Cytosolic Cl\textsuperscript{−} ions in the regulation of secretory and endocytotic activity in melanotrophs from mouse pituitary tissue slices

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Cl\textsuperscript{−} ions are known regulators of Ca\textsuperscript{2+}-dependent secretory activity in many endocrine cells. The suggested mechanisms of Cl\textsuperscript{−} action involve the modulation of GTP-binding proteins, voltage-activated calcium channels or maturation of secretory vesicles. We examined the role of cytosolic Cl\textsuperscript{−} ([Cl\textsuperscript{−}]\textsubscript{i}) and Cl\textsuperscript{−} currents in the regulation of secretory activity in mouse melanotrophs from fresh pituitary tissue slices by using the whole-cell patch-clamp. We confirmed that elevated [Cl\textsuperscript{−}]\textsubscript{i} augments Ca\textsuperscript{2+}-dependent exocytosis and showed that Cl\textsuperscript{−} acts on secretory vesicle maturation. The latter process was abolished by a V-type H\textsuperscript{−}-ATPase blocker (bafilomycin), intracellular 4,4\textsuperscript{′}-diisothiocyanatostilbene-2,2\textsuperscript{′}-disulphonic acid (DIDS), a Cl\textsuperscript{−} channel blocker, and tolbutamide, a sulphonylurea implicated in secretory vesicle maturation. In a small subset of cells, block of plasmalemmal Cl\textsuperscript{−} current by DIDS reversibly enhanced endocytosis. The direct activation of G-proteins by GTP-γ-S, a non-hydrolysable GTP analogue, did not restore the impaired secretion observed in low [Cl\textsuperscript{−}]\textsubscript{i} conditions. The amplitude of voltage-activated calcium currents was unaffected by the [Cl\textsuperscript{−}]\textsubscript{i}. Furthermore, two Cl\textsuperscript{−}-permeable channels, calcium-activated Cl\textsuperscript{−} channels and GAB\textsubscript{A} receptors, appeared as major regulators of intracellular Cl\textsuperscript{−} homeostasis. In conclusion, the predominant underlying mechanism of Cl\textsuperscript{−} action is mediated by intracellular Cl\textsuperscript{−} fluxes during vesicle maturation, rather than activation of G-proteins or modulation of voltage-activated Ca\textsuperscript{2+} channels.

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The modulation of Ca\textsuperscript{2+}-dependent exocytosis of hormone-containing dense core vesicles (DCVs) by cytosolic Cl\textsuperscript{−} ions has been previously described in several neuroendocrine cell types (Rupnik & Zorec, 1992; Garcia et al. 1999; Barg et al. 2001). In these studies, different mechanisms underlying the Cl\textsuperscript{−} dependence of secretion have been proposed, including the modulation of GTP-binding proteins (Rupnik & Zorec, 1995), Ca\textsuperscript{2+} influx (Garcia et al. 1999) and vesicle priming (Barg et al. 2001).

G-proteins appear as likely target molecules for Cl\textsuperscript{−} modulation as Cl\textsuperscript{−} ions increase the affinity of G-proteins for GTP and inhibit the hydrolysis of bound GTP (Higashijima et al. 1987). A high [Cl\textsuperscript{−}]\textsubscript{i} enhances both the rate and amplitude of exocytosis, which is induced by intracellular dialysis with micromolar [Ca\textsuperscript{2+}]\textsubscript{i} in rat melanotrophs from primary culture. This Cl\textsuperscript{−}-dependent stimulation was blocked by inhibitors of heterotrimeric G-proteins (Rupnik & Zorec, 1995).

In experiments on cultured rat lactotrophs, voltage-activated Ca\textsuperscript{2+} currents (VACCs) and the corresponding increase in [Ca\textsuperscript{2+}]\textsubscript{i} were reduced by the application of low Cl\textsuperscript{−} extracellular solution or chloride channel blockers. This inhibitory effect of low extracellular [Cl\textsuperscript{−}] was abolished by pertussis toxin, a G-protein inhibitor (Garcia et al. 1997a). Furthermore, the basal prolactin secretion of lactotrophs was decreased by Cl\textsuperscript{−}-deficient extracellular solution. The replacement of extracellular Cl\textsuperscript{−} by lyotropic anions shifted the activation and peak voltage of T-type Ca\textsuperscript{2+} currents towards more negative potentials (Garcia et al. 1999).

The evidence of anion-dependent modulation of VACCs has also been provided in the retinal photoreceptors of salamander, where lowering of extracellular [Cl\textsuperscript{−}] by lyotropic anions reduced the amplitude of L-type Ca\textsuperscript{2+} currents and caused a hyperpolarizing shift in their activation and peak voltage. These anion effects largely
followed the Hofmeister sequence, suggesting interaction with membrane surface proteins (Thoreson et al. 2000; Thoreson & Stella, 2000). Consistent with this, the replacement of extracellular Cl\(^-\) ions by SCN\(^-\) further reduced secretory activity in cultured rat melanotrophs (Rupnik & Zorec, 1992).

In addition, Cl\(^-\) fluxes were found to play a crucial role in the regulation of pH in cellular organelles such as endosomes and exocytotic vesicles (reviewed by al-Awqati, 1995). In mouse pancreatic β-cells, both a vesicular H\(^+\)-ATPase and vesicular Cl\(^-\) channel (Cl\(^{\text{C-L}}\)) are necessary to build up the low intragranular pH required for hormone processing and vesicle priming. Cl\(^-\) influx into the exocytotic vesicles facilitates vesicular acidification because it allows the activity of a vesicular H\(^+\)-ATPase to be unimpeded by the development of a significant intraluminal electropositivity. Vesicular acidification is regulated by a 65 kDa protein, sensitive to sulphonyleureas and the ATP/ADP ratio (Barg et al. 2001). A pH dependence of Ca\(^{2+}\)-induced secretion has also been described in melanotrophs (Thomas et al. 1993), indicating a similar molecular mechanism for Cl\(^-\) action. Early studies on pancreatic acinar cells provided evidence for the involvement of vesicular Cl\(^-\) conductance in the regulation of enzyme secretion (Fuller et al. 1989).

