Original Paper

Pancreatology

Pancreatology 2005;5:443–449
DOI: 10.1159/000086556

Received: July 6, 2004
Accepted after revision: October 22, 2004
Published online: June 28, 2005

Patch Clamped Single Pancreatic Zymogen Granules: Direct Measurements of Ion Channel Activities at the Granule Membrane

Marie L. Kelly Rania Abu-Hamdah Aleksandar Jeremic Sang-Joon Cho Alina-Elena Ilie Bhanu P. Jena

Department of Physiology, Wayne State University School of Medicine, Detroit, Mich., USA

Key Words

Single secretory vesicle · Patch clamp · Ion channels

Abstract

Background/Aim: Pancreatic acinar cells are involved in the secretion of digestive enzymes. Digestive enzymes in pancreatic acinar cells are stored in membrane-bound secretory vesicles called zymogen granules (ZGs). The swelling of ZGs is implicated in the regulation of the expulsion of intravesicular contents during secretion. The molecular mechanism of ZG swelling has been previously elucidated. It has been further demonstrated that the water channel aquaporin-1, the potassium channel IRK-8, and the chloride channel CLC-2, are present in the ZG membrane and involved in ZG swelling. However, a direct measurement of these ion channels at the ZG membrane in intact ZGs had not been performed. The aim of this study was to investigate the electrical activity of single ZGs and verify the types of channels found within their membrane. ***Methods:*** ZGs from pancreatic acinar cells were isolated from the pancreas of Sprague-Dawley rats. Direct measurements of whole vesicle currents, in the presence and absence of ion channel blockers (quinine, glyburide and DIDS), were recorded following successful patching of single ZGs. ***Conclusion:*** In this study, we were able, for the first time, to patch single ZGs and study ion channels in their membrane. We were able to record currents across the ZG membrane and, utilizing ion channel blockers, confirm the presence of the chloride channels CLC-2 and the potassium channel IRK-8 (Kir6.1), and additionally demonstrate the presence of a second chloride channel CLC-3.

Introduction

The physiological role of pancreatic acinar cells is the secretion of digestive enzymes. Digestive enzymes in these cells are stored in membrane-bound secretory vesicles called zymogen granules (ZGs). The swelling of ZGs is implicated in the regulation of the expulsion of intravesicular contents during secretion [1]. The molecular mechanism of ZG swelling has been previously elucidated [2–4]. It has been further demonstrated that the water channel aquaporin-1, the potassium channel IRK-8, and the chloride channel CLC-2, are present in the ZG membrane and involved in ZG swelling [4]. Individual activity in ZG membranes has also been previously reported using indirect methods [5–10]. These studies report the existence of 2 separate chloride conductances [6, 9–10] and at least 2 cation channel pathways at the ZG membrane. The ZG chloride conductance was activated by ATP and

KARGER

Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

Accessible online at:
www.karger.com/pan

Bhanu P. Jena, PhD, George E. Palade University Professor
Distinguished Professor of Physiology
Department of Physiology, Wayne State University School of Medicine
5245 Gordon Scott Hall, 540 E. Canfield, Detroit, MI 48201 (USA)
Tel. +1 313 577 1532, Fax +1 313 993 4177, E-Mail bjena@med.wayne.edu

blocked by the chloride channel blocker, 4,4′-diisothiocyanato-stilbene-2-2′-disulfonic acid (DIDS). Of the 2 cation channel pathways, one is believed to be through a glyburide-sensitive potassium channel that is blocked by the KCNQ1 channel blocker 239B [6] as well as ATP and quinine. The second pathway is through a nonselective cation channel, which is blocked by flufenamic acid (a mitochondrial uncoupler).

Secretory vesicles, primarily due to their relatively small size, have long presented a challenge for direct measurement of the electrical activity at their membrane. Earlier studies using indirect methods and incorporation of vesicles into lipid bilayers have demonstrated the existence of ion channels in secretory vesicles [5–8, 11–20]. Direct measurements of ion channels at the cell membrane are performed by patch clamp techniques. Patch clamping is achieved by touching the tip of a capillary pipette that has been pulled to create a diameter of a micrometer or less at the tip of the pipette. A voltage is then applied to determine the current directly under the tip of the pipette (cell-attached patch, fig. 1A). The membrane under the tip can be pulled away from the rest of the cell (inside-out patch, fig. 1B) or the membrane can be ruptured by applying a larger voltage pulse (whole-cell patch, fig. 1C). Finally, the plasma membrane of the cell can be looped back by moving the electrode tip to form a small open vesicle under the tip (outside-out patch, fig. 1D). In voltage clamp mode in a patch clamping system, one determines the voltage to be maintained across the cell membrane. The amount of current required to maintain that voltage is equal and opposite to the amount of current moving across the membrane. Thus, the amount of current at different voltages can be measured and plotted. The conductance of the channel or channels in the membrane is the slope of this curve. Patch clamping of synaptic vesicles from Torpedo fish has been accomplished and the existence of channels in the vesicle membrane determined through the use of sub-micrometer patch clamping, a technique in which the capillary pipette electrode tip is pulled to the nanometer range [21]. However, the verification that this patch was of a single synaptic vesicle or a clump of fused vesicles was impossible earlier due to the size of these vesicles (90 nm). Pancreatic ZGs, on the other hand, are an order of magnitude larger, around a micrometer in diameter, hence rendering them visible with a light microscope for sub-micrometer patch clamping.

