Characterization of the phosphatidylinositol-specific phospholipase C isozymes present in the bovine parathyroid and in human kidney HEK293 cells stably transfected with the human parathyroid Ca^{2+} -sensing receptor

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ABSTRACT

The regulation of parathyroid hormone secretion by the chief cells of the parathyroid is mediated by a 7-transmembrane (7-TM) Ca^{2+} -sensing receptor (CaR), which signals via activation of pertussis toxin-insensitive G proteins, causing stimulation of phosphatidylinositol-specific phospholipase C (PI-PLC). We have identified the PI-PLC isoforms expressed in two model systems utilized for studying CaR signal transduction, i.e. dispersed bovine parathyroid cells and a human embryonic kidney cell line (HEK 293) stably transfected with the human parathyroid CaR-cDNA. All of the eight PI-PLC isozymes examined in this study were found to be expressed to varying extents in the bovine parathyroid gland and in the CaR-transfected HEK cells as assessed by immunoblotting. We localized the expression of the more abundant isozymes ($\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, $\gamma 2$, $\delta 2$) to the

chief cells of the bovine parathyroid by immunocytochemistry, while the two less abundant isozymes ($\delta 1$, $\beta 4$) were not detectable in parathyroid sections. G proteins activated by 7-TM receptors are known to activate mainly PI-PLC of the β class. Therefore, $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 4$, all expressed in the bovine parathyroid, are candidate isozymes for coupling to the CaR. A comparison of the levels of expression of PI-PLC isozymes between CaR-transfected HEK cells and non-transfected HEK cells suggested that the expression of the CaR in this human cell line does not cause a significant up-regulation of any of the PLC β and PLC γ isozymes. PLC $\delta 2$, showing predominantly nuclear localization in the parathyroid, was the sole PI-PLC isozyme with higher levels of expression in CaR-transfected HEK cells.

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INTRODUCTION

Parathyroid hormone (PTH), which is secreted by the chief cells of the parathyroid gland, plays a crucial role in Ca^{2+} homeostasis (Brown 1991). PTH secretion is altered in response to small physiological changes in the concentration of extracellular Ca^{2+} , with an unusual inverse relationship between the extracellular Ca^{2+} concentration and PTH release. The property of sensing Ca^{2+} is related to the presence of a 7-transmembrane (7-TM) Ca^{2+} -sensing receptor (CaR), which has been cloned from

both bovine and human parathyroid (Brown *et al.* 1993, Garrett *et al.* 1995a). Mutations of the human CaR cause inherited diseases of Ca^{2+} metabolism (Pollak *et al.* 1993, 1994) and CaR 'knock out' mice were shown to have defects in PTH secretion and Ca^{2+} homeostasis (Ho *et al.* 1995). Genes highly homologous to the parathyroid CaR, which represent species and tissue homologs of the same ancestral gene, have been cloned from human thyroid C cells (Garrett *et al.* 1995b, Freichel *et al.* 1996), rat kidney (Riccardi *et al.* 1995) and rat brain (Ruat *et al.* 1995, Emanuel *et al.* 1996).

At present dispersed bovine parathyroid cells represent the best characterized model for studying the regulation of PTH secretion by the CaR. The CaR signals via activation of pertussis toxin-insensitive heterotrimeric GTP-binding proteins (G proteins) (Hawkins *et al.* 1989), causing activation of phosphatidylinositol specific phospholipase C (PI-PLC) and subsequent breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) with formation of two second messengers, 1,2-*sn*-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Brown *et al.* 1987, Shoback *et al.* 1988). The increase of intracellular Ca^{2+} consequent to the IP_3 -induced release of Ca^{2+} from intracellular stores is an important downstream mediator of intracellular signaling via the CaR, causing inhibition of PTH secretion through mechanisms that remain to be fully defined (Nemeth 1995). In contrast to many other cell systems where DAG activates protein kinase C (PKC) (Nishizuka 1989), high DAG levels are associated with suppressed activity of particulate PKC at high extracellular Ca^{2+} in dispersed bovine parathyroid cells (Nemeth 1995, McKay & Miller 1996). The stimulation of bovine parathyroid cells with extracellular Ca^{2+} is also accompanied by inhibition of cAMP formation via a pertussis toxin-sensitive mechanism likely to be mediated by the CaR and $\text{G}\alpha_i$ proteins (Brown 1991, Nemeth 1995).