The regulation of chloride homeostasis in neuroendocrine cells may have a large impact on the modulation of Ca\(^{2+}\)-dependent secretion. [Cl\(^-\)], in different cell types is regulated by several mechanisms, including different types of Cl\(^-\) channels (Billups & Attwell, 2002; Hull & von Gersdorff, 2004) and cotransporters, anion exchangers (Kakazu et al. 1999) and Cl\(^-\) release from intracellular stores (Garcia et al. 1997b). In particular, a Ca\(^{2+}\)-activated Cl\(^-\) channel (Cl\(^{\text{C-a}}\) channel) as a plausible modulator of [Cl\(^-\)], was found to play a crucial role in fluid and salt secretion in the excretory cells of the respiratory system, intestine and exocrine glands (Kidd & Thorn, 2000). In neurons, Cl\(^{\text{C-a}}\) channels regulate cell excitability and [Cl\(^-\)], (Owen et al. 1986; Hull & von Gersdorff, 2004). The activation of Cl\(^-\) conductance by micromolar Ca\(^{2+}\) has been described in porcine intermediate lobe cells and a pituitary tumour cell line (Korn & Weight, 1987; Taleb et al. 1988; Korn et al. 1991). It has also been speculated, that Cl\(^{\text{C-a}}\) channels are involved in the control of action potential duration and the resulting calcium transients (Korn et al. 1991; Protti et al. 2000).

Another modulator of [Cl\(^-\)], in the neuroendocrine cells is the GABA\(_A\) receptor-linked Cl\(^-\) channel. GABAergic afferent fibres originating from the hypothalamus form synapses with melanotrophs from the rat pituitary intermediate lobe (Oertel et al. 1982). It has been shown that GABAergic innervation modulates the spontaneous electrical activity and hormone output of melanotrophs (Taraskevich & Douglas, 1982; Tomiko et al. 1983; Demeneix et al. 1986; Schneggenburger & Konnerth, 1992), but so far the role of GABA in the regulation of [Cl\(^-\)], has only been demonstrated for neuronal cells (Kuner & Augustine, 2000; Billups & Attwell, 2002; Hull & von Gersdorff, 2004).

The melanotrophs, a homogeneous population of neuroendocrine cells from the rodent pituitary intermediate lobe, release peptide hormones, such as α-MSH and β-endorphin, derived from the precursor pro-opiomelanocortin (Mains & Eipper, 1979). The aim of the present study was to elucidate the mechanism underlying Cl\(^-\)-dependent modulation of Ca\(^{2+}\)-activated exocytosis in mouse melanotrophs in fresh pituitary tissue slices. We suggest that the stimulation of DCV priming by high [Cl\(^-\)\], as in pancreatic β-cells, is a major process in mouse melanotrophs, although different sensitivity to modulatory compounds, i.e. sulphonylureas, suggests a different molecular mechanism. We characterized two sources of cytosolic Cl\(^-\) ions, the GABA\(_A\) receptor and Cl\(^{\text{C-a}}\) current. We describe two populations of melanotrophs that primarily differ in their secretory activity pattern evoked by depolarizations and provide further evidence for the importance of Cl\(^-\) fluxes in the process of endocytotic membrane retrieval in endocrine cells.

Methods

Pituitary tissue slice preparation

The pituitary tissue slices were prepared from adult, male mice (CD-1 and NMRI strains) as previously described (Sedej et al. 2004). The two strains did not differ significantly in the measured parameters, thus data were pooled. Animal handling was according to the regulations of the state of Lower Saxony, Germany. Mice were killed by cervical dislocation, the skull was opened and the brain was removed rapidly. The pituitary gland was carefully dissected out and put into ice-cold extracellular solution 1 (see Solutions section) for up to 1 min. The gland was then embedded in 2.5–3.6% agarose with low-melting point (Seaplaque GTG agarose, BMA Walkersville, MD, USA) in extracellular solution 1. The hardened agarose block was glued with cyanoacrylate (Super Glue, ND Industries, Troy, MI, USA) onto the sample plate of a vibratome VT 1000S (Leica, Nussloch, Germany) and covered with extracellular solution 2 (see Solutions section). Slices 80 μm thick were sectioned at 50–60 Hz and at 0.1 mm s\(^{-1}\). Fresh tissue slices were kept for up to 8 h at 32°C on a nylon mesh in an incubation beaker containing extracellular solution 1.

Solutions

All experiments were performed in extracellular solution 1. Extracellular solution 1 contained (mm): NaCl 125, KCl 2.5, NaH\(_2\)PO\(_4\) 1.25, sodium pyruvate 2,
myo-inositol 3, ascorbic acid 0.5, glucose 10, NaHCO₃ 26, MgCl₂ 1, CaCl₂ 2, lactic acid 6. The slicing procedure was performed in extracellular solution 2 with elevated osmolality (360 ± 10 mosmol kg⁻¹; mm): KCl 2.5, NaH₂PO₄ 1.25, sodium pyruvate 2, myo-inositol 3, ascorbic acid 0.5, sucrose 250, glucose 10, NaHCO₃ 26, MgCl₂ 1, CaCl₂ 2, lactic acid 6. O₂ (95%) and CO₂ (5%) were continuously bubbled through extracellular solutions 1 and 2 to enrich the oxygen content and stabilize the pH to 7.3. The osmolality of the intra/extracellular solutions was 300 ± 10 mosmol kg⁻¹. For pharmacological experiments, GABA (50 µm) and the GABAₐ-receptor antagonist bicuculline (25 µm) were added to extracellular solution 1. The membrane-permeant drugs tolbutamide and diazoxide were added to extracellular solution 1 at a concentration of 100 µm prior to the experiments. In some experiments, 2 mm CaCl₂ was replaced with 10 mm BaCl₂. CdCl₂ (250 µm) was added to block VACCS. Intracellular solutions contained three different [Cl⁻], (mm): 4, 20 and 164. The composition of the intracellular solution with 4 mm [Cl⁻] was (mm): Cs₂SO₄ 105, Hepes 10, MgCl₂ 2, TEA-OH 20, Na₂ATP 2, EGTA 0.05, whereas intracellular solution with 20 mm [Cl⁻] contained (mm): CsCl 16, Cs₂SO₄ 90, Hepes 10, MgCl₂ 2, TEA-OH 20, Na₂ATP 2, EGTA 0.05. Intracellular solution with 164 mm [Cl⁻] contained (in mm): CsCl 140, Hepes 10, MgCl₂ 2, TEA-Cl 20, Na₂ATP 2, EGTA 0.05. CsOH and acetic acid were used to adjust the pH to 7.2. Cs⁺ and TEA⁺ were used to decrease K⁺ conductance. The Cl⁻ channel blocker 4,4′-disothiocyanostilbene-2,2′-disulphonic acid (DIDS; Molecular Probes, Eugene, OR, USA) and the non-hydrolysable GTP analogue GTP-γ-S were both added at a concentration of 100 µm to the intracellular solution with 164 and 20 mm [Cl⁻], respectively. Bafilomycin (200 nm), an inhibitor of the V-type H⁺-ATPase, was included to the pipette solution with high [Cl⁻]. All chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Electrophysiology

