Regulation of intracellular chloride concentration in rat lactotrophs: possible role of mitochondria

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Abstract Increasing evidence is accumulating for the involvement of chloride ions in the stimulus-secretion coupling of pituitary cells. We show that the mean intracellular chloride concentration [Cl^-]i of rat lactotroph cells maintained in culture is high, close to 60 mM (59.4 mM), using the Cl^- sensitive fluorescent probe 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ), coupled with whole-cell patch-clamp recordings. We demonstrate that this high level is correlated with the presence of mitochondrial stores of Cl^- as shown by the release of Cl^- in response to various metabolic inhibitors. We determine that CCP (50 μM) induces a mean [Cl^-]i increase of 15.8 ± 5.8 mM, using combined electrophysiology and microspectrofluorimetry methods. These data strongly suggest that cell metabolism, including the mitochondrial function, modulate [Cl^-]i.

Key words: Intracellular chloride concentration; Mitochondrial chloride store; Metabolic inhibitor; Microspectrofluorimetry; Electrophysiology; Rat lactotroph

1. Introduction

Chloride ions (Cl^-) are mostly known to participate in the modulation of cell excitability. The chloride gradient across cell membranes adjusts the membrane potential [1], regulating action potential firing [2]. Chloride ions are also known to participate in several other cellular processes such as intracellular pH [3] and cell volume regulation [4]. Recent works also suggest their involvement in the secretory process of pituitary cells [5].

The intracellular chloride concentration ([Cl^-]i) is regulated by several processes, including ion channels, anionic exchangers, and co-transporters, but its regulation mechanisms are not clearly understood. In normal rat lactotrophs, a calcium-dependent chloride conductance and a GABA-activated Cl^- conductance have previously been described [6–8]. The opening of GABA-activated Cl^- channels leads to changes in membrane potential and consequently to variations in calcium influx. To further study the regulation of [Cl^-]i, we used a combined approach involving whole cell electrophysiological recordings of membrane conductances and spectrofluorimetric measurements of [Cl^-]i with the Cl^- sensitive fluorescent probe 6-methoxy-*N*-(3-sulfopropyl) quinolinium, inner salt (SPQ) proposed by Verkman [9]. These data suggest that, in addition to membrane channels (and transporters) for Cl^- ions, lactotroph cells also have Cl^- mitochondrial stores.

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2. Materials and methods

2.1. Cell culture

Anterior pituitary cells were obtained from lactating Wistar rats. Dissociation was carried out with collagenase as previously described [10]. Cells grown on 30 mm glass coverslips were cultured in DMEM/F12 supplemented with 0.5 mM pyruvate, 2.5 mM L-glutamine and 10% fetal calf serum. Lactotroph cells were identified as described in a recent work [7].

2.2. Electrophysiology

The whole cell mode of the patch-clamp technique was employed [11]. A RK-400 amplifier (Biologic, Grenoble, France) was used for recordings. Membrane capacitance was obtained directly by compensation. The capacitance averaged 16.3 ± 1.1 pF (n = 39). Recordings in which series resistance varied by more than 25% were discarded.

2.3. Solutions

For the electrophysiological experiments, the extracellular medium (EM) contained 137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 4.3 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 10 mM HEPES, 5 mM glucose. The osmolality and pH of the EM were 310–320 mosmol/kg and 7.3, respectively. When a Cl^- free EM was required, NaCl was replaced by Na-methanesulfonate, $MgCl_2$ by $MgSO_4$ and $CaCl_2$ by Ca-gluconate. The recording pipette (4 mM Cl^-) contained 150 mM K-gluconate, 2 mM $MgCl_2$, 1.1 mM EGTA, 5 HEPES mM and 0.5 mM SPQ. In order to calibrate the fluorescence signal (see Section 3 and microspectrofluorimetric assays), K-Gluconate and KCl were added in appropriate concentrations to give intrapipette solutions containing, 50, 100 or 140 mM Cl^-.

