Title

Effects of cold cardioplegia on pH, Na, and Ca in newborn rabbit hearts

Journal

AJP Heart and Circulatory Physiology, 290(3)

ISSN

0363-6135

Authors

Anderson, Steven E

Liu, Hong

Beyschau, Andrea

et al.

Publication Date

2006-03-01

DOI

10.1152/ajpheart.00776.2004

Peer reviewed

Effects of cold cardioplegia on pH, Na, and Ca in newborn rabbit hearts

Steven E. Anderson, Hong Liu, Andrea Beyschau and Peter M. Cala

Am J Physiol Heart Circ Physiol 290:H1090-H1097, 2006. First published 14 October 2005; doi:10.1152/ajpheart.00776.2004

Am J Physiol Heart Circ Physiol 290: H1090–H1097, 2006.
First published October 14, 2005; doi:10.1152/ajpheart.00776.2004.

Effects of cold cardioplegia on pH, Na, and Ca in newborn rabbit hearts

Steven E. Anderson, Hong Liu, Andrea Beyschau, and Peter M. Cala
Department of Human Physiology, University of California, Davis, California

Submitted 2 August 2004; accepted in final form 6 October 2005

Anderson, Steven E., Hong Liu, Andrea Beyschau, and Peter M. Cala. Effects of cold cardioplegia on pH, Na, and Ca in newborn rabbit hearts. *Am J Physiol Heart Circ Physiol* 290: H1090–H1097, 2006. First published October 14, 2005; doi:10.1152/ajpheart.00776.2004.—Many studies suggest myocardial ischemia-reperfusion (I/R) injury results largely from cytosolic proton (H_i)-stimulated increases in cytosolic Na (Na_i), which cause Na/Ca exchange-mediated increases in cytosolic Ca concentration ($[Ca]_i$). Because cold, crystalloid cardioplegia (CCC) limits $[H]_i$, we tested the hypothesis that in newborn hearts, CCC diminishes H_i, Na_i, and Ca_i accumulation during I/R to limit injury. NMR measured intracellular pH (pH_i), Na_i, $[Ca]_i$, and ATP in isolated Langendorff-perfused newborn rabbit hearts. The control ischemia protocol was 30 min for baseline perfusion, 40 min for global ischemia, and 40 min for reperfusion, all at 37°C. CCC protocols were the same, except that ice-cold CCC was infused for 5 min before ischemia and heart temperature was lowered to 12°C during ischemia. Normal potassium CCC solution (NKCCC) was identical to the control perfusate, except for temperature; the high potassium (HKCCC) was identical to NKCCC, except that an additional 11 mmol/l KCl was substituted isosmotically for NaCl. NKCCC and HKCCC were not significantly different for any measurement. The following were different ($P < 0.05$). End-ischemia pH_i was higher in the CCC than in the control group. Similarly, CCC limited increases in Na_i during I/R. End-ischemia Na_i values (in meq/kg dry wt) were 115 ± 16 in the control group, 49 ± 13 in the NKCCC group, and 37 ± 12 in the HKCCC group. CCC also improved $[Ca]_i$ recovery during reperfusion. After 40 min of reperfusion, $[Ca]_i$ values (in nmol/l) were 302 ± 50 in the control group, 145 ± 13 in the NKCCC group, and 182 ± 19 in the HKCCC group. CCC limited ATP depletion during ischemia and improved recovery of ATP and left ventricular developed pressure and decreased creatine kinase release during reperfusion. Surprisingly, CCC did not significantly limit $[Ca]_i$ during ischemia. The latter is explained as the result of Ca release from intracellular buffers on cooling.

nuclear magnetic resonance; Na/Ca exchanger; Na/H exchanger; ischemia

RESULTS OF NUMEROUS STUDIES (2, 7, 10, 13, 15, 35, 37, 42, 44, 46, 48) are consistent with the interpretation that myocardial ischemia-reperfusion injury is in part caused by the following chain of events: *1*) decreased cytosolic pH (pH_i) stimulates Na-dependent pH regulation, *2*) increased Na uptake increases cytosolic Na (Na_i), *3*) increased Na_i decreases Ca efflux via Na/Ca exchange, and *4*) increased cytosolic Ca concentration ($[Ca]_i$) stimulates a cascade of events that cause injury. Controversy remains concerning whether the bulk of Ca entry occurs during ischemia or reperfusion, but there is near consensus that much of the Ca entry is a consequence of Na loading (13, 41). It has also been repeatedly demonstrated that cold, crystalloid cardioplegia (CCC) decreases the ischemia-induced fall in pH_i (6).

Because newborn and adult hearts respond differently to hypoxia, ischemia, and cardioplegia (6, 9, 11, 19, 37) and the need for basic science studies aimed at understanding and improving cardioplegia for newborns has not diminished (1, 24), we tested the hypothesis that in newborn rabbit hearts, CCC will limit increases in cytosolic [H], Na_i, and $[Ca]_i$ during ischemia-reperfusion and thereby diminish injury. The observed effects of CCC on [H], Na_i, and injury were not surprising and wholly consistent with the hypothesis.

The most important finding of this study, however, is that although $[Ca]_i$ recovered better during reperfusion after CCC, $[Ca]_i$ was not significantly diminished by CCC during ischemia. The latter was not expected and raises at least two major issues that, at least qualitatively, do not appear to be age related. First, given the growing consensus that ischemia-reperfusion-induced increases in $[Ca]_i$ are largely the result of increases in $[Na]_i$, what is the mechanism by which ischemia-induced increases in Na and Ca are dissociated by cooling? Second, if cold cardioplegia increases $[Ca]_i$, how can it limit ischemia-reperfusion injury? The former is explained as the result of an eminently predictable release of Ca from intracellular buffers with cooling (26, 28, 31, 39), such that the observed changes in pH_i, Na_i, and $[Ca]_i$ remain consistent with the general hypothesis modified for changes in temperature. The latter requires further study but has important implications for rational improvement of cardioplegia. Portions of this work have been reported in abstract form (4, 5). To our knowledge, this is the first report in which all ions pertinent to the hypothesis have been measured in ischemic myocardium with and without cold cardioplegia. Finally, by raising the issue of Na_i-independent increases in cytosolic [Ca] with myocardial cooling, this study lays the groundwork for future studies aimed at improving cardioplegia.

METHODS

General. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publications No. 85-23, Revised 1996). Animals used in this study were treated according to the American Physiological Society's "Guiding Principles in the Care and Use of Animals" with a protocal approved by the University of California, Davis. The methods used were modified from those previously reported (6, 10). New Zealand White rabbits (4–7 days old) were anesthetized (pentobarbital sodium 35–65 mg/kg ip) and heparinized (1,000 United States Pharmacopeia units/kg iv). Hearts were removed and Langendorff perfused at a constant rate between 9 and 10 ml/min, except during ischemia and cardioplegia infusion. Control perfusate contained (in mmol/l) 133 NaCl, 4.75 KCl, 1.25 $MgCl_2$, 1.82 $CaCl_2$, 25 $NaHCO_3$, 11.1 dextrose with a pH of 7.35–7.45 after equilibration with 95% O_2-5% CO_2. Perfusion pressure and left ventricular pressure