To directly determine the presence of ion channels at the ZG membrane, we used the sub-micrometer patch clamp technique to investigate whole vesicle patch currents. Whole vesicle patch (fig. 1A) currents were then analyzed and evaluated using chloride and potassium channel blockers. The position of the granule on the electrode tip was demonstrated using atomic force microscopy (AFM) and the presence of chloride channels (CLC-2 and CLC-3) as well as evidence for the presence of IRK-8 (the ATP-sensitive potassium channel Kir6.1) were verified through ion channel blockers and immunoblot analysis.

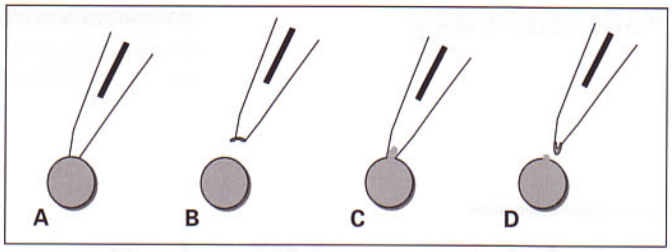

Fig. 1. Patch clamp configurations. **A** The 'cell-attached patch' is achieved immediately after the pipette forms a seal between the glass of the electrode and the cell membrane. **B** The 'inside-out patch' can then be formed by pulling the pipette away from the docked cell while the seal remains between the glass of the pipette and the membrane that was beneath it. **C** The 'whole cell-patch' is formed by breaking through the cell membrane of a 'cell-attached' patch. The solution in the pipette then becomes continuous with the cytosol. **D** After formation of the 'whole-cell patch', an outside-out patch configuration can be fashioned by rocking the pipette back and forth until a seal is made and then backing the pipette away from the cell.

Materials and Methods

Preparation of ZGs and Formation of Granule Patches

ZGs were purified according to a published procedure [2]. Briefly, the pancreas from male Sprague-Dawley rats was dissected and diced into 0.5-mm^3 pieces before being suspended in 15% (wt/vol) ice-cold homogenization buffer (0.3 *M* sucrose, 25 m*M* Hepes, pH 6.5, 1 m*M* benzamidine, 0.01% soybean trypsin inhibitor) and homogenized using 3 strokes of a Teflon glass homogenizer. The homogenate was centrifuged for 5 min at 300 *g* at 4 °C. The supernatant fraction was mixed with 2 volumes of a Percoll-Sucrose-Hepes buffer (0.3 *M* sucrose, 25 m*M* Hepes, pH 6.5, 86% Percoll, 0.01% soybean trypsin inhibitor) and centrifuged for 30 min at 16,400 *g* at 4 °C. Pure ZGs were obtained as a loose white pellet at the bottom of the centrifuge tube and used in the study. The purified granule suspension (0.02–0.18 μg/ml protein) in MES buffer (150 m*M* KCl, 20 m*M* MES, 1 m*M* $CaCl_2$, pH 6.5) was placed on a cover slip (Fisher Scientific). Granules were patched while free floating using negative pressure. Seal strength was at least 1 GΩ in resistance. All patches were performed at room temperature. The pipette solutions and any additions thereof are provided in the figure legends. Current versus voltage traces were recorded for voltages from –80 to +80 mV in order to determine baseline conductance in the 'on vesicle' conformation. After this baseline measurement was recorded, a voltage of 500 mV was applied to the granule to obtain a 'whole vesicle' conformation. A second baseline current versus voltage curve was obtained after the whole granule patch formation was achieved. To test for stability of the patch and the presence of single channel conductances, the granule patch was exposed to 300 m*M* KCl externally and current traces were recorded again for voltages from −80 to +80 mV.

