Intracellular pH regulation in human Sertoli cells: role of membrane transporters

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Abstract

Sertoli cells are responsible for regulating a wide range of processes that lead to the differentiation of male germ cells into spermatozoa. Intracellular pH (pHi) is an important parameter in cell physiology regulating namely cell metabolism and differentiation. However, pHi regulation mechanisms in Sertoli cells have not yet been systematically elucidated. In this work, pHi was determined in primary cultures of human Sertoli cells. Sertoli cells were exposed to weak acids, which caused a rapid acidification of the intracellular milieu. pHi then recovered by a mechanism that was shown to be particularly sensitive to the presence of the inhibitor DIDS (4,4'-diisothiocyanostilbene disulfonic acid). In the presence of amiloride and PSA (picrylsulfonic acid), pHi recovery was also significantly affected. These results indicate that, in the experimental conditions used, pHi is regulated by the action of an Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger and an $\text{Na}^+/\text{HCO}_3^-$ co-transporter and also by the action of the Na^+/H^+ exchanger. On the other hand, pHi recovery was only slightly affected by concanamycin A, suggesting that V-Type ATPases do not have a relevant action on pHi regulation in human Sertoli cells, and was independent of the presence of bumetanide, suggesting that the inhibition of the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporter does not affect pHi recovery, not even indirectly via the shift of ionic gradients. Finally, pHi was shown to be sensitive to the removal of external Cl^- , but not of Na^+ or K^+ , evidencing the presence of a membrane Cl^- -dependent base extruder, namely the Na^+ -independent $\text{HCO}_3^-/\text{Cl}^-$ exchanger, and its role on pHi maintenance on these cells.

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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 (Griswold 1995, 1998, Buzzard *et al.* 2002, Mruk & 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 & Cheng 2004). The effects of testosterone and FSH on the regulation of the above processes in Sertoli cells are mediated by multiple factors/pathways including intracellular calcium level, protein tyrosine kinases such as MAPKs and Src, receptors for androgen and FSH, cyclic nucleotides, and others (Gorczyńska & Handelsman 1995, Lyng *et al.* 2000, Von Ledeber *et al.* 2002, McLachlan *et al.* 2002, Silva *et al.* 2002, Walker & Cheng 2005). Furthermore, it

has been reported that intracellular pH (pHi) regulation is important in 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, the pHi regulation of Sertoli cells should also play a major role in this process (Tuck *et al.* 1970, Mruk & Cheng 2004).

This cellular parameter is kept mainly through the net balance between production and elimination of protons and by intracellular buffers (Roos & Boron 1981). Cells possess in their plasmatic membrane a wide range of ion transporters that participate in pHi regulation, among which are the basic and acidic particles membrane transporters (Boron 2004). These transporters directly involved on the movement basic and acidic particles across the membrane are classified as acid extruders (Na^+/H^+ exchangers, Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ transporters, $\text{Na}^+/\text{HCO}_3^-$ co-transporters, and V-ATPases) or acid loaders (Na^+ -independent $\text{HCO}_3^-/\text{Cl}^-$ transporters and

$\text{Na}^+/\text{HCO}_3^-$ co-transporters), depending on the direction of movement of those particles (Boron 2004).

The goal of this work was to provide a first assessment on the participation of the different membrane transporters (Na^+-H^+ exchangers, Na^+ -driven and Na^+ -independent $\text{HCO}_3^-/\text{Cl}^-$ transporters, $\text{Na}^+/\text{HCO}_3^-$ co-transporter, $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transporters, and V-ATPases) in the regulation of the pHi of human Sertoli cells. The approach used in this study was to follow pHi of cultured human Sertoli cells in Na^+ , K^+ , or Cl^- replete- and free mediums and on pHi recovery in the presence of specific inhibitors (e.g., amiloride, DIDS (4, 4'-diisothiocyanostilbene disulfonic acid), PSA (picrylsulfonic acid), bumetanide, and concanamycin A) after an intracellular acidosis. The acidosis was achieved by adding sodium propionate to cells. To follow the pHi transients, the pH-sensitive fluorescent probe 2', 7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein (BCECF) was used.

Results

pK measurement of BCECF and steady-state average pHi

The determination of the probe's pK was performed *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 (pHe=7.4), the pHi of the Sertoli cells averaged 7.05 ± 0.01 ($n=45$).

