L-type Ca\(^{2+}\) channel function in the avian embryonic heart after cardiac neural crest ablation

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Persistent truncus arteriosus (PTA) represents 2–4% of congenital heart anomalies and is the most severe of the neural crest-associated heart defects (5). PTA is characterized by a failure of the cardiac outflow vessel to septate into aortic and pulmonary arteries, and its presence may indicate a deficiency of the cardiac neural crest (24). The resulting common outflow vessel consequently carries both oxygenated and deoxygenated blood after birth. A divided outflow tract is not needed for embryonic circulation, but surprisingly in both the chick ablation model and the splotech\(^{2H}\) mouse mutant, all embryos with PTA die during mid to late gestation (9, 14). Studies in the chick ablation model and in the splotech\(^{2H}\) mutant in which there is failure of neural crest migration suggest that, along with PTA, a contributing factor to lethality may be impaired cardiac excitation-contraction coupling (EC) stemming primarily from a reduction in the L-type Ca\(^{2+}\) current (2, 8, 14) that appears to stem from abnormal growth factor signaling (see Discussion) (17). Thus neural crest deficiency also leads to intrinsic defects in the myocardium as well as structural defects of the outflow track. The most plausible explanation for decreased L-type current would be a reduction in the number of \(\alpha_{1C}\)-channel subunits also known as dihydropyridine receptors (DHPR). However, measurements of DHPRs in normal hearts and hearts with PTA with a radiolabeled antagonist indicated no difference in the number of DHPRs (2, 11). Therefore, we hypothesize that the diminished L-type calcium current in hearts with PTA is due to reduced function at the single channel level that would be reflected by a decrease in the mean open probability \(P_o\) of L-type calcium channels in myocytes from these hearts. For this study, we compared the single channel properties of myocytes isolated from chick embryos with PTA after cardiac neural crest ablation and from normal hearts from sham-operated embryos at embryonic days (ED) 11 and 15. As predicted by our hypothesis, the \(P_o\) was significantly reduced in hearts with PTA. These results indicate that the cardiac neural crest may influence the development of myocardial Ca\(^{2+}\) channels.

MATERIALS AND METHODS

Animal surgery. Fertilized Arbor Acre chicken eggs (Seaboard Hatchery, Athens, GA) were incubated in forced draft incubators maintained at 90% humidity and 38°C. The eggs for sham-operated and experimental animals were windowed at Hamburger-Hamilton stages 8–10 (~30 h of development). The embryos were stained with neutral red, and the cardiac neural crest (neural crest cells extending from the midline placode to the caudal limit of somite 3) was ablated.
bilaterally with a pulsed nitrogen-dye laser (model VSL-337/DLM-110, Laser Science) as previously described in detail by Kirby and colleagues (25). This lesion produces PTA in >90% of embryos because the cardiac neural crest normally provides ectomesenchymal cells essential for septation of the cardiac outflow tract (24). The microsurgery was performed by a surgery Core Unit, under the supervision of Dr. Margaret L. Kirby, using procedures that are standardized to produce PTA. For sham-operated embryos, the embryos were stained with neutral red, but the laser ablation was not performed. All eggs were sealed with cellophane tape, reincubated, and harvested at ED11 and ED15. The hearts from embryos with neural crest ablations were examined with a dissecting microscope, and only those diagnosed with PTA were used. The presence of PTA served as an indication for successful ablation of the cardiac neural crest. The hearts from sham-operated embryos were considered “normal” if no outflow tract anomalies were detected at the time of macroscopic inspection. By ED15, the mortality of embryos with neural crest ablation was ~70%. PTA was never observed in sham-operated embryos.

Myocyte preparation. The culture method used in this study was as originally established by R. L. DeHaan approximately 20 years ago specifically for the study of Ca$^{2+}$ and Na$^+$ currents in chick heart development (19, 23). After decapitation of the embryos, the hearts were rapidly removed and trimmed of the atria and great vessels, and the ventricles were cleaned of connective tissue and dissociated with brief repeated exposures to collagenase and DNAase at 37°C as previously described (2, 6, 13). The cultures were enriched for myocytes by 20-min incubations in tissue culture flasks before the cells were plated. During these short incubations, other cell types attach to the flasks, whereas myocytes do not. The myocytes were sparsely plated (~5 × 10^5 cells per 35-mm culture dish) in DeHaan’s 21212 medium with 1.8 mM CaCl$_2$ onto plastic petri dishes (Falcon 1008). The cultures were incubated in a humidified 5% CO$_2$-95% air atmosphere at 37°C. The myocytes were cultured overnight and used within 24 h of enzymatic dissociation to allow for attachment and to recover from the cell isolation procedure. The suitability of these myocytes has been demonstrated in previously published reports of cardiac EC coupling using this preparation. These reports indicate EC coupling does not appear to be measurably affected by enzymatic dispersion and overnight culture. This is evident when sarcoplasmic reticulum contributions are compared with electrically stimulated Ca$^{2+}$ transients in isolated myocytes (6, 12) with Ca$^{2+}$ transients elicited in freshly dissected intact cardiac trabeculae (32), which shows that the Ca$^{2+}$ transients from isolated myocytes are virtually identical to those obtained in intact trabeculae whether or not sarcolemmal reticulum function is blocked with ryanodine. Moreover, a high level of colocalization of ryanodine receptors and DHPRs is maintained even after enzymatic dissociation and overnight culture in which the embryonic myocytes, unlike adult myocytes, have typically lost their elongated appearance and have assumed a spherical shape (15). Thus junctional complexes are quite stable and not disrupted under the conditions used in the present study. All experiments were carried out at room temperature (22–24°C).

