

# Infection by *Trypanosoma cruzi* Enhances Anion Conductance in Rat Neonatal Ventricular Cardiomyocytes

Mayra Delgado-Ramírez · Igor I. Pottosin ·  
Valery Melnikov · Oxana R. Dobrovinskaya

Received: 21 July 2010 / Accepted: 29 October 2010  
© Springer Science+Business Media, LLC 2010

**Abstract** Recent studies on malaria-infected erythrocytes have shown increased anion channel activity in the host cell membrane, increasing the exchange of solutes between the cytoplasm and exterior. In the present work, we addressed the question of whether another intracellular protozoan parasite, *Trypanosoma cruzi*, alters membrane transport systems in the host cardiac cell. Neonatal rat cardiomyocytes were cultured and infected with *T. cruzi* in vitro. Ion currents were measured by patch-clamp technique in the whole-cell configuration. Two small-magnitude instantaneous anion currents, outward- and inward-rectifying, were recorded in all noninfected cardiomyocytes. In addition, ~10% of cardiomyocytes expressed a large anion-preferable, time-dependent current activated at positive membrane potentials. Hypotonic (230 mOsm) treatment resulted in the disappearance of the time-dependent current but provoked a dramatic increase of the instantaneous outward-rectifying one. Both instantaneous currents were suppressed by intracellular  $Mg^{2+}$ . *T. cruzi* infection did not provoke new anion currents in the host cells but caused an increase of the density of intrinsic swelling-activated outward current, up to twice in heavily infected cells. The occurrence of a time-dependent current dramatically increased in infected cells in the presence of  $Mg^{2+}$  in the intracellular solution, from ~10 to ~80%, without a significant change of the current density. Our findings represent one further, besides the known *Plasmodium falciparum*, example of an intracellular parasite

which upregulates the anionic currents expressed in the host cell.

**Keywords** Chagas disease · *Trypanosoma cruzi* · Infected cardiomyocyte · Anion · Channel

## Introduction

Intracellular parasites are microorganisms that are capable of growing and reproducing inside host cells. Intracellular phases occur in the life cycle of several protozoa-causative agents of human diseases, such as *Plasmodium* spp., *Leishmania* spp., *Toxoplasma gondii* and *Trypanosoma cruzi*. Situating inside the host cell, the parasite is less susceptible to immune attack and chemotherapy. The host cell provides nutrients essential for parasite proliferation, but the growing requirements of the proliferating parasite cannot always be supplied by internal cell resources. Moreover, intoxication by parasite waste products might result in premature death of the host cell. Therefore, during their evolution, intracellular parasites have to develop specific mechanisms to cope with these challenges. It is widely accepted now that the intracellular malaria parasite induces in the host erythrocyte membrane new permeation pathways (NPPs). These new pathways provide the parasite with essential nutrients and vitamins, dispose of metabolic waste products, modify the electrolyte composition and decrease the colloid osmotic pressure of the infected erythrocyte, preventing its premature hemolysis (Ginsburg et al. 1983; Saliba et al. 1998; Poole and Halestrap 1993; Lew et al. 2003). Based on the results of radiotracer uptake and hemolysis assays, combined with pharmacological studies, some of the NPPs were identified as anion-selective channels, also capable, albeit at lower rates, of transporting electroneutral and cationic solutes

M. Delgado-Ramírez · I. I. Pottosin · O. R. Dobrovinskaya (✉)  
Center for Biomedical Research, University of Colima, Av. 25  
de Julio 965, Villa San Sebastian, C.P. 28045 Colima, México  
e-mail: oxana@uocol.mx

V. Melnikov  
School of Medicine, University of Colima,  
C.P. 28045 Colima, México

(Kirk et al. 1994). These conclusions were confirmed by direct electrophysiological recordings of the channels forming the NPP (see Staines et al. 2007 for review). The whole-cell conductance of mature trophozoite-stage infected erythrocytes is by orders of magnitude higher than that of uninfected cells (Desai et al. 2000; Egee et al. 2002; Huber et al. 2002; Ginsburg and Stein 2004; Bouyer et al. 2006). Although the data of different research groups vary and several single- and multichannel models have been proposed, the consensus is that increased whole-cell conductance of trophozoite-stage infected erythrocytes is dominated by an inwardly rectifying, anion-selective current (Staines et al. 2007). Being crucially important for parasite survival inside the host cell, NPPs were suggested as perspective targets for future antimalaria drugs (Desai et al. 2000). To date, however, *Plasmodium*-infected red blood cells remain a unique model for studies of the nature and function of the NPP, and it is unclear whether other intracellular protozoan parasites adopt this strategy.

*T. cruzi* causes chronic incurable Chagas disease, which affects about 20 million people, with more than 100 million exposed to the risk of infection (WHO 2002). Although this illness is endemic to Latin America, numerous cases also have been reported recently in nonendemic countries, due to tourism and migration (Kirchhoff 1993; Schmunis and Yadon 2009). The parasite life cycle (see Tyler and Engman 2001 for review) involves obligatory passage through vertebrate (usually mammalian, including human) and invertebrate hosts. Two morphologic forms are found in the mammalian host, free mobile trypomastigotes in the peripheral blood and intracellular dividing amastigotes in tissues. Trypomastigotes, following invasion of vertebrate host cells, infect the target host cells and undergo differentiation into amastigotes. After several reproductive cycles in the cytoplasm of the host cell, amastigotes transform to trypomastigotes, the parasitized damaged cell ruptures and numerous parasites leave to disseminate the host body and infect new cells. The majority of *T. cruzi* strains show cardiomyotropism and affect mostly the heart (Andrade and Magalhães 1997).

In this study we applied the patch-clamp technique to infected rat cardiomyocytes in culture, to find out whether intracellular *T. cruzi* parasites, similar to *Plasmodium falciparum* trophozoites, increase anionic current in the host cell.

## Methods

### Ethical Approval

All procedures in this study were approved by the Bioethic and Biosecurity Committee of the University of Colima.

### Cardiomyocyte Isolation and Culture

Neonatal rat ventricular myocytes were isolated and cultured as described previously (Karwatowska-Prokopczuk et al. 1998; Chlopčiková et al. 2001; Sreejit et al. 2008). Neonatal Sprague-Dawley pups (3–5 days old,  $n = 3$ –5 for each experiment) were anesthetized and killed by terminal gaseous (chloroform) anesthesia. Hearts were aseptically removed and collected in  $\text{Ca}^{2+}$ -free Hanks balanced salt solution (HBSS) containing (in mM) 116 NaCl, 5.4 KCl, 0.8  $\text{MgSO}_4$ , 1.0  $\text{NaH}_2\text{PO}_4$ , 20 HEPES, 5.5 glucose (all reagents from Sigma, St. Louis, MO) and supplemented with 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin and 25  $\mu\text{g/ml}$  amphotericin (Invitrogen, Carlsbad, CA), at pH 7.3, 263 mOsm/kg  $\text{H}_2\text{O}$ . After washing, ventricles were minced and transferred to  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS containing 0.38 g/l of EDTA and 0.25% trypsin (Invitrogen, cat. 25200-114) and supplemented with 0.1 mg/ml of bovine deoxyribonuclease I (Sigma, D7691). Digestion was carried out at 37°C in consecutive steps. Cells were then washed with HBSS and resuspended in culture medium containing DMEM/199 (4:1), supplemented with 5% fetal bovine serum (FBS), 10% horse serum, antibiotics and antifungal (all reagents from Invitrogen). To reduce the presence of noncardiomyocytes, cells were preplated for 3 h. Cardiomyocytes were plated at a density of  $1 \times 10^5$  cells in 35-mm tissue culture dishes directly (cultures for parasite maintenance) or on 12-mm coverslips previously treated with poly-L-lysine (cultures for electrophysiological records). To inhibit the growth of nonmuscle cells, 5  $\mu\text{g/ml}$  of cytosine- $\beta$ -D-arabinofuranoside (CDA) hydrochloride (Sigma, C6645) was added to cultures for 3 days. Culture media changes were performed at 24 h and then every 48 h.

