mation of these large and abundantly distributed *H. americanum* cysts in the muscular tissues of experimentally infected dogs was followed by Panceira et al. (1999), who described the gradual encystation process. At 3 wk PI, parasites were visible as inclusions in the cytoplasm of a modified host cell, and at 4.5 wk PI, they were surrounded by a narrow zone of mucopolysaccharide material, which increased considerably in size until 26 wk PI. An attempt to infect 2 dogs by ingestion of muscle from dogs with a natural *H. americanum* infection was unsuccessful (Nordgren and Craig, 1984), but more experiments are needed to examine whether this form of transmission is possible. The *H. canis* cysts differ substantially also from zoites of *H. americanum* observed as smaller subspherical organisms within host macrophagelike cells in granulomas (Vincent-Johnson et al., 1997).

The cystic forms reported in this study in *H. canis*–infected dogs may represent part of a 3-host cycle (Smith, 1996) or may be a relic of such a cycle in which dogs act as transport hosts, preyed upon by intermediate hosts, in addition to themselves being intermediate hosts on which the final tick host feeds on blood. The relative scarcity of the monozoic cysts in the tissues examined may explain why this form has been overlooked in previous studies.

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Upregulation of Cardiac Cell Plasma Membrane Calcium Pump in a Canine Model of Chagas Disease

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ABSTRACT: We have previously demonstrated that cardiac myocytes isolated from the hearts of adult dogs develop rapid repetitive cytosolic Ca^{2+} transients, membrane depolarization, and cell contraction by mobilization of sarcoplasmic reticulum Ca^{2+} stores when exposed to a soluble factor from the trypomastigotes of *Trypanosoma cruzi*. These findings led us to investigate the regulatory mechanisms of cytosolic Ca^{2+} in cardiac tissues from dogs chronically infected with *T. cruzi*. Expression of the plasma membrane calcium pump (PMCA) RNA and protein was determined by Northern and Western blotting, respectively, followed by densitometric analyses. A 642-bp PMCA 1b complementary DNA probe derived from canine epicardial tissue hybridized to 2 major transcripts (7.3 and 5.3 kb) in canine epicardium. Expression of the dominant transcript (7.3 kb) was 77% greater in cardiac tissues obtained from dogs with chronic *T. cruzi* infection (140 days after inoculation) in comparison with constitutive expression levels in normal dogs. Monoclonal antibody 5F10, known to recognize all isoforms of the PMCA, was used to detect expression of the PMCA protein in epicardial tissue. Expression of a 142-kDa protein was increased by 58% in the cardiac tissues of infected dogs when compared with those from uninfected dogs. To establish a link between the upregulation of PMCA in dogs chronically infected with Chagas disease and the ventricular-based arrhythmias and myocardial failure that occur during this stage of disease both in dogs and humans, further study will be required.

Trypomastigotes of *Trypanosoma cruzi* (the etiologic agent of Chagas disease) enter cells through a unique mechanism involving the recruitment and fusion of host lysosomes in a process similar to Ca^{2+}-regulated exocytosis (Andrews, 1995). We have demonstrated that a soluble extract from trypomastigotes elicited rapid repetitive cytosolic Ca^{2+} transients, membrane depolarization, and cell contraction by mobilizing Ca^{2+} from the sarcoplasmic reticulum (SR) in primary canine cardiac myocytes (Barr et al., 1996). Others have demonstrated that a trypomastigote-soluble extract causes similar perturbations in Ca^{2+} homeostasis in several cell lines (Tardieux et al., 1994; Burleigh and Andrews, 1995; Rodriguez et al., 1995) and that a serine oligopeptidase B present in the soluble extract was most likely responsible for the generation of the Ca^{2+}-signaling agonist effect (Burleigh et al., 1997). Deletion of the oligopeptidase B gene from trypomastigotes results in a marked reduction in their ability to induce cytosolic Ca^{2+} signaling and invade cells (Caler et al., 1998).

