J. Membrane Biol. 126, 39–49 (1992)

The Journal of
Membrane Biology

Calcium-Activated Chloride Conductance of Lactotrophs: Comparison of Activation in Normal and Tumoral Cells during Thyrotropin-Releasing-Hormone Stimulation

P. Sartor, L. Dufy-Barbe, P. Vacher, and B. Dufy
Laboratoire de Neurophysiologie, URA CNRS 1200, Université de Bordeaux II, 33076 Bordeaux Cedex, France

Summary. We studied a chloride (Cl^-) conductance activated by calcium (Ca^{2+}) in normal rat lactotrophs and compared its activation during TRH stimulation in normal rat lactrotrophs and in GH3 tumoral lactosomatotrophs cells, using the whole-cell configuration of the patch-clamp technique.

The Cl^- specificity of the conductance was assessed by manipulation of internal and external Cl^- concentrations. The reversal potentials were in agreement with those predicted by the Nernst equation. Ca^{2+} ionophore A23187 and membrane depolarizations activated the Cl^- conductance. However, a feedback effect of Cl^- gradient modifications on Ca^{2+} movements was also observed in normal lactotrophs.

In the latter, TRH (100 nM) mobilization of intracellular Ca^{2+} activated this Cl^- conductance together with the potassium (K^+) conductance when both ions were present in the intracellular medium (IM) or alone when K^+ was absent. Chloride conductance was not activated in the GH3 cells, where mobilization of intracellular Ca^{2+} by TRH (100 nM) activated only Ca^{2+}-dependent K^+ conductance. It seems likely that the activation of Cl^- conductance in these two different cell types involves different mechanisms.

Key Words rat lactotrophs · calcium-activated chloride conductance · TRH stimulation

Introduction

The stimulus-secretion coupling process has been extensively studied in the GH3 pituitary tumoral cell line which secretes prolactin and growth hormone (for review *see* ref. [29]). In these cells, stimulation of release by thyrotropin-releasing hormone (TRH) is a complex process which can be summarized as follows: in an early phase, TRH stimulation leads to a transient rise in $[Ca^{2+}]_i$ as shown by population [33] or single cell microfluorimetric analysis [37]. Electrophysiological recordings have consistently shown that TRH induces a transient hyperpolarization [28]. The use of combined electrophysiological and microfluorimetric techniques in individual cells has established that the transient hyperpolarization is due to the activation of a Ca^{2+}-dependent K^+ current associated with the initial rise in $[Ca^{2+}]_i$ [23, 24, 27].

In normal rat lactotrophs few authors have reported on stimulus coupling mechanisms [11, 12, 31]. Recently we presented data showing that, in primary cultures of normal rat lactotrophs, the electrophysiological response to TRH was not identical to that observed in GH3 cells [31]. The main difference concerned the activation, in normal lactotrophs, of a chloride conductance which interfered with the well-known Ca^{2+}-activated K^+ conductance during the hyperpolarizing phase of the TRH response. The reason for this difference between the tumoral GH3 and normal rat lactotrophs is unclear since chloride conductance activated by Ca^{2+} ions has also been reported in GH3 cells [30].

In these experiments, we studied the chloride conductance of normal rat lactotrophs and show that this conductance can be activated under experimental conditions similar to those reported by Rogawski et al. [30] for GH3 cells. We compare the response to TRH of tumoral and normal lactotrophs and show that TRH can induce activation of a chloride conductance in normal lactotrophs but not in tumoral cells. The biological factors responsible for this key difference could be of importance in understanding the endocrine secretion process. The mechanisms underlying this discrepancy (and its relation to tumoricity) remain to be explored.

Materials and Methods

We found no difference in the involvement of chloride channels in TRH response between pituitary lactotrophs derived from dioestrous or lactating rats [31]. Therefore, we used either lactating or cycling female rats and prepared the primary cultures as reported earlier [31]. Briefly: anterior pituitaries were minced after removal and incubated with collagenase (0.2%, in Ca^{2+}- and

Mg^{2+}-free Hanks' balanced salt solution (HBSS) containing 0.3% of BSA) for 90 min at 36.5°C; isolated cells were then plated in petri dishes (35 mm) and cultured in Ham's F10 or DMEM-F12 complemented with 10% fetal calf serum (FCS) for at least three days before recording began.

GH3 cells, originally provided by Dr. A. Tixier-Vidal and D. Gourdji, College de France, Paris, France, were cultured in Ham's F10 or DMEM-F12 medium containing 12.5% of horse serum (HS) and 2.5% FCS.

Whole-cell recordings (WCR), following the patch-clamp technique reported by Hamill et al. [7], were performed at room temperature (23 ± 2°C) using a DAGAN 8800 amplifier. More technical details were given in a previous article [31]. For microfluorimetric measurements, cells were grown on 30-mm glass coverslides.

Recording media were prepared as follows:

(i) The external bathing solution (EM) was formulated with three different concentrations of Cl^- ions (150, 75 and 30 mM); EM was basically constituted with Ham's F10 supplemented to 10 mM for Ca^{2+}, 2 mM for $MgCl_2$, with 5% FCS, buffered with 10 mM of HEPES. This medium contained 150 mM Cl^-. The 30 mM Cl^- medium was obtained by substituting Na gluconate for NaCl. Ninety mM Cl^- was achieved by mixing 150 mM Cl^- medium with medium where NaCl was substituted by Na gluconate. Osmolarity and pH were adjusted to 300–310 mOsmol/liter and 7.3, respectively.

(ii) The internal recording solution used for filling the patch pipette (IM) was also formulated with three different concentrations of Cl^- ions (4, 30 and 150 mM). Internal media (IM) consisted basically of potassium gluconate (Kgluc 140 mM, or Kgluc 114 + KCl 26 mM), potassium chloride (KCl, 150 mM) or cesium chloride (CsCl, 150 mM) complemented to 2 mM for $MgCl_2$, 1.1 mM for ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), 5 mM for HEPES, 0.1 mM for leupeptine [1]. Osmolarity and pH were adjusted to 300 ± 5 mOsmol/liter and 7.3, respectively.

Stock solution of the calcium ionophore A23187 (10 mM in DMSO) was diluted in the EM at a final concentration of 10 μM. 4-Acetamido-4′-isothiocyanatostilbene-2-2′-disulfonic acid (SITS), a blocker of Cl^- channels, was also diluted in EM at a concentration of 2 mM.

Microspectrofluorimetric measurements of free internal Ca^{2+} was performed using the Ca^{2+}-sensitive probe Indo-1 as described by Mollard et al. [22]. Glass coverslides bearing cells were treated on hollowed petri dishes and bathed in 10 mM Ca^{2+} EM. TRH (100 nM) was applied with the same ejection procedure as for the electrophysiological recordings. Fluorescence was analyzed using a Nikon diaphot inverted microscope fitted with epifluorescence (×100 oil immersion fluorescence objective numerical aperture 1.3). The emitted fluorescence signal of isolated cells was passed through a pinhole diaphragm slightly larger than the selected cell and analyzed by two Nikon photometers (F405 and F480). $[Ca^{2+}]_i$ was calculated following the method reported by Mollard et al. [22]. Coupling of electrophysiological and microspectrofluorimetric techniques were also performed using the method reported by Schlegel et al. [32], adapted by Mollard et al. [22].

Immunocytochemical (ICC) labeling was performed according to the peroxydase anti-peroxydase (PAP) procedure described earlier [34]. Anti-rat PRL S9 was provided by the National Hormone and Pituitary Program (NIDDK). When post-recording ICC labeling was not possible, especially when using glass coverslides, we estimated the percentage of lactotrophs analyzed and found that 90% of cells chosen were TRH responsives (90 cells out of 99). As reported earlier, a bias exists to overcome the heterogeneity of pituitary cell populations in culture. Morphological and spatial arrangement criteria were used to select lactotrophs or lactosomatotrophs.