### Parasites and Cardiomyocyte Infection

The CH4 strain of *T. cruzi*, originated from a patient with chagasic cardiomegaly, was donated to our group by Dr. Mario Barrera (Instituto Hideo Noguchi, Mérida, Yucatan, México). This strain was shown in our previous studies to possess cardiomyotropism (Melnikov et al. 2005). Cell culture-derived trypomastigotes were isolated from the supernatant of previously infected primary culture of cardiomyocytes and used for infection of cultures for electrophysiological recording. Cultures completely recovered from CDA treatment by 24-h incubation in CDA-free medium were used for infection. A parasite–host cell ratio of 20:1 (in some cases 30:1) was used. Cultures were incubated for 48 h in the presence of parasites and washed. To control the efficiency of infection, microscopic analysis of randomly selected cultures was performed. For this assay, cells were fixed with cold methanol for 10 min, stained with Giemsa and observed on an Axioplan 2

microscope connected to Axio Cam MRe5 digital camera (Zeiss, Göttingen, Germany).

### Electrophysiological Recording

All patch-clamp recordings were performed at 27–31°C. Patch pipettes were made from borosilicate glass Kwik-Fil 1B150F-4 capillaries (World Precision Instruments, Sarasota, FL) in four steps on a brown/Flamming model P-97 and fire-polished (Sutter Instruments, Novato, CA) using an LPZ 101 microforge (List Medical Electronics, Darmstadt, Germany). The resistance of patch electrodes was 1–3 MΩ. Patch electrodes were filled with a solution containing (in mM) 112 *N*-methyl-D-glucamine (NMDG), 112 HCl, 10 glucose, 5 EGTA, 10 HEPES, pH 7.4 (290 mOsm/kg H<sub>2</sub>O adjusted with sorbitol). In some experiments 3 mM Mg<sup>2+</sup> or 4 mM ATP was added at pipette solution. Bath solution contained (in mM) 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 34 NMDG, 34 HCl, 3.4 glucose and 10 HEPES, pH 7.4 (230 mOsm/kg H<sub>2</sub>O [hypotonic] and 290 mOsm/kg H<sub>2</sub>O [isotonic]). Some experiments were made with 5 or 118 NMDG-Cl bath solution. Patch-clamp recordings were performed in the whole-cell configuration. The protocol used was as follows: from a holding of −40 mV, a pulse to −80 mV was applied, followed by pulses at different voltages between −150 and 110 mV with an interval of 20 mV. Currents were measured using an Axopatch 200A integrating patch-clamp amplifier (Axon Instruments, Foster City, CA). Records were low pass-filtered at 5 kHz, digitized using a DigiData 1200 Interface (Axon Instruments), transferred to a personal computer and analyzed using pCLAMP 6.0 software (Axon Instruments), ClampFit (Axon Instruments) 9.0 and Graft 3.01 (Erythacus Software, Staines, UK).

### Statistical Analysis

Statistical analysis was done with NPAR1WAY using Wilcoxon scores for the Statistical Analysis System (SAS Institute, Cary, NC). Different experimental groups were compared using the Kruskal–Wallis and Friedman tests, followed by Dunn's test for multiple pairwise comparisons using the macro %DUNN for SAS proposed by Juneau (2007). Current density is expressed as mean ± SE. Differences between values were considered statistically significant at  $P < 0.05$ .

## Results

### General Description of Noninfected and Infected Primary Cultures of Cardiomyocytes

Although the *T. cruzi* parasite is able to infect a broad spectrum of mammalian cells in laboratory conditions in

vitro, cardiomyocytes are its natural target. Initially, we explored the possibility of carrying out this work on cardiomyocytes isolated from infected animals. We thoroughly reviewed the literature and found only two patch-clamp studies carried out on subepicardial cells isolated from chagasic dogs (Pacioretti et al. 1995; Han et al. 1997). We assumed some methodological disadvantages of this model. First of all, not all cells in the chagasic heart are infected. According to our unpublished histopathological data, about 60–80% of cardiac tissue in the acute phase of infection in mice conserves its structure. Furthermore, the procedure of cell dispersion implicates the application of digestive enzymes like trypsin and collagenase. Considering that the plasma membrane of infected cells is significantly more fragile than that of noninfected cells, most parasitized cells are lost during this procedure (our results, not shown). Moreover, in the case of *T. cruzi*-infected cardiomyocytes, it makes it difficult to determine where the living cell is infected if only a few intracellular parasites are present. Therefore, we used a primary culture of cardiomyocytes infected with *T. cruzi* in vitro as an alternative experimental model.

For our purpose, a long-term culture was required because 5–7 days are needed for infection and intracellular proliferation of the *T. cruzi* parasite. However, it was reported previously that up to 70% of the cells isolated from adult rat heart were normally lost during the first days of culture (Schwarzfeld and Jacobson 1981; Haddad et al. 1988; Spahr et al. 1989; Dubus et al. 1990), and surviving cells demonstrated progressive morphological and functional degenerative changes (Banyasz et al. 2008). Therefore, we used neonatal cells instead. This model was successfully utilized in numerous studies of cell morphology, metabolism, cellular ionic exchange and contractile activities (reviewed in Chlopčíková et al. 2001; Sreejit et al. 2008). Although usually short-long cultures were used, experiments with long-term cultures (more than 4 days) of neonatal cardiomyocytes also have been reported. Several studies demonstrated that neonatal cardiomyocytes conserved their properties in culture conditions much better than cardiac cells isolated from adult animals. Yamashita and colleagues (1994) noted that the phenotype of cultured neonatal cardiomyocytes is very stable and that their contractile profile during hypoxia-reoxygenation experiments is comparable with that of in situ hearts. It was shown that, during prolonged cultivation, neonatal ventricular myocytes retained a typical composition of gap junctions (Kwak et al. 1999). The whole-cell peak current density for T-type Ca<sup>2+</sup> current did not change up to 7 days in culture (Pluteanu and Cribbs 2009).

