Introduction

Priming of secretory vesicles prior to Ca<sup>2+</sup>-induced exocytosis involves pairing of SNARE proteins on the vesicles and in the target membrane and biochemical loading by ATP-hydrolysis. Different priming enzymes have been proposed such as N-ethylmaleimide Sensitive Factor (NSF) (Hanson et al., 1997; Sutton et al., 1998) and phosphatidylinositol-4-phosphate-5-kinase (PtdInsP5K) (Hay et al., 1995), but the exact nature of the priming reaction in pancreatic B-cells remains elusive. Insulin secretion is indeed highly dependent on ATP (Eliasson et al., 1997) but NSF failed to reconstitute secretion in insulin-secreting HIT-T15 cells in which the endogenous protein was inactivated by different maneuvers (Kiraly-Borri, 1996). We have previously reported that hypoglycemic sulfonylureas enhance Ca<sup>2+</sup>-dependent exocytosis in pancreatic B-cells (Eliasson et al., 1996). This action is mediated by a 65 kDa granular mdr1-like protein and depends on intracellular Cl<sup>-</sup> fluxes (Barg et al., 1999). The identity and precise role of such Cl<sup>-</sup> fluxes in exocytosis remain elusive, but they are known to play important roles in the pH regulation of cellular organelles (al-Awqati et al., 1992), including exocytotic granules. Mature secretory granules, including those of the B-cell, are in fact strongly acidic with a pH<sub>in</sub>=5 and it is thought that a low intragranular pH is important for processing of the pro-hormone (Hutton, 1989).

Here we report that granular Cl<sup>-</sup> uptake is important not only for the stimulation of exocytosis by sulfonylureas, but also for Ca<sup>2+</sup>-induced B-cell exocytosis in general. Influx of Cl<sup>-</sup> through granular CIC-3 Cl<sup>-</sup> channels stimulates exocytosis by facilitating H<sup>+</sup>-pumping into the granules by a bafilomycin-sensitive V-type H<sup>+</sup>-ATPase. This Cl<sup>-</sup> flux prevents the development of a large electrical gradient over the granule membrane (positive inside) that would otherwise prevent H<sup>+</sup>-pumping and acidification. We further demonstrate that granule acidification is a decisive step in the ATP-dependent priming of insulin granules for exocytosis. Consistent with this idea is the fact that agents that collapse the pH gradient or prevent Cl<sup>-</sup> uptake largely abolish Ca<sup>2+</sup>-induced exocytosis. ATP and ADP reciprocally regulate the CIC-3 channels and their activity increases when the cytoplasmic ATP:ADP ratio is high. By this mechanism, the secretory capacity of the B-cell is influenced by the metabolic state of the cell and this represents an extra level of metabolic regulation of insulin in addition to that mediated by the ATP-regulated K<sup>+</sup>-channels (Ashcroft and Rorsman, 1995). This is functionally significant as it explains why metabolic stimuli exert a stronger stimulatory effect than those merely acting by membrane depolarization (Gembal et al., 1993).

Materials and Methods

Antibody production and expression of hCIC-3

Antibody production and purification was performed by Quality Controlled Biochemicals, Inc. (Hopkinton, MA, USA). Two synthetic peptides coupled with Keyhole limpet hemocyanin (KLH, Pierce, IL, USA) were used to raise antibodies against hCIC-3 in rabbits. The

Summary

ATP-dependent priming of the secretory granules precedes Ca<sup>2+</sup>-regulated neuroendocrine secretion, but the exact nature of this reaction is not fully established in all secretory cell types. We have further investigated this reaction in the insulin-secreting pancreatic B-cell and demonstrate that granular acidification driven by a V-type H<sup>+</sup>-ATPase in the granular membrane is a decisive step in priming. This requires simultaneous Cl<sup>-</sup> uptake through granular CIC-3 Cl<sup>-</sup> channels. Accordingly, granule acidification and priming are inhibited by agents that prevent transgranular Cl<sup>-</sup> fluxes, such as 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and an antibody against the CIC-3 channels, but accelerated by increases in the intracellular ATP:ADP ratio or addition of hypoglycemic sulfonylureas. We suggest that this might represent an important mechanism for metabolic regulation of Ca<sup>2+</sup>-dependent exocytosis that is also likely to be operational in other secretory cell types.

Key words: CIC-3 channels, Exocytosis, Granular pH, Insulin, Sulfonylureas
peptides corresponded to amino acids 59-74 (MTNGGSINSS-
THLLDLC) of the N-terminal domain of hClC-3 and amino acids 730-744 (GSSRVCFAQHTPSLPAE) of the cytoplasmic C-terminal domain. The C-terminal cysteine in the N-terminal sequence and the alanine and glutamic acid residues in the C-terminal sequence were added in order to facilitate antigenicity. After a second boost, anti-hClC-3,74 (anti-hClC-3n) serum was affinity purified using peptide coupled to thiol coupling gel (Quality Controlled Biochemicals, Inc.). The bound IgG was eluted with 3 M MgCl₂, 25% ethylene glycol and immediately neutralized to pH 7.4 with divalent-free PBS. The stock concentration was 0.072 mg/ml. Anti-hClC-3,
730-744 (anti-hClC-3c) serum was affinity purified with peptide bound to amino-linked Sepharose gel and eluted with 0.1 M glycine. The stock concentration was 1.03 mg/ml.

