

Regulation of Intracellular Chloride Concentration in Rat Lactotrope Cells and Its Relation to the Membrane Resting Potential

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Abstract. Rat lactotrope cells in primary culture exhibit physiological properties closely associated with chloride ions (Cl^-) homeostasis. In this work, we studied the regulation of intracellular Cl^- concentrations ($[\text{Cl}^-]_i$) and its relation to the membrane resting potential, using a combination of electrophysiology and spectrofluorimetry. Variations in $[\text{Cl}^-]_i$ resulting from the patch clamp technique, pH_i , antagonists of Cl^- - Ca^{2+} -dependent channels, an anion exchanger antagonist, and an antagonist of K^+ - Cl^- cotransport were considered with respect to their involvement in membrane potential. We show that: (i) The patch-pipette does not always impose its Cl^- concentration. (ii) In rat lactotrope cells, membrane resting potential is partially determined by $[\text{Cl}^-]_i$. (iii) Besides ion channel activity, electroneutral ion transports (cotransports such as K^+ - Cl^- and Na^+ - K^+ - 2Cl^-) participate actively in maintaining a high $[\text{Cl}^-]_i$. (iv) Finally, Cl^- homeostasis is probably linked to cell energetics.

Key words: Rat lactotrope cells — $[\text{Cl}^-]_i$ — Electrophysiology — Microspectrofluorimetry — Cell energetics

Introduction

Most information on contributions of ions to electrophysiological properties of pituitary cell membranes was first obtained in the GH tumoral lactosomatotroph cell line (GH3 or GH4-C1), where the role of potassium ions (K^+) has been widely documented (Dufy and Barker 1982, 1983). In the rat lactotrope cells in primary

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culture, K^+ and the inward-rectifying K^+ current (K_{IR}) were similarly shown to “play a crucial role in the maintenance and modulation of membrane potential” (V_m) (Corrette et al. 1995, 1996; Shwarz and Bauer 1999). However, spontaneous action potentials are largely unaffected by several K^+ channel blockers (Sankaranarayanan and Simasko 1998). This probably means that, along with K^+ , other ions are involved in electrical properties of the lactotrope cell membranes. Since variations of V_m critically affect electrical excitability, activation of voltage-dependent calcium (Ca^{2+}) channels, cell calcium homeostasis, and, finally, hormonal release (see Corrette et al. 1995, for a review), the participation of Cl^- in V_m regulation of the rat lactotrope cells should be more thoroughly investigated. Indeed, convergent data raise the question of participation of chloride ions (Cl^-) in this process. We have reported on the activation of a Ca^{2+} -dependent Cl^- conductance together with a Ca^{2+} -dependent K^+ current in rat lactotrope cells in response to the thyrotropin-releasing hormone (TRH) (Sartor et al. 1990, 1992). In these studies, the experimental reversal potential (E_{rev}) for K^+ and Cl^- was -65 mV, whereas the theoretical E_{rev} was near -90 mV for both ions. An unusual shift of the E_{rev} from the value predicted by the Nernst relationship for Cl^- has already been observed by Rogawski et al. (1988) in the GH3 cell line, as well as by others in other cell types (Mayer 1985; Owen et al. 1986). Indeed, a rather high basal intracellular chloride concentration ($[Cl^-]_i$, 60 mmol/l) was also found in lactating female rat lactotrophs (Garcia et al. 1997b,c). This particular situation explains the stimulating effect of gamma-aminobutyric acid (GABA), mediated by a Cl^- outflow leading to membrane depolarization, as reported by Lorsignol et al. (1994). Moreover, the presence of chloride stores in mitochondria of lactating rat lactotrope cells (Garcia et al. 1997c) adds to the complexity of Cl^- involvement in the rat lactotrope cell physiology.

Obviously, more information on the mechanisms underlying the intracellular Cl^- homeostasis is needed.

Materials and Methods

Cell cultures

Pituitary glands were obtained from lactating female rats a few days after separation from the pups. The anterior lobe was dissected free of the intermediate and posterior lobes, cut into small fragments and incubated for 30 min at $37^\circ C$ in DMEM/F12 medium (Seromed, Strasbourg, France), supplemented with 0.1% trypsin (Sigma, La Verpillière, France) and 0.3% bovine serum albumin (BSA). At the end of incubation, deoxyribonuclease (0.2%, from Boehringer, Mannheim, Germany) was added to the incubation medium. Subsequently enzymes, Ca^{2+} , and Mg^{2+} were removed by rinsing three times in the presence of decreasing concentrations of EGTA before mechanical dispersion with a fire-polished glass pipette in normal DMEM/F12 medium. Cells ($\sim 10^5$ cells *per* dish) were grown on a 30 mm glass coverslip (previously coated with polyornithine) in DMEM/F12, supplemented with 10% fetal calf serum (FCS), 0.5 mmol/l pyruvate, and 2.5 mmol/l

glutamine. Gentamycin (100 µg/ml) was added to the culture for the first 24 h. The medium was then changed every 3 days as well as one day before recordings. Lactotrope cells were easily recognized and patched under these conditions. We determined immunocytochemically that lactotrope cells were continuously present in the cell cultures at the levels of 60 to 68%. As the remaining cells were mostly somatotrophs (20–25%), distinguishable by their size, we assumed that approximately 90% of the recorded cells were lactotrophs (Fahmi et al. 1995).

Electrophysiology

Recordings were made in the whole-cell mode of the patch-clamp technique (Hamill et al. 1981), using an RK400 amplifier (Biologic, Grenoble, France). The liquid junction potential between the external solution and the pipette internal solution was compensated at the beginning of each recording. The junction potential induced by changes of the external solution was minimized by using a salt bridge (potassium gluconate (Kgluc) or a 1/1 mixture of Kgluc and KCl) as the reference electrode and by reducing medium change in the vicinity of the recorded cell, as recommended by Neher (1992). Current-clamp (Iclamp) and voltage-clamp (Vclamp) experiments were performed. Serial resistance and cell capacitance were determined.

Solutions

For electrophysiological experiments, the extracellular medium (EM) contained (in mmol/l): 137 NaCl, 5.4 KCl, 2 CaCl₂, 2 MgCl₂, 0.4 or 10 NaHCO₃, 0.3 Na₂HPO₄, 0.4 KH₂PO₄, 10 HEPES, and 10 glucose. In some experiments, NaCl was replaced equimolarly by KCl to expose the cells to high K⁺.

The intrapipette medium was composed of 140 mmol/l Kgluc, 2 mmol/l MgCl₂, 5 mmol/l 1.1, N-2-hydroxyethyl piperazine-N4-2-ethane sulfonic acid (HEPES), 1.1 mmol/l ethylene glycol-bis (beta-amino ethylether), N,N,N',N'-tetraacetic acid (EGTA), giving a reversal potential for Cl⁻ (E_{revCl⁻}) of approx. -90 mV. When necessary, Kgluc was replaced by N-methyl glucamine methanesulfonate (NMG-methsulf) to eliminate K⁺ from the pipette medium.