Both ventricles and auricles used to be infected by *T. cruzi*. Since the currents present in ventricular and auricular cells differ, we carried out our experiments on



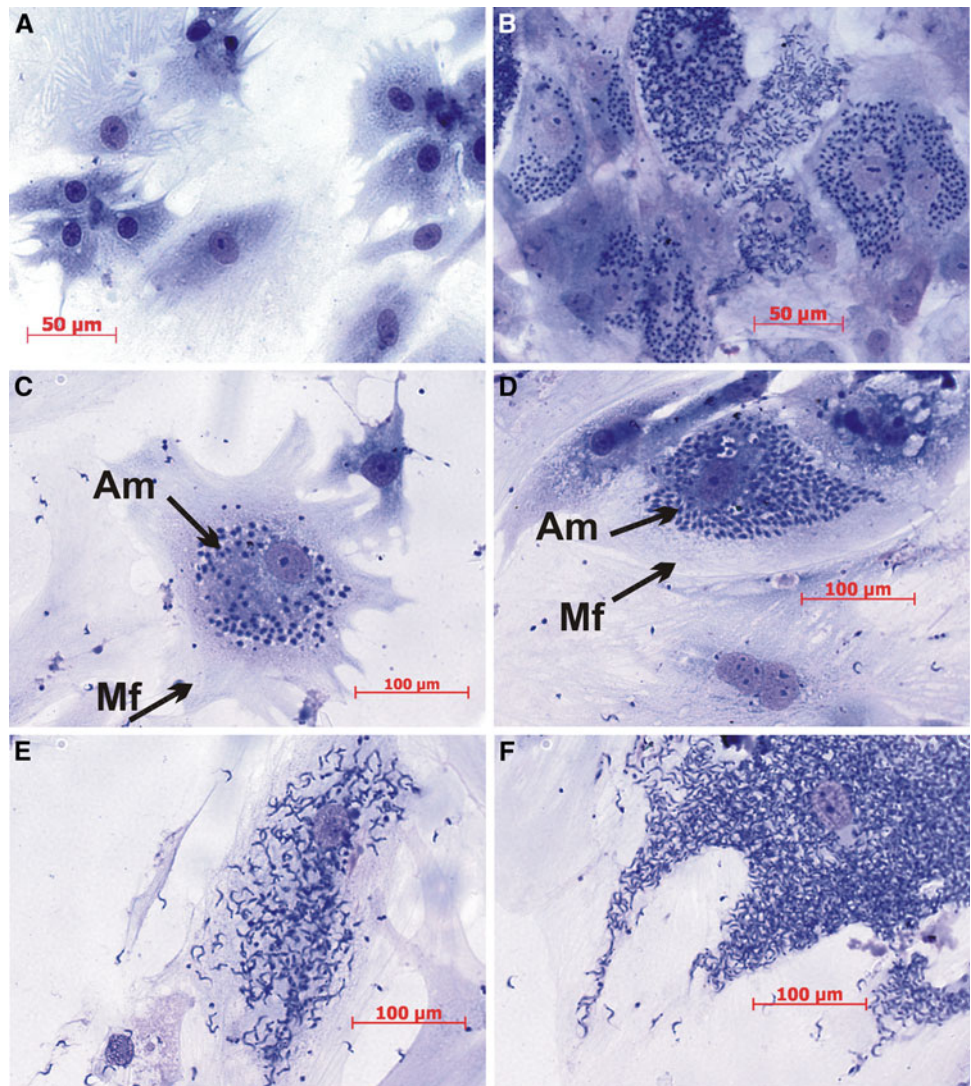
ventricle cells. In our hands, more than 90% of cardiomyocytes survived the dispersion procedure and spread out on the surface of culture dishes (coverslips). Within 3 days, 80–90% of cultured cells manifested spontaneous contractions.

In rats, growth of the heart during postnatal development is due to cell enlargement (hypertrophy) (Claycomb 1977). We did not observe cardiomyocyte proliferation in our cultures. We considered that the number of cardiac cells during culture was constant, which simplified the calculation of the parasite–host cell relationship. Morphologically, cardiomyocytes in culture are well-spread polygonal cells, with numerous extensions (Fig. 1a). In contrast to observations on primary cultures from adult cardiomyocytes (Banyasz et al. 2008), we did not observe any degradation signs in cultivated cells up to 3 weeks after the isolation procedure. Conversely, we observed cell growth and differentiation during cultivation. Cells increased significantly in dimensions and formed numerous extensions and

organelles. At seed density of more than  $1 \times 10^5$  cells in a 35-mm tissue culture dish, a confluent monolayer of beating cardiomyocytes was established. We considered our culture conditions to be appropriate for long-term cardiomyocyte cultivation. The percentage of beating myocardial cells exceeded 90% after 3 days in culture, and at this point the cells were considered to be completely recuperated from the isolation procedure and were used for infection.

We determined such a parasite–host cell relation that resulted in practically 100% infection of the cultured cells, which facilitated patch-clamp recording on infected cultures. Giemsa staining was used in some randomly selected cultures to control the efficiency of infection. At a parasite–host cell ratio of 20:1, the presence of severe intracellular infection was confirmed microscopically in practically 100% of cultured cardiomyocytes at day 8 postinfection (Fig. 1b). Infected cells were bigger than noninfected cells. Cardiac cells with moderate or severe intracellular infection (amastigotes) or even the cells where amastigotes had

**Fig. 1** Primary cultures of uninfected and *T. cruzi*-infected ventricular cardiomyocytes (Giemsa staining). **a** Uninfected cardiomyocytes at day 4 of culture. **b** *T. cruzi*-infected cardiomyocytes culture at day 8 of infection. **c–f** Individual cells of the same preparation as **b**. Cells with early infection (**c**, **d**). Intracellular amastigotes (*Am*) and undamaged myofibrils (*Mf*) are indicated by arrows. Cells with late infection (**e**, **f**). Numerous flagellate trypomastigotes are present, and myofibrils are destroyed

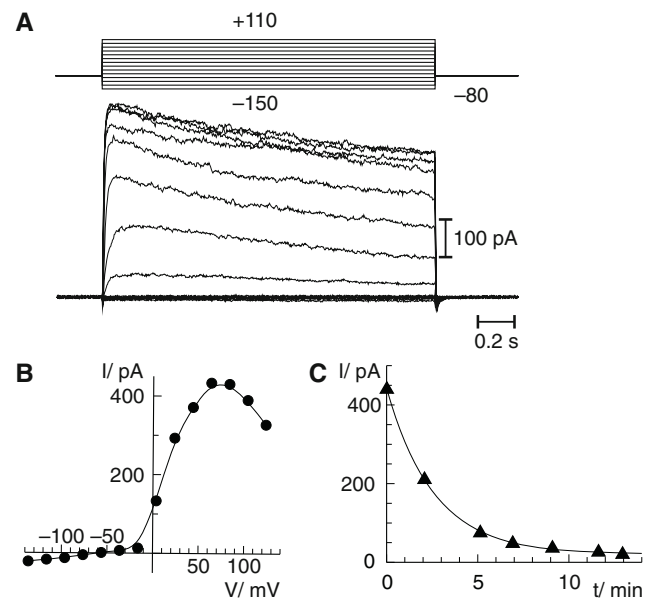


already transformed into trypomastigotes were observed in the same culture dish (Fig. 1c–e). In some cases, completely damaged host cells with disintegrated plasma membrane and numerous liberated trypomastigotes were seen (Fig. 1f). However, intracellular parasites were not always very well detected in the living cells used for patch-clamp experiments. Then, at the end of each patch-clamp recording, the sealed cell was always disintegrated by the patch electrode, to verify how many parasites were coming out. It was easy to do if the infected cell was severely damaged, with practically complete destruction of myofibrils. In general, amastigotic forms were already transformed to flagellate trypomastigotic forms in these cells (Fig. 1e). It is also noteworthy that additional precautions were taken while patching the severely infected cells, to avoid premature cell rupture. These infected cells were classified in our records as “late infection.” In some cases, however, we were unable to rupture the cell with the electrode, evidently due to the presence of undamaged myofibrils (Fig. 1c, d). These cells were classified as “early infection.”

#### Currents Recorded in Cultured Ventricular Cardiomyocytes

To eliminate the contribution of multiple potassium and cationic currents expressed in a cardiomyocyte membrane, we replaced all monovalent cations in the pipette and bath solution by an impermeable cation NMDG. After achievement of the whole-cell configuration, NMDG-containing patch pipette solution started to perfuse the cell. Whereas in the first moment the cell membrane conductance was dominated by a sustained delayed rectifying  $K^+$  current (Fig. 2), replacement of  $K^+$  by  $NMDG^+$  caused a gradual decrease of this current. Due to the plane geometry and large size of single cardiomyocytes, the substitution of  $K^+$  by  $NMDG^+$  took a relatively long time (Fig. 2c); but in all cases it was completed after 15 min of internal perfusion. At this moment actual measurements of intrinsic anion ( $Cl^-$ )-conducting currents were initiated.