2.4. Microspectrofluorimetric assays

2.4.1. Chloride measurements. Fluorescence was analyzed using an inverted microscope (Nikon, Paris, France) equipped for microspectrofluorimetry. The experiments were performed using the fluorescent probe SPQ [9]. Excitation wavelength was 350 ± 10 nm and emission wavelength 470 ± 10 nm. Cells plated on round (30 mm) glass coverslips were loaded using the hypotonic method proposed by Chao et al. [12]. This loading procedure led to an SPQ intracellular concentration of 0.5 mM (see below). For the calibration of chloride measurements we employed an approach combining micro-spectrofluorimetry and electrophysiology, as calibration by the double ionophore technique described by Chao et al. [12] was not possible. First, the intracellular concentration of SPQ was estimated. For this purpose, cells loaded with SPQ, as described above, were dialysed with increasing concentrations of SPQ through the patch pipette. The patch pipette concentration of SPQ was set at 0, 0.25, 0.5, 1 and 2 mM with a fixed pipette Cl^- concentration (0 mM). SPQ loaded cells were bathed in 0 mM Cl^- EM and fluorescence variations following patch rupture were analyzed. Under these conditions, the equilibrium between pipette and cell SPQ was obtained for 0.5 mM. This intrapipette SPQ concentration was then routinely used. Second, cells loaded with SPQ were dialysed through the patch pipette with a fixed Cl concentration, thus clamping [Cl]i to different intracellular chloride concentrations (4, 50, 100 and 140 mM). Fluorescence values for Cl^- concentrations of 4, 50, 100, and 140 mM were thus determined.

[Cl^-]i variations induced by metabolic drugs (carboxyl cyanide *m*-chlorophenyl hydrazone (CCP), dinitrophenol (DNP) and iodoacetate (IAA)) were studied. The maximal absorbance for CCP and DNP was at 350 nm, exactly the excitation wavelength used for SPQ. To rule out possible artifacts due to fluorimetric interference with the SPQ

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excitation wavelength, we estimated the filter effect of these metabolic drugs on SPQ emission. Taking into account the concentrations of the metabolic drugs and those of SPQ, we established, using a Hitachi F2000 spectrofluorimeter, that the filter effect in quartz cuvettes was less than 1% for CCP and 4% for DNP. Moreover, as this estimation cannot be directly applied to single cell micro-spectrofluorimetry, we performed additional controls in which the effect of the drugs was tested under conditions similar to those used in single cell microspectrofluorimetry. Fluorescence of the bathing medium containing SPQ, with or without one of the uncouplers, was recorded in the single cell recording situation. Under these conditions, SPQ fluorescence was not affected by the presence of either drug.

2.4.2. Calcium measurements. The fluorescent probe Indo-1 was used to follow intracellular calcium concentrations, as previously described [13]. After loading, cells were rinsed with HBSS. The ratio of the fluorescence at the two wavelengths (405 and 480 nM), proportional to $[Ca^{2+}]i$, was obtained directly using an analog divider. Online recordings were made on an IBM computer, fitted with a labmaster TL-1 interface. Absorbance of CCP or DNP at the excitation wavelength of Indo-1 cannot be a source of artifact as Indo-1 is a ratio probe. Data were analyzed using p-clamp and Axotape software (Axon Instruments, USA).

2.5. Radiotracer techniques

In order to validate the measurement of free $[Cl^-]i$ found with SPQ, additional experiments were also performed using the ^{36}Cl technique. 3×10^6 pituitary cells were plated in 60 mm Petri dishes. On the 5th day of culture, cells were incubated with ^{36}Cl (0.2 μCi/ml) until equilibration (1 h at room temperature) in normal saline, rinsed 3 times with saline at 4°C, then cells were scraped into 1.5 ml of a solution containing 200 mM KCl, 1 mM EGTA, 2 mM phosphate, 2 mM ATP, 2 mM $MgCl_2$, pH 7.3. Crude extract was then ground with a teflon-glass tissue homogenizer. After centrifugation ($800\times g$, 10 min) 100 μl of the supernatant was aliquoted. Radioactivity was counted and compared to that of 100 μl of the incubation medium (150 mM Cl^-), harvested before rinsing to obtain the intracellular (cytosolic and mitochondrial) ^{36}Cl content. The remaining supernatant was centrifuged at $15000\times g$ for 10 min (4°C), to obtain the mitochondrial pellet. This pellet was lysed with 300 μl of 7.5% perchloric acid (PCA) and the supernatant counted after centrifugation. Cell and mitochondrial volumes were estimated using the method described by Espie et al. [14] adapted for pituitary cells. Briefly, cells were incubated at 2°C with 0.2 μCi/ml of the volume indicator ^3HOH (45 min) and the extracellular space marker ^{14}C labeled inulin (1 min), previously saturated with cold inulin (0.2 mg/ml) for 30 min. Cells were lysed with 1 ml of 7.5% of PCA and the radioactivity of the lysate counted. To determine the mitochondrial volume, [^{14}C]inulin was replaced by [^{14}C]mannitol. In this way, we found that cell volume was 2.06 μl per 10^6 cells ($n=3$), and that mitochondrial volume was estimated at 0.37 μl per 10^6 cells (18% of cell volume, $n=4$). The number of cells was determined with a cell counter channelyser (Coultronics).

