubtedly are due to a variety of mechanisms (Penefsky, 1984; Fabiato, 1982), yet it seems possible that they might be due in part to differences in the structure, activity, and/or regulation of the myofilaments. Neonatal hearts have a reduced myofibrillar mass compared to the adult (Olivetti et al., 1980) and the pCa-myofilament force relation of skinned cells of rat myocardium has also been shown to change with development (Fabiato, 1982). Studies in some species have shown that there are differences in the thick filaments that may account for some of the observed functional differences with age (Lompre et al., 1981; Chizzonite and Zak, 1984). Ca++-dependent myosin adenosine triphosphatase (ATPase) activity and Ca++-stimulated actomyosin Mg-ATPase activity of heart myofilaments vary with age (Martin et al., 1983; Rupp, 1982), and these changes have
been associated with variations in the population of isoenzymic forms of myosin heavy chain, especially in rodents. Developmental differences in thin filament function and structure have been less extensively studied.

In experiments reported here, we have focused on the possibility that functional differences between adult and immature hearts are due to variations in myofilament activity and regulation. Our results show that, in contrast to the case with adult hearts (Blanchard and Solaro, 1984), Ca$$^{++}$$-activation of myofilament preparations from fetal and newborn dog hearts are unaffected by a reduction in pH from 7.0 to 6.5. We also present evidence that this relative insensitivity of the immature cardiac myofilaments to acidic conditions may reside in thin filament structure and activity.

Methods

Preparations

Cardiac myofibrils were prepared from ventricular tissue of adult, near-term, and newborn dog hearts, as previously described (Soloro et al., 1971; Pagani and Solaro, 1984). Adult animals and puppies were killed by injection with pentobarbital. Fetuses were delivered by cesarean section and killed by intraperitoneal injection of pentobarbital. Hearts were excised immediately, placed in ice-cold saline, and immediately processed for preparation of myofibrils. In some cases, the myofibrillar preparations were stored at -20°C in 60 mM KCl, 30 mM imidazole, pH 7.0, 2 mM MgCl$_2$ (standard buffer) containing 50% glycerol. Before use, the myofibrils were washed by centrifugation and were resuspended in standard buffer. The same experimental results were obtained with fresh or stored preparations. The myofibrils also were prepared and stored in the presence of inhibitors of proteolytic enzymes (5 μg/ml leupeptin, 5 μg/ml pepstatin, and 2 μM phenylmethyl sulfonyl fluoride), although we obtained the same experimental results and the same gel electrophoresis profiles of myofibrillar proteins prepared with and without these inhibitors. Myosin was prepared from rabbit longissimus dorsi by the procedure of Kielly and Harrington (1959) after initial extraction of the tissue by the procedure of Perrie and Perry (1970). Actin was prepared from the same muscle as described by Spudich and Watt (1971), and cardiac troponin C (TnC) was prepared as described previously (Holroyde et al., 1980). Fractions enriched in thin and thick filaments were prepared by the method described by Fukazawa et al. (1970).

Assays

Ca$$^{++}$$-binding and Mg-ATPase activity of the myofibrillar preparations were measured as described previously (Blanchard et al., 1984; Blanchard and Solaro, 1984). 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$$^{++}$$, free Mg$$^{++}$$, MgATP$$^{-}$$, and ionic strength using stability constants compiled by Fabiato (1981). The calcium contamination of reaction solutions was determined by atomic absorption spectroscopy as described previously (Blanchard et al., 1984). Incubation conditions are given in the figure legends.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE in the presence of sodium dodecyl sulfate (SDS) was performed by the procedure of Laemmli (1970). Alkaline-urea PAGE was done as described by Perrie et al. (1973) and Blanchard and Solaro (1984). Acid-urea PAGE was carried out in gels containing 7.5% acrylamide, 0.9 M acetic acid, pH 3.5, and 6.35 M urea. The electrode buffer was 0.9 M acetic acid. Samples were dialyzed against 0.9 M acetic acid and contained 8 M urea, 0.01% methyl green, 10% sucrose, 10 mM β-mercaptoethanol, and 2 mM EGTA. Electrophoresis was performed for 3.5 hours at a constant 150 V. The gels were stained as described by Laemmli (1970). Isoelectric focusing gels were run by a modification of the method of Righetti and Drysdale (1973). The gels contained 4.2% acrylamide, 2% Triton X-100, 9 M urea, and 1.6% ampholines (pH 4.5–2.5). Electrophoresis was performed for 4 hours with an initial 2 mA/tube constant current followed by constant 400 V. The gels were fixed in 15% trichloroacetic acid (TCA) for 1 hour and washed with 10% acetic acid, 15% methanol, until clear. The gels were stained overnight in 25% isopropanol, 10% acetic acid, 0.1% coomassie blue R-250, and then were destained. Isoenzymic forms of myosin were separated by nondissociating PAGE, using a modification (Martin et al., 1982) of the procedure of Hoh et al. (1977).

