Biophysical Properties of ClC-3 Differentiate It from Swelling-activated Chloride Channels in Chinese Hamster Ovary-K1 Cells*

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Preparation of ClC-3 Expression Vector—A full-length ClC-3 transcript (GenBank™ accession number AF142778), corresponding to the

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CIC is a highly conserved voltage-gated chloride channel, which together with CIC-4 and CIC-5 belongs to one subfamily of the larger group of CIC chloride channels. Whereas CIC-5 is localized intracellularly, CIC-3 has been reported to be a swelling-activated plasma membrane channel. However, recent studies have shown that native CIC-3 in hepatocytes is primarily intracellular. Therefore, we reexamined the properties of CIC-3 in a mammalian cell expression system and compared them with the properties of endogenous swelling-activated channels. Chinese hamster ovary (CHO)-K1 cells were transiently transfected with rat CIC-3. The resulting chloride currents were Cl\(^{-}\) > I\(^{-}\) selective, showed extreme outward rectification, and lacked inactivation at positive voltages. In addition, they were insensitive to the chloride channel blockers, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and 4,4'-diirothiocyanostilbene-2,2'-disulfonic acid (DIDS) and were not inhibited by phorbol esters or activated by osmotic swelling. These properties are identical to those of CIC-5 but differ from those previously attributed to CIC-3. In contrast, nontransfected CHO-K1 cells displayed an endogenous swelling-activated chloride current, which was weakly outward rectifying, inactivated at positive voltages, sensitive to NPPB and DIDS, and inhibited by phorbol esters. These properties are identical to those previously attributed to CIC-3. Therefore, we conclude that when expressed in CHO-K1 cells, CIC-3 is an extremely outward rectifying channel with similar properties to CIC-5 and is neither activated by cell swelling nor identical to the endogenous swelling-activated channel. These data suggest that CIC-3 cannot be responsible for the swelling-activated chloride channel under all circumstances.

Chloride channels are widely distributed in eukaryotic cells, and three major superfamilies have been identified (1, 2). In recent years great progress has been made in understanding the CIC family of voltage-gated chloride channels. Mutations in some family members have been proven to cause human diseases, such as Dent’s disease (hereditary nephrolithiasis) and myotonia congenita. Nonetheless, the precise function of the majority of the chloride channels remains unclear (3).

One important function of chloride channels is to allow the efflux of cell chloride as part of cell volume regulation. However, the identities of the swelling-activated chloride channels involved have not been established (3, 4). This channel activity has aroused attention because it is present in nearly all cells, is critical for cell volume regulation, and may play a role in other physiological and pathophysiological processes, including stabilization of membrane potential, initiation of apoptosis, and epithelial transport (5, 6). Accordingly, great efforts have been focused on determining the molecular identity of this channel. Several candidates have been proposed, such as pICln (7), P-glycoprotein (8), and ClC-2 (9), but in each case further investigation has shown that the proposed channel is not responsible for the observed currents (4, 10).

CIC-3 has been proposed to be the swelling-activated chloride channel (11). Heterologous expression of CIC-3 has been reported by two groups (11, 12). These groups (11, 12) observed large currents in stably transfected cell lines. However, the properties of the currents reported are markedly different from those of CIC-4 and CIC-5, the two most closely homologous family members (13). Because endogenous swelling-activated currents are inevitably present in all expression systems and there are no highly selective inhibitors of the volume-activated chloride channel, there is a possibility that the currents attributed to CIC-3 reflect in part the properties of endogenous channel molecules.