2.6. Chemicals

SPQ and Indo-1 were from Molecular Probes (Leiden, The Netherlands). CCP, DNP, antimycin A, oligomycin B, ionomycin, and iodoacetate (IAA) were from Sigma (France).

2.7. Statistics

Results were expressed as mean ± S.E.M. Statistical comparisons were made using one way ANOVA and Fisher least significant difference post tests.

3. Results and discussion

3.1. Determination of $[Cl^-]i$

As SPQ is a single excitation single emission probe without isosbestic wavelength, an accurate determination of $[Cl^-]i$ cannot be obtained from a direct fluorescence reading. However, the relative variation (Fo/F) is independent of the intracellular concentration of the dye and of cell size. Fluorescence was measured in the on-cell situation (Fo) and after patch rupture (F). For $Fo/F=1$, Cl^- concentrations in the cell and pipette were identical, making it possible to estimate Fo. After patch rupture, the time course to peak was 69 ± 5.5 s ($n=39$), showing a rapid dialysis of the Cl^- patch pipette. Fo/F values obtained with 4 mM ($n=16$), 50 mM ($n=8$), 100 mM ($n=6$) and 140 mM Cl^- ($n=9$ cells) in the pipette showed a linear relationship (Fig. 1). It was therefore possible to estimate the $[Cl^-]i$ basal mean value of rat lactotroph cells at 59.4 mM ($n=39$ cells) for $Fo/F=1$. To strengthen these data concerning free Cl^- in rat lactotrophs, we carried out additional experiments with radiolabeled chloride (^{36}Cl). The cell and mitochondrial volumes were also estimated separately (see Section 2). The activity found for intracellular ^{36}Cl gave a Cl^- concentration (cytosolic and mitochondrial) of 78 mM. The cytosol value was estimated at 50% of the cell volume, on the basis of the pioneering ultrastructural description of lactotroph cells [15] and the morphometric data reported by Bolender for exocrine cells [16]. Thus the SPQ fluorescence technique gave a free cytosolic $[Cl^-]i$ concentration of 59.4 mM whereas the radiolabeled Cl^- technique gave an intracellular Cl^- concentration (cytosolic and mitochondrial) of 78 mM.

The basal $[Cl^-]i$ of rat lactotrophs was higher than that found by Kock et al. [17] in the GH4 C1 tumor cell line (31 mM) and by our team in the GH3 tumor cell line (around 20 mM, unpublished results). Conversely, Korn et al. [18] estimated $[Cl^-]i$ close to 50 mM in the tumoral corticotrope cell line At-T20. Such a high basal $[Cl^-]i$ for rat lactotrophs is in agreement with other findings of this laboratory showing that GABA led to membrane depolarization by opening chloride channels, thus inducing an outward flux of chloride ions [8]. Therefore $[Cl^-]i$ has to be well above 30 mM which corresponds to a Cl^- reversal potential of −40 mV, the mean resting potential of these cells.