Address for reprint requests and other correspondence: S. E. Anderson, Dept. of Physiology & Membrane Biology, Univ. of California, 1 Shields Ave., Davis, CA 95616-8644 (e-mail: seanderson@ucdavis.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

were monitored continuously by strain-gauge transducers. Left ventricular developed pressure (LVDP = end-systolic pressure − end-diastolic pressure) was measured by a fluid-filled balloon, continuously adjusted to maintain end-diastolic pressure between 0 and 10 cmH_2O throughout the experiment. The control ischemia protocol was 30 min baseline perfusion, followed by 40 min global ischemia, followed by 40 min reperfusion, all at 37°C. Hearts in the cold, crystalloid cardioplegia (CCC) groups were treated the same, except that ice-cold CCC was infused for 5 min before ischemia and the variable temperature control on the spectrometer was set to 12°C during cardioplegia infusion and ischemia. For these hearts the temperature fell to ~25°C during the cold infusion and then fell to 12°C during the first 15 min of ischemia where it remained for the rest of the ischemic interval. During reperfusion, the spectrometer variable temperature control was returned to 37°C and heart temperature recovered to 33°C in 5 min and 37°C within 10 min. Solutions used for baseline and reperfusion were the same in all hearts. Two CCC solutions were tested: a normal potassium solution (NKCCC) and a high potassium solution (HKCCC). NKCCC was identical to control perfusate except for temperature; HKCCC was identical to NKCCC except an additional 11 mmol/l KCl was added (isosmotically substituted for NaCl). Initiation of ischemia was designated at $t = 0$ min. ^{23}Na, ^{19}F, and ^{31}P NMR were used to measure Na_i, $[Ca]_i$, and pH_i and high-energy phosphates, respectively. To measure Na_i, 7.5 mmol/l dysprosium triethylenetetraminehexaacetic acid was substituted isosmotically for NaCl in the perfusate and Ca was added to reach a perfusate concentration of 1.8–2 mmol/l (37). To measure $[Ca]_i$, hearts were perfused for 35 min before the baseline interval with perfusate containing the acetoxymethyl ester of 5-fluoro-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (FBAPTA) at 2.5 μmol/l (10). FBAPTA was then washed out of the extracellular space with control solution for 15 min before measurement of baseline $[Ca]_i$. After perfusions were completed, hearts in which ^{23}Na was measured were weighed wet, dried to constant weight (at least 48 h) at 65°C, and reweighed to determine dry weight. Their wet and dry weights were 0.555 ± 0.020 and 0.085 ± 0.003 g, respectively.

NMR spectroscopy. ^{23}Na, ^{19}F, and ^{31}P spectra were generated from the summed free induction decays of 1,000, 1,500, and 148 excitation pulses (90°, 45°, and 60°) with the use of 2K, 2K, and 4K word data files and ±4,000, ±5,000, and ±4,000 Hz sweep widths, respectively, with the use of a Bruker AMX400 spectrometer. Data files were collected over 5-min intervals, but to improve signal to noise for ^{19}F measurement of $[Ca]_i$, two 5-min ^{19}F files were added together, except for the 5-min interval in which CCC was infused (9). Because the spectra for each of the nuclei are generated by using signal averaging for 5-min (^{23}Na and ^{31}P) or 10-min (^{19}F) acquisition intervals, the data are represented in figures as points located in time midway between the beginning and end of the acquisition interval. Only one NMR nucleus (Na, P, or F) was measured from each heart.

Na_i (in meq/kg dry wt) was calculated from the calibrated area under the unshifted intracellular peak as previously described (9). Of note, in contrast to pH_i and $[Ca]_i$ measurements, the NMR method measures Na_i as an amount that we normalize to dry weight. Unless otherwise stated, to minimize assumptions and data manipulation, Na_i is reported in this way.

$[Ca]_i$ (in nmol/l cell water) was calculated as the product of the Ca-FBAPTA dissociation constant (K_d) and the ratio of the areas of the Ca-bound and Ca-free peaks (10). The amount of FBAPTA per area of ^{19}F spectrum was calibrated by using a 1-ml heart-sized phantom filled with 150 mmol/l KCl containing 1 μmol of FBAPTA (potassium salt). The area under the spectrum acquired by using parameters identical to those used for hearts was thus calibrated to represent 1 μmol of FBAPTA in heart spectra. With the use of this calibration and assuming cytosolic volume is 2.5 l/kg dry wt (7), the total [FBAPTA] in the cytosol (bound + free) was calculated as 27.5 ± 8.0 μmol/l, similar to values previously reported (33, 38). Rather than use K_d for Ca-FBAPTA as previously taken from the literature (10), it was determined from an Arrhenius plot, where K_d (in mol/l) = 10 exp[(1,964.2/K) − 13.02] (see APPENDIX). This equation describes the increase in K_d that occurs as temperature falls (27). Of note, FBAPTA has been previously shown not to enter intracellular organelles significantly, and the K_d for FBAPTA was not adjusted for changes in pH_i because the effect is not substantial over the pH range observed in these studies (38).

pH_i was determined from the chemical shift of P_i (6). High-energy phosphates are reported as percentage of control peak intensity (10). The intensity of the β-ATP peak was taken as representative of ATP.

To assess ischemic injury, total creatine kinase (CK) was measured from timed collections of perfusate exiting the heart. CK (in IU/g dry wt) was measured spectrophotometrically (43).

Unless stated otherwise, results are reported as means ± SE. Repeated-measures ANOVA was used to test for differences between treatments. Also, to test for differences between treatments rather than between animals, ATP and LVDP were compared after each was normalized to the percentage of its baseline value. When differences between treatments were found, the Student-Newman-Keuls multiple comparison test was used to determine the times at which differences occurred. Only the latter are indicated in the figures. Because overpowered studies waste resources (23) and raise serious ethical questions when animal or human subjects are involved (36), we have limited sample sizes to those necessary to test our hypotheses. Differences were considered significant when $P < 0.05$.

RESULTS

Intracellular proton accumulation. When compared with control, both NKCCC ($P < 0.0001$) and HKCCC ($P < 0.0003$) diminished the fall in pH_i during ischemia. The data in Fig. 1 demonstrate that at the end of 40 min of ischemia, pH_i was higher in CCC hearts than in control hearts [NKCCC (7.07 ± 0.12) vs. HKCCC (6.89 ± 0.13) vs. control (5.76 ± 0.08)]. In terms of pH_i, neither cardioplegia treatment was different from control except during ischemia, and there was no difference between the two cardioplegia treatments at any time.

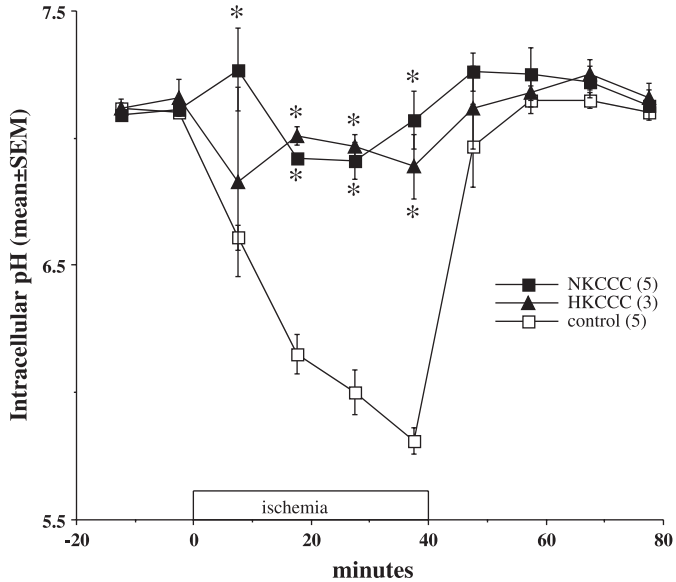

Fig. 1. In newborn rabbit hearts, cold, crystalloid cardioplegia (CCC) diminishes fall in intracellular pH during ischemia. Intracellular pH is plotted vs. time before, during, and after ischemia without cardioplegia (open squares) and after normal K CCC (NKCCC, 4.75 mmol/l, closed squares) and high K CCC (HKCCC, 15.75 mmol/l, closed triangles). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses.