The full-length hClC-3 was subcloned into pcDNA3.1zeo+ vector (Invitrogen, CA, USA) and transfected into large-T-antigen-stabilized HEK 293 cells (tsA) using a SuperFect reagent kit (Qiagen, CA, USA). Stably transfected clonal cell lines were selected using zeocin (Invitrogen, CA, USA) at 400 μg/ml and maintained at 200 μg/ml. Expression of hClC-3 was detected by immunoblotting and the homogeneity of the stable cell lines was confirmed by immunostaining. HEK 293 cell lines stably expressing hClC-1, hClC-4 and rat ClC-2 were kindly provided by Christoph Fahlke at the University of Cincinnati College of Medicine (rat ClC-2), respectively. The expression of hClC-1, hClC-4 and rat ClC-2 was confirmed by immunoblotting and electrophysiology studies.

Immunoblot analysis
Cultured stably transfected tsA cells were harvested in H20E1D1 solution (20 mM Hepes, pH 8.0, 1 mM EDTA and 1 mM dithiothreitol) in the presence of protease inhibitor cocktail (Boehringer-Mannheim, IN, USA). In cells stably expressing hClC-3, the membrane and cytosolic fractions were crudely separated by centrifugation of the whole-cell lysate at 70,000 rpm for 30 minutes at 4°C using Optima™ TL ultracentrifuge (Beckman Instruments, Inc., CA, USA). For the evaluation of antibody specificity, whole-cell lysate of HEK 293 cells expressing hClC-1, hClC-4 or rat ClC-2 was used. Protein denaturation was accomplished in 1/10 V of 10% SDS and 1 M DTT at 95°C for 10 minutes. The protein thus obtained (100 μg) was incubated with 5 of N-glycanase F (Oxford GlycoSciences Ltd, UK) for 18 hours at 37°C. Total protein (10 or 100 μg) was loaded on 7.5% SDS-PAGE gels, separated by electrophoresis and transferred to polyvinylidene difluoride membrane. Blots were incubated overnight at 4°C with the affinity-purified polyclonal anti-hClC-3n at 1:500 dilution, or anti-hClC-3c at 1:6000 dilution, and treated subsequently with donkey anti-rabbit purified polyclonal anti-hClC-3n at a 1:500 dilution, or anti-hClC-3c

Electron microscopy
Intact pancreatic islets were fixed in 4% paraformaldehyde supplemented with 0.5% glutaraldehyde for 1 hour and embedded in Lowicryl® to before being cut into ultrathin sections (60-80 nm) using an LKB MKIII ultratome. Immunoreactivity was detected using the anti-hClC-3c-antibody at 1:200 dilution and a donkey anti-rabbit secondary antibody conjugated with 12 nm gold particles, and visualized using a Philips CM10 electron microscope. The density of gold particles was calculated using software package from Scion Corporation, MD, USA.

To determine the number of lysosomes per B-cell, the volume density was estimated according to procedures of DeHoff and Rhines (1961), and averaged 0.035±0.003 lysosomes/μm³. By multiplying this value with the average B-cell volume (1376±84 μm³) an estimate of the number of lysosomes per B-cell was obtained.

Cells and electrophysiology
Mouse pancreatic B-cells were isolated and exocytosis monitored by combining the patch-clamp technique with measurements of cell capacitance as previously described (Barg et al., 1999). The standard whole-cell configuration was used in all experiments and the cells were voltage-clamped at –70 mV to prevent activation of the voltage-gated Ca²⁺ channels. Exocytosis was elicited either by infusion of a Ca²⁺-EGTA buffer through the recording electrode (see Figs 1, 4, 6A) or by trains of ten 500-millisecond voltage-clamp depolarizations (rate: 1 Hz) from the holding potential to zero (Fig. 7A,B).

Solutions
The extracellular solution consisted of (in mM) 138 NaCl, 5.6 KCl, 1.2 MgCl₂, 2.6 CaCl₂, 5 D-glucose and 5 Hepes (pH 7.4 with NaOH). In Fig. 5, a Ca²⁺-free extracellular solution, supplemented with 200 μM EGTA, was used to prevent initiation of exocytosis by the addition of tolbutamide. In Fig. 7A,B, 20 mM of NaCl was replaced equimolarly with TEA-Cl to facilitate the detection of the Ca²⁺ currents. All measurements were carried out at 32°C except those of Fig. 5 and 6C,F, which were performed at room temperature. The standard electrode (intracellular) solution (Figs 1, 4, 6A,B) contained (in mM) 125 K-glutamate, 10 KCl, 1 MgCl₂, 5 Hepes, 10 EGTA, 3 Mg-ATP, 0.1 cAMP and 9 CaCl₂ (pH 7.15 with KOH). The free [Ca²⁺] was estimated as 1.5 μM using the binding constants of Martell and Smith (1974). All chemicals were from Sigma. Compounds were dissolved directly in the pipette solution, or added from fresh stock solutions (DSMO, final concentration <0.1%) as indicated. In Fig. 1C, the [Cl⁻] in the pipette was either increased to 165 mM by equimolar replacement of K-glutamate with KCl, or lowered to 2 mM by substituting KCl, NaCl and CaCl₂ with K-glutamate, Na-glucuronate and Ca(OH)₂, respectively. In Fig. 7A,B the electrode solution contained (in mM) 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 MgCl₂, 0.05 EGTA, 3 Mg-ATP, 0.1 cAMP and 9 CsCl (pH 7.15 using CsOH). 5 mM Mg-ADP, 0.1 mM tolbutamide, anti-hClC-3c (1:2000) or anti-hClC-3n (1:500) was added to the electrode solution as indicated.

