J Membrane Biol (2009) 227:49–55
DOI 10.1007/s00232-008-9139-z

SHORT COMMUNICATION

Membrane Transporters and Cytoplasmatic pH Regulation on Bovine Sertoli Cells

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Received: 12 June 2008 / Accepted: 5 November 2008 / Published online: 3 December 2008

Abstract Sertoli cells are responsible for regulating a wide range of processes that lead to the differentiation of male germ cells into spermatozoa. Cytoplasmic pH (pH_i) has been shown to be an important parameter in cell physiology, regulating namely cell metabolism and differentiation. However, membrane transport mechanisms involved in pH_i regulation mechanisms of Sertoli cells have not yet been elucidated. In this work, pH_i was determined using the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF). Addition of weak acids resulted in rapid acidification of the intracellular milieu. Sertoli cells then recovered pH_i by a mechanism that was shown to be sensitive to external Na^+. pH_i recovery was also greatly reduced in the presence of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and amiloride. These results point toward the action of an Na^+-driven HCO_3^-/Cl^- exchanger and/or an Na^+/HCO_3^- cotransporter and the action of the Na^+/H^+ exchanger on pH_i regulation in the experimental conditions used. pH_i recovery was only slightly affected by ouabain, suggesting that the inhibition of Na^+/K^+-ATPase affects recovery indirectly, possibly via the shift on the Na^+ gradient. On the other hand, recovery from the acid load was independent of the presence of concanamycin A, a specific inhibitor of the V-type ATPases, suggesting that these pumps do not have a relevant action on pH_i regulation in bovine Sertoli cells.

Keywords Sertoli cells · Intracellular pH · Membrane ion transporter · 2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein

Abbreviations

BCECF	2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
DIDS	4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
FSH	Follicle Stimulating Hormone
$HBSS_f$	Calcium- and magnesium-free Hank's Balanced Salt Solution
PBS	Phosphate Buffered Solution
pH_e	Extracellular pH
pH_i	Intracellular pH
SEM	Standard error of the mean

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Introduction

Sertoli cells face the lumen of the seminiferous tubule, providing structural support and creating an immunologically protected space for germ cells. They facilitate the progression of spermatogenesis and are responsible for the phagocytosis of degenerating germ cells and their remains (Buzzard et al. 2002; Griswold 1995, 1998; Mruk and Cheng 2004). Sertoli cells provide germ cells with necessary nutrients, hormones and growth factors and control the composition of the seminiferous fluid (Griswold 1995; Mruk and Cheng 2004). The effects of testosterone and follicle-stimulating hormone (FSH) on the regulation of the above processes are mediated by the intracellular Ca^{2+} concentration of Sertoli cells (Gorczynska and Handelsman 1995; Lyng et al. 2000; McLachlan et al. 2002; Silva et al. 2002; Von Ledebur et al. 2002; Walker and Cheng 2005). Furthermore, it has been reported that intracellular pH (pH_i) regulation is important in the cellular response to hormones involved in raising cytosolic Ca^{2+} (Conlin et al. 1993). In addition, as the control of the pH of the seminiferous fluid is crucial for male fertility, pH_i regulation of Sertoli cells should also play a major role in this process (Mruk and Cheng 2004; Tuck et al. 1970).

pH_i is kept mainly through the net balance between production and elimination of protons and by intracellular buffers (Roos and Boron 1981). The goal of this work was to provide enlightenment on the possible role of the different membrane transporters of Sertoli cells (Na^+–H^+ exchangers, Na^+-driven and Na^+-independent HCO_3^-/Cl^- transporters, Na^+/K^+-ATPase, V-ATPases) in the regulation of pH_i. The approach used in this study was to study pH_i recovery in the presence and absence of specific inhibitors (e.g., amiloride, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid [DIDS], ouabain and concanamycin A) in response to intracellular acidosis of cultured bovine Sertoli cells. Acidosis was achieved by adding sodium or potassium propionate to cells. To follow the pH_i transients, the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) was used.

