DARPP-32 (dopamine and cAMP-regulated phosphoprotein, M_r 32,000) is a membrane protein in the bovine parathyroid

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Received 1 March 1995; revised version received 21 March 1995

Abstract A distinct form of DARPP-32, a protein phosphatase-1 inhibitor, has been identified in bovine calf parathyroid glands. Immunoblot analysis of parathyroid tissue revealed a 32 kDa protein present predominantly in a particulate fraction; it remained particulate after treatment with 1.0 M NaCl or 0.1 M Na_2CO_3. Metabolic labeling of parathyroid cells with mevalonolactone demonstrated that DARPP-32 is isoprenylated. Immunocytochemical localization studies demonstrated that DARPP-32 is present in vesicles throughout the cytoplasm of parathyroid cells, and that protein phosphatase-1γ is concentrated in the region of the plasma membrane. Thus, in contrast to the predominately soluble form of DARPP-32 that has been characterized in selected areas of the central nervous system, the parathyroid form is tightly associated with intracellular membranes.

Key words: Parathyroid; DARPP-32; PTH secretion; Protein phosphatase-1

1. Introduction

The parathyroid glands maintain the blood calcium concentration within a very narrow range by sensing small changes in free Ca^{2+} via the Ca^{2+} receptor [1] and responding with altered secretion of parathyroid hormone (PTH) [2]. The parathyroid cell is unusual among secretory systems in exhibiting an inverse secretory response to Ca^{2+}_e. Low extracellular calcium (Ca^{2+}_e) stimulates PTH secretion, and high Ca^{2+}_e inhibits secretion [3,4].

PTH secretion from dispersed bovine parathyroid cells is also stimulated by agents that increase intracellular cAMP [5–7]. The parathyroid cell has dopamine receptors of the D_1 subclass that share pharmacological and structural homology with striatal neuron D_1 receptors [8,9]. Dopamine can act independently of Ca^{2+}_e to activate adenylyl cyclase and increase cAMP production [10,11]. Ca^{2+} and cAMP interact to modulate short-term changes in PTH release. The intracellular Ca^{2+} (Ca^{2+}_i) concentration determines the sensitivity to cAMP; a greater increase in cAMP is necessary at high Ca^{2+}_i than at low Ca^{2+}_i to achieve the same level of PTH secretion [12–14]. Thus, signals that act via Ca^{2+} may attenuate cAMP-regulated processes in the parathyroid [15]. The proteins that are the targets of regulation by Ca^{2+} and cAMP are not yet well defined.

DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) is a regulated phosphoprotein; phosphorylated DARPP-32 is an inhibitor of protein phosphatase-1 [16], and the dephosphorylated form is inactive. DARPP-32 integrates the signals of intracellular messengers [15]; in neural tissue several signal transduction pathways have been demonstrated to converge upon DARPP-32, including those stimulated by dopamine, VIP and isoproterenol [17–19]. The firing rate of striatal neurons is regulated by inhibitory dopaminergic input from the substantia nigra and excitatory glutamatergic input from the neocortex. Dopamine acting through the D_1 receptor leads to an increase in cAMP, activation of cAMP-dependent protein kinase, and phosphorylation of DARPP-32. Glutamate activates the NMDA subclass of glutamate receptors to increase Ca^{2+} influx, which leads to the activation of calcineurin, a Ca^{2+} and calmodulin-dependent phosphatase, and results in the dephosphorylation of DARPP-32. The degree of phosphorylation of DARPP-32, and thus its ability to inhibit protein phosphatase-1, is controlled by the antagonistic actions of Ca^{2+} and cAMP [15].

DARPP-32 and its target, protein phosphatase-1, may be involved in the interaction of the Ca^{2+}_i and cAMP signaling pathways in the parathyroid cell and in regulating PTH secretion. DARPP-32 has been localized to the bovine parathyroid by radioimmunoassay [17] and to human parathyroid chief cells by immunofluorescence microscopy [20]. The present study examines the subcellular location of parathyroid DARPP-32 and demonstrates that parathyroid DARPP-32, in contrast to brain DARPP-32, is not predominantly a soluble protein, but rather is tightly bound to intracellular membranes.

2. Materials and methods

Bovine parathyroid glands were collected from newborn calves at a local abattoir (DeMartino's, Seymour, CT), or were shipped on wet-ice from Florida Biologicals, Highland City, FL. Human parathyroid adenoma tissue was obtained at surgery in accordance with Yale human subjects protocols. Ca^{2+}, Mg^{2+}-free MEM (Minimal Essential Medium (Eagle)) was formulated by Gibco, Grand Island, NY. Ham's F12 media, fetal calf serum and penicillin/streptomycin were also purchased from Gibco. Electrophoresis reagents were purchased from Bio-Rad, Richmond, CA. Immobilon was purchased from Millipore, Bedford, MA. ^{125}I-Labeled rabbit anti-mouse secondary antibody and *RS*-[5-^{3}H(N)]mevalonolactone were purchased from New England Nuclear (DuPont), Boston, MA. Chemiluminescent reagents were purchased

