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Expression and Immunolocalization of Calcium Transport Proteins in the Canine Duodenum, Kidney, and Pancreas

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ABSTRACT

The aim of the study was to elucidate expression and localization of several calcium handling proteins and the regulating vitamin D receptor (VDR) in the dog using RT-PCR and immunohistochemistry. The results of RNA expression and antibody staining were compared to ensure specificity. We found several antibodies to be useful for further studies on calcium homeostasis. Anat Rec, 293:770–774, 2010. © 2010 Wiley-Liss, Inc.

Key words: dog; beta cells; calcium homeostasis; immunohistochemistry; kidney; duodenum

The regulation of intracellular calcium levels depends on uptake, intracellular buffering, and clearance of Ca^{2+}. Important calcium handling proteins in epithelial cells are the calcium-binding protein calbindin D-28k, the plasma membrane calcium ATPase (PMCA1), the sodium calcium exchanger (NCX1), and the epithelial Ca^{2+} channels TRPV5 and TRPV6. TRPV5 is predominantly expressed in the distal convoluted tubules (DCT) of the kidneys, whereas TRPV6 is expressed in the duodenum; they both mediate the calcium uptake at the apical surface of epithelial cells (Hoenderop et al., 2001). Calbindin is an intracellular protein that binds calcium ions and moves them from the apical to the basolateral membrane, where PMCA1 and NCX1 are located to extrude them into the extracellular space (Lambers et al., 2006; Schlatter, 2006). Also involved in calcium handling is the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which moves free cytosolic Ca^{2+} back into the endoplasmic and sarcoplasmic reticulum. There is evidence for control of these transport systems by calcitriol ($1,25(OH)_2D_3$), the activated form of vitamin D (Okano et al., 2004). The widespread endocrine, autocrine, and paracrine responses to calcitriol are mediated via the vitamin D receptor (VDR). Recently, calcium transport proteins which were first detected in epithelial cells of the intestinal tract and the kidneys have been found to play a role in the insulin secretion mechanisms of pancreatic β-cells (Chen et al., 2003). Defects of calcium handling in pancreatic β-cells are known to contribute to insulin secretion deficits, which can lead to diabetes mellitus (Marie et al., 2001). Dogs are often affected by diabetes and are considered to represent a non-rodent model for latent autoimmune diabetes of the adult in man (Catchpole et al., 2005). This study addresses the presence of calcium handling proteins and their mRNA in canine kidney, duodenum, and pancreatic islets, because nothing is known about the relevance and distribution of these targets in the dog. Kidneys and duodenum were chosen as positive control tissues, since expression of the proteins examined in this study has been proven in these tissues in other species. None of the available antibodies had been previously tested on canine tissue; therefore RNA expression was assessed as a control for the immunohistochemical data to detect possible false positive or false negative staining.

Samples of canine pancreas (left lobe), kidneys (section containing renal cortex and medulla), and duodenum (close to the stomach) were obtained from four Beagle dogs from a control group of a toxicological research experiment (Boehringer Ingelheim Pharma GmbH, Biberach, Germany), which was approved and conducted