Pituitary slices were viewed under an upright Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a 10 × DIC air objective (NA 0.3, WD 16 mm) and a 60 × DIC water immersion objective (NA 1, WD 2 mm) with a mounted CCD camera (Cohu, San Diego, CA, USA). The slices were held at the bottom of the recording chamber by a nylon-mesh stretched on a U-shaped platinum wire. Pituitary gland morphology and particularly densely packed clusters of cells from the intermediate lobe could be easily distinguished (Schneeggenburger & Lopez-Barneo, 1992; Sedej et al. 2004). In contrast to anterior pituitary, the intermediate lobe consists of a homogeneous population of melanotrophs (Tong & Pelletier, 1992). By applying a gentle positive pressure to the pipette, the connective tissue envelope surrounding the clusters was broken and a seal could be formed on a single cell within the cluster. During all electrophysiological experiments the recording chamber was constantly perfused with extracellular solution 1 at 1–2 ml min⁻¹. Pipettes were pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) on a P-97 puller (Sutter Instruments, Novato, CA, USA) and heat polished to obtain a resistance of 2–4 MΩ. All recordings were done in standard whole-cell patch-clamp configuration (Hamill et al. 1981) at 31–33°C. A patch-clamp lock-in amplifier (SWAM IIC, Celica, Ljubljana, Slovenia) was used to record membrane capacitance changes (ΔC_m) and membrane currents in the voltage-clamp mode (Neher & Marty, 1982). Membrane capacitance changes were stimulated by trains of depolarizing square pulses (see Results). Current–voltage relations (I–V plots) were obtained from the analysis of current responses to voltage ramp protocols. The liquid junction potentials caused by different [Cl⁻] were corrected. Signals were filtered at 300 Hz (4-pole Bessel) and transferred to a standard PC via an A/D converter (PCI-6035E, National Instruments, Austin, TX, USA). Zero current membrane potential was measured in current-clamp mode. Data acquisition, voltage protocol generation and basic analysis were done by WinWCP v 3.2.6 software written by J. Dempster (Strathclyde University, Glasgow). For data analysis and figure preparation we used Matlab (The MathWorks, Novi, MI, USA), Matview (Matlab WinWCP extension, Wise Technologies, Ljubljana, Slovenia) and SigmaPlot (SPSS, Chicago, IL, USA). Statistics are given as means ± s.e.m. and statistical significance was tested by Student’s t test. One-way ANOVA was performed by using SigmaStat (SPSS) to determine whether differences between the various groups existed. ANOVA on ranks was tested by Dunn’s test and the significance level was chosen at P < 0.05.

Ca²⁺ measurements

Simultaneous measurements of [Ca²⁺]i during electrophysiological recordings were possible by including 125 µm fura-6F (Molecular Probes, Eugene, OR, USA) in the pipette solution. By using the monochromated light of a Polychrome IV (TILL Photonics, Graeffeling, Germany; dichroic mirror 400 nm) the fluorescence was excited at 380 nm and the emitted light was detected by a photodiode (TILL Photonics) at wavelengths longer than 420 nm. The fluorescence signal was recorded together with the electrophysiological data and analysed as previously described (Carter & Ogden, 1994). The equation used in the calculation is:

$$[\text{Ca}^{2+}]_i = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$$
where \(K_d\) is the apparent dissociation constant for fura-6F (5.3 \(\mu\)M; Gee et al. 2000), \(F_{\text{max}}\) is the autofluorescence in a cell-attach configuration, \(F_{\text{min}}\) is the fluorescence in a resting whole-cell recording and \(F\) is fluorescence during the voltage protocol.

**Results**

**Two different patterns of exocytotic and endocytotic activity in mouse melanotrophs**

\(Ca^{2+}\)-dependent secretion from melanotrophs in fresh pituitary tissue slices can be triggered effectively by a train of depolarizing pulses (Sedej et al. 2004). In our experiments, we used a train of 50 depolarizations from the holding potential of \(-80\) mV to \(-10\) mV for 40 ms at 10 Hz (Fig. 1A) to stimulate a rise in cytosolic \(Ca^{2+}\) to low micromolar levels (Fig. 1A). All control experiments were performed in high \(Cl^-\)-containing pipette solution. A simultaneous increase in membrane capacitance from resting level was interpreted as \(Ca^{2+}\)-induced exocytosis (Fig. 1Ab and b). No major changes in membrane conductance were observed during the train (Fig. 1A). As shown in Fig. 1B, a marked difference in the distribution of the endocytotic maximal rates clearly separated the cells into two groups. In the majority of cells the capacitance increase was followed by a slower decrease, reflecting endocytosis (Fig. 1Aa). However, a small group of cells was characterized by a significantly faster post-stimulatory decrease in membrane capacitance (Fig. 1Ab). The maximal rate of capacitance decrease was slower than \(125\) fF s\(^{-1}\) in 93% of the cells (Fig. 1B); the remaining 7% showed faster endocytosis (\(>125\) fF s\(^{-1}\)) up to about \(400\) fF s\(^{-1}\). The cumulative capacitance increase triggered was significantly higher in cells with fast endocytosis (\(2.19\pm0.23\) pF, \(n=10\)) compared to cells with slow endocytosis (\(0.77\pm0.05\) pF, \(n=132\); \(P<0.001\); Fig. 1C). Resting membrane capacitance as a parameter proportional to the membrane surface area was \(7.3\pm0.2\) pF (\(n=142\)) and did not vary between the cell groups.