Cultured cardiomyocytes under these ionic conditions mostly expressed relatively small instantaneous outward- or inward-rectifying currents, conducting  $Cl^-$  into or outside the cell, respectively (Fig. 3a, b). However, ~10% of cardiomyocytes expressed a relatively large time-dependent current activated at positive membrane potentials (Fig. 3c). In the literature we found no indication of such an anion current in cardiomyocytes, which forced us to verify its selectivity. Figure 4 demonstrates that the time-dependent current reversed at +14 and +28 mV, respectively, when bath solution with 40 mM  $Cl^-$  was changed for that with 5 mM  $Cl^-$ . Positive reversal potential (the pipette always contains  $Cl^-$  at higher concentration, 118 mM) and



**Fig. 2** Internal perfusion with NMDG-Cl solution eliminates the outward-rectifying  $K^+$  current. **a** Original record of the outward-rectifying  $K^+$  current in response to a series of depolarizing pulses immediately after achievement of the whole-cell configuration. Cell capacitance was 91 pF. **b** Peak current–voltage relationship for the record in **a**. **c** Decay of membrane current at +90 mV as a function of time after achievement of the whole-cell configuration

a positive shift of the reversal after decreasing  $Cl^-$  in the bath means that the time-dependent current preferentially conducts  $Cl^-$ . As several anionic currents expressed in cardiomyocytes are sensitive to volume changes (Tseng 1992; Sorota 1992; Du and Sorota 1997; Duan et al. 1997; Walsh and Zhang 2005; Yamamoto et al. 2008), we decreased the osmolality of the external medium from 290 to 230 mOsm. Such a challenge resulted in complete disappearance of the time-dependent current within a few minutes ( $n = 4$ , result not shown). The same hypotonic treatment in a majority of cases provoked a slow but dramatic increase of the instantaneous outward-rectifying current (Fig. 5). This current was carried by  $Cl^-$  as upon a change from symmetric 118 NMDG-Cl conditions to a lower (40 mM) NMDG-Cl in the bath the reversal was shifted by +25 mV, approaching an equilibrium potential for  $Cl^-$  (+28 mV), and the outward current ( $Cl^-$  influx) decreased without a significant change of the conductance for the inward component (Fig. 5c). All anionic currents registered in the present work were not sensitive to internal ATP (4 mM, data not shown).

#### Current Variation Due to *T. cruzi* Infection

The density of the most frequently expressed instantaneous currents varied according to the age of the cell culture.

**Fig. 3** Three types of ion currents are detected in cultured cardiomyocytes.

**a** Instantaneous inward-rectifying current and current-voltage ( $I$ - $V$ ) relationship. **b** Instantaneous outward-rectifying current and  $I$ - $V$  relationship. **c** Time-dependent outward-rectifying current and  $I$ - $V$  relationship. All three cells have a comparable plasma membrane area ( $c \sim 90$  pF). Bath and pipette solutions are  $K^+$ -free (with the exception of 2 mM  $Ca^{2+}$  in the bath, NMDG is the only cation present at both membrane sides)

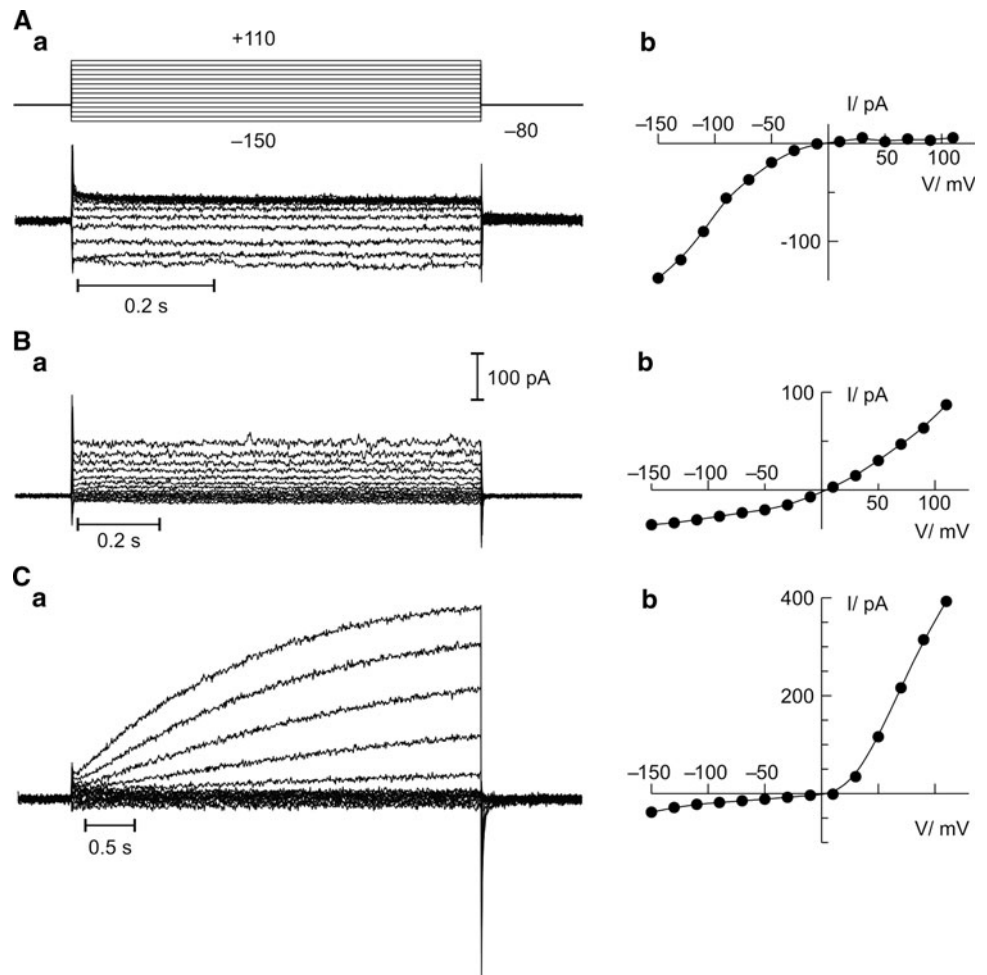
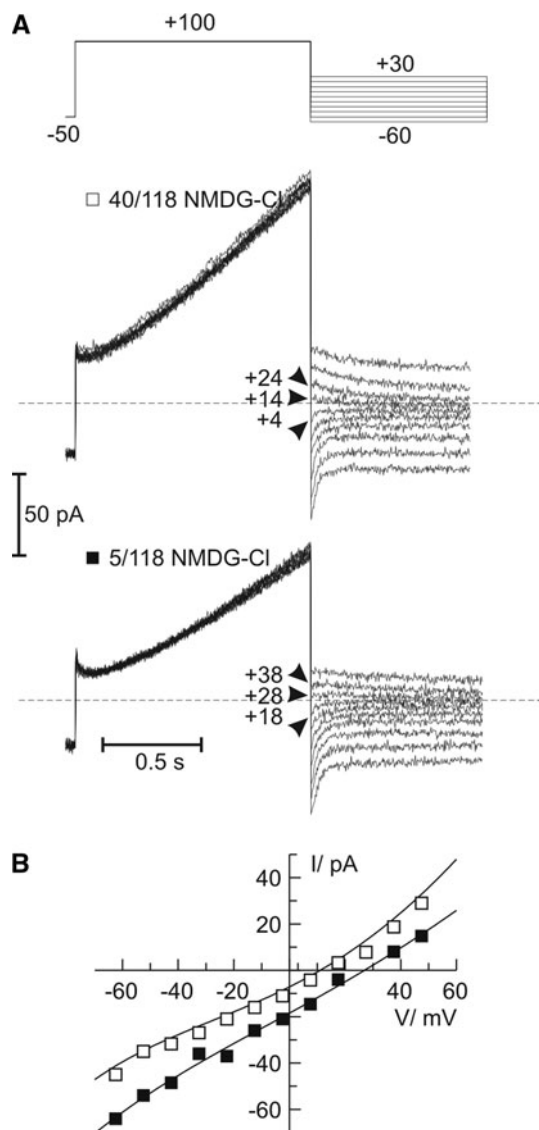


