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LABORATORY INVESTIGATION

Distribution of dopamine- and cAMP-dependent phosphoprotein (DARPP-32) in the developing and mature kidney

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Distribution of dopamine- and cAMP-dependent phosphoprotein (DARPP-32) in the developing and the mature kidney. DARPP-32 is a dopamine- and cAMP-regulated inhibitor of protein phosphatase-1 (PP-1). Dopamine and DARPP-32 regulate sodium reabsorption in renal tubules by inhibiting the activity of Na^+,K^+-ATPase. We here report the pre- and postnatal distributions of DARPP-32 in the kidney as demonstrated by immunoblotting and immunohistochemistry. With immunoblotting we examined the abundance of DARPP-32 and the functionally similar but more widespread inhibitor of PP-1, inhibitor-1 (I-1). We compared their relative abundance in the renal cortex, renal medulla and neostriatum from the brain, where DARPP-32 is greatly enriched. DARPP-32 levels in the adult rat were fourfold higher in the neostriatum than in the renal medulla and 13-fold higher than in the renal cortex. I-1 levels were approximately the same in the neostriatum and in the renal medulla and 2.5-fold higher in neostriatum than in the renal cortex. Between postnatal day 10 (PN10) and 40 (PN40) DARPP-32 abundance increased 1.3-fold in the neostriatum, 1.4-fold in the renal cortex and sixfold in the medulla. The abundance of I-1 did not increase in the striatum from PN10 to PN40 but increased 1.5-fold in the renal cortex and threefold in the renal medulla. Thus, during the time of maturation of tubular transport function, the levels of both PP-1 inhibitors increased in the kidney, the largest increase being found in the renal medulla. With immunohistochemistry strong DARPP-32-like-immunoreactivity (DARPP-32-LI) was detected in the ureteral buds from gestational day 18 and up to postnatal day 8 when nephrogenesis was completed. No I-1-like immunoreactivity (I-1-LI) was found in the ureteral buds. From gestational day 21, DARPP-32-LI was identified in the proximal convoluted tubules. After postnatal day 8, DARPP-32-LI increased greatly in the medullary tubules of the thick ascending limb of Henle. These results suggest two separate roles for DARPP-32 in renal function. During tubulogenesis, DARPP-32 may participate in differentiation/proliferation. In the mature kidney, DARPP-32 participates in the regulation of sodium excretion.

Reversible protein phosphorylation is one of the major molecular mechanisms by which extracellular signals produce their biological response. Target phosphoproteins change their biological activity when phosphorylated by protein kinases and they return to the basal state when dephosphorylated by protein phosphatases [reviewed in 1]. The activity of the widespread protein phosphatase-1 (PP-1) is regulated by a few phosphoproteins, including DARPP-32 (dopamine- and cAMP-regulated phosphoprotein with a M_r of 32 kDa on SDS-PAGE) and the similar, but more widespread, inhibitor-1 (I-1). Both are potent inhibitors of PP-1 when phosphorylated by cAMP-dependent protein kinase [2, 3].

In renal tubules, dopamine, cAMP and phospho-DARPP-32 inhibit sodium reabsorption by inhibiting the activity of Na^+,K^+-ATPase [4–8]. We have previously shown that dopamine regulation of Na^+,K^+-ATPase activity undergoes postnatal changes [9, 10]. In this study we investigated, with Western blotting, the postnatal abundance of DARPP-32 and I-1 in the renal medulla and cortex and compared the findings with those in the neostriatum, where both inhibitors are present. The abundance of both I-1 and DARPP-32 increased postnatally in the kidney, the most substantial increase of DARPP-32 being seen in the renal medulla.

Since it has been suggested that neurotransmitters participate in differentiation/growth [11, 12] and can modulate gene transcription [13–15] we also studied the prenatal distribution of DARPP-32. With immunohistochemistry we show that DARPP-32 is present at an early stage of gestation and is specifically localized to differentiating and proliferating cells during early renal development.

Methods

Animals

Sprague-Dawley rats (ALAB, Sollentuna, Sweden) were used for all studies. Adult rats were fed with standard rat chow and water *ad libitum*. Pups were kept with their dams until 20 days of age. Rats were anesthetized with inactin (80 mg/kg i.p.; Byk-Gulden, Constanze, Germany).