Cell-attached patch clamp. Depolarizing and pipette solutions for single Ca$^{2+}$ channel recordings were as described by Lew et al. (31). In each experiment, the culture medium was replaced by a depolarization solution containing (in mM) 120 potassium aspartate, 20 MgCl$_2$, 0.01 isoproterenol, 10 EGTA, and 10 HEPES; pH to 7.4 with NaOH. High potassium was used to depolarize the cell. From calculations using the Nernst equation, 120 mM potassium depolarized the cells to approximately ~2.0 mV at room temperature. As indicated in Lew et al. (31), isoproterenol was used to enhance the detection of single channel events especially in experimental embryos where the $P_o$ was very low (see RESULTS). The overall conclusions were not likely to be affected because we have found that isoproterenol increases the whole cell L-type Ca$^{2+}$ current by 50% in both sham-operated embryos and embryos with PTA (12). Moreover, these conditions reflect life in ovo that there is a high level of circulating catecholamines in embryos, which is unaffected by cardiac neural crest ablation (26). To avoid Ca$^{2+}$-dependent inactivation and to increase the conductance to better visualize single channels events, Na$^+$ was used as the charge carrier (31). The pipette solution contained 150 mM NaCl, 5 mM EDTA, 5 mM HEPES, and 3 μM tetrodotoxin (~500 times the inhibitory constant for the chick cardiac Na channel). Single channel recordings consisted of 50 consecutive 200-ms sweeps from a holding potential of −80 to 0 mV. An Axopatch 200B integrating patch-clamp amplifier was used and zeroed to compensate for the offset potential after the electrode was in the bath but before seal formation. The data were collected and analyzed using pCLAMP 6.0 software from Axon Instruments. The amplitude of each channel opening was measured as the difference between the baseline and the height at the top of the opening. $P_o$ for each cell was determined as the total amount of time the channel is opened over the total duration of the recording. Open time frequency histograms were plotted and fitted to a single exponential using Origin 7.0 software.

Statistics. Statistical comparisons were made using ANOVA for a single factor. Individual comparisons were made using the Student’s $t$-test. A $P$ value < 0.05 was considered significant.

RESULTS

Data were collected from ventricular myocytes isolated at ED11 and ED15 from normal hearts and from cardiac neural crest-ablated embryo hearts diagnosed with PTA for a total of four groups. These ages were chosen primarily for comparison with our previously published data that have specifically identified a reduction in the L-type Ca$^{2+}$ current in hearts with PTA at these ages, and second, because PTA is readily identifiable by ED11 with visual inspection during the dissection (2, 11, 12, 14). The presence of PTA indicated successful ablation of the cardiac neural crest. Data were recorded and analyzed from a total of 50 patches with at least 7 patches and 3 embryos for each group (Fig. 1).

Open channel probability. Example traces from four consecutive 200-ms depolarizing episodes to 0 mV are shown in Fig. 1A from a sham-operated embryo at ED11. In the sham-operated controls, most episodes showed one or more channel opening events of relatively short duration with intervening blank episodes with no openings. The magnitude of the single channel current was about 1.7 pA and was not different among the four groups. For sham-operated controls, most episodes showed one or more channel opening events of relatively short duration with intervening blank episodes with no openings. The magnitude of the single channel current was about 1.7 pA and was not different among the four groups. A similar magnitude with Na$^+$ as the charge carrier was reported for the rabbit ventricle (31). However, the number of blank episodes was greater, and the number of events per episode was noticeably fewer in myocytes from hearts with PTA at both ED11 and ED15. This was verified by quantifying the number of opening events for each of the groups (Fig. 1B). The number of single channel events declined by 39% with normal development between the ages of ED11 and ED15. However, there was no significant decline in the number of events between these ages in hearts with PTA. More strikingly was a substantial decrease in the number of events per episode in hearts with PTA compared with normal hearts of the same age from sham-operated controls. The decreases were 71% and 48% for ED11 and ED15, respectively. These data indicated a reduction in the mean $P_o$ for L-type Ca$^{2+}$ channels.