Results

Figure 1 shows results of experiments in which we compared the effects of a reduction of pH from 7.0 to 6.5 on the relation between pCa and the relative activation of Mg-ATPase activity of myofibrils prepared from adult and perinatal dog hearts. Whereas the adult heart myofibrils show about a 0.4 pCa unit shift in the pCa$_{50}$ for activation of ATPase activity with acidic pH, the immature heart myofibrils were activated by Ca$$^{++}$$ to the same extent at pH 7.0 and pH 6.5. A similar result was reported in a communication by Donaldson (1980), who studied Ca$$^{++}$$-activated force of skinned fibers from adult and embryonic rabbit heart.

Our previous studies with preparations from adult animals have shown a correlation between effects of acidic pH on the pCa$_{50}$ for Ca$$^{++}$$ activation of cardiac (Blanchard and Solaro, 1984) and skeletal myofibrils (Blanchard et al., 1984) and Ca$$^{++}$$ binding to myofibrillar TnC. To examine whether acidic pH has a similar effect on Ca$$^{++}$$-binding to immature cardiac myofibrils, we determined their TnC content and measured Ca$$^{++}$$ binding at pH 7.0 and 6.5 (Fig. 2). In agreement with the lack of effect of acidic pH on Ca$$^{++}$$ activation of the ATPase activity, acidic pH had no significant effects on Ca$$^{++}$$ binding by myofibrils of immature dog hearts (Fig. 2). Based on our determination of the TnC content of the immature cardiac myofibrils (Fig. 3), the data in Figure 2 are expressed as mol Ca$$^{++}$/mol TnC (right ordinate), and indicate that the maximum activity of the ATPase activity of the immature cardiac myofibrils occurs in a pCa range that is associated with the binding of about 3 mol Ca$$^{++}$/mol TnC. This agrees with previous findings from our studies of TnC in
FIGURE 1. The relation between pCa and Mg-ATPase activity of perinatal (near term) and adult dog heart myofibrils at pH 7.0 (●) and pH 6.5 (▲). Myofibrils were incubated in 2.14 to 2.3 mM Na₂ATP, 4.0 mM MgCl₂, 83-89 mM KCl, 30 mM imidazole to obtain a constant 2 mM Mg-ATP⁻, 2 mM Mg²⁺, and ionic strength of 0.12. Various pCa values were achieved by varying the total EGTA at constant 100 μM CaCl₂. Data are presented as means ± SEM for six preparations.

adult heart myofibrils (Blanchard and Solaro, 1984) and pure cardiac TnC or Tn complex (Holroyde et al., 1981). The amount of TnC (0.4 nmol TnC/mg protein) was the same in adult and perinatal dog cardiac myofibrils. The adult and perinatal cardiac myofibrils thus appear similar, not only in their relative amounts of TnC, but also with respect to the stoichiometry of Ca²⁺ binding at maximum activation of the myofibrils at pH 7.0. We also com-

FIGURE 2. Calcium binding by perinatal (near term) dog cardiac myofibrils at pH 7.0 (●) and pH 6.5 (▲). Incubation conditions were the same as those described in the legend to Figure 1 except for the addition of 0.3 μCi/ml [³²P]glucose and 0.3 μCi/ml ⁴⁵CaCl₂, 2 mM creatine phosphate, and 0.2 mg/ml creatine phosphokinase. Data are means ± SEM for six measurements.

