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Identification of calcium channel $\alpha 1$ subunit mRNA expressed in retinal bipolar neurons

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Abstract

Purpose—Glutamate release from goldfish bipolar cell terminals is driven by Ca^{2+} influx through L-type calcium channels that exhibit several uncommon features, including rapid kinetics of activation and deactivation, slow inactivation, and activation at an unusually negative voltage range for L-type channels. The purpose of this study was to establish the molecular identities of the $\alpha 1$ subunits responsible for these distinctive properties.

Methods—Transcripts for calcium channel $\alpha 1$ subunits expressed in individual goldfish ON-type bipolar cells were identified using single-cell reverse transcriptase polymerase chain reaction (RT-PCR). After cloning the goldfish homologs of the zebrafish and mammalian subunits, we designed sets of nested primers that are specific for $\text{Ca}_v 1.3a$, and $\text{Ca}_v 1.3b$ L-type calcium channels.

Results—Large-terminal, ON-type bipolar cells express transcripts of $\text{Ca}_v 1.3a$ and/or $\text{Ca}_v 1.3b$.

Conclusions—The endogenous expression of only one or both subunits in a single cell raises the possibility of functionally distinct classes of bipolar cells that differ in calcium current properties.

Glutamate release from goldfish bipolar cell terminals is driven by L-type calcium channels with uncommon biophysical properties [1]. The Ca^{2+} current in these cells activates at a more negative voltage range than is typical for L-type current, it activates and deactivates more rapidly, and it exhibits slower calcium-dependent inactivation [2,3]. These features, shaped by the $\alpha 1$ and auxiliary subunits, allow the cell to drive sustained neurotransmitter release, a requirement at the bipolar cell terminal. Although the synaptic terminal of the goldfish bipolar cell is a widely used model for neurotransmitter release at ribbon synapses, the molecular composition of the calcium channels that drive release is unknown.

The $\alpha 1$ subunit is the largest and most significant among the calcium channel subunits. Each of its four repetitive domains consists of six transmembrane segments. Together, the $\alpha 1$ subunit domains give rise to the pore-forming region, selectivity filter, voltage sensor, gating apparatus, and the channel's functional binding sites. Therefore, the $\alpha 1$ subunit shapes the channel's primary biophysical characteristics and is sufficient to form a functional calcium channel [4]. To date, there are 10 known $\alpha 1$ subunits in mammals. $\text{Ca}_v 3.1$ ($\alpha 1G$), $\text{Ca}_v 3.2$ ($\alpha 1H$), and $\text{Ca}_v 3.3$ ($\alpha 1I$) form T-type channels, and $\text{Ca}_v 2.1$ ($\alpha 1A$), $\text{Ca}_v 2.2$ ($\alpha 1B$) and $\text{Ca}_v 2.3$ ($\alpha 1E$) are present at P/Q, N, or R-type channels. The remaining four subunits, $\text{Ca}_v 1.1$ ($\alpha 1S$), $\text{Ca}_v 1.2$ ($\alpha 1C$), $\text{Ca}_v 1.3$ ($\alpha 1D$), and $\text{Ca}_v 1.4$ ($\alpha 1F$), form the L-type calcium channels. One or more of the L-type subunits may be expressed in the bipolar cell.

Based on the observed properties of heterologously expressed $\text{Ca}_v 1.2$ [5,6], all L-type calcium channels had been thought to share the distinctive properties of the native cardiac calcium current, including activation at high voltages, high sensitivity to dihydropyridines (DHPs),

slow activation and deactivation kinetics, and calcium-dependent inactivation [7]. Calcium currents mediated by $Ca_v1.1$ and $Ca_v1.2$ exhibit many of the classic L-type current characteristics. Compared to the native bipolar cell current, both channels are highly sensitive to DHPs, activate more slowly and at potentials 20–25 mV more depolarized, deactivate more slowly, and show strong calcium-dependent inactivation [8]. These properties make both $Ca_v1.1$ and $Ca_v1.2$ unlikely candidates for the presynaptic calcium channels at the bipolar cell synapse.