Figure 6 shows that both inward and outward conductance decreased at day 9 in culture compared to day 4, remained relatively constant up to day 15 and gradually increased for 30-day-old cultures. Keeping in mind this behavior and that *T. cruzi* infection is relatively slow to develop, we assayed cells in 10- to 15-day-old cultures, in control conditions and infected at day 5 of culture by *T. cruzi*. The results presented in Figure 7 show that the average density of the instantaneous outward current increased by approximately twofold at the late stages of infection (cells containing dozens to hundreds of trypomastigotes). The occurrence (percentage of cells expressing respective current) and density of the instantaneous outward current substantially decreased when the pipette solution was supplemented by  $Mg^{2+}$ , a known natural inhibitor of the  $ClC$ -3-mediated, volume-sensitive, outward-rectifying anion current in cardiomyocytes (Yamamoto-Mizuma et al. 2004). However, also in the presence of  $Mg^{2+}$ , infection by *T. cruzi* provoked a significant increase of the occurrence and density of the outward-rectifying instantaneous current (Fig. 7b, d).  $Mg^{2+}$  also suppressed the

inward-rectifying instantaneous currents, but in this case the variation of current density in infected cells compared to control was statistically insignificant at  $P < 0.05$  (Fig. 7a, c). When it comes to the voltage-dependent, slowly activating anion current (Fig. 3a, b), its density was also unaffected by *T. cruzi* infection and its occurrence was low in both control and infected cells with an  $Mg^{2+}$ -free intracellular solution (Fig. 8). However, the occurrence of this current dramatically increased in infected cells in the presence of  $Mg^{2+}$  in the intracellular solution, from  $\sim 10$  to  $\sim 80\%$ , without a significant change of the current density (Fig. 8). No such change in occurrence was observed in control cardiomyocytes. Our results suggest that the two types of anion currents were altered by *T. cruzi*: the instantaneous outward current (mainly due to an increase in the current density) and the time-dependent outward current (mainly due to the increase of percentage of cells expressing it). Therefore, bulk conductance of plasma membrane to anions and, possibly, to small neutral solutes would increase upon *T. cruzi* infection.



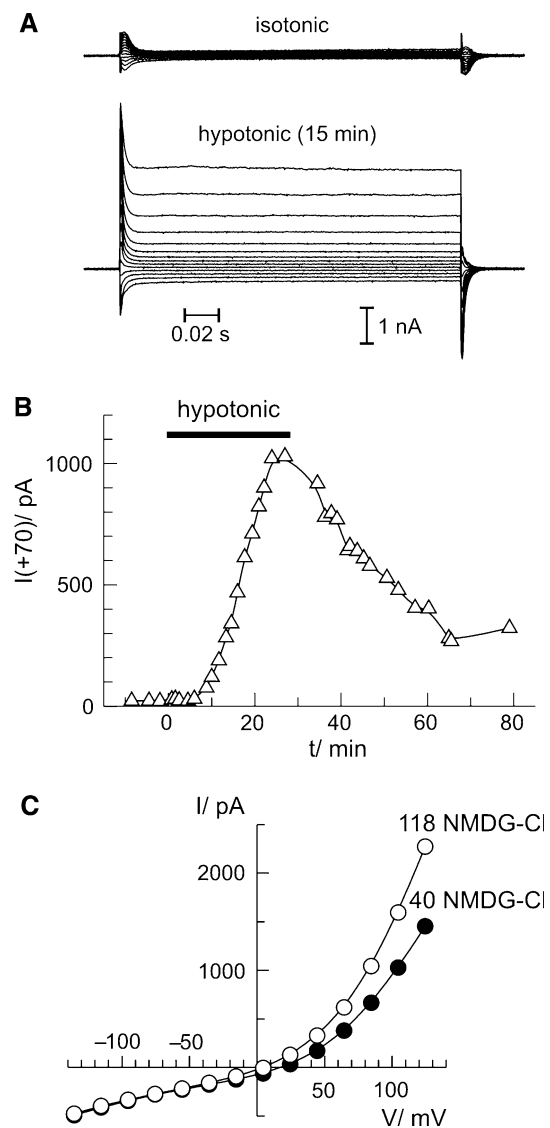


**Fig. 4** The time-dependent current is anion-selective. **a** Tail currents are evoked by a prepulse to +100 mV, followed by a series of steps to lower potentials. The concentration of salt (NMDG-Cl) was lowered from 40 to 5 mM, resulting in a positive shift of the reversal potential of the tail currents from approximately +14 to +28 mV. **b** Current-voltage relations of the tail currents presented in **a**; the instantaneous inward-rectifying whole-cell current was subtracted. Cell capacitance was 69 pF

## Discussion

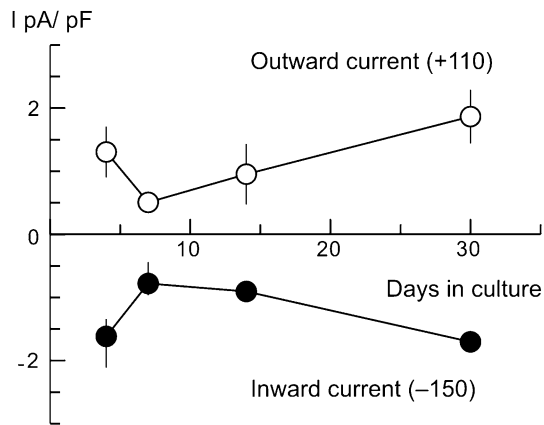
### Endogenous Anion Currents in Noninfected Cultured Ventricular Cardiomyocytes Isolated from Neonatal Rats

It is widely accepted that the functional properties, including excitability, action potential, contractility and relaxation, differ in immature and mature hearts. Although previous studies have identified a range of anion currents in



**Fig. 5** The instantaneous outward current is swelling-activated and anion-selective. **a** Application of hypotonic (60 mOsm difference) stress activates the outward-rectifying current. **b** Time course of the swelling-induced outward current activation; same experiment as in **a**. **c** Swelling-activated current-voltage relationships under symmetrical (118 NMDG-Cl) and asymmetrical (bath 40 NMDG-Cl) conditions. A positive ~20-mV shift of the reversal and a decrease of the outward current conductance imply anionic preference

cardiac myocytes (Duan 2009), one may expect that the pattern of currents in cardiac cells alters from birth through perinatal development. Indeed, developmental changes in the inwardly rectifying background potassium current were described earlier (Kilborn and Fedida 1990). Equivalent data about anionic currents are absent in the literature. However, large-scale genetic analysis revealed the developmental alterations in the levels of expression of various genes coding cationic and anionic ion channels in the mouse heart (Harrell et al. 2007). Besides, we presumed that the long-term culture conditions may also affect the