Measurements of granular pH
Granular pH was monitored semi-quantitatively by supplementing the extracellular solution with 5 μM of the fluorescent probe Lysosensor™Green DND-189® (Molecular Probes), which is
fluorescent only in acidic compartments. Excitation was effected using the 488 nm line of a Zeiss 510 confocal microscope and emitted light collected using a 63x/1.3NA oil objective and a >505 nm filter. It was ascertained that the initial fluorescence signal was stable before the experiments commenced and that the initial signal, using identical microscope settings, was similar in all experiments. The fluorescence was measured over the entire cell with low spatial resolution to prevent photo-bleaching of the probe. The data are presented as changes of LysoSensor-fluorescence (in per cent) over initial fluorescence after addition of tolbutamide (Fig. 5), or establishment of the standard whole-cell configuration and dialysis of cell interior (Fig. 6C-F). The latter experiments were performed at room temperature (22°C), where exocytosis is largely abolished (Renström et al., 1996), to exclude the possibility of an artefactual decrease in measured granular pH caused by cell swelling during exocytosis.

**Data analysis**

In Figs 1, 4 and 6, rates of increases in cell capacitance and changes in LysoSensor-fluorescence were determined for the first minute after establishment of the standard whole-cell configuration. In Fig. 7A,B, the increases in cell capacitance elicited by the entire trains are expressed as in (A) at 165 mM (n=10), 40 mM (n=8), and 2 mM (n=9) intracellular [Cl\(^-\)] as indicated. (D) Average ΔC/Δt ± s.e.m. Statistical significance was evaluated using ANOVAR followed by Dunnet’s test.

**RESULTS**

**Intracellular Cl\(^-\) fluxes and exocytosis**

Hypoglycemic sulfonylureas potentiate Ca\(^{2+}\)-dependent exocytosis by an action that involves intracellular Cl\(^-\) fluxes (Barg et al., 1999). We now explore the role of Cl\(^-\) fluxes in Ca\(^{2+}\)-induced exocytosis in general. Fig. 1A shows the exocytotic responses obtained using the standard whole-cell configuration in which the pipette-filling electrode solution replaces the cytosol. Exocytosis was evoked by the inclusion of a Ca\(^{2+}\)/EGTA-buffer (free intracellular [Ca\(^{2+}\)]) 1.5 μM) to the pipette solution, which was also supplemented with 3 mM ATP and 0.1 mM cAMP to maximize the exocytotic response (Renström et al., 1996). Under control conditions, the rate of capacitance increase averaged 32±1 fF/s during the first 60 seconds. This corresponds to the release of 19 granules/s as every granule contributes 1.7 fF (Åmmålå et al., 1993). Inclusion of 10 μM of the Cl\(^-\) channel blocker DIDS in the pipette solution reduced the rate of capacitance increase by 80% (Fig. 1A,B). Niflumic acid (100-500 μM), an inhibitor of Ca\(^{2+}\)-activated Cl\(^-\) channels, likewise inhibited exocytosis, albeit less potently. Other Cl\(^-\) channel blockers such as 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 9AC were ineffective (not shown). To exclude the possibility that the effects of DIDS and niflumic acid are due to actions unrelated to inhibition of Cl\(^-\) channels, we varied the cytoplasmic Cl\(^-\) concentration (Fig. 1C,D). Lowering the cytoplasmic Cl\(^-\) concentration ([Cl\(^-\)]) from the standard 40 mM to 2 mM reduced exocytosis by 93%. Increasing [Cl\(^-\)] to 165 mM tended to increase (=15% stimulation, not statistically different from control rate) exocytosis beyond that observed at 40 mM. Varying the extracellular Cl\(^-\) concentration between 150 mM and zero did not influence the rate of exocytosis (not shown). Substituting Cs\(^+\) or N-methyl-D-glucamine\(^+\) for K\(^+\) as the major intracellular cation only marginally affected exocytosis (not shown), as did intracellular addition of the broad-spectrum K\(^+\)-channel blocker tetraethylammonium\(^+\) (TEA, not shown). The data suggest the participation of (granular) Cl\(^-\), but not K\(^+\)-channels, in exocytosis.

**Granular ClC-3 channels in the B-cell**

To detect Cl\(^-\) channels in mouse insulin granules, we produced affinity-purified polyclonal antibodies directed against different cytoplasmic epitopes of the ClC-3 Cl\(^-\) channel. The antibodies anti-hClC-3n and anti-hClC-3c, raised against synthetic peptides corresponding to residues 59-74 in the N-terminus and residues 730-744 in the C-terminal region of hClC-3, respectively, were evaluated in large-T-antigen stabilized HEK 293 cells (tsA) stably expressing full-length human ClC-3 (Fig. 2A). Anti-hClC-3c detected a 120 kDa protein in membrane isolates. After deglycosylation by digestion with N-glycanase for 18 hours, both antibodies detected a single ~90 kDa immunoreactive band, which is close to the 88 kDa predicted for the hClC-3. It was verified that the antibodies specifically interact with ClC-3 by immunoblotting in HEK 293 cells stably transfected with hClC-1, hClC-3, hClC-4 or rat ClC-2 (Fig. 2B).