To date ten enzymes have been identified as members of the PI-PLC family. On the basis of sequence homology and structural similarities, these isozymes have been classified into three main classes, i.e. $\text{PLC}\beta$ ($\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$), $\text{PLC}\gamma$ ($\gamma 1$ and $\gamma 2$) and $\text{PLC}\delta$ ($\delta 1$, $\delta 2$, $\delta 3$, and $\delta 4$) (reviewed in Lee & Rhee 1995). A variety of studies have described PLC isoforms differentially expressed in particular mammalian tissues. In order to establish which isozymes are available for the coupling to the CaR in the bovine parathyroid we have employed a combination of two methods: Western blotting as a sensitive technique for identifying the presence of different PLC isozymes in parathyroid tissue and in dispersed parathyroid cells; and immunocytochemistry for attributing unequivocally the expression to the chief cells of the parathyroid.

When cultured for several days, dispersed parathyroid cells show a progressive decrease in CaR messenger RNA and protein (Mithal *et al.* 1995). For this reason a new experimental model has been developed by stably transfecting the human parathyroid CaR into human embryonic kidney cells (HEK293) (Rogers *et al.* 1995). The parental HEK cells, which do not *per se* express the CaR, have been previously demonstrated to express functional forms of the receptor in transient transfection studies used to characterize mutated

form of the human CaR (Bai *et al.* 1996). The stably CaR-transfected HEK cells were shown to acquire new properties in comparison to the parental HEK cells, that is the capacity to respond to stimulation by high extracellular Ca^{2+} with: (i) activation of membrane non-selective cation channels; (ii) increase in IP_3 production; and (iii) elevation in intracellular Ca^{2+} (Ye *et al.* 1996, Kifor *et al.* 1997). Because of these data suggesting that the CaR also couples to PI-PLC in CaR-transfected HEK cells, we have identified the set of PI-PLC isozymes present in this experimental model and, by comparing CaR-transfected HEK cells to non-transfected HEK cells, we have evaluated a possible up-regulation of PI-PLC isozymes induced by the expression of the CaR.

MATERIALS AND METHODS

Tissues and cell culture

Bovine parathyroid and liver were obtained from local abattoirs. Dispersed bovine parathyroid cells were isolated from 20 calf parathyroid glands pooled together, following a procedure previously described (Brown *et al.* 1976). These primary cultures were incubated overnight in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 medium (Gibco, Life Technologies AB, Täby, Sweden) supplemented with 10% fetal calf serum (FCS) at 37 °C in a 5% CO_2 incubator before the protein extraction. Cerebellum was dissected out from three Sprague-Dawley rats.

Non-transfected human embryonic kidney HEK293 cells were grown in DMEM (Gibco) supplemented with 10% FCS. The stably CaR-transfected HEK293 cells (clone 7) were kindly provided by Dr Kimberly Rogers of NPS Pharmaceuticals, Inc., Salt Lake City, UT, USA. This clonal line was originated by stably transfecting HEK cells with a plasmid containing the full-length cDNA of the human parathyroid CaR (Rogers *et al.* 1995, Kifor *et al.* 1997). As the plasmid also carries the hygromycin resistance gene, these transfected cells were grown in the same medium used for the non-transfected HEK cells supplemented with 200 $\mu\text{g}/\text{ml}$ hygromycin. The human promyelocytic cell line, HL-60, was grown in RPMI 1460 medium (Gibco) supplemented with 10% FCS. All these cell lines were grown at 37 °C in a 5% CO_2 incubator.