AFM

After electrical measurements of the patched vesicle, the electrode with the patched ZG was imaged using the AFM (Nanoscope IIIa; Digital Instruments, Santa Barbara, Calif., USA) in the contact mode. The AFM micrograph in this manuscript was obtained using a silicon nitride tip with a spring constant of 0.06 $N \cdot m^{-1}$, and an imaging force of <200 pN. The image was obtained at line frequencies of 1.9 Hz, with 128 lines per image, and constant image gains. Topographical dimensions were analyzed using the software nanoscopeIIIa4.43r8 supplied by Digital Instruments.

Western Blot Analysis

Isolated ZGs were incubated in Laemmli sample preparation buffer and boiled. Twenty micrograms of protein was loaded in each lane. Proteins were resolved using 12.5% SDS-PAGE, and then electrotransferred to nitrocellulose for immunoblot analysis using specific antibodies to CLC-2, CLC-3 and IRK-8 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA). The antibodies have been raised against a synthetic peptide, representing a conserved domain of the channel in question. When the antibody is used in combination with the peptide, the immunodetectable band representing the protein and other proteolytic degradations containing the conserved domain are immunochemically undetectable. The nitrocellulose was incubated for 1 h at room temperature in blocking buffer (5% nonfat milk in PBS containing 0.1% Triton X-100 and 0.02% NaN_3), and immunoblotted for 1 h at room temperature with the specific antibody at a dilution of 1:200 (CLC-2 and CLC-3) and 1:500 (IRK-8) in blocking buffer. The immunoblotted nitrocellulose sheets were washed in PBS containing 0.1% Triton X-100 and 0.02% NaN_3 and incubated for 1 h at room temperature in horseradish peroxidase-conjugated second antibody at a dilution of 1:3,000 in blocking buffer. The immunoblots were then washed in PBS buffer, processed for enhanced chemiluminescence and developed using a Kodak 440 image station.

Results

AFM demonstrated the presence of single patched ZG at the tip of the glass pipette (fig. 2). The majority of granule patches presented a mixture of channel types. In order to elucidate which currents were present in each individual patch, a current versus voltage trace was obtained before and after exposure of the granule to 3 channel blockers: 20 μ*M* DIDS, the chloride channel blocker; quinine, a general potassium channel blocker; and glyburide (glibenclamide), the ATP potassium channel blocker. Baseline conductances were compared in the presence and absence of the blockers (fig. 3). Since individual ZG patches displayed different levels of ionic activity, calculated conductances were normalized to account for these differences and are represented as bar graphs (inset).

Application of the chloride channel blocker, DIDS, at a concentration of 20 μ*M* resulted in a general reduced baseline conductance (~18% within 3 min of addition (fig. 3A, n = 5) with 7 out of 9 patches experiencing an overall decrease in conductance. Increasing the concentration of DIDS to 40 μ*M* further decreased this conductance to nearly 50% of the conductance recorded 1 min after exposure to asymmetric KCl (this time point was used as the standard baseline conductance for all blocker experiments). The dramatic overall decrease supported the presence of chloride channels in the ZG patches.

Addition of quinine, a general potassium channel blocker, diminished baseline conductance (fig. 3B, n = 7) by ~14% initially and further decreased conductance within 3 min of addition. By increasing the concentration of quinine to 40 μ*M* (n = 6), a greater decrease in baseline conductance was obtained. These results confirm the presence of potassium channels in the ZG membrane. Finally, addition of 20 μ*M* glyburide resulted in a slightly greater decrease in baseline conductance than quinine (15%, fig.3C, n = 6). All channel blockers were dissolved in 50% ethanol making the final bath concentration 2% ethanol. Addition of 2% ethanol to the bath resulted in virtually no change in the baseline conductance.

Single channel currents were observed in approximately one quarter of all patches (n = 27). The inset in figure 2 provides a current versus time trace of the electrical activity of a whole granule conductance observed in an isolated ZG patch. The channel observed was fairly nonselective after exposure to a 300-m*M* KCl bath, and 150-m*M* KCl internal pipette gradient without ATP. Channel conductance was ~37 pS with no apparent voltage dependence. However, the channel was sensitive to DIDS, temporarily turning off the channel and diminishing baseline conductance to zero immediately after the first exposure of 20 μ*M* DIDS (fig. 4). Baseline conductance returned to 63% of the pre-DIDS value just over a second later. This new baseline conductance level was further decreased by the addition of both quinine and glyburide (data not shown), thus suggesting that the underlying currents observed following addition of DIDS are from a potassium and/or a nonselective potassium-channel-blocker-sensitive cation channel.