Materials and Methods

Chemicals

BCECF-AM was purchased from Molecular Probes (Eugene, OR). Sodium propionate, potassium propionate, nigericin, collagenase, pancreatin, DNAse, amiloride, DIDS, ouabain and concanamycin A were purchased from Sigma (St. Louis, MO). Propionate choline was purchased from Bioniqs (Heslington, UK). Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 were purchased from GIBCO (Grand Island, NY). Stock solutions of BCECF-AM and concanamycin A were prepared in dimethyl sulfoxide (DMSO) and kept at −20°C. Stock solutions of nigericin were prepared in ethanol and kept at −20°C. DMSO and ethanol were always used in concentrations below 0.1% (v/v).

Sertoli Cell Culture

Experiments were done using bovine testes from animals up to 1 year of age obtained from the slaughterhouse. Once excised, testes were transferred to ice-cold phosphate-buffered saline (PBS) containing penicillin and streptomycin until cell isolation. These experiments complied with the "Principles of Animal Care" (publication 86.23, revised 1985) of the National Institutes of Health and with the current laws of Portugal.

Sertoli cells were isolated from bovine testes using an adaptation of the enzymatic procedure described by Welsh and Wiebe (1975), modified by Majumdar et al. (1995) and evaluated by Valdes-Gonzalez et al. (2005).

Testes were transferred to cold calcium- and magnesium-free Hanks balanced salt solution ($HBSS_f$) containing 50 U/ml of penicillin and 50 μg/ml of streptomycin sulfate (pH 7.4) immediately after removal.

Decapsulated tissue (2 g) was washed twice and cut into small square pieces (2–4 mm) in a sterile Petri dish. Minced tissue was suspended in $HBSS_f$ (25 ml/g of tissue) in a glass-stoppered 100-ml Erlenmeyer and shaken vigorously for 1 min to disperse tubules. The tissue was left to settle for 5 min on ice, and the supernatant was discarded. This procedure was repeated twice to mechanically remove red blood cells and free Leydig cells. The resulting pellet was digested in 25 ml of HBSS with collagenase type I (1,000 U; C0130, Sigma) and DNAse (500 U; D4263, Sigma) and continuously shaken (100 rpm) at 32°C for 25–35 min. The formed aggregate was removed, washed in $HBSS_f$ and discarded. The washing $HBSS_f$ was added to the cellular suspension resulting from the digestion.

The resulting suspension was washed twice and left to settle completely at 4°C. The resulting pellet was suspended in 20 ml $HBSS_f$ with 5 mg pancreatin (P3292, Sigma) and DNAse (500 U, D4263) and digested at 32°C with continuous shaking (100 rpm) for 15–25 min. The new aggregate formed was discarded, and 0.4 ml of fetal bovine serum was added to the cellular suspension, which was left to rest at 4°C for 5 min. The suspension was then centrifuged at 100*g* for 5 min. The pellet was gently suspended in 30 ml $HBSS_f$. This procedure was repeated twice, and the resulting pellet was suspended in 20 ml $HBSS_f$. This suspension was passed through a glass Pasteur pipette in order to loosen germ cells from the clusters and then pelleted at 200*g* for 5 min. This procedure was

repeated twice. The resulting pellet was suspended 10 ml Sertoli culture medium (DMEM + Ham's F-12 [HF12]; 1:1, containing 50 U/ml of penicillin and 50 μg/ml of streptomycin sulfate, 0.5 μg/ml fungizone and 5% heat-inactivated fetal bovine serum) and forced through a 19G needle in order to disaggregate large Sertoli clusters.

For culture of Sertoli cells, the concentration of clusters on the cellular suspension obtained from the procedure described above was adjusted to 1,000 clusters/ml plated on 25 cm^2 culture flasks (Cell+; Sarstedt, Leicester, UK) and incubated at 37°C in an atmosphere of 5% CO_2:95% O_2. The day of plating was considered day 0 of culture. Cultures were left undisturbed until day 2.

pH_i Measurements

Cells were loaded with the fluorescent probe for 15 min at 37°C with 1 ml Sertoli Ringer (in mM: NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 10, glucose 1, HEPES 10; pH 7.4) containing 1 μM BCECF-AM (Molecular Probes). Cells were transferred to the imaging chamber and washed with Sertoli Ringer using a gravity perfusion system (1 ml/min).