Results

Ca^{2+} Activates a Chloride Conductance in Normal Rat Lactotrophs

In this series of experiments, the contribution of K^+ ions to membrane macroscopic currents was eliminated by loading the cell, through the patch pipette, with an ion impermeant to K^+ channels. We used cesium (Cs^+). The demonstration of a Ca^{2+}-dependent chloride conductance in normal lactotrophs was achieved by ejecting the calcium ionophore A23187 onto lactotrophs recorded with CsCl (150 mM) filled pipettes (Fig. 1). In the voltage-clamp configuration, the cell being clamped at −40 mV, application of A23187 (10 μM) was followed within 5 sec by the development of an inwardly directed current associated with an increase in membrane conductance (Fig. 1*A*). Conductance changes were measured by stepping the voltage to −10 mV for 0.6 sec every 5 sec (upward-directed bars, Fig. 1*A*). The reversal potential (E_{rev}) of the ion carrying the induced current was calculated following the equation $E_{rev} = V_h - \Delta I/\Delta g$, where V_h is the holding potential, ΔI is the variation of the intensity of the current and Δg the variation of the conductance during the step. Using this method, E_{rev} was +3 ± 0.05 mV, which is in close agreement with the Nernst prediction for Cl^- ions. Under these experimental conditions (both extracellular and intracellular concentrations of Cl^- ions being 150 mM), the equilibrium potential for Cl^- ions is 0 mV. When the voltage was clamped at +30 mV, the A23187-induced current turned outward, as expected for a chloride current entering the cell (Fig. 1*B*). E_{rev} was estimated at −2 ± 3 mV, which is again in agreement with the Nernst theoretical E_{rev} for Cl^- ions in these conditions. The plot of the current as a function of V_h or the I/V chord relationship following Owen, Segal and Barker [27] (Fig. 1*C* and *D*) gave an approximate value between −10 and 0 mV. The shift towards more negative values than expected (Fig. 1*C*) may result from Ca^{2+} current activation near 0 mV.

To show that in fact a Cl^- conductance was activated in response to A23187, the external chloride concentration was reduced to 30 mM, whereas the internal concentration of Cl^- was maintained at 150 mM Cl^-. As shown in Fig. 2*A*, with the cell

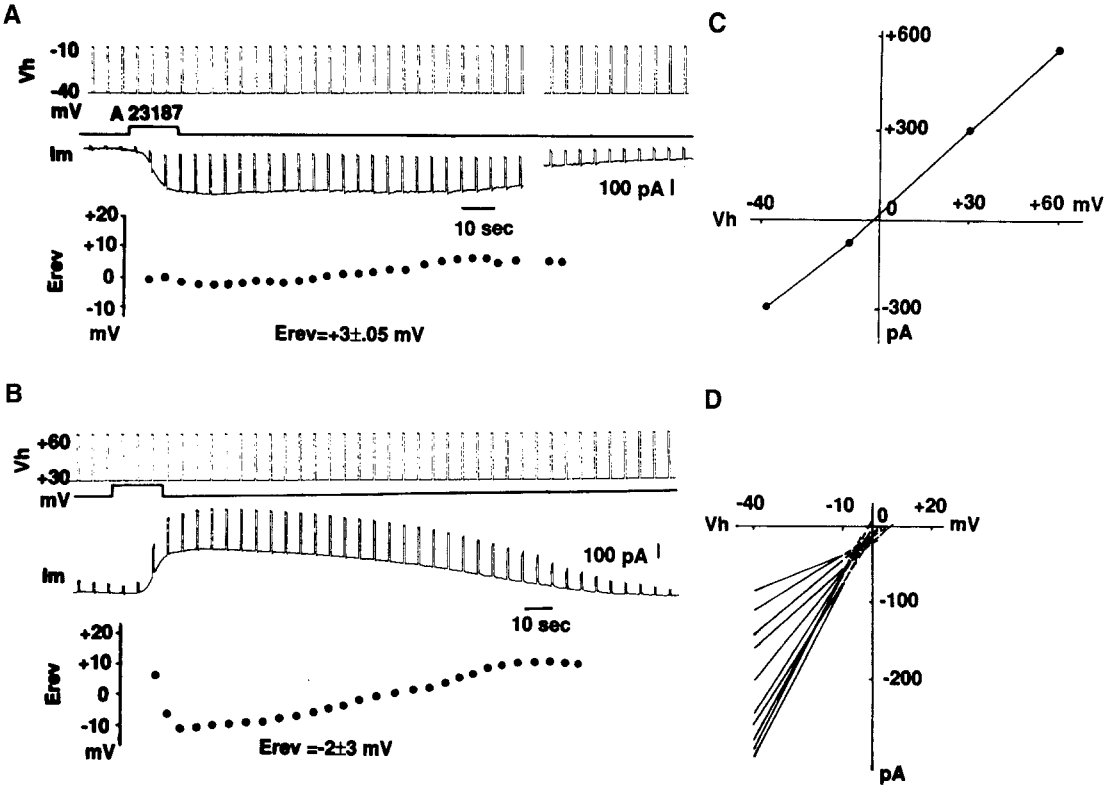

Fig. 1. Effects of the Ca^{2+} ionophore A23187 on the chloride current in normal lactotrophs. (IM = 150 mM CsCl; EM contains 150 mM Cl^-; voltage-clamp analysis.) (*A*) Holding potential (V_h) was maintained at −40 mV. Depolarizing steps, 30 mV in amplitude (upper trace), were applied to the recorded cell. A23187 gave rise to an inwardly directed (Cl^- outward) current (downward deflection of the current on the middle trace), with great increase of conductance (upward bars during the steps). Reversal potential (E_{rev}) for this cell was estimated at +3 ± 0.05 mV (lower part of the graph). Mean E_{rev} for the cells tested was estimated at +0.7 ± 2.1 mV (n = 5 cells). (*B*) When V_h was maintained at +30 mV, A23187 induced an outward current (Cl^- inward). A similar increase in conductance was observed (upper and middle traces). In this case also, the calculated E_{rev} (lower part) estimated at −2 ± 3 mV, was close to 0 mM which is the theoretical equilibrium potential for Cl^- ions, according to the Nernst equation. (*C*) and (*D*) The plot of the currents as a function of V_h (*C*) or *I*/*V* chord relationship (*D*) gave graphic estimations of E_{rev} close to zero in both cases.

clamped at V_h = −40 mV, the Ca^{2+} ionophore induced an inward current and E_{rev} was estimated at +24.9 ± 1.5 mV. The *I*/*V* chord graphic representation (Fig. 2*B*) confirmed this value (~+20 mV).

In order to further substantiate the Ca^{2+}-dependence of the Cl^- conductance, tail currents were studied using long depolarizing steps (from 0.5 sec to more than 5 sec), with a holding potential stepping from −70 to 0 mV.

We first verified that results similar to those reported by Rogawski et al. [30] could be obtained with the GH3/B6 cells used in our laboratory. Ca^{2+}-dependent Cl tail currents were consistently obtained with CsCl or N-methylglucamine Cl^- (NMG-Cl^-) formulated internal medium (*not shown*).

The ionic nature of the tail current in normal lactotrophs was confirmed. Tail current was blocked when the solution in the pipette patch contained Kgluc 140 mM instead of CsCl. TEA (10 or 20 mM) and TTX (5 or 20 μM) added to the bathing medium had no effect, indicating that the only ion which contributes to the tail current was Cl^-, since the tail of Ca^{2+} current deactivates within 10 msec [20], whereas tail deactivation in the present experiments lasted several seconds. Combined electrophysiological and fluorimetric techniques clearly showed that the duration of the depolarizing step was directly correlated to cytosolic Ca^{2+} and Cl^- tail amplitude (Fig. 3*A*). Chloride tail was already activated by a 0.5-sec depolarizing step (Fig. 3*B*) and reached its maximal value for a 4- to 5-sec step. Lowering the external Ca^{2+} (2 mM instead of 10 mM) (Fig. 3*B*), or buffering the internal calcium by high concentration of EGTA (10 mM instead of 1.1) in the patch pipette solution strongly decreased the depolarization-induced tail current (*not shown*).