Western blot analysis was performed to verify the presence of both chloride and potassium channels in the gran-

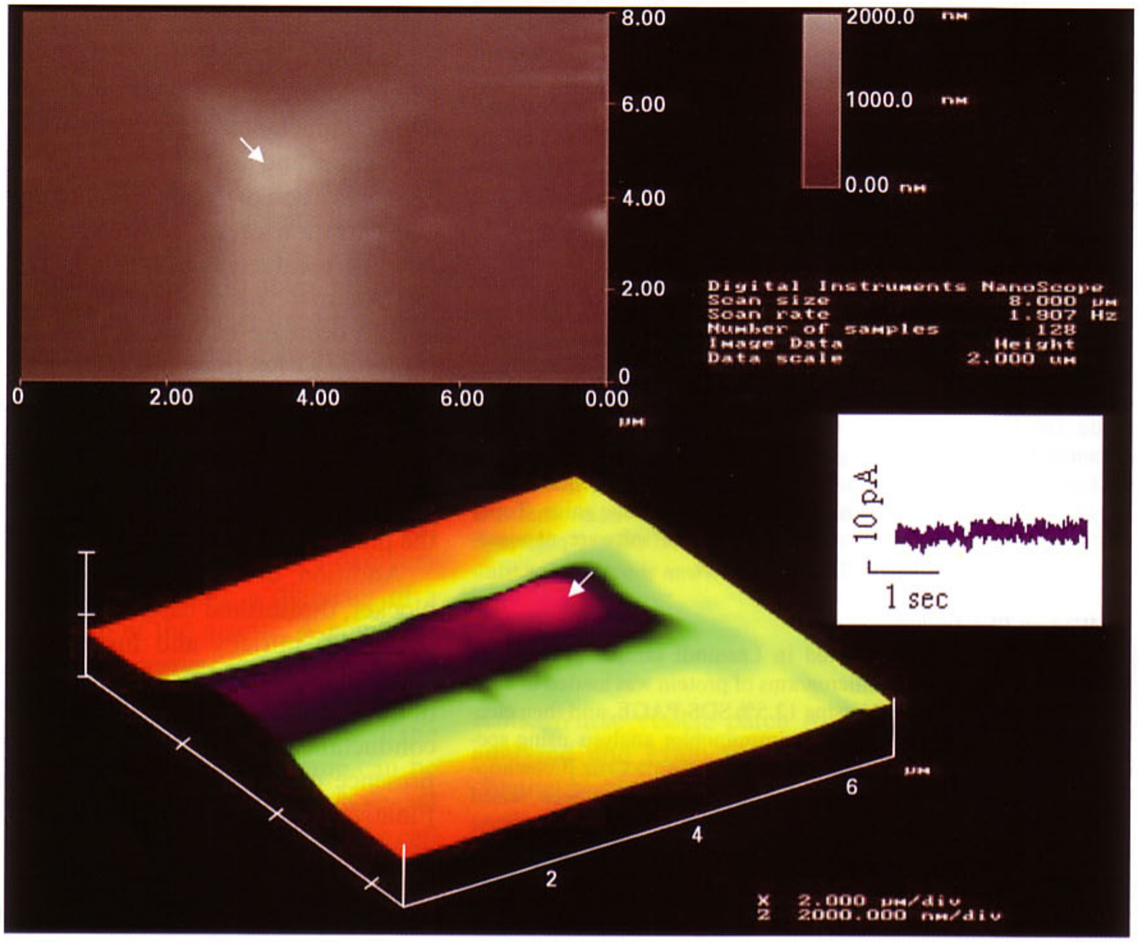

Fig. 2. AFM micrograph of a ZG patch. AFM image of a ZG patched to a patch pipette tip. The granule was fixed by adding formaldehyde to the bath after making contact with the granule membrane and forming a loose seal (200 MΩ). The patched granule (white arrow) was then removed from the bath and scanned in order to produce the AFM image (in 2D, top left; and 3D bottom left in color). On the right is an inset of the electrical recordings obtained earlier, from the patched ZG.

ules used for patching. Western blot analyses using specific antibodies to a number of potassium and chloride channels, demonstrate the presence of 2 types of chloride channels, CLC-2 and CLC-3, and the presence of the ATP-sensitive potassium channel, IRK-8 (fig. 5).