ClC-3 expression in mouse pancreatic islets was investigated by immunoblotting. Both anti-hClC-3n (Fig. 2A) and anti-hClC-3a (Fig. 2C) detected the glycosylated protein, and to a minor extent the deglycosylated form of hClC-3 (Fig. 2A). In factorized insulin-secreting cells, ClC-3 immunoreactivity was confined to the secretory granules (not shown). Because of the scarcity of material these experiments were made using clonal RINm5F cells. The subcellular localization of ClC-3 immunoreactivity in B-cells was detected by confocal immunofluorescence and revealed a granular distribution. The co-localization with insulin immunoreactivity was 73% (Fig. 3A), reflecting the fact that, although insulin is released upon exocytosis, ClC-3 is retained in the granule membrane. The B-
Cell distribution of the ClC-3 channels was also investigated by electron microscopy combined with immunogold labeling of the binding sites (Fig. 3B). In the B-cells, ClC-3 primarily localized to the insulin-containing large dense core granules. The immunogold density in the secretory granules (39±2 particles/μm²) was fivefold higher than the background activity (8±3 particles/μm²; P<0.001 vs in the insulin granules, n=11). These results confirm that ClC-3 channels are expressed in mouse B-cells and preferentially localize to the insulin granules. No expression of ClC-2 channels was detectable (not shown).

**Importance of granular pH for exocytosis**

Granular Cl⁻ uptake has previously been suggested to promote exocytosis by an osmotic mechanism resulting in increased hydrostatic pressure inside the granule (Zimmerberg and Whittaker, 1985; Day and Hinkle, 1988). However, exocytosis was unaffected by elevating the cytosolic osmolarity with 0.3-1 M sucrose (Fig. 4A,B) or mannitol (not shown), which should counteract or even reverse possible osmotic gradients over the granular membrane. It is therefore unlikely that osmotic effects participate in the stimulation of exocytosis via Cl⁻ fluxes and alternative mechanisms must therefore be considered. It is known that insulin-containing secretory granules acidify during their course of maturation (Hutton, 1989) and Cl⁻ fluxes have been implicated in cellular and organellar pH regulation by charge neutralization (Al-Awquati et al., 1992). A possible role of granular pH was tested by supplementing the electrode solution with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) (100 μM) and this maneuver inhibited Ca²⁺-induced secretion by 80% (Fig. 4A,B). It should be noted that the electrode contained 3 mM ATP and the suppression of exocytosis can therefore not be accounted for by inhibition of mitochondrial ATP production. Importantly, the ability of CCCP to suppress exocytosis is was dependent on cytoplasmic pH. At pH 6.6, CCCP only reduced exocytosis by 45%, from 24±5 fF/s (n=5) to 13±4 fF/s (n=4). Inhibition of exocytosis was likewise observed in the presence of the K⁺/H⁺-exchanger nigericin (1 μM) (Fig. 4A,B) or after addition of bafilomycin (200 nM), an inhibitor of the vesicular H⁺-ATPase (Fig. 4C,D). Inhibition of

![Image](https://example.com/image.png)

**Fig. 2.** Antibody characterization and ClC-3 expression in mouse B-cells. Immunoblots of mouse islet homogenate and cells overexpressing ClC-3 isoforms. (A) Whole-cell lysates (10 μg/lane), crude membrane and cytosol isolates (100 μg/lane) were prepared from tsA cells stably transfected with full-length hClC-3 or vector alone (mock), or from mouse islets. Anti-hClC-3c detected a 120 kDa protein in the membrane fractions of the transfected cells. After deglycosylation with N-glycanase for 18 hours immunoreactivity was detected as a single band at ≈90 kDa in the tsA cells. In islets, both the glycosylated form (double band at 115-130 kDa) as well as the deglycosylated protein (≈90 kDa) were detected. (B) Isoform specificity of anti-hClC-3c was verified by immunoblotting of deglycosylated whole-cell lysates (10 μg/lane) obtained from HEK 293 cells stably expressing hClC-3, hClC-1, hClC-4 or rat ClC-2. (C) and (D), as in (A) and (B), except that anti-hClC-3n was used.

![Image](https://example.com/image.png)

**Fig. 3.** ClC-3 channels localize to the B-cell granule membrane. (A) Distribution of anti-hClC-3c binding, insulin immunoreactivity and overlay of anti-hClC-3c and insulin immunoreactivity visualized by confocal immunocytochemistry. Bar, 2 μm. (B) Immunogold detection of ClC-3 localization to insulin-containing secretory granules (SG) visualized by electron microscopy. The 12 nm gold particles appear as small black dots (arrow). Bar, 100 nm. Ly, lysosome.
Fig. 4. Exocytosis requires granular acidic pH. (A) Capacitance increases (ΔC) elicited by infusion of the Ca\(^{2+}\)- and ATP-containing control electrode solution (ctrl, n=21), after addition of sucrose (sucr, n=6) and in the presence of CCCP (n=6) or nigericin (nig, n=5). (B) Average rates of capacitance increase (ΔC/Δt) ± s.e.m. (C) ΔC elicited by the control solution (ctrl, n=9), in the presence of the Na\(^+/\)H\(^+\)-exchanger inhibitor EIPA (n=10) and the v-1'-H\(^+\)-ATPase inhibitor bafilomycin (bafi, n=11). (D) Mean ΔC/Δt ± s.e.m.