Immunohistochemistry

The kidneys from fetuses (gestational age 18, $N = 3$, and 21, $N = 3$), neonatal litter (within 24 hr of birth; $N = 3$) and from rats at the age of 3, 5, and 8 days ($N = 3$) were removed and immediately fixed by immersion in an ice-cold formalin-picric acid mixture (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9) [16] for three to four hours. Rats at the age of 20, 40 and 80 days ($N = 3$) were retrogradely perfused via the descending aorta with Ca^{2+}-free Tyrodes solution, followed by ice-cold fixative (as above). Tissue was rinsed for at

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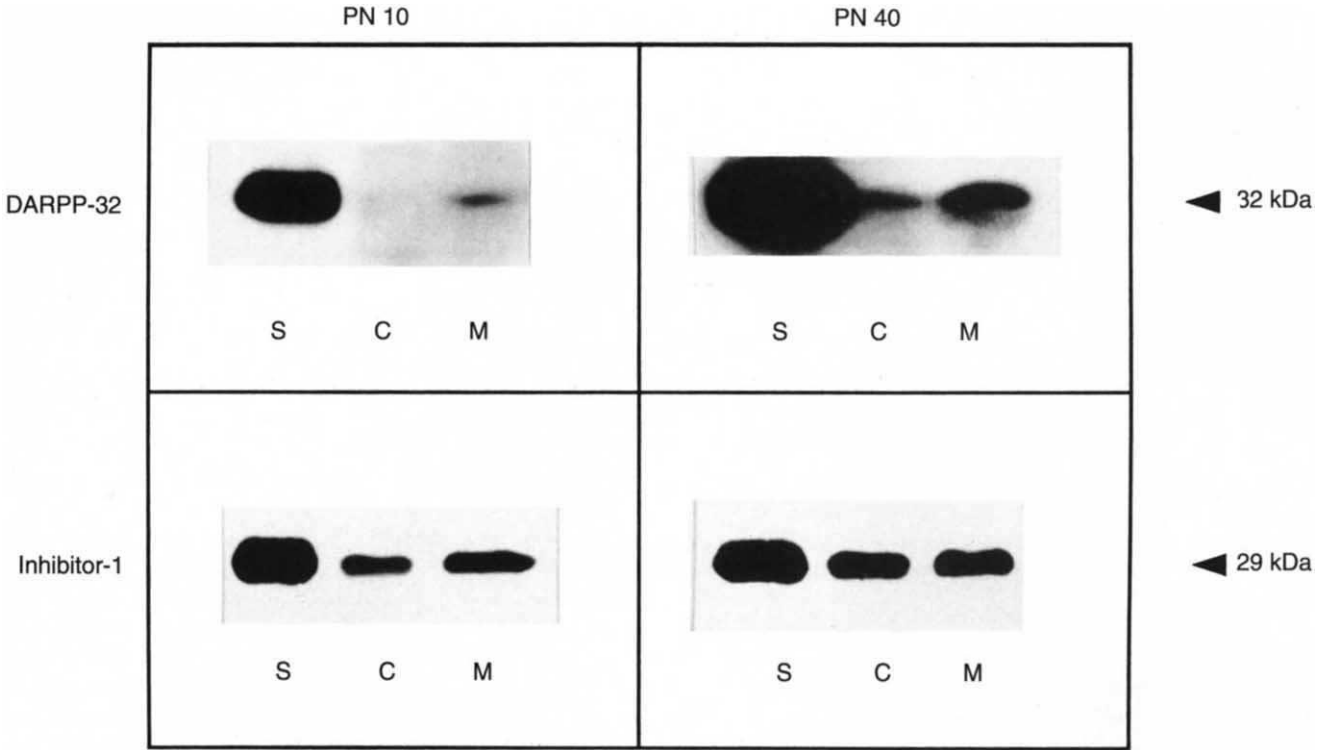

Fig. 1. *Abundance of DARPP-32 and I-1 in corpus striatum (S), renal cortex (C) and renal medulla (M) at postnatal ages of 10 (PN10) and 40 days (PN40).* Tissue samples of 100 μg were separated on SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with a DARPP-32 and an I-1 antibody, respectively. After incubation with a secondary antibody, proteins were detected with ECL, recorded on an autoradiography film and photographed. Tissues from the two ages were separated on the same gel and blotted on the same filter to enable comparisons. This necessitated an over-exposure of the striatal tissue to envision the much weaker signal from the kidney tissue. This photograph shows representative blots from six experiments.