On the other hand, $Ca_v1.4$, found at the rod photoreceptor ribbon synapse, does not exhibit the classic kinetic or pharmacological profile of the L-type channel and has similar kinetics and DHP sensitivity to the bipolar terminal calcium current. Heterologous expression of $Ca_v1.4$ indicates however, that the channel's inactivation is insensitive to calcium [9,10]. This is unlike the bipolar cell calcium current, which inactivates in a calcium-dependent manner, albeit slowly. $Ca_v1.3$ is the closest in similarity to the native calcium current observed in bipolar cells. It is less sensitive to DHPs than classic L-type channels, has a threshold of activation that is physiologically relevant in the retina, and shows little calcium-dependent inactivation when depolarized [8,11]. The kinetic profile permits the channel to activate at subthreshold voltages and to mediate sustained calcium entry when the cell is depolarized. As the bipolar cell presynaptic terminal requires a similar kinetic and pharmacological profile to sustain rapid and long-lasting release, it is likely that $Ca_v1.3$ is the primary channel found in this cell type.

Previously detected in neuronal and endocrine cells [12], $Ca_v1.3$ has been shown by immunostaining to be present in both the outer and inner plexiform layers of the retina, where photoreceptor and bipolar cell terminals are located, respectively [13]. $Ca_v1.3$ expression has also been shown at the chick hair cell ribbon synapse, where mounting evidence suggests it is responsible for sustained neurotransmitter release [14]. Furthermore, studies in zebrafish hair cells revealed that a $Ca_v1.3$ -like channel mediates release at the hair cell ribbon synapse. While the mammalian and avian genomes contain only one copy of the $Ca_v1.3$ gene, the zebrafish genome contains two genes, both belonging to the 1.3 class: $Ca_v1.3a$ and $Ca_v1.3b$ [15]. As part of the extensive gene duplication in teleosts [16], $Ca_v1.3$ apparently underwent duplication and subsequently diverged, giving rise to the paralogs $Ca_v1.3a$ and $Ca_v1.3b$. The zebrafish mutant, *Gem*, is deaf and imbalanced as a result of a loss of calcium influx at hair cell ribbon synapses. The mutated gene giving rise to the phenotype, *Gemini*, was shown to encode $Ca_v1.3a$, thus supporting this channel's role in sustained neurotransmission at the hair cell ribbon synapse [15]. In the same study, $Ca_v1.3a$ expression was localized to the inner plexiform, inner nuclear and ganglion cell layers of the retina, whereas $Ca_v1.3b$ was localized to the outer plexiform and photoreceptor layers [15]. It is unclear, however, which of these paralogs might be expressed in the bipolar cell.

We used single-cell reverse transcriptase polymerase chain reaction (RT-PCR) to determine if $Ca_v1.3a$ and $Ca_v1.3b$ transcripts are expressed in individual morphologically distinct bipolar neurons enzymatically dissociated from goldfish retina. We show that single bipolar cells express transcripts of the L-type calcium channel pore-forming subunits $Ca_v1.3a$ and $Ca_v1.3b$. Different combinations of $\alpha 1$ subtype expression may alter the specific function of the cell within the network.

METHODS

Cloning $Ca_v1.3a$ and $Ca_v1.3b$ from goldfish retina using RT-PCR

All animal procedures were carried out in accordance with National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee. Initial $\alpha 1$ sequences were generated by RT-PCR from goldfish retina total RNA using random hexamers (Invitrogen, Carlsbad, CA) for reverse transcription. Goldfish equivalents of

Ca_v1.3a and Ca_v1.3b were amplified with degenerate primers designed against known zebrafish and mammalian sequences. The first round primers used to detect Ca_v1.3a were 5'-AYT TGG CWG ACG CWG ARA G-3' and 5'-GAA RAA RAT GGA GAT SWC CAC-3'. The second round primers were 5'-TCT TCT TGG CCA TYG CTG TRG-3' and 5'-CGA ART TGA AAT CAC TRT TYT SCC-3'. The first round primers used to detect Ca_v1.3b were 5'-CTT GGM ATG CAG CTS TTT GG-3' and 5'-GTT RAA RTC ACT GTT SWC CCA G-3'. The second round primers used were 5'-GTC TTT CAG ATY YTG ACN GGW GAG G-3' and 5'-CTG MAC CAC ATG YTT GAG KCC-3'. Subsequently, subunit-specific primer sets were designed to extend the sequences 5'. The Ca_v1.3a primers were 5'-CGT CGC TGG AAC CGG TTG-3' and 5'-CTC GTC TCC AGC CTT GGC AT-3'. The Ca_v1.3b primers were 5'-CAG AAG CTT CGR GAG AAR CAG CAG-3' and 5'-CAG ATG CCT TGA CGT CTC CAT-3'. The GenBank accession numbers of the corresponding goldfish Cav1.3a and Cav1.3b sequences are DQ314779 and DQ314780, respectively. The goldfish sequences were then used to design primers for single-cell RT-PCR.