Na\(^{+}\)/H\(^+\) exchanger activity did not significantly affect Ca\(^{2+}\)-induced secretion. Although ethylisopropylamilorid (EIPA), tended to suppress exocytosis by ~20% (ns vs. control; Fig. 4C,D), another inhibitor 3-methylsulfonyl-4-piperidinobenzoylguanidine methanesulfonate (HOE694) was completely without effect (not shown).

Modulation of granular pH by hypoglycemic sulfonylureas

Tolbutamide, an inhibitor of B-cell K\(_{\text{ATP}}\) channels that acts by binding to the sulfonylurea receptor SUR1, stimulates exocytosis in pancreatic B-cells at a stage distal to the elevation of [Ca\(^{2+}\)]\(_{\text{i}}\). This effect depends on activation of intracellular Cl\(^{-}\) fluxes and is inhibited by DIDS (Barg et al., 1999). These earlier observations, taken together with the findings shown in Figs 1, 3 and 4, suggest that the stimulatory effect of tolbutamide on Ca\(^{2+}\)-dependent exocytosis may involve an effect on secretory granule pH. In Fig. 5 we have tested whether tolbutamide affects intragranular pH by using the fluorescent probe LysoSensor Green DND-189, which reveals a pH-dependent fluorescence. Isolated B-cells were perfused using a Ca\(^{2+}\)-free extracellular solution to prevent initiation of insulin secretion. After the addition of 100 μM tolbutamide to the perfusate the fluorescence signal increased promptly, indicating further acidification of the insulin granules. On average, tolbutamide increased the initial fluorescence intensity by 35±11% (P<0.01; n=9). To verify that changes in LysoSensor Green fluorescence accurately report the intragranule pH, we estimated the number of lysosomes per B-cell by electron microscopy (not shown). The average B-cell contained only 48±7 (n=14) lysosomes, which is negligible compared to the ≈11,000 insulin granules estimated by Dean (1973).

Effects of granular Cl\(^{-}\) channel modulation on granular pH and exocytosis

The stimulatory action of tolbutamide on Ca\(^{2+}\)-elicited exocytosis can be antagonized by diazoxide, an activator of K\(_{\text{ATP}}\)-channels (Barg et al., 1999). Fig. 6A,B shows that intracellularly applied diazoxide (0.1 mM) also inhibited Ca\(^{2+}\)-induced exocytosis by 44%. Pinacidil, an activator of vascular K\(_{\text{ATP}}\)-channels containing the SUR2A sulfonylurea receptor (Shindo et al., 1998), was ineffective (not shown).

B-cell K\(_{\text{ATP}}\)-channels are physiologically regulated by the ADP:ATP ratio via SUR1. It is interesting that the ATP:ADP ratio also modulates Ca\(^{2+}\)-elicited exocytosis in a similar fashion. After supplementing the intracellular solution with Mg-ADP (5 mM) in the presence of Mg-ATP (3 mM), Ca\(^{2+}\)-induced secretion was strongly inhibited (75%; green trace and bar in Fig. 6A,B). Thus, access to ATP alone is not sufficient to sustain exocytosis. For comparison, the effect of DIDS (cf. Fig. 1A,B) has been included (magenta trace and bar in Fig. 6A,B). The inhibitory action of ADP was fully counteracted by tolbutamide, and the rate of capacitance increase was the same as that seen under control conditions (navy blue trace and bar in Fig. 6A,B). Tolbutamide was ineffective when applied under conditions associated with close to maximum priming (intracellular solution containing 3 mM ATP, 0.1 mM cAMP and 1.5 μM Ca\(^{2+}\); Renström et al., 1997). In a separate series of experiments, the rate of exocytosis measured in the presence of tolbutamide averaged 97±12% (n=5) of that seen under control conditions. The same negative observations were made when priming was suppressed by withdrawal of cytoplasmic ATP (not shown). These data allow us to discard the possibility that tolbutamide stimulates exocytosis by an unspecific mechanism. They further suggest that tolbutamide is only effective under conditions that permit priming to proceed at a submaximal velocity.