Na_i accumulation. The data in Fig. 2 demonstrate that when compared with the control group, CCC diminished Na_i during ischemia and reperfusion (NKCCC, $P < 0.008$; and HKCCC, $P < 0.002$). During 40 min of ischemia, Na_i in the control group rose from 21 ± 4 to 115 ± 16 meq/kg dry wt and recovered to 64 ± 18 meq/kg dry wt during reperfusion. In NKCCC and HKCCC groups, the corresponding values were 25 ± 7, 49 ± 13, and 32 ± 4 and 27 ± 5, 37 ± 12, and 28 ± 3 meq/kg dry wt, respectively. For Na_i, NKCCC and HKCCC were not significantly different.

$[Ca]_i$ accumulation. The data in Fig. 3 demonstrate that when compared with control, cardioplegia treatments improved recovery of $[Ca]_i$ during reperfusion (NKCCC, $P < 0.038$; and HKCCC, $P < 0.033$). In the control group, $[Ca]_i$ rose from 186 ± 17 to 381 ± 62 nM during ischemia and recovered to 302 ± 50 nM during reperfusion. Corresponding values for NKCCC and HKCCC were 170 ± 12 to 467 ± 102 nM (with recovery to 145 ± 13 nM) and 191 ± 32 to 792 ± 237 nM (with recovery to 182 ± 19 nM), respectively. Possible reasons for this phenomena are included in DISCUSSION along with an explanation of the EqCal program simulation data (Fig. 3, closed circles). Of note, although $[Ca]_i$ is nominally greater in HKCCC than NKCCC during cooling and ischemia, the Student-Newman-Keuls test showed no significant difference between these two groups at any time. It is not clear whether this is due to small sample sizes and/or a lack of precision in measurement and/or a lack of effect of high K on $[Ca]_i$.

Myocardial high-energy phosphates. One prediction based on the preceding results is that CCC-diminished increases in Na_i and $[Ca]_i$ should result in diminished ATP consumption by active Na and Ca transport processes, and therefore CCC should diminish high-energy phosphate depletion during ischemia-reperfusion. Consistent with previous observations in adult and newborn hearts, the results shown in Fig. 4 demonstrate that this prediction was born out; compared with control, both CCC treatments diminished ATP depletion during ischemia and reperfusion (NKCCC, $P < 0.016$; and HKCCC, $P < 0.002$). In the control groups, ATP fell to 19 ± 3% of baseline

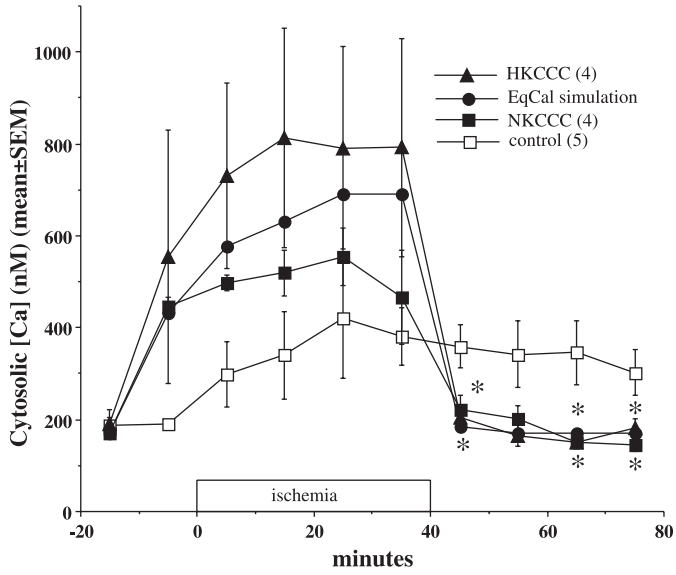

Fig. 3. In newborn rabbit hearts, CCC improves recovery of intracellular [Ca] during reperfusion. Cytosolic [Ca] (in nmol/l cell water) is plotted vs. time before, during, and after ischemia without cardioplegia (open squares), and after NKCCC (4.75 mmol/l, closed squares) and HKCCC (15.75 mmol/l, closed triangles). *$P < 0.05$ vs. control; number of experiments are shown in parentheses. NKCCC and HKCCC are not significantly different. Cold cardioplegia data are reasonably well fit by EqCal simulation (closed circles) using simplifying assumptions to calculate $[Ca]_i$ based on our measurements of temperature-dependent changes in the Ca-5-fluoro-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (FBAPTA) K_d combined with those of literature reports describing release of Ca from cytosolic buffers with cooling. All data, except closed circles, are corrected for temperature-dependent changes in Ca-FBAPTA K_d (see METHODS and APPENDIX).

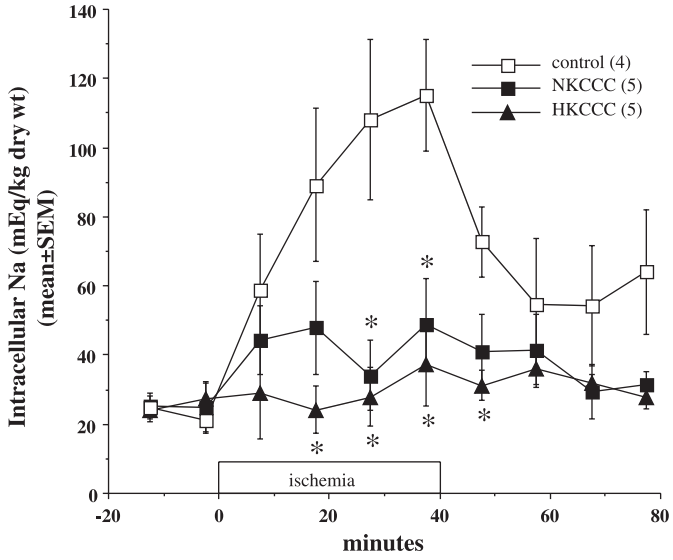

Fig. 2. In newborn rabbit hearts, CCC diminishes increase in intracellular Na during ischemia and improves recovery of Na early during reperfusion. Intracellular Na (in meq/kg dry wt) is plotted vs. time before, during, and after ischemia without cardioplegia (open squares) and after NKCCC (4.75 mmol/l, closed squares) and HKCCC (15.75 mmol/l, closed triangles). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses.