The $P_o$ for L-type channel activity was determined for each of the groups by dividing the total time in which the channels remained open by the total duration of all the episodes. The
data are summarized in Fig. 2. The $P_o$ declined significantly by 34% between ED11 and ED15 in normal hearts from sham-operated embryos. These results are consistent with a similar decline in $P_o$ for Ca$^{2+}$ channel activity described in an earlier report of normal avian heart development (36). When compared with hearts with PTA, there was a significant decline in $P_o$ compared with the sham controls. There were 75% and 43% reductions in $P_o$ at ED11 and ED15, respectively. In the hearts with PTA, the small difference in $P_o$ between ED11 and ED15 was not significant at the 0.05 level (Fig. 2).

Open time frequency histograms. To better characterize single channel events, ensemble open time frequency histograms were constructed by combining the data from all the myocytes in each of the four groups and plotting the number of channel openings and durations in 2-ms bins (Fig. 3). The great majority of the duration times were less than 20 ms, although there were a few openings (<5%) of much longer duration with rare events lasting as long as 70 ms. In all groups the data were best fitted by single exponentials, indicating a single gating mode. The very long open durations suggested an additional mode of gating, but these events were too rare to affect the curve fitting. These openings with very long duration times are known to be much more prevalent in very early heart development (i.e., ED3 in the chick embryo) and are thought to reflect “mode 2” L-type Ca$^{2+}$ channel gating (36).

The open duration time constant declined in normal hearts between ED11 and ED15 by 25%, indicating that the observed decline in $P_o$ between these ages was due in part to channel openings of shorter duration as well as fewer events per 200-ms episode. There was no apparent difference in the open channel duration time constants in hearts with PTA from ED11 and ED15, and these were very similar to the time constant for the sham control at ED11. These data indicate that the decrease in the $P_o$ in hearts with PTA is due to a reduction in the number of channel opening events rather than a shortening of the duration in which the channel remains open. In hearts with PTA there was no indication of a developmental decrease in the open duration time as was observed in the sham-operated embryos. These observations indicate abnormal development of L-type Ca$^{2+}$ channels after cardiac neural crest ablation.

**DISCUSSION**

The Ca$^{2+}$ channel $P_o$ declines with normal development. In normal hearts from sham-operated embryos, there was a 34% reduction in the $P_o$ with development from ED11 to ED15. These results are consistent with previous reports indicating that the $P_o$ of L-type channels declines with development (35) and that this decline is consistent with an overall reduction in the magnitude of the whole cell L-type Ca$^{2+}$ current that occurs with development (13, 28). The magnitude of the single channel current measured in the present study was very similar to the single channel current for L-type Ca$^{2+}$ channels in a rabbit ventricle measured under essentially identical conditions (31). The open time frequency histograms were best fitted by a single exponential, which further indicated a population of channels among the four groups with similar kinetics consistent with L-type channel gating.

Decreased $P_o$ in hearts after neural crest ablation. In the hearts with PTA, the mean $P_o$ was significantly reduced at both
ED11 and ED15, and there was no observable decline in the $P_o$ between these ages as was evident in the sham-operated controls. Similarly, there was an absence of a normal decline in the open time duration between ED11 and ED15 in hearts with PTA. These data suggested possible retention of an embryonic Ca$^{2+}$ channel isoform with longer open time kinetics. Studies in mouse embryonic heart indicate the presence of mRNA transcripts for three dihydropyridine-sensitive L-type Ca$^{2+}$ channel isoforms (29). These isoforms are $\alpha_{1S}$, $\alpha_{1C}$, and $\alpha_{1D}$. $\alpha_{1S}$, the skeletal muscle isoform, probably does not form functional Ca$^{2+}$ channels in the embryonic heart because currents with kinetics characteristic of this channel have not been detected. Expression of $\alpha_{1C}$ increases threefold between 9.5 and 15.5 days postcoitum (dpc) and becomes the predominant cardiac isoform with embryo development (34). In mouse mutants with functional knockout of $\alpha_{1C}$, the embryos have a normally beating heart at 12.5 dpc apparently due to upregulation of a splice variant of $\alpha_{1D}$ (29). However, the embryos die suddenly from a failing heart by 14.5 dpc, indicating that $\alpha_{1C}$ expression is essential for survival in late fetal development. Interestingly, mouse embryos homozygous for the splotch$^{2H}$ mutation have hearts with PTA along with reduced Ca$^{2+}$ current similar to the chick embryo after cardiac neural crest ablation (8). As is the case for the $\alpha_{1C}$ knockout mutants, the splotch$^{2H}$ embryos also die suddenly of apparent heart failure by 14.5 dpc (9) and similarly, chick embryos after cardiac neural crest ablation never hatch and few survive beyond ED15. Taken together these observations are suggestive of cardiac Ca$^{2+}$ channel isoform misexpression in the absence of cardiac neural crest.