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SSDI 0014-5793(95)00359-2

from Amersham, Arlington Heights, IL. Collagenase D was purchased from Boehringer Mannheim, Indianapolis, IN, and type 3 collagenase from Worthington, Freehold, NJ. DNAse I, *n*-octyl β-D-glucopyranoside (β-octylglucoside) and proteinase K were purchased from Sigma, St. Louis, MO. Trans-^{35}S-label was purchased from ICN Biomedicals, Irvine, CA. Percoll was purchased from Pharmacia, Uppsala, Sweden. Compactin, purified from the culture broth of *Penicillum citrinum* [21], was a generous gift from Dr. A. Endo, Tokyo, Japan.

Rabbit anti-PTH was purchased from Biomeda, Foster City, CA. Mouse monoclonal anti-DARPP-32 (5a) and rabbit antiserum to DARPP-32 (R2) have been previously characterized [17]. Rabbit polyclonal antibody specific for the gamma isoform of protein phosphatase-1 (RU-31) has been characterized (da Cruz e Silva and Greengard, manuscript in preparation). TRITC (tetramethylrhodamine isothiocyanate)-labeled goat anti-mouse immunoglobulin and FITC (fluorescein isothiocyanate)-labeled goat anti-rabbit F(ab')$_2$ were purchased from Tago, Burlingame, CA.

2.1. Immunofluorescence and electron microscopy

Dissociated parathyroid cells were incubated overnight on glass coverslips in Ham's F12 medium supplemented with 2 mM glutamine, 10% fetal calf serum, and 10 units/ml penicillin, and 10 μg/ml streptomycin at 37°C in an atmosphere of 95% air/5% CO_2. The cells were fixed in 2% formaldehyde made from paraformaldehyde in 75 mM sodium phosphate, pH 7.4 for 45 min, permeabilized with 0.05% (w/v) saponin, incubated for 2 h with monoclonal anti-DARPP-32, followed by TRITC-labeled anti-mouse for 1 h.

Cryosections (4–6 μm) of calf parathyroid glands were fixed as described in [22] and modified in [23]. The fixed tissue was incubated in 3% goat serum in PBS for 1 h, permeabilized with 0.1% Triton X-100 in PBS for 10 min, then incubated for 2 h simultaneously with rabbit anti-PTH (1:300) and mouse anti-DARPP-32 (1:1000) followed by FITC-labeled anti-rabbit (1:200) and TRITC-labeled anti-mouse (1:300). Slides were photographed on a Zeiss Axiophot or a Zeiss Axiovert 10 microscope equipped with a Bio-Rad (Richmond, CA) MRC-600 confocal attachment.

Parathyroid fractions were prepared for transmission electron microscopy by fixation with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C, followed by postfixation for 1 h in 1.0% osmium tetroxide in the same buffer, dehydration through a graded ethanol series and were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 300 electron microscope.

2.2. Tissue preparation and fractionation

Parathyroids were trimmed free of connective tissue, minced to a fine slurry and homogenized by hand with 10 strokes of a ground-glass homogenizer at 20% (w/v) in homogenization buffer consisting of 0.3 M sucrose, 10 mM Tris-HCl, pH 7.6, 2.0 mM ethylenediamine tetracetic acid (EDTA), 1.0 mM dithiothreitol (DTT), and a cocktail of protease inhibitors composed of leupeptin, chymostatin, pepstatin, soybean trypsin inhibitor and aprotinin [24]. The homogenate was centrifuged at $600 \times g$ for 20 min and the pellet discarded, resulting in a postnuclear supernatant. Discontinuous sucrose gradients consisted of steps of 0.8 M, 1.3 M, 1.7 M sucrose above a 3.0 M cushion in 10 mM Tris-HCl, pH 7.6, and 2.0 mM EDTA [25]. Gradients were centrifuged at $145{,}000 \times g$ for 1 h in an SW40 rotor at 4°C. Rat midbrain was homogenized as previously described [26].

2.3. Primary parathyroid cell preparation

Cells were prepared from calf parathyroids by previously described methods [27,28] with the following modifications. Trimmed parathyroid tissue (3.0–3.5 g, i.e. approximately 36 glands) was digested with 0.036% collagenase D and 0.004% DNAse I in 50 ml of Ca^{2+}/Mg^{2+}-free MEM supplemented with 0.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 3.2 mM L-methionine and 10 mM HEPES, pH 7.47. The resulting bovine parathyroid cells were separated from debris, non-viable cells and red cells on a 30% (v/v) self-forming Percoll gradient [13]. The optimal dispersion conditions for human parathyroid cells differed slightly from those used to obtain bovine cells. Human tissue (1–2 g) was digested with 0.032% type 3 collagenase and 0.002% DNAse I in 50 ml of Ca^{2+}/Mg^{2+}-free MEM supplemented as described above; the parathyroid cells were then isolated on a 30% Percoll gradient.