The fluorescence intensities, excited at 490 and 440 nm (emission 515 nm), were continuously measured with an epifluorescence system (DeltaRam; PTI, Princeton, NJ) while the cells were perfused with different solutions.

Background fluorescence was determined at the end of each experiment by removing the cells and perfusing the empty chamber with Ringer Sertoli. Background fluorescence intensity was measured for each wavelength. The ratios (F_{490}/F_{440}) were calculated after subtracting the background fluorescence intensities for each measurement at each wavelength.

The calibration procedures were done using the method described by Thomas (1986). In order to convert the fluorescence signals into pH_i values, at the end of each protocol an in vivo calibration procedure was performed, using solutions of known pH (5.5 and 9.0), to which nigericin (10 μM) was added.

To convert the measured fluorescence ratio (F_{490}/F_{440}) to pH_i values, the following equation was used:

$$pH = pK + \log\left(\frac{(R - R_A)}{(R_B - R)} \times \frac{F_{440}^{Ac}}{F_{440}^{Bas}}\right)$$

where K is the BCECF dissociation constant, R is the fluorescence ratio (F_{490}/F_{440}) of the sample, R_A is the fluorescence ratio (F_{490}/F_{440}) of the pH 5.5 solution, R_B is the fluorescence ratio (F_{490}/F_{440}) of the pH 9.0 solution, F_{440}^{Ac} is the fluorescence intensity (440 nm) of the pH 5.5 solution and F_{440}^{Ba} is the fluorescence intensity (440 nm) of the pH 9.0 solution.

To determine the BCECF dissociation constant value, we performed in vivo measurements of the fluorescence intensity signals emitted at 515 nm for the excitation wavelengths of 490 and 440 nm in cells loaded with BCECF and perfused with a series of five calibration solutions of increasing pH (5.5, 6.5, 7.4, 8.0 and 9.0) where nigericin (10 μM) was added previously. The pH data were plotted as a function of $log((R - R_A)/(R_B - R).(F_{440}^{AC}/F_{440}^{BAS}))$, and the value of pK was estimated from the straight line intercept.

Intracellular Buffering Capacity

In order to characterize changes in pH_i, cells were acidified with sodium propionate Ringer (in mM: NaCl 90, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 10, glucose 1, HEPES 10, sodium propionate 40; pH 7.4), potassium propionate Ringer (in mM: KCl 95, $MgCl_2$ 1, $CaCl_2$ 2, $KHCO_3$ 10, glucose 1, HEPES 10, potassium propionate 40; pH 7.4) or choline propionate Ringer (in mM: choline cloride 90, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, choline bicarbonate 10, glucose 1, HEPES 10, choline propionate 40; pH 7.4). At physiological pH, propionate is in equilibrium with its non-ionized form, propionic acid, which rapidly diffuses into the cell and promptly ionizes causing intracellular acidosis. The response to this intracellular acid load was followed in time.

Cells were initially perfused with Sertoli Ringer and, after a steady-state period of 180 s, the propionate was added. The subsequent alkalinization (pH_i recovery) of the cells was followed by the presence or absence of (1) specific inhibitors (amiloride 1 mM, DIDS 0.5 mM, ouabain 0.5 mM and concanamycin A 10 μM) and (2) external Na^+.

Analysis of Data

All data are presented as arithmetic means ± standard error of the mean (SEM). For all situations analyzed, quintuplicates of five different animals were used. The initial pH_i recovery rate was determined by linear regression of the data. For statistical analysis, a one-way ANOVA was performed followed by a Newman–Keuls multiple comparison test. Results were considered significantly different from control value if $P < 0.05$.