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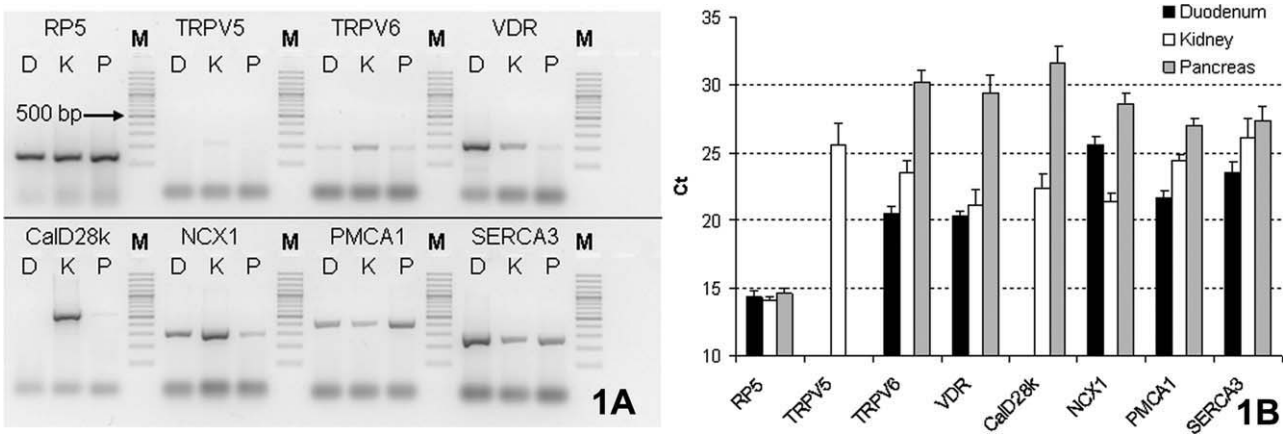

Fig. 1. Qualitative (**1A**) and quantitative (**1B**) mRNA expression of calcium handling proteins in canine duodenum (D), kidneys (K), and pancreas (P). Quantitative analysis was done on $n = 4$ independent samples. The loading control RP5 shows that comparable amounts of mRNA were used for all tissues. Ct = cycle threshold.

according to the German animal welfare law. All samples were taken immediately after euthanasia and kept in RNAlater solution (Qiagen, Hilden, Germany) for RNA extraction or fixed in 4% paraformaldehyde solution (PFA) and embedded in paraffin.

Total RNA was extracted from kidneys, duodenum, and pancreas from the four dogs using the RNeasy Midi Kit (Qiagen). Four microgram of total RNA were reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). For the qualitative PCR, cDNA from one dog was amplified by PCR for all targets using the HotStarTaq Master Mix (Qiagen). For the quantitative PCR, all targets were amplified in parallel from a single reverse transcription reaction. The cDNA template was mixed with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the appropriate primer pair. Samples were run in duplicates in a 96-well plate using a 7300HT Real Time PCR system (Applied Biosystems). No template controls (NTCs) were included, and a melting curve analysis was run to make sure that only a single PCR product was amplified and no primer dimer were present. Specificity of the primer pairs was assessed by sequencing the PCR products (Seqlab, Göttingen, Germany). Ribosomal protein S5 (RP5) was used as a reference gene and loading control. For all measurements, mean values, and standard deviations (SD) for the threshold cycle (Ct) were calculated using the four independent samples of each organ.

Immunohistochemistry was done on deparaffinized sections which were autoclaved in 0.01 M citrate buffer pH 6.0 for 20 min for antigen retrieval and incubated with the primary antibody overnight at 4°C. The primary antibodies used were targeted against calbindin D-28k (Mouse monoclonal to Calbindin-Neuronal Marker, clone 300, 1:100, abcam, Cambridge, UK), VDR (Rat monoclonal to VDR Ab-1, clone 9A7γ.E10.E4, 1:100, Dianova, Hamburg, Germany), SERCA3 (Mouse monoclonal to SERCA3 ATPase, 1:500, abcam), PMCA1-ATPase (Mouse polyclonal to Calcium Pump PMCA1 ATPase, 1:200, abcam), NCX1 (Mouse monoclonal to NCX1, clone 6H2, 1:200, abcam), TRPV 5 (Sheep polyclonal to TRPV 5, 1:1000, abcam), and TRPV 6 (Rabbit polyclonal to TRPV 6, 1:1000, abcam) and against Insulin (Guinea pig anti Insulin, 1:100, DAKO, Hamburg, Germany).