FIGURE 3. Typical gels used to determine the TnC content of adult and perinatal cardiac myofibrils. Varying amounts of pure TnC were used as standards to quantify the TnC separated from myofilament proteins by electrophoresis on 7.5% polyacrylamide gels at pH 8.3 in the presence of 2 mM EGTA and 8 M urea. See Methods for details. Loadings were 3 μg protein for TnC and 275 μg for myofibrils.
pared the properties of TnC from the adult and immature hearts by electrophoresis on isoelectric focusing gels. As shown in Figure 4, isoelectric focusing gels were loaded on top of the isoelectric focusing (IEF) gels. The isoelectric pH of the TnC was 4.1.

Although our studies comparing TnC from adult and immature myocardium are not exhaustive, they do indicate that the differential response of the immature and adult heart myofibrils to acidic pH may reside in myofibrillar proteins other than TnC. For example, this possibility is indicated by the alkaline urea gels shown in Figure 3 in which there are apparent differences in the protein profile between adult and perinatal preparations. We therefore examined the adult and perinatal cardiac myofilaments by a variety of electrophoretic techniques. In view of evidence for age-related changes in the relative population of isoenzymic forms of myosin heavy chain (Lompre et al., 1981; Chizzonite and Zak, 1984), we analyzed the dog heart preparations for such differences by electrophoresis on native polyacrylamide pyrophosphate gels (Hoh et al., 1977). Clark et al. (1982) reported previously that adult dog ventricular myosin runs as a single band on pyrophosphate gels, which has been immunologically identified as a V3 isomyosin. Our studies, exemplified by the gels shown in Figure 5, indicated no consistent or big differences between myofibrillar proteins of adult and perinatal myocardium, at least as reflected by their relative mobilities on the pyrophosphate gels. In Figure 6A, we show SDS-PAGE profiles of the myofibrillar proteins of adult and immature dog hearts. Major and consistent differences were seen among these preparations. It has been reported that myofibrils from hearts of animals in the perinatal period contain "fetal" light chains (Price et al., 1980; Srihari et al., 1982), which are evident in the gels shown in Figure 6 as a band running with a mobility just ahead of light chain 1 (LC1). There are also four unidentified, low-molecular-weight bands below TnC, which are prominent in the myofibrils from perinatal dog hearts. These bands are associated with the thin filaments as they copurify with the myosin free I-Z-I brush preparation of Fukazawa et al. (1970) (Fig. 6B). The gels also show a possible variant of TnI in the immature myofibrillar preparations. Troponin I in the adult cardiac myofibrils migrates as a single prominent band, whereas, in the immature cardiac myofibrils, we consistently found that the corresponding band is relatively faint or associated with a second band of slower mobility. This slower-running band appears to correspond with a weakly staining band in the adult myofibrils. To test further for a possible variant in thin-filament regulatory proteins, we compared fetal and adult myofibrils by urea-PAGE at pH 3.5, a condition in which the basic proteins TnI and TnT have high relative mobilities (Fig. 7). As demonstrated by these gels, fetal myofibrils show a profile of relatively basic proteins that is different from that of the adult. Both the TnI and TnT bands in fetal myofibrils corresponding to
those of the adult preparation are faint, and a band of higher mobility is apparent. Thus, examination of the preparations by gel electrophoresis provides evidence for thin filament protein variabilities which might be responsible for the lack of effect of acidic pH on Ca++ activation of Mg-ATPase activity of immature myofibrils. Yet, it is also possible that myosin light chain variants in the fetal myofilaments may be important. Wagner (1984) has presented data indicating that myosin light chains may be important in the Ca++-sensitive binding of cross-bridges to the thin filaments.