The potential dependence of the Cl^- tail were studied in cells, clamped at −70 mV, on which depo-

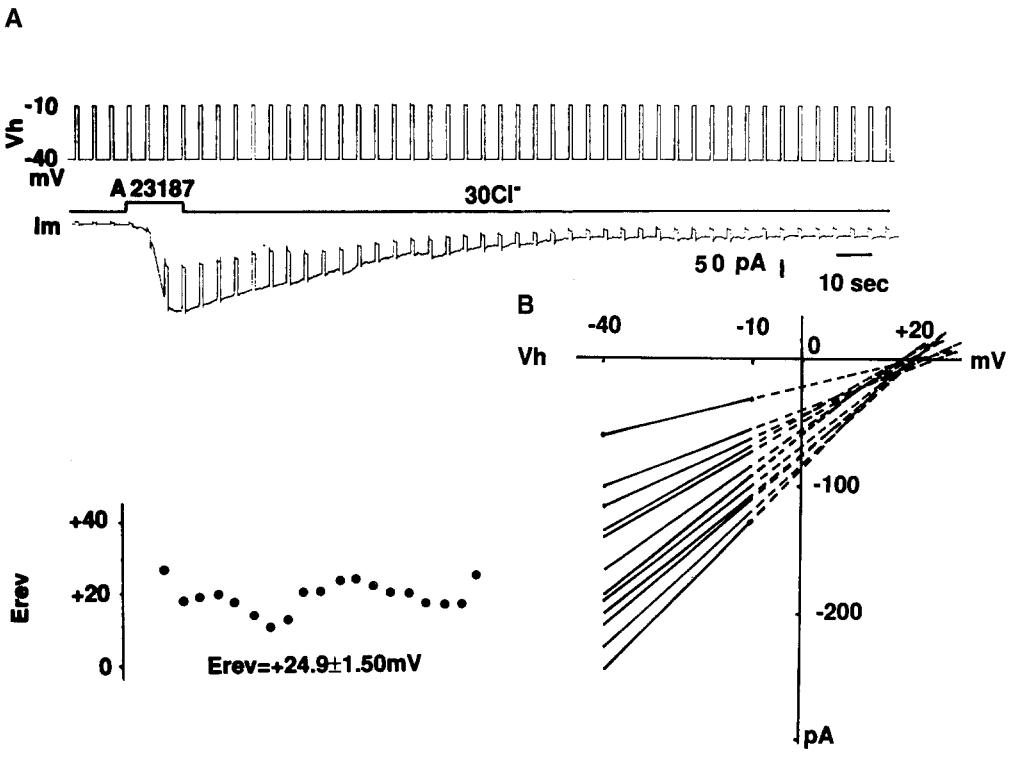

Fig. 2. Effect of external chloride concentration modification (30 mM Cl^- instead of 150 in the EM) on A23187 response. (IM = 150 mM CsCl; EM contains 150 mM Cl^-; voltage-clamp analysis.) (*A*) EM containing 30 mM Cl^- was ejected during and after A23187 application onto a cell recorded at V_h of −40 mV, inducing an inward current (middle trace). The increased conductance elicited by 30-mV depolarizing steps was less than in Fig. 1*A*. E_{rev} in this case was estimated to +24.9 ± 1.5 mV (lower part). Mean E_{rev} for all the cells tested was estimated at +17.8 ± 5.1 mV (n = 5 cells). (*B*) *I/V* chord relationship gave approximately the same value for E_{rev} (~ +20 mV).

larizing steps of increasing amplitude were applied (Fig. 4). The *I/V* relationship between Ca^{2+} currents and Cl^- tail amplitude are represented in Fig. 4*A*. Maximal amplitude of the Cl^- tail amplitude are represented in Fig. 4*A*. Maximal amplitude of the Cl^- tail peaked at 0 mV and closely followed the *I/V* plot of the slow inactivating Ca^{2+} current (L).

The presence of an important leak current (70 pA when the cell was depolarized to 0 mV) and the combination of calcium inward current and Cl^- inwardly or outwardly directed currents during voltage steps to 0 mV (Fig. 4*B*), make accurate evaluation of Ca^{2+} current amplitudes difficult. However, it seems likely that the potential dependence of the Cl^- tail reflects the potential dependence of the L-type calcium current.

Manipulations of Extracellular Cl^- Concentration Affect Membrane Ion Conductances

Manipulation of the Cl^- gradient affect membrane ion conductances and resting properties. Modification of the external Cl^- concentration (150 mM Cl^- inside and 90 mM Cl^- outside) led to a decrease of the Cl^- tail instead of an increase as expected from Nernst. Indeed reducing the external chloride concentration from 140 to 90 mM decreased the Ca^{2+} inward current induced during the depolarizing step, thus lowering the Ca^{2+}-dependent Cl^- tail (Fig. 5 where the peak Ca^{2+} currents were plotted and Fig. 6*A,b* as compared to *A,a* and *A,c*). This effect is independent of Ca^{2+} current rundown occurring in the whole-cell configuration and clearly demonstrates that manipulation of external Cl^- concentration has a direct effect on Ca^{2+} current in these experimental conditions. A similar Cl^- dependence of the depolarization-induced Ca^{2+} influx was observed in GH3 cells (*data not shown*). In order to show the Cl^- dependence of the tail current, we used the Cl^- channel blocker SITS (2 mM in the EM) and obtained a 66% decrease in the Cl^- tail (five cells). On occasions the decrease was also partly due to a direct effect on the depolarization-induced Ca^{2+} current. However when SITS was ejected at the end of the depolarization step, a specific effect on the Cl^- tail current (Fig. 6*B,a B,b*) was shown.