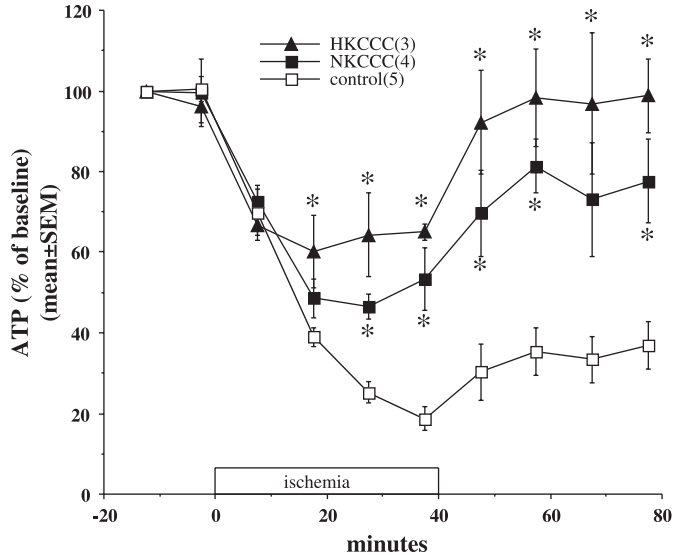

Fig. 4. In newborn rabbit hearts, CCC diminishes depletion of ATP during ischemia and improves its recovery during reperfusion. β-ATP (% of baseline intensity) is plotted vs. time before, during, and after ischemia without cardioplegia (open squares) and after NKCCC (4.75 mmol/l, closed squares) and HKCCC (15.75 mmol/l, closed triangles). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses.

during ischemia and recovered to 37 ± 6% during reperfusion. The corresponding values for NKCCC and HKCCC were 53 ± 8 to 78 ± 10% and 65 ± 2 to 99 ± 9%, respectively. There were no significant differences in ATP between NKCCC and HKCCC.

Functional recovery. As also predicted by the hypothesis and as previously observed in newborn and adult hearts, two measures of functional recovery were improved by the cardioplegia treatments. When compared with the control group, LVDP (Fig. 5) recovered better in both NKCCC ($P < 0.003$) and HKCCC ($P < 0.021$) groups. Similarly, CK release during reperfusion (Fig. 6) was diminished compared with the control group in NKCCC ($P < 0.040$) and HKCCC ($P < 0.029$). Again, for both LVDP and CK release, there were no significant differences between the HKCCC and NKCCC solutions.

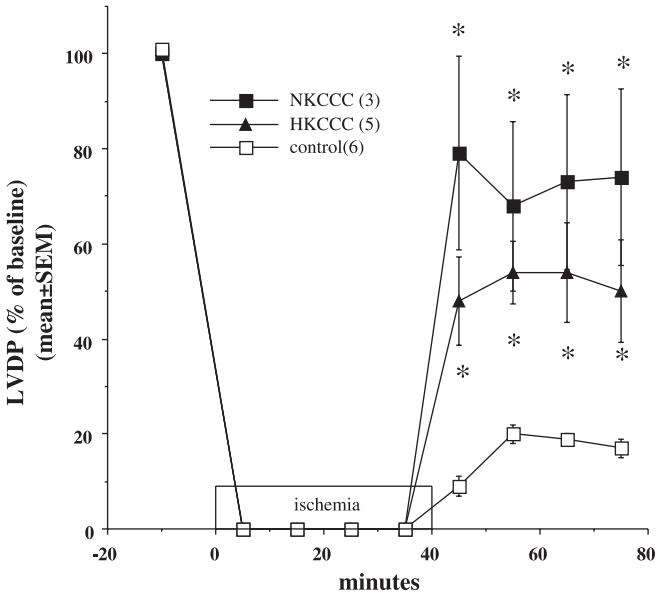

Fig. 5. In newborn rabbit hearts, CCC improves recovery of left ventricular developed pressure (LVDP) during reperfusion. LVDP (% of control) is plotted vs. time before, during, and after ischemia without cardioplegia (open squares) and after NKCCC (4.75 mmol/l, closed squares) and HKCCC (15.75 mmol/l, closed triangles). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses.

DISCUSSION

It is beyond question that appropriately formulated cardioplegia improves recovery of adult and newborn myocardium after ischemia. However, further improvements in cardioplegic formulations must be based on understanding the mechanism(s) of ischemia-induced injury. The aim of this study was not to improve on state-of-the-art cardioplegia per se but rather to provide a firmer foundation for understanding the mechanism(s) of cardioplegia. Thus we tested two very simple cardioplegia solutions within the context of the broadly accepted general hypothesis that hypoxic/ischemic injury is the result of the following chain of events: *1*) anaerobic metabolism increases $[H]_i$, *2*) Na-dependent pH-regulatory mechanisms (e.g., Na/H exchange and Na-dependent HCO_3 transport) are stimulated, *3*) Na uptake and Na_i are increased, *4*) the driving force for Ca efflux via Na/Ca exchange is decreased or reversed, *5*) $[Ca]_i$ is increased, and *6*) Ca-dependent processes

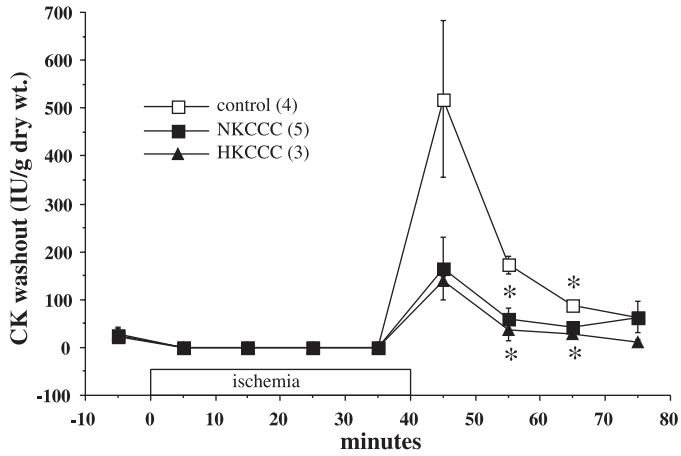

Fig. 6. In newborn rabbit hearts, CCC decreases release of creatine kinase (CK) during reperfusion. CK release (IU/g dry wt) is plotted vs. time before ischemia and during reperfusion without cardioplegia (open squares) and after NKCCC (4.75 mmol/l, closed squares) and HKCCC (15.75 mmol/l, closed triangles). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses.

lead to injury (2, 7, 10, 13, 15, 35, 37, 42, 44, 46, 48). From this hypothesis, one predicts that the well-known effect of cardioplegia in limiting the fall in pH_i during ischemia would thus also limit increases in, and improve recovery of, Na_i and $[Ca]_i$ during ischemia-reperfusion, which should, in turn, limit injury and improve functional recovery.

The studies described here are the first to report results for all the relevant ions (H, Na, and Ca) consistent with the prediction in newborn hearts. As such, it provides a stronger foundation for understanding the benefits of cardioplegia. Perhaps more importantly, however, the results demonstrate that CCC limitation of Na_i accumulation during ischemia is not accompanied by significant limitation of $[Ca]_i$. One simple explanation for this result is provided in *Cardioplegia does not significantly limit increases in $[Ca]_i$ during ischemia* and raises questions about the limitations of cold cardioplegia. On the other hand, contrary to a growing consensus that a major portion of ischemia-reperfusion-induced injury is the result of increases in $[Ca]_i$, this work suggests that the protective effects of cold cardioplegia are not the result of limiting increases in $[Ca]_i$.

Cardioplegia limits ischemia-induced changes in pH_i and Na_i. Our results (Fig. 1) reconfirm that CCC diminishes the fall in pH_i otherwise observed during ischemia (6, 50). Again, one predicts from the general hypothesis that, after cardioplegia, diminished proton accumulation will decrease stimulation of Na-dependent pH regulation and thereby diminish Na uptake (16, 49) and Na_i accumulation during ischemia (7, 10). These predictions are born out by the data in Fig. 2.