Microspectrofluorimetry

Intracellular Cl⁻ studies

Cells were loaded with 6-methoxy-N (3 sulfopropyl) quinolinium (SPQ), (from Molecular Probes, Europe, BV, Leiden, Netherlands) by exposure to a low osmolality external medium (150 mosmoles/l) containing 7.5 mmol/l SPQ for 4 min, as proposed by Chao et al. (1989) and Verkman et al. (1989). Microspectrofluorimetry was combined with electrophysiology using the technique described earlier (see Garcia et al. 1997b,c for more details) and pipette media were supplemented with 0.5 mmol/l SPQ in order to compensate for cellular probe dialysis. This concentration statistically equilibrated the cell SPQ concentration. Since SPQ is a single-excitation, simple-emission, Cl⁻-sensitive molecule, relative variations in fluorescence are usually attributed to changes in Cl⁻ concentrations (Verkman et al. 1989). Under these conditions, we assumed that the initial rise in SPQ fluorescence

(F_{SPQ}), observed after patch rupture, was due to Cl^- dialysis towards the pipette, where the Cl^- concentration was 4 mmol/l (Kgluc).

Since Vasseur et al. (1993) reported that SPQ quenching was pH-sensitive, we verified that this property did not interfere with the recordings. In our experiments, it should have produced the opposite effect to that which was actually observed. Moreover, since cell volume changes may modify apparent F_{SPQ} , we measured the apparent diameters of round or oval-shaped isolated cells that constitute the majority of the recorded cell population under the usual patch-clamp recording conditions. We evaluated also the effect of the $[\text{Cl}^-]_i$ decrease induced by the intra-pipette medium (4 mmol/l Cl^-) on cell volume estimated in parallel by diameter measurement and volume sensitive fluorescence changes using the calcein non-invasive technique developed by Crowe et al. (1995). Cells on glass coverslips were loaded by short term (10 min) incubation with bathing medium containing calcein acetoxymethyl ester (AM) (2 $\mu\text{mol/l}$, from Molecular Probes Europe; excitation wavelength – 490 nm, emission wavelength – 560 nm). Cell volumes were derived from mean radius values obtained by measuring two perpendicular diameters. No significant cell volume changes (<2%) occurred after 5 or 15 min of patch rupture. In addition, we demonstrated that in fluorescence recording conditions without patch-clamping the cells, a decrease of $[\text{Cl}^-]_i$ induced by external isoosmotic Cl^- -free medium was followed by only a very slight decrease in calcein fluorescence ($\sim 2\%$) and a non significant volume change ($\sim 1\%$). Finally, the relevance of the morphometric data was assessed by concomitant observation of significant diameters and calcein fluorescence changes for cells exposed to hypoosmotic (200 mosmoles/l) external medium (not shown).

We also assumed that SPQ loading with hypotonic shock had no impact on cell volume during recordings, in agreement with the kinetics reported by Foskett (1990) for regulatory volume decrease and regulatory volume increase mechanisms. In addition, Foskett concluded that the possible role of non- Cl^- cellular SPQ quenchers, such as organic anions, was of little importance compared to the effect of Cl^- itself. Finally, the patch-clamp is more likely to cause shrinking than swelling. For all these reasons, and given the fact that the cytosolic SPQ concentration and osmolality were balanced by the pipette, we assumed that the changes in F_{SPQ} were due to intracellular Cl^- SPQ interaction rather than cell volume variation.

pH_i studies

The fluorescent probe seminaphthorodafluor (SNARF-1) (Molecular Probes Europe, Leiden, Netherlands) was used to measure intracellular pH (pH_i) as reported earlier (Mariot et al. 1991). Cells on their glass coverslips were incubated at $35 \pm 1^\circ\text{C}$ for 30 min with the permeant form SNARF-1/AM (excitation wavelength was 514 nm (10 nm bandwidth), emission centered at 580 nm for the acidic form of the probe and 640 nm for the alkaline form). The F_{580}/F_{640} ratio was recorded online by an analog divider and used to calculate the pH_i of individual cells. Ratio traces were recorded on a chart recorder.

Results

Ion regulation of membrane resting potential in lactotrope cells

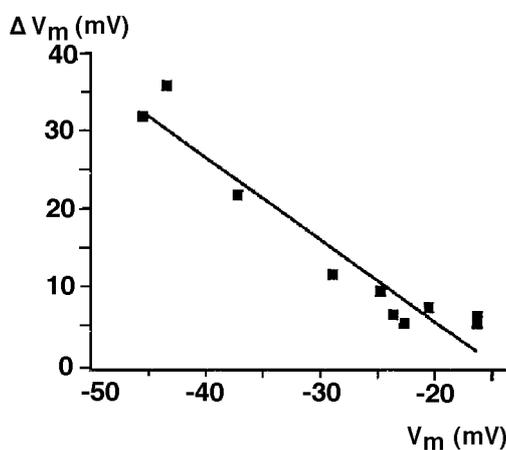
Effect of external K⁺ increase on membrane potential (V_m)

The membrane resting potential of lactotrope cells recorded in this study was -28.6 ± 3.2 mV ($n = 11$). These cells responded to exposure to high-K⁺ extracellular medium (140 mmol/l) by a variable depolarization with a mean amplitude of $+13.8 \pm 3.2$ mV. The relationship between K⁺-induced depolarization and cell membrane potential is shown in Fig. 1. The amplitude of K⁺-induced depolarization was inversely related to the membrane resting potential ($R = 0.95$). If we assume that the intracellular K⁺ concentration imposed by the patch pipette is close to 140 mmol/l, these data indicate that K ions are variably involved in maintaining membrane potential in individual lactotrope cells. As we demonstrated that Cl⁻ is implicated, together with K⁺, in the electrophysiological response of lactotrope cells to TRH (Sartor et al. 1990, 1992) and played an important role in the basal secretion of prolactin (Garcia et al. 1999), we further investigated the role played by Cl⁻ in establishing and maintaining the V_m.

Relation between Cl⁻ movements and V_m

Lactotrope cells were loaded with the chloride-sensitive molecule, SPQ. Membrane potential and intracellular F_{SPQ} were recorded simultaneously. The patch pipette medium contained 4 mmol/l Cl⁻, considerably less than the expected intracellular Cl⁻ concentration of normal lactotrope cells (50–60 mmol/l) (Garcia et al. 1997b,c). Fluorescence increased rapidly after patch rupture, indicating a fast dialysis of intracellular Cl⁻ by the recording pipette medium. The cell was depolarized

Figure 1. Relationship between the membrane potential (V_m) measured just before KCl injection and V_m relative variations (ΔV_m) induced by KCl. 140 mmol/l KCl was injected in the vicinity of cells recorded in the whole-cell configuration (WCR) under current-clamp (Iclamp) conditions (potassium-gluconate, (Kgluc), inside the pipette).