The next day, sections were incubated with the appropriate biotinylated secondary antibody 1:200 (anti-mouse/anti-rat/anti-sheep/anti-rabbit IgG, Rockland, Gilbertsville, PA) or HRP-conjugated anti-guinea pig IgG (DAKO) for 30 min at room temperature. The sections were washed again and incubated with a peroxidase-conjugated streptavidin-biotin complex (Vectastain Elite ABC Standard Kit, Vector Laboratories, Burlingame, CA). Vector VIP (Vector Laboratories) was used as substrate, and nuclei were counterstained with methyl green. For the VDR, signal amplification was required using biotinyl tyramide (1:50) for 30 min at room temperature (Renaissance indirect tyramide reagent, Perkin Elmer, Waltham, MA). For the calbindin D-28k/insulin double staining Vector VIP was used as a substrate for the insulin antibody, and DAB (Sigma-Aldrich, Hamburg, Germany) was used for the calbindin antibody. Images were made using a Zeiss Axioskop with a digital camera (Zeiss Axio Vision, Munich, Germany).

For each antibody, immunohistochemistry was performed in sections from duodenum, kidneys, and pancreas. As negative controls, sections from all tissues were stained using the same staining protocols with omission of the first antibodies.

RESULTS AND DISCUSSION

Since tissue samples can yield highly variable results for mRNA expression due to variability in tissue composition, mRNA loss during handling, or inadequate cDNA yield after reverse transcription, the expression of the ribosomal protein S5 (RP5) was used as a loading control. Qualitative PCR shows that RP5 is expressed abundantly in all three organs. Duodenum, kidney, and pancreas express TRPV6, VDR, NCX1, PMCA1, and SERCA3, while TRPV5 is only expressed in the kidney and calbindin D28k is not expressed in the duodenum (Fig. 1A). Quantitative PCR confirms the results of the qualitative PCR and shows the different expression levels of the targets and comparable amounts of the RP5