Given the profound perturbations in cytosolic Ca^{2+} in primary canine cardiac myocytes induced by exposure to extracts from trypomastigotes, we were curious as to what changes might occur in the regulatory mechanisms of cytosolic Ca^{2+} in cardiac tissues from dogs chronically infected with *T. cruzi*. The regulation of cytosolic Ca^{2+} within cardiac myocytes is dependent on the integration of several carefully orchestrated effector mechanisms. Excitation–contraction coupling in cardiac myocytes initially depends on Ca^{2+} influx into the cell through plasma membrane Ca^{2+} channels. Ca^{2+} influx leads to the release of sufficient Ca^{2+} (10–65 times more than that entering through the plasma membrane Ca^{2+} channels) from the SR to bind with actin and myosin to activate contraction (Fabiato, 1985; Bers et al., 1993). Cytosolic-free Ca^{2+} levels are then returned to baseline levels very quickly (given that a myocyte may need to produce a Ca^{2+} transient and a subsequent contraction as often as 200 times a minute) by pumping Ca^{2+} back into the SR using SR Ca^{2+} pumps. Two additional important pathways for calcium extrusion from the cell are the Na–Ca^{2+} exchanger and the plasma membrane calcium pump (PMCA). In humans, the PMCA is made up of several isoforms of which PMCA 1b and PMCA 4b are reputed to be the main ones constitutively expressed in cardiac cells. The isoforms of canine cardiac PMCA have not been characterized. Changes in the regulation of PMCA in the cardiac tissue under pathologic conditions are poorly understood generally and have not been elucidated in myocardial tissue from humans or animals with Chagas disease. As a first step to better characterize the effectors of Ca^{2+} homeostasis in cardiac tissue from dogs with Chagas disease, we proposed to determine if changes occur in the PMCA during the chronic stages of *T. cruzi* infection in dogs by measuring the expression of messenger RNA (mRNA) and the protein levels of the PMCA.

Cardiac tissues were obtained from 22 Beagle dogs divided into 2 equal age- and sex-matched groups. Eleven dogs were experimentally infected with *T. cruzi* and monitored throughout their infection until they reached the chronic stage of disease (between 140 and 150 days postinfection [DPI]), as described previously by our group (Barr, Gossett, and Klei, 1991; Barr, Schmidt et al., 1991; Barr et al., 1992). The other 11 dogs acted as uninfected controls. The numbers of parasites contained in cardiac myocytes from tissues from chronically infected dogs are extremely low, as noted previously (Barr, Schmidt et al., 1991). Cardiac tissues were processed immediately after harvest (RNA studies and Northern blots) or stored at −80 C until use (Western blots).

Total RNA was isolated from canine cardiac tissue, as described previously (Chomczhinsky and Sacchi, 1987), using the Trizol reagent (Life Technologies, Grand Isle, New York). Poly-A^+ RNA was selected with the PolyAtract mRNA Isolation System (Promega, Madison, Wisconsin). Purified RNA integrity was excellent, judging from banding patterns of 18S and 28S ribosomal RNA (rRNA) after ethidium bromide staining of total RNA preparations. Ribonucleic acid was quantitated from the 260:280 ratio determined with a Beckman spectrophotometer. Poly A^+ RNA was reverse transcribed with Superscript RT II (Life Technologies) using the downstream (antisense) primer 5′-TCAGAGTGATGTTTCCAAAC-3′. First-strand products were amplified using polymerase chain reaction (PCR) with *Pfu* polymerase (Stratagene, San Diego, California), the downstream primer, and the upstream (sense) primer 5′-GCCATCTTCTGCACAATTGT-3′. These primers are homologous to the 3′ coding region of the human teratoma PMCA 1b and encompass the calmodulin-binding portion of the encoded protein. Polymerase chain reaction conditions were as follows: 100 ng of each primer, reaction buffer (final concentration 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), and 1 mM of each of the deoxynucleotides (deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, final concentraion 1 mM; New England Biolabs, Beverly, Massachusetts), to a final volume of 49 μl with DEPC water and *Taq* polymerase (5 U; Perkin–Elmer, Foster City, California). The samples were placed in a thermal cycler (PTC-100, MJ Research, Watertown, Massachusetts) for 40 cycles (1 time at 95 C for 2 min denaturation, followed by cycles of 95 C for 1 min and 60 C for 1 min annealing, and 72 C for 1 min for extension, with a 2-min final extension at 72 C). A single PCR product of approximately 650 bp was obtained, and this was cloned into the *Eco*R1–*Not*I site of PCRscript (Stratagene). The sequence of this cloned product was determined with an automated Perkin–Elmer/Applied Biosystems 372A Stretch DNA sequencer by Cornell University DNA Services.