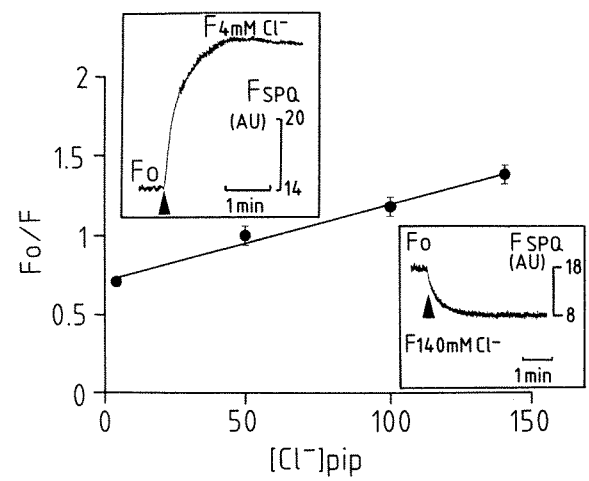

Fig. 1. Calibration of intracellular chloride concentration ($[Cl^-]i$) in normal rat lactotrophs. Normal rat lactotrophs were loaded for 4 min in a hypotonic external medium (150 mosmol) containing 7.5 mM SPQ. Then the cell was patched with a pipette containing a fixed concentration of Cl ions and 0.5 mM SPQ. Fluorescence was measured in the on-cell situation (Fo) and at equilibrium, after patch rupture (F). The Fo/F ratio was calculated for each cell in each of the four experimental conditions (4, 50, 100 and 140 mM Cl^-), giving four mean values, used to construct a Stern-Volmer plot. The data fitted a regression deduced by the equation $y=0.716+0.0047x$; $r=0.99$. For $y=1$, intracellular and pipette Cl^- concentrations were identical. The inserts show representative recordings of fluorescence variations for two cells recorded with pipette solutions containing 4 mM Cl^- (top left) and 140 mM Cl^- (bottom right), respectively. Arrowhead = patch rupture; Fo = basal $[Cl^-]i$ fluorescence; F = clamped $[Cl^-]i$ fluorescence.

With a resting membrane potential at about −40 mV [10], a mean [Cl⁻]i estimated at around 60 mM, giving a reversal potential for Cl^- ions at −23 mV, implies that an active mechanism for Cl^- movement must be present to provide the cytoplasm with Cl^- ions. This high [Cl⁻]i may be due to an influx of external Cl^- ions, through cotransporters such as $Na^+/K^+/Cl^-$ and/or Cl^-/HCO_3^-, or a release from intracellular stores.

3.2. Effect of metabolic inhibitors on SPQ fluorescence

In view of de Weille and Lazdunski's report on a link between cellular ATP and intracellular Cl^- concentrations [19], and the description of a mitochondrial anionic channel by Sorgato et al. [20], we decided to explore the hypothesis of intramitochondrial stores. To study the putative involvement of mitochondria, we used two mitochondrial uncouplers, CCP and DNP, and IAA, a glycolysis inhibitor.

In a first series of experiments, [Cl⁻]i modifications were estimated as SPQ fluorescence variations and thus given in arbitrary units (AU). Cells were bathed in a medium containing 140 mM Cl^-, CCP (50 μM), decreased SPQ emission: −2.8 ± 0.33 AU (n = 19) and DNP (2 mM) emission: −1.36 ± 0.36 AU (n = 7) (Fig. 2A1,A2). When cells were bathed in a Cl^- free EM, CCP still decreased fluorescence (−3.76 ± 0.44 AU; n = 22) and DNP gave an even more pronounced decrease (−5.66 ± 0.64 AU; n = 5) (Fig. 2B1,B2). Similarly, IAA (10 mM) induced a decrease in fluorescence when cells were bathed in 140 mM Cl EM (−3.45 ± 0.6 AU, n = 6). This effect was also enhanced when extracellular chloride was eliminated (−7.45 ± 1.15 AU, n = 10).

In Cl^- free EM, the existence of an internal source of Cl^- was further suggested by decreased response amplitudes following repetitive uncoupler administrations (Fig. 2C), thus indicating that the intracellular reserve was gradually diminishing. Amplitudes of responses to the metabolic inhibitors decreased only when the cells were bathed in Cl^- free medium (Fig. 3). The effect of CCP, DNP and IAA, observed even in

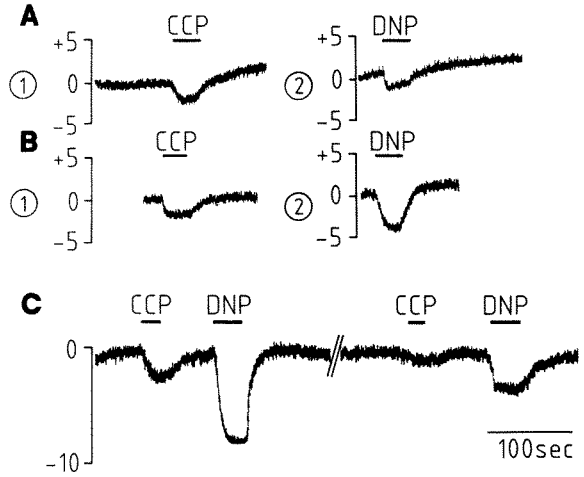

Fig. 2. Effects of uncouplers on SPQ fluorescence. A: Effect of DNP and CCP on SPQ fluorescence for cells bathed in normal (140 mM Cl^-) EM. CCP (50 μM) (A1) and DNP (2 mM) (A2) decreased SPQ fluorescence thus indicating that [Cl⁻]i increased. B: Effect of uncoupler on individual cells bathed in 0 mM Cl^- medium. CCP (50 μM) (B1) and DNP (2 mM) (B2) were still able to increase SPQ fluorescence. However, DNP responses were consistently higher. Repetitive applications of CCP and DNP continued to increase [Cl⁻]i, although with a less marked effect (C).