least 24 hours in a 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% bacitracin (Sigma Chemical Co., St. Louis, Missouri, USA) and 0.01% sodium azide (Merck, Darmstadt, Germany). Sections were cut at 14 μm thickness in a cryostat (Dittes, Heidelberg, Germany) and processed for indirect immunofluorescence histochemistry [17]. Kidneys obtained from pre- and postnatal animals were processed for immunohistochemistry in the same experiment in order to eliminate variations in fluorescence intensity. Briefly, the sections were incubated with a mixture of three mouse monoclonal antibodies (C24a-4D7, C24a-5a, and C-24a-6a) raised against purified DARPP-32 (diluted 1:800) [18, 19] for 24 hours at 4°C, rinsed in phosphate-buffered saline (PBS), and incubated for 30 minutes at 37°C with fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse secondary antibodies (Amersham Ltd., Amersham, UK), and rinsed again in PBS. For control purpose, the DARPP-32 antibodies were preabsorbed with purified bovine brain DARPP-32. For detection of I-1, a rabbit polyclonal antibody, G187, diluted 1:200 was used as a primary antibody [20] and an FITC-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Stockholm, Sweden) was used as a secondary antibody. The sections were finally mounted in a mixture of glycerol and PBS (3:1) containing *p*-phenylenediamine to reduce the fading of immunofluorescence [21, 22], and examined in a Nikon Microphot-FX epifluorescence microscope equipped with filter combinations for FITC-induced fluorescence (450 to 490 nm excitation filter, 520 to 560 nm barrier filter and 520 to 550 nm extra barrier filter). Tri-X (Kodak, Rochester, New York, USA) black-and-white film was used for photography.

Immunoblotting

Kidney and brain were removed from rats at a postnatal age of 10 days (PN10) and 40 days (PN40). Neostriatum from the brain, renal medulla and renal cortex were dissected on ice, and stored at −80°C. Tissue was homogenized by sonication in 1 ml of boiling SDS 1% (BDH Chemicals Ltd., Poole, UK) and maintained in a boiling water bath for 10 minutes. Protein content was determined with Bio-Rad protein assay (Bio-Rad, Richmond, California, USA) using BSA (Boehringer Mannheim, Germany) as a standard. Sample buffer was added to a final concentration of SDS 1%, Tris-HCL 68 mM, glycerol 10%, β-mercaptoethanol 5%, trace amounts of Pyronin Y, and samples were boiled for two minutes.

Samples of equal amounts of protein (100 μg) were separated by SDS-polyacrylamide gel electrophoresis containing 12% polyacrylamide, and transferred to a nitrocellulose filter 0.45 μ (Schleicher and Schrell, Dassel, Germany). In each experiment samples were used for both I-1 and DARPP-32 detection. Samples from the two age groups were detected on the same filter to enable comparisons. Proteins were detected with immunoblotting as modified from Girault et al [23]. In brief, filters were rehydrated in PBS after transfer and incubated in blocking