(Fig. 2A) and F_{SPQ} reached a maximum, consistent with the new $[Cl^-]_i$ imposed by the patch pipette (4 mmol/l). However, after 1–2 min F_{SPQ} began to decrease in the majority of cells tested ($n = 117$ of 167). This decrease in F_{SPQ} indicated that, shortly after patch rupture, $[Cl^-]_i$ increased again. This increase in $[Cl^-]_i$ was accompanied by hyperpolarization. Fig. 2A shows a representative illustration of the kinetics observed. Fig. 2B illustrates the relation between the variation in F_{SPQ} , as measured by $\Delta F_0/F$, and membrane potential. The recorded cells repolarized concomitantly with the decrease in F_{SPQ} ($R = 0.53$). These data demonstrate that both K and Cl ions may equally control the membrane resting potential.

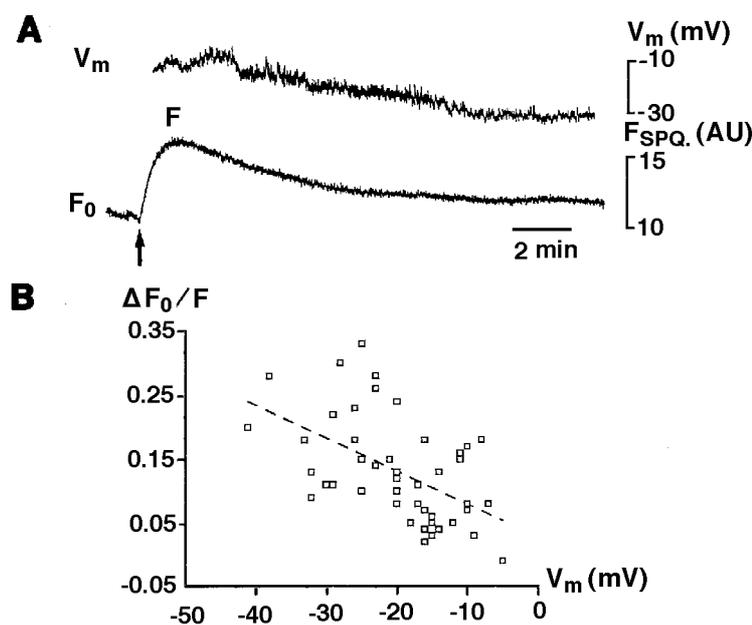


Figure 2. Combined electrophysiology and microspectrofluorimetry. **A.** V_m and fluorescence of cells loaded with SPQ (F_{SPQ}) were recorded under WCR conditions in Iclamp. Kgluc in the pipette (4 mmol/l Cl^-) contained 0.5 mmol/l SPQ. After rupture of the patch under the pipette (arrow) F_{SPQ} (lower trace) increased for 1–2 min, indicating cell Cl^- dialysis by the pipette. A decrease in F_{SPQ} was then observed in most cells. V_m recorded concomitantly suggested a direct relation between the decrease in F_{SPQ} and hyperpolarization. AU, arbitrary units. **B.** Relation between F_{SPQ} and V_m . Each point represents values from 39 individual cells (for 8 cells, two different values obtained at different times have been plotted). $\Delta F_0/F$ corresponds to the variation in the $F_0/F_2 - F_0/F_1$ ratio, where F_0 is F_{SPQ} before patch rupture, F_1 is the maximum value, and F_2 the value(s) obtained during recording after the maximum value. V_m represents the corresponding membrane potential.

Consequence of intracellular K⁺ and Cl⁻ removal

To further study the involvement of K⁺ and Cl⁻ in the membrane resting potential, N methyl-glucamine (NMG) was substituted for K⁺ and methanesulfonate for Cl⁻, both impermeant through K⁺ and Cl⁻ channels (Garcia et al. 1999). V_m then exhibited erratic values, ranging from -28 to +40 mV. Intense depolarization probably reflected the new K⁺ gradient (4 mmol/l outside, 0 mmol/l inside). Under voltage-clamp (Vclamp) conditions (voltage holding, V_h = -50 mV), hyperpolarizing steps to -80 mV led to an increase in F_{SPQ}, whereas depolarizing steps to 0 mV induced a noticeable decrease in F_{SPQ} indicating Cl⁻ entry (Fig. 3A). We know that Ca²⁺-dependent Cl⁻ currents are elicited during short depolarizing episodes, so their activation during voltage ramps gives a good approximation of E_{revCl⁻}, indicative of [Cl⁻]_i variation. Protocols using voltage ramps (-80 to +20 mV, 1024 ms) were thus used to estimate E_{revCl⁻}. SPQ-loaded cells, patched with pipettes containing NMG-methsulf and held at -80 mV, did not develop any appreciable voltage-dependent current in 6 cells out of 9, as illustrated in Fig. 3Ba,b,c (ramps 1 and 2), and F_{SPQ} was stable (Fig. 3Ba,d). On the contrary, depolarization to 0 mV induced a huge decrease in F_{SPQ}, indicating Cl⁻ entry, that was correlated with an outwardly directed current (Fig. 3Ba,d). The voltage ramps allowed us to determine E_{rev} to be -35.8 mV (n = 6) (Fig. 3Bc, ramp 3). This demonstrates that the membrane Cl⁻ influx triggered by long depolarization episodes exceeds the pipette Cl⁻ dialysis. The use of impermeant ions in the pipette medium made it necessary to investigate in more detail the link between F_{SPQ} and [Cl⁻]_i in the presence of Kgluc under more physiological membrane potential conditions than those used with NMG-methsulf.

Link between F_{SPQ} and [Cl⁻]_i in the presence of Kgluc*Relationship between F_{SPQ} and E_{revCl⁻}*

We again used the potential ramp technique to analyze concomitant changes in currents and F_{SPQ}. After patch rupture, in cells recorded with Kgluc as the pipette medium we consider that [Cl⁻]_i is close to the pipette concentration (4 mmol/l) at the maximum increase in F_{SPQ}. The first ramp triggered at this time serves as a control. The intercept of the following ramps gives a good estimate of E_{revCl⁻}. When cells were permanently maintained at -80 mV, the third ramp showed a significant shift towards more depolarized potentials than the second ramp (Fig. 4A, E_{revCl⁻} = -51.6 ± 3 and -41.4 ± 3.9 for ramps 2 and 3, respectively; Fig. 4B, p < 0.05, n = 9, paired non-parametric test). In both cases, the shift of the intercept towards the right correlated with a decrease in F_{SPQ} (91.4 ± 0.95 and 80 ± 2.45% of maximum F_{SPQ} for ramps 2 and 3, respectively, p < 0.01, Mann-Whitney U-test).