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation of DARPP-32 was performed in RIPA buffer [29] using rabbit polyclonal anti-DARPP-32. For immunoblot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gels) [30] and transferred to Immobilon [31]. The membranes were blocked with 5% non-fat dry-milk in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% (v/v) Triton X-100. Primary antibody incubations were performed overnight at 4°C with a 1:2000 dilution of monoclonal anti-DARPP-32 or a 1:1000 dilution of anti-protein phosphatase-1γ. Antibody detection was performed either by incubation for 1 h in a solution of ^{125}I-labeled goat anti-mouse IgG diluted 1:1000, or by chemiluminescent detection according to the manufacturer's instructions. Autoradiography was performed with Kodak XAR or XRP film.

2.5. Radioisotopic labeling

Parathyroid cells were prepared and 1.5×106 cells/ml were incubated overnight in Ham's F12 medium, 10% (v/v) dialyzed (vs. 0.15 M NaCl) fetal calf serum, 10 units/ml penicillin, 10 μg/ml streptomycin, 50 μCi/ml *RS*-[5-^{3}H(N)]mevalonolactone and 100 μM compactin [32]. In parallel, 1.0×106 cells/ml were incubated in methionine-free MEM, 10% dialyzed fetal calf serum, penicillin/streptomycin and 300 μCi/ml Trans-^{35}S-label. After labeling, the cells were washed in Ham's F12 medium and solubilized with RIPA buffer in preparation for immunoprecipitation.

3. Results

3.1. Immunofluorescent localization of parathyroid DARPP-32

Cells dissociated from bovine parathyroid tissue were 92–97% viable as assessed by Trypan blue exclusion. Fig. 1A depicts a cluster of dispersed bovine parathyroid cells labeled with monoclonal anti-DARPP-32 and visualized by indirect immunofluorescence. DARPP-32 immunoreactivity was specifically localized to small vesicular structures distributed throughout the cell cytoplasm, and absent from the nucleus (n). Fig. 1B depicts a similar cluster of dispersed human parathyroid cells; DARPP-32 immunoreactivity was observed in a vesicular compartment that is indistinguishable from that observed in bovine cells.

Frozen sections of an intact bovine parathyroid gland were double labeled with mouse anti-DARPP-32 (Fig. 1C,E) and rabbit anti-PTH (Fig. 1D,F). PTH was present in secretory granules of the chief cells. DARPP-32 was observed primarily in parathyroid cells, and was also observed rarely in nerve terminals in the parathyroid, but was not detected in the endothelium, adipose tissue or other connective tissue in the gland. DARPP-32 immunoreactivity in the parathyroid was preserved and the vesicular signal appeared identical after fixation of the tissue with acetone, 2% formaldehyde, formaldehyde-lysine-periodate [33], or formaldehyde-PIPES-tetraborate [22].

The intracellular distribution of DARPP-32 is predominantly vesicular, but is not identical with that of PTH. The arrows in Fig. 1E,F point to selected regions in which DARPP-32 and PTH appeared to be associated with the same granule. The degree of overlap is difficult to quantify at the light level even with confocal optics because of the large number of PTH and DARPP-32 positive organelles; however, DARPP-32 appeared to colocalize with only a small fraction of the total PTH granule population.

3.2. Parathyroid DARPP-32 is localized to a particulate subcellular fraction

The presence of immunoreactive DARPP-32 in bovine parathyroid was examined by immunoblot analysis, and its subcel-

