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Parvalbumin 3 is an Abundant Ca^{2+} Buffer in Hair Cells

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ABSTRACT

Ca^{2+} signaling serves distinct purposes in different parts of a hair cell. The Ca^{2+} concentration in stereocilia regulates adaptation and, through rapid transduction-channel reclosure, underlies amplification of mechanical signals. In presynaptic active zones, Ca^{2+} mediates the exocytotic release of afferent neurotransmitter. At efferent synapses, Ca^{2+} activates the K^+ channels that dominate the inhibitory postsynaptic potential. A copious supply of diffusible protein buffer isolates the three signals by restricting the spread of free Ca^{2+} and limiting the duration of its action. Using cDNA subtraction and a gene expression assay based on *in situ* hybridization, we detected abundant expression of mRNAs encoding the Ca^{2+} buffer parvalbumin 3 in bullfrog saccular and chicken cochlear hair cells. We cloned cDNAs encoding this protein from the corresponding inner-ear libraries and raised antisera against recombinant bullfrog parvalbumin 3. Immunohistochemical labeling indicated that parvalbumin 3 is a prominent Ca^{2+}-binding protein in the compact, cylindrical hair cells of the bullfrog's sacculus, and occurs as well in the narrow, peanut-shaped hair cells of that organ. Using quantitative Western blot analysis, we ascertained that the concentration of parvalbumin 3 in saccular hair cells is approximately 3 mM. Parvalbumin 3 is therefore a significant mobile Ca^{2+} buffer, and perhaps the dominant buffer, in many types of hair cell. Moreover, parvalbumin 3 provides an early marker for developing hair cells in the frog, chicken, and zebrafish.

Keywords: afferent synapse, efferent synapse, electrical resonance, mechanoelectrical transduction

INTRODUCTION

To subserve its functions of transduction, tuning, and transmission, a hair cell makes use both of signals that spread throughout the cell and of those confined to specific subcellular domains. Because the cell is electrically compact, lacking the elongated axon and protruding dendrites that give many neurons their complex electrical characteristics, its membrane potential reflects the summation of ionic currents from at least three spatially distinct sources that contribute to the electrical response. First, the current through the hair bundle's mechanoelectrical transduction channels initiates the receptor potential. Next, the Ca^{2+} current at presynaptic active zones triggers the release of afferent synaptic transmitter. In many hair cells, this current additionally interacts with K^+ currents to mediate electrical resonance, a process that abets frequency selectivity. Finally, the Ca^{2+}-dependent K^+ current across the postsynaptic membrane at efferent synapses produces a long-lasting hyperpolarization that desensitizes the hair cell.

Local signaling in hair cells is mediated primarily by Ca^{2+}. The efficacy of mechanoelectrical transduction, for example, depends upon the Ca^{2+} concentration near the stereociliary tips (Lumpkin and Hudspeth 1998; Ricci et al. 1998). Ca^{2+} that enters through transduction channels regulates the molecular motors responsible for adaptation (reviewed in Eatock 2000; Holt and Corey 2000). This influx also mediates rapid

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reclosure of these channels (Howard and Hudspeth 1988), a phenomenon that constitutes a fast form of adaptation (reviewed in Holt and Corey 2000; Fettiplace et al. 2001). Both processes power active hair-bundle motility, a source of mechanical amplification in the hair cells of nonmammalian tetrapods (Martin and Hudspeth 1999, 2001; Martin et al. 2000; reviewed in Hudspeth 1997; Hudspeth et al. 2000).

As at other synapses using quantal transmission, the Ca^{2+} concentration at the hair cell's presynaptic active zones regulates the release of afferent neurotransmitter. When Ca^{2+} enters through L-type channels congregated at an active zone (Roberts et al. 1990; Issa and Hudspeth 1994), the local Ca^{2+} concentration rises sufficiently to be imaged with fluorescent indicators (Issa and Hudspeth 1994, 1996; Tucker and Fettiplace 1995; Hall et al. 1997). Clustered with Ca^{2+} channels are *Slo*-type, high-conductance K^+ (BK) channels (Roberts et al. 1990; Issa and Hudspeth 1994). Gated by the local increase in Ca^{2+} concentration, these channels bear the dominant current in electrical resonance. Coclustering ensures that the K^+ channels experience large, rapid increases in Ca^{2+} concentration when the Ca^{2+} channels are activated (Roberts 1994).

At efferent synapses, acetylcholine released from presynaptic terminals activates receptors comprising α9 and α10 subunits (Elgoyhen et al. 1994, 2001). The high Ca^{2+} permeability of these receptors results in an increase in the Ca^{2+} concentration sufficient to activate small-conductance K^+ (SK) channels, producing a protracted hyperpolarization (Housley and Ashmore 1991; Shigemoto and Ohmori 1991). The accumulation of Ca^{2+} again suffices for imaging with fluorescent Ca^{2+} indicators (Shigemoto and Ohmori 1990).

While facilitating electrical signaling, the compact nature of a hair cell poses a problem: How can the three physiological processes described above function independently when each relies upon cytoplasmic Ca^{2+} as a critical intermediate? To isolate the local Ca^{2+} signals from one another, hair cells make use of a high concentration of cytoplasmic Ca^{2+} buffer (reviewed in Lenzi and Roberts 1994; Jaramillo 1995). By intercepting Ca^{2+} near its site of entry, this buffer restricts the diffusive spread of the free ion. Buffering additionally limits the period during which the concentration of Ca^{2+} is great enough to trigger the ion's physiological effects. Physiological assays reveal that the endogenous Ca^{2+} buffer is highly mobile (Roberts 1993; Issa and Hudspeth 1996; Hall et al. 1997), and hence a relatively small molecule, presumably a protein. The speed of Ca^{2+} abstraction and the cytoplasmic buffering capacity are mimicked by an exogenous chelator such as BAPTA at a concentration of 1–2 mM (Roberts 1993; Tucker and Fettiplace 1996; Ricci et al. 1998).