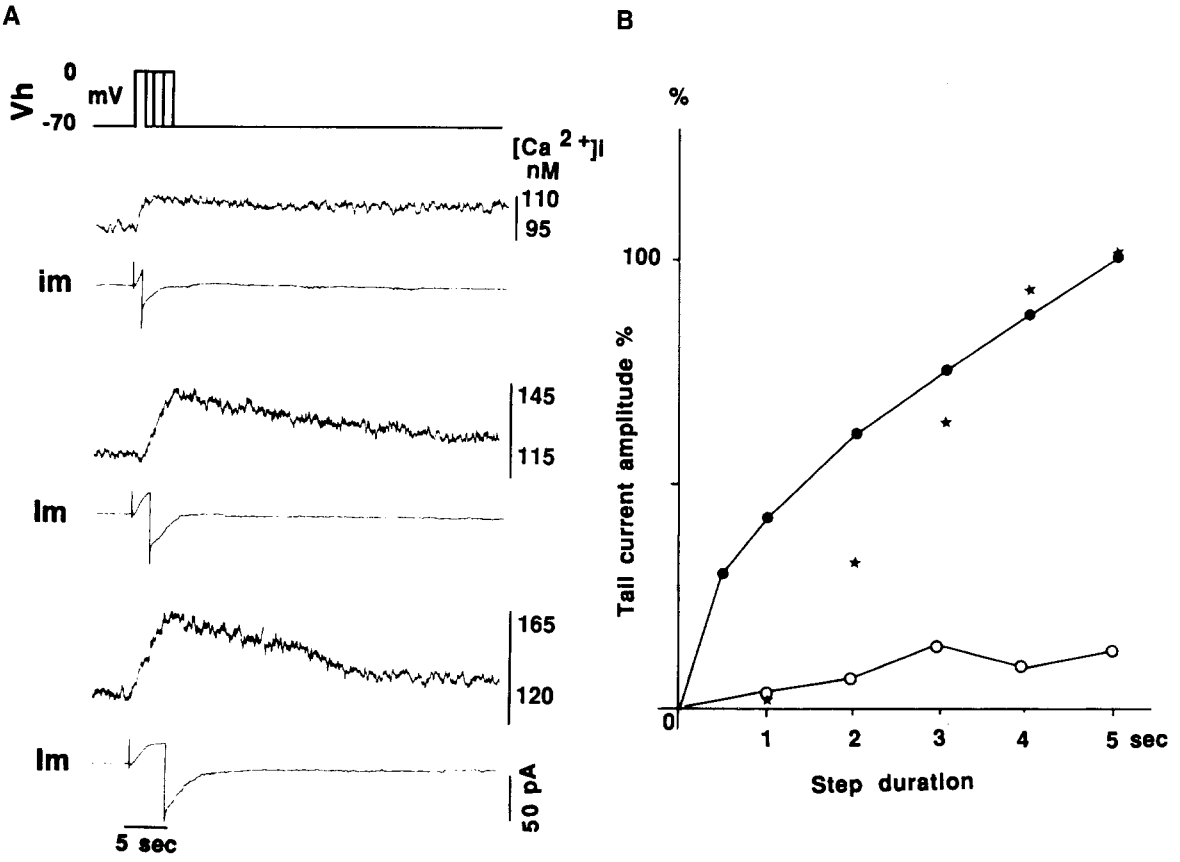

Fig. 3. Ca^{2+} dependence of chloride tail current in normal lactotrophs. (IM = 150 mM CsCl; EM contains 150 mM NaCl, 10 to 20 mM TEA.) It was shown that 5 or 20 μM TTX had no effect on the tail current. (*A*) Combined electrophysiology and microspectrofluorimetry. Cells loaded with Indo-1 were patched, and simultaneous electrophysiological and fluorimetric data were recorded in voltage-clamp conditions (V_h = −70 mV). Depolarizing steps (+70 mV) were applied to the cell for increasing periods (1 to 5 sec). The amplitude of $[Ca^{2+}]_i$ and tail current increases were dependent on the step duration. Ca^{2+} rise was strictly correlated with the step length. (*B*) The graph of the tail current amplitude increase as a function of the depolarizing step duration. The increase is calculated as a percentage of the maximal increase (100%) attained after 5 sec (closed symbols, n = 4 cells). Lowering external Ca^{2+} (2 mM instead of 10 mM) led to a drastic decrease (open symbols). The leak current has been integrated in the calculation. Soon after the beginning of cell depolarization (0.5 sec), tail current is 29.5% of the maximal amplitude, and after 1 sec 42%. In GH3 cells Rogawski et al. [30] reported less than 5% increase after 1 sec. For comparison, the stars represent tail activation obtained by Rogawski in GH3 cells. When K gluconate replaced CsCl, Cl^- tail current did not occur (*not shown*). When 10 mM of EGTA were used instead of 1.1 mM, thus buffering the internal calcium, no tail current occurred within 5 sec (*not shown*).

To demonstrate further that manipulations of the Cl^- gradient affect membrane resting properties, we studied the response of lactotrophs to the ejection of low Cl^- external medium (90 mM Cl^-) in current-clamp recordings. Figure 7 shows that decreasing the external Cl^- concentration from 150 to 90 mM hyperpolarized a cell recorded at the resting potential. Under present recording conditions (Kgluc in the recording pipette), this response reversed around 0 mV, a potential which neither corresponds to K^+ (~ −80 mV) nor to Cl^- reversal potential (~ −90 mV). Concomittantly with the membrane potential change, the membrane resistance also increased, especially when the cell was depolarized (−18 mV), suggesting that complex ion movements are elicited, including K^+ outward movements and the possible blockade of a Cl^- conductance functioning in basal conditions at this membrane potential. The activation of action potentials during the induced hyperpolarization was often observed even when the membrane potential was around −60 mV. The same results were obtained with GH3 cells (Fig. 7*B*).

It is obvious that manipulation of the external Cl^- concentration affects membrane ion traffic and cell calcium homeostasis. Further experiments to explore this question are clearly necessary.

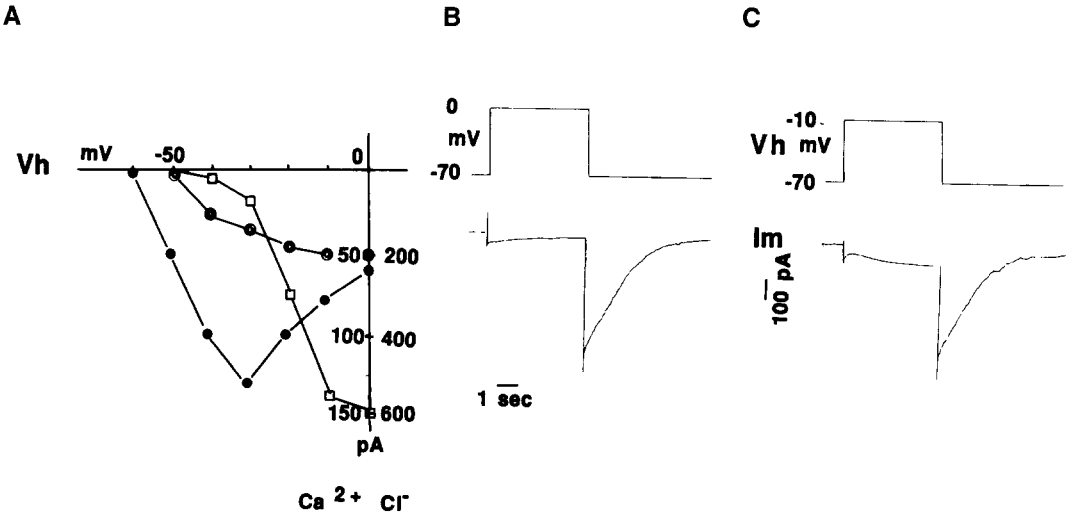

Fig. 4. I/V relationships for calcium currents and chloride tail. (IM = 150 mM CsCl; EM contains 150 mM NaCl, 10 mM TEA.) (*A*) *I/V* curves of Ca^{2+} currents and Cl^- tail ($V_h = -70$ mV). Depolarizing steps (5-sec, 10-mV increments) were applied to a cell maintained at −70 mV up to 0 mV. Leak current was evaluated for each step by applying hyperpolarization of the same voltage. Peak value of the inward current (filled circles) was then calculated as the sum of the peak current and of the leak current. An approximation of slow inactivating Ca^{2+} current (L current, open circles) was made by plotting the amplitude 200 msec after the beginning of the step. Peak amplitudes of the tail (open squares) were reported. Increased amplitude of the tail seems to be correlated with L current curve. This curve is representative of responses obtained in five cells. (*B* and *C*) Interaction between calcium and chloride tail currents. (*B*) Effect of depolarizing steps bringing the cell around the reversal potential of Cl^- (from −70 to 0 mV). The Ca^{2+} current is not affected by the activation of the Cl^- tail after the first 0.5 sec. (*C*) When the depolarizing step brings the cell to −10 mV, the activation of the Cl^- current give an inwardly directed deflexion of the Ca^{2+} current, starting 0.5 sec after the beginning of the depolarizing step. These results illustrate the difficulty of separating Ca^{2+} and Cl^- currents when using long-lasting depolarizing steps.

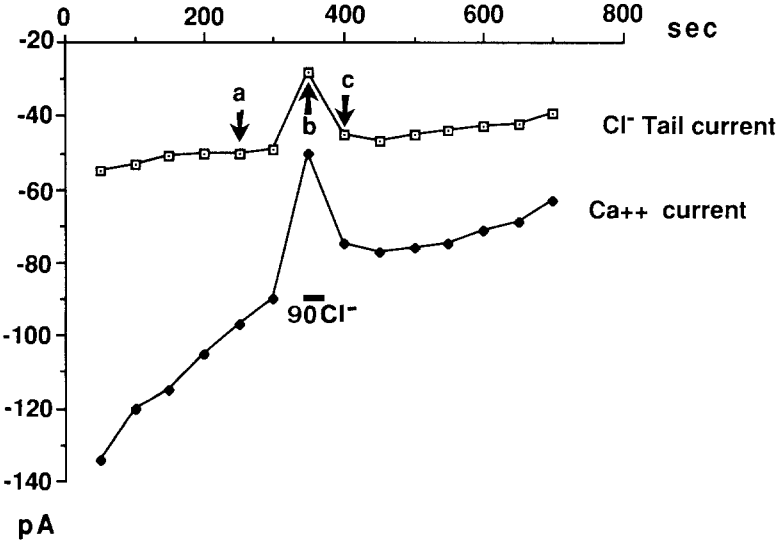

Fig. 5. Effect of Cl^- gradient modification on Ca^{2+} and Ca^{2+}-dependent Cl^- current rundown. (IM = 150 mM CsCl; EM contains 150 mM Cl^- + 10 mM TEA; voltage-clamp analysis; Vh = −70 mV; 3-sec depolarizing steps (+70 mV) every 50 sec.) Peak amplitude of Ca^{2+} current and Cl^- tail were plotted as a function of time. Ca^{2+} rundown (filled squares) was greater than tail Cl^- current rundown (open squares). A 20-sec ejection of EM containing only 90 mM Cl^- (60 mM NaCl were replaced by 60 mM Na gluconate) induced a rapid decrease in both Ca^{2+} and Cl^- tail currents. The points a, b and *c*, which are represented in Fig. 6*A*,*a*,*b* and *c*, illustrate the current amplitude variations before, during and after ejection. The leak current (~20 pA) has been taken into account in calculation of the amplitudes.