**Intracellular \(Cl^-\) ions stimulate \(Ca^{2+}\)-dependent exocytosis**

In order to investigate the role of [\(Cl^-\)], in \(Ca^{2+}\)-dependent exocytosis, we replaced intracellular \(Cl^-\) ions by sulphate. This resulted in inhibition of depolarization-induced exocytosis by more than 50%, without changing the time profile of post-stimulatory endocytosis (Fig. 2A). The change in cumulative capacitance at 4 mm [\(Cl^-\)], \((0.36\pm0.09\) pF, \(n=11\)) was significantly lower compared to the change when 164 mm [\(Cl^-\)] was used \((0.77\pm0.05\) pF, \(n=132\)). However, 20 mm [\(Cl^-\)] did not significantly increase the cumulative capacitance \((0.39\pm0.09\) pF, \(n=13\); one-way ANOVA; \(P<0.05\); Fig. 2B). We pooled 4 mm and 20 mm experiments as low [\(Cl^-\)], and 164 mm as high [\(Cl^-\)].

To differentiate which kinetic phase of secretion is modulated by [\(Cl^-\)], we analysed the membrane capacitance change evoked by single pulses during the depolarization train (Fig. 2C). The capacitance response to the first 40 ms depolarization, reflecting the release of preocked and fusion competent vesicles spatially colocaled with VACCs (immediately releasable pool, IRP; Horrigan & Bookman, 1994), was not statistically different at low [\(Cl^-\)], \((11\pm3\) fF, \(n=25\)) with respect to high [\(Cl^-\)], \((10\pm2\) fF, \(n=54\)). After subsequent depression in the secretory activity a second phase of exocytosis was elicited. The increase in capacitance evoked by subsequent pulses was enhanced by high [\(Cl^-\)], (Fig. 2C, upper left). At high [\(Cl^-\)], maximal \(\Delta C_m\) per pulse was observed.
after 24 pulses and was significantly larger (26 ± 3 pF; \( n = 54 \)) compared to low [Cl\(^{-}\)] conditions (peak after 28 pulses, 10 ± 2 pF; \( n = 25 \), \( P < 0.002 \). The fusion rate slowly decreased towards a steady-state level, regardless of the cytosolic [Cl\(^{-}\)] used.

Granular acidification driven by a V-type H\(^{+}\)-ATPase in the granular membrane, which requires simultaneous Cl\(^{-}\) uptake through granular ClC-3 Cl\(^{-}\) channels, is a decisive step in vesicle priming in \( \beta \)-cells (Barg et al. 2001). A possible role of granular pH involved in the regulation of exocytosis in melanotrophs was tested by adding 200 nm bafilomycin, an inhibitor of the vesicular H\(^{+}\)-ATPase, to the pipette solution. In the presence of high Cl\(^{-}\) and bafilomycin, exocytosis mimicked the exocytotic response when low Cl\(^{-}\) was used (Fig. 2C, upper right). The change in the cumulative capacitance was significantly reduced and measured 0.43 ± 0.11 pF (\( n = 9 \); Fig. 2B).

Since it was previously described that the modulation of GTP-binding proteins may account for the Cl\(^{-}\)-dependent stimulation of secretion (Rupnik & Zorec, 1995), we dialysed the cells with GTP-\( \gamma \)-S (100 \( \mu \)M) for 2 min before the train was applied. We found that the activation of G-proteins by GTP-\( \gamma \)-S did not significantly stimulate the \( \Delta C_m \) per pulse at low [Cl\(^{-}\)] (Fig. 2C, lower left).

The block of intracellular Cl\(^{-}\) channels by DIDS (100 \( \mu \)M) inhibited the stimulatory effect of high [Cl\(^{-}\)], on the second phase of exocytosis (Fig. 2C, lower right) and analysis of the \( \Delta C_m \) per pulse showed similar kinetics and amplitude as in low [Cl\(^{-}\)]. The capacitance response to the first depolarization pulse remained unaffected. Extracellularly applied DIDS did not affect the secretory response in the majority of melanotrophs (not shown). In a small subset of melanotrophs (7\% of all cells investigated), which showed fast endocytotic response, elevated [Cl\(^{-}\)] significantly augmented the secretory activity. However, this augmentation was not sensitive to DIDS (see also Fig. 4B). The density of VACCs was not statistically different between low (6.1 ± 1.3 pA pF\(^{-1}\), \( n = 17 \)) and high [Cl\(^{-}\)], (8.6 ± 1.2 pA pF\(^{-1}\), \( n = 12 \)) and high [Cl\(^{-}\)],

![Figure 2. Cl\(^{-}\)-dependent facilitation of vesicle priming](image)