Single-cell RT-PCR

For single-cell RT-PCR, goldfish retinal cells were dissociated by mechanical trituration after papain digestion, as described in the literature [1]. Cells were plated on flamed polished glass coverslips and washed thoroughly with goldfish Ringer's solution to eliminate debris. For each experiment, several negative controls were collected to assess the possibility of false positives. In addition, two bipolar cells per experiment were collected and processed normally, except that reverse transcriptase was omitted. These "RT" cells test that detected transcripts were derived from cellular mRNA. To check for possible contamination in reagents, one water-only sample underwent PCR and was designated "no DNA." The contents of single bipolar cells and controls were aspirated into a whole-cell patch pipette containing approximately 1 μl RNase-free solution. The contents of the pipette were then expelled into a siliconized 0.5 ml microfuge tube containing 10.5 μl nuclease-free water (Ambion, Austin, TX). We immediately added 4 μl 5X first strand buffer, 0.5 μl 0.1 M DTT, 2 μl RNAsin (2 units/μl), 1 μl dNTPs (10 mM each), and 1 μl random hexamer primers (3 mg/ml). All reagents were obtained from Invitrogen. Following a 10 min incubation at room temperature, 1 μl (200 units) of Superscript II reverse transcriptase was added, and cDNA synthesis was carried out at 42 °C for 1 h. Amplification of a region of a specific subunit was achieved with two rounds of PCR using Platinum Taq DNA polymerase and 2 μl reverse-transcribed cDNA in 50 μl PCR buffer and reactants (Invitrogen). The PCR protocol was as follows: 95 °C for 5 min, 40 cycles of 95, 55, and 72 °C for 1 min each, and 72 °C for 4 min at completion. The first round primers were degenerate and designed to span Domain II, the II-III linker, and a piece of Domain III. This region was chosen based on the high degree of homology across subunits and species, thus allowing the first-round primers to effectively amplify both Ca_v1.3a and Ca_v1.3b. The degenerate forward primer was 5'-GKC TGC AGG CMT AYT TTG TGT C-3' and the degenerate reverse primer was 5'-GAG GAT GAG RTT GGT GAA GAT STG RTG-3' (expected size of Ca_v1.3b 896 bp, Ca_v1.3a 830 bp). The second-round specific primers were designed to span the II-III linker region of a specific target gene and nest within the first round primers. This region was chosen for the high degree of divergence between Ca_v1.3a and Ca_v1.3b while preserving homology within a subunit across species. The specific forward primer designed to target Ca_v1.3b was 5'-TTG CTT GGC ATG CAG CTC-3', and the specific reverse primer was 5'-AGG CTC AGC TCT GAC AGC CT-3' (512 bp). The specific forward primer designed to target Ca_v1.3a was 5'-GTG GTG TGT GGA GGC ATC AC-3', and the specific reverse primer was 5'-CGT CTC CAG CCT TGG CAT-3' (577 bp). All first and second round primer sets are predicted to span introns based on the mammalian Ca_v1.3 genomic sequence. PCR products of the correct size were gel-purified, subcloned in pGEM-T Easy (Promega, Madison, WI), and sequenced to verify their identity.