Comparison of the Activation of Cl^- Channels by TRH in GH3 Cells and Normal Lactotrophs

We subsequently studied the effect of TRH on normal rat lactotrophs. To show that the fast transient response of normal lactotrophs to TRH involved a chloride conductance, manipulation of internal concentration of Cl^- ions were performed.

Figure 8*A* summarizes data concerning the effect of changes in the intracellular Cl^- concentration on TRH responses in normal lactotrophs. External Cl^- concentration at 150 mM and intracellular Cl^- concentration at 4 mM produced the classical TRH-

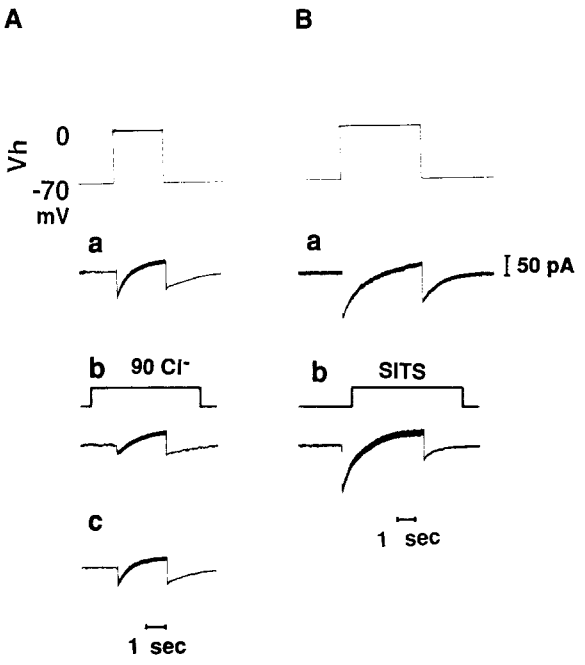

Fig. 6. Calcium and chloride current interactions. (IM = 150 mM CsCl; EM contains 150 mM Cl^- and 10 mM TEA; voltage-clamp analysis; $V_h = -70$ mV.) (*A*) Modification of Cl^- gradient had an effect on the calcium current induced during depolarizing steps. Decreasing external Cl^- from 150 mM (*a*) to 90 mM (*b*) induced a decrease of the Ca^{2+} inward current. The expected increase of the Cl^- tail current did not occur following gradient modification. Recovery (*c*) showed that Cl^- tail varied in parallel with Ca^{2+} current amplitude. (*B*) The chloride blocker 4 SITS : 2 mM blocked the Cl^- tail current without any effect on the Ca^{2+} inward current, provided it was ejected after the beginning of the depolarizing step (Fig. 6*B,a* compared to *B,b*).

induced hyperpolarization resulting under voltage-clamp recording conditions from the activation of an outward current (Fig. 8*A,1*). However the application of hyperpolarizing steps from -40 to -70 mV suggested the probable involvement of an inward current during the TRH response (downward deflection of the conductance bars, Fig. 8*A,1*). When intracellular Cl^- concentration was raised to 30 mM, the current response was clearly inwardly directed at both voltage levels (-40 and -70 mV) (Fig. 8*A,2*). Finally, when the recording pipettes were filled with CsCl (150 mM Cl^-, 0 mM K^+), the amplitude of the inwardly directed current was greatly increased (Fig. 8*A,3*).

Comparisons of the TRH responses in GH3 cells under the same Cl^- gradients are given in Fig. 8*B*. In GH3 cells recorded at -40 mV (voltage-clamp conditions) with Kgluc (Cl^- 150 mM outside, 4 mM inside), TRH responses were characterized by the rapid rise of an outward current known to be carried by the outflow of K^+ ions. Hyperpolarizing voltage jumps ($V_h = -70$ mV) did not reveal any inward current (Fig. 8*B,1*), as indicated by the baseline of the conductance bars. The reversal potential of the TRH-induced outward current was near -70 mV, i.e., close to the K^+ equilibrium potential. Addition of 26 mM Cl^- to the intracellular medium (Fig. 8*B,2*) did not fundamentally change the response to TRH (*cf.* Fig. 8*A,2* for normal lactotrophs). Thus, in GH3 cells, the transient response mainly involves a K^+ conductance change. Moreover, when GH3 cells were recorded with pipettes filled with CsCl (Cl^- 150 mM inside, 150 mM outside), the TRH transient response no longer occurred. It should be noted that K^+ channels are not permeable to Cs ions. In 19 cells recorded with either CsCl (150 mM) or K-gluconate (140 mM) filled pipettes, those recorded with CsCl were unresponsive (10/10) (Fig. 8*B,3*), whereas those recorded with Kgluc exhibited the classical outward current associated with an increase in membrane conductance (8/9).

Microspectrofluorimetric measurements of $[Ca^{2+}]_i$ variations were performed using the calcium-sensitive probe Indo-1. When TRH was applied to pituitary cells in primary culture, most of the cells (36/42) exhibited a rapid increase in $[Ca^{2+}]_i$. Mean level of $[Ca^{2+}]_i$ reached during the peak (0.700 ± 0.120 μM) (mean $\pm$ SEM, $n = 36$) was significantly lower than that obtained for GH3 cells (2.512 ± 0.416 μM) ($n = 39$). However dispersion of TRH-induced peak $[Ca^{2+}]_i$ in normal lactotrophs was wide (0.191 to 2.907 μM), indicating that micromolar concentrations can also be reached in this cell type.

Discussion

Calcium-activated chloride currents have been found in several cell types including neurones [21, 25], lizard retinal cells [18], lacrimal gland cells [3–5], pituitary intermediate lobe cells [35, 36], corticotrophs (AtT-20 cells) [8, 15], the tumoral pituitary cell line GH3 which are lactosomatotroph cells and bovine lactotrophs [10].

In these experiments, we studied chloride membrane conductance of normal rat lactotrophs and show its Ca^{2+} dependence. These data confirm and extend to normal lactotrophs the description of a calcium-dependent chloride conductance already reported in the GH3 tumoral cell line by Rogawski et al. [30]. Experiments with the Ca^{2+} ionophore A23187, high intracellular EGTA concentration, modulation of $[Ca^{2+}]_i$ by depolarizing step duration and manipulation of external calcium demonstrate that in rat normal lactotrophs, as in GH3 cells, chloride ion movements are calcium dependent. In GH3 cells, longer depolarizing steps are needed to acti-