Results

pK Measurement of BCECF and Steady-State Average pH_i

The probe's pK was determined in vivo using a range of solutions of increasing pH (5.5, 6.5, 7.4, 8.0 and 9.0) to

which nigericin (10 μM) was added previously. pK had an average value of 7.01 ± 0.21 ($n = 3$). At the conditions used in these experiments ($pH_e = 7.4$), the pH_i of the Sertoli cells averaged 7.04 ± 0.02 ($n = 30$).

Acidification and Recovery

Cells equilibrated in Sertoli Ringer were suddenly exposed to sodium propionate Ringer, causing a rapid decrease in pH_i, corresponding to an average fall of 0.66 ± 0.01 ($n = 5$) units (Fig. 1, Table 1). The half-time of the acidification process was approximately 36 s. Following the acidification, pH_i slowly recovered to 78% of its initial value with an initial recovery rate of 0.0079 ± 0.002 pH units s^{-1} (Table 1).

Effects of External Na^+ on pH_i Recovery

Cytoplasmic acidification and subsequent alkalinization were dependent on external Na^+. ΔpH_i observed with either potassium propionate or choline propionate in the absence of external Na^+ was statistically different from values observed with sodium propionate. When cells were exposed to potassium propionate, pH_i decreased 0.76 ± 0.01 units ($n = 5$) (Fig. 1, Table 1). pH_i then recovered to 58% of its initial value, resulting in a value at steady state lower than that observed in the presence of Na^+ with an initial recovery rate of 0.0045 ± 0.002 pH units s^{-1} (Table 1). When using choline propionate, pH_i decreased approximately 0.66 ± 0.01 units ($n = 5$) (Fig. 1, Table 1) and then recovered to 45% of the control value at an initial rate of 0.0027 ± 0.001 pH units s^{-1} (Table 1).

Effects of Inhibitors on pH_i Recovery

Acidification of Sertoli cells with sodium propionate was unaffected by the presence of inhibitors of membrane ion transporters (Fig. 2). Recovery of pH_i, however, was altered, although in different degrees, by all the inhibitors used, as can be seen in Fig. 2 and Table 1.

DIDS (0.5 mM), an inhibitor of several bicarbonate membrane transporters (Boron 2001), caused the most significant decrease in both the recovery extension (34% of the initial value) and the initial recovery rate (Fig. 2, Table 1), supporting the notion that this compound inhibits the major base transport systems in Sertoli cells.

Addition of amiloride (1 mM), an inhibitor of Na^+–H^+ exchange (Ahearn et al. 1994, 1999), and concanamycin A (10 μM), a specific inhibitor of the V-type ATPases (Huss et al. 2002), also caused significant decreases with similar magnitudes of the initial pH_i recovery rates (Table 1). On the other hand, the recovery extension in the presence of amiloride was significantly lower (46% of the initial value) than that in the presence of concanamycin A (64% of the initial value), as can be seen in Fig. 2 and Table 1.

The presence of ouabain (0.5 mM), a specific inhibitor of the Na^+–K^+ pump (Schneider et al. 1998; Shimizu et al. 1983), also affected the pH_i recovery. Although this compound does not inhibit any acid/base membrane transporter, it diminished the pH_i recovery extension to 59% (Fig. 2, Table 1).