Antibodies

The antibody against $\text{PLC}\delta 1$ is a monoclonal antibody purchased from UBI (Lake Placid, NY, USA) (documented species cross-reactivity: bovine,

rat, mouse and human). The rest of the primary antibodies employed in this study were affinity-purified, rabbit polyclonal antibodies purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA): PLC β 1 epitope: amino acids 1204–1216 mapping at the carboxy terminus of PLC β 1 of rat origin (tested species cross-reactivity: mouse, rat and human); PLC β 2 epitope: amino acids 1170–1181 mapping at the carboxy terminus of PLC β 2 of human origin (tested species cross-reactivity: mouse, rat and human); PLC β 3 epitope: 20 amino acids mapping at the carboxy terminus of PLC β 3 of rat origin (tested species cross-reactivity: mouse, rat and human); PLC β 4 epitope: amino acids 1159–1176 mapping at the carboxy terminus of PLC β 4 of rat origin (tested species cross-reactivity: mouse, rat and human); PLC γ 1 epitope: amino acids 1249–1262 mapping at the carboxy terminus of PLC γ 1 of bovine origin (tested species cross-reactivity: bovine, rat, mouse and human); PLC γ 2 epitope: amino acids 1213–1232 mapping at the carboxy terminus of PLC γ 1 of human origin (tested species cross-reactivity: bovine, rat, mouse and human); PLC δ 2 epitope: amino acids 746–764 mapping at the carboxy terminus of PLC δ 2 of rat origin (tested species cross-reactivity: mouse, rat and human).

Control peptides (PLC β (1–4) and PLC γ (1–2)), that is peptides having the same amino acid sequence of the epitope used to raise the antisera, were also purchased from Santa Cruz Biotechnologies.

Protein extraction and Western blotting

Monolayers of dispersed parathyroid cells were washed quickly with phosphate-buffered saline (PBS solution, Gibco) and immediately extracted by adding boiling lysis solution (1% SDS, 10 mM Tris pH 7.4) to the Petri dishes. The cells were scraped from the dishes, transferred to tubes and boiled for an additional 5 min. The HL-60 cells, which grow in suspension, were washed with PBS by centrifugation, and the pellet was then extracted with lysis solution as described above. In the case of pieces of tissues, the sample was first homogenized in lysis buffer using an electric homogenizer, then boiled for 5 min. The viscosity of the samples was reduced by several passages through a 26 gauge needle, and debris were removed by centrifugation at 12 000 r.p.m. for 10 min in a microcentrifuge. An aliquot of each extract was used for protein determination by the Micro BCA Protein Assay reagent kit (Pierce, Rockford, IL, USA), the rest of the extract was mixed with an equal volume of $2 \times$ concentrated electrophoresis sample buffer ($1 \times = 125$ mM

Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 1.25×10^{-3} % (w/v) pyronin Y), and β -mercaptoethanol was added (5 μ l β -mercaptoethanol/100 ml of protein extract). The protein concentration of the samples in loading buffer was measured again employing the Schaffner–Weissmann method (Schaffner & Weissmann 1973).

The proteins were separated on 5.5% gels by SDS-PAGE (Laemmli 1970) in Tris-glycine buffer pH 8.3 using the Miniprotean II System (BioRad, Hercules, CA, USA) and then electroblotted onto nitrocellulose membranes at 75 V for 1 h using as transfer buffer 20% (v/v) methanol, 186 mM glycine, 26 mM Tris pH 8.3. The blots were incubated with blocking solution (4% non-fat dry milk, 0.1% Tween-20, 137 mM NaCl, 20 mM Tris-HCl, pH 7.6) at room temperature for 2 h. The membranes were then incubated with the primary antibody diluted in blocking solution as specified in Results at 4 °C overnight. After washing three times for 15 min with washing solution (0.1% Tween-20, 137 mM NaCl, 20 mM Tris pH 7.6), the blots were incubated with a donkey horseradish peroxidase-labeled secondary antibody, anti-rabbit IgG (Amersham) diluted 1/2000 in blocking solution at room temperature for 1 h. They were then washed again three times as described above, rinsed twice for 2 min with 137 mM NaCl, 20 mM Tris pH 7.6, and finally incubated with the ECL reagents for chemiluminescence (Amersham, Little Chalfont, Bucks, UK) and exposed to autoradiography film (Hyperfilm, Amersham). In the case of PLC δ 1, Tween-20 was omitted from the solutions and the washes of the membranes were shorter, as recommended by the antibody supplier. Three independent experiments were performed for each PLC isozyme-specific antibody.