If the compounds used in Fig. 6A,B act by modulating granular Cl\(^{-}\) fluxes and thus proton pump activity, then their effects on granular pH should echo the changes in exocytotic capacity. In the experiments shown in Fig. 6C,D we monitored intragranular pH using LysoSensor Green DND-189. Following establishment of the whole-cell configuration and intracellular dialysis with 3 mM ATP and 1.5 μM [Ca\(^{2+}\)]\(_{\text{i}}\) (i.e. control conditions in Fig. 1; red trace and bar), the signal increased slightly during the first minute, indicating a net
Fig. 6. $K_{ATP}$-channel effectors modulate exocytosis by influencing granular pH. (A) Increases in cell capacitance ($\Delta C$) under control conditions (red, $n=26$), after addition of Mg-ADP (green, $n=12$), tolbutamide and Mg-ADP (blue, $n=12$), diazoxide (cyan, $n=19$) or DIDS (magenta, $n=8$). (B) Average rate of changes in cell capacitance ($\Delta C/\Delta t$) ± s.e.m. ***$P<0.01$; ****$P<0.001$. (C) Changes in granular pH estimated as the relative changes (in per cent) of the initial LysoSensor-fluorescence intensity ($F/F_0$) after establishment of the standard whole-cell configuration, using the $Ca^{2+}$- and ATP-containing control solution (red, $n=5$), after addition of ADP (green, $n=5$), tolbutamide and ADP (blue, $n=5$) and DIDS (magenta, $n=5$). The arrow indicates the establishment of the standard whole-cell configuration. (D) Average changes (per cent of initial) in LysoSensor fluorescence after 60 seconds recording ($\Delta F/F_0$). Data are mean values ± s.e.m. ***$P<0.001$. Statistical significances were evaluated comparing the responses in the respective groups with the responses obtained with the ATP-containing control (red). (E), as in (C), but after supplementing the control pipette solution with: CCCP (red, $n=14$), ATP and CCCP (green, $n=9$), tolbutamide, ADP and CCCP (blue, $n=10$), diazoxide and CCCP (cyan, $n=7$), DIDS and CCCP (magenta, $n=5$), anti-hClC-3c and CCCP (yellow, $n=9$) and anti-hClC-3n and CCCP (brown, $n=6$). The arrow indicates the establishment of the standard whole-cell configuration. (F), as in (D), but statistical significance was evaluated by comparing the responses in the respective groups with the responses obtained when CCCP was added to the control solution (red). dz, diazoxide; tb, tolbutamide. Data are mean values ± s.e.m. **$P<0.01$, ***$P<0.001$.

granule acidification under these conditions. Intragranular acidification was almost abolished by the inclusion of either 100 $\mu$M DIDS (magenta trace and bar in Fig. 6C,D) or 5 mM Mg-ADP (green trace and bar in Fig. 6C,D) in the pipette solution. Interestingly, inclusion of tolbutamide on top of Mg-ADP (navy blue trace and bar in Fig. 6C,D), restored granule acidification, thereby mimicking the effects of the compound on exocytosis. The same results were observed when a $Ca^{2+}$-free pipette solution was used, suggesting that the regulation of granular Cl$^-$ fluxes and pH is not affected by [$Ca^{2+}$].

To ascertain that the effects on granule pH observed in Fig. 6C,D are secondary to an effect on granular Cl$^-$ uptake and not the result of a direct action on proton uptake, the protonophore CCCP was introduced to the cytosol (Fig. 6E,F). In keeping with the idea that the interior of the granule is acidic (pH=5; Hutton, 1989), addition of CCCP (100 $\mu$M; red trace and bar in Fig. 6E,F) to the pipette solution reduced the fluorescence as the granular pH approached that of the cytosol (pH=7.15). CCCP-mediated H$^+$ efflux from the granule is electrogenic. In the absence of a counterion conductance a large electrical potential will rapidly develop when the H$^+$ permeability is increased and this can be envisaged to prevent pH equilibration across the granule membrane. If CIC-3 Cl$^-$ channel activity is required for proton translocation over the granule membrane, then Cl$^-$ channel inhibition should also affect H$^+$-efflux via the protonophore. Indeed, when the Cl$^-$ channel inhibitor DIDS (magenta trace and bar in Fig. 6E,F) was supplemented to the ATP- and $Ca^{2+}$-containing pipette solution, the decrease in fluorescence evoked by CCCP was prevented. This effect of DIDS was mimicked by diazoxide (cyan trace and bar), Mg-ADP (green) and by anti-hClC-3c (yellow), whereas anti-hClC-3n was ineffective (brown). Tolbutamide counteracted the inhibitory action of ADP on collapsing the pH gradient fully, and in the simultaneous presence of CCCP, ADP and tolbutamide (navy blue trace and bar in Fig. 6E,F), the rate of fluorescence was almost identical to that observed in the presence of CCCP alone. Taken together, these results strongly support the idea that Cl$^-$ ions act as counterions for H$^+$ transport over the granule membrane and thereby modulate the secretory capacity of the B-cell.