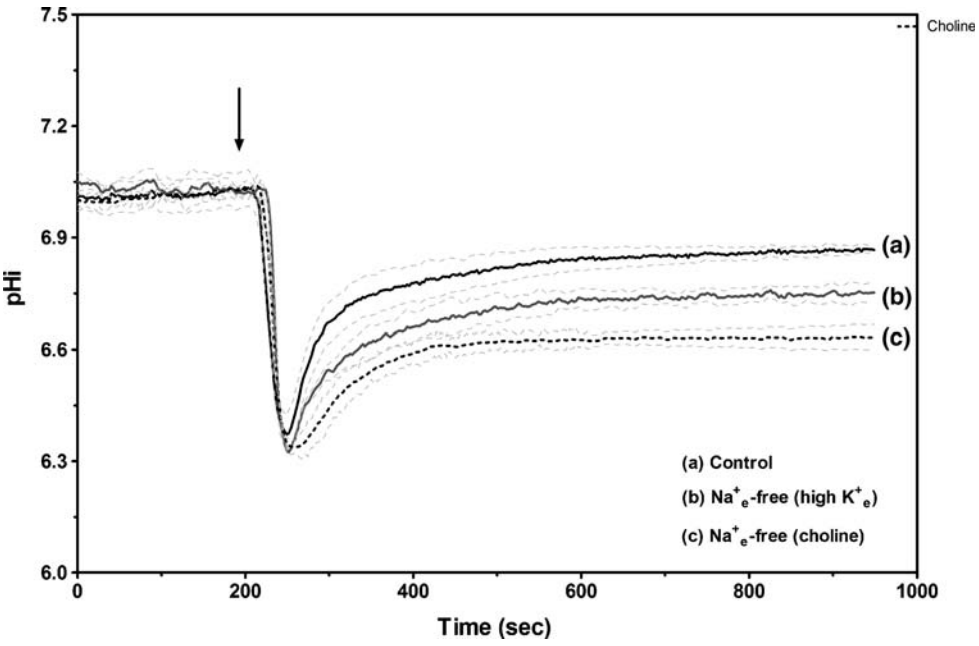

Fig. 1 pH_i of bovine Sertoli cells acidified with a weak acid in the presence (line *a*) or absence of extracellular sodium: Na^+ replaced by K^+ (line *b*) or choline (line *c*). *Arrow* represents the addition of sodium, potassium or choline propionate. Cells were preloaded with BCECF and placed in Ringer Sertoli, and pH_i was measured until a steady state of at least 180 s was observed (see "Materials and Methods" for detail). pH_i is represented as average ± SEM ($n = 5$)

Table 1 Experimental pH_i recovery parameters

Inhibitor	$[Na^+]_e$ (mM)	$[K^+]_e$ (mM)	pH_i recovery (%)	Initial pH_i recovery rate (10^3 pH units s^{-1})
None	140	5	77	7.9 ± 0.2
None	0	145	58*	4.5 ± 0.2*
None	0	5	45*	2.7 ± 0.1*
DIDS	140	5	34*	1.6 ± 0.1*
Amiloride	140	5	46*	2.8 ± 0.1*
Ouabain	140	5	59*	2.3 ± 0.1*
Concanamycin A	140	5	64*	2.6 ± 0.1*

Values are presented as mean ± SEM. pH_i recovery was calculated as the percentage of recovery at steady state after acid load recovery; initial pH_i recovery rate was calculated by linear regression of the data (see "Materials and Methods" for detail)