Acidification and recovery

Cells equilibrated in Sertoli Ringer were suddenly exposed to sodium propionate Ringer causing a rapid decrease in pHi, corresponding to an average fall of 0.63 ± 0.01 ($n=5$) units (Fig. 1; Table 1). The half-time of the acidification process was ~ 295 s. Following the acidification, pHi slowly recovered to 100% of its initial value with an initial recovery rate of 0.0069 ± 0.0002 pH units/s (Table 1).

Effects of inhibitors on pHi recovery

Acidification of Sertoli cells with sodium propionate was not significantly affected by the presence of specific inhibitors of some membrane ion transport systems (Figs 1 and 2). Recovery of pHi, however, was altered, although in different degrees, by all the inhibitors used, as can be seen in Table 1.

DIDS (0.5 mM), an inhibitor of several bicarbonate membrane transporters usually classified as acid extruders, Na^+ -driven and Na^+ -independent $\text{HCO}_3^-/\text{Cl}^-$ transporters and $\text{Na}^+/\text{HCO}_3^-$ co-transporter, used at concentrations from 0.5 to 1 mM (LD_{50} =not available; IC_{50} =20–200 μM ; Grassl & Aronson 1986, Helbig *et al.*

Figure 1 Intracellular pH of human Sertoli cells acidified with a weak acid in the presence of specific inhibitors. The arrow represents the addition of (a) sodium propionate, (b) sodium propionate and concanamycin A 10 μM , (c) sodium propionate and PSA 0.5 mM, (d) sodium propionate and amiloride 1 mM, and (e) sodium propionate and DIDS 0.5 mM. pHi is represented as average $\pm$ S.E.M. ($n=5$). *Significantly different from control value if $P<0.05$.

1988, Boron 2001) caused the most significant decrease both on the recovery extension (39% of the initial value) and on the initial recovery rate (Fig. 1; Table 1), supporting the notion that this compound is inhibiting the major transport systems involved in pHi regulation in Sertoli cells.

Addition of amiloride (1 mM), an inhibitor of the Na^+/H^+ exchange, used at doses from 0.1 to 1 mM (LD_{50} (rat/oral)=36–85 mg/Kg; LD_{50} (mouse/oral)=56 mg/Kg; IC_{50} =5–25 μM ; Delvaux *et al.* 1990, Otani *et al.* 1990, Ahearn *et al.* 1994, Good & George 1995, Ahearn *et al.* 1999), caused a similar decrease on the initial pHi recovery rates (Table 1). On the other hand, the recovery extension in the presence of amiloride was significantly lower (56% of the initial value) than in the presence of DIDS as can be seen in Fig. 1 and Table 1.

PSA (0.5 mM), a specific inhibitor of the Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger, used at the concentration 0.5 mM (LD_{50} =not available; IC_{50} =not available; Knauf & Rothstein 1971, Madshus & Olsnes 1987, Frelin *et al.* 1988, Marvao *et al.* 1994), also affected the pHi recovery. Although to a smaller extent than DIDS,

Table 1 Experimental pHi recovery parameters in human Sertoli cells.

Inhibitor	pHi recovery (%)	pHi recovery rate (10^3 pH units/s)
None	100	6.9 ± 0.2
Bumetanide	100	$3.6 \pm 0.1^*$
Concanamycin A	95*	$1.6 \pm 0.1^*$
PSA	79*	$1.3 \pm 0.1^*$
Amiloride	56*	$1.2 \pm 0.1^*$
DIDS	39*	$1.2 \pm 0.1^*$

Values are presented as mean $\pm$ S.E.M.; *significantly different from control value if $P<0.05$; pHi recovery was calculated as the percentage of recovery at steady state after acid load recovery; Initial pHi recovery rate was calculated by linear regression of the data (see Materials and Methods for detail).

Figure 2 Intracellular pH of human Sertoli cells acidified with a weak acid in the presence of specific bumetanide. The arrow represents the addition of sodium propionate (black line) or sodium propionate and bumetanide 100 μ M (dashed line). pHi is represented as average $\pm$ S.E.M. ($n=5$). * Significantly different from control value if $P<0.05$.

which also inhibits this membrane transporter, PSA caused a 21% decrease in pHi recovery and an 81% decrease in the initial recovery rate (Fig. 1; Table 1).