To distinguish between the contribution of thin and thick filament proteins to the differential effects of acidic pH on Mg-ATPase activity of the perinatal and adult myofibrils, we did "filament displacement" studies as lines described by Lehman and Szent-Gyorgyi (1975). The approach involved preparation of hybrid myofilaments generated by addition of a 10-fold molar excess of either pure rabbit skeletal muscle actin or pure rabbit skeletal muscle myosin to myofibrillar preparations from adult and immature hearts. In these preparations, the excess actin or myosin competes with the endogenous proteins for reaction sites on the thin or thick filaments. We measured the Ca++-dependent Mg-ATPase activity of these hybrid preparations as functions of pCa at pH 7.0 and at pH 6.5. Results depicted in Figure 8 show that displacement of the native thin filaments of immature cardiac myofibrils with unregulated pure skeletal muscle actin results in a loss of Ca++ regulation, i.e. lack of thick filament related Ca++ regulation in the immature cardiac myofibrils. Data shown in Figure 9 summarize results obtained from studies of displacement of the native thick filaments...
of adult and immature dog cardiac myofibrils with pure skeletal myosin. As expected, Ca++ regulation is retained in these preparations, but it is significant that in both the adult and immature hybrid preparations, the change in pCa50 with acidic pH remains about the same as that obtained in the native preparations (Fig. 1).

Discussion

The principal new finding reported here is that cardiac myofilaments in the perinatal period possess alterations in thin filament regulation that appear to account for their relative insensitivity to acidic pH. We found no evidence for thick filament related Ca++ regulation in the perinatal myofilament preparations (Fig. 8); and, in our studies of the pCa-myofibrillar Mg-ATPase activity relation, we obtained the same effects of acidic pH following displacement of the native thick filaments with pure rabbit skeletal myosin (Figs. 1 and 9). These results not only show that the different effects of acidic pH on adult and perinatal heart myofibrils are due to a variation in thin filament structure and activity, but also agree with earlier work from our laboratory (Blanchard et al., 1984; Blanchard and Solaro, 1984).
indicating that the effect of acidic pH on Ca** activation of cardiac and skeletal myofilaments is most likely due to an effect on thin-filament regulation.

Whereas developmental changes in cardiac thick filaments have been reported for hearts of a variety of species (Lompre et al., 1981; Chizzonite and Zak, 1984), there have been few studies addressing the question of alterations in thin filaments of the developing heart. In general, changes in the population of myosin heavy chain isoforms have not been associated with changes in Ca** regulation as reflected in the pCa50 for activation of myofilament Mg-ATPase activity (Solaro et al., 1984). Humphreys and Cummins (1984) isolated TnI and tropomyosin from human and bovine adult and fetal hearts, and found no evidence for polymorphic forms of TnI during development; neither was there evidence for unique fetal forms of the α- and β-subunits of tropomyosin in their experiments. The ratio of α:β forms of tropomyosin decreased upon maturation of the heart, but this change was not associated with any variations in the pCa50 for activation of myofibrilar Mg-ATPase activity at pH 7.0, which remained nearly the same during development. This result is different from that reported by Fabiato (1982) and that obtained in this study, in which the pCa50 for activation of myofilament activity at pH 7.0 was shifted to the right by about 0.3 pCa unit in the perinatal compared to the adult dog heart myofibrils. The pCa-ATPase activity curves of adult and perinatal myofibrils do, in fact, overlay each other at pH 6.5, since the pCa-activity relation of the adult, but not the immature, preparations is shifted to the right by a reduction in pH from 7.0 to 6.5. Thus, data such as those of Humphreys and Cummins (1984), indicating no shifts in pCa-myofilament activity relations with development, may result from the particular conditions of the measurements or may be species related.

Although more experiments must be done to show clearly the exact thin filament variations that account for our observations, our present results point to the possibility that the lack of effect of acidic pH on calcium activation of the perinatal heart myofilaments may be due to a variant of TnI or TnT. As we argued previously, shifts in the pCa-myofilament force or ATPase activity relations induced by acidic pH are more likely to be due to thin filament proteins other than TnC. For example, studies with pure preparations of TnC indicate that protons and Ca** do not compete for the same binding site. Potter et al. (1977) reported that upon addition of Ca** to calcium-free TnC at neutral pH, there was a small release of protons that was not proportional to the amount of Ca** bound by the TnC. Moreover, Ogawa (1985) reported that Ca** binding to TnC is unaffected by changes in pH between 7.2 and 6.5. In the present work, the charge and general calcium-binding properties appear the same in the TnC of adult and immature cardiac myofilaments—a result arguing against a direct role for TnC as being responsible for differences in pCa50 for activation at pH 7.0, and in the differential responses of adult and immature cardiac myofilaments to acidic pH.