Numerous Ca^{2+}-binding proteins that might serve as buffers have been identified in hair cells (for comprehensive studies see Kerschbaum and Hermann 1993; Slepecky and Ulfendahl 1993; Pack and Slepecky 1995; Baird et al. 1997). Calbindin, which is also called cholecalcin or vitamin D-dependent calcium-binding protein (CaBP-28K), has been observed in the receptors of several organs of the inner ear (Sans et al. 1986). Calretinin occurs in hair cells, including those of the mammalian cochlea (Dechesne et al. 1991; Edmonds et al. 2000). Hair cells also display immunoreactivity to conventional parvalbumin (Kerschbaum and Hermann 1993) and the related protein oncomodulin (Henzl et al. 1997; Sakaguchi et al. 1998). Found more commonly in supporting cells, S-100 is expressed by some hair cells as well (Saidel et al. 1990). Although calmodulin has been localized to hair cells (Shepherd et al. 1989), this protein serves less as a buffer than as a Ca^{2+} detector for effector molecules with which it interacts (Walker and Hudspeth 1996). Consistent with such a role, calmodulin occurs in hair bundles at a concentration of only 70 μM (Walker et al. 1993), most of it bound to plasmamembrane Ca^{2+}–ATPase (PMCA; Yamoah et al. 1998). Calsequestrin, which displays a patchy distribution in some hair cells (Slepecky and Ulfendahl 1993), is also unlikely to serve as a mobile Ca^{2+} buffer. This protein is ordinarily concentrated within calciosomes, where it binds Ca^{2+} accumulated by the smooth endoplasmic reticulum Ca^{2+}–ATPase (SERCA; Tucker and Fettiplace 1995, 1996).

Despite the wealth of reports on Ca^{2+}-binding proteins, hair cells must employ additional Ca^{2+} buffers that have not yet been recognized. The most striking evidence for this stems from physiological studies of the compact, cylindrical hair cells of the frog's sacculus. Although the prodigious Ca^{2+}-buffering capacity of these cells has been characterized extensively (Roberts 1993, 1994), no Ca^{2+} buffer has been identified at the concentration necessary to explain the results. In the present study, we have used molecular–biological techniques to identify a Ca^{2+} buffer that occurs at a high concentration in these and many other hair cells.

METHODS

Cloning of cDNAs encoding the bullfrog's parvalbumin 3

The complete coding sequence of chicken parvalbumin 3 (GenBank accession No. U03850) was used as a ^{32}P-dCTP-labeled cDNA probe in plaque hybridization on 5×10^5 clones of a bullfrog saccular cDNA library. Constructed from 0.5 μg of poly(A)+ RNA derived from 200 dissected sacculi, the library com-

prises 1.25×10^6 primary clones in the lambda bacteriophage vector ZAP Express (Stratagene, La Jolla, CA). We purified eight cDNA clones from more than 100 hybridization-positive colonies; sequencing revealed that each of these represented a full-length isolate encoding bullfrog parvalbumin 3.

The cDNA sequence for the bullfrog's parvalbumin 3 has been deposited in GenBank under accession No. AY049967.

Expression analysis

Northern blot analysis was performed with ^{32}P-dCTP-labeled cDNA probes corresponding to the complete coding sequence and 252 nucleotides of the 3′-untranslated region of the chicken's parvalbumin 3 mRNA. *In situ* hybridization was conducted on frozen sections of the chicken's or the bullfrog's inner ear with digoxigenin-labeled cRNA representing, respectively, the same sequence of the chicken's parvalbumin 3 mRNA or the complete coding sequence and 263 nucleotides of the 3′-untranslated region of the bullfrog's parvalbumin 3 mRNA. In control experiments conducted under identical conditions, the respective sense probes consistently yielded no detectable labeling.

Production of antisera

The complete coding sequence of bullfrog parvalbumin 3 was subcloned into the pFastBac HTa expression vector (Life Technologies, Carlsbad, CA). Recombinant bullfrog parvalbumin 3, tagged with a hexahistidine moiety at its amino terminus, was expressed in insect cells (SF9, Life Technologies) and purified under native conditions with Ni^{2+}-conjugated agarose beads (Ni^{2+}–NTA, Qiagen, Valencia, CA). The histidine affinity tag was cleaved from the fusion protein by treatment with recombinant TEV protease (Life Technologies) that itself bore a hexahistidine motif. After the proteolyzed affinity tag and protease had been removed by affinity chromatography, the recombinant parvalbumin 3 was purified by gel filtration (Superdex 75 HR 10/30, Amersham Pharmacia Biotech, Inc., Piscataway, NJ). The product differed from the predicted native protein only by the addition at its amino terminus of a glycine and an alanine residue, which formed part of the target for specific proteolytic cleavage of the fusion protein.

Two female rabbits were each initially immunized with 200 μg of bullfrog parvalbumin 3; additional boost injections were given at 2–3-week intervals (Covance Inc., Princeton, NJ). The sera of both animals displayed strong reactivity against the immunogen. All experiments described in this publication were done with the final-bleed serum of one rabbit.

Detection of parvalbumin 3 by immunohistochemistry and Western blotting

Specimens to be used in immunohistochemical procedures were fixed overnight at 4°C with 4% (wt/vol) paraformaldehyde in PBS. The specimens were then cryoprotected for 48 h at 4°C in 30% (wt/vol) sucrose in PBS; the solution was renewed every 12–16 h. Sections 14 μm thick were cut with a cryomicrotome (CM3000, Leica Instruments GmbH, Nussloch, Germany), collected on silylated slides (PGC Scientific, Gaithersburg, MD), dried at 37°C for 45 min, and stored frozen at −80°C.