We recently cloned two isoforms of the CIC-3 channel from rat hepatocytes (14). The long form has the identical amino acid sequence as the mouse CIC-3 and has 98% identity to the human CIC-3. The short form has 99.9% identity to the guinea pig CIC-3. Immunofluorescence and immunoblotting demonstrated that native CIC-3 in hepatocytes was localized both intracellularly and in association with the canalicular membrane (14). In the present study, we expressed a FLAG-tagged CIC-3 construct in Chinese hamster ovary (CHO)-K1 cells, examined its activation by cell swelling, and compared its properties to those of endogenous swelling-activated chloride currents. In contrast to previous studies, we found that the kinetic and pharmacological properties of CIC-3 are nearly identical to those of CIC-4 and CIC-5 (13) but differ markedly from the properties previously reported for CIC-3 (11, 15). Furthermore, the CIC-3 channel is not activated by cell swelling.

EXPERIMENTAL PROCEDURES

Preparation of ClC-3 Expression Vector—A full-length ClC-3 transcript (GenBank™ accession number AF142778), corresponding to the

1 The abbreviations used are: CHO, Chinese hamster ovary; DIDS, 4,4'-diirothiocyanostilbene-2,2'-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; GFP, green fluorescent protein.
short form of rat ClC-3 with a FLAG epitope that was inserted at the 3' end of the open reading frame, was prepared by reverse transcriptase-polymerase chain reaction, ligated into pcDNA3.1+ (Invitrogen), and sequenced using eight sequencing primers by the sequencing facility at the University of Texas Medical Branch as described previously (14). An additional expression vector consisting of ClC-3 without the FLAG epitope was prepared by introducing a stop codon before the FLAG epitope using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol and confirmed by sequencing.

**Transfection**—CHO-K1 cells were cultured in Dulbeco's modified Eagle's medium/F12 medium and grown on glass coverslips. Cells were transfected at 40–60% confluency with the CIC-3 cDNA using FuGENE™6 (Roche Molecular Biochemicals) according to the manufacturer's protocol. To identify the transfectants, a green fluorescent protein construct (pEGFP, CLONTECH) was cotransfected at a CIC-3-GFP ratio of 30:1. Cells expressing GFP fluorescence at 40–70 h were selected for the experiments. In the control experiments, cells were transfected with pEGFP only.

**Patch Clamp**—Whole cell patch clamp was performed by the methods previously described in detail (14, 16). The bath solution contained (in mM) 114 NaCl, 5.4 CaCl₂, 1 MgSO₄, 1.5 CaCl₂, 10 HEPES, 10 glucose, pH 7.4, adjusted with NaOH. In some experiments, NaCl was substituted by tetramethylammonium chloride, which did not alter the observed results. Osmolarity was measured with a vapor pressure osmometer (Model 5500, Wescor, Inc., Logan, UT) and adjusted to 300 mosmol/kg by adding sucrose. Hypotonic bath solutions were the same with the exception that the osmolality was reduced to 240 mosmol/kg by omitting sucrose. A pipette solution contained (in mM) 120 CsCl, 3 MgSO₄, 1 CaCl₂, 11 EGTA, 3 Na₂ATP, 10 HEPES, 10 glucose, pH 7.2, using CsOH. Osmolality was adjusted to 290 mosmol/kg with sucrose. For determining anion selectivity, NaCl in the bath solution was substituted with NaI as indicated. GFP-expressing cells were visualized with epifluorescence on a Nikon Diaphot microscope and selected for patching. Whole cell currents were measured for up to 1 h after whole cell formation by sequential ramp voltage protocols from −90 to +90 mV in 200 ms at a frequency of 0.1 Hz. Data presented were obtained 3–5 min after whole cell formation. B, examples of currents are shown for control and ClC-3 cells using 2-s voltage steps from −100 to +100 mV at 20-mV increments. C, effect of the lack of chloride channel inhibitors on CIC-3 currents is shown. Control, n = 7; DIDS (300 μM), n = 5; NPPB (300 μM), n = 5. D, effect of a 5-min incubation with the phorbol ester, phorbol 12,13-dibutyrate (PDBu) (100 nM) on CIC-3 currents, n = 7.