To confirm the involvement of Cl⁻ in the ramp-induced currents, we tested the response to a GABA-receptor channel agonist. When muscimol (10⁻⁵ mol/l) was injected on the recorded cell (not shown), it induced a shift of E_{rev} towards depolarized potentials in cells maintained at -80 mV (-52.3 ± 6 vs. -37 ± 5 mV,

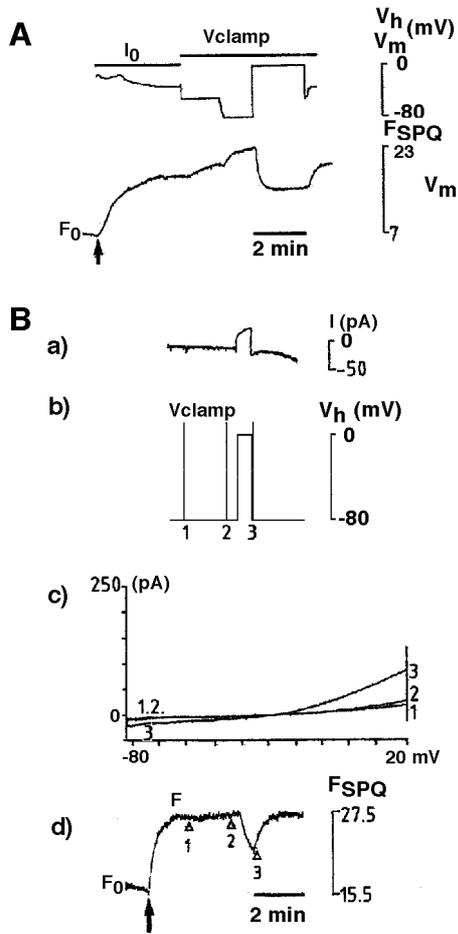
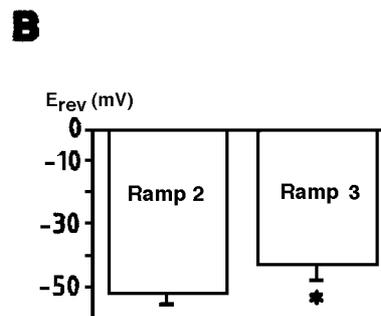
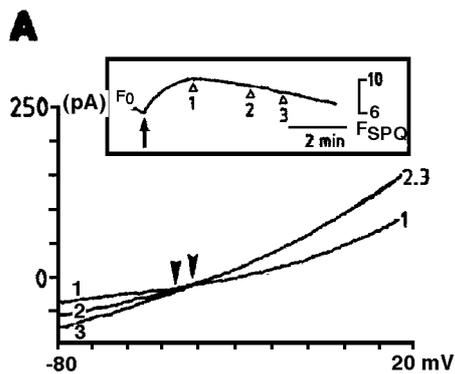


Figure 3. Effect of K^+ and Cl^- withdrawal from the internal medium (NMG-methane-sulphonate in the pipette instead of Kgluc) on membrane electrical properties and F_{SPQ} . **A.** The cell was first recorded under I_0 conditions (I_0). V_m reached a plateau at -28 mV (upper trace). F_{SPQ} also reached a plateau (lower trace). In the voltage-clamp (V_{clamp}) mode at -50 and -80 mV (upper trace), F_{SPQ} increased (lower trace), indicating a probable Cl^- outflow. On the contrary, at 0 mV F_{SPQ} decreased due to rapid Cl^- entry. The E_{rev} for Cl^- in this cell is probably between -50 and 0 mV. **B.** Application of voltage ramps to a cell maintained in V_{clamp} (voltage holding: $V_h = -80$ mV, amplitude: 100 mV, duration: 1024 ms). Electrical recordings (Ba, current; Bb, application of ramps during V_h ; Bc, individual ramps) and F_{SPQ} (Bd). After patch rupture, F_{SPQ} increased rapidly and remained at its maximum value (Bd). It then decreased when the cell was held at 0 mV, following Cl^- inflow (outward current in Ba). No current was elicited by voltage ramps No. 1 and 2, whereas ramp No. 3 showed a current which reversed near -30 mV following depolarization to 0 mV (Bc). Open arrowheads indicate voltage ramp applications.



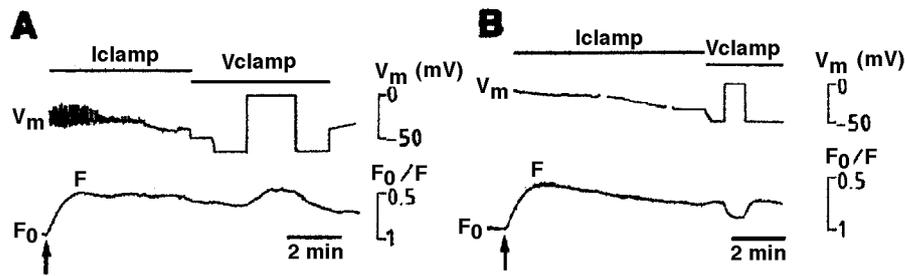


Figure 5. Combined recordings of two cells, displaying opposite F_{SPQ} responses in the Vclamp situation when depolarized to 0 mV. **A.** Increase of F_{SPQ} . **B.** decrease of F_{SPQ} .

$p < 5\%$, $n = 7$) for control (intercept between the first and second ramps) and muscimol-treated cells, respectively.

We conclude that the decrease in F_{SPQ} correlated with an increasing Cl^- influx which bypassed the Cl^- dialysis by the pipette ($[Cl^-]_{pip}$, 4 mmol/l) enabling the cells to reach a higher $[Cl^-]_i$ than that predicted by the Nernst equation at equilibrium, (approx. 35 mmol/l after muscimol injection rather than 25–30 mmol/l). This conclusion was confirmed by the current amplitude analysis. Under Vclamp conditions (V_h maintained at -80 mV), the normalized current amplitude (third ramp) at -80 mV was 8.53 ± 1.80 pA/pF ($n = 9$), whereas it was only 1.25 ± 0.17 pA/pF ($n = 11$) when the cells, recorded under current clamp conditions, were maintained at -80 mV only during the voltage ramp application. This indicates that $[Cl^-]_i$ is capable of increasing against the gradient theoretically imposed by the pipette.

Heterogeneity of F_{SPQ} responses to changes in V_h

Two types of F_{SPQ} responses could be distinguished from the voltage clamp studies (Fig. 5). The Nernst equation predicts that, under our recording conditions (150 mmol/l $[Cl^-]_o$, 4 mmol/l $[Cl^-]_i$), Cl^- should enter at 0 mV. This was not always the case. In the cell population shown in Fig. 5A, a voltage step to 0 mV induced an increase in F_{SPQ} in a major part of the cells, indicating a Cl^- outflow (type 1, $n = 33$ of 76 cells). A decrease was observed in other cells (type 2, $n = 20$ of 76 cells), suggesting a Cl^- inflow (Fig. 5B). The remaining cells (23 of

Figure 4. Interaction between V_h , F_{SPQ} , and Cl^- movements (Combined recordings). Determination of the current (I_{Cl^-}) inversion (E_{rev}) elicited by voltage ramps in combined recordings (Kgluc + 0.5 mmol/l SPQ in the pipette). **A.** Voltage ramps and F_{SPQ} (inset) recordings. Cells loaded with SPQ were recorded in Vclamp ($V_h = -80$ mV). The first ramp started as close as possible to the maximum F_{SPQ} increase. Arrow: patch rupture. Arrowheads: intercepts. **B.** Mean values for E_{rev} of ramps No. 2 and. 3. * $p < 5\%$.