For immunodetection, the sections were thawed and nonspecific binding sites were blocked for 1 h in 10% (vol/vol) heat-inactivated goat serum in PBS. The slides were then incubated overnight at room temperature in antiserum, diluted 1:30,000 to 1:50,000 for antiparvalbumin 3 and 1:2000 for anticalretinin (Edmonds et al. 2000) in 290 mM NaCl, 3 mM KCl, 6.5 mM NaH_2PO_4, and 1.5 mM KH_2PO_4 at pH 7.3 (high-salt PBS). Unbound protein was removed by three PBS washes for 15 min each at room temperature. FITC-conjugated antirabbit and TRITC-conjugated antimouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were diluted 1:400 in high-salt PBS. A 2-h incubation in secondary antibody mixture preceded three washes for 15 min each in PBS. The coverslipped slides were analyzed by confocal microscopy (MRC-1024ES, Bio-Rad Laboratories, Hercules, CA).

Western blots were incubated for 1 h at room temperature in 2.5% (vol/vol) Liquid Block (Amersham Pharmacia Biotech, Inc.) and 0.1% (vol/vol) Tween-20 in PBS. Parvalbumin 3 antiserum was diluted 1:20,000 for detection of the parvalbumin 3 content of whole sacculi and for protein expression analysis; a dilution of 1:10,000 was used for evaluation of the parvalbumin 3 content of individual hair cells. Blots were incubated overnight at 4°C in diluted antiserum in 2.5% (vol/vol) Liquid Block and 0.1% (vol/vol) Tween-20 in PBS and washed four times for 15 min each at room temperature in 0.1% Tween-20 in PBS. Bound primary antibodies were detected with horseradish peroxidase-conjugated antirabbit serum (Amersham Pharmacia Biotech, Inc.) at a dilution of 1:7500 in 0.1% Tween-20 in PBS. Unbound antibodies were removed by two washes of 15 min each in 0.1% Tween-20 in PBS and two washes for 15 min each in PBS. Detection was performed with chemiluminescence substrate (ECL plus) and exposure to Hyperfilm ECL (Amersham Pharmacia Biotech, Inc.). Quantification of bands on scanned films was conducted with NIH Image software (version 1.61, http://rsb.info.nih.gov/nih-image/).

We confirmed the concentration of the recombinant protein employed as a standard by two independent analyses of individual amino acids and by measurement of the optical density of the protein solution at 280 nm (Gill and von Hippel 1989; http://www.expasy.ch/tools/). Both amino acid analyses agreed well with the optical density measurements. By contrast, because no parvalbumin 3-related reference protein was available, colorimetric assays of the concentration of parvalbumin 3 in solutions varied among multiple determinations and yielded inexact results that we accordingly disregarded.

Isolation of hair cells

To collect individual hair cells for Western blot analysis of their parvalbumin 3 content, we isolated cells from the bullfrog's sacculus, as described previously (Lumpkin and Hudspeth 1995). After dissociation of hair cells onto an uncoated glass coverslip, we observed them with Nomarski optics to identify single cells that were characteristic of two categories: the compact, cylindrical cells and the narrow, peanut-shaped cells. Each selected hair cell was sucked into an unpolished micropipette with a 15–20-μm orifice. The pipette's contents were then expelled by positive pressure and its tip was crushed in the sample tube to ensure complete recovery of the sample.

Estimation of hair–cell volume

To estimate the volumes of hair cells, we isolated cells as described above with the following modifications. To obtain a fluorescence image of each cell's plasmalemma, we exposed the cells to one of two lipophilic dyes, di-8-ANEPPS or Calcium Green-C_{18} as the hexapotassium salt (Molecular Probes, Inc., Eugene, OR). After protease treatment, maculae were placed in standard saline solution (Lumpkin and Hudspeth 1995) with the Ca^{2+} concentration reduced to 100 μM and exposed for either 40–120 min to 150 μM di-8-ANEPPS or 10 min to 10 μM. Calcium Green-C_{18}. Di-8-ANEPPS solutions were made by dilution of a 1-mg/mL stock solution in DMSO containing 0.05% (wt/vol) of the detergent Pluronic F-127 (Molecular Probes, Inc.). Hair cells were then dissociated into chambers with glass coverslip bottoms that had previously been treated for 40 min with a 60 μg/mL aqueous solution of poly-L-lysine to promote adhesion.

We estimated hair cell volume from serial images acquired with a laser-scanning confocal microscope (LSM510, Carl Zeiss, Jena, Germany) equipped with Nomarski optics and a 40× C-Apochromat water–immersion objective lens of numerical aperture 1.2. During confocal scanning of cells treated with di-8-ANEPPS, the standard saline solution in the chamber included 50 μM di-8-ANEPPS, which does not fluoresce when free in an aqueous environment. Confocal images were acquired at intervals of either 0.5 μm or 1.0 μm and with a pinhole setting that held the optical slice thickness below 0.6 μm. We determined a hair cell's cross-sectional area within each slice by outlining the fluorescent signal from the plasmalemma by eye and using the confocal system's software to ascertain the enclosed area. The cell's volume was then approximated by summation of its cross-sectional area in each slice over the image stack and multiplying the result by the imaging interval.