Discussion

For almost 20 years now, the existence of ion channels in synaptic vesicle and other secretory vesicle membranes has been debated. Although the presence of ion channels has been indirectly demonstrated, a direct demonstration has been difficult primarily due to the difficulty in patching them. Most of our understanding of these channels is based on pharmacological analysis without direct evidence of ionic current. In this study, we report the presence of ion channels at the ZG membrane, the secretory vesicles of the exocrine pancreas, by being able to for the first time directly patch isolated ZGs. Our study confirms earlier findings of the presence of both potassium and chloride ion channels at the ZG membrane. In our study, the electrical activity at the ZG membrane displays a range of sensitivity both to chloride and potassium channel blockers (whole vesicle conductance was decreased with the addition of the chloride channel blocker, DIDS, and the ATP K^+ channel blocker, glyburide, in both vesicles patches and indirect analysis), supporting the hypothesis for the presence of more than 1 channel type. This finding was further confirmed by Western blot analysis,

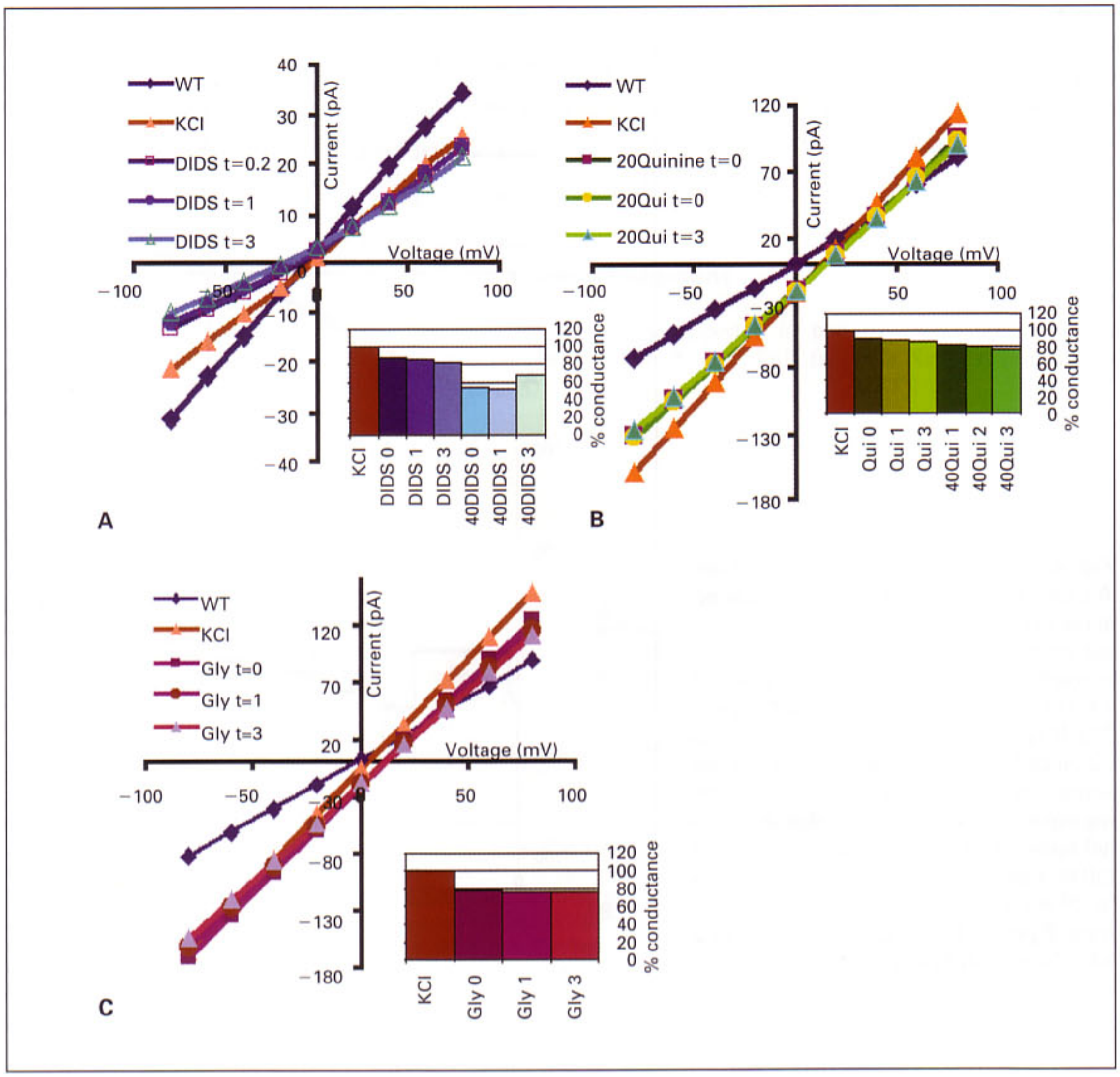