RESULTS

To characterize the molecular identity of the partial cDNAs obtained from goldfish retina, the predicted amino acid sequences were aligned with known zebrafish and mammalian sequences (ClustalW). The percent identity, based on the partial sequence, between goldfish $Ca_v1.3a$, and zebrafish $Ca_v1.3a$ is 95%, and the percent identity to human $Ca_v1.3$ is 82%. The percent identity between goldfish $Ca_v1.3b$ and zebrafish $Ca_v1.3b$ is 82%, and the percent identity to human $Ca_v1.3$ is 71% (NCBI BLAST). Figure 1 is an alignment across subunits and species of the II-III linker amino acid sequences (ClustalW). The regions in red highlight amino acid motifs that are highly conserved in $Ca_v1.3a$ and $Ca_v1.3b$ across species. Although $Ca_v1.2$ shares similar conserved motifs as $Ca_v1.3$, the overall homology between goldfish $Ca_v1.3a/b$ and zebrafish $Ca_v1.2$ is significantly lower compared to zebrafish $Ca_v1.3a/b$. Other $\alpha 1$ subunits such as $Ca_v1.1$, 1.4, 2.1 and 2.2 have low homology and do not share these conserved motifs. Therefore, we conclude that the subunits we have detected in goldfish are orthologs of zebrafish $Ca_v1.3a$ and $Ca_v1.3b$.

To determine the subunits present in mixed bipolar cells, we utilized the single cell RT-PCR technique described in Methods. Figure 2 shows an example of amplicons detected in the second round of PCR for a single experiment. Each sample was collected in the order that it appears on the agarose gel. Lanes 1, 4, 6, 7, 9, and 10 are results from single bipolar cell cDNA synthesis. The remaining lanes show results of control experiments: Lanes 2 and 11 are -RT control cells, lanes 3, 5, and 8 are bath-fluid +RT controls, and lane 12 is a no DNA (water) control. In this experiment, $Ca_v1.3a$ and $Ca_v1.3b$ were detected both individually and together in single cells. One bipolar cell expressed only $Ca_v1.3a$ (lane 1), another expressed only $Ca_v1.3b$ (lane 7), and one cell expressed both $Ca_v1.3a$ and $Ca_v1.3b$ (lane 9). Experiments were included in the analysis only if all negative controls failed to yield PCR products and at least one sample was clearly positive for one or both subunits. PCR products of the correct size were gel-purified, subcloned in pGEM-T Easy, and sequenced, which confirmed the identity of the detected transcripts.

In 11 experiments that met the criteria, 25% of all cells collected were positive for $Ca_v1.3a$, $Ca_v1.3b$, or both. In 17 cells that were negative for both transcripts, the original RT sample underwent another two rounds of PCR to determine if negative cells would become positive when retested. All 17 samples were again negative for both transcripts, which suggests that detection failure occurred at the RT stage.

Overall, $Ca_v1.3a$ or $Ca_v1.3b$ transcripts were detected in 18 bipolar cells. Figure 3 shows 16 of the 18 positive products, after two rounds of PCR. All amplicons were of the expected size except the $Ca_v1.3a$ amplicon detected in lane 11, which was smaller than the expected size of 577 bp. Sequencing revealed that the detected transcript in this case was $Ca_v1.3a$, suggesting a possible splice variant. Figure 4A shows the percentage of the total number of positive cells expressing each $\alpha 1$ subunit: 33% expressed only $Ca_v1.3b$, 39% expressed only $Ca_v1.3a$, and 28% of cells expressed transcripts of both subunits. Figure 4B is a bright field image of an ON-type mixed bipolar cell harvested for single cell RT-PCR and treated as a -RT negative control. All of the cells sampled were large terminal mixed bipolars, and we did not examine small-terminal, presumptive cone bipolar cells.

DISCUSSION

Using single cell RT-PCR, we have shown that mRNA transcripts of the L-type calcium channel subunits $Ca_v1.3a$ and/or $Ca_v1.3b$ are expressed in bipolar cells of the goldfish retina. Though mRNA expression does not always assure protein synthesis, the similarities between the bipolar calcium current and chick and mammalian IHC $Ca_v1.3$ currents support the