RESULTS

Cloning of cDNAs encoding parvalbumin 3

In a search for genes restricted in their expression to the inner ear, we used subtractive hybridization to enrich cDNAs from the chicken's cochlea for those absent from other organs (Heller et al. 1998). Employing these cDNA mixtures as probes, we isolated cDNA clones from a chicken cochlear library and verified the expression pattern of the corresponding genes by *in situ* hybridization. We identified one cDNA whose associated mRNA apparently occurred only in hair cells of the chicken's cochlea and vestibular organs. Sequencing revealed that this cDNA encoded the Ca^{2+}-binding protein parvalbumin 3 (pv3; Henzl et al. 1991). A small protein of 109 amino acids (Fig. 1) and a calculated molecular mass of 12.4 kDa, parvalbumin 3 displays sequence homology to both muscle parvalbumin and avian thymic hormone, or parvalbumin 2, a secreted protein found in the chicken's thymus (Hapak et al. 1996). Chicken parvalbumin 3 also resembles mammalian oncomodulin, a Ca^{2+}-binding protein that has been observed in cochlear outer hair cells (Henzl et al. 1997; Sakaguchi et al. 1998).

Because the endogenous Ca^{2+} buffer in hair cells of the bullfrog's sacculus has been studied in detail, we asked whether parvalbumin 3 is expressed in this organ as well. Using chicken parvalbumin 3 cDNA as a probe, we screened a cDNA library derived from bullfrog sacculi and isolated eight plasmid clones encoding bullfrog parvalbumin 3. The predicted anuran protein closely resembles that from the chicken (Fig. 1).

Expression of parvalbumin 3

Northern blot analysis using total RNA prepared from the cochleae of chicken hatchlings revealed an abundance of a single parvalbumin 3 mRNA transcript of 0.8 kb (Fig. 2A). No expression of parvalbumin 3 mRNA was observed in total RNA derived

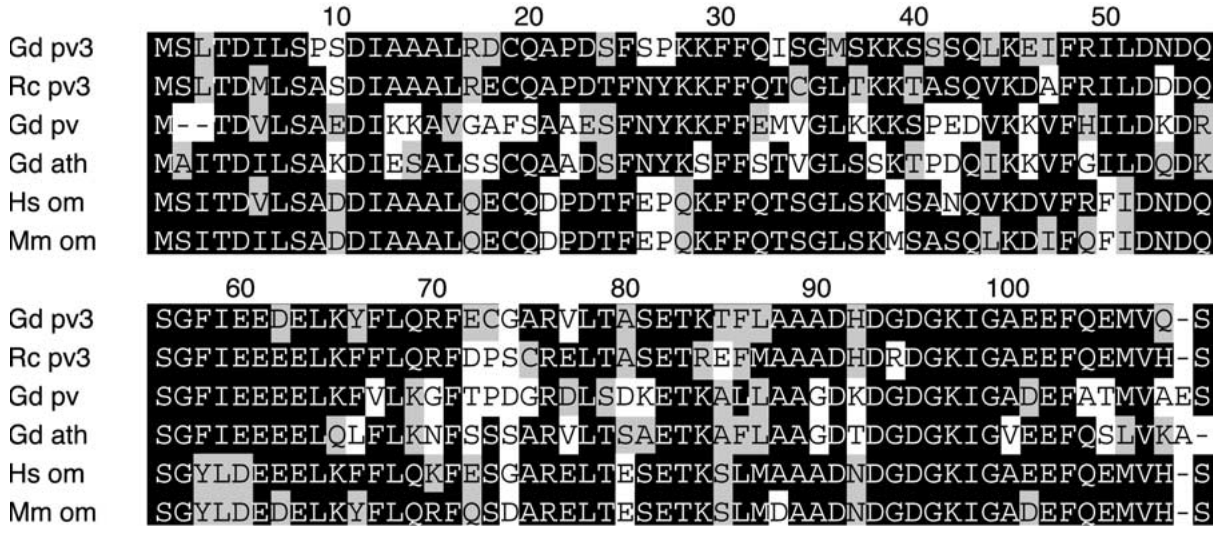

Fig. 1. Comparison of the amino acid sequences of chicken (Gd) and bullfrog (Rc) parvalbumin 3 (pv3) with those of chicken parvalbumin (pv) and avian thymic hormone (ath) and with human (Hs) and murine (Mm) oncomodulin (om). Residues that occur in at least half of the six sequences are marked in black, conservative variations are indicated by gray, and significant differences are unshaded. Chicken and bullfrog parvalbumin 3 are 74% identical and each displays about 70% identity with mammalian oncomodulin.

from any other organ analyzed. *In situ* hybridization on sections of the chicken's cochlea and bullfrog's sacculus demonstrated that parvalbumin 3 mRNA is confined to hair cells (Fig. 2B–D).

An antiserum against recombinant bullfrog parvalbumin 3 identified a single, nominally 13-kDa protein on Western blots from extracts of chicken cochlear ducts and bullfrog sacculi (Fig. 3A,B). The antiserum also detected immunoreactivity in the chicken's cerebellum (Fig. 3B). Western blots of inner-ear proteins resolved by isoelectric focusing revealed a single immunopositive protein with an isoelectric point of approximately 4.5 (Fig. 3C), a value consistent with the large fraction of acidic residues in parvalbumin 3. At a lower dilution, the serum detected in muscle extracts a second, 11-kDa protein that was evidently absent from the ear. Preincubation with the antigen eliminated the serum's capacity to detect either protein. Despite its weak cross-reactivity with the muscle protein, which is presumably conventional parvalbumin, the antiserum appears to detect only parvalbumin 3 in the inner ear.

Because Ca^{2+} buffering has been studied extensively in anuran hair cells, we conducted an immunohistochemical analysis of the presence of parvalbumin 3 in the bullfrog's sacculus. Extremely strong labeling was observed throughout the cytoplasm of hair cells (Fig. 4A). The labeling extended into hair bundles, as would be expected for a mobile Ca^{2+} buffer free to diffuse into stereociliary cytoplasm. Nuclei appeared not to be immunolabeled.