DHPRs versus number of functional channels. We have previously reported that the number of L-type Ca$^{2+}$ channel $\alpha_{1C}$-subunits as determined by radiolabeled DHPR binding was not different when normal hearts are compared with PTA hearts, although L-type Ca$^{2+}$ current is reduced by about 50% in PTA hearts (2). The present report indicates that the decreased Ca$^{2+}$ current in PTA is due to reduced L-type channel function as measured at the single channel level. The number of DHPRs in the heart is relatively constant throughout most of normal development in the chick (33). In an earlier study of a normal heart at ED11, we determined the single channel properties for L-type channels and calculated the density of functional channels using nonstationary fluctuation analysis (3). We concluded that there were far fewer functional L-type channels than indicated by the DHPR binding, which is in contrast to Lew et al. (31), who reported that the surface density of L-type channels and the number of DHPRs in adult ventricle were similar. The fluctuation analysis used in the earlier study may have lacked the sensitivity to determine the $P_o$ accurately. None the less, the substitution of the directly measured $P_o$ value from the present study into our earlier calculations indicates a functional channel density of about 3.5/µm$^2$ for ED11, which is still considerably less than the DHPR density (~25/µm$^2$) in the embryonic myocytes (3). Therefore, we still maintain that there is a relatively large population of “silent” channels or channels that exhibit a gating mode with an extremely low and difficult to detect $P_o$. A possible explanation for the apparent disparity in the density of DHPRs and functional L-type channels is that the silent channels are the skeletal muscle $\alpha_{1S}$-isoform because the mRNA transcript is present in the embryonic heart but the channels appear nonfunctional (29). Thus radiolabeling of DHPRs by itself does not provide an accurate reflection of L-type Ca$^{2+}$ channel function in the embryonic heart whether in normal or diseased states.

Fibroblast growth factor 8 in neural crest and myocardial development. Cardiac neural crest-ablated embryos show reduced contractility and Ca$^{2+}$ transients by stage 16 (~ED2.5), which is 2 days before the migrating neural crest would normally have reached the heart (27, 30, 37). The likely cause of decreased myocardial function appears to be over stimulation by fibroblast growth factor 8 (FGF8), which is highly

Fig. 3. Open time frequency histograms plotting the number of events versus the duration of openings. Data were a well-fitted single exponential indicating mostly a single mode of L-type Ca$^{2+}$ channel activity. $\tau$ is the duration time constant calculated from the fit. Note that there are a few events with very long duration times (>25 ms). These rare events exhibit gating characteristic of mode 2 channel behavior (see text).
expressed in the ectodermal and endodermal tissues adjacent to the developing heart tube (17). FGF8 and its likely receptor in the heart FGFR1 are essential for early cardiogenesis and myocyte differentiation (4, 16, 22), and FGF8 is necessary for migration, proliferation, and survival of the relatively large population of neural crest cells seeding and migrating through the pharyngeal arches (1, 18, 21). Farrell et al. (17) have shown that antibody against FGF8 rescues the Ca\textsuperscript{2+} transient in explant cultures of stage 14 pharynx with the heart tube from neural crest-ablated embryos. More recent evidence (20) indicated that in ovo treatment with either antibody specific for FGF8 or an FGFR1 inhibitor (SU-5402) rescued the Ca\textsuperscript{2+} transient phenotype in at least 75% of neural crest-ablated embryos. These data provide strong evidence that FGF8 signaling continues to influence myocardial development even after early cardiogenesis and myocyte specification. More work is needed to determine the influences of FGF8 and other growth factors in the surrounding mesenchyme during early heart formation on the development of myocardial function.

Finally, we note that although both PTA and defective EC coupling result from deficiencies in the cardiac neural crest, these phenomena are otherwise not likely to be related. PTA is a structural defect of the outflow tract, whereas the EC coupling defect is intrinsic to cardiac myocytes and apparent early in development before the period of normal outflow septation. Both defects are likely to contribute to the demise of embryos observed during mid to late gestation in chick and mouse.

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REFERENCES

6. Broetto MAD and Creazzo TL. Ca\textsuperscript{2+} transients in embryonic chick heart: contributions from Ca\textsuperscript{2+} channels and the sarcoplasmic reticulum. Am J Physiol Heart Circ Physiol 270: H518–H525, 1996.