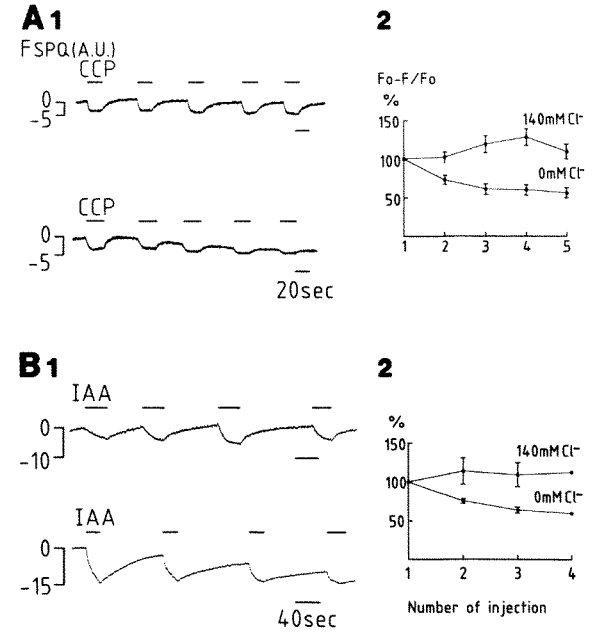

Fig. 3. Comparative effect of multiple applications of metabolic inhibitors on SPQ fluorescence in either 140 mM Cl^- (1) or 0 mM Cl^- EM (2). A: CCP (50 μM). A1: upper trace 140 mM Cl^- EM; lower trace 0 mM Cl^- EM. A2: normalized responses ($Fo-F/Fo$ expressed in %) in both media, where Fo=fluorescence before application and F=fluorescence during application of the metabolic inhibitor in absolute values (n = 17, 17, 14, 10 and 6 for normal EM 13, 13, 13, 11 and 5 for 0 mM Cl^- EM for each application, respectively). B: IAA (10 mM). B1: upper trace = normal EM; lower trace: 0 mM Cl^- EM. B2: normalized responses in both media (see A2) (n = 5 for application 1, 2 and 3 for both media, n = 2 for the 4th application for both media).

the absence of extracellular Cl^- ions, indicated that the increase in [Cl⁻]i could not be of external origin. It follows that, even if present, co-transport systems cannot be responsible for these changes in [Cl⁻]i. Experiments with repetitive application of CCP and IAA for cells bathed in normal or Cl^- free EM seem to indicate that these stores are refilled quickly only in the presence of external chloride. As pointed out in Section 2, the filter effect of CCP and DNP was studied and estimated to be negligible in the case of CCP. In fact Verkman [9] proposed CCP for analyzing Cl movements with Cl^- sensitive probes. As for DNP, the filter effect observed must be taken into account. However, the data obtained with other metabolic inhibitors (antimycin, oligomycin, IAA, see below), which do not interfere with SPQ fluorescence, reinforce those reported with CCP.

Rat lactotroph cells possess Cl^- channels that are also Ca^{2+} dependent. For this reason, two types of experiments were performed to exclude the possibility that these channels participate in the effects observed.

3.3. Effect of metabolic inhibitors on [Ca^{2+}]i

First, we studied a putative interference with changes in intracellular Ca^{2+}, as CCP and DNP have also been reported to release Ca^{2+} from isolated mitochondria [21] and mitochondria from smooth muscle cells [22]. We show here that similar effects can be observed in rat lactotroph cells.

In order to study a putative direct effect of Ca^{2+} on mitochondrial uncoupler induced variations of [Cl⁻]i, we tested the effect of the Ca^{2+} ionophore, ionomycin. The injection of

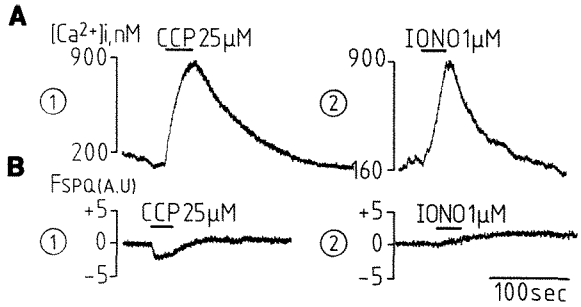

Fig. 4. Effects of CCP (25 μM) and ionomycin (1 μM) on $[Ca^{2+}]i$ as compared to their effect on SPQ fluorescence. CCP and ionomycin were injected in the vicinity of the cell. CCP and ionomycin induced similar effects on $[Ca^{2+}]i$ of individual cells (see Fig. 3A) whereas they had the opposite effect on SPQ fluorescence (see Fig. 3B).