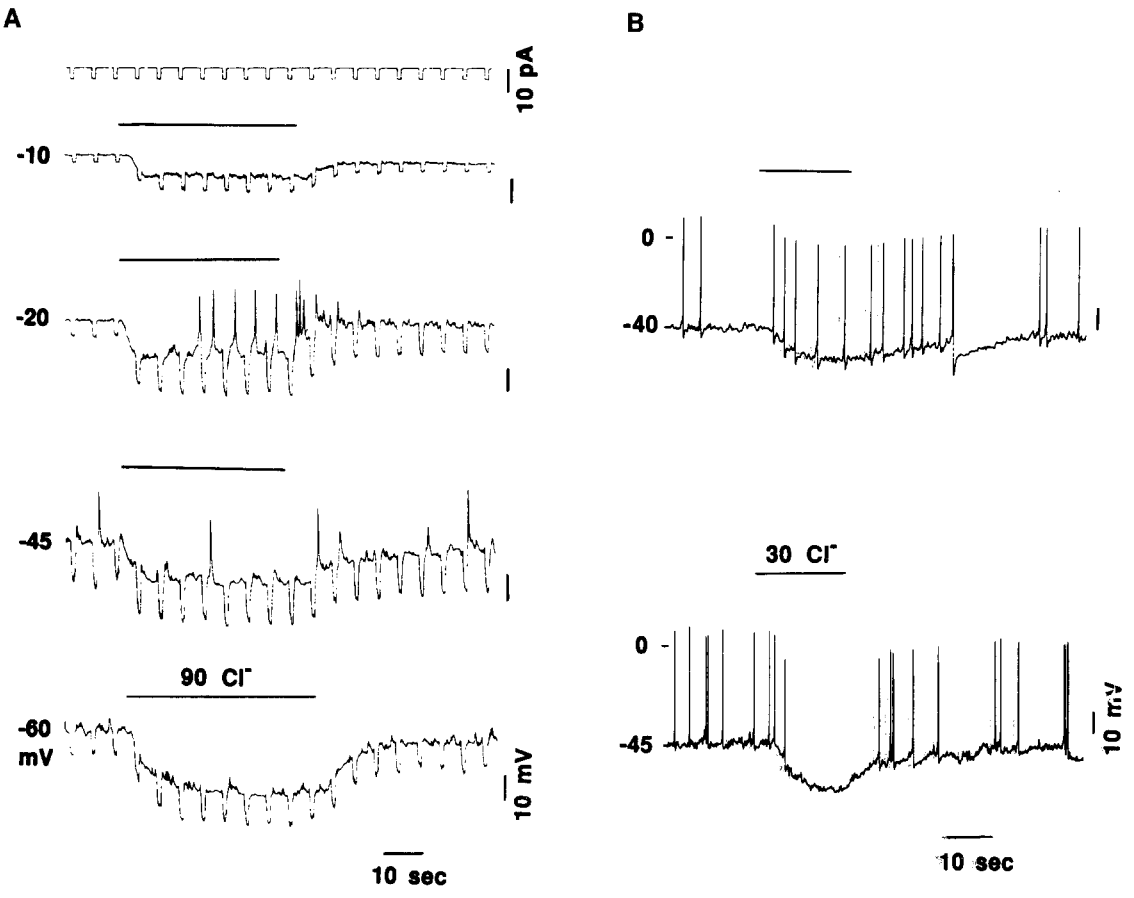

Fig. 7. Effect of external chloride lowering on membrane potential of normal and tumoral lactotrophs. Current-clamp condition recordings (IM = Kgluc 150 mM; EM contains 150 mM NaCl ± 10 mM TEA). (*A*) External medium formulated with 90 mM of Cl^- ions instead of 150 was ejected onto a normal lactotroph maintained at different potentials by hyperpolarizing or depolarizing currents. The modification of external chloride concentration induced hyperpolarization of the cell membrane which increased when the cell was clamped at −60 mV. Resistance variations, slight at −60 or −45 mV, or considerable at −18 mV, seem to indicate that K^+ movements are linked with blockade of another conductance, probably Cl^-, during the hyperpolarization response. (*B*) Similar effect of low Cl^- EM ejection (30 mM) on the resting membrane potential of GH3 cells. Two examples showing hyperpolarization with or without accompanying action potentials.

vate a Cl^- tail current as compared with normal lactotrophs. Similarly, as found by Rogawski et al. [30], we obtained a potential dependence curve of the tail current tightly correlated with the potential dependence of the Ca^{2+} currents. Moreover Ca^{2+} currents are stronger in GH3 cells than in normal lactotrophs. Therefore comparison between Cl^- tail activation data reported by Rogawski and our results shows that Ca^{2+} is less effective in activating Cl^- current in GH3 cells than in normal lactotrophs. In AtT-20 cells, Korn and Horn [14] have reported similar data concerning the chloride tail activation.

Our results demonstrate a direct and complex effect of the extracellular Cl^- concentrations on Ca^{2+} currents and on resting membrane properties. They also show that the calcium-dependent chloride current, though present, is not activated during the TRH response in GH3 cells.

The question arises as to the biological role of this Cl^- current in the two kinds of lactotrophs and its possible involvement in the hormone release process. It is clear that Cl^- ion movements play an essential role in membrane resting potential. We have shown that modification of external Cl^- concentration alters the resting membrane potential. However, the mechanism is complex since it probably involves an outward K^+ current together with the blockade of a conductance which could be a Cl^- resting conductance. The differential involvement of Cl^- channels in the response of GH3 cells and normal lactotrophs to TRH may correspond to a different involvement of Cl^- ions in the resting potential of each kind of cell. The resting potential of

