Functional Role of the Carboxyl Terminal Domain of Human Connexin 50 in Gap Junctional Channels

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Abstract. Gap junction channels formed by connexin 50 (Cx50) are critical for maintenance of lens transparency. Because the C-terminus of Cx50 can be cleaved post-translationally, we hypothesized that channels formed by the truncated Cx50 exhibit altered properties or regulation. We used the dual whole-cell patch-clamp technique to investigate the macroscopic and single-channel properties of gap junctional channels formed by wild-type human Cx50 and a truncation mutant (Cx50A294stop) after transfection of N2A cells. Our results show that wild-type Cx50 formed functional gap junctional channels. The macroscopic $G_{mac}V_j$ relationship was well described by a Boltzmann equation with $A$ of 0.10, $V_0$ of 43.8 mV and $G_{min}$ of 0.23. The single-channel conductance was 212 ± 5 pS. Multiple long-lasting substates were observed with conductances ranging between 31 and 80 pS. Wild-type Cx50 gap junctional channels were reversibly blocked when pH_i was reduced to 6.3. Truncating the C-terminus at amino acid 294 caused a loss of pH_i sensitivity, but there were no significant changes in single-channel current amplitude or $G_{mac}V_j$ relationship. These results suggest that the C-terminus of human Cx50 is involved in pH_i sensitivity, but has little influence over single-channel conductance, voltage dependence, or gating kinetics.

Key words: Lens — Acidification — Gating — Substate — Connexin 50 — Gap junction

Introduction

Gap junctional channels are low-resistance intercellular pathways between adjacent cells for the exchange of ions, metabolites, second messengers and other molecules with molecular weight up to 1 kDa (Bruzzone, White & Paul, 1996). They are made of two connexons, or hemichannels, each contributed by two adjoining cells. Connexons are composed of six subunits called connexins (Cx). The connexins belong to a multigene family composed of at least 15 human members (Beyer & Willecke, 2000).

The lens is an avascular organ that is highly dependent on intercellular communication for volume regulation and metabolic homeostasis. Three connexins have been identified in the mammalian lens: Cx43, Cx46 and Cx50 (Beyer, Paul & Goodenough, 1987; Paul et al., 1991; White et al., 1992). Cx43 is expressed primarily in epithelial cells; Cx46 and Cx50 are expressed in fiber cells. Previous studies have shown that disruption of either Cx46 or Cx50 leads to cataract formation (Gong et al., 1997; White et al., 1998). Furthermore, certain types of congenital cataracts in humans and mice are associated with mutations in the genes for these connexins (Mackay et al., 1999; Berry et al., 1999; Shiels et al., 1998; Steele et al., 1998). However, the specific roles of these two connexins in the lens are still not completely understood.

Two different size forms of Cx50 have been detected in gap junction-enriched membrane preparations from ovine lens (Kistler & Bullivant, 1987). A 70-kDa isoform corresponds to the full-length protein and has been detected in fiber cell membranes from the cortex of the lens. A lower-M, form (38 kDa) has been detected in isolated membranes from the lens core region. This Cx50 isoform lacks much of the carboxyl tail and is thought to be generated from
the long form by posttranslational proteolytic cleavage by a calpain (Lin et al., 1997).

The carboxyl tail of Cx50 may play an important role in the regulation of gap junctional channel function in the vertebrate lens. It has been previously shown that removal of most of the carboxyl tail of ovine Cx50 causes loss of pH sensitivity when studied in Xenopus oocyte pairs (Lin et al., 1998). Several studies have implicated the carboxyl tail of connexins in the regulation of gap junctional function. Expression studies of a number of connexins in paired Xenopus oocytes have suggested a role of their carboxyl tails in channel gating in response to cytoplasmic acidification (Ek-Vitorin et al., 1996; Morley, Taffet & Delmar, 1996). Analysis of SKHep-1 cells stably transfected with truncated Cx43 constructs has suggested that single-channel conductance is influenced by the carboxyl tail (Fishman et al., 1991).

In the present study, we used a dual whole-cell patch-clamp technique to examine the macroscopic and single-channel properties of gap junctional channels formed by wild-type human Cx50 or truncated variants in transfected N2A neuroblastoma cells.