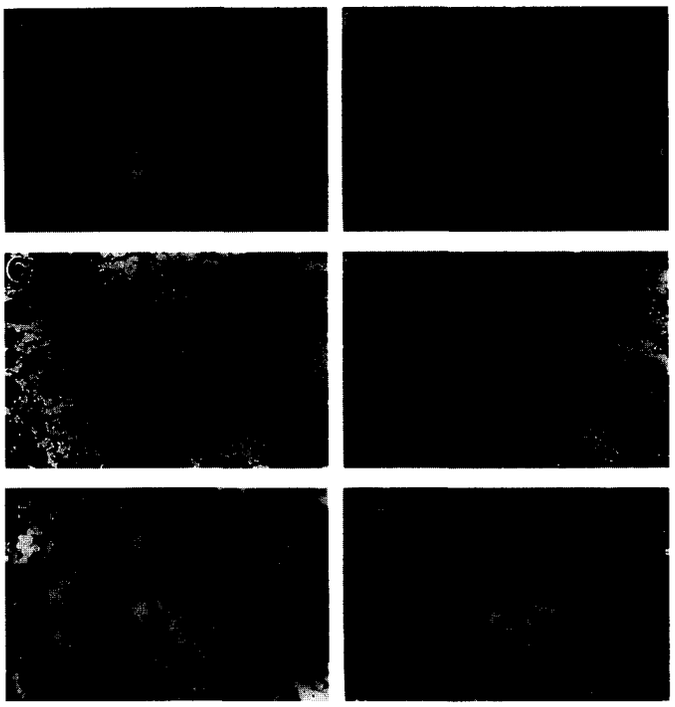

Fig. 1. (A,B) Indirect immunofluorescence localization of DARPP-32 in dissociated bovine (A) and human (B) parathyroid cells. Fixed, permeabilized cells were labeled with monoclonal anti-DARPP-32 (1:600), the signal was detected with TRITC-labeled anti-mouse antibody, and visualized using confocal microscopy. Magnification = 1200×. (C,D,E,F) Double indirect immunofluorescence localization of DARPP-32 and PTH in bovine parathyroid. A cryosection of parathyroid tissue was incubated simultaneously with mouse anti-DARPP-32 (C,E) and rabbit anti-PTH (D,F), and the signal was detected with a mixture of TRITC-anti-mouse and FITC anti-rabbit secondary antibodies. The arrows in (E) and (F) denote regions of colocalization between DARPP-32 and PTH. Magnification (C and D) = 630×; (E and F) = 1260×.

lular distribution compared to that of rat midbrain (Fig. 2). In the rat brain, as shown previously, DARPP-32 was present predominantly in the supernatant as compared to the pellet; the 'particulate' DARPP-32 has been shown to be in the cytosol of synaptosomes, which sediment under the centrifugation conditions used [34]. In contrast, in the parathyroid gland most of the DARPP-32 was found in the pellet. This difference in subcellular location was unexpected, and suggests that parathyroid DARPP-32 may be associated with either cytoskeleton or membrane-bound organelles. The apparent molecular mass of particulate bovine parathyroid DARPP-32 was the same as that of rat brain DARPP-32, but the small amount of DARPP-32 in the bovine parathyroid soluble fraction had a slightly higher molecular mass. On some gels soluble parathyroid DARPP-32 appeared as a doublet, and on some gels as a triplet; the band of lowest molecular mass always co-migrated with particulate bovine parathyroid DARPP-32.

Further subcellular fractionation was performed to localize parathyroid DARPP-32 more specifically. DARPP-32 purified from bovine caudate nucleus [26] is shown in Fig. 3A, lane 1. DARPP-32 present in a P10 fraction prepared by centrifuging bovine parathyroid postnuclear supernatant 10,000 × *g* for 10 min migrated with a slightly higher molecular mass than the bovine brain DARPP-32 (lane 2). This is consistent with the observations that: (a) rat brain DARPP-32 migrated with a higher molecular mass than bovine brain DARPP-32 [17]; and (b) bovine parathyroid particulate DARPP-32 co-migrated with rat brain DARPP-32 (Fig. 2). Lane 3 shows three forms of parathyroid DARPP-32 present in the soluble S100 fraction prepared by centrifuging the S10 supernatant of the P10 pellet at 100,000 × *g* for 1 h. The predominant band had a molecular mass several kilodaltons greater than in the P10 fraction; two minor bands were visible at a lower molecular mass, the smaller of which co-migrated with P10 DARPP-32. No DARPP-32 was observed in the P100 fraction (the pellet formed after centrifugation of the S10 for 100,000 × *g* for 1 h) (lane 4). The P10 fraction (lane 2), which contained most of the parathyroid DARPP-32, was subfractionated on a discontinuous sucrose gradient resulting in four fractions shown in order of increasing density (lanes 5–8). Lane 6 contained material sedimenting at

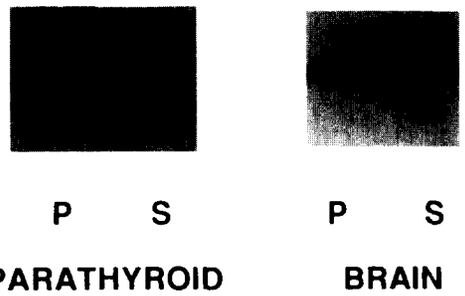

Fig. 2. DARPP-32 is predominantly soluble in brain and particulate in parathyroid. Rat midbrain postnuclear supernatant and bovine parathyroid postnuclear supernatant were centrifuged at 100,000 × *g* for 1 h and the resulting supernatants (S) and pellets (P) were subjected to immunoblot analysis with monoclonal anti-DARPP-32 followed by horseradish peroxidase labeled sheep anti-mouse immunoglobulin; the complex was detected by enhanced chemiluminescence. Parathyroid, 50 mg protein/lane; brain 5 mg protein/lane.