conclusion that Ca_v1.3a and/or Ca_v1.3b are translated into functional proteins in bipolar cells. Whole-cell patch clamp experiments have revealed that the bipolar cell calcium current rapidly activates at low voltages and is less sensitive to DHPs compared to Ca_v1.1 and Ca_v1.2. Also, inactivation of the calcium current in bipolar cells occurs only after an extended depolarization and completes with a time course of several seconds, which is about 100 fold slower than in many other cells expressing L-type calcium channels [2]. Nonetheless, inactivation eventually takes place in a calcium-dependent manner, unlike heterologously expressed Ca_v1.4. These characteristics are closely mimicked by the Ca_v1.3-mediated calcium current recorded in mammalian and avian systems. Whole-cell patch clamp recordings of mouse inner hair cells (IHCs) reveal calcium currents reminiscent of the L-type current found in bipolar cells: rapidly activating at low voltages and slowly inactivating in a calcium-dependent manner [17]. In the same study, a Ca_v1.3^{-/-} mouse was generated to test the function of this L-type calcium channel subunit at the inner hair cell ribbon synapse. The null mouse lost 90% of its calcium current density, was resistant to DHPs, and had congenital deafness [17]. In another study, Ca_v1.3^{-/-} mice exhibited reduced exocytosis in the organ of corti, indicating a direct role for Ca_v1.3 in neurotransmission [18]. These experiments provide evidence that Ca_v1.3 is the primary L-type calcium channel mediating neurotransmitter release at the IHC ribbon synapse. The resemblance of calcium current characteristics among ribbon synapses of the cochlea and of the retina lends credence to the idea that Ca_v1.3a and/or Ca_v1.3b mRNA expression predicts functional protein expression in the goldfish bipolar cell.

In goldfish bipolar cells, approximately 90% of the whole-cell calcium current is generated by calcium channels residing in the synaptic terminal, and the remaining 10% arises from the soma [1]. We found that at least some bipolar cells express transcripts for both Ca_v1.3a and Ca_v1.3b calcium channels, and it is possible that the two channel subtypes differ in subcellular localization, and thus in function. For instance, Ca_v1.3a may be associated with synaptic ribbons and drive transmitter release, whereas Ca_v1.3b may represent the somatic calcium current. However, the specific properties of Ca_v1.3b are not known, nor is it known whether the two genes encode channels with functionally distinct properties. Our results suggest that subsets of bipolar cells may express either Ca_v1.3a or Ca_v1.3b alone, which raises the possibility of functionally distinct classes of bipolar cells that may differ slightly in calcium current characteristics. Clarification of these issues of isoform distribution both within and across cells awaits the availability of subtype-specific antibodies to localize the Ca_v1.3a and Ca_v1.3b alpha subunits in fish retina.

Acknowledgements

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GfCav1.3a	--DAESLNTD	SDDKKKGDEN	EE-----	-----
ZfCav1.3a	--DAESLNT-	-DDTKKPDEI	DE-----	-----
GfCav1.3b	--DAESLNSA	QKEE-EKRNK	RKKSRSMSIY	KRDG-----
ZfCav1.3b	--DAESLNSA	QKEE-EKRNK	RRKSRSMSIY	KRDG-----
hCav1.3	--DAESLNTA	QKEEAEKER	KKIARKESLE	NKKN-NKPEV
ZfCav1.4	--GGDGDNK--	---KNEEK--	-KEGE-----	AEPA-----
hCav1.4	SGDAGTAKDK	GGEKSNEKDL	PQENEGLVPG	VEKE-----
ZfCav1.1	--EAESLAAA	QKERAEERAR	KKLMKPL-PE	KTEE-EKALM
ZfCav1.2	--DAESL TSA	QKEEEEEKER	KKLARTASPE	KRQNSEKPPL
ZfCav2.1	--NAQELTKD	EQEEEQAANK	KMALQTAKEV	AEVSPLSAAN
GfCav1.3a	-----	-----	-----NEED	AKAGDEDEKD
ZfCav1.3a	-----	-----	-----IEDE	AKAGEBEDEKD
GfCav1.3b	-----	-----	-----DDGD	VKASGDQEGD
ZfCav1.3b	-----	-----	-----DDRE	LKAPDDQEAS
hCav1.3	NQIAN-----	-----	-SDNKVTIDD	YR-EEDEDKD
ZfCav1.4	-----	-----	-----EBEN	GEVK-VDMDE
hCav1.4	-----	-----	-----EEEG	ARREGADMEE
ZfCav1.1	AKRLMESRQK	-----TEG-M	PTTAKLKIDE	FESNVNEVKD
ZfCav1.2	EDEKKBEKIE	LKSITSDGET	PTATKINIDE	YTGEDNEEKN
ZfCav2.1	LSIAAKEQOK	NHKG-----	-CKSVWEQRT	SELRRQTLVN
GfCav1.3a	N-----A	EEDEDL----	----PDVPAG	PRPM--
ZfCav1.3a	N-----A	EEDEEE----	----PDVPAG	PRPK--
GfCav1.3b	QDQTEDVIDS	SEEDDE----	--DEPEVPSG	PRPPR-
ZfCav1.3b	RDPAEELADS	SEEEEE----	--EEPEVPSG	LRAQR-
hCav1.3	PYPDCDVPVG	EEEEEEEE----	-EDEPEVPSG	PRPRR-
ZfCav1.4	EYE-----E	EEEEPE----	----GDDEE-	-----
hCav1.4	EEE-----E	EEEEEE----	----EEEEEG	AGGVEL
ZfCav1.1	PFPADFP-G	DDEEEEE----	----PEIPIS	PRPRP-
ZfCav1.2	PYPVNDFPAG	EDDEEE----	----PEMPVG	PRPRP-
ZfCav2.1	SREALYNELD	PEDRWKVSYS	RHIRPDMKTH	LDRPL-