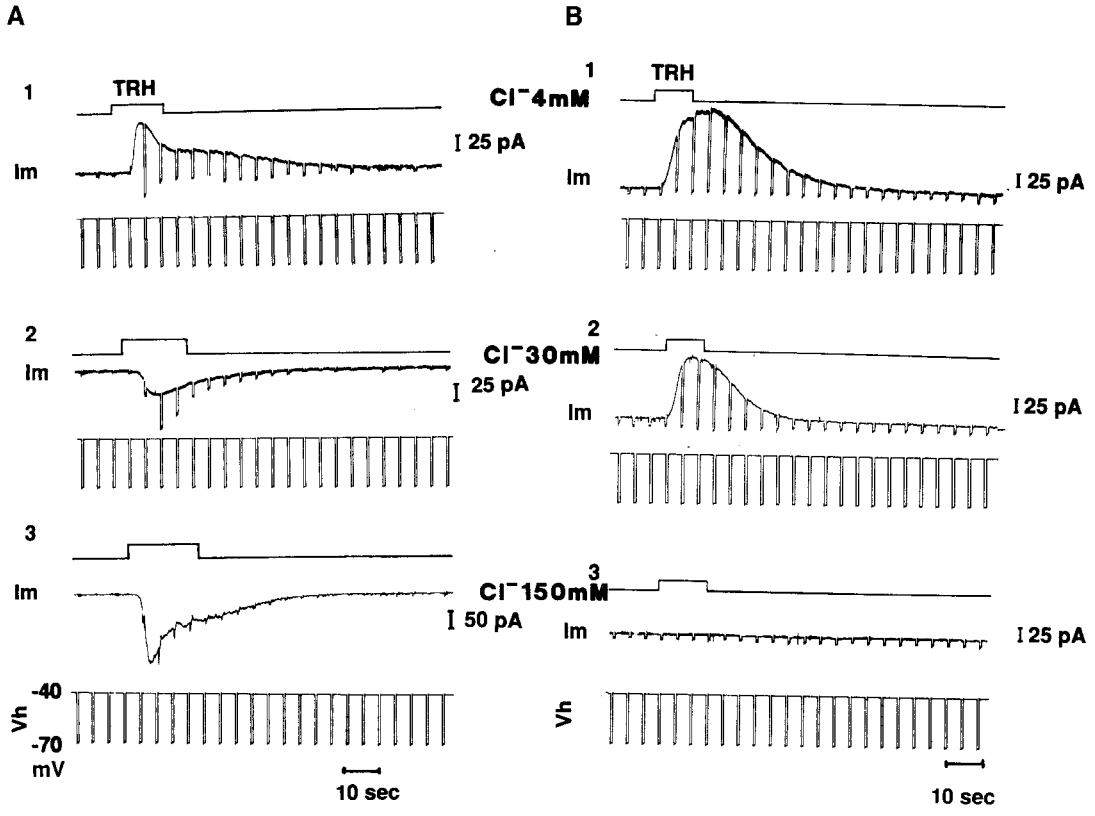

Fig. 8. Comparison of TRH cell responses (10^{-7} M) in normal lactotrophs and GH3/B6 recorded with differently formulated IM. (V_h = −40 mV, hyperpolarizing steps 30 mV, 0.6 sec.) (*A*) Normal lactotrophs. *A1:* When the cells were recorded with 140 mM of Kgluc in the IM, TRH ejected for 20 sec, induced an outward current at −40 mV. However the current during the hyperpolarizing steps (−70 mV) was inwardly directed. *A2:* When the cells were recorded with 140 mM K^+ plus 30 mM Cl^- in the IM, TRH induced an inward current at −40 mV. The amplitude of the current was greatly increased during the steps (−70 mV). *A3:* Total replacement of K^+ by Cs^+ (150 mM CsCl) in the IM was followed by enhancement of the inward current. (*B*) GH3 cells. *B1* (as in *A1*). Typical outward current after TRH application. The current induced during the hyperpolarizing step (−70 mV) appears to be either outward or nil. *B2* (as in *A2*). The addition of 30 mM Cl^- in the IM did not change the response to TRH. *B3* (As in *A3*). However, total replacement of K^+ by Cs^+ ions (CsCl in the patch pipette) caused the disappearance of the TRH response, indicating that in GH3 cells, K^+ ions are the only ones involved in the current response.

normal lactotrophs is more depolarized (~ −30 mV) [31] than that of GH3 cells (40–45 mV). Depolarized membrane potentials have also been observed in normal lactoctrophs using the membrane potential marker oxonol (J. Barker, personal communication). We do not at present know the intracellular concentration of Cl^- ions in normal lactotrophs. We have evidence that individual values may vary widely, as shown by the range of reversal potentials for the ions carrying the current in response to TRH (−48 to −86 mV) in normal rat lactotrophs [31]. It is unlikely that the mean value of 30 mM determined for GH4/C1 population by Kock, Breck Balock and Schonbrunn [13] is representative of the normal lactotrophs situation since we have ourselves reported on a great variability in this cell type, but this point remains to be clarified. However, it should be noted that Cl^- movements depend on $[Ca^{2+}]_i$ concentrations and in these cells Ca^{2+} entry through the slowly inactivating Ca^{2+} channels begins to increase at potentials near −30 mV [17]. Moreover, the direct effect of external Cl^- concentrations on Ca^{2+} movements and on resting membrane potential, here described, suggest a constant interplay between Cl^- and Ca^{2+}. This feedback system may in part contribute to stabilization of the membrane resting potential close to −30 mV. The findings of Korn and Horn [14] showing that sodium-calcium exchange affects the calcium-dependent chloride currents in pituitary cells provide more evidence for such a regulating system using constant feedback interplays at the membrane level. This mechanism, acting directly on $[Ca^{2+}]_i$ or on Ca^{2+} changes near plasma membrane and chloride channels as suggested by Korn and Horn [14], could explain the direct effect of external chloride on Ca^{2+} currents.

Finally, one needs to explain why the chloride current was not activated in GH3 during the TRH response since GH3 chloride channels are activated by a $[Ca^{2+}]_i$ rise during long depolarizing steps [30] and since we know that TRH rapidly increases $[Ca^{2+}]_i$ simultaneously with the induced hyperpolarization [23]. Three hypotheses can be advanced: (i) lack of activation of Cl^- conductance in GH3 cells is due to difference in the amplitude of the rise of $[Ca^{2+}]_i$ during the first phase of TRH response in GH3 as compared with normal lactotrophs; (ii) sensitivity of Cl^- channels to Ca^{2+} is different in GH3 cells as compared with normal lactotrophs; (iii) the membrane transducing systems of GH3 and normal rat lactotrophs are not entirely similar, although TRH binding to its receptor induces a rise in $[Ca^{2+}]_i$ able to activate Cl^- conductances in both cell types. One part of the coupling system, such as a G protein, may act directly on the chloride channel either to facilitate or to block the Ca^{2+} activation.

As regards the first hypothesis, previous reports of the fluorescent probe Fura-2 used to monitor Ca^{2+} levels [37] have shown a rise in $[Ca^{2+}]_i$ in response to TRH in normal lactotrophs and in GH3 cells [23]. We found an increase in $[Ca^{2+}]_i$ similar to or higher than that reported by Winiger et al. [37] in normal cells. Since Ca^{2+} transient induced by TRH is higher in GH3 cells than in normal cells, it must be sufficient to activate the Ca^{2+}-dependent Cl^- channel as in normal lactotrophs.

In these experiments, we found that depolarizing steps of longer duration are needed to activate a Cl^- tail in GH3 cells compared with normal rat lactotrophs. This observation may therefore explain the difference in the response to TRH between GH3 cells and normal rat lactotrophs.

Taleb et al. [35, 36] have described two kinds of Cl^- channels on cells of the porcine intermediate lobe, which have different Ca^{2+} sensitivities. These findings are relevant to our second hypothesis. These authors report on the presence of spontaneous and gaba-evoked Cl^- channels, with a conductance of 25–30 pS, activable for low $[Ca^{2+}]_i$ concentrations, i.e., between 100 and 10 nM [36]. A second kind of Cl^- channel [35] had a smaller conductance (2–3 pS) and was activated with higher $[Ca^{2+}]_i$ concentrations (100 nM to 1 μM). If such a duality is present in rat lactotrophs, differences in the relative proportions of both kinds of channels in normal lactotrophs and GH3 cells could explain the difference in TRH responses reported here.

As discussed by Rogawski et al. [30], the third hypothesis concerning the involvement of a G protein cannot be ruled out, and the positive effect of nonhydrolyzable GTP analogs on the rundown of the Cl^- current indicates that in GH3 cells a GTP-binding protein may participate in the "activation or the recovery from activation" of the current. Thus, differences in the efficiency of such a system in GH3 cells and in normal lactotrophs remain possible. Conversely, a direct effect of Cl^- ions on GTPγ-S binding to G_o protein extracted from bovine brain, has been described by Higashijima, Ferguson and Sternveis [9] in a range of concentrations from 1 to 100 mM, suggesting that chloride concentration variations may play an important role.

The second and the third hypotheses remain likely and are not incompatible.

As regards the role of membrane permeability to Cl^- ions in the secretory process, a direct effect of Cl^- ions on the G protein system as reported by Higashijima et al. [9] is possible. Moreover, it has been suggested that membrane permeation by Cl^- ions is involved in exocrine secretion [19] and endocrine secretion processes [2, 8, 35]. In salivary acinar cells, volume is controlled by Cl^- movements [6]. In the response to TRH in normal rat lactotrophs, Cl^- and K^+ ion movements are linked. As reported for Ehrlich ascites tumor cells [16], K-Cl electroneutral cotransport is essential for cell volume regulation, since functional ionic channels are not available. However, in cases where Ca^{2+}-activated K^+ and Cl^- channels are functional, as in normal lactotrophs in response to TRH, ionic movements may modify cell water content. As suggested by Taleb et al. [35], enlargement of extracellular space may facilitate extrusion of secretory granules as well as access of exogenous endocrine factors, thus favoring the endocrine function.

The authors acknowledge the National Hormone and Pituitary Program, NIDDK, for supplying the antibodies used in these studies. They also thank G. Gaurier, D. Varoqueaux and S. Ciotta for their help in various parts of these studies. They are indebted to P. Mollard and N. Guérineau for data on intracellular calcium levels in GH3 cells and helpful comments and to B. Lussier and J. McKenna for revision of the manuscript.

This work was supported by grants from Centre National de la Recherche Scientifique (URA 1200), Université de Bordeaux 2, Etablissement Public Régional, and Fondation pour la Recherche Médicale.