**Materials and Methods**

**Preparation of Wild-Type Cx50 and C-Terminal-Truncated Cx50 Variants**

Wild-type human Cx50 (Shiels et al., 1998; Pal et al., 1999) was subcloned into the EcoRI site of the eukaryotic expression vector pSFFV-neo (Fuhlbrigge et al., 1988). Carboxyl terminal deletion mutants of Cx50 were obtained by PCR using a sense primer, 5'-ggaattcattggtggcactg-3', and two antisense primers, 5'-cgggaattctactctattctaggg-3' and 5'-cgggaattctctatggtgctgtgg-3' to generate Cx50 polypeptides that ended at valine-284 (Cx50V284stop) and alanine-294 (Cx50A294stop), respectively (Fig. 1A). An EcoRI site was introduced at the 5'-end of both sense and antisense primers. The PCR products were digested with EcoRI and subcloned into pSP64TH or pSFFV-neo. The constructs were sequenced (DNA sequencing facility, Iowa State University, Ames, IA) to confirm that the designed truncation sites had been incorporated and to ensure that no random mutations were introduced in the sequence.

**In Vitro Translation in Rabbit Reticulocyte Lysate**

RNAs for human Cx50 wild type, Cx50V284stop and Cx50A294stop were prepared from pSP64TH templates as previously described (Ebihara et al., 1999). In vitro transcribed cRNAs were in vitro translated in a cell-free rabbit reticulocyte lysate system (Life Technologies, Rockville, MD) containing [35S]-methionine, according to the manufacturer’s protocol. Translated proteins were resolved by SDS-PAGE. The gel was dried, and the translated proteins were detected by autoradiography.

**Cell Culture**

N2A mouse neuroblastoma cells were grown in DMEM (Life Technologies) containing 10% fetal bovine serum, 2 mm l-Glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 5% CO2 at 37°C.

For stable transfections, 2 μg of linearized pSFFV-neo plasmid DNA containing wild-type Cx50 was transfected into communication-deficient N2A cells using Lipofectin (Life Technologies, Rockville, MD) (Vermont et al., 1992). Compatible-transfected clones were selected for their resistance to 0.25 mg/ml active G418.

For transient transfections, N2A cultures (50–90% confluent) grown in 35-mm dishes were cotransfected with 1–3 μg of cDNA for green fluorescent protein (GFP) and 4.5–6 μg of pSFFV-neo DNA containing wild-type Cx50, Cx50V284stop or Cx50A294stop using Lipofectamine (Life Technologies). N2A cells were incubated for the subsequent 10–11 hr in OptiMEM (Life Technologies); then, the medium was replaced with regular growth medium containing 10% fetal bovine serum. Cells were incubated for an additional 12–18 h, and then split onto polylysine-treated coverslips for electrophysiological experiments (which were performed after 12–48 h). Cell pairs expressing introduced connexins were identified by expression of GFP and emission of green light when viewed under UV illumination.

**Immunohistochemical Analysis**

A bacterial fusion protein containing amino acids 231–433 of human Cx50 linked to glutathione-S-transferase (GST-Cx50) was constructed in pGEN-3X (Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (Berthoud et al., 1997). The fusion protein was purified by affinity chromatography on glutathione-agarose and used to immunize rabbits. Sera were depleted of anti-GST antibodies by passing through a GST column, and then affinity-purified by chromatography using GST-Cx50 coupled to a Sulfo-Link column (Pierce, Rockford, IL) as done previously (Berthoud, Cook & Beyer, 1994).

Immunoblots were performed using homogenates of human lenses or of cultured cells. Frozen human lenses were obtained from the Lions Eye Bank of Oregon. Lens homogenates were prepared in PBS containing 4 mM EDTA and 2 mM PMSF using a glass-glass Dounce homogenizer, sonicated, aliquoted, and frozen. Cell cultures were rinsed with PBS, harvested by scraping in Laemmli sample buffer (Laemmli, 1970), and stored at 4°C. Electrophoresis and immunoblotting were performed as previously described (Berthoud et al., 1994).

For immunofluorescence studies, N2A cells seeded on multiwell slides (LAB-TEK, Nalge Nunc International, Naperville, IL) were fixed with 50% methanol-50% acetone for 2 min at room temperature, and incubated sequentially in primary (rabbit polyclonal anti-Cx50) and secondary (Cy3-conjugated goat anti-rabbit IgG) antibodies according to Berthoud et al. (2000). The preparations were observed using a Zeiss Axioplan 2 microscope equipped for epifluorescence.

**Electrophysiological Measurements and Analysis**

The dual whole-cell patch-clamp technique was applied on N2A cell pairs using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and an L/M-EPC7 amplifier (List-electronic, Germany). All experiments were performed at room temperature (22–24°C). Patch pipettes were pulled from glass capillaries with 1.5 OD/1.0 ID (World Precision Instruments, Sarasota, FL), using a Brown-Fleming micropipette puller (Sutter Instruments, San Francisco, CA). The tips of micropipettes were fire-polished. The resistance of the micropipettes was 2–6 MΩ when the pipettes were filled with a pipette solution containing (in mM): 130 CsCl, 10...