The majority of receptors in the frog's sacculus, which are compact, cylindrical hair cells, were strongly immunopositive for parvalbumin 3 (Fig. 4A). Because these cells express neither calbindin, calretinin, nor conventional parvalbumin (Edmonds et al. 2000), parvalbumin 3 is a significant and perhaps the only Ca^{2+}-buffering protein in them. Calretinin has been identified as a Ca^{2+} buffer in a minority subpopulation of saccular receptors, the narrow, peanut-shaped cells (Fig. 4B; Edmonds et al. 2000). Because parvalbumin 3 mRNA appeared to be present throughout the bullfrog's sacculus, we inquired whether parvalbumin 3 immunoreactivity is also detectable in these cells. Double immunofluorescence labeling with monoclonal anticalretinin and polyclonal antiparvalbumin 3 antibodies confirmed that parvalbumin 3 occurs in calretinin-expressing hair cells (Fig. 4C).

Hair cells of the chicken's basilar papilla, the sensory epithelium of the cochlea, were also strongly immunopositive for parvalbumin 3 (Fig. 4D). Although no striking difference in signal intensity was observed in hair cells along the tonotopic gradient of the chicken's cochlea, we did not attempt to quantitate this observation. Labeling was absent from supporting cells and other components of the inner ear.

The observation of abundant parvalbumin 3 in the sacculi of anuran tadpoles (Fig. 4E) led us to examine the protein's expression during inner-ear development. In the developing cochlea of the chicken, parvalbumin 3 was detectable as early as embryonic day 6 (developmental stage 28; Hamburger and Hamilton 1951) in cells that appeared to be primordial hair cells (Fig. 4F). Vestibular hair cells, which differentiate earlier than the cochlear receptors (Bartolami et al. 1991), were already parvalbumin 3 positive at the same developmental stage (Fig. 4G). In

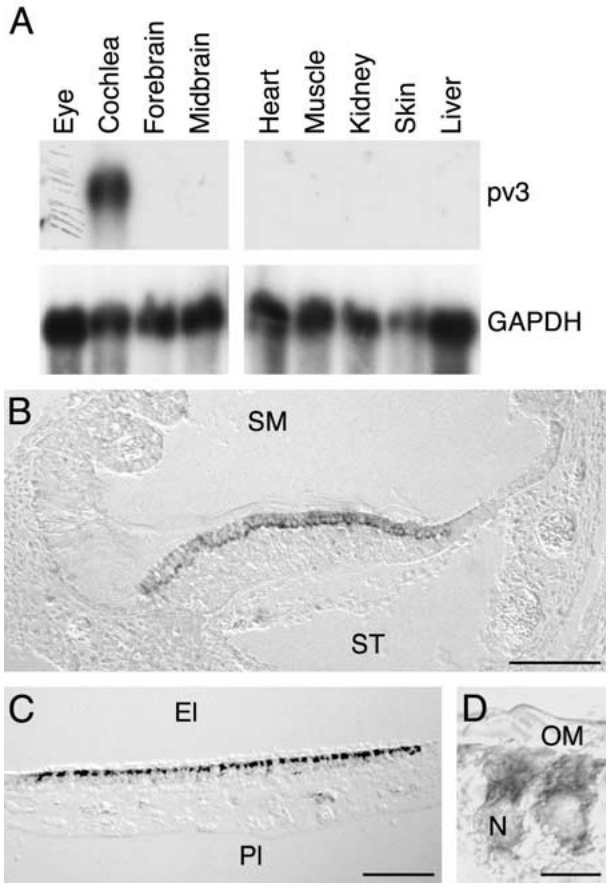

Fig. 2. Expression analysis of parvalbumin 3 mRNA. **A.** Northern blot analysis reveals a single parvalbumin 3 transcript (pv3) in total RNA prepared from organs of two-week-old chicks. Each lane was loaded with 10 μg of total RNA; as a control for equal loading, the blots were rehybridized to a probe specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **B.** *In situ* hybridization on a cross section of a hatchling's basilar papilla shows an abundance of parvalbumin 3 mRNA in cochlear hair cells. SM, scala media; ST, scala tympani; scale bar, 10 μm. **C.** A probe specific for anuran parvalbumin 3 labels hair cells in a cross section of the bullfrog's sacculus. EI, endolymphatic compartment; PI, perilymphatic compartment; scale bar, 100 μm. **D.** At a higher magnification, the labeling of two bullfrog saccular hair cells for parvalbumin 3 mRNA is clearly apparent. OM, otolithic membrane; N, nucleus; scale bar, 10 μm.

some instances we observed labeling of thin cytoplasmic strands connecting spindle-shaped cells with the basement membrane. These were presumably cells that had recently assumed a hair cell identity and were captured in the process of withdrawing from the basement membrane to form the pseudostratified epithelium of the mature basilar papilla. At later embryonic stages, after hatching, and in adult animals, parvalbumin 3 was abundant in hair cells of the cochlea, utriculus, sacculus, lagena, and the semicircular canals' ampullae.

Immunohistochemical examination of whole-mounted zebrafish embryos revealed a similar early expression pattern of parvalbumin 3. Zebrafish hair cells expressed the Ca^{2+}-binding protein as soon as they detached from the basement membrane. After 22 h of embryonic development, the anterior and the posterior macula each contained two labeled cells (data not shown), probably the tether cells that pioneer the receptors of the utriculus and the sacculus, respectively (Riley et al. 1997). The specificity of the antiparvalbumin 3 labeling of early hair cells was corroborated by visualizing the massive increase in the number of immunopositive hair cells in zebrafish bearing the *mind bomb* (*mib*) mutation, which deflects nascent supporting cells to a hair cell fate (Haddon et al. 1998). Following 30 h of embryonic development, wild-type embryos held only four to six immunopositive hair cells in the two developing maculae of each ear (Fig. 4H), whereas *mind bomb* mutants displayed numerous parvalbumin 3-positive cells per macula (Fig. 4I). A few spindle-shaped cells immediately beneath the maculae, probably eighth-nerve ganglionic neurons delaminating from the sensory epithelium, were also immunolabeled.