Cardioplegia improves recovery of $[Ca]_i$ during reperfusion. In addition, as predicted by the general hypothesis, limiting cytosolic proton and Na accumulation during ischemia with NKCCC and HKCCC (Figs. 1 and 2) is associated with improved recovery of $[Ca]_i$ during reperfusion (Fig. 3), which, in turn, leads to improved recovery of LVDP (Fig. 5) and diminished injury (CK release) during reperfusion (Fig. 6).

Cardioplegia does not significantly limit increases in $[Ca]_i$ during ischemia. As outlined by the general hypothesis, the effect of CCC-limiting increases in Na_i during ischemia is

predicted to limit entry of Ca into the cytosol via Na/Ca exchange. At first glance, one might conclude that the calcium data (Fig. 3) are inconsistent with that prediction; i.e., although Na_i is limited by CCC, $[Ca]_i$ rises rapidly during NKCCC to a level significantly greater than the level during control perfusion, and there is no significant difference between either CCC and control $[Ca]_i$ groups at the end of ischemia. However, the simplest explanation for this observation is that increases in $[Ca]_i$ during CCC are the result of Ca release from Ca_i buffers, of which the K_d values increase with cooling. Literature (26, 28, 30, 31) reports for the effects of temperature on Ca binding by the most important buffers [e.g., cardiac troponin C (TnC)] are consistent with this argument; i.e., with cooling, Ca will dissociate from passive buffer sites. Thus, to most effectively interpret the $[Ca]_i$ data, we must assess the contribution of changes in the K_d values of FBAPTA (experimentally added Ca indicator) and important physiological Ca buffers to cooling-induced changes in $[Ca]_i$. Given a passive buffer capacity for Ca_i in adult and newborn hearts of 50–200 $\mu mol \cdot l^{-1} \cdot pCa^{-1}$ (14, 22, 39) and literature values characterizing the major passive buffers (26, 28, 30, 39), one can use the EqCal program to calculate equilibrium [Ca] for multiple buffers (see APPENDIX). In this case, literature values for high-affinity sarcolemmal binding (hiSARC) and TnC along with those measured in this laboratory for FBAPTA were inserted in the program to create the EqCal data indicated by closed circles in Fig. 3. The closed circles represent how $[Ca]_i$ would change with cooling if no Ca transport occurred, but, rather, Ca was only released from intracellular passive buffers. The EqCal data are similar to data for both CCC protocols at the end of ischemia. Furthermore, if FBAPTA is removed from the model, $[Ca]_i$ rises even higher with cooling (to 732 nmol/l without FBAPTA vs. 690 nmol/l with FBAPTA). If the model is accurate, this means FBAPTA is modestly limiting cooling-induced increases in $[Ca]_i$, so the value we measure is less than that which would otherwise occur at the end of ischemia after cooling. This occurs, in part, because the FBAPTA K_d is near the physiological $[Ca]_i$ range and it increases less than the K_d for major physiological buffers as temperature falls (26, 39); i.e., to some extent, when the physiological buffers release Ca on cooling, a portion of it is taken up by FBAPTA.

Furthermore, a thermodynamic analysis of the data is consistent with the hypothesis that the observed increase in $[Ca]_i$ during ischemia after CCC is not due to Ca entry via Na/Ca exchange; i.e., the force driving flux through the exchanger would cause net Ca efflux during ischemia after both of the CCC protocols. We can calculate the equilibrium or reversal potential for Na/Ca exchange based on the following: *1*) before ischemia, extracellular concentrations of Na and Ca ($[Na]_o$ and $[Ca]_o$, respectively) are equal to perfusate concentrations; *2*) we measure Na_i and $[Ca]_i$ before, during, and after ischemia; and *3*) because the perfused heart is a closed system during ischemia, we can calculate changes in $[Na]_o$ and $[Ca]_o$ from changes in Na_i and $[Ca]_i$. Having done so, we can predict for each data point whether the Na/Ca exchanger can mediate net Ca influx or efflux. More specifically, we make the following simplifying assumptions. First, in the crystalloid perfused heart intra- and extracellular (intravascular plus interstitial) volumes are equal, so changes in $[Na]_i$ and $[Na]_o$ are equal and opposite. Second, changes in $[Ca]_o$ are negligible (7). Finally, if the Na/Ca exchanger has a stoichiometry of three Na for one Ca, the equilibrium or reversal potential ($E_{Na/Ca}$) = $RT/F \ln([Ca]_i/[Ca]_o) - 3RT/F \ln([Na]_i/[Na]_o)$, where R is ideal gas constant, T is absolute temperature, and F is Faraday's constant. Assuming the exchanger is active, if the cell membrane potential (E_m) is more negative than $E_{Na/Ca}$, the Na/Ca exchanger will mediate net Ca efflux; if $E_m = E_{Na/Ca}$, there will be no net flux (equilibrium); and if E_m is more positive than $E_{Na/Ca}$, the Na/Ca exchanger will mediate net Ca influx. The data presented in Figs. 2 and 3 can thus be used to demonstrate the following. Under baseline conditions, $E_{Na/Ca}$ is not significantly different between groups and varies between −28 and −35 mV. Under these conditions, the heart is beating and the average E_m must be more negative than $E_{Na/Ca}$. Thus the Na/Ca exchanger mediates net Ca efflux. However, during the four consecutive 10-min intervals of ischemia after HKCCC, $E_{Na/Ca}$ was −8, 8, −3, and −25 mV, respectively. For HKCCC, E_m is likely to be a few millivolts more positive than E_K {$E_K \approx -53$ mV because $[K]_o$ starts at 15.75 mmol/l (45)} but always more negative than $E_{Na/Ca}$. Therefore, the driving force for the Na/Ca exchanger during ischemia after HKCCC would tend to drive Ca out of the cell. Similarly, for NKCCC during ischemia, $E_{Na/Ca}$ remains near, or more positive than, E_m (40) as $E_{Na/Ca}$ falls from −50 to −59 mV. This means that during ischemia the driving force for the Na/Ca exchanger is not directed so as to mediate Ca uptake for either of the CCC protocols. In comparison, $E_{Na/Ca}$ for the control group falls from −85 to −130 mV during ischemia. Given the consensus that ischemia causes depolarization, the latter values indicate that for the control group, the driving force for the Na/Ca exchanger would cause Ca uptake throughout ischemia [consistent with the general hypothesis and previous reports (47)]. {Please note, assumptions regarding intra- and extracellular volumes are based on our previous work as well as that of others (8–10, 21, 29). However, if there were a 10–20% increase in cell volume during ischemia (32), our calculations would overestimate changes in $[Na]_i$ and underestimate changes in $[Na]_o$. The latter would also be true if extracellular (nonbath) volume decreased regardless of changes in intracellular volume. Either way, the net result would be that our calculations would overestimate the driving force for Ca entry via Na/Ca exchange. Thus including cell swelling and/or decreased vascular/interstitial volumes in our estimates for the force driving Ca entry via Na/Ca exchange would provide more support for the above argument that the observed increase in $[Ca]_i$ is not due to influx via Na/Ca exchange.}