CCP (25μM) raised $[Ca^{2+}]i$ to 923±339 nM ($n=8$) (Fig. 4A1), close to the value obtained with ionomycin $[Ca^{2+}]=680\pm110$ nM ($n=4$) (Fig. 4A2). CCP increased $[Cl^-]i$ ($\Delta Fl_{SPQ}=-1.27\pm0.30$, $n=9$), whereas the increase in SPQ fluorescence ($\Delta Fl_{SPQ}=+1.6\pm0.29$ AU, $n=12$) showed that ionomycin decreased $[Cl^-]i$, probably by activating Ca^{2+} dependent Cl^- channels, thus resulting in a Cl^- efflux. DNP also increased $[Ca^{2+}]i$ to 1020±166 nM ($n=12$; not shown). Thus, CCP and DNP increased $[Ca^{2+}]i$, as did ionomycin. As an increase in $[Ca^{2+}]i$ is associated with a Cl^- efflux, the amount of Cl^- mobilized by these uncouplers may be underestimated.

3.4. *Effect of metabolic inhibitors on membrane conductances*

Second, we studied a putative direct effect of the mitochondrial uncouplers on membrane electrophysiological properties, by combining electrophysiological and microspectrofluorimetric experiments. In current clamp mode, CCP produced a depolarization concomitant with a reduced membrane input resistance. The depolarization induced by CCP was probably due to an increased Cl^- efflux and cannot result from Cl^- influx. As expected, CCP also induced a decrease in SPQ fluorescence, corresponding to an increase of $[Cl^-]i$ (Fig. 5A). Taking into account that the mean resting $[Cl^-]i$ of the lactotrophs recorded was 59.4 mM and that $Fo-F$ (4 mM) corresponded to 55.4 mM (59.4–4 mM) of Cl^-, we estimated the $[Cl^-]i$ increase due to CCP (50 μM) at 15.8±5.8 mM ($n=4$).

This estimation also agreed with the values obtained using radiolabeled ^{36}Cl which gave an intracellular Cl concentration (cytosolic and mitochondrial) of 78 mM.

Taking into account the relative volume of mitochondria, this would imply a mitochondrial chloride concentration of about 70–80 mM. These values are compatible with the osmotic equilibrium of mitochondria. Morphologists report that a hyperosmotic fixation medium is required to protect the mitochondrial ultrastructure [15].

In voltage clamp mode, voltage ramps applied from a holding potential of −80 mV (−80 to +20 mV, 400 ms) showed that CCP induced an inward current for hyperpolarized potentials (Fig. 5B) (−77±9.8 pA; Vh = −80 mV, $n=4$ cells). Its reversal potential was −35±6.7 mV ($n=4$). Activation of the Ca^{2+} dependent Cl^- conductance described in lactotroph cells [6,10] provides a partial explanation for the increase in membrane permeability. However, as shown in the cell illustrated in Fig. 5B, at potentials higher than −40 mV, complex ionic mechanisms were activated (Ca^{2+} current at least). Subtraction of control current from that recorded during exposure to CCP (Fig. 5B, lower trace) showed that the CCP-induced current was not linearly related to changes in membrane potential. The observed current was unlikely to be carried by potassium ions, as the equilibrium potential of the current induced by CCP was not shifted in conditions where potassium ions were removed from the internal medium (Cs gluconate). Conversely, the inward current was inhibited with 9-anthracene carboxylic acid (9-AC), a chloride conductance blocker (not shown). The current developing between −80 mV and −35 mV was thus essentially represented by Cl^- ions (efflux of Cl^- ions). This current may result from the new equilibrium potential for chloride ions (−50 mV for a −15.8 mM mean $[Cl^-]i$ increase under CCP). The shift between −35 and −50 mV may be explained by the participation of cations around −40 mV. In fact Brismar and Collins [23] have reported on the involvement of cations in metabolic