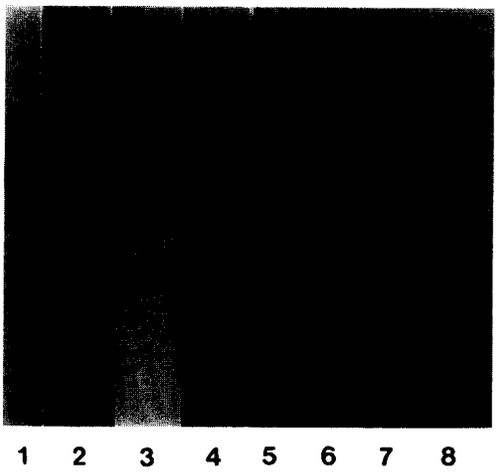

Fig. 3. (A) Distribution of DARPP-32 in subcellular fractions of bovine parathyroid. Bovine parathyroid postnuclear supernatant was fractionated, and the fractions analyzed by immunoblot analysis with monoclonal anti-DARPP-32 followed by [^{125}I]rabbit anti-mouse immunoglobulin. Lane 1 = bovine caudate nucleus DARPP-32 (64 ng protein); lane 2 = P10 (100 μg protein); lane 3 = S100 (200 μg protein); lane 4 = P100 (100 μg protein); lanes 5–8 = 4.4 mg of P10 protein was loaded on the top of a 4-step sucrose density gradient, and centrifuged for 1 h at 145,000 × g in an SW40 rotor at 4°C. The protein at the interfaces was collected, and each fraction was examined (100 μg protein). Lane 5 = < 0.8 M sucrose (density < 1.10); lane 6 = 0.8–1.3 M sucrose (density 1.10–1.17); lane 7 = 1.3–1.7 M sucrose (density 1.17–1.21); lane 8 = > 1.7 M sucrose (density > 1.21). (B) Morphological characterization of the 0.8–1.3 M sucrose gradient fraction. Magnification = 12,500×.

the 0.8 M–1.3 M interface, and has the majority of parathyroid DARPP-32.

This gradient fraction represents 64% of the protein in the P10 fraction and approximately 15% of the total parathyroid protein. The majority of PTH is detected by immunoblot in this fraction (not shown), the density of which (1.17–1.21) corresponds approximately to the density of the heavier two of the three major subpopulations of PTH granules isolated on metrizamide sucrose gradients previously described by Bajpai and Hamilton [35]. Electron microscopic examination (Fig. 3B) confirms that it is enriched in secretory granules (sg), as well as mitochondria (m), multivesicular bodies (mvb) and unidentified vesicles and cytoskeletal fragments.

3.3. *Parathyroid DARPP-32 has a cytoplasmic exposure*

To determine if DARPP-32 is present within a membrane-bound structure, or is exposed to the cytoplasmic surface, limited protease digestion was used to selectively degrade proteins which are exposed to the cytoplasmic surface. Proteinase K, a non-specific protease, was added to the salt-washed P10 frac-

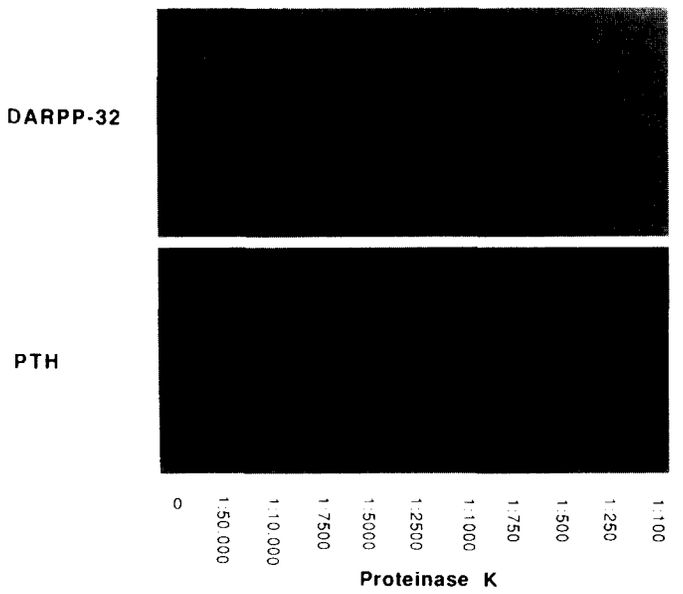

Fig. 4. DARPP-32 has a cytoplasmic exposure in the bovine parathyroid. The P10 fraction was suspended in 250 mM NaCl, resedimented at 10,000 × *g* for 20 min, then treated on ice for 1 h with proteinase K. The proteinase K:protein ratio (w/w) ranges from 0:100 to 1:100. Immunoblot of DARPP-32: 100 μg protein/lane, 10% polyacrylamide gel. Immunoblot of PTH: 30 μg protein/lane, 15% polyacrylamide gel.

tion for 1 h on ice [36]. The sample was divided between two polyacrylamide gels, then DARPP-32 and PTH were each detected by immunoblot (Fig. 4).

In the absence of proteinase K, or in the presence of low concentrations of proteinase K (0–1:10,000), neither DARPP-32 nor PTH was degraded. Intermediate concentrations (1:7500–1:250) result in progressively increasing DARPP-32 degradation, while PTH remains intact. High concentrations (1:100) of proteinase K presumably disrupted the secretory granules, and began to degrade PTH. Almost no digestion products of DARPP-32 were observed, presumably either because the fragment containing the monoclonal epitope was too small to detect by immunoblot or the antigenic site was lost. Thus, at certain protease concentrations DARPP-32 was accessible to digestion while the intragranular PTH remained inaccessible. This suggests that DARPP-32 has a cytoplasmic orientation in or on the membrane, as opposed to an intravesicular distribution.