Cellular concentration of parvalbumin 3

We estimated the amount of parvalbumin 3 in bullfrog saccular hair cells by resolving their proteins on polyacrylamide gels and conducting quantitative Western blotting. The densities of the stained 13-kDa protein bands were compared with those of protein standards consisting of recombinant bullfrog parvalbumin 3 run on the same gels (Fig. 5). A single sacculus contained 183 ± 12 ng of parvalbumin 3 [mean ± standard deviation (SE), $n = 4$ experiments]. If the protein were evenly distributed among the organ's 2500 hair cells, the average hair cell would hold 73 pg of parvalbumin 3. For a cell about 10 μm in diameter and 40 μm in length, and hence with a volume of approximately 3 pL, this quantity corresponds to a parvalbumin 3 concentration of roughly 2 mM.

We estimated the concentration of parvalbumin 3 with greater precision in individually picked hair cells of known type (Table 1). Western blot analysis revealed an average of 103 ± 11 pg (mean ± SE, $n = 4$ experiments) of parvalbumin 3 in each compact, cylindrical hair cell (Fig. 5). Utilizing three-dimensional reconstruction of confocal image stacks of living, isolated hair cells labeled with a fluorescent membrane marker, we determined the average volume of such a cell to be 5.5 ± 1.8 pL (mean ± SE, $n = 12$ cells). If the Ca^{2+} buffer is uniformly distributed throughout the cytoplasm, the intracellular parvalbumin 3 concentration of a compact, cylindrical hair cell is therefore at least 1.51 ± 0.52 mM. The average narrow, peanut-shaped hair cell was found to contain 12 ± 2 pg (mean ± SE, $n = 4$ experiments) of parvalbumin 3 (Fig. 5); distributed throughout the estimated cellu-

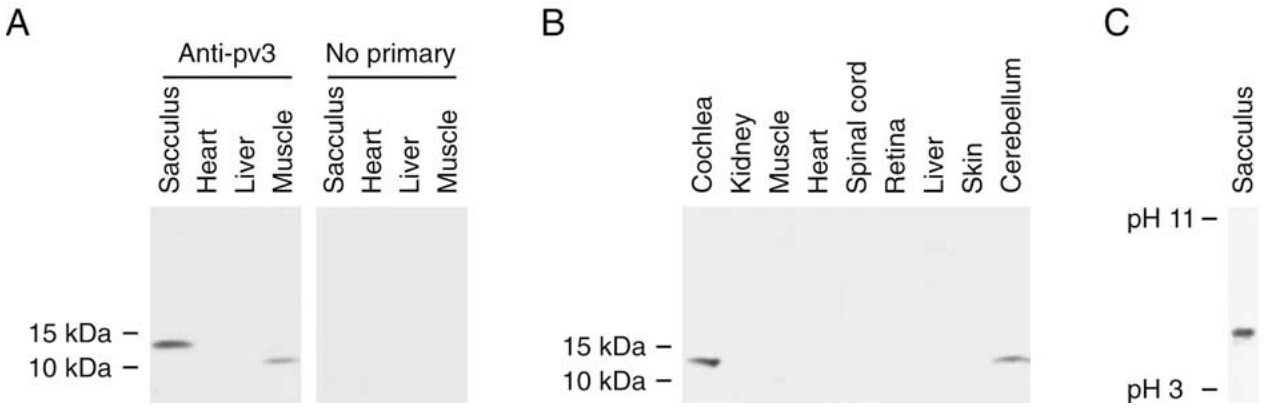

Fig. 3. Western blot analysis of parvalbumin 3 expression. **A.** A protein of approximately 13 kDa is detected by an antibody against parvalbumin 3 (Anti-pv3) in an extract of the bullfrog's sacculus. A weaker signal of lower molecular mass is observed in anuran muscle. In a control experiment, omission of the primary antibody (No primary) prevents labeling. **B.** Parvalbumin 3 is detectable in the chicken's cochlea and cerebellum. A weak signal corresponding to a protein of lower molecular mass is apparent in muscle. **C.** Western blot analysis of proteins from the bullfrog's sacculus resolved on an isoelectric-focusing gel reveals a single band at an isoelectric point of 4.5.