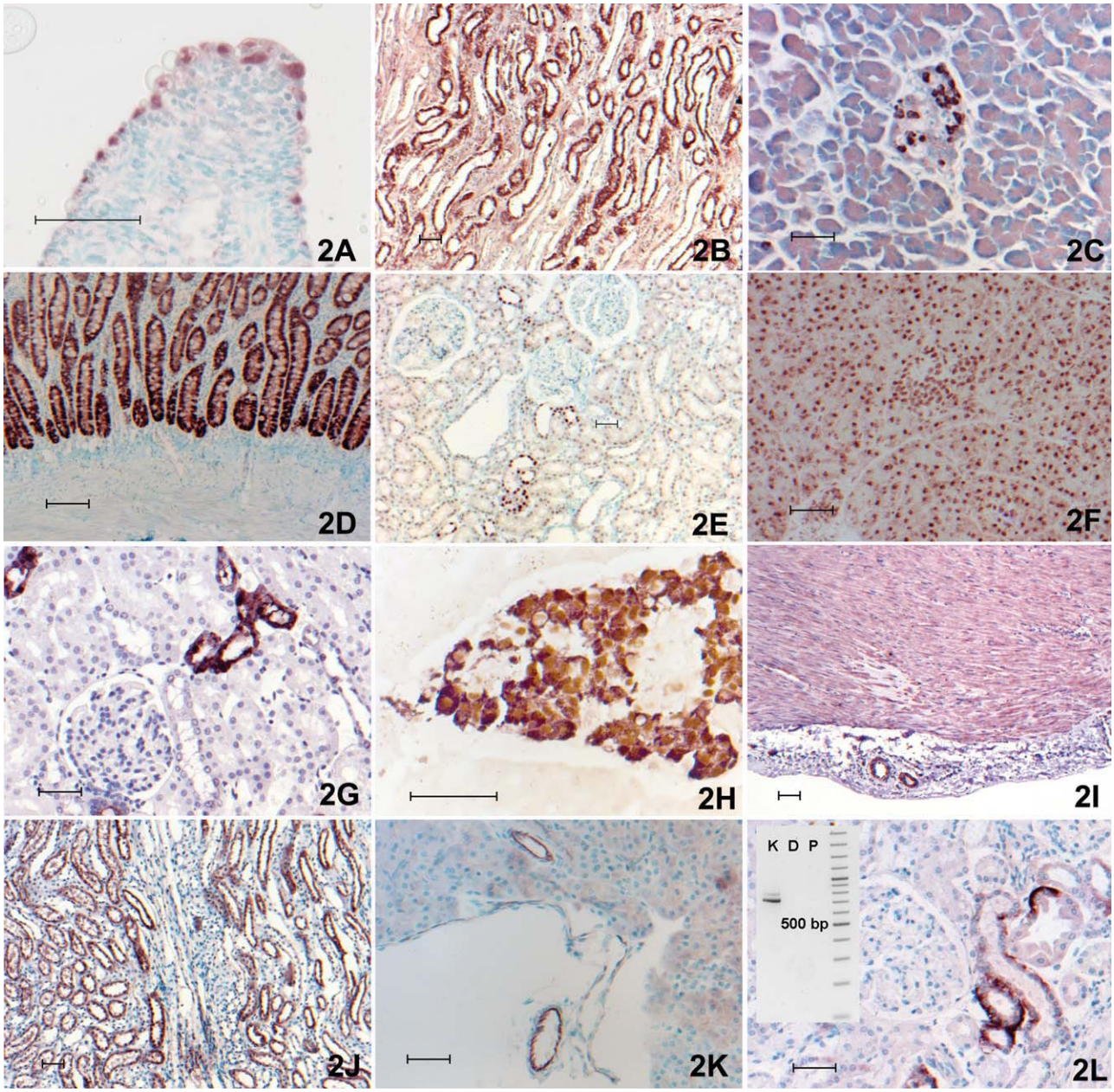

Fig. 2. Immunhistochemical staining of Ca^{2+}-regulating proteins in the canine duodenum, kidneys, and pancreas: TRPV6 staining in apical epithelial cells of the duodenum (**2A**), epithelial cells of the kidney collecting ducts (**2B**), and cells of the pancreatic islets (**2C**). Nuclear VDR staining in duodenal crypt cells (**2D**), in renal tubular cells (**2E**), and endocrine and exocrine pancreas (**2F**). CaID28k staining in the kidney cortex (**2G**) and in pancreatic β-cells (**2H**). The pancreatic islet is double-stained with insulin (blue) and CaID28k (brown). SERCA3 staining in the muscular layer of the duodenum (**2I**), kidney collecting ducts (**2J**), and blood vessel endothelia of the pancreas (**2K**). NCX1 staining in distal convoluted tubules of the kidney (**2L**) and expression of NCX splice variants in the kidney [2L inset; kidneys (K), duodenum (D), pancreas (P)]. All scale bars 50 μm.

loading control. Low Ct values equal high expression (Fig. 1B).

The two highly homologous and very selective Ca^{2+} channels TRPV5 and TRPV6 provide an apical transport of Ca^{2+} ions into epithelial cells of the kidneys and the duodenum. Using polyclonal antibodies against TRPV5 and TRPV6 in the dog, the results were remarkably similar for both antibodies in duodenum, kidneys, and pancreas. In the duodenum, apical enterocytes showed strong immunhistochemical staining for TRPV6 (Fig. 2A), which resembles findings in other species. Apical enterocytes have been found to express TRPV6 in human and mouse tissue (Peng et al., 1999). Since there is no mRNA expression of TRPV5 in the duodenum, the immunohistochemical staining of enterocytes using this antibody is most probably an artefact. In the canine kidneys, the TRPV6 transporter showed a widespread expression in the cytoplasm of epithelial cells from the

distal convoluted tubule to the collecting duct (Fig. 2B). For TRPV5 background staining was very high with some tubuli showing stronger immunoreactivity. Comparable results were found in murine kidneys with widespread expression of TRPV6 in the kidneys from the distal convoluted tubule to the collecting duct, whereas TRPV5 was only found in the distal convoluted tubule and connecting tubule (Nijenhuis et al., 2003).