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  1 GCC ATC TTC TGC ACA ATT GTT TTA GGC ACT TTT GTG GTA
 40 CAG ATA ATA ATT GTG CAG TTT GGT GGA AAA CCT TTC AGT
 79 TGT TCA GAA CTT TCA ATA GAA CAG TGG CTA TGG TCA ATA
118 TTC CTA GGA ATG GGG ACA TTA CTC TGG GGC CAG CTT ATT
157 TCA ACA ATT CCA ACT AGC CGT TTA AAA TTC CTA AAA GAA
196 GCT GGC CAT GGA ACA CAA AAG GAA GAA ATC CCA GCG GAG
235 GAG CTC GCA GAG GAT GTG GAA GAG ATT GAC CAT GCT GAA
274 AGG GAA CTG CGG CGT GGC CAG ATC TTA TGG TTT AGA GGT
313 CTG AAC AGA ATC CAA ACT CAG ATT CGA GTG GTG AAT GCA
352 TTT CGT AGT TCT TTA TAT GAA GGG TTA GAA AAA CCG GAA
391 TCA AGA AGT TCG ATT CAC AAC TTT ATG ACA CAT CCT GAG
430 TTT AGG ATA GAG GAT TCA GAG CCT CAT ATC CCC CTA ATT
469 GAT GAC ACT GAT GCC GAA GAT GAT GCT CCT ACA AAA CGT
508 AAC TCC AGT CCT CCA CCC TCT CCC AAC AAA AAT AAC AAT
547 GCT GTT GAC AGC GGG ATT CAC CTT ACA ATA GAA ATG AAC
586 AAG TCT GCT ACC TCT TCA TCC CCA GGA AGC CCA CTA CAT
625 AGT TTG GAA ACA TCA CTC

AIFCTIVLGTFVVQIIIVQFGGKPFSCSELSIEQWLWSIFLGMGTLLWGQLISTI
PTSRLKFLKEAGHGTQKEEIPAEELAEDVEEIDHAERELRRGQILWFRGLNRIQT
QIRVVNAFRSSLYEGLEKPESRSSIHNFMTHPEFRIEDSEPHIPLIDDTDAEDDA
PTKRNSSPPPSPNKNNNAVDSGIHLTIEMNKSATSSSPGSPLHSLETSL
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FIGURE 1. Nucleotide sequence of the 642-bp fragment of the canine PMCA 1b isoform and the predicted amino acid sequence given below. The predicted amino acid sequence contains a calmodulin-binding domain (underlined) and the serine site of phosphorylation by the cyclic adenosine monophosphate–dependent protein kinase (boldface).

For Northern analysis, total RNA was isolated as described above from the canine cardiac tissue of 8 *T. cruzi*–infected dogs and 8 normal dogs and was quantified from the 260:280 ratio determined with a Beckman spectrophotometer. Thirty micrograms of RNA was size fractionated on 1.2% agarose–formaldehyde gels and then transferred overnight by capillary transfer to a nitrocellulose membrane (Genescreen-Plus, NEN-DuPont, Wilmington, Delaware). Equality of total RNA applied to each well was assessed by analyses of images of ethidium bromide–stained samples. Bands of 18S rRNA were stained on agarose gels before transfer to assess uniformity of sample loading. Actin mRNA was also probed (using a highly specific actin complementary DNA [cDNA] probe) to judge the uniformity of sample loading and transfer. Complementary DNA probes were labeled with ^{32}P by the random prime method (GIBCO, Grand Island, New York). Prehybridization of membranes was carried out in a solution of 1 M NaCl, 50% formamide, 1% sodium dodecyl sulfate (SDS), and 10% dextran sulfate at 42 C for 2 hr. Labeled probe and salmon sperm (final concentration: 100 μg/ml) were combined and boiled for 10 min and then added to the prehybridization solution; membranes were incubated at 42 C overnight. Genescreen was then washed twice in 2× standard saline citrate (SSC) for 5 min at room temperature, followed by 2 washes in 2× SSC, 1% SDS at 60 C for 30 min, and finally twice in 0.1× SSC at room temperature for 30 min. Genescreen was allowed to air-dry; then, autoradiography was conducted (X-Omat Scientific Imaging film, Eastman Kodak Co., Rochester, New York). Images were saved on computer disk with Photoshop (Corel, Ottawa, Canada). Band density was analyzed with Scan Analysis 2.5.