3.4. *Parathyroid DARPP-32 is a membrane protein*

To determine the nature of the association of parathyroid DARPP-32 with the membrane, the P10 fraction was treated with agents known to release membrane-associated peripheral, but not integral membrane proteins. Following treatment, equal amounts of the pellet and supernatant proteins were examined for DARPP-32 immunoreactivity by immunoblot (Fig. 5).

All of the immunoreactive DARPP-32 remained in the pellet under control conditions (lanes 1 and 2) and after treatment with 1.0 M NaCl (lanes 3 and 4), or 0.1 M Na_2CO_3, pH 11 (lanes 5 and 6). It was partially removed to the soluble fraction by 1.0% β-octylglucoside (lanes 7 and 8). It was removed to the soluble fraction by 1.0% SDS (lanes 9 and 10). Parathyroid DARPP-32 was recovered in the pellet after treatment with 1.0% Triton X-114 followed by centrifugation at 45,000 × *g* for 30 min (data not shown). By these criteria, parathyroid DARPP-32 behaved as a membrane protein, and not as a classically defined membrane-associated protein.

3.5. *Parathyroid DARPP-32 is isoprenylated*

To determine if parathyroid DARPP-32 is isoprenylated, isolated bovine parathyroid cells were labeled overnight with

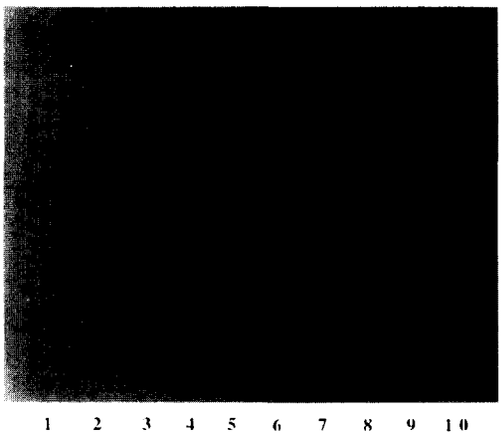

Fig. 5. DARPP-32 is tightly bound to membranes of bovine parathyroid cells. The P10 pellet was prepared by centrifugation at 10,000 × *g* for 20 min at 4°C, then resuspended in homogenization buffer with 1.0 mM phenylmethylsulfonylfluoride and the following additions: lanes 1 and 2 = no addition; lanes 3 and 4 = 1.0 M NaCl; lanes 5 and 6 = 0.1 M Na_2CO_3, pH 11; lanes 7 and 8 = 1.0% (w/v) β-octylglucoside; lanes 9 and 10 = 1.0% (w/v) SDS. Supernatant fractions after centrifugation at 180,000 × *g* for 10 min are compared to the resulting pellets. Eighty mg protein/lane were examined by immunoblot with monoclonal anti-DARPP-32. Lanes 1, 3, 5, 7 and 9 = supernatant; lanes 2, 4, 6, 8 and 10 = pellet.

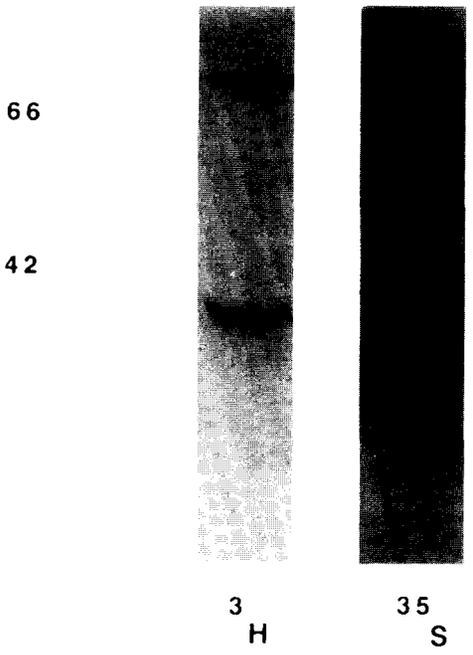

Fig. 6. Parathyroid DARPP-32 is isoprenylated. Cells labeled with *RS*-[5-^{3}H(N)]mevalonolactone or Trans-^{35}S-label were subjected to immunoprecipitation with polyclonal anti-DARPP-32. The major immunoprecipitated bands were 32 and 70 kDa.

50 μCi/ml *RS*-[5-^{3}H(N)]mevalonolactone, a precursor of mevalonic acid, in the presence of compactin, an inhibitor of HMG Co-A reductase. The cells remained 94% viable as assessed by Trypan blue after the compactin treatment. The labeled cells were subjected to immunoprecipitation with polyclonal anti-DARPP-32. A predominant protein of 32 kDa, and a minor 70 kDa protein were observed (Fig. 6). The 70 kDa band was observed consistently in immunoprecipitations and occasionally in immunoblots; its identity is not known at this time. DARPP-32 immunoprecipitated from [^{35}S]methionine-labeled cells co-migrated with that immunoprecipitated from *RS*-[5-^{3}H(N)]mevalonolactone-labeled cells. Thus, parathyroid DARPP-32 was isoprenylated. This modification of DARPP-32 may be responsible in part for the association of DARPP-32 with intracellular membranes.