In the murine pancreas, immunohistochemical TRPV6 staining has been demonstrated in the exocrine tissue, but not in pancreatic islets (Zhuang et al., 2002). In contrast, a strong cytoplasmic TRPV6 staining in the canine pancreatic islets, but only faint staining of exocrine tissue was found in this study (Fig. 2C). Colocalization of TRPV5 with insulin as seen in the rat could not be confirmed for the dog, where pancreatic tissue is devoid of TRPV5 mRNA expression. The results for both antibodies are ambiguous; TRPV5 antibody most certainly is not specific in canine tissue, but TRPV6 antibody might also bind to another closely related epitope and exhibit unspecific staining. It is therefore uncertain whether the TRPV6 antibody is useful for the detection of this protein in canine tissue.

High expression of the VDR (both mRNA and protein) can be found in duodenum and kidneys. The VDR is necessary in these organs to regulate systemic calcium levels, and several calcium-handling proteins are regulated via vitamin D. The distribution of VDR-positive cells in duodenal crypts (Fig. 2D) and kidney epithelial cells of the DCT (Fig. 2E) in the dog is similar to findings in many other species in which the same antibody has been used successfully (Clemens et al., 1988; Kumar et al., 1994). In the pancreas, signal amplification with biotinyl tyramide was necessary to show immunoreactivity for the VDR. The receptor was expressed in the nuclei of the exocrine and endocrine cells of the pancreas (Fig. 2F). Other species, like the zebrafish, express VDR in pancreatic acinar cells (Craig et al., 2008), but it has also been found in β-cells of rats and humans (Johnson et al., 1994; Bourlon et al., 1996). In β-cells, calcitriol appears to enhance insulin secretion by activating calcium influx (Billaudel et al., 1993), an effect mediated through the VDR.

Calbindin D-28k has been extensively studied in several species as a neuronal marker and as a calcium binding protein in the kidneys (Biner et al., 2002).

In the dog, the results of the immunohistochemical staining of calbindin D-28k are in agreement with the expression data. In the kidneys, specific staining could be seen in the DCT and cortical collecting ducts, whereas the glomerula remained unstained (Fig. 2G). This finding is comparable to the expression seen in other species (Hoenderop et al., 1999).

In the pancreas, calbindin D-28k could be detected in the cytoplasm and nuclei of the majority of cells in the pancreatic islets. A double staining with insulin showed that calbindin-positive cells are also insulin-positive, characterizing these cells as β-cells (Fig. 2H). These results match the findings in human and rat pancreas (Johnson et al., 1994). In the rat, calbindin D-28k has been shown to influence insulin secretion through the modulation of intracellular Ca^{2+} concentration (Parkash et al., 2002). Ca^{2+} handling in the canine β-cells may follow a similar pathway.

In the duodenum, no immunoreactivity against calbindin D-28k was detected in the dog. It is known from other species that the closely related protein calbindin D-9k instead of calbindin D-28k is responsible for intestinal intracellular calcium binding, and that calbindin D-28k is not expressed in the duodenum (Opperman et al., 1990).

SERCA3 immunoreactivity in sections of the duodenum was found in smooth muscle cells of the intestinal walls and the blood vessels (Fig. 2I). Strong immunoreactivity was also found in the epithelial cells of collecting ducts in the kidneys (Fig. 2J). In the pancreas, only blood vessel endothelia were stained (Fig. 2K). SERCA3 has been described as the sarco(endo)plasmic Ca^{2+} ATPase of non-muscle tissue because it has been detected in vascular endothelial cells, tracheal epithelial cells, pancreatic β-cells, and many other tissues. The closely related proteins SERCA1 and SERCA2 are predominantly found in skeletal and cardiac muscle cells (Wuytack et al., 1992). Several splice variants have been detected for SERCA3, which are expressed in different tissues and vary between humans and rodents (Martin et al., 2002). In our study, a strong expression in the smooth muscle cells of the duodenal tunica muscularis and the vessel walls was found. Together with the detection of specific mRNA in the duodenum this indicates that SERCA3 is expressed in smooth muscle cells, and therefore can not longer be labelled as the non-muscle SERCA. Its localization in kidney epithelial cells of the collecting ducts has not been reported before and might be important for intracellular Ca^{2+} handling in these cells. SERCA3 was not detected in pancreatic β-cells, although others consider it an important factor in Ca^{2+}-signalling and insulin stimulus-secretion coupling (Beauvois et al., 2006). The only prominent staining in the canine pancreas was found in blood vessel endothelia. Despite some unexpected results, it appears likely that the staining is specific for canine SERCA3. Detection of specific mRNA in all tissues tested supports this notion.