On the other hand, unlike TnC, cardiac troponin I (TnI) and troponin T (TnT) are basic proteins relatively rich in amino acid residues, such as histidine, which titrate protons in the range of pH 7.0 to 6.5. Moreover, analysis of adult and immature myofilaments by gel electrophoresis under various conditions suggests that variants of these proteins may exist. For example, evidence for a variant of TnI is indicated by the SDS-PAGE profiles shown in Figure 6. In the myofilaments prepared from perinatal dog hearts, there was a doublet in the region of migration of the adult TnI. These profiles on PAGE do not vary with protein phosphorylation, since we have obtained the same pattern shown in Figure 6 in preparations phosphorylated by incubation with cyclic adenosine 3',5'-monophosphate (cAMP) and cAMP-dependent protein kinase (data not shown). As mentioned above, the pattern, including the four low-molecular-weight thin-filament-associated polypeptides seen with the perinatal myofilaments, was obtained when they were prepared in the presence of proteinase inhibitors. In addition, limited autolysis of the adult myocardium and myofibrils never produced a pattern on SDS-PAGE, acid-urea, or alkaline-urea gels like that obtained with the perinatal myofilaments (Blanchard, 1983). Adult dog cardiac myofilaments contain 100% of the α-subunit of tropomyosin (Leger et al., 1976), and we have no evidence that the results presented here are due to alterations in the relative amounts of the β-unit subunit of tropomyosin. Although we know of no data on developmental transitions of isoforms of cardiac TnT, multiple TnT species have been identified in cardiac (Risnik et al., 1985) and in fast skeletal muscle (Briggs et al., 1984), and evidence for multiple splicing of the TnT gene suggests that many more isoforms could exist (Redford et al., 1984).

The developmental differences in thin filament regulation that are revealed by the differential sensitivity of the adult and perinatal cardiac myofilaments to Ca** and pH may account in part for differences in function during cardiac development. For example, in the experiments of Jarmakani et al. (1982), maximum inotropic effects of Ca** on adult rabbit hearts were achieved at 15 mM extracellular Ca**, but required 30 mM extracellular Ca** in the newborn heart. Although the factors responsible for this difference are likely to be multiple, it is possible this result is due partly to a relatively low Ca** sensitivity of the newborn heart myofilaments (Fig. 1). Differences in the Ca** sensitivity of the newborn heart myofilaments may also account in part for the fact that relaxation times of adult ventricular preparations are significantly slower than those of newborn hearts (Park et al., 1982).

The reduced effect of acidic pH on Ca** sensitivity...
of perinatal vs. adult heart myofilaments may also be important with regard to the ability of perinatal and neonatal dog hearts to maintain function during hypoxic episodes that impair function of adult hearts. Jarmakani et al. (1978a) reported that newborn, but not adult, hearts are capable of maintaining mechanical function during a 30-minute period of hypoxia. It was also shown that during the hypoxic episode, ATP concentrations were not different from controls in the newborn, but fell significantly in the adult hearts (Jarmakani et al., 1978b). This maintenance of ATP concentration was associated with a relatively greater glycolytic flux in newborn vs. adult hearts as lactate production increased significantly more in the newborn hearts during the hypoxia. This would presumably result in a relatively greater acidification of the newborn hearts, and it seems possible that the reduced sensitivity of the newborn cardiac myofilaments to acidic pH could be an important feature of their relative insensitivity to the hypoxic stress. Along these lines, Allen and Orchard (1983) have reported that intracellular Ca++ transients, measured using the bioluminescent protein aequorin, are under some conditions essentially unaffected in cardiac muscle preparations subjected to hypoxic episodes, although the amplitude of twitch force is depressed. They concluded that the hypoxia is associated with a reduction of the sensitivity of the cardiac myofilaments to free Ca++. Thus, in the case of the perinatal hearts, the lack of effects of acidic pH on the Ca++ sensitivity of the myofilaments may indeed be the major factor which permits these hearts to maintain function during hypoxia and intracellular acidification.

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