Figure 1.

Alignment of II-III linker amino acid sequences across subunits and species. The red regions highlight amino acid motifs that are highly conserved among Ca_v1.3a and Ca_v1.3b across species. The zebrafish α 1 subunits Ca_v1.1, Ca_v1.2, Ca_v1.4, Ca_v2.1, and human Ca_v1.4 do not share the conserved motifs.

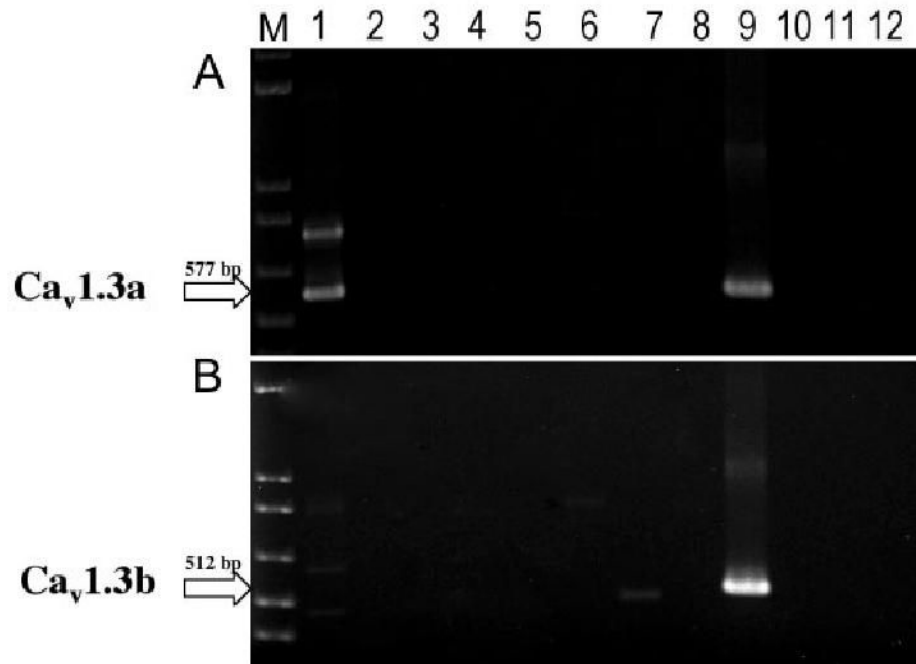


Figure 2.