For Western blot analysis, purified plasma membranes were prepared from cardiac tissues from 11 *T. cruzi*–infected dogs and 7 normal dogs. The tissue was placed into a solution of 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride and homogenized with a polytron (Virtishear, VirTis, Gardiner, New York) on ice, first with a heavy cutter and then with a fine cutter. The homogenate was sonicated at a high setting for 1 min and then centrifuged (7,000 *g*, 10 min). All centrifugations were carried out at 4 C. The pellet was discarded, and the supernatant was centrifuged at 10,000 *g* for 20 min. The pellet was resuspended in the Tris–EDTA buffer with a polytron (fine cutter) and then centrifuged (43,000 *g*, 20 min). The pellet consists of purified plasma membrane and was resuspended in Tris–EDTA and stored at −80 C. Partially purified human erythrocyte membranes were prepared as discussed above after washing 3× in PBS and also stored at −80 C before use in all assays as a control of positive 5F10 antibody (Bioaffinity Reagents, Golden, Colorado) binding to the PMCA. To ensure uniformity of sample loading, 100 μg of each sample was loaded into

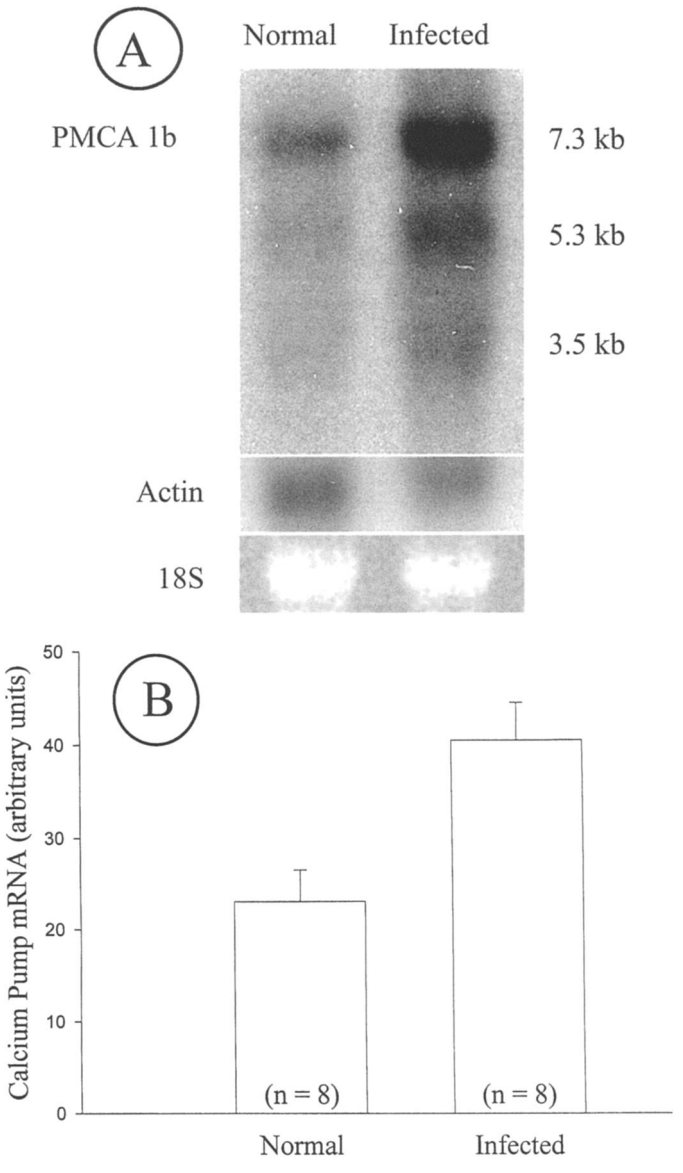

FIGURE 2. **A.** Northern analysis shows that the canine PMCA 1b cDNA hybridizes to 3 RNA transcripts (7.3, 5.3, and 3.5 kb) in canine cardiac tissue from normal (n = 8) and *Trypanosoma cruzi*–infected (n = 8) dogs. Bands of 18S rRNA on stained agarose gel before transfer indicate uniform sample loading. Actin mRNA was also probed to judge uniformity of sample loading and transfer. **B.** Mean expression levels of the 7.4-kb transcripts as measured by densitometric analysis in tissue from the 8 *T. cruzi*–infected dogs were 77% greater than levels from 8 normal dogs (2-sample *t*-test, $P < 0.05$; bars represent standard deviation).

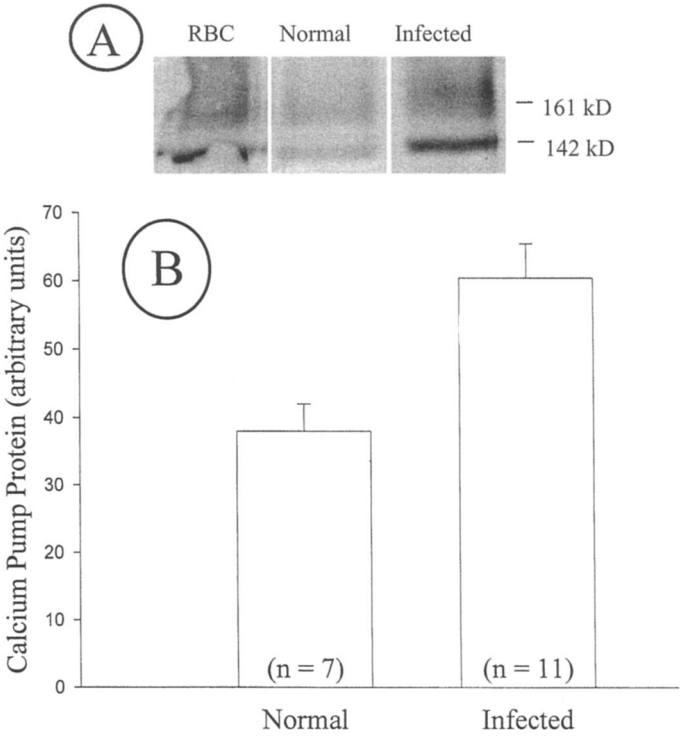