Granular pH controls the replenishment of secretory granules for release

Secretory granules in B-cells can be divided into the readily releasable pool (RBP) and the reserve pool (Renström et al., 1996; Renström et al., 1997; Eliasson et al., 1997). The process in which granules proceed from the reserve pool into the RRP is referred to as mobilization and involves priming by ATP hydrolysis. To determine at which stage granular ion fluxes mediate exocytosis, we recorded the secretory responses elicited by trains of ten 500 millisecond voltage-clamp depolarizations to 0 mV using the standard whole-cell configuration. Mg-ADP, tolbutamide, anti-hClC-3c, anti-hClC-3n or CCCP were added as indicated in Fig. 7A.B. Under control conditions (3 mM Mg-ATP and 0.1 mM cAMP), exocytosis proceeded throughout the train and the total capacitance increase averaged 36±36fF. Consistent with the observations in Fig. 6A,B, addition of Mg-ADP (5 mM) reduced the
Fig. 7. Granular Cl\(^{-}\) fluxes and pH affect the recruitment of secretory granules for release. (A-B) Increases in cell capacitance (ΔC) evoked by a train of ten voltage-clamp depolarizations from −70 mV to zero (500 milliseconds, 1 Hz) under control conditions (n=9), after addition of Mg-ADP (n=4), in the presence of simultaneously added tolbutamide (tb) and Mg-ADP (n=6), after inclusion of anti-hClC-3c (n=7), anti-hClC-3n (n=17) or CCCP (n=6) as indicated. (B) Average total increases in cell capacitance (ΔC) ± s.e.m. for experiments in (A). *P<0.05; **P<0.01, ***P<0.001.

The magnitude of the capacitance increased evoked by the train by 72% (100±16 fF). This effect was counteracted by tolbutamide (0.1 mM) and the magnitude of the capacitance increase evoked by the train averaged 258±54 fF. It is of interest that the capacitance increase evoked by the first depolarization, reflecting mainly exocytosis of RRP, was not much affected by ADP or tolbutamide and averaged ~100 fF under all conditions. Thus, ADP seems to interfere with the refilling of RRP, but not with exocytosis of granules that have already progressed into this pool.

Inhibition of Cl\(^{-}\) fluxes by intracellular application of the antibody anti-hClC-3c had effects similar to those produced by ADP and reduced the total increase in cell capacitance during the train to 29% of that seen under control conditions. Again, the capacitance increase evoked by the first pulse was only marginally affected. Similar results were obtained with DIDS (not shown). We considered the possibility that anti-hClC-3c exerts its inhibitory effect by steric hindrance. However, this seems unlikely because supplementing the pipette solution with anti-hClC-3n failed to affect B-cell exocytosis. The latter antibody also recognizes a cytoplasmic epitope of ClC-3 while not (unlike anti-hClC-3c) affecting the Cl\(^{-}\) currents flowing through the channel (Huang et al., 2001). We also tested the role of granular pH in rapid and sustained depolarization-evoked exocytosis by supplementing the pipette solution with CCCP (100 μM). Under these conditions, the protonophore abolished the late component of release whereas exocytosis evoked by the first depolarization was reduced by 70% (Fig. 7A,B).

The different exocytotic responses evoked by the trains cannot be accounted for by a decrease in the Ca\(^{2+}\) current as the integrated currents were essentially unaffected by the experimental maneuvers throughout the trains. The total Ca\(^{2+}\)-current charge entry during the train averaged 48±12 pC (n=9), 62±33 pC (n=4), 65±33 pC (n=6), 59±22 pC (n=7), 53±5 pC

Fig. 8. Model for the molecular mechanisms underlying regulated granular Cl\(^{-}\) fluxes and their actions on intragranular pH and exocytosis. (A) Replenishment of the RRP of secretory granules depends on intragranular acidification. (B) Metabolically regulated granular Cl\(^{-}\) uptake through an ion channel complex comprising of ClC-3 Cl\(^{-}\) channels (grey) and a mdr1-like 65 kDa regulatory protein (white), determines the rate of the intragranular pH decrease driven by the V-type H\(^{+}\)-ATPase (black). Tolbutamide (tb), diazoxide (dz) and ADP are likely to exert their actions by interacting with the regulatory 65 kDa protein, whereas ATP, DIDS and anti-hClC-3c bind to CIC-3 directly. See text for details.
DISCUSSION

The recruitment of secretory granules to the RRP by ATP-dependent priming is essential for sustained secretory activity in endocrine cells and neurons. The cytosolic ATPase NSF has previously been implicated in this process (Hanson et al., 1997), but increasing evidence suggests the participation of additional or other mechanisms in the insulin-releasing B-cell (Kiraly-Borri et al., 1997). Here we propose that the acidification of the granule interior by a V-type H+-ATPase represents a novel ATP-dependent reaction in priming. A few particularly interesting aspects of this finding will be discussed below.

Cl⁻ uptake controls granular acidification and exocytosis

It seems safe to conclude that Cl⁻ fluxes are involved in the priming of secretory granules for Ca²⁺-elicited exocytosis in the pancreatic B-cell. Replacing cytosolic Cl⁻ with larger anions, pharmacological interference with intracellular Cl⁻ channels and application of a specific antibody against hCIC-3 all severely affected replenishment of the RRP, but not exocytosis per se. This does not seem to be limited to the B-cell, as Cl⁻ is required for hormone release in a variety of other cells, like pancreatic acinar cells (Fuller et al., 1989) and pituitary melanotrophs. In the latter cell type, an apparent K₉ of 12 mM was reported (Rupnik and Zorec, 1992). Influx of Cl⁻ into the granule can be envisaged to have osmotic and electrochemical effects. The latter effect appears more important in this context given the present observations that exocytosis was strongly suppressed by agents interfering with the granular pH, such as CCCP and baflomycin, whereas it was resistant to inverting the osmotic gradient over the granular membrane (Fig. 4).