Immunocytochemistry

Bovine parathyroid glands embedded in OCT compound (Tissue-Tek, Elkhart, IN, USA) were frozen in cold isopentane (-80 °C), following a procedure previously described (Kifor *et al.* 1996). Sections of the glands (5 μ m) attached to slides were fixed with 5% formaldehyde in PBS for 10 min. After a quick rinse with PBS, the sections were incubated with 1% Triton X-100 in Serum Free Blocking Solution (DAKO, Carpinteria, CA, USA) at room temperature for 1 h. Endogenous peroxidase activity was neutralized by incubating the sections with Peroxidase Blocking Reagent (DAKO) for 10 min, followed by a quick wash with Serum Free Blocking Solution. The sections were then incubated separately with the primary antibodies against the various PLC isotypes in a humidified

incubation chamber at 4 °C overnight. The antibodies were diluted at the concentration indicated in Results in 0.3% Triton X-100 Serum Free Blocking Solution. For the pre-adsorption with the corresponding blocking peptide, the antibody and the peptide were incubated in a small volume of PBS at a ratio of 1:10 (w/w), as recommended by the reagent supplier, at room temperature for 45 min. The antibody-control peptide complex was then diluted in 0.3% Triton X-100 Serum Free Blocking Solution, and the sections were incubated overnight.

All the sections were subsequently washed three times for 20 min each with Triton buffer (0.5% BSA, 0.3% Triton X-100 in PBS) at room temperature and then incubated with a donkey horseradish peroxidase-labeled secondary antibody (anti-rabbit Ig or anti-mouse Ig) (Amersham), at room temperature for 2 h. The secondary antibody was diluted 1/100 in Triton buffer. After washing three times for 20 min with Triton buffer, and twice for 2 min each with PBS, the sections were incubated with a solution containing 1 mg/ml DAB (DAKO) and 0.02% H_2O_2 in PBS for 5–10 min. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted in Pertex (Histolab). Photomicrographs were taken with an Axiophot microscope (Zeiss) using a Kodak Ektachrome 64T film at a magnification of $\times 100$ and $\times 157.5$. The glands of three different animals were analyzed separately and the staining of sections with isozyme-specific PI-PLC antibodies was repeated in three independent experiments.

RESULTS

Western blotting

For identification of the PI-PLC isozymes expressed in bovine parathyroid, total proteins were extracted from the whole glands. In the bovine parathyroid gland the chief cells are the main cellular component. However, other cell types, e.g. endothelial cells, fat cells, blood cells and fibroblasts, are also present in significant quantities. In order to obtain an homogeneous chief cell preparation, dispersed bovine parathyroid cells were isolated from the glands and cultured overnight, thereby reducing the contamination with blood cells prior to the extraction of the total proteins. Calf parathyroid glands contain little connective tissue compared with the glands of the cow and were chosen, therefore, as a source for the preparation of dispersed cells (Brown *et al.* 1976). The immunocytochemistry experiments were performed with the larger parathyroids obtained from cows. Because of

the use of animals of different ages, calf and cow parathyroids were always compared in the Western blots.

The amino acid sequence of each member of the PI-PLC family is highly conserved in different tissues and species (Lee & Rhee 1995). However, the same isozyme may be present in alternative-spliced forms and may generate multimers. Protein phosphorylation and proteolytic degradation are also common and contribute to the presence of multiple bands in Western blotting. The results obtained with the antibodies specific for the four PLC β isozymes are shown in Fig. 1. PLC β 1, initially isolated from bovine brain, has not been thoroughly investigated in other bovine tissues. PLC β 1 has been reported to exist in 150, 140 and 100 kDa forms, which have similar catalytic properties (Lee *et al.* 1993, Bahk *et al.* 1994). The 150 and 140 kDa subtypes, produced by alternative splicing from the same gene, have been either detected together or not detected in rat tissues and their ratios differ in different rat tissues, showing variation during development (Bahk *et al.* 1994). The anti-PLC β 1 antibody used here detected the three forms mentioned above in the two human HEK cell lines and two forms (140 and 100 kDa) in the bovine tissues, i.e. parathyroid and liver (Fig. 1a). A band of intermediate size was found in the parathyroid gland preparation but was not expressed in the dispersed chief cells, and might be either a PLC β 1 form expressed in other cell types present in the gland or a PLC β 1 form which is lost when culturing the cells. An additional band of high molecular weight was detected in extracts of the HEK cell lines. This band was also present in protein extracts obtained from mouse embryo Balb/3T3 cell but not in protein extracts from human kidney (data not shown). Therefore, it is not a tissue-specific subtype and its expression is probably related to an altered state of differentiation in immortalized cell lines.