Thus the simplest interpretation of our results is that there is little net Ca transport into or out of the cytosol during CCC, but rather, the observed increase in $[Ca]_i$ is the result of dissociation from passive buffers on cooling. This is consistent with the simple EqCal calculations for Ca dissociation from FBAPTA, TnC, and a hiSARC site shown in Fig. 3. More complicated explanations for this result are too numerous to list, but because the data are well fit by temperature-dependent changes in passive buffer K_d, all other explanations must entail a balanced addition and removal of Ca from the cytosol, e.g., Ca efflux from sarcoplasmic reticulum (SR) balanced by efflux via sarcolemmal Na/Ca exchange (or vice versa) and/or various combinations of balanced uptake and release from other cytosolic Ca buffer sites. Furthermore, this simple explanation is consistent with explanations given for hypothermic inotropy that argue that cooling increases $[Ca]_i$, whereas flux via Ca

channels and Na/Ca exchange are temperature limited and SR Ca content is essentially unaltered (14). Thus, although we cannot rule out contributions of Ca channels and Na/Ca exchange to increases in $[Ca]_i$ during CCC, experts have argued their contribution is not large enough to account for the observed data (14). The interpretation is also consistent with the finding that Ca binding to TnC (a major Ca buffer) has positive enthalpy (31), which, according to Le Chatelier's principle, provides further evidence that the K_d for TnC-Ca binding increases as temperature falls. This interpretation is also supported by recent results demonstrating that the $K_{1/2}$ for bovine cardiac TnC-Ca binding rises from 3.8 μmol/l at 37°C to 10.7 μmol/l at 7°C (26). Not surprisingly, temperature-induced changes in Ca buffer binding have also been identified as an important determinant of the temperature dependence of skinned fiber sensitivity to [Ca] (28). Therefore, the increase in $[Ca]_i$ observed during CCC need not be inconsistent with the hypothesis that CCC limits transport of Ca into the cytosol. Instead, because the observed increase can be explained almost completely as the result of dissociation from cytosolic buffers, the data are consistent with the argument that there is no important net entry of Ca into the cytosol during ischemia after CCC; i.e., if net entry occurred, $[Ca]_i$ measured after CCC would have had to increase even more.

Not to be overlooked, however, the data in Fig. 3 demonstrate that both NKCCC and HKCCC improve $[Ca]_i$ recovery during reperfusion. This result is not only consistent with the general hypothesis (see INTRODUCTION) but also with those of investigators who have argued limiting intracellular proton and Na_i accumulation during ischemia should improve recovery of $[Ca]_i$ during reperfusion; i.e., it has been postulated that during reperfusion, kinetic and thermodynamic limitations on Na/H exchange and Na-dependent HCO_3 transport are removed to allow a surge of Na uptake in response to intracellular proton buildup (3). Limiting proton buildup with cardioplegia will diminish this response. Furthermore, limiting Na_i accumulation during ischemia would allow Na_i recovery earlier during reperfusion (41) (after Na/K pump function is restored), so the amount of time the thermodynamic driving force for Na/Ca exchange favors Ca influx is minimized and that favoring Ca efflux is maximized. This will limit increases in $[Ca]_i$ and improve its recovery. Data in Figs. 1–3 are all consistent with these postulates.

Further studies are required to explain how increases in $[Ca]_i$ during ischemia after cardioplegia, which are not significantly different from control, can be associated with better recovery and less CK release than control. One could argue that this is consistent with reports (3) concluding the most damaging Ca influx occurs during reperfusion, which, in this case, is limited as described above by relatively low $[Na]_i$ and $[H]_i$. On the other hand, there is evidence that in addition to limiting proton production, cooling limits Ca-dependent events that cause injury. For example, in skeletal muscle the activity of calpains 1 and 2 both decrease as temperature falls from 25 to 4°C (18), and the autolysis rate of μ-calpain increases with temperature as well as proton concentration, such that cold cardioplegia would limit generation of subunits with Ca K_m values in the low micromolar range (34). Furthermore, it is not unreasonable to speculate that, as previously shown for cytosolic Ca buffers, K_d values for Ca binding to degradative enzymes may increase as temperature falls and thus limit the otherwise injurious

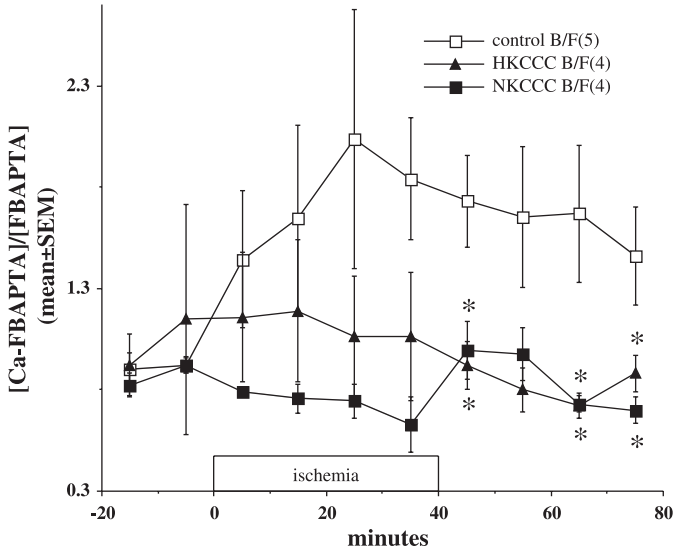

Fig. 7. In newborn rabbit hearts, ratio of Ca-bound FBAPTA to Ca-free FBAPTA (B/F) is nominally greater during ischemia and significantly greater during reperfusion under normothermic conditions (control, open squares) than after cooling with NKCCC (4.75 mmol/l, closed squares) or HKCCC (15.75 mmol/l, closed triangles). Because Ca-FBAPTA K_d increases as temperature falls (as do K_d values for other major intracellular Ca buffers), relative amount of FBAPTA bound to Ca changes little even though $[Ca]_i$ rises with cooling (see Fig. 3). NKCCC and HKCCC are not significantly different. *$P < 0.05$ vs. control; number of experiments are shown in parentheses. See DISCUSSION for more detailed explanation and implications of this phenomenon.

effects of increased $[Ca]_i$ observed during cold cardioplegia. This possibility is illustrated using FBAPTA as an example in Fig. 7, which shows the ratio of Ca-bound FBAPTA to Ca-free FBAPTA (i.e., [Ca-FBAPTA]/[FBAPTA]) for the control group and the two cardioplegia treatments. The data shown in Fig. 7 are derived from the same raw data as that shown in Fig. 3 but graphed to illustrate Ca binding rather than free Ca. Figure 7 illustrates that even though $[Ca]_i$ rises nominally during ischemia after cardioplegia, because the FBAPTA K_d increases as temperature falls, the change in Ca binding to FBAPTA is relatively small and in this case not measurable. Thus, if Ca-binding to Ca-dependent degradative enzymes changes with temperature as it does for FBAPTA (or TnC or hiSARC), cooling could limit increases in Ca binding to degradative enzymes and thus limit injury even if $[Ca]_i$ rises.