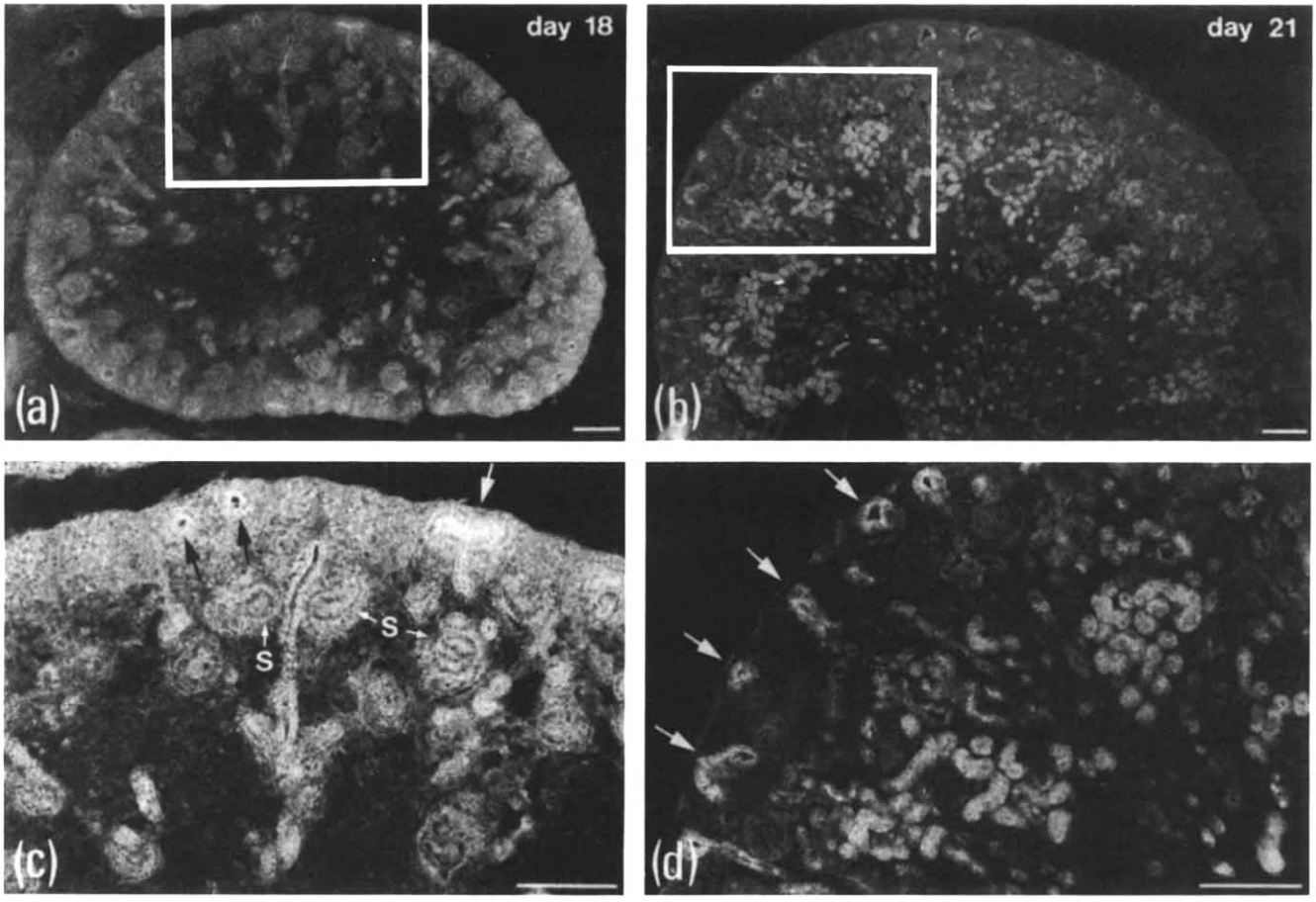

Fig. 2. *Immunofluorescence photomicrographs of sections of rat kidney on gestational days 18 (a,c) (G18) and 21 (G21) (b,d) after incubation with monoclonal antibodies to DARPP-32.* Rectangles in a and b represent higher magnifications seen in c and d. On G18, strong DARPP-32-LI is seen in the ureteral buds (see arrows in c). On prenatal day 21, DARPP-32-LI is found in ureteral buds (arrows in d) and in proximal tubule cells in the inner cortex (b, d). S = S-shaped bodies. Bars = 100 μm.

solution [5% dry milk (Semper, Stockholm, Sweden)] and 0.1% TWEEN (Merck-Schochardt, Hohenbrunn bei München, Germany) in PBS. After rinsing, filters were incubated with primary antibody. The DARPP-32 antibody 4D7 was used in a 1/1000 dilution. The I-1 antibody G 187 was a rabbit polyclonal antibody raised against purified I-1 [20] and used in a 1/500 dilution. Filters were then incubated with secondary antibody in PBS after thorough rinsing. The secondary antibody was a horseradish peroxidase (HRP)-linked anti-mouse or anti-rabbit antibody (Amersham Ltd.). All procedures were performed at room temperature and with agitation. Detection was performed with an ECL detection kit (Amersham Ltd.) and electrochemical luminescence was recorded on autoradiography film (Hyperfilm, Amersham Ltd.). Films were scanned with a densitometer (LKB Ultrascan XL laser densitometer) using LKB software. Integrated areas under the appropriate peaks were calculated using plain film density as a uniform background. Abundance was compared to that in the striatum from 40-day-old rats as an internal standard in each experiment. The values presented are median of six experiments.

All chemicals, unless otherwise stated, were from Sigma Chemical Co.