Table 1. Effect of chronic estradiol treatment (10^{-8} mmol/l during the cultures) on F_{SPQ} response of cells statistically of type 2 (decrease in F_{SPQ} at 0 mV).

F_{SPQ}	type 2 cells $n = 14$			type 2 E_2 -treated cells $n = 13$		
	increase	plateau	decrease	increase	plateau	decrease
$V_h = 0$ mV	1	5	8	5	5	3

The control and treated cells were from the same source and processed simultaneously. EM contained 4 mmol/l bicarbonate. SPQ loaded cells were patched with Kgluc-containing pipettes. F_{SPQ} responses were analyzed during voltage steps (>30 s) at $V_h = 0$ mV. Number of cells showing each kind of response is reported.

76) showed a stable F_{SPQ} . We will refer to type 1 and type 2 cells in the following paragraphs. To exclude the possibility of a dialysis effect, we compared the serial resistance of recorded cells from cultures showing statistically either a type 1 or a type 2 response. No difference was detected (not shown). Conversely, type 2 cell capacitance (12.6 ± 1.8 pF, $n = 16$) was significantly lower than that of type 1 cells (15.9 ± 0.66 pF, $n = 63$, $p < 0.05$). These data suggest a different intracellular Cl^- concentrations and (or) a different $[\text{Cl}^-]_i$ regulation mechanisms in the two groups of lactotrope cells.

The heterogeneity of rat lactotrope cells has already been described. Lledo et al. (1991) reported the existence of two populations, low- and high-density cells, with different electrophysiological and secretion properties. A transition between these two lactotrope cell phenotypes under the effect of estradiol (E_2) was reported by Zhang et al. (1990). We also observed that under treatment with 10^{-8} mol/l E_2 , the cell cultures, statistically exhibiting a type 2 response, had an inverse F_{SPQ} response to V_h at 0 mV as compared to controls (Table 1), and similar to that of type 1. Moreover, the cell capacitance of 10^{-8} mol/l E_2 -treated cells increased to 14.38 ± 1.8 pF ($n = 13$).

These results strongly suggest that, apart from passive mechanisms of Cl^- distribution, active mechanisms are probably present in the rat lactotrope cell $[\text{Cl}^-]_i$ regulatory machinery. In the third part of our study, we further examined the $[\text{Cl}^-]_i$ regulatory mechanisms in both cell types, paying special attention to a possible link with cell energetics.

Cl^- transport mechanisms involved in $[\text{Cl}^-]_i$ variations

Nature of mechanisms that differentiate type 1 from type 2 cells. Involvement of pH_i

pH_i , measured in both cell populations, was more alkaline in type 1 (7.34 ± 0.01 , 12 experiments, $n = 132$ cells, Fig. 6A column (col.) 1) than in type 2 cells (7.15 ± 0.02 , $n = 20$ experiments with 20–30 cells measured for each experiment, Fig. 6A col. 3). When type 2 cells were exposed to a Cl^- -deprived (0 Cl^-) medium containing

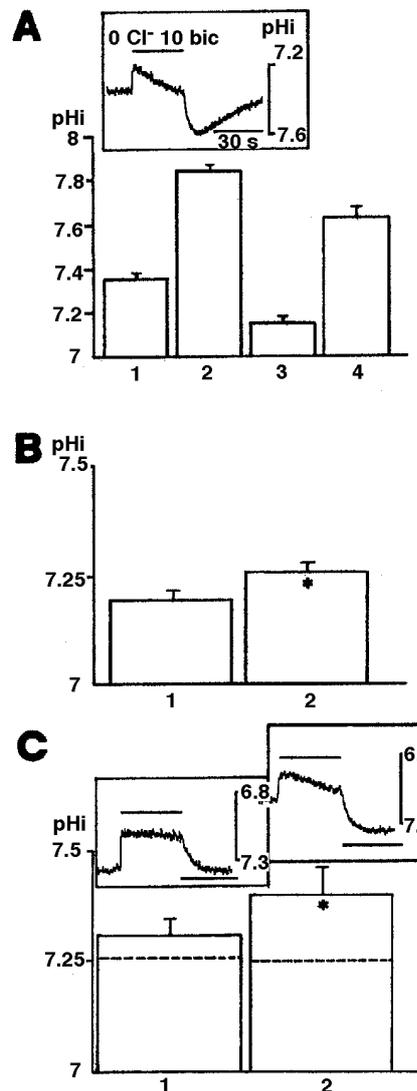


Figure 6. Comparison of pH_i in cells responding either as type 1 or 2. Functionality of anion exchangers and effect of E₂ (10⁻⁸ mol/l). **A.** Comparison of pH_i in type 1 (column (col.), col. 1, basal pH_i; col. 2, pH_i in 0 Cl⁻ 10 bic medium) and type 2 cells (col. 3, basal pH_i; col. 4, pH_i in 0 Cl⁻ 10 bic medium). Inset: induction of anion exchange activation in response to CO₂ acidification during and after 0 Cl⁻ 10 bic medium injection in a type 1 cell. **B.** Effect of E₂ (10⁻⁸ mol/l) on basal pH_i of type 2 cells (col. 1, control cells; col. 2, cells treated with E₂ during culture). * *p* < 5%. **C.** Effect of short application of 0 Cl⁻ 10 bic medium (30 s) on activation of anion exchangers. Col. 1, pH_i of control cells (7.31 ± 0.04, *n* = 7); col. 2, pH_i of E₂-treated cells exposed to 0 Cl⁻ 10 bic, at maximum alkalinization (7.41 ± 0.05, *n* = 8). Dotted lines; basal pH_i. Insets as in A; * *p* < 5%.

10 mmol/l bicarbonate (10 bic), pH_i increased to 7.625 ± 0.038 (20 experiments as for controls, Fig. 6A col. 4), vs. 7.84 ± 0.01 in type 1 cells (3 experiments, *n* = 50 cells, Fig. 6A col. 2). Thus, type 2 cells were significantly less alkaline in a 0 Cl⁻ 10 bic medium than type 1.

The addition of E₂ (10⁻⁸ mol/l) during culture induced alkalinization of the cytosol (7.18 ± 0.024, vs. 7.24 ± 0.023 in control and E₂-treated cells, respectively; 17 experiments, *n* > 200, *p* < 0.05, Fig. 6B). We then tested the functionality

of the $\text{Cl}^-/\text{HCO}_3^-$ exchange (anionic exchange AE) by injecting 0 Cl^- 10 bic. Activation of AE led to a significant alkalization in E_2 -treated cells (Fig. 6C col. 2, $\text{pH}_i = 7.23 \pm 0.03$ before injection *vs.* 7.41 ± 0.05 at its maximum value, $n = 8$). In control cells, the anion exchange was not significantly activated at similar pH_i values (7.26 ± 0.04 before the injection, *vs.* 7.31 ± 0.04 at its maximum value, $n = 7$, Fig. 6C col. 1) (note the difference in the response slopes in the insets and compare also to the inset in Fig. 6A). Thus, it seems likely that pH_i may also be involved in the mechanisms differentiating type 1 from type 2 cells.