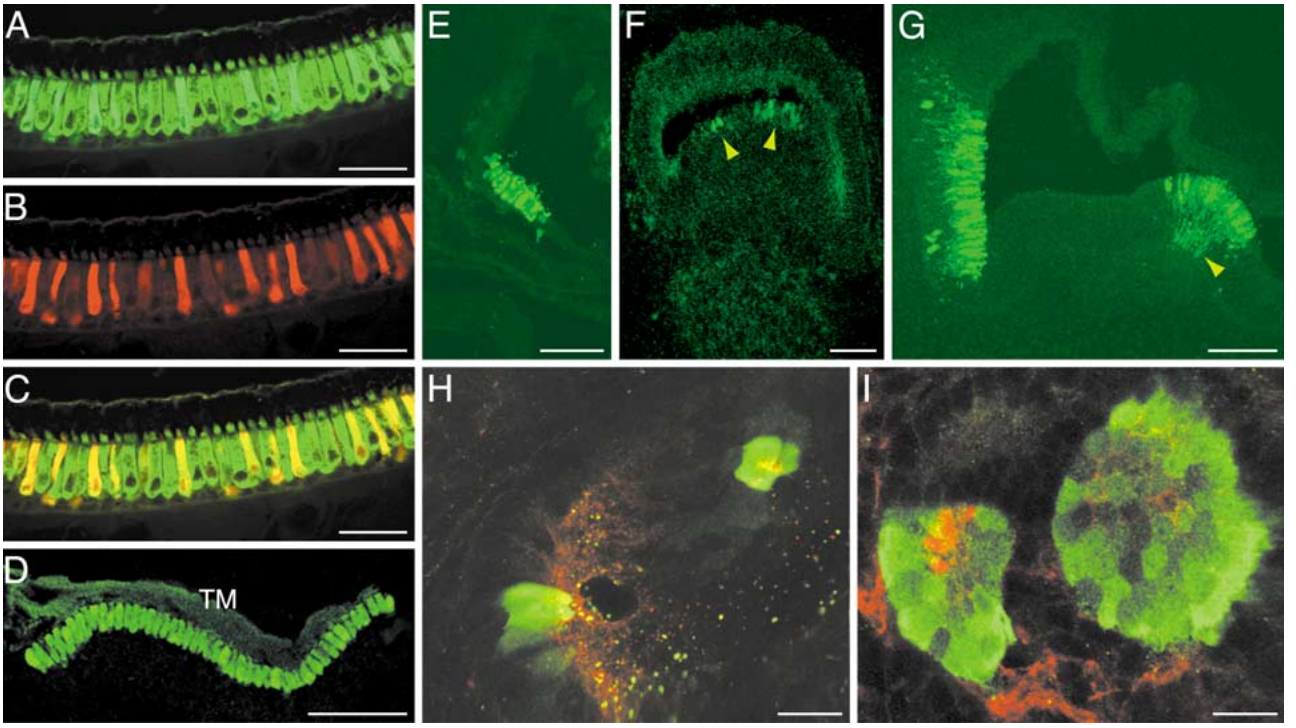

Fig. 4. Immunohistochemical localization of parvalbumin 3. **A.** On a cross section of the bullfrog's sacculus, strong antiparvalbumin 3 labeling extends throughout the cytoplasm and into the hair bundles; nuclei are not labeled; scale bar, 50 μm. **B.** Anticalretinin immunoreactivity marks the narrow, peanut-shaped hair cells of the bullfrog's sacculus in the same section as that illustrated in panel **A**; scale bar, 50 μm. **C.** Double immunolabeling for calretinin (red) and parvalbumin 3 (green) demonstrates that the hair cells expressing calretinin also contain parvalbumin 3 and accordingly appear yellow; scale bar, 50 μm. **D.** On a cross section of the chicken's basilar papilla, strong labeling is confined to hair cells. A trace of nonspecific labeling occurs in the tectorial membrane (TM); scale bar, 100 μm. **E.** A transverse section of the developing sacculus from a *Xenopus laevis* tadpole reveals intense labeling of hair cells; scale bar, 50 μm. **F.** In the developing cochlear duct of a six-day-old (stage 28) chicken embryo, a few immunopositive cells are detectable in the developing auditory epithelium (arrowheads); scale bar, 50 μm. **G.** At the same stage, labeling is both more extensive and more intense in the vestibular system. Some cells display immunopositive cytoplasmic processes that likely reflect the detachment of hair cells from the basement membrane (arrowhead); scale bar, 50 μm. **H.** In a whole-mount preparation of a wild-type zebrafish embryo at 30 h of development, a few immunopositive hair cells are found in the anterior and posterior sensory maculae; scale bar, 20 μm. **I**. In the otocyst of a *mind bomb* mutant, in which supporting cells have adopted a hair cell fate, far more labeled cells are detectable at the same stage; scale bar, 20 μm. The preparations in **H** and **I** were counterstained with rhodamine-conjugated antibody against acetylated tubulin to delineate microtubules.

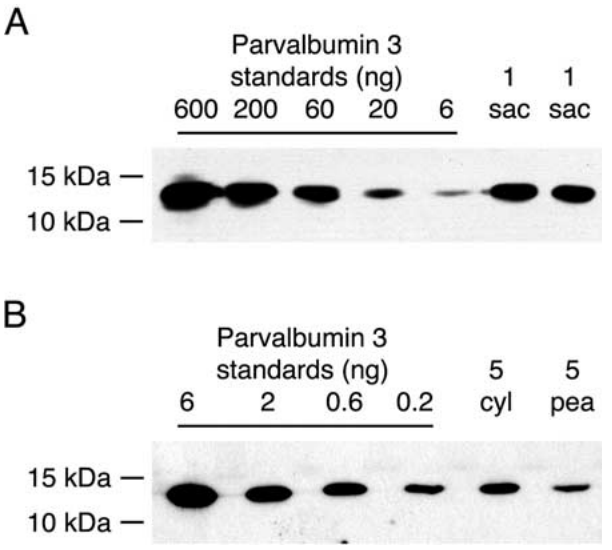

Fig. 5. Quantification of the amount of parvalbumin 3 in bullfrog sacculi and in isolated hair cells. **A.** The parvalbumin 3 from single sacculi (1 sac), detected in electrophoretically separated total protein, is compared on a Western blot with standards of recombinant parvalbumin 3. **B.** The parvalbumin 3 from five compact, cylindrical hair cells (5 cyl) and that from five narrow, peanut-shaped hair cells (5 pea) is compared with parvalbumin 3 standards. The different sensitivities in panels **A** and **B** were achieved by varying the exposure times.

lar volume of 2.6 ± 0.8 pL (mean ± SE, n = 9 cells), this corresponds to a minimal buffer concentration of 0.36 ± 0.13 mM.