Calcium channel expression pattern in a single experiment. Gel electrophoresis of second round reverse transcriptase polymerase chain reaction (RT-PCR) products. Six negative controls were included to eliminate the possibility of false positives (lanes 2, 3, 5, 8, 11, 12). Six bipolar cells were collected for subunit detection by RT-PCR (lanes 1, 4, 6, 7, 9, 10). Expected band sizes (indicated by arrows): **A** Ca_v1.3a-577 bp. **B**: Ca_v1.3b-512 bp. One cell expressed only Ca_v1.3a (lane 1), one expressed only Ca_v1.3b (lane 7), and one expressed both subunits (lane 9). On average across experiments, about 25% of all collected bipolar cells expressed one or more calcium channel subunits. M denotes the 1 kb plus DNA ladder (Invitrogen).

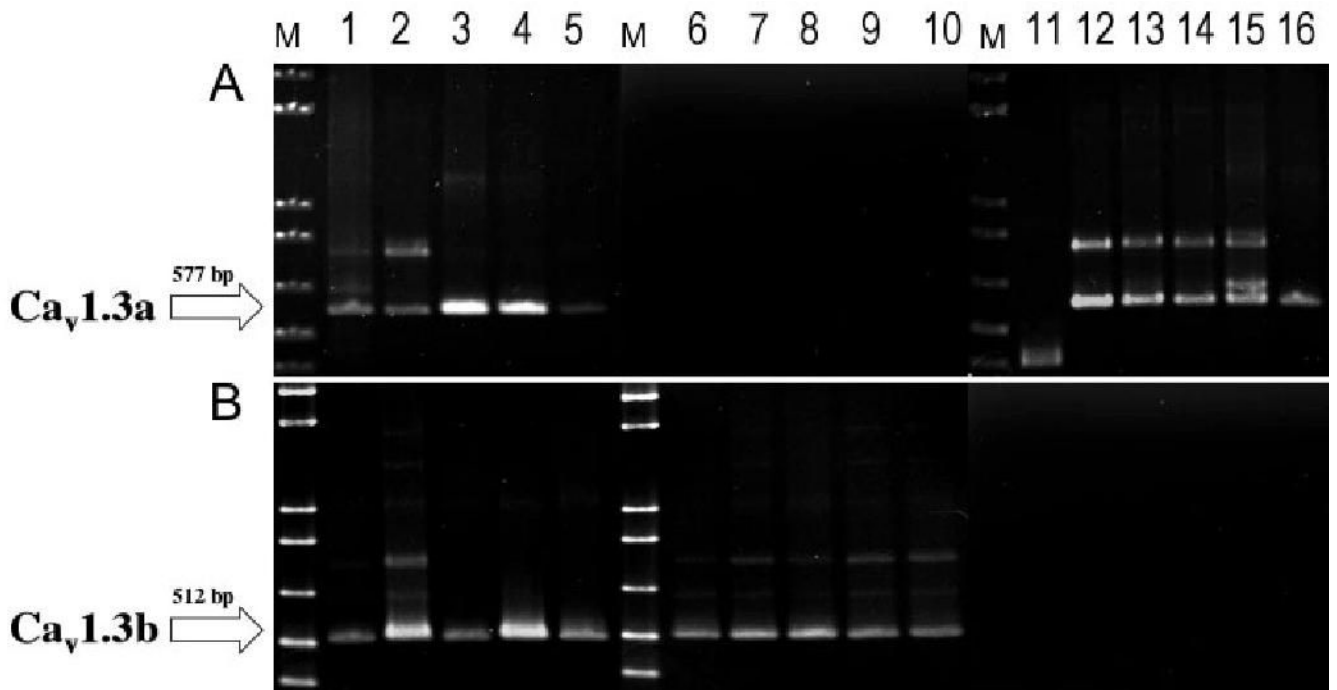


Figure 3.

Bipolar cells express transcripts of Ca²⁺ channel subunits Ca_v1.3a, Ca_v1.3b, or both. Gel electrophoresis of reverse transcriptase polymerase chain reaction (RT-PCR) products. cDNA from 16 of 18 positive cells are shown. **A:** Ca_v1.3a PCR products. Samples in lanes 1–5 and 11–16 were run on the same gel. Samples in lanes 6–10 were run on a separate gel. **B:** Ca_v1.3b products. Samples in lanes 1–10 were run on the same gel. Samples in lanes 11–16 were run on a separate gel. Bands that appear in the same lane in both panels **A** and **B** indicate cells that express both subunits. Correct band size is indicated with an arrow. Sample number 11 is smaller in size than the rest of the Cav1.3a products, but sequencing verified that this product is Cav1.3a. It is not known whether this represents a Cav1.3a splice variant. M denotes the 1 kb plus DNA ladder (Invitrogen).