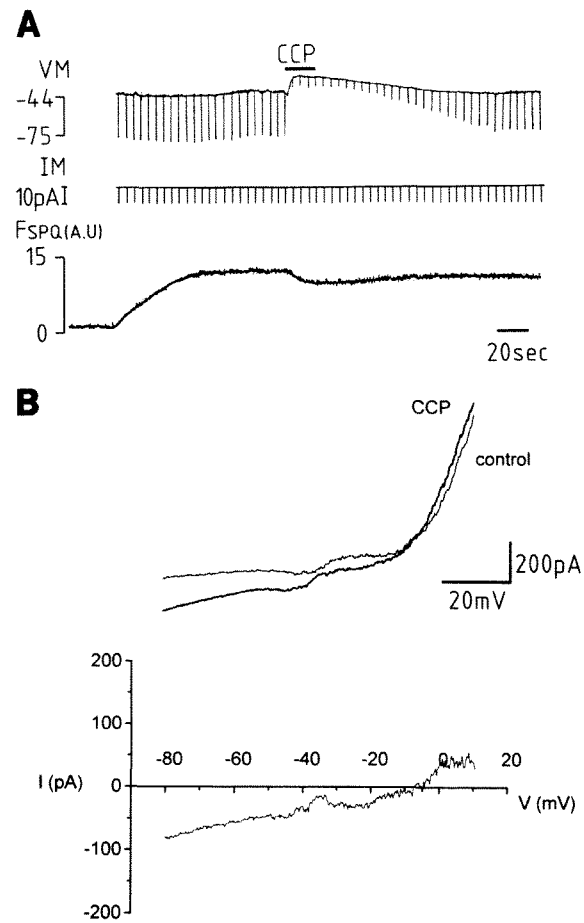

Fig. 5. Combined microspectrofluorimetry and electrophysiology. Cells were bathed in normal external medium. Pipettes were filled with K gluconate containing 0.5 mM SPQ. A: Effect of CCP (50 μM) on membrane electrical properties and SPQ fluorescence in a cell patched with a 4 mM Cl^- patch solution. The patch was ruptured at the arrow. CCP induced a depolarization associatd with a decrease in membrane resistance (upper trace). SPQ fluorescence also decreased (lower trace). B: In another series of experiments, in response to a voltage ramp (−80 to +20 mV for 400 msc) an inward current was activated (upper trace). It reversed at around −40 mV and was associated with the $[Cl^-]i$ increase induced by CCP. Net current is plotted in the lower trace.

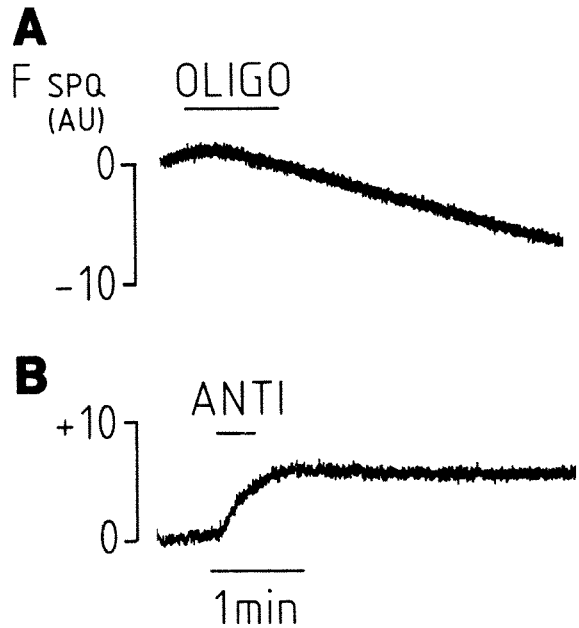

Fig. 6. Effect of metabolic inhibitors on individual cells. Application of oligomycin and antimycin gave opposite results on SPQ fluorescence.

inhibitor induced depolarizations. It thus follows that the $[Cl^-]i$ increase observed under exposure to CCP resulted from Cl^- mobilization associated with a Cl^- efflux through Cl^- channels. As our aim was to exclude a putative entry of Cl^- ions during uncoupler applications, we did not investigate the nature of this type of membrane effect more thoroughly. The mean $[Cl^-]i$ increase (15.8 ± 5.8 mM) included release from mitochondria and Cl^- efflux, probably through Ca^{2+} activated chloride channels. DNP and IAA, which induced $[Cl^-]i$ increases of 37.5 ± 7.6 mM ($n = 5$) and 20.0 ± 6.5 mM ($n = 9$), respectively, did not greatly affect membrane conductance (not shown).