DISCUSSION

Mechanoelectrical transduction, afferent synaptic signaling, and efferent synaptic responsiveness in hair cells all involve Ca^{2+} signals. Despite the compact nature of hair cells, the Ca^{2+}-entry sites are largely isolated from one other by fast-acting and highly mobile Ca^{2+} buffer, presumably one or more small proteins each with several Ca^{2+}-binding sites (reviewed in Lenzi and Roberts 1994). We have determined that the Ca^{2+}-binding protein parvalbumin 3 is abundant in the cytoplasm of hair cells in receptor organs of chickens, frogs, and zebrafish. The mammalian ortholog of parvalbumin 3 may be oncomodulin (Henzl et al. 1991), which has also been reported to occur in hair cells (Henzl et al. 1997; Sakaguchi et al. 1998). Parvalbumin 3 and related proteins should therefore be regarded as candidates to explain the Ca^{2+}-buffering capacity of many varieties of hair cells.

The concentrations of parvalbumin 3 calculated for the two major types of hair cells in the bullfrog's sacculus represent minimal estimates, for these values rest on the assumption that the buffer has access to each cell's entire volume. The actual cytoplasmic concentration must be greater, for the protein appears in immunohistochemical preparations to be excluded from nuclei. Moreover, the buffer is very probably excluded from the numerous membrane-bounded organelles in the hair cell's soma, especially mitochondria, lysosomes, vesicles, and elements of the endoplasmic reticulum and Golgi apparatus. We estimate that membrane-bounded organelles and the nucleus occupy half of the cellular volume. With correction for this factor and on the assumption that the nucleus wholly excludes the buffer, the cytoplasmic concentration of parvalbumin 3 in a compact, cylindrical hair cell is approximately 3 mM. With the same corrections, the concentration of parvalbumin 3 in a narrow, peanut-shaped hair cells is about 0.7 mM.

Oncomodulin displays one high-affinity binding site for which Ca^{2+} and Mg^{2+} compete and a second that is more selective for Ca^{2+} (Henzl et al. 1996; reviewed in Pauls et al. 1996). If the Ca^{2+}-binding properties of parvalbumin 3 resemble those of its homolog and are subject to the volume corrections noted above, the compact, cylindrical hair cells that constitute the majority of receptors in the frog's sacculus contain a concentration of Ca^{2+}-binding sites of about 6 mM attributable to their parvalbumin 3. On the same assumptions, the narrow, peanut-shaped hair cells that make up the balance of the saccular population contain roughly 1.4 mM of Ca^{2+}-binding sites owing to parvalbumin 3.

The extensive studies of Ca^{2+} buffering in hair cells of the frog's sacculus (Roberts 1993, 1994; Roberts et al. 1990; Hall et al. 1997) permit comparison of the concentration of Ca^{2+}-binding sites on parvalbumin 3 with the buffer concentration necessary to explain the physiological observations. In narrow, peanut-shaped hair cells, the 1.4 mM Ca^{2+}-binding sites owing to parvalbumin 3 appears to provide only a modest supplement to the 6 mM of sites residing on calretinin (Edmonds et al. 2000). Functional assays indicate that compact, cylindrical hair cells contain the equivalent of 8 mM Ca^{2+}-binding sites on small, mobile proteins (Roberts 1994). Our estimate of 6 mM binding sites on parvalbumin 3 suggests that this protein constitutes the dominant Ca^{2+} buffer in such cells. For several reasons, however, it remains uncertain whether other proteins also play significant buffering roles in these cells. As noted above, the present results rest upon an estimate of the free cytoplasmic volume and upon an assumption about the Ca^{2+}-binding sites of parvalbumin 3. Next, there may be a difference in the Ca^{2+}-buffer concentrations in the grass frog hair cells used in the physiological measurements and the bullfrog hair cells employed in the present study. Finally, the physiological or biochemical estimate may be somewhat in error. We cannot exclude the possibility, for example, that the antibody

TABLE 1

Quantification of parvalbumin 3 in sacculi and hair cells

Sample	*Parvalbumin 3 content (ng)*	*Number of cells*	*Average content per cell (pg)*
Individual saccular maculae			
	198.7	~2500	79.5
	176.9	~2500	70.8
	171.7	~2500	68.7
	187.7	~2500	75.1
Isolated compact, cylindrical hair cells			
	0.527	5	105.4
	0.568	5	113.5
	0.527	5	105.4
	0.433	5	86.5
Isolated narrow, peanut-shaped hair cells			
	0.066	5	13.1
	0.041	5	8.1
	0.066	5	13.1
	0.062	5	12.4

used to quantify the parvalbumin 3 content of hair cells recognizes native parvalbumin 3 less well than the exogenously expressed protein used in Western blotting.

The intracellular concentration of free Ca^{2+} in resting hair cells is 50–100 nM (Chabbert et al. 1994; Lumpkin and Hudspeth 1996). This low level is achieved by avid Ca^{2+} pumps, or Ca^{2+}–ATPase molecules, which occur at high density throughout the hair cell's plasma membrane (Crouch and Schulte 1995; Yamoah et al. 1998; Dumont et al. 2001) and actively extrude Ca^{2+} into both the endolymphatic and the perilymphatic fluid compartments. If the dissociation constants for the binding sites on parvalbumin 3 are as expected, these sites should largely be free of Ca^{2+} in the absence of stimulation. Most of the protein's buffering capacity should therefore be available to deal with the Ca^{2+} influxes resulting from mechanical stimulation or synaptic activity. By analogy to oncomodulin (Henzl et al. 1996), however, parvalbumin 3 may have complex ion-binding properties; a definitive understanding of the protein's role in Ca^{2+} buffering will require direct measurement of its binding kinetics.

Parvalbumin 3 appears early in the development of hair cells, before they display mechanosensitive hair bundles or synaptic specializations. Because of its strong immunoreactivity, high concentration, and ubiquitous occurrence in hair cells, parvalbumin 3 should therefore prove an effective marker for the study of hair cell differentiation in the developing sensory epithelium. The restricted cellular expression of parvalbumin 3 also implies that the control elements of the cognate gene would be useful in expressing transgenes specifically in hair cells.

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