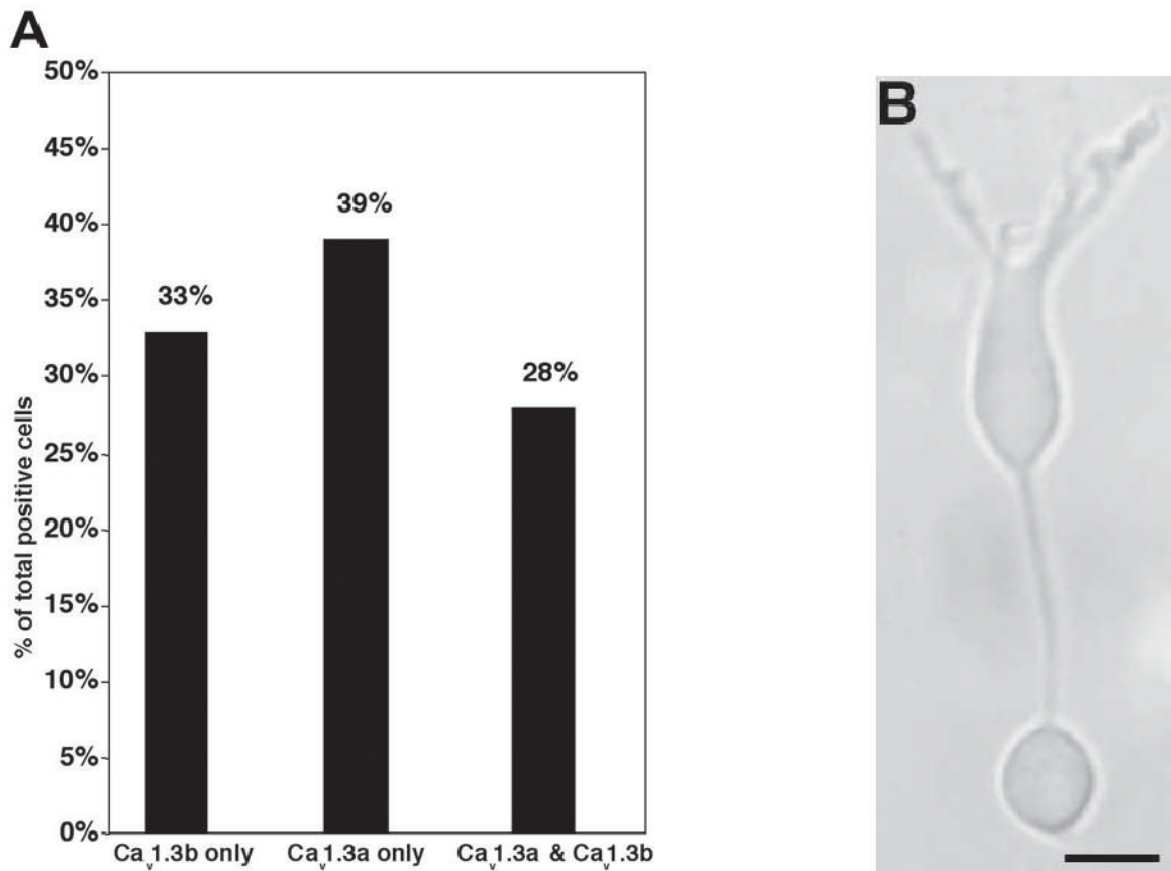


Figure 4. Goldfish bipolar cells form L-type Ca²⁺ channels composed of one or more subunits. **A:** Percentage of total positive cells expressing $\alpha 1$ subunits. Of the 18 cells positive for $\alpha 1$ subunits, 33% expressed only Ca_v1.3b, 39% express only Ca_v1.3a, and 28% expressed both subunits. **B:** Bright field image of an ON-type goldfish bipolar cell. Scale bar represents 10 μ m.