Fig. 3. Effect of ion channel blockers on conductance of ZG membrane. **A** Addition of 20 μ*M* DIDS (DIDS 0–3), final concentration, to a bath containing 300 m*M* KCl, 20 m*M* MES, and 1 m*M* CaCl, after formation of a whole granule patch. Patch electrode solution is 150 m*M* KCl , 20 m*M* MES, and 1 m*M* CaCl. Conductance was measured immediately following addition, and 1 min and 3 min after addition. Values are percent of the whole cell conductance (conductance point 1 min after 300 m*M* KCl was introduced to the bath, approximately 3 min before addition of blocker). The same procedure was followed after increasing DIDS concentration to 40 μ*M* (40DIDS 0–3). **B** Effect of 20 (Qui 0–3) and 40 μ*M* quinine (40Qui 1–3) on baseline activity of the whole granule patch. Procedure for addition was the same as for DIDS. **C** Effect of the addition of 20 μ*M* glyburide (Gly 0–3) on baseline conductance.

and as speculated, the presence of 2 chloride channels, CLC-2 and CLC-3, was observed. Also consistent with pharmacological evidence was the presence of ATP-sensitive potassium channel, Kir6.1 in our Western blot analysis of the ZGs. This is surprising, since Kir6.2 is the predominant form in β cells of the exocrine pancreas.

endocrine

Earlier, indirect measurements had also suggested the existence of a nonspecific cation channel. Although our study demonstrates the presence of such a current, the identity of the nonspecific channel remains to be established. Since this channel is blocked by a mitochondrial uncoupler, it may be suggested that this channel does not represent a channel at all, but rather a transporter in the