References

1. Armstrong, D., Eckert, R. 1987. Voltage-actived calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA* **84:**2518–2522
2. Day, R.N., Hinkle, P.M. 1988. Osmotic regulation of prolactin secretion. Possible role of chloride. *J. Biol. Chem.* **263:**15915–15921
3. Evans, M.G., Marty, A. 1986. Calcium dependent chloride currents in isolated cells from rat lacrimal glands. *J. Physiol.* **378:**437–460

4. Evans, M.G., Marty, A., Tan, Y.P., Trautmann, A. 1986. Blockage of Ca-activated Cl conductance by furozemide in rat lacrimal glands. *Pfluegers Arch.* **406:**65–68
5. Findlay, I., Petersen, O.H. 1985. Acetylcholine stimulates a Ca^{2+} dependent Cl^- conductance in mouse lacrimal acinar cells. *Pfluegers Arch.* **403:**328–330
6. Foskett, J.K., 1990. $[Ca^{2+}]_i$ modulation of Cl^- content controls cell volume in single salivary acinar cells during fluid secretion. *Am. J. Physiol.* **259:**C998–C1104
7. Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch clamp techniques for high-resolution current recording from cell and cell free membrane patches. *Pfluegers Arch.* **391:**85–100
8. Heisler, S., Jeandel, L. 1989. 4-Acatamido-4'isothiocyano stilbene-2,2'-disulfonic acid inhibits adrenocorticotropin secretion from anterior pituitary cells. *Endocrinology* **125:**1231–1238
9. Higashijima, T., Ferguson, K.M., Sternveis, P.C. 1987. Regulation of hormone-sensitive GTP-dependent regulatory proteins by chloride. *J. Biol. Chem.* **262:**3597–3602
10. Inenaga, K., Mason, W.T. 1987. γ-aminobutyric acid modulates chloride channel activity in cultured primary bovine lactotrophs. *Neuroscience* **23:**649–660
11. Ingram, C.D., Bicknell, R.J., Mason, W.T. 1986. Intracellular recordings from bovine anterior pituitary cells: Modulation of spontaneous activity by regulators of prolactin secretion. *Endocrinology* **119:**2508–2518
12. Israel, J.M., Kirk, C., Vincent, J.D. 1987. Electrophysiological responses to dopamine of rat hypophysial cells in lactotroph enriched primary cultures. *J. Physiol.* **390:**1–22
13. Koch, B.D., Breck Blalock, J., Schonbrunn, A. 1988. Characterization of the cyclic AMP-independent action of somatostatin in GH cells. I. An increase in potassium conductance is responsible for both the hyperpolarization and the decrease in intracellular free calcium produced by somatostatin. *J. Biol. Chem.* **263:**216–225
14. Korn, S.J., Horn, R. 1989. Influence of sodium-calcium exchange on calcium current rundown and the duration of calcium-dependent chloride currents in pituitary cells, studied with whole cell and perforated patch recording *J. Gen. Physiol.* **94:**789–812
15. Korn, S.J., Weight, F.F. 1987. Patch-clamp study of the calcium-dependent chloride current in AtT-20 pituitary cells. *J. Neurophysiol.* **58:**1431–1451
16. Kramhoft, B., Lambert, J.H., Hoffmann, E.K., Jorgensen, F. 1986. Activation of Cl^- dependent K^- transport in Ehrlich ascites tumor cells. *Am. J. Physiol.* **251:**C369–C379
17. Lewis, D.L., Goodman, M.B., St.John, P.A., Barker, J.L. 1988. Calcium currents and fura-2 signals in fluorescence activated cell sorted lactotrophs and somatotrophs of rat anterior pituitary. *Endocrinology* **123:**611–621
18. Marricq, A.V., Korenbrot, J.I. 1988. Calcium and calcium dependent chloride currents generate action potentials in solitary photoreceptors. *Neuron* **1:**503–515
19. Marty, A., Tan, Y.P., Trautmann, A. 1984. Three types of calcium-dependent channel in rat lacrimal glands. *J. Physiol.* **357:**293–325
20. Matteson, D.R., Armstrong, C.M. 1986. Properties of two types of calcium channels in clonal pituitary cells. *J. Gen. Physiol.* **87:**161–182.
21. Mayer, M.L. 1985. A calcium activated chloride current generates the after-depolarization of rat sensory neurons in culture. *J. Physiol.* **364:**217–239
22. Mollard, P., Guérineau, N., Audin, J., Dufy, B. 1989. Measurement of Ca^{2+} transients using simultaneous dual emission microspectrofluorimetry and electrophysiology in individual pituitary cells. *Biochem. Biophys. Res. Commun.* **164:**1045–1052
23. Mollard, P., Vacher, P., Dufy, B., Winiger, B.P., Schlegel, W. 1988. Thyrotropin-releasing hormone induced rise in cytosolic calcium and activation of outward K^+ current monitored simultaneously in individual GH3/B6 pituitary cells. *J. Biol. Chem.* **263:**19570–19576
24. Mollard, P., Vacher, P., Dufy, B., Winiger, B.P., Schlegel, W. 1989. Inhibitors of 1,2 diacylglycerol kinase potentiate the TRH-induced stimulation of Ca^{2+} activated K^+ current. *Eur. J. Pharmacol.* **172:**71–79
25. Owen, D., Segal, M., Barker, J.L. 1984. A Ca-dependent Cl^- conductance in cultured mouse spinal neurones. *Nature* **311:**567–570
26. Owen, D., Segal, M., Barker, J.L. 1986. Voltage clamp analysis of a Ca^{2+} and voltage-dependent chloride conductance in cultured mouse spinal neurons. *J. Neurophysiol.* **55:**1115–1135
27. Ozawa, S. 1985. TRH-induced membrane hyperpolarization in rat clonal anterior pituitary cells. *Am. J. Physiol.* **248:**E64–E69
28. Ozawa, S., Kimura, N. 1979. Membrane potential changes caused by thyrotropin-releasing hormone in the clonal GH3 cell and their relationship to secretion of pituitary hormones. *Proc. Natl. Acad. Sci. USA* **76:**6017–6020
29. Ozawa, S., Sand, O. 1986. Electrophysiology of excitable endocrine cells. *Physiol Rev.* **66:**887–952
30. Rogawski, M.A., Inoue, K., Susuki, S., Barker, J.L. 1988. A slow calcium-dependent chloride conductance in clonal anterior pituitary cells. *J. Neurophysiol.* **59:**1854–1870
31. Sartor, P., Dufy-Barbe, L., Corcuff, J.B., Taupignon, A., Dufy, B. 1990. Electrophysiological response to thyrotropin-releasing hormone of rat lactotrophs in primary culture. *Am. J. Physiol.* **258:**E311–E319
32. Schlegel, W., Winiger, B.P., Mollard, P., Vacher, P., Wuarin, F., Zahnd, G., Wollheim, C.B., Dufy, B. 1987. Oscillations of cytosolic Ca^{2+} in pituitary cells due to action potentials. *Nature* **329:**719–721
33. Schlegel, W., Wollheim, C.B. 1984. Thyrotropin-releasing hormone increases cytosolic free Ca^{2+} in clonal pituitary cells (GH3 cells): Direct evidence for the mobilization of cellular calcium. *J. Cell. Biol.* **99:**83–87
34. St. John, P.A., Dufy-Barbe, L., Barker, J.L. 1986. Anti-prolactin cell-surface immunoreactivity identifies a subpopulation of lactotrophs from the rat anterior pituitary. *Endocrinology* **119:**2783–2795
35. Taleb, O., Feltz, P., Bossu, J.L., Feltz, A. 1988. Small conductance chloride channels activated by calcium on cultured endocrine cells from mammalian pars intermedia. *Pfluegers Arch.* **412:**641–646
36. Taleb, O., Trouslard, J., Demeneix, B.A., Feltz, P., Bossu, J.L., Dupont, J.L., Feltz, A. 1987. Spontaneous and Gaba evoked chloride channels on pituitary intermediate lobe cells and their internal calcium requirements. *Pfluegers Arch.* **409:**620–631
37. Winiger, B.P., Wuarin, F., Zahnd, F., Wollheim, C.B., Schlegel, W. 1987. Single cell monitoring of cytosolic calcium reveals subtypes of rat lactotrophs with distinct responses to dopamine and thyrotropin-releasing hormone. *Endocrinology* **121:**2222–2228

Received 17 April 1991; revised 23 September 1991