Results

Immunoblotting

The monoclonal DARPP-32 antibody detected a protein migrating at 32 kDa in the neostriatum, as previously described, and a protein of the same molecular weight in the renal cortex and medulla (Fig. 1). DARPP-32 levels in the adult rat were fourfold higher in the neostriatum than in the renal medulla and 13-fold higher than in the renal cortex. The I-1 antibody detected a protein with a M_r of 29 to 30 kDa. This corresponds well to the M_r previously reported for rat I-1 [20, 24]. The differences in abundance were much less pronounced for I-1 than for DARPP-32. I-1 levels were approximately the same in the neostriatum and in the renal medulla and 2.5-fold higher in the neostriatum than in the renal cortex.

In the neostriatum DARPP-32 abundance increased 1.3-fold from PN10 to PN40. In the renal tissue DARPP-32 was barely

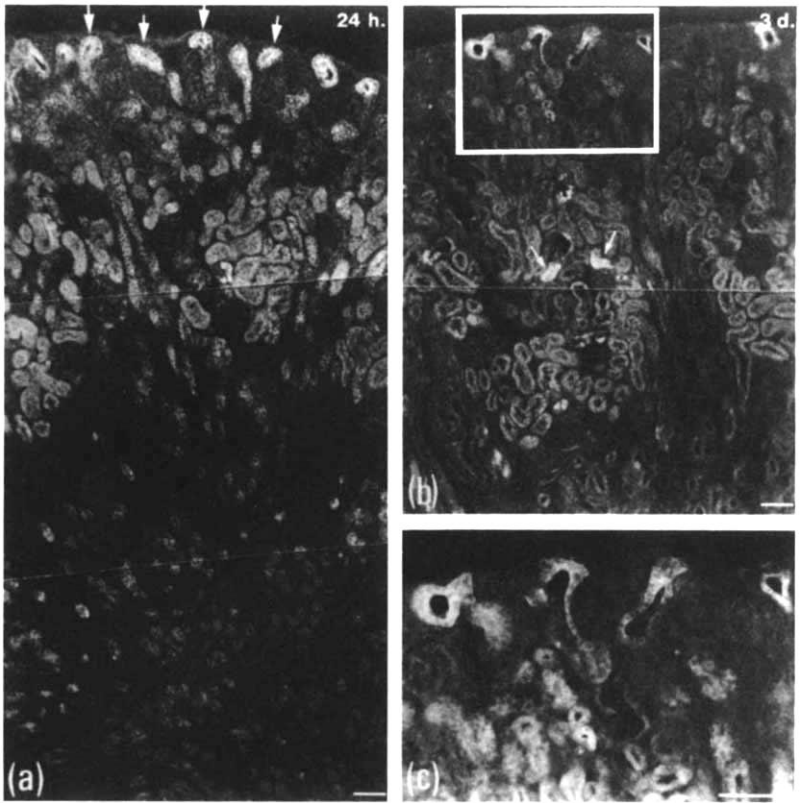

Fig. 3. *Immunofluorescence photomicrographs of sections of rat kidney within 24 hours of birth (a) and on postnatal day 3 (b,c) after incubation with monoclonal antibodies against DARPP-32.* Strong DARPP-32-LI is seen in the ureteral buds within 24 hours of birth (arrows in a) and on 3 days postnatally (b,c). Proximal tubule cells display DARPP-32-LI, some of them strongly (see arrows in b). Bars = 100 μm.

detectable at PN10 with immunoblotting. The levels increased 1.4-fold in the renal cortex and sixfold in the medulla. I-1 was present in substantial amounts already at PN10. The abundance of I-1 did not increase in the striatum from PN10 to PN40. In the renal cortex it increased 1.5-fold, and in the medulla it increased threefold.

Immunohistochemistry

On gestational age day 18 (G18) (Fig. 2 a and c) strong DARPP-32-LI was detected in the ureteral bud, and in the surrounding undifferentiated mesenchyme. No immunoreactivity was seen in the PCT.

On gestational day 21 (G21) (Fig. 2 b and d) strong DARPP-32-LI was localized to the ureteral buds (Fig. 2d). Most of the ureteral buds were located at the outer border of the cortex. In the inner cortex, approximately one-half of the proximal convoluted tubule (PCT) cells displayed DARPP-32-LI. Surrounding PCT showed weaker immunoreactivity.