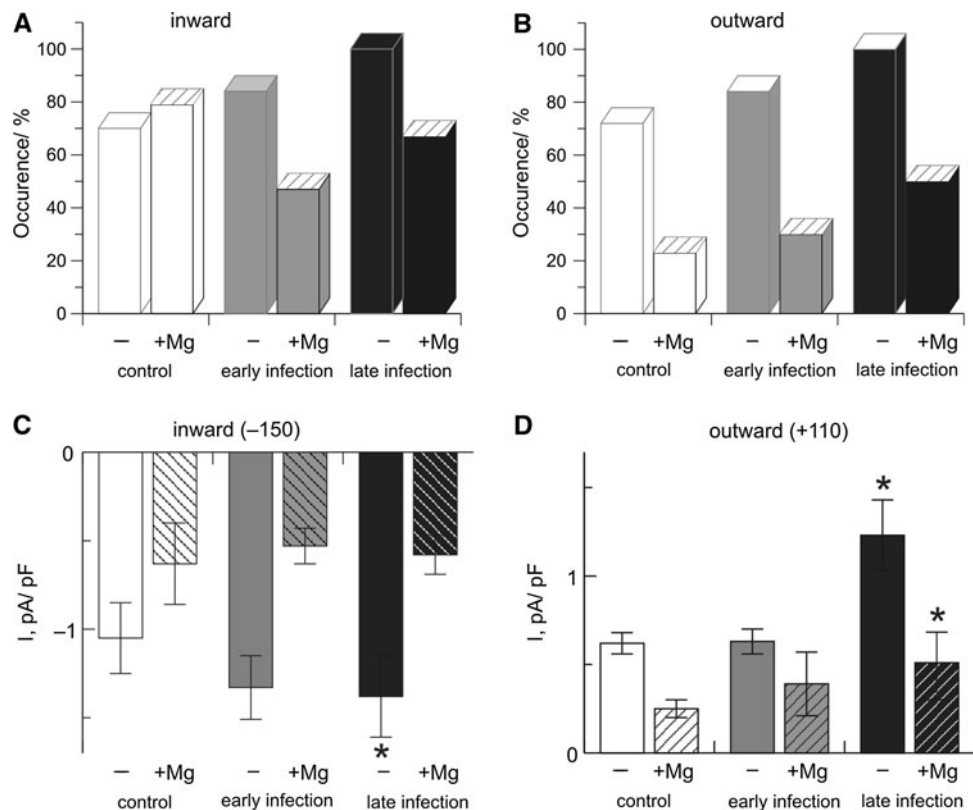


**Fig. 6** Expression of instantaneous currents depends on cardiomyocyte culture age. The magnitude of outward- and inward-rectifying currents was evaluated at +110 and -150 mV, respectively, and divided by cell capacitance (68 pF as an average). Data are means  $\pm$  SE, 10–15 individual cells assayed for each point

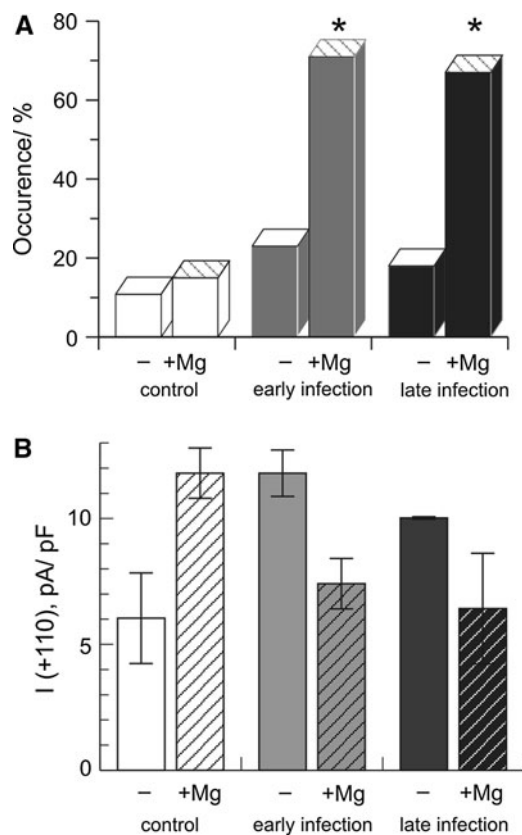
ion channel expression. For instance, the expression of genes encoding major histocompatibility complex (MHC), sarco-/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA2) and ryanodine receptor (RyR) was significantly decreased in 10-day monolayer ventricular cell culture compared to freshly isolated cells (Khait and Birla 2009). Then, to reveal the feasible changes during prolonged cultivation, we undertook patch-clamp experiments in noninfected

neonatal cardiac cells cultured in vitro for different periods (4, 7, 14 and 30 days). In our experimental conditions, two small instantaneous anionic currents, inwardly and outwardly rectifying, were present in every recorded cell (Fig. 3a, b). Hypotonic stress caused dramatic activation of the outward current (Fig. 5). A swelling-activated, outwardly rectifying  $\text{Cl}^-$  current ( $I_{\text{Clswell}}$ ) was described previously in mammalian cardiac myocytes (Tseng 1992; Sorota 1992; Du and Sorota 1997; Duan et al. 1997), including ventricular cells isolated from neonatal rats (Tilly et al. 1996; Walsh and Zhang 2005). The most likely molecular candidate proposed for  $I_{\text{Clswell}}$  is the *CIC-3* gene (Duan et al. 1997, 1999), a member of the large superfamily of volume-sensitive osmolyte and anion channels. As for the inward rectified instantaneous current, we did not find any report describing this type of current in neonatal ventricular cardiomyocytes. On the other hand, a volume-regulated, hyperpolarization-activated  $\text{Cl}^-$  inward rectifier channel was recently reported in adult rat ventricular myocytes and identified as *CIC-2* (Britton et al. 2005). Anionic channels activated by swelling were suggested to be important for cardiac function under physiological and pathophysiological conditions, like cardiomyopathies, ischemia, reperfusion, myocardial injury and mechanical stress during cellular hypertrophy (reviewed by Baumgarten and Clemons 2003; Duan 2009). We observed a slight decrease in the density of both instantaneous currents during the first days of

**Fig. 7** Infection by *T. cruzi* increases the expression of instantaneous currents. **a**, **b** Percentage of cells (6–18 were tested for each condition) expressing inward- and/or outward-rectifying instantaneous currents. **c**, **d** Current densities for inward- and outward-rectifying currents for the same cells as in **a** and **b**. Data are means  $\pm$  SE







**Fig. 8** Occurrence, but not the density, of the time-dependent anion current is strongly upregulated by *T. cruzi* infection in the presence of cytosolic  $Mg^{2+}$ . **a** Percentage of cells expressing outward-rectifying, time-dependent anion current. **b** Current density of a steady-state, time-dependent current at +110 mV. Only cells expressing measurable outward-rectifying, time-dependent currents were taken into account. Data are means  $\pm$  SE ( $n = 3$ –10 cells for each condition)

culture. The main factors contributing to this phenomenon might be the dispersion procedure itself and the presence, in the first 3 days of culture, of the cytostatic drug CDA, added for inhibition of mesenchymal cells.

The weakly selective, time-dependent anion current (Fig. 3c) was not previously reported in studies on cardiac cells and requires further characterization. The cells expressing this current were similar in morphology to other cultivated cells, and their proportion in the population was relatively constant during the entire experimental period (4–30 days).