As PLC β 2 was originally cloned from the human promyelocytic HL-60 cell line (Kritz *et al.* 1990, Park *et al.* 1992), when analyzing PLC β 2 we have used protein extracts of HL-60 cells as a positive control (Fig. 1b). In spite of the fact that the bovine PLC β 2 has not yet been cloned, the high homology between the rat and the human PLC β 2 (only two amino acid residues are different) (Park *et al.* 1992) suggests that a high degree of homology in amino acid sequence would also be present between the bovine and human forms. The two bands detected by the PLC β 2 antibody in the HL-60 extract (140 and 100 kDa) were present in the bovine extracts and in the two HEK cell lines. The PLC β 2 antibody also recognized a band of about 150 kDa,

FIGURE 1. Identification of PI-PLCβ isozymes by Western blotting. Total protein extracts (20 μg/lane) were separated by SDS-PAGE using 5.5% polyacrylamide gels. (a) PLCβ1 antibody diluted 1/200. (b) PLCβ2 antibody diluted 1/200. (c) PLCβ3 antibody diluted 1/200. (d) PLCβ4 antibody diluted 1/150. CALF LIV. = calf liver; COW PT. GL. = cow parathyroid gland; CALF PT. GL. = calf parathyroid gland; CALF D. PT. C. = dispersed calf parathyroid cells; CaR-T. HEK = CaR-transfected HEK cells; NT. HEK = non-transfected HEK cells; HL60 = human promyelocytic HL-60 cell line; RAT CEREB. = rat cerebellum. Molecular weight standards (Kaleidoscope Marker, High Range Marker and Low Range Marker, BioRad) were run along with protein extracts.

which was abundant in all of the bovine tissues analyzed but was only weakly detected in the extracts obtained from the human HEK cell lines (Fig. 1b). This is likely to be an alternative-spliced or tertiary-modified form of PLCβ2 that is either more common in bovine tissues or is even species specific for the bovine. Similar to our findings, it has recently been shown that when comparing extracts of rat brain, vascular smooth muscle and liver, PLCβ2 has three different apparent molecular weights in SDS-PAGE (Labelle & Polyak 1996).

PLCβ3 appears to be the most ubiquitously expressed of the PLCβ isoforms in rat and human tissues (Jhon *et al.* 1993, Weber *et al.* 1994). Previous investigations have demonstrated the presence of PLCβ3 in bovine tissues, e.g. bovine retina (Lee *et al.* 1993). Here we found PLCβ3 expression both in dispersed bovine parathyroid

cells and in the two human HEK cell lines (Fig. 1c). As shown in Fig. 1c, the PLCβ3 antibody detected the expected 145 kDa band in all the protein extracts. Considering that the same amount of total protein was loaded in all of the lanes (20 μg), the difference in the signal between the human and bovine samples might represent either variation in species cross-reactivity of the antibody or different levels of expression. The difference in the signal in the lanes with cow gland extract, calf gland extract and dispersed cell extract might be related to the different percentage of chief cells in the various preparations, depending on the age of the animals and the purity of the chief cell dispersion. In all of the lanes a 110 kDa band was also visible, likely a proteolytic fragment of PLCβ3 (Blank *et al.* 1993).

PLCβ4 is known to be highly expressed in bovine, rat and mouse retina and in cerebellum

(Ferreira *et al.* 1993). Recent studies with knock out mice have suggested that this particular PLC plays a role in the mechanisms of visual signal transduction (Jiang *et al.* 1996). The PLC β 4 antibody detected the expected band in the protein extract of rat cerebellum that we employed as a control (Fig. 1d) (Lee *et al.* 1993). This band was also visible when 10 μ g protein extract were loaded in the lane. In contrast, in order to see signals in the other extracts we had to employ at least 40 μ g total protein/lane and expose the autoradiography films for longer times. Bands of about 90 kDa were observed in calf parathyroid gland and in extracts of dispersed parathyroid cells, as well as in the HEK cell lines (Fig. 1d). Moreover, the bovine extracts showed a high molecular weight band of about 200 kDa. The calf parathyroid also demonstrated a weak 140 kDa band when 100 μ g protein extract were loaded in the lane (data not shown). Previous studies have indicated that PLC β 4 has different forms that originate through alternative splicing and, possibly, by proteolysis. For example, in the bovine cerebellum two forms, of 130 and 97 kDa, have been described (Min *et al.* 1993). Moreover, a total of four alternatively spliced forms are expected in this tissue on the basis of sequence analysis of cloned cDNAs (Ferreira & Pak 1994).