Concanamycin A (10 μ M), a specific inhibitor of the V-type ATPases, used at concentrations from 1 to 5 μ M (LD_{50} (mouse/oral)=21 mg/Kg; IC_{50} =0.2–0.7 μ M; Muroi *et al.* 1993, Huss *et al.* 2002, Oliveira *et al.* 2004), caused a very small, although significant, decrease in the pHi recovery extension, as can be seen in Fig. 1 and Table 1. On the other hand, in the presence of this inhibitor the initial pHi recovery rate was significantly lower than in the control situation (77% of the initial value; Table 1).

Bumetanide (10 μ M) was the only inhibitor of a non-acid/base membrane transporter used. Used at the concentration 0.1 mM, it specifically inhibits the Na^+ – K^+ – Cl^- co-transporters (LD_{50} (rat/oral)>6000 mg/Kg; LD_{50} (mouse/oral)>4000 mg/Kg; IC_{50} =0.4–5 μ M; Paris & Pouyssegur 1986, Vigne *et al.* 1994, Gamba 2005). The inhibition of this transporter did not affect the pHi recovery extension, although the initial recovery rate was significantly decrease to 52% of the control value (Fig. 2; Table 1).

Effects of external Na^+ , K^+ , and Cl^- on pHi

Cytoplasmic pH was also monitored in the presence and absence of external Na^+ , K^+ , and Cl^- . Isosmotic removal of external Na^+ (by replacement with K^+) or K^+ (by replacement with Na^+) did not cause any effects on pHi (Fig. 3). On the other hand, isosmotic removal of Cl^- (by replacement with gluconate) caused a rapid increase in pHi of 0.13 ± 0.02 units (Fig. 3). Reperfusing of external Cl^- caused a recovering of pHi to values identical of the control situation.

Figure 3 Intracellular pH of human Sertoli cells acidified with a weak acid in the presence or absence of extracellular Na^+ (gray line), extracellular K^+ (dashed line) or extracellular Cl^- (black line). The bars represent the removal of the specific ion and the readdition of the ion. pHi is represented as average $\pm$ S.E.M. ($n=5$).

Discussion

Several membrane transport systems participate in pHi 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 (Swallow *et al.* 1990, Beyenbach & Wieczorek 2006, Breton & Brown 2007), the Na^+/H^+ exchangers (Orlowski & Grinstein 1997, Counillon & Pouyssegur 2000), the Na^+ -driven HCO_3^-/Cl^- exchangers (Russell & Boron 1976, Wang *et al.* 2000), the Na^+/HCO_3^- exchangers, and the Na^+/HCO_3^- co-transporter 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).

The nature of the ion membrane transporters involved in pHi regulation in Sertoli cell is not very clear in the literature. As the ability of the Sertoli cell to regulate pHi is an important aspect of its physiology, especially because this cellular parameter may play a major role on the response to hormonal stimulation as well as on the determination of the pH of the seminiferous fluid, the enlightenment of the participation of diverse membrane transporters on the mechanisms of pHi regulation in these cells is of great relevance and was the main purpose of this study. For that, cells were loaded with a pH-sensitive fluorescent probe (BCECF) and subjected to an acid load in the presence of specific inhibitors.

Under the experimental conditions used, human Sertoli cells presented a mean value of pHi of 7.05 ± 0.01 . Amiloride, a high-affinity inhibitor of the Na^+/H^+ exchanger (Ahearn *et al.* 1994, 1999) significantly decreased the pHi initial recovery rate and extension (Table 1). These results are consistent with the

participation of the Na^+/H^+ exchanger on pH_i regulation mechanisms.

DIDS is an inhibitor of several bicarbonate transport systems (Boron *et al.* 1997, Boron 2001), namely the ones that involve base loading at the expense of the Na^+ gradient, such as the $\text{Na}^+/\text{HCO}_3^-$ co-transporters and the Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger. In the presence of this inhibitor, the initial pH_i recovery rate and recovery extension were drastically reduced (to 17% of the control value and 39% of the initial value respectively), therefore we could presuppose that one or both these transporters could be involved in the pH_i regulation after an acid load.

In order to further elucidate the participation of these two bicarbonate transporters on the pH_i recovery we used PSA, a specific inhibitor of the Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger (Knauf & Rothstein 1971, Madshus & Olsnes 1987). This compound significantly reduced the pH_i initial recovery rate and recovery extension after the acid load. Therefore, we could assume the presence of the Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchangers on the membrane of the human Sertoli cells. Nevertheless, the magnitude of the effect of PSA on the recovery extension was much smaller than that of DIDS, suggesting to us the presence of the $\text{Na}^+/\text{HCO}_3^-$ co-transporters on the plasma membrane of these cells and their participation in pH_i regulation after an acid load.