Involvement of anion exchange and cotransport in $[\text{Cl}^-]_i$ variations

Role of anion exchange. We previously demonstrated that $\text{Cl}^-/\text{HCO}_3^-$ exchangers (AE_2 and AE_3) are active in these cells (Garcia et al. 1997a). In fact, the type 1 lactotrope cells, maintained in a medium containing 140 mmol/l Cl^- and 0 bic and

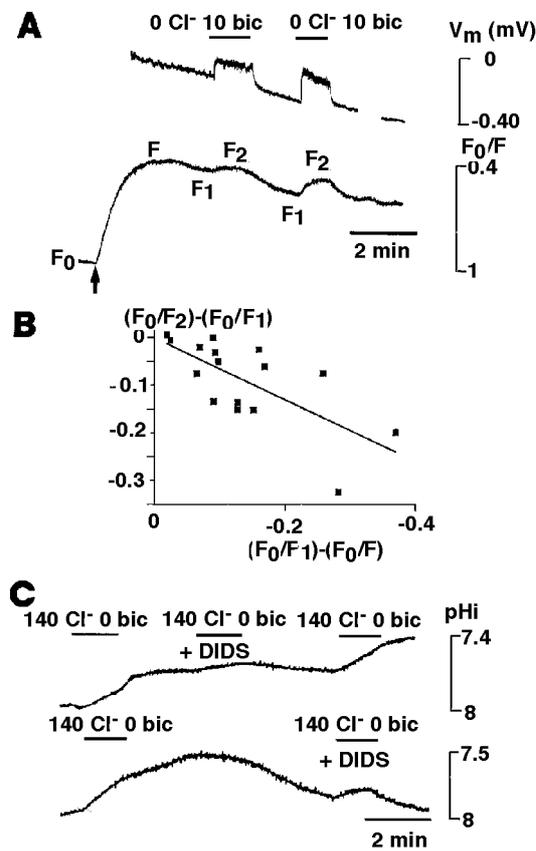


Figure 7. Effect of anion exchange on F_{SPQ} evolution. **A.** Combined recording showing that injection of Cl^- -free medium with 15 bic onto cells exhibiting an F_{SPQ} decrease (Cl^- entry) induced a Cl^- outflow, revealed by the inversion of F_{SPQ} during injection. The response was enhanced when a second injection was made at a lower F_{SPQ} level. Concomitant recording of V_m revealed a depolarization during the injection (Cl^- outflow). **B.** Relation between the amplitude of the F_{SPQ} response to 0 Cl^- 15 bic. $F_0/F_2 - F_0/F_1$ and the amplitude of F_{SPQ} decrease during the recording $F_0/F_1 - F_0/F$ are correlated (correlation coefficient $r = 0.71$, $p < 0.01$). **C.** Anion exchange promoted H^+ loading of alkalized lactotrope cells (cells bathing in Cl^- -free medium with 10 bic). $\text{H}_2\text{-DIDS}$ inhibited the activation of AE_3 during and after injection and partly blocked acidification. The remaining H^+ loading may be attributed to AE_2 .

Table 2. Effect of furosemide (1 mmol/l) on F_{SPQ} response of cells which are statistically of type 2 (decrease in F_{SPQ} at 0 mV).

F _{SPQ}	type 2 cells n = 20			type 2 furosemide-treated cells n = 10		
	increase	plateau	decrease	increase	plateau	decrease
V _h = 0 mV	2	5	13	8	2	0

Furosemide was added to the EM (4 bic) at the beginning of each recording. SPQ loaded cells were patched with Kgluc-containing pipettes and processed as in Table 1. Number of cells showing each type of response is reported.

exposed to acute injection of a 0 Cl⁻ medium containing 10 or 15 bic responded by the alkalization of the cytosol, concomitant with a Cl⁻ outflow and an HCO₃⁻ inflow. Fig. 7A shows a representative example of the increase in F_{SPQ} (Cl⁻ outflow) associated with membrane depolarization. In addition, the amplitude of the Cl⁻ outflow was inversely related to F_{SPQ} levels (F₁) (Fig. 7A,B).

However, incubating cells in a Cl⁻-free bathing medium with 10 mmol/l bic brought basal pH_i to around 7.8 (Garcia et al. 1997a, see also Fig. 6A), and injecting 140 Cl⁻ 0 bic solution under these conditions, induced H⁺ loading, both during and after injection. This acidification decreased during 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) application, an anion exchange blocker, (Fig. 7C). ΔpH_i was evaluated at -0.258 ± 0.04 U pH_i/min ($n = 25$). These data demonstrate that AE (AE₂ and AE₃) works both ways, inducing either Cl⁻ inflow or outflow. Thus, a Cl⁻/HCO₃⁻ exchanger is also a good candidate for promoting a Cl⁻ inflow capable of exceeding the dialysis imposed by the pipette. The role of each has yet to be determined.

Role of cotransport. The cation chloride cotransports K⁺-Cl⁻ (KCC) and Na⁺-K⁺-2Cl⁻ (NKCC) are influenced by pH_i (Russel 2000) and may also be responsible for Cl⁻ entry. To test this hypothesis, we used furosemide, a KCC and NKCC cotransport blocker. When type 2 cells bathing medium was supplemented with furosemide, depolarization from V_h = -80 to 0 mV induced an increase in F_{SPQ}, indicating a chloride outflow similar to the type 1 response (Table 2). When furosemide was acutely injected on the recorded cell, F_{SPQ} increased in 6 out of 9 cells. This suggests that the gradient imposed by the pipette which produced a decrease in [Cl⁻]_i was active provided that the cation chloride cotransports were blocked by furosemide.

Link between ion concentrations and cell metabolic status

To test the hypothesis of a putative link between Cl⁻ homeostasis and metabolic cell status, we injected H₂-DIDS, the non-fluorescent form of DIDS, on the recorded cell during voltage ramps. Cells left at I₀ between the ramps or maintained at -80 mV exhibited a shift of E_{rev} towards more hyperpolarized potentials (Fig. 8A). The shift, around -8 mV in either case, was not quite significant (-40.2 ± 6.5

mV for controls, -48.2 ± 6.5 mV for treated cells, $n = 5$ for cells left at I_0 , and -35.6 ± 5.3 mV for controls and -43.25 ± 5.6 mV for treated cells held at -80 mV, $n = 8$). We re-examined this aspect by incubating the cells in the presence of H_2 -DIDS. When the cells were incubated with 0.1 mmol/l of H_2 -DIDS during the recordings, F_{SPQ} response was variable, from a slight increase (Fig. 8B) to a slight decrease (not shown). Voltage ramps did not activate ion currents for negative potentials of up to -20 mV (Fig. 8B). E_{rev} shift towards hyperpolarized potentials increased (-74.4 ± 4.3 mV, $n = 7$) and was thus significantly different from controls (-40.7 ± 5.2 mV, $n = 6$, $p < 0.001$) and acute DIDS-treated cells (-49 ± 5 mV, $n = 6$, $p < 0.01$, Fig. 8C). These mechanisms basically reflect the status of the Cl^- gradient. Correlated with the variations in F_{SPQ} , the ramps clearly show that $[Cl^-]_i$ was close to the Cl^- pipette concentration. One effect of DIDS was a blockade of Ca^{2+} -dependent Cl^- channels. However, DIDS also inhibited the anion exchanger, AE_3 (Cl^-/HCO_3^-), which regulates pH_i .