* Significantly different from control value if $P < 0.05$

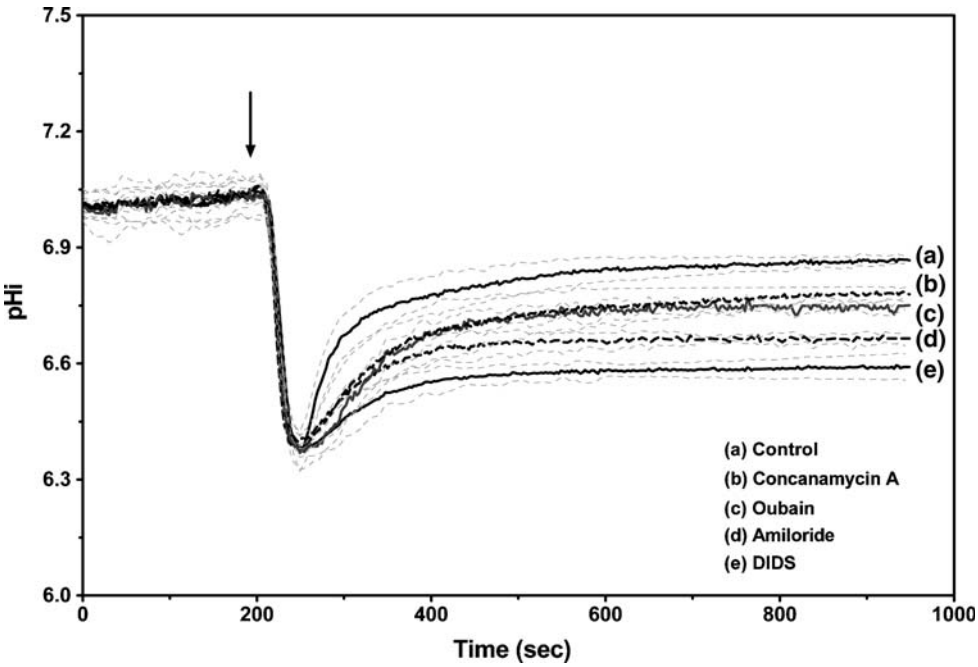

Fig. 2 pH_i of bovine Sertoli cells acidified with a weak acid in the presence of specific inhibitors. Cells were preloaded with BCECF and placed in Ringer Sertoli, and pH_i was measured until a steady state of at least 180 s was observed (see "Materials and Methods" for detail). pH_i is represented as average ± SEM ($n = 5$). *Arrow* represents addition of sodium propionate (line *a*), sodium propionate and concanamycin A 10 μM (line *b*), sodium propionate and ouabain 0.5 mM (line *c*), sodium propionate and amiloride 1 mM (line *d*) and sodium propionate and DIDS 0.5 mM (line *e*)

Discussion

Several membrane transport systems participate in pH_i regulation mechanisms and have been classified as acid extruders or acid loaders. Acid extruders require energy to move H^+ from the cell or to take up HCO_3^- (Boron 2004). The V-type H^+-ATPases (Beyenbach and Wieczorek 2006; Breton and Brown 2007; Swallow et al. 1990), the Na^+–H^+ exchangers (Counillon and Pouyssegur 2000; Orlowski and Grinstein 1997), the Na^+-driven HCO_3^-/Cl^- exchangers (Russell and Boron 1976; Wang et al. 2000), the Na^+/HCO_3^- exchangers and the Na^+/HCO_3^- cotransporter have been classified as acid extruders (Boron 2001, 2004). The so-called acid loaders mediate the exit of weak bases, usually HCO_3^- or CO_3^{2-}, or the entry of H^+. The Na^+-independent HCO_3^-/Cl^- and the electrogenic Na^+/HCO_3^- exchangers have been classified as acid loaders (Boron 2001, 2004). On the other hand, for some membrane transport systems, like the K^+/HCO_3^- cotransporter (Boron 2001; Hogan et al. 1995a, b), it has been reported that the ability to function either as an acid loader or as an acid extruder depends on the ionic gradients established through the membrane.

The nature of the ion membrane transporters involved in pH_i regulation in Sertoli cells is not very clear in the literature. As the ability of Sertoli cells to regulate pH_i is an important aspect of its physiology, specifically as this cellular parameter may play a major role in the response to hormonal stimulation as well as the determination of the pH of the seminiferous fluid, determination of the participation of diverse membrane transporters in the mechanisms of pH_i regulation on these cells is of great relevance and

was the main purpose of this study. For a first approach to the participation of some membrane transport systems on those mechanisms, cells were loaded with a pH-sensitive fluorescent probe (BCECF) and subjected to an acid load in the presence of specific inhibitors and in the absence or presence of external Na^+.

Under the experimental conditions used, bovine Sertoli cells presented a mean pH_i value of 7.04 ± 0.02. Amiloride, a high-affinity inhibitor of the Na^+/H^+ exchanger (Ahearn et al. 1994, 1999) significantly decreased the pH_i initial recovery rate and extension (see Table 1). These results are consistent with the participation of the Na^+/H^+ exchanger in pH_i regulation mechanisms.

Although proton pumps have been described in a wide variety of plasma membrane acid-secreting epithelial cells (Ehrenfeld and Klein 1997; Harvey 1992; Martinez-Zaguilan et al. 1993; Oliveira et al. 2004; Rebelo da Costa et al. 1999), its presence has not yet been confirmed in Sertoli cells (Herak-Kramberger et al. 2001). The present study indicates that the pH_i recovery mechanisms of Sertoli cells involve the action of the H^+ pump as a statistically significant difference was observed on initial pH_i recovery rate and extension after the acid load in the presence of concanamycin A, a specific inhibitor of this kind of pump (Huss et al. 2002).