Although proton pumps have been described in a wide variety of plasma membranes of acid-secreting epithelial cells (Harvey 1992, Martinez-Zaguilan *et al.* 1993, Ehrenfeld & Klein 1997, Rebelo da Costa *et al.* 1999, Oliveira *et al.* 2004), 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 in Sertoli cells involve the action of the H^+ pump, although to a small extent, 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).

Bumetanide is a specific inhibitor of the $\text{Na}^+-\text{K}^+-\text{Cl}^-$ co-transporter, expressed in a broad spectrum of tissues and implicated in cell volume regulation and in ion transport by secretory epithelial tissue (O'Grady *et al.* 1987), namely in the seminiferous epithelium (Pace *et al.* 2000). The presence of this inhibitor did not affect the recovery extension of the pH_i. As one can see, this membrane transporter is not directly implicated on the extrusion or loading of acid particles thus, unless it had a major role on the maintenance of the intracellular ionic gradients in human Sertoli cells, its effect on pH_i recovery should not be very significant, as is the case. Nevertheless, the presence of bumetanide on the external bathing solution causes a decrease on initial pH_i recovery rate, probably due to the referred disturbance of the intracellular ionic gradients.

Finally, ionic replacement experiments were performed in order to further access on the presence of the several membrane transporters discussed. Only the removal of chloride ion caused a shift to basic values of the basal pH_i. Neither the removal of external Na^+ , nor of K^+ , caused any effect. The reasoning of these results could be the following: the removal of external Cl^- affected the functioning of either the Na^+ -independent $\text{HCO}_3^-/\text{Cl}^-$ exchanger or the Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger that will lead to the intracellular accumulation of bicarbonate and consequent rise of pH_i.

In conclusion, human Sertoli cells, in the presence of external Na^+ and bicarbonate, seem to regulate pH_i after an acid load mainly by the action of an Na^+ -driven $\text{HCO}_3^-/\text{Cl}^-$ exchanger and a $\text{Na}^+/\text{HCO}_3^-$ co-transporter and also by the action of the Na^+/H^+ exchanger (Fig. 4). Also, our work, although it suggests the presence in Sertoli cells of V-type ATPases, points to a minor participation on these mechanisms of the regulation of pH_i after an acid load. In addition, human Sertoli cells seem to also have on their plasma membranes the $\text{Na}^+-\text{K}^+-\text{Cl}^-$ co-transporter, which, as one could expect, does not have a major role on pH_i regulation mechanisms but its absence (inhibition) affects those mechanisms, probably due to ionic gradient alterations.

Materials and Methods

Chemicals

BCECF-AM was purchased from Molecular Probes (Carlsbad, CA, USA). Sodium propionate, potassium propionate, nigericin, collagenase, pancreatin, PSA, DNase, amiloride, DIDS, and concanamycin A were purchased from Sigma. DMEM and Ham's F-12 were purchased from Gibco. 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). All other chemicals were purchased from Sigma.

Figure 4 Diagram of the Sertoli cells' membrane transport systems considered.

Ethical issues

Human Sertoli cells were obtained from testicular biopsies of infertile patients under treatment for recovery of male gametes at the IVF Unit of the Department of Genetics, Faculty of Medicine and University of Porto.

Patients with normal spermatogenesis were selected. In accordance with the Guidelines of the Local, National and European Ethical Committees, in the present study only cells left in the tissue culture plates after treatment of the patients by intracytoplasmic sperm–spermatid injection were used. In all cases, Sertoli cells were used only after informed patient consent.

Sertoli cell culture

Each biopsy (0.1 to 0.2 g), once obtained, was transferred to sperm preparation medium (SPM; Medicult, Copenhagen, Denmark) containing penicillin and streptomycin until cell isolation.

Sertoli cells were isolated using an adaptation of the enzymatic procedure adapted from the one described by Welsh & Wiebe (1975), modified by Majumdar *et al.* (1995) and evaluated by Valdés-González *et al.* (2005).