FIGURE 3. **A.** Western analysis shows that the monoclonal antibody 5F10, raised against human teratoma PMCA, recognizes 1 sharp band of 142 kDa and 1 larger diffuse band with differential staining of approximately 160 kDa. Because expression levels of the PMCA-immunoreactive protein from canine cardiac tissue (142 kDa) were closest in molecular weight to the human PMCA protein (134 kDa), densitometric measurements of the 142-kDa band were taken from Western blot analysis from 11 *Trypanosoma cruzi*–infected dogs and compared with tissue from 7 normal dogs. **B.** Mean expression levels of the 142-kDa protein in tissue from the *T. cruzi*–infected dogs were 58% greater than those from normal dogs (2-sample *t*-test, $P < 0.05$; bars represent standard deviation).

each lane. Furthermore, after transfer, acrylamide gels were stained with Coomasie to ensure that all proteins transferred to the nitrocellulose membrane.

The nucleotide sequences of vertebrate PMCA 1b isoform from a variety of tissues share a high degree of homology (Strehler, 1991). We used primers based on this homology to carry out reverse transcription of mRNA with subsequent amplification by PCR to clone a 642-bp cDNA fragment (GenBank accession number: AF287015) from canine cardiac tissue (Fig. 1), which shared 96.5% nucleotide identity with the human PMCA 1b isoform and 99.5% identity at the amino acid level (Verma et al., 1988). The canine PMCA 1b described in this study also retains important regulatory sequences such as the calmodulin-binding domain and the serine site of phosphorylation by the cyclic adenosine monophosphate–dependent protein kinase (Fig. 1) (Verma et al., 1988). In humans, the PMCA 1b and PMCA 4b isoforms are reputed to be the main constitutively expressed isozymes in cardiac cells. Unfortunately, we were unable to amplify a cDNA fragment from dog tissue for the PMCA 4b isoform. It may be that this isoform is not likewise highly expressed in dog cardiac tissue.