Both exocytosis and H⁺ fluxes over the granular membrane were stimulated by tolbutamide, but inhibited by DIDS, Mg-ADP and diazoxide (Figs 5, 6). Interestingly, a similar pharmacology has been reported for a Cl⁻ conductance in exocrine pancreatic zymogen granules (Thévenod et al., 1992), which depends on the activity of an ion channel complex consisting of a 65 kDa regulatory protein and CIC-2 channels (Braun et al., 1997; Carew and Thorn, 1996). This links granular Cl⁻ uptake to the control of intragranular pH and thereby exocytosis, and argues that the physiological and pharmacological modulators of exocytosis (such as ADP and tolbutamide) act via changes of the intragranular pH.

How can H⁺ pumping by the V-type ATPase, or H⁺-efflux via CCCP (Fig. 5), be controlled by the granular Cl⁻ conductance? In this context it is worth considering that the proton transport over the granular membrane results in the development of a considerable membrane potential. The development of a positive voltage inside the granule can be expected to prevent H⁺ pumping before substantial acidification has occurred (al-Awqati et al., 1992). For example, even in an unbuffered sphere of insulin granule size (diameter 0.2 μm), a reduction in the pH from 7.15 to 5 (requiring translocation of only 20 protons) produces an electrical potential of 135 mV across the granular membrane. The fact that the insulin granule interior is indeed highly buffered (Hutton, 1989) only emphasizes the necessity of a granular counter conductance for acidification to proceed. Passive Cl⁻ fluxes over the granular membrane may well serve this role in the pancreatic B-cell because the cytoplasmic [Cl⁻] in vivo is 75 mM (Sehlin, 1978), which is marginally higher than estimates of the intragranular concentration (Foster et al., 1993). Theoretically, the same effect can be achieved by cations moving out of the granules. Our experimental approach does not allow us to unequivocally discard the involvement of a granular cation conductance in the modulation of intragranular pH. However, the fact that the broad K⁺-channel inhibitor TEA has little effect on Ca²⁺-elicited exocytosis (not shown), taken together with the pronounced effects of intervening with the granular Cl⁻ fluxes, indicates that granular Cl⁻ uptake is quantitatively more important in this respect.

Regulation of granular Cl⁻ fluxes, pH and exocytosis by K⁺-channel agonists

The mere presence of ATP is not sufficient to ensure protracted Ca²⁺-dependent secretion in the B-cell. In fact, addition of ADP in the continued presence of ATP potently inhibited exocytosis. This effect was mimicked by diazoxide and could be antagonized by tolbutamide (Figs 6, 7). We have previously reported that sulfonylureas directly stimulate exocytosis by a mechanism that depends on granular Cl⁻ fluxes and involves a 65 kDa mdr1-like protein in the vesicular membrane (Barg et al., 1999). A regulatory protein with identical properties has been studied in greater detail in zymogen granules. In these granules, Cl⁻ uptake is accelerated by ATP, tolbutamide and is inhibited by ADP or diazoxide (Thévenod et al., 1992; Braun et al., 1997). The present finding that H⁺ fluxes over the insulin granule membrane are strongly reduced by ADP, diazoxide and DIDS (Fig. 6C-F) clearly indicates the importance of such metabolically regulated granular Cl⁻ fluxes for the control of granular pH, and thus exocytosis. Interestingly, potentiation of Ca²⁺-dependent exocytosis by sulfonylureas was recently also found in catecholamine-secreting PC12 cells (Taylor, 1999), indicating that the mechanism may be widely applicable.

Priming of insulin granules for exocytosis by granule acidification

Agents that prevent granular Cl⁻ uptake or prevent acidification affected only the refilling of the RRP, whereas granules that had already proceeded into this pool remained releasable when [Ca²⁺] was subsequently elevated (Fig. 7). This suggests that priming of granules for exocytosis depends on intragranular acidification, and we propose that the ATP dependence of priming in B-cells also reflects the activity of the V-type H⁺-ATPase in the granule membrane. A role for low vesicular pH in the control of slow-phase glutamate release has recently been reported in synaptosomes (Zoccarato et al., 1999), suggesting that the mechanisms we describe here for the B-cell has its counterpart in other cells exhibiting regulated exocytosis. The finding that CCCP interferes with both the early (triggered by the first depolarization) and late components of exocytosis (Fig. 7A) further suggests that a low intragranular pH is required not only for the priming of the
granules but also for the maintenance of the granules in a releasable state. These observations are in good agreement with those made in other secretory cell types, like pituitary melanotrophs (Thomas et al., 1993), synaptosomes (Zoccorato et al., 1999) and glucagon-releasing pancreatic α-cells (Hoy et al., 2000), but appear at variance with early reports in permeabilized catecholamine-releasing chromaffin cells (Knight and Baker, 1985).

A model for metabolic modulation of rate of insulin granule priming

A model that accounts for our observations is presented in Fig. 8. The refilling of RRP depends on the priming of granules that originally belonged to the reserve pool. Our data suggest that the secretory granules gain release competence by intragranular acidification (Fig. 8A). This depends on the simultaneous operation of the V-type H+ -A TPase and the ClC-3 Cl− channel that originally belonged to the reserve pool. Our data suggest that the refilling of RRP depends on the priming of granules that (Knight and Baker, 1985).

REFERENCES


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