**Results**

**Properties of CIC-3 Currents**—In this study, we performed whole cell patch clamp to compare CIC-3 and swelling-activated currents. We have previously shown that cotransfection of CHO-K1 cells with CIC-3/FLAG and GFP results in the expression of the CIC-3 protein both in the cytoplasm and at the plasma membrane. The presence of GFP fluorescence accurately identifies those cells that express CIC-3 (14). Fig. 1 demonstrates that nontransfected CHO-K1 cells have a very small linear chloride conductance, but large chloride-selective currents are readily observed after CIC-3 transfection. Transfection of the GFP plasmid alone did not result in detectable chloride currents that were different from the results in untransfected cells. The CIC-3/FLAG currents exhibited the following properties: 1) strong outward rectification and failure to conduct at negative voltages (Fig. 1A); 2) lack of inactivation after 2 s at positive voltages above 80 mV (Fig. 1B); 3) insensitivity to the chloride channel blockers DIDS and NPPB even at high concentrations of 300 μM (Fig. 1C); 4) lack of an effect of the activation of protein kinase C by the addition of the phorbol ester, phorbol 12,13-dibutyrate (PDBu) (100 nM) on CIC-3 currents, n = 7.

**Properties of Endogenous Swelling-activated Currents**—Because each of the above properties differs from previous reports for CIC-3, we next examined the endogenous swelling-activated chloride currents in nontransfected CHO-K1 cells (17). These results are demonstrated in Fig. 2, and show that the swelling-activated chloride current is larger in magnitude and is only weakly outward rectifying (Fig. 2A). The current inactivates at positive holding voltages (Fig. 2B), is sensitive to chloride channel blockers (Fig. 2C), and is inhibited by the activation of protein kinase C with phorbol ester (Fig. 2D). It has a halide selectivity of outward currents of Cl⁻ > I⁻.

CIC-3-mediated Chloride Currents Are Not Swelling-activated—We next examined the effect of osmotic cell swelling on chloride currents in the control and CIC-3 transfected cells. Bath osmolality was changed from isotonic 300 mosmol/kg to hypotonic 240 mosmol/kg after the whole cell mode was established. The activation of currents began anywhere from 6–12 min after the solution change and plateaued 3–5 min later. Fig. 3 compares the results of osmotic swelling on currents in transfected and nontransfected cells. In both instances, the currents were greatly increased after swelling, but no difference was observed between the two cell types (Fig. 3A). After osmotic swelling, the currents from the CIC-3 transfected cells exhibited inactivation at positive voltages (Fig. 3B). NPPB completely abolished the swelling-induced component of the current. The residual current had almost exactly the same

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1 Of the more than 30 cells tested with the protocol, none showed inactivation. This finding differs from swelling-activated chloride currents in CHO-K1 cells and in most other cells, which inactivate under these conditions (6).

2 Swelling-activated currents are generally sensitive to both inhibitors.
amplitude and rectification as it had before these same cells had undergone swelling (Fig. 3C). The swollen NPPB-inhibited ClC-3-expressing cells again displayed non-inactivating currents that were identical to those currents before cell swelling (Fig. 3B). These results were uniformly reproducible and clearly demonstrate that the currents present in the swollen and transfected cells are just the arithmetic sum of ClC-3 currents and endogenous swelling-activated currents.

The Effect of the FLAG Epitope on ClC-3 Current Properties—To determine if our results could be accounted for by the presence the FLAG epitope in our ClC-3 construct, we prepared a ClC-3 expression vector without the FLAG. Fig. 4A demonstrates the currents observed. Cells transfected with this construct also displayed strongly outward rectifying currents, which did not conduct in the inward direction, failed to inactivate at strong positive voltages, and were insensitive to NPPB. All of the tested properties were identical to the construct with the C-terminal FLAG epitope. Fig. 4B compares the halide selectivity of ClC-3 (without the FLAG epitope) to that of the endogenous swelling-activated current. Because the ClC-3 currents are so strongly outward rectifying, it is not possible to determine the reversal potential under these conditions. Nonetheless, the endogenous currents displayed outward current selectivity of I\(^-\) > Cl\(^-\), whereas ClC-3 currents were selective for Cl\(^-\) > I\(^-\). This finding was identical for ClC-3/FLAG construct (data not shown).