A, representative \( C_m \) responses at low (middle trace) and high (lower trace) [Cl\(^{-}\)], stimulated by the train of depolarizing pulses. Changes in [Cl\(^{-}\)] did not alter the time course of post-stimulatory endocytosis. B, \( \Sigma \Delta C_m \), in cells at 4, 20 and 164 mM [Cl\(^{-}\)], and 164 mM [Cl\(^{-}\)] in the presence of 200 nm bafilomycin (bafi) (\( n = 11, 13, 132, 9 \), respectively; one-way ANOVA; \( P < 0.05 \)). C, membrane capacitance changes evoked by the single pulses of the depolarization train (\( \Delta C_m/pulse \)) for low [Cl\(^{-}\)], high [Cl\(^{-}\)], high [Cl\(^{-}\)] + GTP-\( \gamma \)-S (\( \bullet \), \( n = 25 \), upper left panel), high [Cl\(^{-}\)] + bafi (\( \bullet \), \( n = 25 \), upper right panel); low [Cl\(^{-}\)], high [Cl\(^{-}\)] + 100 \( \mu \)M GTP-\( \gamma \)-S-\( \Delta \)-S (\( \square \), \( n = 25 \), lower left panel); high [Cl\(^{-}\)] + 100 \( \mu \)M DIDS in the pipette solution (\( \bullet \), \( n = 22 \), lower right panel). Activation of G-proteins by GTP-\( \gamma \)-S does not significantly stimulate the \( \Delta C_m/pulse \), while the stimulatory Cl\(^{-}\)-effect is inhibited by DIDS. D, leak subtracted Ca\(^{2+}\) current \( I-V \) relationship from cells dialysed with high [Cl\(^{-}\)] (1): low [Cl\(^{-}\)], high [Cl\(^{-}\)] + DIDS (3) and low [Cl\(^{-}\)] and GTP-\( \gamma \)-S (4). E, VACC density at low [Cl\(^{-}\)] (\( n = 17 \)) and high [Cl\(^{-}\)] with (\( n = 6 \)) or without bafilomycin (\( n = 12 \)). F, estimation of size of IRP. The actual pool size lies between \( \Delta C_m \) and \( B_{max} \), and is not statistically different at low (left panel, \( n = 15 \)) and high (right panel, \( n = 26 \)) [Cl\(^{-}\)].
with bafilomycin (9.6 ± 1.8 pA pF⁻¹, n = 7; Fig. 2D and E).

To further assess whether Cl⁻ changes the pool size of membrane-docked and fusion competent vesicles, which are located close to the VACCs, we used a dual-pulse stimulation as previously described (Gillis et al. 1996). The actual size of this pool of vesicles extends between Cm1, the capacitance increase evoked by the first pulse, and Bmax, given by S/(1−R²), where S is the sum (∆Cm1 + ∆Cm2) and R is the ratio (∆Cm1/∆Cm1) of the capacitance increases. For the correct estimation of Bmax the exocytic activity must show a significant depression (R < 0.7). We used paired pulses of 40 ms duration from the holding potential of −80 mV to −10 mV and to 0 mV, at 100 ms intervals. There was no statistical difference in the estimated pool size in cells with low [Cl⁻]i; (Cm1 18 ± 5 fF to Bmax 27 ± 8 fF, n = 15) compared to cells with high [Cl⁻]i; (Cm1 14 ± 1 fF to Bmax 19 ± 2 fF, n = 26; Fig. 2F).

Our findings are consistent with previous reports from pancreatic β-cells, suggesting that the function of vesicular Cl⁻ channels is crucial for granular acidification during the priming of exocytic vesicles (Barg et al. 2001).

As priming in β-cells is also sensitive to sulphonylureas, we tested if such a mechanism plays a role in melanotrophs. To test the potential involvement of a sulphonylurea receptor in Cl⁻-dependent vesicular priming, we used tolbutamide (100 µM) and diazoxide (100 µM). In contrast to the report from Barg et al. (2001), tolbutamide was inhibitory, almost completely abolishing the Cl⁻-dependent augmentation (Fig. 3A). On the other hand, diazoxide did not affect secretory activity in high [Cl⁻]i conditions (Fig. 3B). In low [Cl⁻]i, neither diazoxide nor tolbutamide showed a significant effect on the ∆Cm per pulse (data not shown).

The block of plasmalemmal Cl⁻ channels enhances fast post-stimulatory endocytosis

In cells displaying fast endocytosis, the block of Cl⁻ channels by DIDS enhanced the post-stimulatory membrane capacitance decrease (Fig. 4A) without affecting the cumulative capacitance increase (Fig. 4B). The maximal rate of endocytosis was significantly higher in cells with high [Cl⁻]i, and 100 µM DIDS in the pipette solution (655 ± 173 fF; n = 7) with respect to cells with high [Cl⁻]i, (234 ± 25 fF s⁻¹, n = 10, P < 0.02). Remarkably, the stimulation of endocytosis was also present during the extracellular perfusion of slices with 100 µM DIDS (Fig. 4C). Endocytosis was up to eightfold

**Figure 3. Reduced priming in tolbutamide**

Membrane capacitance changes evoked by the single pulses of the depolarization train (∆Cm/pulse). A, ∆Cm/pulse for high [Cl⁻]i (○, n = 52) and high [Cl⁻]i + 100 µM tolbutamide (●, n = 9). Tolbutamide inhibits Cl⁻-dependent stimulation. B, ∆Cm/pulse at high [Cl⁻]i (○, n = 52) and high [Cl⁻]i + 100 µM diazoxide (●, n = 8). ∆Cm/pulse with diazoxide is not statistically different from the control at high [Cl⁻]i.

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**Figure 4. Stimulation of fast post-stimulatory endocytosis by DIDS**

A, representative Cm response of a cell with fast endocytosis at high [Cl⁻]i + 100 µM DIDS in the pipette solution. ∆Cm decreased to the resting level within a few seconds after stimulation. B, ∑ΔCm in cells at 164 mM [Cl⁻]i (white, n = 10) and 164 mM [Cl⁻]i in the presence of 100 µM DIDS (grey, n = 7). C, Cm responses recorded from a cell at high [Cl⁻]i in control conditions (black, 1), after 2 min of perfusion with 100 µM DIDS (dark grey, 2) and after 6 min of DIDS washout with standard extracellular solution (grey, 3). For comparison of the kinetics of post-stimulatory endocytosis, Cm traces were normalized to ∆Cm max. D, maximal endocytotic rate (−dCm/dt) under control conditions, during DIDS perfusion and DIDS washout (colour and number coding as in B). −dCm/dt was normalized to the initial −dCm/dt in control conditions. Extracellular application of DIDS led to an eightfold increase in endocytotic rate.
faster than in control conditions, when measured after 2 min of DIDS application. An interval of 2 min was allowed between two consecutive trains. This effect was partially reversible after the washout of Cl\(^-\) channel blocker (Fig. 4D).