3.6. Protein phosphatase-1γ is located at the periphery of the parathyroid cell

The parathyroid was examined for the presence of the regulatory target of DARPP-32, protein phosphatase-1, with an antibody to the γ isoform. Fig. 7A shows that a 37 kDa protein corresponding to the molecular mass of the catalytic subunit of PP-1γ was specifically detected in the parathyroid by immunoblot. In the P10, P100 and S100 fractions there was a predominant immunoreactive protein of approximately 37 kDa that was relatively more abundant in the soluble fraction.

To further determine the intracellular localization of PP-1γ in the parathyroid, cryosections of bovine parathyroid were labeled with anti-PP-1γ (Fig. 7B). PP-1γ immunoreactivity was present in the majority of chief cells in the cords of parathyroid tissue, and was predominantly located at the periphery of the cell, in the vicinity of the plasma membrane. Blood vessels and nerves also reacted intensely with anti-PP-1γ. The distribution and intensity of the signal was similar after fixation with acetone or paraformaldehyde. In control tissue treated identically to that shown in Fig. 7B with the exception that no primary antibody was used, there is no detectable background fluorescence (Fig. 7C). The PP-1γ detected by immunofluorescence in this tissue most likely corresponds to that in the P10 or P100 fractions, since much of the soluble enzyme may have been lost from the cryosection during processing for immunocytochemistry.

4. Discussion

This study used a combined immunocytochemical and biochemical approach to characterize bovine parathyroid DARPP-32 as an isoprenylated, tightly membrane bound protein with a cytoplasmic exposure on an intracellular granule compartment that is not identical to the PTH secretory granule compartment. Subcellular fractionation localizes DARPP-32 to a heterogeneous gradient fraction that contains the majority of PTH secretory vesicles. The immunocytochemical data show that parathyroid DARPP-32 is associated with vesicular structures distributed throughout the cytoplasm of the cell; however, these vesicles are on average slightly smaller than bovine PTH-containing secretory granules, which are 0.3–0.5 μm in diameter [37]. While there is a small degree of overlap between DARPP-32 and the secretory granule compartment, the majority of the DARPP-32 positive vesicles do not co-localize with secretory granules. Thus, although parathyroid DARPP-32 and PTH positive organelles co-sediment to similar densities, DARPP-32 is not primarily a secretory granule membrane component. DARPP-32 may selectively associate only with a small subset of PTH-containing secretory granules, such as ones destined for secretion or for degradation. Alternatively, DARPP-32 may be associated with a secretory product other than PTH, or may have a function unrelated to secretion.

Parathyroid DARPP-32 is isoprenylated, a modification that may play a role in localizing the protein to specific intracellular membranes and mediate reversible membrane binding and directed vesicular trafficking. Consensus sequences for isoprenylation contain cysteine residues and are present at the carboxyl-terminus [38]. Neither bovine brain nor rat brain DARPP-32 possess these sequences, nor do they have hydrophobic sequence domains that span the lipid bilayer [39–41]. Thus, there may be species and tissue-specific forms that result from alternative gene splicing or differing posttranslational modifications that are responsible for the localization of parathyroid DARPP-32 to intracellular membranes.

A small amount of parathyroid DARPP-32 is soluble. The molecular mass of this soluble form(s) is slightly greater than that of the particulate form, and of brain DARPP-32. A possible explanation for this observation is the existence of post translational modification(s) such as lipid addition, proteolytic cleavage or phosphorylation. The apparent molecular mass on SDS-PAGE of caudate DARPP-32 decreases upon phosphorylation of serine-137 by casein kinase I [42]. Thus, if parathyroid DARPP-32 is similar to caudate DARPP-32 in this respect, the soluble higher molecular mass band(s) may represent dephosphorylated or partially phosphorylated forms.

DARPP-32 is specifically localized to neurons in dopaminoceptive regions of the brain and peripheral tissues with the