DISCUSSION

We have found that ClC-3, when transiently expressed in CHO-K1 cells, produces a characteristic chloride current with properties unlike those seen for endogenous chloride channels. These unique properties are extreme outward rectification, lack of inactivation at positive voltages, insensitivity to NPPB, DIDS, or phorbol esters, and outward currents that are greater with Cl\(^-\) than I\(^-\) in the bath solution. In every one of these instances, the properties of ClC-3 appear to be identical to that reported for ClC-5 and ClC-4 (13). Because ClC-3 shares an 80% amino acid identity with ClC-5, these similar biophysical properties are not unexpected. In a sharp contrast, the endogenous swelling-activated chloride currents in the CHO-K1 cells differ in each of these properties. The previously reported properties of ClC-3 (11) appear to be nearly identical to the endogenous currents of CHO-K1 cells and differ markedly from the currents we see associated with ClC-3.

The activation of endogenous channels is always a possibility in heterologous transfection studies, and it is necessary to...
consider whether the currents observed in our studies result directly from ion flow through CIC-3 molecules or from the activation of endogenous channels. For two other putative ion channels pICln and P-glycoprotein, the initial reports of channel activity were later shown to result from the protein in question activating endogenous chloride channels (4, 10). Nonetheless, there are several facts that suggest we are observing the current flow through the expressed channel. First, as detailed above, the CIC-3 currents had very specific biophysical and pharmacological properties. We never observed this channel activity either in untransfected CHO-K1 cells, CHO-K1 cells transfected with GFP alone, or CHO-K1 cells transfected with another membrane protein, such as the apical sodium bile acid cotransporter (18). In contrast, the currents associated with pICln and P-glycoprotein can occasionally be seen in untransfected cells and can always be induced by cell swelling in untransfected cells. Second, we have documented that at least some of our expressed proteins are present on the plasma membrane of the transfected cells (17). Third, the properties we observed are identical to the properties of CIC-5 and CIC-4, the two most closely related family members (13). Taken together, these observations strongly support the conclusion that we have expressed CIC-3 channel activity.

Two groups have previously reported the heterologous expression of CIC-3. Duan et al. (11, 19) described CIC-3 currents in fibroblasts, which were stably and transiently transfected with a guinea pig CIC-3 cDNA construct that was very similar to the short form of rat CIC-3 used in the present study. These currents displayed slight outward rectification, inactivated at positive voltages, and were sensitive to DIDS and phorbol 12,13-dibutyrate. Similar CIC-3 currents were reported by Kawasaki et al. (12, 20) who described rat ClC-3 currents in oocytes (20) and stably transfected CHO-K1 cells (12). Their currents exhibited somewhat different properties in the different expression systems, but they also showed only a weak outward rectification and sensitivity to DIDS as well as phorbol esters that were similar to that reported by Duan et al. (11).

The results of the present study, therefore, do not agree with these previous findings and raise the possibility that endogenous channels may account for some of the results. In support of this conclusion, the single channels reported by Kawasaki et al. (12) show extreme outward rectification in contrast to the weakly rectifying whole cell currents that were observed in those same experiments. Because the properties that were previously attributed to CIC-3 are present in endogenous channels and are very similar to what had once been attributed to pICln and before that P-glycoprotein, the possibility that these results reflect endogenous channel activation needs to be considered.