**Depolarization-induced Ca\(^{2+}\) entry activates a Cl\(^-\) current**

To gain insight into the source of modulatory Cl\(^-\) ions in melanotrophs, we further analysed the current response evoked by a train of depolarizing pulses. During the train stimulation, only the first few depolarizations induced an inward current due to Ca\(^{2+}\) influx through VACCs (Sedej et al. 2004). Cells with high [Cl\(^-\)]\(_i\) developed an inward current at \(-80\) mV, which increased from the resting level to its maximum after 25 pulses (Fig. 5A, middle trace). The inward current was sustained or declined slowly during the subsequent pulses and returned to resting current within a few seconds after the end of stimulation. Reduction of [Cl\(^-\)]\(_i\) to 4 mM inhibited the development of inward current (Fig. 5A, lower trace). The peak amplitude of inward current measured from resting level in cells with high [Cl\(^-\)]\(_i\) (68 ± 8 pA, \(n = 69\)) was significantly higher compared to the peak inward current in cells with low [Cl\(^-\)]\(_i\) (17 ± 4 pA, \(n = 12\); \(P < 0.006\), Fig. 5B), suggesting that this current reflects an increase in membrane conductance for Cl\(^-\) ions. The depolarization-induced increase of [Ca\(^{2+}\)]\(_i\) measured by fura-6F closely correlated with Cl\(^-\) current (Fig. 5C). In addition, the replacement of extracellular Ca\(^{2+}\) by Ba\(^{2+}\) or block of VACCs by extracellular Cd\(^{2+}\) resulted in the complete inhibition of Cl\(^-\) current at \(-80\) mV (Fig. 5D). With Ba\(^{2+}\) as a charge carrier for currents through VACCs, the inward current at \(-10\) mV showed amplitudes of several hundred picoamps and slow inactivation during pulse stimulation. After the application of extracellular Cd\(^{2+}\) all inward currents disappeared and the current response exhibited a small outward current at \(-10\) mV, most probably due to a technical leak (Fig. 5D, lower trace). The application of DIDS, either intra- or extracellularly, tended to diminish the Cl\(_{Ca}\) current by around 30%, although no statistical difference was found.

[Cl\(^-\)]\(_i\) determines the GABAergic effect on electrical activity

A recent report shows that under physiological conditions the GABAergic effect on the electrical activity of neuronal cells can be inhibitory or excitatory, depending on the [Cl\(^-\)]\(_i\) (Gulledge & Stuart, 2003). In our experiments, the changes of membrane current and membrane potential evoked by the application of GABA were modulated by [Cl\(^-\)]\(_i\) (Fig. 6). Perfusion with 50 µM GABA stimulated a small inward current in cells with low [Cl\(^-\)]. Resting membrane current was \(-29 ± 5\) pA at \(-80\) mV and increased to \(-50 ± 6\) pA (\(n = 9\), Fig. 6A, upper left trace). In current-clamp recordings the membrane potential in cells with low [Cl\(^-\)], was \(-7 ± 4\) mV and transiently

**Figure 5. Activation of Cl\(^-\) current by depolarization-induced Ca\(^{2+}\) entry**

A, leak subtracted membrane current \(I_m\) responses at 164 mM (middle trace) and 20 mM (lower trace) [Cl\(^-\)]\(_i\), evoked by the train of depolarizing pulses (upper trace). During the stimulation, the development of inward current measured as a difference from resting \(I_m\) is inhibited by low [Cl\(^-\)]. Zero lines are shown as dotted grey lines. B, maximal inward \(I_m\) measured as the difference from resting \(I_m\) at 4 mM (\(n = 12\)) and 164 mM (\(n = 69\)) [Cl\(^-\)], \(P < 0.01\). C, representative changes of [Ca\(^{2+}\)]\(_i\) (grey) and inward Cl\(_{Ca}\) current \(I_{ClCa}\) (black) during a train stimulation. D, \(I_m\) responses with extracellular Ca\(^{2+}\) replaced by Ba\(^{2+}\) (upper trace) and block of VACCs by extracellular Cd\(^{2+}\) (lower trace). Note that activation of Cl\(_{Ca}\) is abolished under both conditions.
hyperpolarized during GABA application to $-36 \pm 2 \text{ mV}$. The spiking activity was abolished (Fig. 6A, lower left trace). In cells with high $[\text{Cl}^-]_i$, GABA induced an increase of inward current at $-80 \text{ mV}$ from $111 \pm 41 \text{ pA}$ to $-417 \pm 52 \text{ pA}$ ($n = 6$), which was significantly higher compared to cells in low $\text{Cl}^-$ conditions (Fig. 6A, upper right trace; $P < 0.001$). The membrane potential in cells with high $[\text{Cl}^-]_i$ was slightly depolarized by GABA (Fig. 6A, lower right trace). These GABAergic effects were mediated by GABA$_A$-receptor activation, because they were reversed by 25 $\mu\text{M}$ bicuculline, a specific GABA$_A$-receptor antagonist (Fig. 6A, lower left trace). Interestingly, there was a statistical difference in resting membrane current at the holding potential of $-80 \text{ mV}$ between cells with high $[\text{Cl}^-]_i$ ($134 \pm 11 \text{ pA}$, $n = 85$) and cells with low $[\text{Cl}^-]_i$ ($30 \pm 5 \text{ pA}$, $n = 33$, $P < 0.001$). In addition, a decrease in resting membrane current due to the application of bicuculline alone was observed in some cells (data not shown). The contribution of $\text{Cl}^-$ conductance to the resting membrane conductance might be mediated by constitutive activation of GABA$_A$ receptors by GABA release from functional nerve endings maintained in the fresh slice preparation. At low and high $[\text{Cl}^-]_i$, we observed a GABA-mediated shift of membrane potential towards the $\text{Cl}^-$ equilibrium potential calculated by Nernst's equation: $-92 \text{ mV}$ at 4 $\text{mM}$ $[\text{Cl}^-]_i$, and $5 \text{ mV}$ at 164 $\text{mM}$ $[\text{Cl}^-]_i$ (Fig. 6B). The reversal potential for both GABA$_A$ and $\text{Cl}^-_{Ca}$ channels did not entirely match these calculated values, probably due to a contribution of other anions in the extracellular solution, e.g. bicarbonate. The latter ion has a calculated equilibrium potential at $-12 \text{ mV}$ at intracellular pH 7.2. Bicarbonate permeability through GABA$_A$ receptors is small relative to the $\text{Cl}^-$ permeability and therefore it shows a modest contribution to $E_{\text{GABA}}$ (Kaila, 1994). Comparison of GABA evoked currents with $\text{Cl}^-_{Ca}$ currents revealed that the two currents showed a similar current–voltage relationship (Fig. 6C, filled symbols; Owen et al. 1986). Extrapolated equilibrium potentials for the $\text{Cl}^-_{Ca}$ current indicate that the permeability of these channels might be less specific for $\text{Cl}^-$ ions than the permeability of GABA$_A$ receptor-linked $\text{Cl}^-$ channels.