Northern analyses showed that the canine PMCA 1b cDNA probe hybridizes to 2 major transcripts (Fig. 2A). The sizes of these transcripts are approximately 7.3 and 5.3 kb. These transcripts are comparable with the 2 most abundant human PMCA 1b mRNA species (7.6–7.8 kb and 5.5–6.0 kb) (Greeb and Shull, 1989). A minor band of approximately 3.5 kb was also observed. Similar sizes and patterns of expression were seen in poly A^+ RNA samples from infected and control dog hearts (data not shown). Levels of expression of the dominant transcript (7.3 kb) were determined by subjecting Northern blots to densitometric analyses. The level of expression, as judged by the density of the bands, was 77% greater in tissue from 8 chronically *T. cruzi*–infected dogs than in tissue from 8 uninfected dogs (Fig. 2B; $P < 0.05$).

We next used the monoclonal antibody 5F10 (Bioaffinity Reagents), raised against human teratoma PMCA, to investigate the expression levels of the canine PMCA in the cardiac tissues from 11 *T. cruzi*–infected dogs and 7 uninfected dogs. Antibody 5F10 recognizes all known isoforms of the PMCA (Stauffer et al., 1995; Caride et al., 1996). In our study, antibody 5F10 recognized 1 sharp band of 142 kDa and 1 larger diffuse band of approximately 160 kDa (Fig. 3A). Because the denser staining band was closest to the actual size of the PMCA (134 kDa), we chose to measure the levels of expression of the most im-

munoreactive protein (142 kDa) as determined by densitometric analysis of Western blots, for this protein was also identical in mass to our positive control preparation (prepared from human red blood cells). The densitometric measurements showed that PMCA expression was 58% greater in tissue from chronically *T. cruzi*–infected dogs than in tissue from uninfected dogs (Fig. 3B; $P < 0.05$).

The upregulation of canine myocyte PMCA at 140 DPI does not appear to correspond to generalized cellular injury because 140-DPI myocytes exhibit normal cellular topology at the light microscope level and normal action potential duration and morphology (Pacioretty et al., 1995). Furthermore, both rapid and slow phases of recovery of the transient outward potassium current (I_{to}) from inactivation were normal in cardiac myocytes taken from dogs with chronic Chagas disease (140 DPI) (Pacioretty et al., 1995). The cause of the upregulated PMCA levels is unknown. However, the result of an upregulated PMCA may be to decrease diastolic cytosolic Ca^{2+} levels. It is tempting to suppose that upregulation of PMCA is tied in some way to the mechanisms this particular parasite uses to enter cells and the need for repeated Ca^{2+} transients for the parasite to do this. However, it is equally possible that upregulation of PMCA may be a cellular response to added workload on the remaining healthy cells in a failing heart. To further understand the mechanisms of Ca^{2+} disruption in the chagasic myocyte, it will be necessary to investigate the activity and expression of the SR pump, intracellular Ca^{2+} channels, Na–Ca^{2+} exchanger, as well as additional PMCA isoforms.

The reduction in epicardial I_{to} and the increase in epicardial PMCA RNA and protein expression in the canine model of Chagas disease have parallels to changes in membrane current and Ca^{2+} handling in canine tachycardia-induced heart failure and human heart failure. In both the latter cases, I_{to} is reduced in epicardial or myocardial cells. Furthermore, SR Ca^{2+} adenosine triphosphatase RNA and protein expression are reduced, whereas Na^{+}–Ca^{2+} exchange RNA and protein expression are significantly increased (Winslow et al., 1999). Our observations of upregulation of PMCA RNA and protein expression add an additional element to the dynamics of Ca^{2+} regulation that may impinge on canine heart failure.

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