#### Changes in Expression of Anionic Currents Induced by *T. cruzi* Infection in Cardiomyocytes

In our preparations *T. cruzi* infection did not provoke new anionic currents in the host cells. In ventricular cardiomyocytes with intracellular infection we observed the same three types of anionic currents as in noninfected cells, but the density of both instantaneous currents in severely

infected cells (late infection with numerous trypomastigotes and completely destroyed myofibers) on day 6 postinfection (day 11 of culture) was increased (Fig. 7). The density of the outward current in heavily infected cells was twice that in noninfected cells (Fig. 7d). Noteworthy is that the effect of *P. falciparum* infection in erythrocytes was significantly more pronounced: Whole-cell currents registered in infected cells were some 100- to 150-fold larger than those measured in noninfected cells (reviewed in Staines et al. 2007). Different factors could explain this phenomenon, including the level of anionic channel expression in healthy cells, the level of metabolic activity of the host cell and the rate of parasite proliferation. Erythrocytes are significantly smaller than cardiac cells, contain fewer nutrients, do not possess a nucleus and do not synthesize proteins de novo. Thus, a rapidly proliferating malaria parasite needs for its survival a significant modification in membrane transport to activate nutrient uptake. Cardiac cells, unlike erythrocytes, are metabolically active. Most likely these cells possess all of the nutrients necessary for parasite survival up to the moment when the host cell is damaged severely, and multiple parasite cells are present. The level of expression of anionic currents in healthy cardiac cells is significant and contributes to a variety of important cardiac cell functions, including control of resting and action potentials (Ehara and Hasegawa 1983; Fossard and Lee 1976), volume and pH regulation and transport of organic osmolytes (Vandenberg et al. 1996). Hence, the modification in membrane transport is needed at late phases of infection, when the internal resources of the host cell are used up but the parasite still requires some period to complete the differentiation and to be liberated. It is remarkable that swelling-activated inwardly and outwardly rectifying  $Cl^-$  currents were shown to be activated persistently in hypertrophic cardiomyocytes isolated from animals with congestive heart failure (Clemo et al. 1999). In our experiments, swelling-activated channels in infected cells may be activated by a mechanical stretch at the moment of parasite overload and significant cell enlargement.

Although it has been known for several decades that *P. falciparum* infection greatly increases the permeability of the host cell membrane, many questions remain to be answered. It is not yet known which channel types precisely are responsible for transport of different organic solutes or ions, what are the mechanisms of their regulation and which channels are essential for parasite survival; it is also a matter of debate whether *P. falciparum* infection induces completely new currents or provokes the upregulation and/or overexpression of the constitutively expressed ones (Staines et al. 2007). Another intracellular parasite, *Leishmania amazonensis*, was reported to provoke the changes in the inward- and outward-rectified potassium currents of the macrophage host cell (Forero et al. 1999;

Camacho et al. 2008; Quintana et al. 2010). The authors speculated that these changes would prevent macrophage activation and phagocytosis rather than affect the uptake of solutes. In the present work on cardiomyocytes infected with *T. cruzi* we were able to demonstrate that intracellularly the *T. cruzi* parasite upregulates anionic currents, present constitutively in noninfected cardiomyocytes. Future studies are needed to confirm the hypothesis that these currents are important for solute exchange and parasite survival.

**Acknowledgments** This work was funded by the Mexican National Council for Science and Technology (CONACyT-SEP-2004-CO1-46731) and the Ramon Alvarez-Buylla Foundation (University of Colima, 332/05). We are grateful to O. Montesinos-López for statistical analysis.

## References

- Andrade SG, Magalhães JB (1997) Biodems and zymodems of *Trypanosoma cruzi* strains: correlations with clinical data and experimental pathology. *Rev Soc Bras Med Trop* 30:27–35
- Banyasz T, Lozinskiy I, Payne CE, Edelmann S, Norton B, Chen B, Chen-Izu Y, Izu LT, Balke W (2008) Transformation of adult rat cardiac myocytes in primary culture. *Exp Physiol* 93:370–382
- Baumgarten CM, Clemo HF (2003) Swelling-activated chloride channels in cardiac physiology and pathophysiology. *Prog Biophys Mol Biol* 82:25–42
- Bouyer G, Egee S, Thomas SL (2006) Three types of spontaneously active anionic channels in malaria-infected human red blood cells. *Blood Cells Mol Dis* 36:248–254
- Britton FC, Wang GL, Huang ZM, Ye L, Horowitz B, Hume JR, Duan D (2005) Functional characterization of novel alternatively spliced CIC-2 chloride channel variants in the heart. *J Biol Chem* 280:25871–25880
- Camacho M, Forero ME, Fajardo C, Niño A, Morales P, Campos H (2008) *Leishmania amazonensis* infection may affect the ability of the host macrophage to be activated by altering their outward potassium currents. *Exp Parasitol* 120:50–56
- Chlopčková S, Psotová J, Míketová P (2001) Neonatal rat cardiomyocytes—a model for the study of morphological, biochemical and electrophysiological characteristics of the heart. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 145:49–55
- Claycomb WC (1977) Differentiation and growth of the heart cell during development. *Biochem J* 168:599–601
- Clemon HF, Stambler BS, Baumgarten CM (1999) Swelling-activated chloride current is persistently activated in ventricular myocytes from dogs with tachycardia-induced congestive heart failure. *Circ Res* 84:157–165
- Desai SA, Bezrukov SM, Zimmerberg J (2000) A voltage-dependent channel involved in nutrient uptake by red blood cells infected with the malaria parasite. *Nature* 406:1001–1005
- Du XY, Sorota S (1997) Cardiac swelling-induced chloride current depolarizes canine atrial myocytes. *Am J Physiol Heart Circ Physiol* 272:H1904–H1916
- Duan D (2009) Phenomics of cardiac chloride channels: the systemic study of chloride channel function in heart. *J Physiol* 587:2163–2177
- Duan D, Hume JR, Nattel S (1997) Evidence that outwardly rectifying  $\text{Cl}^-$  channels underlie volume regulated  $\text{Cl}^-$  currents in heart. *Circ Res* 80:103–113
- Duan D, Cowley S, Horowitz B, Hume JR (1999) A serine residue in CIC-3 links phosphorylation–dephosphorylation to chloride channel regulation by cell volume. *J Gen Phys* 113:57–70
- Dubus I, Samuel JL, Marotte F, Delcayre C, Rappaport L (1990)  $\beta$ -Adrenergic agonists stimulate the synthesis of noncontractile but not contractile proteins in cultured myocytes isolated from adult rat heart. *Circ Res* 66:867–874
- Egee S, Lapaix F, Decherf G, Staines HM, Ellory JC, Thomas SL (2002) A stretch-activated anion channel is up regulated by the malaria parasite *Plasmodium falciparum*. *J Physiol* 542:795–801
- Ehara T, Hasegawa J (1983) Effects of hypertonic solution on action potential and input resistance in the guinea-pig ventricular muscle. *Jpn J Physiol* 33:151–167
- Forero ME, Marín M, Corrales A, Llano I, Moreno H, Camacho M (1999) *Leishmania amazonensis* infection induces changes in the electrophysiological properties of macrophage-like cells. *J Membr Biol* 170:173–180
- Fossard HA, Lee CO (1976) Influence of changes in external potassium and chloride ions on membrane potential and intracellular potassium ion activity in rabbit ventricular muscle. *J Physiol* 256:663–689
- Ginsburg H, Stein HD (2004) The new permeability pathways induced by the malaria parasite in the membrane of the infected erythrocyte: comparison of results using different experimental techniques. *J Membr Biol* 197:113–122
- Ginsburg H, Krugliak M, Eidelman O, Cabantchik ZI (1983) New permeability pathways induced in membranes of *Plasmodium falciparum* infected erythrocytes. *Mol Biochem Parasitol* 8:177–190
- Haddad J, Decker ML, Hsieh LC, Lesch M, Samarel AM, Decker RS (1988) Attachment and maintenance of adult rabbit cardiac myocytes in primary cell culture. *Am J Physiol Cell Physiol* 255:C19–C27
- Han W, Barr SC, Paciorek LM, Gilmour RF Jr (1997) Restoration of transient outward potassium current by noradrenaline in chagasic canine epicardium. *J Physiol* 500:75–83
- Harrell MD, Harbi S, Hoffman JF, Zavadil J, Coetzee WA (2007) Large-scale analysis of ion channel gene expression in the mouse heart during perinatal development. *Physiol Genomics* 28:273–283
- Huber SM, Uhlemann AC, Gamper NL, Duranton C, Kremsner PG, Lang F (2002) *Plasmodium falciparum* activates endogenous  $\text{Cl}^-$  channels of human erythrocytes by membrane oxidation. *EMBO J* 21:22–30
- Juneau P (2007) Pharmaceutical statistics using SAS. In: Dmitrienko A, Chuang-Stein C, D’Agostino RD (eds) Nonparametric methods in pharmaceutical statistics. A Practical Guide, Cary SAS Press, pp 117–149
- Karwatowska-Prokopczuk E, Nordberg JA, Li HL, Engler RL, Gottlieb RA (1998) Effect of vacuolar proton ATPase on  $\text{pHi}$ ,  $\text{Ca}^{2+}$ , and apoptosis in neonatal cardiomyocytes during metabolic inhibition/recovery. *Circ Res* 82:1139–1144
- Khait L, Birla RK (2009) Changes in gene expression during the formation of bioengineered heart muscle. *Artif Organs* 33:3–15
- Kilborn MJ, Fedida D (1990) A study of the developmental changes in outward currents of rat ventricular myocytes. *J Physiol* 430:37–60
- Kirchhoff LV (1993) American trypanosomiasis (Chagas’ disease)—a topical disease now in the United States. *N Engl J Med* 329:634–644
- Kirk K, Horner HA, Elford BC, Ellory JC, Newbold CI (1994) Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. *J Biol Chem* 269:3339–3347
- Kwak BR, van Kempen MJ, Théveniau-Ruissy M, Gros DB, Jongsma HJ (1999) Connexin expression in cultured neonatal rat