DIDS is an inhibitor of several bicarbonate transport systems (Boron 2001; Boron et al. 1997), namely the ones that involve base loading at the expense of the Na^+ gradient, such as the Na^+/HCO_3^- cotransporters and the Na^+-driven HCO_3^-/Cl^- exchanger. In the presence of this inhibitor, the initial pH_i recovery rate and recovery extension were drastically reduced (to 20% of the control value and 34% of the initial value, respectively); therefore, we can assume that one or both of these transporters could be involved in pH_i regulation after an acid load.

To analyze the role of the presence of external Na^+ on pH_i recovery, cells were perfused using propionate salts that replaced Na^+ (potassium or choline). Choline has long been used to replace iso-osmotically external Na^+ (Koketsu et al. 1958). When extracellular sodium was removed, intracellular acidification magnitude was similar to that of the control situation. On the other hand, the initial pH_i recovery rate and recovery extension diminished significantly (to 34% of the control value and 45% of the initial value, respectively). If, as we predict, the membrane transporters using the Na^+ gradient are the major systems involved in these pH_i recovery mechanisms, when removing external Na^+, they would be nonfunctional and pH_i recovery would be severely compromised. As seen, the magnitude of the effect of the replacement of external Na^+ by choline on pH_i recovery parameters (initial rate and extension) is similar to that of the inhibitor amiloride (see Table 1). When replacing the external Na^+ by K^+, the magnitude of the cytoplasmic acidification was 20% higher and, after intracellular acidification, the initial pH_i recovery rate and recovery extension diminished significantly (to 57% of the control value and 58% of the initial value, respectively). However, the magnitude of the effect of the replacement of Na^+ by potassium on the recovery parameters is markedly lower than the effect of the inhibitor DIDS or even of the inhibitor amiloride (see Table 1).

The reasoning for these results could also be one or a combination of the following: (1) the increase of extracellular K^+ (replacing the external Na^+) could result in membrane depolarization influencing the functioning of some membrane transporters or (2) as the removal of external Na^+ was accomplished by isotonic replacement with K^+, not only the Na^+ gradient but also the K^+ gradient was altered. Hogan et al. (1995a) reported that, in a similar situation, the functioning of the K^+/HCO_3^- cotransporter could be reversed and it would function as a base loader. Thus, the action of this cotransporter, possibly with the joint action of the V-type ATPases, could be the mechanism responsible for the pH_i recovery in the absence of external Na^+ and the presence of high concentrations of external K^+.

Finally, ouabain is a potent specific inhibitor of Na^+/K^+-ATPase, a ubiquitous pump in animal cell membranes (Schneider et al. 1998; Shimizu et al. 1983). The presence of this inhibitor slightly diminished the initial rate and extension of the pH_i recovery, as could be expected. This membrane transporter is not directly implicated in the extrusion or loading of acid particles; thus, its effect must be explained on the basis of the alteration of the ionic gradients. The inhibition of Na^+/K^+-ATPase would lead to a decrease in pH_i recovery indirectly, mainly via the shift in the Na^+ gradient it causes.

In conclusion, our main purpose was to provide insight on the mechanisms involved in pH_i regulation in bovine Sertoli cells, so our results would function as the starting point for the planning of additional experiments to further confirm the presence of the several membrane transporters here suggested. Hence, our results indicate that, in the presence of external Na^+ and bicarbonate, these cells seem to regulate pH_i mainly by the action of an Na^+-driven HCO_3^-/Cl^- exchanger and/or an Na^+/HCO_3^- cotransporter and by the action of the Na^+/H^+ exchanger. Also, our work, although it suggests the presence in Sertoli cells of V-type ATPases, points to a minor participation of these mechanisms of pH_i regulation after an acid load.

Acknowledgement P. F. Oliveira was supported by Fundação para a Ciência e a Tecnologia (SFRH/BPD/20965/2004).

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