**Discussion**

Cytosolic $\text{Cl}^-$ ions enhance Ca$^{2+}$-regulated secretion in a variety of endocrine cell types, including mammalian melanotrophs, lactotrophs and pancreatic $\beta$-cells (Rupnik & Zorec, 1992; Garcia et al. 1999; Barg et al. 2001). In previous studies, three major mechanisms for $\text{Cl}^-$-dependent stimulation of secretion have been proposed: (i) $\text{Cl}^-$ modulates the activity of G-proteins involved in the regulated exocytosis (Rupnik & Zorec, 1995); (ii) $\text{Cl}^-$ influences Ca$^{2+}$ entry through VACCs by either direct interaction with channel proteins or by modulation of cytosolic effectors such as G-proteins (Garcia et al. 1999); (iii) $\text{Cl}^-$ uptake into exocytotic vesicles during granular acidification is crucial for vesicle priming (Barg et al. 2001).

Here, we demonstrate that elevated $[\text{Cl}^-]_i$ stimulated Ca$^{2+}$-dependent exocytosis induced by membrane
depolarization in melanotrophs from fresh pituitary tissue slices. This phenomenon resulted from Cl\(^-\)–dependent acceleration of large DCV ‘priming’ and ‘maturation’ (Barg et al. 2001). As shown in Fig. 2B and C, baflodipromycin, a V–type H\(^+\)-ATPase blocker, prevented the acidification of secretory granules and subsequently the secretory augmentation. In addition, consistent with previous reports on pancreatic β-cells (Barg et al. 2001) and exocrine acinar cells (Fuller et al. 1989), we found that the stimulatory effect of cytosolic Cl\(^-\) ions can be effectively inhibited by intracellular dialysis with the Cl\(^-\) channel blocker, DIDS, suggesting an analogy with regulatory mechanisms. Enhanced secretion in melanotrophs is present only at high [Cl\(^-\)], while a concentration of 20 mM [Cl\(^-\)] is not sufficient to significantly stimulate exocytosis. This indicates a lower Cl\(^-\) sensitivity of secretory apparatus in the slice preparation, compared to single cells, where exocytosis is half stimulated at around 12 mM [Cl\(^-\)]. (Rupnik & Zorec, 1992).

Since the changes in [Cl\(^-\)] did not influence the release of vesicles from the RRP, we conclude that the target of the Cl\(^-\) effect on secretion must lie upstream from the actual vesicle fusion process. We found Cl\(^-\)–mediated augmentation of regulated exocytosis only during prolonged stimulation. Several possible explanations might account for this. First, this increase might be due to facilitated fusion of primed vesicles from the readily releasable pool (RRP). Second, a promoted priming and mobilization of vesicles from the so-called unprimed pool (UPP) to the RRP or a stabilized primed state (i.e., slower de-priming reaction) would lead to an increased number of fusion-competent vesicles. A kinetic model for the release of large DCVs from chromaffin cells (Sorensen, 2004) shows that a destabilized priming reaction, i.e., as a result of low [Cl\(^-\)], or blocking of vesicular Cl\(^-\) channels, could lead to a decrease in the initial size of RRP. Furthermore, a subset of secretory UPP vesicles is found to undergo a parallel secretory pathway, which could be another target for Cl\(^-\) action. The low micromolar levels of [Ca\(^{2+}\)] do not change significantly during the whole period of stimulation, which leads to a slow, but constant rate of fusion from the RRP (Sorensen, 2004). Under these conditions an increase in RRP size would not affect the number of vesicles fusing during the stimulation. We favour the explanation that high [Cl\(^-\)] enhances the fusion of vesicles through a parallel secretory pathway (Rupnik et al. 2000; Sorensen, 2004). Further experiments will be needed to clarify this issue.

Our results contrast with those in β-cells (Barg et al. 2001), where tolbutamide, a hypoglycaemic sulphonylurea, blocked Cl\(^-\) augmentation of secretion in melanotrophs. On the other hand, diazoxide, a sulphonylurea antagonist, showed no significant effect on stimulated secretion. In melanotrophs, the regulation of vesicular Cl\(^-\) uptake during maturation is not mediated by a sulphonylurea receptor as described for β-cells (Barg et al. 2001).

The Cl\(^-\)–dependent modulation of Ca\(^{2+}\) entry described in lactotrophs (Garcia et al. 1999) is not predominant in melanotrophs. In fact, the amplitude of VACCs remained unaffected by the changes in [Cl\(^-\)].