- myocytes reflects the pattern of the intact ventricle. *Cardiovasc Res* 44:370–380
- Lew VL, Tiffert T, Ginsburg H (2003) Excess hemoglobin digestion and the osmotic stability of *Plasmodium falciparum*-infected red blood cells. *Blood* 101:4189–4194
- Melnikov V, Fierro F, Espinoza F, Guzmán F, Dobrovinskaya O (2005) Pathologic changes in lungs caused by Mexican isolates of *Trypanosoma cruzi* in the acute phase of infection in mice. *Am J Trop Med Hyg* 73:301–306
- Pacioretty LM, Barr SC, Han WP, Gilmour RF Jr (1995) Reduction of the transient outward potassium current in a canine model of Chagas' disease. *Am J Physiol Heart Circ Physiol* 268:H1258–H1264
- Pluteanu F, Cribbs LL (2009) T-type calcium channels are regulated by hypoxia/reoxygenation in ventricular myocytes. *Am J Physiol Heart Circ Physiol* 297:H1304–H1313
- Poole RC, Halestrap AP (1993) Transport of lactate and other monocarboxylates across mammalian plasma membranes. *Am J Physiol Cell Physiol* 264:C761–C782
- Quintana E, Torres Y, Alvarez C, Rojas A, Forero ME, Camacho M (2010) Changes in macrophage membrane properties during early *Leishmania amazonensis* infection differ from those observed during established infection and are partially explained by phagocytosis. *Exp Parasitol* 124:258–264
- Saliba KJ, Horner HA, Kirk K (1998) Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. *J Biol Chem* 273:10190–10195
- Schmunis GA, Yadon ZE (2009) Chagas disease: a Latin American health problem becoming a world health problem. *Acta Trop*. doi:10.1016/j.actatropica.2009.11.003
- Schwarzfeld TA, Jacobson SL (1981) Isolation and development in cell culture of myocardial cells of the adult rat. *J Mol Cell Cardiol* 13:563–575
- Sorota S (1992) Swelling-induced chloride-sensitive current in canine atrial cells revealed by whole-cell patch-clamp method. *Circ Res* 70:679–687
- Spahr R, Jacobson SL, Siegmund B, Schwartz P, Piper HM (1989) Substrate oxidation by adult cardiomyocytes in long-term primary culture. *J Mol Cell Cardiol* 21:175–185
- Sreejit P, Kumar S, Verma RS (2008) An improved protocol for primary culture of cardiomyocyte from neonatal mice. *In Vitro Cell Dev Biol Anim* 44:45–50
- Staines HM, Alkhalil A, Allen RJ, De Jonge HR, Derbyshire E, Egée S, Ginsburg H, Hill DA, Huber SM, Kirk K, Lang F, Lisk G, Oteng E, Pillai AD, Rayavara K, Rouhani S, Saliba KJ, Shen C, Solomon T, Thomas SL, Verloo P, Desai SA (2007) Electrophysiological studies of malaria parasite-infected erythrocytes: current status. *Int J Parasitol* 37:475–482
- Tilly BC, Bezstarosti K, Boomaars WE, Marino CR, Lamers JM, de Jonge HR (1996) Expression and regulation of chloride channels in neonatal rat cardiomyocytes. *Mol Cell Biochem* 157:129–135
- Tseng GN (1992) Cell swelling increases membrane conductance of canine cardiac cells: evidence for a volume-sensitive  $\text{Cl}^-$  channel. *Am J Physiol Cell Physiol* 262:C1056–C1068
- Tyler KM, Engman DM (2001) The life cycle of *Trypanosoma cruzi* revisited. *Int J Parasitol* 31:472–481
- Vandenberg JI, Rees SA, Wright AR, Powell T (1996) Cell swelling and ion transport pathways in cardiac myocytes. *Cardiovasc Res* 32:85–97
- Walsh KB, Zhang J (2005) Regulation of cardiac volume-sensitive chloride channel by focal adhesion kinase and Src kinase. *Am J Physiol Heart Circ Physiol* 289:H2566–H2574
- WHO (2002) The world health report. World Health Organization, Geneva
- Yamamoto S, Ichishima K, Ehara T (2008) Regulation of volume-regulated outwardly rectifying anion channels by phosphatidylinositol 3,4,5-triphosphate in mouse ventricular cells. *Biomed Res* 29:307–315
- Yamamoto-Mizuma S, Wang GX, Liu LL, Schegg K, Hatton WJ, Duan D, Horowitz TL, Lamb FS, Hume JR (2004) Altered properties of volume-sensitive osmolyte and anion channels (VSOACs) and membrane protein expression in cardiac and smooth muscle myocytes from *Clcn3<sup>-/-</sup>* mice. *J Physiol* 557:439–456
- Yamashita N, Nishida M, Hoshida S, Kuzuya T, Hori M, Taniguchi N, Kamada T, Tada M (1994) Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 h after preconditioning. *J Clin Invest* 94:2193–2199