As pH_i is linked to cellular production of CO_2 , we decided to block the CO_2 (H_2CO_3 reaction driven by carbonic anhydrase by incubating the cells with acetazolamide (0.1 mmol/l) for four hours (Garcia et al. 1997a). This metabolic inhibitor blocks anion exchange (Cl^-/HCO_3^-) and disturbs the respiratory chain. Voltage ramps revealed a large ion current decrease at -80 mV, ranging from -40 to -85% for ramps 1 to 3 respectively (not shown).

Thus, in rat lactotrope cells, Cl^- homeostasis may depend partly on metabolic processes linked to anion exchange.

Discussion

In this study, we investigated the Cl^- homeostasis of rat lactotrope cells in primary culture, using the patch-clamp technique in the whole-cell configuration, combined with microspectrofluorimetry of SPQ, a Cl^- -sensitive dye. We show that the membrane potential of rat lactotrope cells is partially determined by $[Cl^-]_i$. We also show that the patch pipette does not always impose its ion concentration. We confirm that the Cl^- homeostasis depends, at least partly, on active chloride entry against the cytosol-pipette gradient. In addition, we have identified two mechanisms that may perform this function: the pH_i regulator system, based on anion exchange (Cl^-/HCO_3^-), and the cation chloride cotransport system (CCC). We also provide physiological evidence for lactotrope cell heterogeneity. Finally, we present indications that these mechanisms may be linked to cell energetics.

Whole cell patch-clamp recording and volume changes

We demonstrate here that dialysis of the rat lactotrope cytosol by intrapipette medium is without any real incidence on cell volume in the absence of osmotic shock, as estimated by morphometric analyses. These observations are in agreement with data reported by others for different cell types (Bond et al. 1999; Voets et al. 1999). On the contrary, the F_{SPQ} decrease appearing in a majority of patched cells a few minutes after patch rupture is positively correlated with hyperpolarization of

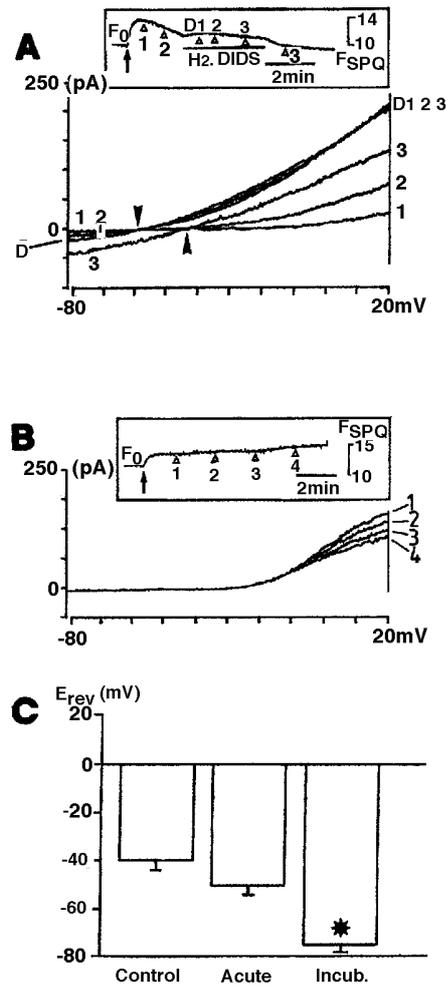


Figure 8. Effects of DIDS on electrophysiological properties and F_{SPQ}. Voltage ramps on cells recorded in Vclamp mode ($V_h = -80$ mV). **A.** Acute effect of H₂-DIDS. 1, 2, and 3 normal ramps and D1, 2, 3 ramps during H₂-DIDS application. Inset: changes in F_{SPQ} following DIDS application. **B.** A cell recorded in the presence of 0.1 mmol/l H₂-DIDS. No current activated at negative potentials up to -30 to -20 mV. Inset: F_{SPQ}. No decrease in F_{SPQ}. An increase was even observed at the end of the recording. Arrows and arrowheads as in Fig. 4. **C.** Mean values for E_{rev} in acute and incubation experiments (* $p < 5\%$, Tukey-Kramer post-test).

the membrane due to Cl⁻ entry. We can thus postulate that, in these conditions, membrane Cl⁻ influx overrides the cytosol-Cl⁻ dialysis by the recording pipette.

Cl⁻ homeostasis mechanisms working against a 4 mmol/l Cl⁻ concentration in the recording pipette

In the whole-cell patch-clamp configuration with Kgluc in the pipette, we found an experimental E_{revCl^-} near -40 mV. This value was consistent with the data obtained by microspectrofluorimetric analyses. Thus, the [Cl⁻]_i of about 35 mmol/l in the cytosol of recorded cells was very different from the 4 mmol/l in the pipette medium. We have identified several mechanisms capable of by-passing the pipette dialysis (Fig. 9).

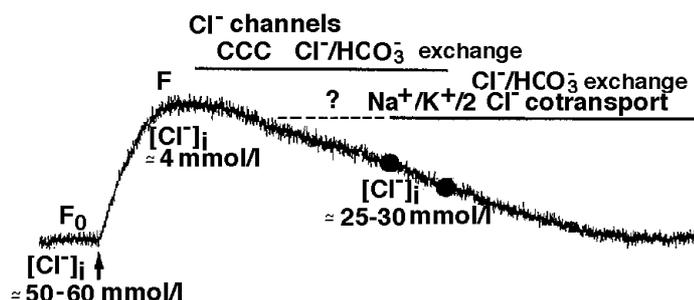


Figure 9. Interactions between the different $[\text{Cl}^-]_i$ modulating mechanisms in rat lactotrope cells. Possible sequence of events occurring after patch rupture. NKCC and AE activities might explain the uphill Cl^- flux.