Finally, our results for pH_i, Na_i, and functional recovery are consistent with previously published results for adult hearts exposed to cold cardioplegia (6, 12). On the other hand, changes in $[Ca]_i$ during ischemia after cold cardioplegia are less predictable due in part to great variation in treatments that could alter $[Ca]_i$ (and, therefore, buffer loading) before cooling. To our knowledge, the results we report are the first measurements of changes in Na_i and $[Ca]_i$ in the intact newborn heart during and after cold cardioplegia. Although Camara et al. (17) used a protocol very different from ours, their results in adult guinea pigs are also predicted from the general hypothesis. They perfused at 37°C with and without high magnesium, high potassium cardioplegia (CP and KR, respectively) with and without the Na/H exchanger inhibitor eniporide before cooling to 17°C. With CP, $[Ca]_i$ fell before cooling, but, consistent with our results, $[Ca]_i$ rose during cooling both with and without CP. Although they did not measure changes

in Na_i, as predicted by the general hypothesis, eniporide improved recovery of $[Ca]_i$ and function. This result is also similar to previously reported results from rat hearts demonstrating Na/H exchange inhibition during normothermic cardioplegic arrest-limited Na_i accumulation during ischemia and improved recovery during reperfusion (20). Our result is also consistent with a more recent report (25) that lowering $[Ca]_o$ in, or adding the Na/H exchange inhibitor cariporide to, the cardioplegic solution is equally effective in improving function during reperfusion after ischemia.

In conclusion, data presented in this study are the first to demonstrate that in the newborn rabbit heart, both NKCCC (4.75 mmol/l) and HKCCC (15.75 mmol/l) at 12°C diminished intracellular proton and Na accumulation during ischemia and that limiting increases in these ions was associated with improved recovery of ATP, $[Ca]_i$, and ventricular function and decreased injury (CK release) during reperfusion. The results are consistent with the hypothesis that, as shown previously for normothermic conditions, ischemia-reperfusion-induced injury is secondary to increases in $[Ca]_i$ and that these can be mitigated by limiting Na uptake (41). The data are further consistent with the hypothesis that increases in $[Ca]_i$ during ischemia after cold cardioplegia are to a large extent the result of Ca release from passive intracellular buffers, such as TnC and hiSARC sites (26, 39). Thus, although cooling induces increases in $[Ca]_i$, to a large extent, low temperature must also limit Ca-dependent chemical reactions and protect against their consequences. Nevertheless, our results lead us to further speculate that because increased $[Ca]_i$ is an unavoidable physical consequence of cooling cytosolic Ca buffers, manipulations that limit Na and Ca uptake in warmer cardioplegia hold more promise for cytoprotection and should be exploited.

APPENDIX

Modeling Changes in Ca_i Buffering With Temperature

The data indicated by closed circles in Fig. 3 were generated by using the program EqCal (Biosoft, Cambridge, UK) and the following assumptions with starting values from the literature and this laboratory. Only three Ca buffers were included in the model: FBAPTA, hiSARC site, and TnC (30). K_d values (in mol/l) for the buffers were calculated from Arrhenius plots: FBAPTA $K_d = 10 \exp[(1964.2/K) - 13.02]$, analogous to that previously reported for dibromo-BAPTA (27); hiSARC $K_d = 10 \exp[(2856.1/K) - 16.61]$ (39); TnC $K_d = 10 \exp[(1297.8/K) - 9.6389]$ (26). Rather than again using a K_d for FBAPTA from the literature (10), the equation for FBAPTA used here was generated from the mean of three experiments in which $[Ca]_i$ was measured between 12° and 37°C by calcium electrode and the Ca-free-to-Ca-bound FBAPTA ratios for the same temperatures were measured by NMR as described in methods. These measurements were made in a solution at pH 7.0 containing 150 mmol/l KCl, 10 mmol/l HEPES, the cell impermeant potassium salt of FBAPTA, and ≈150 nmol/l free [Ca] (the latter varying with temperature). Equations for hiSARC and TnC were derived by using the data from Marengo et al. (39) and Gillis et al. (26). Total concentrations for calcium, hiSARC, and TnC were 48.5, 40, and 28 μmol/l, respectively (30), and [FBAPTA] was measured as described in METHODS as 27.5 μmol/l.

GRANTS

This work was supported during the tenure of a Postdoctoral Research Fellowship from the American Heart Association, California Affiliate and by National Heart, Lung, and Blood Institute Grants HL-07682 and 1-RO1-HL-56681. NMR spectrometer expense was funded in part by National Institutes of Health RR-02511 and National Science Foundation PCM-8417289.

REFERENCES

1. **Allen BS, Barth MJ, and Ilbawi MN.** Pediatric myocardial protection: an overview. *Semin Thorac Cardiovasc Surg* 13: 56–72, 2001.
2. **Allen DG, Cairns SP, Turvey SE, and Lee JA.** Intracellular calcium and myocardial function during ischemia. *Adv Exp Med Biol* 346: 19–29, 1993.
3. **Allen DG and Xiao XH.** Role of the cardiac Na^+/H^+ exchanger during ischemia and reperfusion. *Cardiovasc Res* 57: 934–941, 2003.
4. **Anderson S, Beyschau A, Liu H, and Cala PM.** Cardioplegia, Na, and Ca in ischemic newborn myocardium (Abstract). *FASEB J* 12: A710, 1998.
5. **Anderson S and Cala PM.** Are increases in cytosolic [Ca] during cold cardioplegia Na dependent? (Abstract) *FASEB J* 17: A1265, 2003.
6. **Anderson SE, Carr LJ, Schierling TD, and Kost GJ.** Are age-related differences in response to myocardial ischemia and cardioplegia pH dependent? *Biol Neonate* 65: 25–35, 1994.
7. **Anderson SE, Dickinson CZ, Liu H, and Cala PM.** Effects of Na-K-2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion. *Am J Physiol Cell Physiol* 270: C608–C618, 1996.
8. **Anderson SE and Johnson JA.** Tissue-fluid pressure measured in perfused rabbit hearts during osmotic transients. *Am J Physiol Heart Circ Physiol* 252: H1127–H1137, 1987.
9. **Anderson SE, Liu H, Ho HS, Lewis EJ, and Cala PM.** Age-related differences in Na^+-dependent Ca^{2+} accumulation in rabbit hearts exposed to hypoxia and acidification. *Am J Physiol Cell Physiol* 284: C1123–C1132, 2003.
10. **Anderson SE, Murphy E, Steenbergen C, London RE, and Cala PM.** Na/H exchange in myocardium: effects of hypoxia and acidification on Na and Ca. *Am J Physiol Cell Physiol* 259: C940–C948, 1990.
11. **Artman M, Ichikawa H, Avkiran M, and Coetzee WA.** Na^+/Ca^{2+} exchange current density in cardiac myocytes from rabbits and guinea pigs during postnatal development. *Am J Physiol Heart Circ Physiol* 268: H1714–H1722, 1995.
12. **Askenasy N, Vivi A, Tassini M, and Navon G.** Efficient limitation of intracellular edema and sodium accumulation by cardioplegia is dissociated from recovery of rat hearts from cold ischemic storage. *J Mol Cell Cardiol* 31: 1795–1808, 1999.
13. **Avkiran M, Gross G, Karmazyn M, Klein H, Murphy E, and Ytrehus K.** Na^+/H^+ exchange in ischemia, reperfusion and preconditioning. *Cardiovasc Res* 50: 162–166, 2001.
14. **Bers DM.** *Excitation-Contraction Coupling and Cardiac Contractile Force.* Dordrecht: Kluwer, 1991.
15. **Cala PM, Anderson SE, and Cragoe EJ.** Na/H exchange-dependent cell volume and pH regulation and disturbances. *J Comp Biochem Physiol* 90A: 551–555, 1988.
16. **Cala PM and Maldonado HM.** pH regulatory Na/H exchange by Amphiuma red blood cells. *J Gen Physiol* 103: 1035–1054, 1994.
17. **Camara AK, An J, Chen Q, Novalija E, Varadarajan SG, Schelling P, and Stowe DF.** Na^+/H^+ exchange inhibition with cardioplegia reduces cytosolic $[Ca^{2+}]$ and myocardial damage after cold ischemia. *J Cardiovasc Pharmacol* 41: 686–698, 2003.
18. **Cena P, Jaime I, Beltran JA, and Roncales P.** Proteolytic activity of isolated lamb calpains on myofibrils under the conditions of pH, Ca^{2+} concentration and temperature existing in postmortem muscle. *Z Lebensm Unters Forsch* 194: 248–251, 1992.
19. **Chen F, Jarmakani JM, and Van Dop C.** Developmental changes in mRNA encoding cardiac Na^+/H^+ exchanger (NHE-1) in rabbit. *Biochem Biophys Res Commun* 212: 960–967, 1995.
20. **Choy IO, Schepkin VD, Budinger TF, Obayashi DY, Young JN, and DeCampli WM.** Effects of specific sodium/hydrogen exchange inhibitor during cardioplegic arrest. *Ann Thorac Surg* 64: 94–99, 1997.
21. **Clarke K, Anderson RE, Nedelec JF, Foster DO, and Ally A.** Intracellular and extracellular spaces and the direct quantification of molar intracellular concentrations of phosphorus metabolites in the isolated rat heart using ^{31}P NMR spectroscopy and phosphonate markers. *Magn Reson Med* 32: 181–188, 1994.
22. **Creazzo TL and Godt RE.** Calcium buffering and excitation-contraction coupling in developing heart (Abstract). *FASEB J* 17: A954, 2003.
23. **Daniel WW.** *Biostatistics: A Foundation for Analysis in the Health Sciences* (5th ed.). New York: Wiley, 1991.