Around the time of birth (PN1) and postnatal day 3 (PN3) (Fig. 3 a to c) the ureteral buds had uniformly reached the outer cortex. Strong DARPP-32-LI was localized to the ureteral buds. Weak immunoreactivity was found in all PCT cells in the cortex (Fig. 3a).

On postnatal day 8 (PN8) (Fig. 4 a and b), most nephrons had been induced and no ureteral buds were detected. Weak DARPP-32-LI was found throughout the cortex in PCT cells, and in the medulla in TAL cells.

On postnatal day 20 (PN20) (Fig. 5 a and b), postnatal day 40 (PN40) (Fig. 5c) and on day 80 (not shown), strong DARPP-32-LI was demonstrated in mTAL tubule cells. Weak immunoreactivity was localized to PCT cells. Glomeruli, blood vessels and inner medulla were devoid of immunoreactivity.

No immunoreactivity was demonstrated after the addition of purified DARPP-32 (1 μM) to the DARPP-32 antibodies (not shown).

I-1-like immunoreactivity (I-1-LI) was not found in the ureteral buds at PN1, but it was present in PCT cells (Fig. 6 a and b).

Discussion

In this study we have confirmed, by immunoblotting, the presence of DARPP-32 in the renal medulla and renal cortex. We furthermore compared the abundance of DARPP-32 and I-1 to the known abundance in neostriatal tissue and found that DARPP-32 was approximately fourfold more enriched in the striatum than in renal medulla and 13-fold more enriched than in the renal cortex. I-1 had a similar abundance in striatum and renal medulla but was less abundant in renal cortex, although the difference was much less pronounced than for DARPP-32. This is the first time phosphatase inhibitors have been quantitatively studied in the kidney. A general conclusion is that the renal medulla is more rich in these inhibitors than the cortex. Over a period of functional maturation of the kidney tubules, the abundance of both DARPP-32 and I-1 increased, and the most remarkable increase was seen in the renal medulla. We furthermore studied the pre- and postnatal distribution of DARPP-32 with immunohistochemistry and confirmed the postnatal localization to the PCT and the TAL demonstrated by