Parathyroid cells and the HEK cell lines were then investigated for the expression of PLC γ 1 and PLC γ 2 (Fig. 2). PLC γ 1 is known to be expressed in almost every mammalian tissue, while PLC γ 2 seems to be rather specific for hematopoietic cells (Katan 1996). The PLC γ 1 antibody detected the expected 145 kDa band in all of the lanes (Fig. 2a) (Lee *et al.* 1993). This protein appeared to be very abundant in the human cell lines, which also showed an additional weak band of about 120 kDa. PLC γ 2 was also expressed in all of the samples analyzed (Fig. 2b). The size of the major band was about 140 kDa, in agreement with previous studies with other bovine tissues (Lee *et al.* 1993). The expression level was higher in the dispersed cells compared with the parathyroid glands, and the signal was very strong in extracts of the HEK cell lines. Additional bands in the lanes are likely to be due to non-specific binding of the antibody.

PLC δ 1 was expressed at similarly low levels in all of the bovine extracts tested as the expected 97 kDa band (Fig. 3a). The HEK cell lines expressed both a 150 kDa and an 80 kDa band (Ryu *et al.* 1987). With the anti-PLC δ 2 antibody (Fig. 3b) a 85 kDa band was detected in all of the samples (Meldrum *et al.* 1991), but it was clearly more abundant in the dispersed cells than in the parathyroid gland and was also more highly

FIGURE 2. Determination of the expression of PI-PLC γ isozymes. Immunoblots were obtained employing 5·5% polyacrylamide gels and 20 μ g total protein/lane. (a) PLC γ 1 antibody diluted 1/200. (b) PLC γ 2 antibody diluted 1/200. CALF LIV. = calf liver; COW PT. GL. = cow parathyroid gland; CALF PT. GL. = calf parathyroid gland; CALF D. PT. C. = dispersed calf parathyroid cells; CaR-T. HEK = CaR-transfected HEK cells; NT. HEK = non-transfected HEK cells. Kaleidoscope Marker (BioRad) was used as the molecular weight standard.

expressed in the CaR-transfected HEK cells than in the non-transfected HEK cells. After verifying the amount of protein in the extracts with two different methods, serial dilutions of the extracts obtained from the two HEK cell lines were compared by immunoblotting. The difference in expression of PLC δ 2 was estimated to be about 5-fold (data not shown).

In summary, we have detected four PLC β isoforms, two PLC γ isoforms and two PLC δ isoforms in parathyroid gland, dispersed parathyroid cells and the two HEK cell lines. When

FIGURE 3. Western blotting pattern of expression of PI-PLC δ isozymes. Total proteins were separated with 5-5% polyacrylamide gels. (a) 40 μ g total protein/lane, PLC δ 1 antibody diluted 1/300. (b) 20 μ g total protein/lane, PLC δ 2 antibody diluted 1/200. CALF LIV. = calf liver; COW PT. GL. = cow parathyroid gland; CALF PT. GL. = calf parathyroid gland; CALF D. PT. C. = dispersed calf parathyroid cells; CaR-T. HEK = CaR-transfected HEK cells; NT. HEK = non-transfected HEK cells; RAT CEREB. = rat cerebellum. Kaleidoscope Marker and Low Range Marker (BioRad) were employed as molecular weight standards.

loading the same amount of protein, the patterns of expression of PI-PLC isozymes were virtually identical in the CaR-transfected HEK cells and in the parental HEK cells, with the sole exception of PLC δ 2, which was about five times more abundant in the CaR-transfected cells.

Immunocytochemistry

Once we established the presence of the various PLC isotypes in the dispersed parathyroid cell

preparation by Western blotting, sections of the glands were analyzed using the indirect immunocytochemical technique based on peroxidase staining to verify that the signal was specific for the chief cells.