3.5. Mechanisms involved

Additional experiments were performed to clarify the metabolic events involved in the release of chloride ions by mitochondria. Oligomycin (60 μM) (Fig. 6A), an ATP synthase activity inhibitor, only induced a slight, slow decrease, which was difficult to quantify. The slow decay of SPQ fluorescence in the presence of oligomycin may be explained by its low diffusion rate across the cell membrane. Conversely, antimycin (100 μM), a respiratory chain inhibitor [21], applied (Fig. 6B) to cells bathed in 0 mM Cl^- EM, led to a decrease in $[Cl^-]i$ in all cells tested (($\Delta Fl = +6.9 \pm 1.07$ AU, $n = 5$). The rapid decrease in $[Cl^-]i$ in the presence of antimycin suggests that a mitochondrial Cl^- flux activated in 0 mM external chloride was blocked, as already proposed by Kinally et al. [24] and Campo et al. [25]. When we tried to measure intramitochondrial Cl^- stores, after cell disruption with radiolabeled ^{36}Cl, we found Cl^- in that organelle only when antimycin was added to the medium (not shown). The fact that antimycin has been described as a potent inhibitor of anion inner mitochondrial membrane channel activity is in agreement with this putative mechanism.

The mitochondrial function depends on proton electrochemical gradient ($\Delta\mu H^+$) for ATP production, and $\Delta\mu H^+$ is essentially due to mitochondrial membrane potential [26]. In addition, mitochondria possess a high capacity to concentrate ions such as Ca^{2+} [21,27] and chloride, as demonstrated here. These data suggest that mitochondrial Cl^- transport and oxidative phosphorylation are closely related. The rapid kinetics of $[Cl^-]i$ increase following uncoupler action may be explained by this interaction.

In this work, we compared the effects of different drugs, acting on mitochondrial oxidative phosphorylation, on $[Cl^-]i$ changes. The uncouplers antimycin and oligomycin drastically decreased mitochondrial ATP production. However, the inhibition mechanisms are different: uncouplers dissipate the $\Delta\mu H^+$ and increase respiratory rate; antimycin stops the electron transfer through the respiratory chain and decreases $\Delta\mu H^+$, while oligomycin slows down respiratory rate but considerably increases $\Delta\mu H^+$ [28]. The fact that uncouplers and oligomycin have similar effects on $[Cl^-]i$ changes and that antimycin does the opposite indicates that $\Delta\mu H^+$ size is not directly involved in Cl^- movement through mitochondrial membranes.

In the metabolic pathway, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase are close to equilibrium, the cytosolic ATP/ADP and $NADH/NAD^+$ ratio changes are strictly related, i.e. increase in $NADH/NAD^+$ decreases ATP/ADP. As the level of the $NADH/NAD^+$ ratio depends on the coenzyme reoxidation rate by the respiratory chain, it must be higher in the presence of antimycin than in the presence of either uncoupler or oligomycin. The response obtained with IAA supports the hypothesis of the involvement of the metabolic pathway in Cl^- homeostasis.

Chloride permeability across liver [29], heart [30], and brown adipose tissue [31] mitochondria has been reported and a potential dependent anionic channel has been described in the internal wall of mouse mitochondria [20], allowing Cl^- fluxes. Together with the report from de Weille and Lazdunski [19] on the ATP-dependent K^+ channels in an insulinoma cell line, these data support the proposal that chloride release from mitochondria is intimately related to ATP levels.

A Cl^- channel regulated by cell energy level may be present in mitochondria. These results give a new insight into the role of Cl^- ions in lactotroph cell energy processes.

In conclusion, the effects reported here of inhibitors of oxidative phosphorylation and/or of the glycolytic pathway suggest strongly that cell metabolism, including mitochondrial functions, modulate $[Cl^-]i$. Interaction of this type has already been proposed for cell calcium homeostasis [21]. Finally, these results highlight the need for more investigation into the energy status of these cells, in order to enhance our understanding of these mechanisms.

It has been suggested that variations in $[Cl^-]i$ lead to altered cell functions [5,19,20]. The physiological relevance of the high $[Cl^-]i$ found in normal rat lactotrophs is emphasized by the observation of increased exocytosis in the presence of high intracellular Cl^- in rat melanotrophs [5] and lactotrophs [32]. Thus the involvement of mitochondria in $[Cl^-]i$ regulation is certainly an important process which will need further study.

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