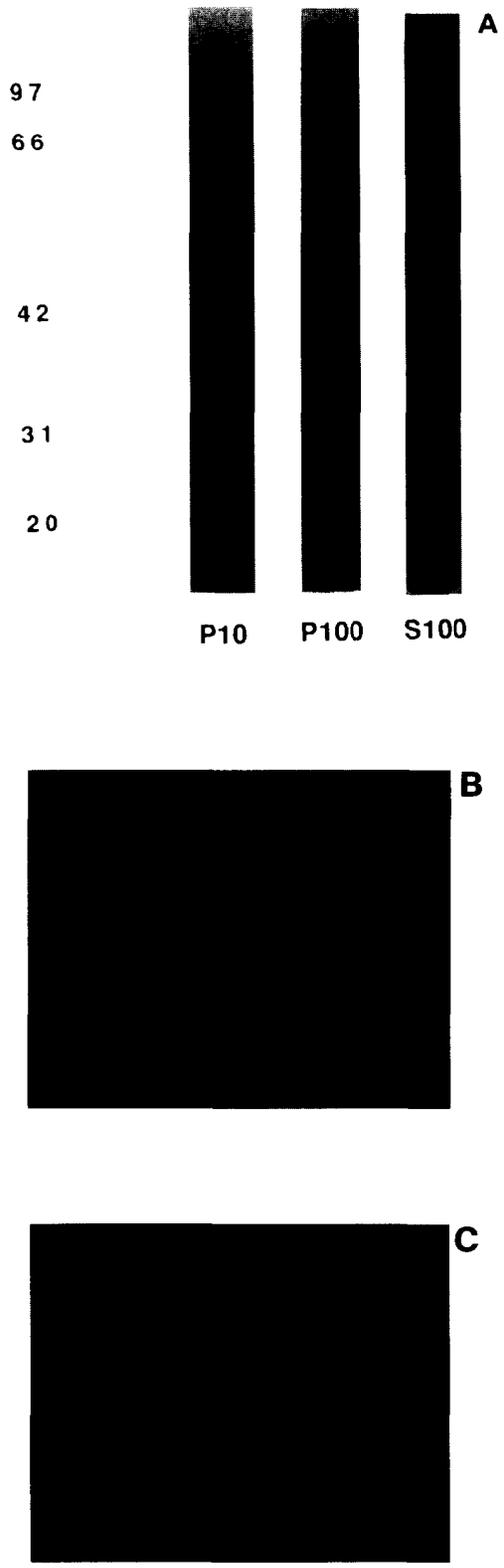

Fig. 7. (A) Protein phosphatase-1γ is present in the bovine parathyroid. Lane 1 = P10; lane 2 = P100; and lane 3 = S100 (100 μg protein each) were subjected to immunoblot with anti-PP-1γ. The P10, P100 and S100 fractions were prepared as described for Fig. 3. (B,C) Indirect immunofluorescence localization of PP-1γ in bovine parathyroid tissue. (B) A bovine parathyroid cryosection was incubated with anti-PP-1γ, then FITC-labeled anti-rabbit antibody. (C) In the absence of anti-PP-1γ, the secondary antibody produced no signal. Magnification = 500×.

D_1 subclass of dopamine receptors such as the parathyroid, secretory ciliary epithelium, brown adipose tissue, and the renal tubule cells of the thick ascending limb of the Loop of Henle [20,34,43–44]. The parathyroid cell and non-pigmented ciliary epithelium secrete PTH and aqueous humor respectively in response to stimulation by dopamine. Dopamine results in thermogenesis in brown adipose tissue, and may mediate natriuresis by the thick ascending limb. Parathyroid D_1 receptors act through the stimulatory guanine nucleotide regulatory protein (G_s) to activate adenylyl cyclase and activate cAMP-dependent protein kinase [45]. Bovine parathyroids contain high levels of dopamine [46]. Nerves innervating rat parathyroids contain tyrosine hydroxylase, the enzyme which catalyses dopamine synthesis [47]; and catacholamine-containing nerve fibers have been demonstrated to be present in the bovine parathyroid [48]. Intravenous infusion of high concentrations of dopamine into both cattle [48] and humans [49] results in transient increases in serum PTH levels. This suggests that dopamine may play a previously unappreciated role in regulating parathyroid function.

The primary known regulatory target of DARPP-32 is protein phosphatase-1. The data above demonstrate that at least one isoform, PP-1γ, is present in both cytosolic and particulate fractions of the parathyroid; this is consistent with observations of PP-1γ in varying ratios in the soluble and particulate fractions in cortex, cerebellum, testis, spleen and adrenal [50]. Type 1 protein phosphatases have a broad range of physiological functions, including the regulation of exocytosis [51]. Parathyroid PP-1γ is concentrated at the cell periphery in the vicinity of the plasma membrane; this is consistent with a role in the modulation of PTH secretion. Identification and characterization of parathyroid-specific forms of the components of signal transduction pathways may lead to a better understanding of the specialized regulation of parathyroid hormone secretion.

Acknowledgements: The authors offer special thanks to Dr. Paul Greengard for monoclonal and polyclonal antibodies to DARPP-32, antibodies to protein phosphatase-1γ, and his generous support and guidance; to Ms. Jean F. Schaefer for her expert technical assistance, and to Dr. Fred S. Gorelick for invaluable ideas and resources. The authors thank Dr. Edgar F. da Cruz e Silva for antibodies to protein phosphatase-1γ. Tissue cryosections and photographic work were provided by the VA Research Electron Microscopy and Tissue Preparation Laboratory and Ms. Lillemor Wallmark. This work was funded in part by BRSG Fluid Research Funds to L.M.M., and a Merit Review Award from the Department of Veteran's Affairs to B.K.K.

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