The PLC β 4 and the PLC δ 1 antibodies, which detected very weak bands in Western blotting, did not give any signal on immunocytochemistry (Fig. 4). All of the other antibodies, which readily detected the expected band in the blots, also specifically recognized antigens present in the chief cells, as shown by the peroxidase staining (Fig. 4). No staining occurred when the antibodies were pre-adsorbed with the corresponding blocking peptide. Most of the antibodies recognized their antigen in the cytoplasm of the chief cells. The anti-PLC δ 2 antibody showed a peculiar pattern of staining, indicating predominant nuclear localization of PLC δ 2 (Fig. 4f). In each microscopic field a low percentage of the nuclei were not stained.

DISCUSSION

The similarity in the structure of the CaR to other 7-TM receptors suggests a likely coupling of this receptor to isoforms of PI-PLC of the β class via G-protein activation (Exton 1996). Indeed high extracellular Ca^{2+} activates PI-PLC in CaR-expressing bovine parathyroid cells as well as in CaR-transfected but not in non-transfected HEK cells (Kifor *et al.* 1997). The members of the PLC β family are activated both by the α and the $\beta\gamma$ subunits of stimulated G-proteins (Katan 1996, Singer *et al.* 1997). The pertussis toxin-insensitive G proteins that stimulate PLC β belong to the G_q family and their α subunits can directly activate PLC β by binding to its carboxy terminal region. In contrast, the activation of PLC β by pertussis toxin-sensitive G proteins of the G_o/G_i family is mediated by $\beta\gamma$ subunits, which bind to the amino terminus of PLC β . Our results show that parathyroid cells express all of the four PLC β isozymes, with predominant expression of PLC β 1, PLC β 2 and PLC β 3. These three isozymes appear to be abundant in the parathyroid gland and in dispersed parathyroid cells in Western blotting (Fig. 1), and the immunocytochemistry pattern indicates their specific localization in the chief parathyroid cells (Fig. 4). PLC β 4 was present at levels hardly detectable by immunoblotting (Fig. 1d), and we were not able to obtain any signal in immunocytochemistry, probably because of the lower sensitivity of this technique. In the case of the two HEK cell lines, we performed Western analysis only, because these are homogeneous clonal lines. Figure 1 shows

that the four PLC β isozymes were likewise present in the HEK cell lines, again with a lower level of expression of PLC β 4.

It is possible that the CaR activates divergent pathways by stimulating both G_i and G_q proteins. Subunits of the α_q (such as α_{11} and α_q) and the α_i classes (such as α_{i2}) are present in bovine parathyroid gland (Varrault *et al.* 1995). α_{i2} is a candidate for CaR-mediated inhibition of cAMP formation, while α_q and α_{11} could promote activation of PLC β (Brown 1991). However, $\beta\gamma$ subunits of G_i could also stimulate PLC β , therefore these two pathways might recruit different PLC β isozymes based on their differing mechanisms of activation. The sensitivity of PLC β to α_q activation is different for the four isozymes of this class, with the following order of magnitude: $\beta 1 > \beta 3 > \beta 4 > \beta 2$ (Smrcka & Sternweis 1993). In the case of $\beta\gamma$ activation, PLC β 2 and PLC β 3 are more sensitive than PLC β 1, while PLC β 4 cannot be activated by $\beta\gamma$ (reviewed in Katan 1996).

It has recently become evident that some 7-TM receptors can activate PLC γ , but not PLC β (Lee & Rhee 1995, Marrero *et al.* 1996). The PLC γ isozymes have the peculiarity of containing Src homology domains and are typically activated by tyrosine phosphorylation mediated either by receptor tyrosine kinases or by soluble non-receptor tyrosine kinases. Here we demonstrate that parathyroid and HEK cells express both the two known PLC γ isozymes, γ 1 and γ 2 (Figs 2 and 4). Therefore PIP $_2$ breakdown induced in the bovine parathyroid by increased extracellular Ca^{2+} could conceivably be linked to activation of PLC γ by soluble tyrosine kinases stimulated by the CaR, although this possibility has not been studied to date.