In canine tissue, expression of NCX1 mRNA was detected in kidneys, duodenum, and pancreas, while immunohistochemical staining was only found in the kidneys. Staining was found at the basolateral membrane of cells in the DCT, but not in glomeruli or proximal tubular segments (Fig. 2L). Other studies show that NCX1 is widely distributed in various tissues. It influences calcium uptake in the duodenal mucosa and is considered to control calcium reabsorption in the kidneys (Loffing et al., 2001; Dong et al., 2005). Some findings support an important role of NCX1 in Ca^{2+}-extrusion from pancreatic β-cell (Herchuelz et al., 2007). The lack of staining in duodenum and pancreas in the dog, despite expression of mRNA, might be due to expression of NCX1 splice variants in these tissues. Multiple species-specific and tissue-specific splice variants have been described (Quednau et al., 1997), which might change the epitope the antibody recognizes. We therefore used an additional primer pair for NCX1 splice variants [as described in (Van Eylen et al., 2001)] and detected two PCR products (about 700 bp and 770 bp long) in the kidney, but no PCR products in duodenum and pancreas (Fig. 2L, inset). The antibody appears to be useful for the NCX1 variant of canine kidney but seems to lack specificity for other forms of NCX1.

Immunohistochemistry using an antibody against PMCA1 did not show any reaction in the kidneys, duodenum, or pancreas. PMCA1 expression is known to be widespread but so far, protein content in several tissues

could only be identified by Western blot. The immunogen used to generate the PMCA1 antibody is 100% homologue in many species (human amino acid 1121–1220), but it seems that this antibody is not suitable for formalin or methacarn fixed paraffin-embedded tissue sections and can only be applied to analyze fresh tissue samples. Specific mRNA expression could be detected in the canine kidneys, duodenum, and pancreas, but the cellular localization remains unclear.

No staining was detected on all control slides using only secondary antibodies.

In conclusion, several Ca^{2+}-regulating proteins were identified in canine tissues and the cellular localization using commercially available antibodies for which cross-reactivity with canine proteins was assessed for the first time. Apart from the main tissues of systemic calcium regulation, the expression in the pancreas was also investigated, primarily to determine the localization of these proteins in β-cells, since Ca^{2+}-handling in these cells is crucial for insulin secretion. The mRNA and protein expression results show that the canine β-cell is equipped with calbindin D-28k for intracellular Ca^{2+}-buffering, and that the VDR can mediate hormonal regulation by calcitriol. For Ca^{2+}-entry at least some cells of the islets appear to express TRPV6, whereas TRPV5 is not expressed in the canine pancreas. For uptake into the endoplasmic reticulum, SERCA3 is most probably not an important factor in canine β-cells. It should be investigated whether other SERCA proteins can serve this function. For Ca^{2+}-extrusion, NCX1 and PMCA1 are expressed in canine pancreas and might be functional, but their cellular distribution could not be localized.

Detection of calbindin D-28k, VDR, TRPV6, SERCA3, and NCX1 in the canine kidneys and duodenum emphasizes the importance of these proteins for systemic calcium regulation. The antibodies used could prove useful in studying dysregulation of calcium homeostasis in the dog.

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