A key question in this field is whether CIC-3 is a swelling-activated chloride channel. Duan et al. (11) observed that stable CIC-3 transfection of NIH/3T3 fibroblasts produced outward rectifying chloride currents, which could be further increased about 2-fold by cell swelling. In the present study, transiently transfected CHO-K1 cells demonstrated a unique and strong rectifying current that was not further activated by swelling. Furthermore, in our experiments, transfected and nontransfected CHO-K1 cells had nearly identical swelling-activated currents, which were biophysically and pharmacologically similar to the CIC-3-associated currents observed by Duan et al. (11).

There are two possible explanations for these differences. Either CIC-3 has very different properties in the different expression systems or one or both of these studies were examining endogenous channels, which were activated by CIC-3 expression. As explained above, we favor the latter explanation because the CIC-3 currents observed in the present study are dissimilar from endogenous currents and identical to those seen for the close family members CIC-4 and CIC-5. On the other hand, the currents previously attributed to CIC-3 have biophysical and pharmacological properties that are identical to endogenous currents. These currents can be seen spontaneously in multiple different cell types including NIH/3T3 cells (21) and have a different chloride/sodium selectivity from that of any other described CIC family channel (3). Therefore, it is possible that there was some degree of basal activation of the swelling-activated channels in the studies of Duan et al. (11).

The strongest evidence in support of the function of CIC-3 as a swelling-activated channel comes from mutagenesis studies (11, 19) showing that CIC-3 mutations can result in changes in rectification and phorbol ester sensitivity. Although this evidence appears compelling, it is based exclusively on the analysis of whole cell currents, which could represent the sum of activities of different channel types. Changes in the proportions of active channels could produce apparent changes in rectification, selectivity, and even in phosphorylation dependence of the net whole cell current. The mutagenesis studies would therefore be more definitive if they were replicated in single channels.

One of the difficulties in identifying the swelling-activated channel is that the heterologous expression of some proteins can clearly activate an endogenous outward rectifying chloride channel. This has been shown conclusively for pICln, an intracellular protein, as well as for the putative chloride channel CIC-6 (22). The possibility that CIC-3 may also play a role in modulating endogenous swelling-activated channel activity is further suggested by the recent study of Wang et al. (23). These authors examined the role of CIC-3 in swelling-activated chloride currents of nonpigmented ciliary epithelial cells using antisense technology. Reduction of the CIC-3 expression did reduce the swelling-activated chloride conductance, but it also delayed activation lag time. In addition, nearly complete elimination of the CIC-3 protein expression only reduced the magnitude of the swelling-activated current by 60%. These authors concluded that although CIC-3 could be a swelling-activated channel in their system, there certainly had to be other channels as well, and CIC-3 also had an ability to regulate the activation of other channels.

We have also observed a situation in which CIC-3 might be activating endogenous channels. In our previous studies (14), we had shown that both the long and short forms of CIC-3 produced chloride currents. The short form currents described in the present study are clearly different from swelling-activated currents in these cells. However, the long form-associated currents described previously (14) were quite similar to the endogenous swelling-activated currents. We have recently determined that unlike the short form, the long form currents are sensitive to NPPB. This finding suggests that the ion pore structure itself may be different in these two situations. Therefore, it is possible that the currents we observed that were associated with the long form of CIC-3 resulted from the activation of endogenous channels. In our particular experimental system, this occurred only with the long form but not the short form of CIC-3. Possibly in other systems, the short form may have channel-activating properties as well.

In conclusion, we have transiently expressed CIC-3 derived from rat hepatocytes in CHO-K1 cells. The resulting currents are not activated by cell swelling and are essentially identical to those produced by CIC-4 and CIC-5. Therefore, CIC-3 is not a swelling-activated channel in all cell systems. Because of its localization in hepatocytes (14) and its similarity to CIC-5, we propose that CIC-3 is primarily an intracellular ion channel.
The evidence suggests that like ClC-6, ClC-3 may also have the
ability to activate endogenous channels under some conditions.
This phenomenon may contribute to the difficulty in determin-
ing the role of ClC-3 in swelling-activated chloride conductance.

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