24. **Feng J, Li H, and Rosenkranz ER.** K(ATP) channel opener protects neonatal rabbit heart better than St. Thomas' solution. *J Surg Res* 109: 69–73, 2003.
25. **Fukuhiro Y, Wowk M, Ou R, Rosenfeldt F, and Pepe S.** Cardioplegic strategies for calcium control: low Ca(2+), high Mg(2+), citrate, or Na(+)/H(+) exchange inhibitor HOE-642. *Circulation* 102, 19 *Suppl* 3: III319–III325, 2000.
26. **Gillis TE, Marshall CR, Xue XH, Borgford TJ, and Tibbits GF.** Ca^{2+} binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. *Am J Physiol Regul Integr Comp Physiol* 279: R1707–R1715, 2000.
27. **Harrison SM and Bers DM.** The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim Biophys Acta* 925: 133–143, 1987.
28. **Harrison SM and Bers DM.** Modification of temperature dependence of myofilament Ca sensitivity by troponin C replacement. *Am J Physiol Cell Physiol* 258: C282–C288, 1990.
29. **Ho HS, Liu H, Cala PM, and Anderson SE.** Hypertonic perfusion inhibits intracellular Na and Ca accumulation in hypoxic myocardium. *Am J Physiol Cell Physiol* 278: C953–C964, 2000.
30. **Hove-Madsen L and Bers DM.** Passive Ca buffering and SR Ca uptake in permeabilized rabbit ventricular myocytes. *Am J Physiol Cell Physiol* 264: C677–C686, 1993.
31. **Jacobson AL, Devin G, and Braun H.** Thermal denaturation of beef cardiac troponin and its subunits with and without calcium ion. *Biochemistry* 20: 1694–1701, 1981.
32. **Jennings RB, Reimer KA, and Steenbergen C.** Myocardial ischemia revisited. The osmolar load, membrane damage, and reperfusion. *J Mol Cell Cardiol* 18: 769–780, 1986.
33. **Kirschenlohr HL, Metcalfe JC, Morris PG, Rodrigo GC, and Smith GA.** Ca^{2+} transient, Mg^{2+}, and pH measurements in the cardiac cycle by ^{19}F NMR. *Proc Natl Acad Sci USA* 85: 9017–9021, 1988.
34. **Koohmaraie M.** Effect of pH, temperature, and inhibitors on autolysis and catalytic activity of bovine skeletal muscle mu-calpain. *J Anim Sci* 70: 3071–3080, 1992.
35. **Lazdunski M, Frelin C, and Vigne P.** The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J Mol Cell Cardiol* 17: 1029–1042, 1985.
36. **Lenth RV.** Some practical guidelines for effective sample size determination. *American Statistician* 55: 187–193, 2001.
37. **Liu H, Cala PM, and Anderson SE.** Ethylisopropylamiloride diminishes changes in intracellular Na, Ca, and pH in ischemic newborn myocardium. *J Mol Cell Cardiol* 29: 2077–2086, 1997.
38. **Marban E, Kitakaze M, Koretsune Y, Yue DT, Chacko VP, and Pike MM.** Quantification of $[Ca^{2+}]i$ in perfused hearts. Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. *Circ Res* 66: 1255–1267, 1990.
39. **Marengo FD, Wang SY, and Langer GA.** The effects of temperature upon calcium exchange in intact cultured cardiac myocytes. *Cell Calcium* 21: 263–273, 1997.
40. **Mitani A and Shattock MJ.** Role of Na-activated K channel, Na-K-Cl cotransport, and Na-K pump in $[K]_e$ changes during ischemia in rat heart. *Am J Physiol Heart Circ Physiol* 263: H333–H340, 1992.
41. **Murphy E, Cross H, and Steenbergen C.** Sodium regulation during ischemia versus reperfusion and its role in injury. *Circ Res* 84: 1469–1470, 1999.
42. **Pike MM, Luo CS, Clark MD, Kirk KA, Kitakaze M, Madden MC, and Cragoe EJ Jr, and Pohost GM.** NMR measurements of Na^+ and cellular energy in ischemic rat heart: role of Na^+-H^+ exchange. *Am J Physiol Heart Circ Physiol* 265: H2017–H2026, 1993.
43. **Ramasamy R, Liu H, Anderson S, Lundmark J, and Schaefer S.** Ischemic preconditioning stimulates sodium and proton transport in isolated rat hearts. *J Clin Invest* 96: 1464–1472, 1995.
44. **Scholz W and Albus U.** Na^+/H^+ exchange and its inhibition in cardiac ischemia and reperfusion. *Basic Res Cardiol* 88: 443–455, 1993.
45. **Snabaitis AK, Shattock MJ, and Chambers DJ.** Comparison of polarized and depolarized arrest in the isolated rat heart for long-term preservation. *Circulation* 96: 3148–3156, 1997.
46. **Steenbergen C, Fralix TA, and Murphy E.** Role of increased cytosolic free calcium concentration in myocardial ischemic injury. *Basic Res Cardiol* 88: 456–470, 1993.
47. **Steenbergen C, Perlman ME, London RE, and Murphy E.** Mechanism of preconditioning. *Circ Res* 72: 112–125, 1993.
48. **Tani M and Neely JR.** Role of intracellular Na^+ and Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. *Circ Res* 65: 1045–1056, 1989.
49. **Vandenberg JI, Metcalfe JC, and Grace AA.** Mechanisms of pH_i recovery after global ischemia in the perfused heart. *Circ Res* 72: 993–1003, 1993.
50. **Whitman GJ, Roth RA, Kieval RS, and Harken AH.** Evaluation of myocardial preservation using ^{31}P NMR. *J Surg Res* 38: 154–161, 1985.