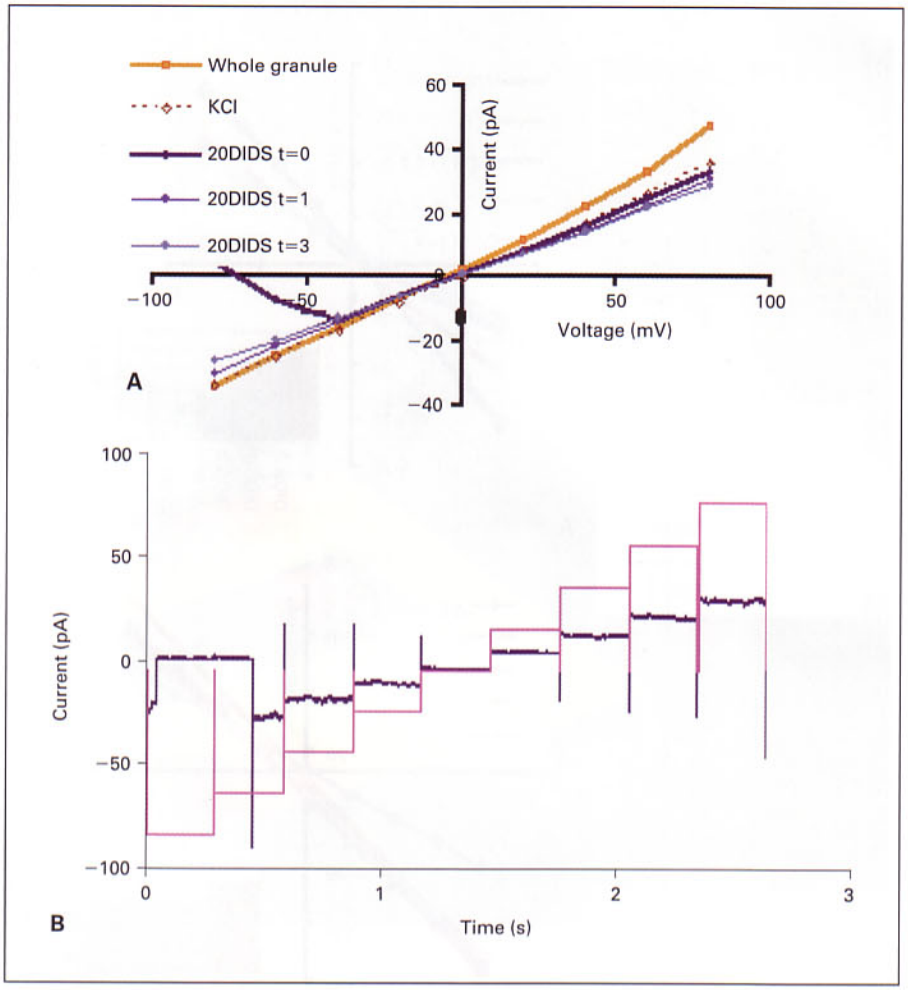

Fig. 4. Ion channels at the ZG membrane. **A** Current versus voltage traces of a channel at the ZG membrane during whole-cell granule patch (squares). The channel was then exposed to 300 m*M* KCl, 20 m*M* MES, and 1 m*M* $CaCl_2$ (dashed line) before addition of 20 μ*M* DIDS (circles) immediately after addition and 1 and 3 min there after. **B** Current versus time trace of channel activity following exposure to DIDS. Notice that the channel turns off immediately after addition of DIDS, followed by recovery. Voltage line is in 20 mV steps starting below the current trace. Pipette solution was standard 150 m*M* KCl, 20 m*M* MES, and 1 m*M* $CaCl_2$.

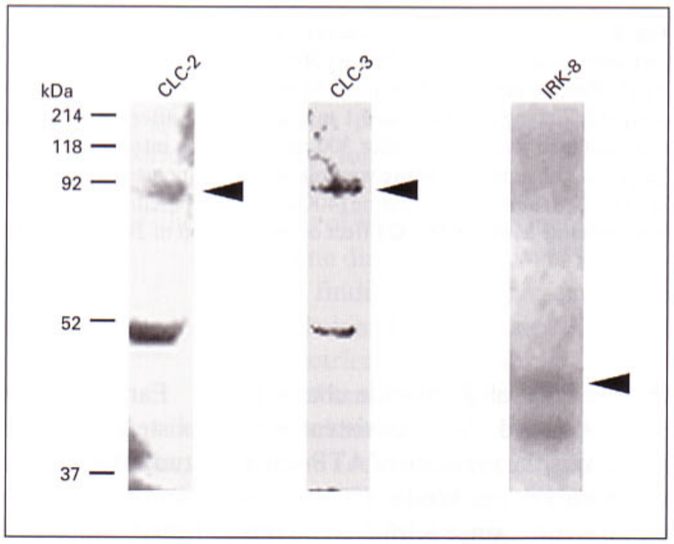

Fig. 5. CLC-2, CLC-3 and IRK-8 (Kir6.1) channel are present in ZGs of rat pancreatic acinar cells. 20 μg of ZGs protein were resolved by 12.5% SDS-PAGE, and then electrotransferred to nitrocellulose for immunoblot analysis using specific antibodies to CLC-2, CLC-3 and IRK-8. The extreme left panel demonstrates the position of the various molecular weight markers. Presence of CLC-2, CLC-3 and IRK-8 channel in the ZG membrane is demonstrated (arrowheads). Lower bands are possibly proteolytic degradation products of the specific protein.

absence of its substrate, or a combination of potassium channel and the water channel [4, 22–25], aquaporin 1, that is known to be present in the ZG membrane. This will be the subject of future studies.

This is the first demonstration of the presence of the 2 chloride channels at the ZG membrane. The presence of CLC-2 has previously been demonstrated in pig pancreatic acini [26] and CLC-3, in β-cell granule membranes, and synaptic vesicles [27, 28].

The use of sub-micrometer patch clamping has made it possible for the first time to make direct measurements of the electrical activity of a single subcellular organelle, in this case a single secretory vesicle, and determine channels in its membrane. Thus further work to determine the regulatory mechanisms and role of ion channels at the secretory vesicle membrane, in the secretory process, is now possible. Also, the direct examination of prospective drugs for pancreatic disorders can be screened and studied by this approach.

Acknowledgment

This work was supported in part by grants DK56212 and NS39918 to Bhanu P. Jena, PhD from the National Institutes of Health.