Tissue samples were washed twice in cold HBSS_f (calcium- and magnesium-free HBSS, containing 50 U/ml of penicillin and 50 µg/ml streptomycin sulfate (pH 7.4)) and minced in HBSS_f (2.5 ml per 0.1 g tissue) in a glass-stoppered Erlenmeyer, shaken vigorously during 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 5 ml of HBSS with collagenase type I (200 U; C0130, Sigma) and DNase (100 U; D4263, Sigma) continuously shaken (100 r.p.m.) at 32 °C during 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 5 ml HBSS_f with 1 mg pancreatin (P3292, Sigma) and DNase (100 U; D4263) and digested at 32 °C with continuous shaking (100 r.p.m.) during 15–25 min. The new aggregate formed was discarded and 0.1 ml fetal bovine serum (FBS) 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* during 5 min. The pellet was gently suspended in 5 ml HBSS_f. This procedure was repeated twice and the resulting pellet was suspended in 5 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 Sertoli culture medium (DMEM + Ham's F-12 [HF12]; 1:1, containing 50 U/ml penicillin and 50 µg/ml streptomycin sulfate, 0.5 µg/ml fungizone, and 5% heat inactivated FBS) and forced through a 19 G 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 1000 clusters/ml plated on 25 cm²

culture flasks (Cell+, Sarsted), and incubated at 37 °C in an atmosphere of 5% CO₂: 95% O₂. The day of plating was considered day 0 of culture. The cultures were left undisturbed until day 2. Sertoli cell isolation procedures had an average yield of 5 × 10⁴ cell per gram of tissue.

Intracellular pH measurements

Cells were loaded with the fluorescent probe during 15 min at 37 °C with 1 ml Sertoli Ringer (NaCl 130 mM; KCl 5 mM; MgCl₂ 1 mM; CaCl₂ 2 mM; NaHCO₃ 10 mM; Glucose 1 mM; HEPES 10 mM (pH 7.4)) containing 1 µM BCECF-AM (Molecular Probes). The 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) 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 Sertoli Ringer. The 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 a *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}) in intracellular pH values, the following equation was used:

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

Where K is the BCECF K_d , 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}^{Bas} is the fluorescence intensity (440 nm) of the (pH 9.0) solution.

To determine the BCECF K_d 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

$$\log \left((R - R_A)/(R_B - R) \times (F_{440}^{\text{Ac}}/F_{440}^{\text{Bas}}) \right)$$

and the value of pK was estimated from the straight line intercept.

Intracellular buffering capacity

In order to characterize changes in pHi, cells were acidified with sodium propionate Ringer (NaCl 90 mM; KCl 5 mM; MgCl₂ 1 mM; CaCl₂ 2 mM; NaHCO₃ 10 mM; glucose 1 mM; HEPES 10 mM; 40 mM sodium propionate (pH 7.4)) or potassium propionate Ringer (KCl 95 mM; MgCl₂ 1 mM; CaCl₂ 2 mM; KHCO₃ 10 mM; glucose 1 mM; HEPES 10 mM; 40 mM potassium propionate (pH 7.4)). At physiological pH, propionate is in equilibrium with its non-ionized form propionic acid that rapidly diffuses into the cell and promptly ionizes causing an intracellular acidosis. The response to this intracellular acid load was followed in time.

The cells were initially perfused with Sertoli Ringer and, after a steady-state period of 180 s, the propionate was added. The subsequent alkalization (pHi recovery) of the cells was followed in the presence or absence of specific inhibitors (amiloride 1 mM, bumetanide 100 µM, DIDS 0.5 mM, PSA 0.5 mM, and concanamycin A 10 µM).

Effect of external ion removal on pHi

The cells were initially perfused with Sertoli Ringer and, after a steady-state period of 180 s, Na⁺, K⁺, or Cl⁻ were removed from the external medium and replaced isosmotically by K⁺, Na⁺, or gluconate respectively. Sodium-free medium was composed by KCl 135 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, KHCO₃ 10 mM, glucose 1 mM, HEPES 10 mM (pH 7.4). Potassium free-medium was composed by NaCl 135 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, NaHCO₃ 10 mM, glucose 1 mM, HEPES 10 mM (pH 7.4). Chloride free-medium was composed by sodium gluconate 130 mM, potassium gluconate 135 mM, magnesium gluconate 1 mM, calcium gluconate 2 mM, NaHCO₃ 10 mM, glucose 1 mM, HEPES 10 mM (pH 7.4). The pHi of the cells was followed in the presence or absence of the specific ions.

Analysis of results

All data are presented as arithmetic means ± S.E.M. of five replicates of five different biopsies. The initial pHi recovery rate was determined by linear regression of the data.

For statistical analysis a one-way ANOVA was performed followed by a Dunn's multiple comparison test. Results were considered significantly different from control value if $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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