The mechanisms of activation of PLC γ isozymes are not known. Some investigations have suggested that neither G-protein stimulation nor tyrosine kinase phosphorylation plays a major role in PLC δ activation (Lee & Rhee 1995, Katan 1996). PLC δ isozymes, like the other PLC isoforms, are activated by Ca^{2+} , and they are the only members of the PI-PLC family with EF-hand domains, known to be Ca^{2+} -binding motifs (Katan 1996). Being

stimulated by transient increases in cytoplasmic Ca^{2+} , PLC δ could amplify intracellular signals initiated by receptors activating other PLC isoforms by producing more second messengers, e.g. DAG, IP $_3$ and Ca^{2+} . We have investigated the presence of two out of four PLC δ isozymes, δ 1 and δ 2. PLC δ 1, which is known to be rather ubiquitous, was expressed at a very low level in bovine parathyroid and in the human HEK cell lines (Fig. 3), and we have not been successful in detecting it in the parathyroid glands by immunocytochemistry. In contrast, PLC δ 2 was readily detected in all of the extracts analyzed by Western blotting, and it was very abundant in the HEK cell lines (Fig. 3). Only in the case of PLC δ 2 did we observe a nuclear-specific expression pattern in bovine parathyroid by immunocytochemistry (Fig. 4). In agreement with the presence of a signal peptide for nuclear localization in the amino acid sequence of PLC δ 2 (Meldrum *et al.* 1991), we show for the first time preferential localization of this isozyme in the nucleus. PLC δ 2 is the only PI-PLC isozyme presenting a dissimilar level of expression in the two HEK cell lines. The 5-fold increased expression of PLC δ 2 could be induced by the overexpression of the CaR. However, we cannot exclude clonal differences between the two HEK cell lines and further investigations are required to establish whether the hygromycin selective pressure alters the gene regulation or the cell cycle. Interestingly, PLC δ 4, another member of the PLC δ class sharing a high degree of sequence homology with PLC δ 4, is reported to be maximally expressed during S/M phase of the cell cycle in the nucleus of Swiss 3T3 cells (Liu *et al.* 1996). The expression of PLC δ 3 and PLC δ 4 in the bovine parathyroid and in the HEK cell lines remains also to be analyzed.

In conclusion, bovine parathyroid cells and the HEK cell lines studied here express the same eight PLC isoforms, with considerable amounts of PLC β 1, PLC β 2, PLC β 3, PLC γ 1, PLC γ 2 and PLC δ 2, but low levels of PLC β 4 and PLC δ 1. In most cases, the CaR-transfected HEK and the parental non-transfected HEK cells express specific

FIGURE 4. Immunocytochemical detection of PI-PLC δ isozymes expressed in the bovine parathyroid. Frozen sections of the glands were fixed with 4% formaldehyde, incubated first with the anti-PLC isozyme specific primary antibody, then with a peroxidase-conjugated, secondary antibody. DAB was used as peroxidase substrate, generating a dark brown precipitate around the bound antibodies. The sections were counterstained with Mayer's hematoxylin, which stains the cell nucleus a blue color. (a) PLC β 1 antibody diluted 1/100 (scale bar represents 66.3 μm). (b) PLC β 2 antibody diluted 1/100 (magnification $\times 100$). (c) PLC β 3 antibody diluted 1/50. (d) PLC γ 1 antibody diluted 1/100 (magnification $\times 100$). (e) PLC γ 2 antibody diluted 1/100 (magnification $\times 100$). (f) PLC δ 2 antibody diluted 1/50 (magnification $\times 157.5$). (g) Control experiment where the PLC β 1 antibody (diluted 1/100) was preadsorbed for 1 h with a 10-fold excess (w/w) of the blocking peptide (corresponding to the antibody epitope) prior to being used for the incubation of the sections (magnification $\times 100$).

PI-PLC isozymes at similar levels. Thus heterologous expression of the CaR does not up-regulate the PLC β (1–4), PLC γ (1,2) and PLC δ 1 isozymes. PLC δ 2 was 5-fold more abundant in CaR-transfected cells and was also the sole PI-PLC preferentially expressed in the nucleus of the parathyroid cell. These data lay the groundwork for future investigation of the steps leading to CaR-mediated activation of PI-PLC. Knocking out the four PLC β isozymes that are the best candidate for the immediate coupling to the stimulated CaR, via microinjection of blocking antibodies and/or anti-sense oligonucleotide technology, will allow definitive identification of the molecules involved in CaR signal transduction.

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