References

1 Jena BP: Discovery of the porosome: Revealing the molecular mechanism of secretion and membrane fusion in cells. J Cell Molec Med 2004;8:1–21.
2 Jena BP, Schneider SW, Geibel JP, Webster P, Oberleithner H, Sritharan KC: Gi regulation of secretory vesicle swelling examined by atomic force microscopy. Proc Natl Acad Sci USA 1997;94:13317–13322.
3 Cho S J, Sattar AK, Jeong EH, Satchi M, Cho JA, Dash S, Mayes MS, Stromer MH, Jena BP: Aquaporin 1 regulates GTP-induced rapid gating of water in secretory vesicles. Proc Natl Acad Sci USA 2002;99:4720–4724.
4 Abu-Hamdah R, Cho WJ, Cho SJ, Jeremic A, Kelly M, Ilie AE, Jena BP: Regulation of the water channel aquaporin-1: Isolation and reconstruction of the regulatory complex. Cell Biol Int 2004;28:7–17.
5 El Far O, Betz H: Synaptophysins: Vesicular cation channels? J Physiol 2002;1:539(Pt 2): 332.
6 Thevenod F: Ion channels in secretory granules of the pancreas and their role in exocytosis and release of secretory proteins. Am J Physiol Cell Physiol 2002;283:C651–C672.
7 Kidd JF, Thorn P: Intracellular Ca^{2+} and Cl^- channel activation in secretory cells. Ann Rev Physiol 2000;62:493–513.
8 Thevenod F, Braun M, Roussa E, Fuller CM: Molecular characterisation of pancreatic zymogen granule ion channel and regulator proteins involved in exocytosis. J Korean Med Sci 2000;15(suppl):S51–S52.
9 Braun M, Thevenod F: Photoaffinity labeling and purification of ZG-16p, a high-affinity dihydropyridine binding protein of rat pancreatic zymogen granule membranes that regulates a K^+-selective conductance. Mol Pharmacol 2000;57:308–316.
10 Thevenod F, Hildebrandt JP, Striessnig J, de Jonge HR, Schulz I: Chloride and potassium conductances of mouse pancreatic zymogen granules are inversely regulated by a approx 80-kDa mdr1a gene product. J Biol Chem 1996; 271:3300–3305.
11 Kicinska A, Debska G, Szewczyk A: Intracellular ion channels: Properties, function and experimental methodology (in Polish). Postepy Hig Med Dosw 2000;54:307–316.
12 Cho SJ, Kelly M, Rognlien KT, Cho J-A, Horber JKH, Jena BP: SNAREs in opposing bilayers interact in a circular array to form conducting pores. Biophys J 2002;23:2522–2527.
13 Rahamimoff R, Butkevich A, Duridanova D, Ahdut R, Harari E, Kachalsky SG: Multitude of ion channels in the regulation of transmitter release. Phil Trans R Soc London B 1999;354: 281–288.
14 Kelly ML, Woodbury DJ: Ion channels from synaptic vesicle membrane fragments reconstituted into lipid bilayers. Biophys J 1996;70: 2593–2599.
15 Woodbury DJ: Evaluation of the evidence for ion channels in synaptic vesicles. Mol Memb Biol 1995;12:165–171.
16 Yakir N, Rahamimoff R: The non-specific ion channel in Torpedo ocellata fused synaptic vesicles. J Physiol 1995;485(Pt 3):683–697.
17 Kopelman H, Gauthier C: Cyclic AMP-sensitive chloride efflux in rabbit pancreatic acini. Pediatric Res 1999;29:529–533.
18 Spruce AE, Breckenridge LJ, Lee AK, Almers W: Properties of the fusion pore that forms during exocytosis of a mast cell secretory vesicle. Neuron 1990;4:643–654.
19 Rahamimoff R, Deriemer SA, Ginsburg S, Kaiserman I, Sakmann B, Shapira R, Stadler H, Yakir N: Ionic channels in synaptic vesicles: Are they involved in transmitter release? J Exp Physiol 1989;74:1019–1031.
20 Stanley EF, Ehrenstein G, Russell GT: Evidence for anion channels in secretory vesicles. Neuroscience 1988;25:1035–1039.
21 Horber JKH, Mosbacher J, Haberle W, Ruppersberg JP, Sakmann B: A look at membrane patches with a scanning force microscope. Biophys J 1995;68:1687–1693.
22 Anthony TL, Brooks HL, Boassa D, Leonov S, Yanochko G, Regan JW, Yool AJ: Cloned Human Aquaporin-1 Is a Cyclic GMP-Gated Ion Channel. Mol Pharmacol 2000;57:576–588.
23 Brooks HL, Regan JW, Yool AJ: Inhibition of aquaporin-1 water permeability by tetraethylammonium: Involvement of the loop E pore region. Mol Pharmacol 2000;57:1021–1026.
24 Saparov SM, Kozono D, Rothe U, Agre P, Pohl P: Water and ion permeation of aquaporin-1 in planar lipid bilayers. J Biol Chem 2001;276: 31515–31520.
25 Yool AJ, Weinstein AM: New roles for old holes: Ion channel function in aquaporin-1. News Physiol Sci 2002;17:68–72.
26 Carew MAC, Thorn P: Identification of CLC-2 like chloride currents in pig pancreatic acinar cells. Pflugers Archiv 1996;433:84–90.
27 Al-Awqati Q: Chloride channels of intracellular organelles. Curr Opin Cell Biol 1995;7:504–508.
28 Szewczyk A: The intracellular potassium and chloride channels: Properties, pharmacology and function. Mol Memb Biol 1998;15:49–58.