The membrane Cl^- influx (φ_1) may vary, whereas chloride flux at the pipette tip (φ_2) is solely dependent on the cell-pipette gradient and the pipette tip section, which is fixed for a given cell. As illustrated in Fig. 9, in the first part of the recordings the electrochemical gradient alone might explain the increase in $[\text{Cl}^-]_i$, provided that φ_1 is higher than φ_2 . Ca^{2+} -dependent Cl^- conductance activation (Sartor et al. 1992) might be sufficient to induce this influx. However, we must remember that low $[\text{Cl}^-]_i$ may also decrease $[\text{Ca}^{2+}]_i$ and, consequently, affect the Cl^- channel function (Garcia et al. 1997b). It was therefore necessary to investigate other mechanisms participating in the Cl^- membrane influx, including the CCC. Two isoforms of KCC, (KCC_1 and KCC_2), have been described (Payne 1997). KCC_1 is ubiquitous (Gillen et al. 1996), whereas KCC_2 is brain-specific (Payne et al. 1996). KCC_1 is involved in volume regulation (Payne 1997) and operates near equilibrium: “depending on $[\text{Cl}^-]_i$ and $[\text{K}^+]_o$, the transport will extrude or accumulate Cl^- ” (Jarolimek et al. 1999).

A balance between KCC and NKCC cotransport activation should also be considered, as KCC is activated by an increase in $[\text{Cl}^-]_i$, whereas NKCC is inhibited (see a review of this question in Russell 2000). KCC_1 may extrude or accumulate intracellular Cl^- . NKCC is essentially responsible for Cl^- influx due to the residual energy carried by Na^+ . Even at a furosemide concentration of 1 mmol/l that blocked both cotransports (Gillen et al. 1996), the K^+ gradient prevented KCC from acting as a potent Cl^- accumulator. On the contrary, Garcia (1996) found that, in rat lactotrope cells, Na^+ was necessary to activate the Cl^- entry mechanism blocked by bumetanide and furosemide. This clearly shows that NKCC is a good candidate for maintaining a high $[\text{Cl}^-]_i$ in rat lactotrope cells in primary culture. The fact that estradiol receptor activation may, in turn, inactivate KCC and activate NKCC (Lytle and Forbush 1992; Lytle 1997; Jennings 1999; Katzenellenbogen 2000) is in agreement with this suggestion.

Another mechanism participating in the $[\text{Cl}^-]_i$ control is pH_i . Olsnes et al. (1987) first reported a $[\text{Cl}^-]_i$ increase in Vero cells when pH_i was raised from 7

to 7.5. In human embryo kidney cells, transfected with the AE₂ gene, Sekler and co-authors found a huge increase in [Cl⁻]_i between pH 7 and 7.2. AE₂-induced Cl⁻/HCO₃⁻ exchanges may intervene when pH_i is below or near 7.2–7.3, and the direction of the flux is determined by the inside and outside Cl⁻ and HCO₃⁻ concentrations (Sekler et al. 1996). However, Sterling and Casey (1999) compared the anion transport of AE₂ and AE₃ in HEK-293 transfected cells and found a differential regulation by pH_i. AE₂ was negatively regulated by acid pH_i, whereas AE₃ was pH_i-independent. Acidic pH_i may, therefore, decrease the involvement of Cl⁻/HCO₃⁻ in the pH_i regulation. Most cultured cells had an [Cl⁻]_i near equilibrium (30–40 mmol/l) when pH_i was under 7.2 (data not shown). This means that, at acidic pH_i, lactotrope cells do not have the molecular machinery, even in the presence of a functional K⁺/Cl⁻ cotransport, to actively regulate [Cl⁻]_i and pH_i. In this case, the Na⁺/H⁺ exchanger regulates pH_i, with no effect on [Cl⁻]_i. Our data add to previous reports on the phenotypic transition of rat lactotrope cells (Zhang et al. 1990) and provide physiological evidence that the phenomenon depends on pH_i and [Cl⁻]_i.

The existence and functionality of these two mechanisms (CCC and anion exchangers) infer that the Nernst equation cannot be used to explain Cl⁻ movements with reference to only two compartments (extracellular (EM) and intracellular – including the pipette). When active or passive entry mechanisms override pipette dialysis, at least three compartments are involved: (i) the EM, (ii) the cytosol, and (iii) the pipette. Our interpretation is not in contradiction with the fundamental study by Hamill et al. (1981), who proposed that the whole-cell patch clamp “allows at least partial control of the cell interior”. In lactotrope cells, pipette control is only partial. Several mechanisms, including active transports, are capable of overriding this control.

Cl⁻ homeostasis is linked to cell energetics

The results reported in the last part of our paper illustrate a probable interaction between [Cl⁻]_i regulation and cell energetics. Voltage ramps recorded in the presence of the anion exchanger antagonist, DIDS, were not able to develop large-amplitude currents. Although the effects of acute injections and incubations in the presence of DIDS indicate AE₃ inhibition, as previously described (Garcia et al. 1997a), they do not exclude a possible intervention of the Ca²⁺-dependent Cl⁻ channels (Sartor et al. 1992; Fahmi et al. 1995). Obviously, the effect of DIDS may, in part, result from the anion exchanger (AE₃) inhibition that affects the equilibrium of the reaction driven by carbonic anhydrase. We also observed that DIDS increased cell oxygen consumption (unpublished results). Recent findings indicate two other modes that may also interfere with the cell machinery. Firstly, in human red blood cells, DIDS reduces CO₂ permeability through its effect on a membrane transport protein (Forster et al. 1998). Secondly, Vince and Reithmeier (2000) proposed that AE₂ was capable of binding with a region of carbonic anhydrase, as does AE₁ (Vince et al. 2000), so we can speculate that AE₃ has the same property.

Finally, the application of acetazolamide, which inhibits carbonic anhydrase (CA), produced data that reinforce the concept of a close interaction between chloride and cell energetics. Little has been published in this area. Henry (1996) noted that CA “is one of the few enzymes occupying a central role in both transport and metabolism”. To our knowledge, the first data were presented by De Weille and Lazdunski (1990), who showed that removal of intracellular chloride modified the properties of K^+ -ATP-dependent channel activity in an insulinoma cell line. We have presented evidence that, in rat lactotrope cells, chloride is stored in mitochondria (Garcia et al. 1997c) and intracellular chloride depletion is followed by a decrease in intracellular ATP (Sartor et al. 2004), increase in aerobic glycolysis, and activation of the respiratory chain in type 1 cells, whereas the last two parameters are inhibited in type 2 cells (Sartor et al. 1999, 2001, 2004). These data must be viewed in relation to the results of Erecinska et al. (1991) showing that depolarization increased respiratory chain activity. In rat lactotrope cells, we found evidence indicating that a balance between aerobic and anaerobic glycolysis pathways modulated cell ATP production, with a preferential utilization of the lactate pathway to meet major, urgent ATP requirements (Sartor et al. 2004).

In conclusion, the results reported here emphasize the plasticity of physiological properties of lactotrope cells in culture. Several reports have noted that chloride movements do not always reflect the values indicated by the Nernst fundamental equation (Mayer et al. 1985; Owen et al. 1986; Rogawski et al. 1988; Sartor et al. 1990). We now know that this apparent discrepancy probably reveals a fundamental physiological issue in rat lactotrope cells in primary cultures: the interaction of chloride with cell energetics. This interaction emphasizes the importance of chloride stores in mitochondria (Garcia et al. 1997c) and deserves further investigation.

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