The activation of GTP-binding proteins by a non-hydrolysable GTP analogue failed to stimulate the depolarization-induced Ca\(^{2+}\)–dependent secretion in melanotrophs at low [Cl\(^-\)]. This suggests that in the majority of cells the Cl\(^-\) effect is not mediated by G-proteins. Nevertheless, in a small fraction of melanotrophs, a significant Cl\(^-\)–mediated increase in secretory activity, insensitive to intracellular DIDS, was observed. Thus, we could not exclude a possible effect of Cl\(^-\) on the activation of G-proteins independent of the function of vesicular Cl\(^-\) channels (Higashijima et al. 1987; Rupnik & Zorec, 1992, 1995). The cAMP-mediated, PKA–independent activation of Rab proteins, a family of small G-proteins involved in vesicle fusion (Oberhauser et al. 1992), has been described in several endocrine cell types (Ozaki et al. 2000; Eliasson et al. 2003; Holz, 2004). In addition, in the majority of cultured rat melanotrophs a G-protein-dependent effect of Cl\(^-\) modulation of regulated secretion has been previously described, using whole-cell Ca\(^{2+}\) dialysis experiments (Rupnik & Zorec, 1995). The observed discrepancy might therefore reflect differences in preparation, approach used or animal species, but we did not further explore this issue in the present study.

A recent report on retinal bipolar cells shows that the block of Cl\(^-\) entry through GABA-receptor linked Cl\(^-\) channels reveals a fast component of post-stimulatory endocytosis (Hull & von Gersdorff, 2004). It is speculated that transiently increased [Cl\(^-\)] in local areas close to the plasma membrane, rather than global changes in [Cl\(^-\)], inhibits fast endocytosis. These putative ‘high Cl\(^-\) microdomains’ are maintained by Cl\(^-\) influx through ionotropic GABA receptors and are spatially and temporally restricted, because of Cl\(^-\) extrusion mechanisms such as Cl\(^-\) efflux through Cl\(_{\text{ca}}\) channels and the K\(^{+}\)–Cl\(^-\) cotransporter. The pharmacological manipulations of GABA\(_{\text{A}}\) receptor in our experiments did not affect the properties of post-stimulatory endocytosis. Interestingly, the block of Cl\(_{\text{ca}}\) channels by DIDS increased the rate of fast endocytosis. This effect was mediated by a Cl\(^-\) channel located in the plasma membrane, because it was elicited by DIDS application both extracellularly and intracellularly. DIDS tended to decrease the amplitude of the Cl\(_{\text{ca}}\) current, suggesting that Cl\(_{\text{ca}}\) channels may increase or decrease [Cl\(^-\)] depending on the reversal potential for Cl\(^-\). However, the block of Cl\(^-\) channels by DIDS did not affect the time profile of slow post-stimulatory endocytosis, observed in ~93% of the cells, indicating that the majority lack this pathway of fast membrane retrieval.

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or that high subplasmalemmal \([\text{Cl}^-]\) was maintained by other mechanisms. So far, little is known about regulation of post-stimulatory endocytosis in endocrine cells. Further studies on slow and fast endocytosis and their modulating factors in melanotrophs from tissue slices may elucidate the underlying mechanism.

The broad spectra of \(\text{Cl}^-\) effects on the electrical activity of cells and on the exocytotic and endocytotic processes underline the importance of regulation of \([\text{Cl}^-]\). As our study demonstrates, \(\text{Cl}_{\text{Ca}}\) currents represent a plausible component for the modulation of \(\text{Cl}^-\) homeostasis in melanotrophs from fresh pituitary tissue slices. Consistent with previous findings in other neuroendocrine cell types, \(\text{Cl}^-\) conductance is activated by the increase of \(\text{Ca}^{2+}\) (Taleb et al. 1986; Korn & Weight, 1987; Taleb et al. 1988). It has been suggested that \(\text{Cl}_{\text{Ca}}\) currents are involved in the regulation of electrical activity and \(\text{Ca}^{2+}\) transients in endocrine cells of the pituitary (Korn et al. 1991). At high resting \([\text{Cl}^-]\), the activation of \(\text{Cl}_{\text{Ca}}\) current by \(\text{Ca}^{2+}\) entry during spontaneous action potentials leads to a sustained depolarization by \(\text{Cl}^-\) efflux and to increased \(\text{Ca}^{2+}\) transients. \(\text{Ca}^{2+}\)-activated \(\text{Cl}^-\) influx at low resting \([\text{Cl}^-]\) would ‘shunt’ the inward current and thereby promote repolarization and limitation of \(\text{Ca}^{2+}\) entry. In addition to the modulation of electrical activity, the resulting changes in the subplasmalemmal \([\text{Cl}^-]\) would alter secretion by enhancing or inhibiting the priming of exocytotic vesicles and the endocytotic membrane retrieval. The resting \([\text{Cl}^-]\) in melanotrophs from pituitary slices, estimated by two-photon imaging with the fluorescent \(\text{Cl}^-\) indicator, MQAE, was found to be around 9 mM (authors’ unpublished data). Thus, we propose that the \(\text{Cl}^-\) influx through \(\text{Cl}_{\text{Ca}}\) channels may be a dominant mechanism to control electrical and secretory activity of melanotrophs.

In summary, we examined the role of cytosolic \(\text{Cl}^-\) ions in the regulation of electrical and secretory activity in mouse melanotrophs from fresh tissue slices. We show that a major mechanism of \(\text{Cl}^-\) action in melanotrophs, as in other endocrine cells, is the \(\text{Cl}^-\) influx into exocytotic granules during the maturation steps preceding vesicle fusion. We further demonstrate that modulation of vesicle priming by hypoglycaemic sulphonylureas, a group of substances widely used in the treatment of diabetes, also takes place in melanotrophs; however, sulphonylureas would chronically reduce peptide hormone secretion. We conclude that, although distinct neuroendocrine cell types show similarities in the regulation of secretion, the underlying mechanisms may not be identical. The \(\text{Cl}_{\text{Ca}}\) and \(\text{GABA}_B\) currents are major regulators of \(\text{Cl}^-\) homeostasis in melanotrophs. It is possible that subplasmalemmal \([\text{Cl}^-]\) concentrations in the form of putative ‘\(\text{Cl}^-\) microdomains’ can account for the modulatory effect of \(\text{Cl}^-\) on exocytosis and endocytosis.

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