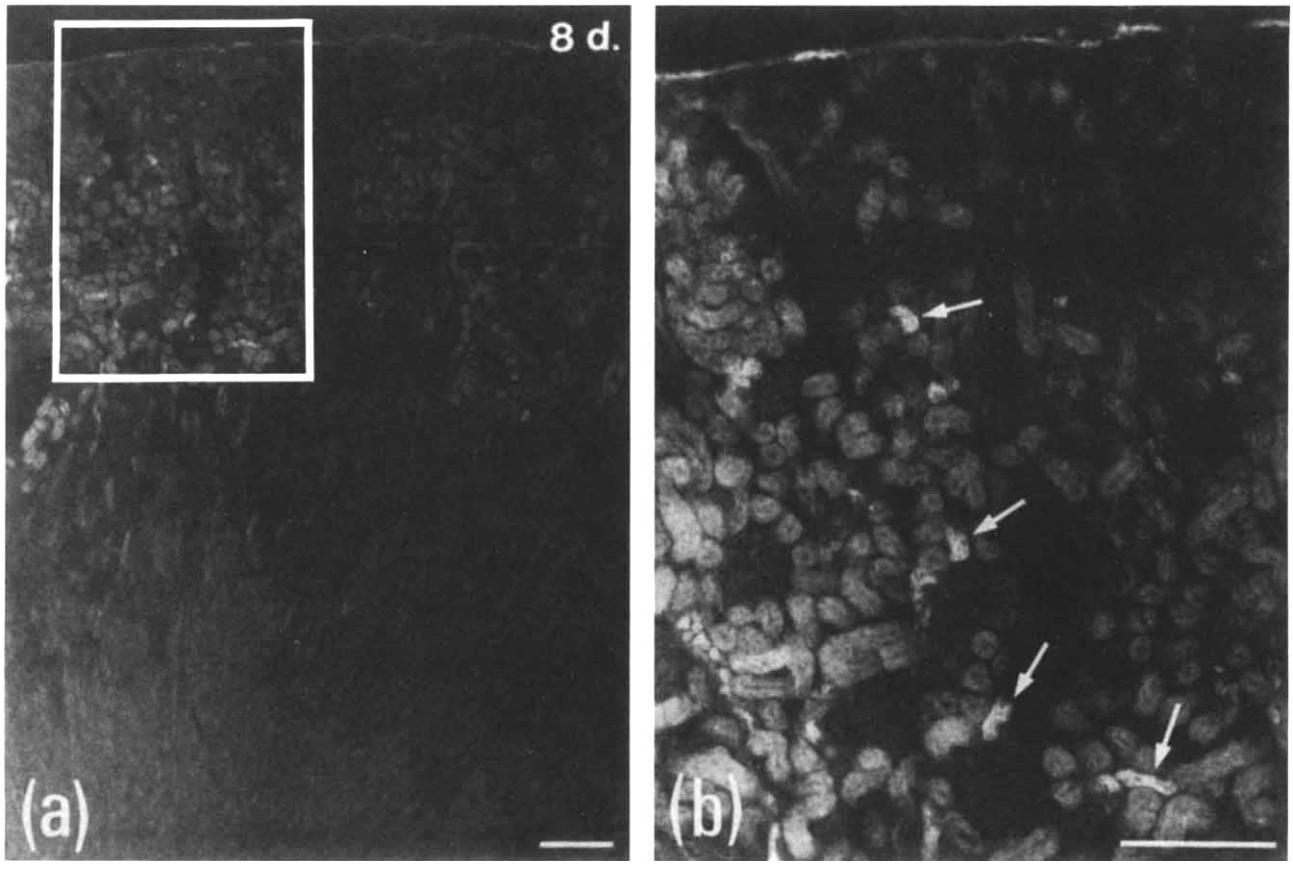

Fig. 4. *Immunofluorescence photomicrographs of sections of rat kidney on postnatal day 8 (PN8) (a,b) after incubation with monoclonal antibodies against DARPP-32.* Rectangle in a indicate higher magnifications seen in b. On day 8 DARPP-32-LI is present in tubular cells in the cortex and medulla. Bars = 100 μm.

immunoblotting. In addition, DARPP-32 was localized to the ureteral bud during the last half of nephrogenesis. This was not seen for I-1, indicating a specific role for dopamine/DARPP-32 in the ureteral bud.

DARPP-32 has a restricted distribution and is highly enriched in dopaminoceptive cells in the basal ganglia. It has been identified in a few peripheral tissues with DA_1 receptors [3]. I-1 is partially co-localized with DARPP-32, but it has been identified in high concentrations in several peripheral tissues [24–26]. DARPP-32 levels in medium spiny neurons in the neostriatum have been calculated at 20 to 50 μM [19]. The levels in renal tubular cells from this study can be extrapolated into the micromolar range. In the neostriatum I-1 levels have been estimated at more than 1 to 2 μM [20]. The concentration in renal tissues should be in the same range according to the present results. The calculated K_m for DARPP-32 (2.4 μM) and for I-1 (5.0 μM) for phosphorylation by cAMP-dependent protein kinase [27] is similar to the approximated concentrations of DARPP-32 and I-1, making these reactions kinetically possible in renal tubular cells. In an earlier study [19], DARPP-32 was not detected in the kidney using an RIA assay. However, the whole kidney was used in this study and considering the low content or absence of DARPP-32 in some kidney cells, the DARPP-32 content was probably diluted below the detection level in the RIA assay.

Both DARPP-32 and I-1 are transformed to potent inhibitors of PP-1 activity when phosphorylated by cAMP-dependent protein kinase [27]. In the renal mTAL cell, phospho-DARPP-32 inhibits Na^+,K^+-ATPase activity [8]. The proposed mechanism involves the DA_1 receptor, activation of adenyl cyclase, cAMP-dependent protein kinase, and phospho-DARPP-32 [4, 5, 9]. DARPP-32 is also phosphorylated by cGMP-dependent protein kinase [27], casein kinase [28] and is dephosphorylated by Ca^{2+}/calmodulin-regulated protein phosphatase 2B (calcineurin) [29]. Since Na^+,K^+-ATPase activity is regulated by several hormones in renal tubules cells, DARPP-32 is a possible regulatory site where different signals could converge [30].

DARPP-32 levels increase slightly in the neostriatum from PN10 to PN40, whereas I-1 levels do not. These results are in accordance with those of Ehrlich et al [31] and of Hemmings et al [24] who showed that there was a large increase in DARPP-32 protein and in mRNA levels mainly before two weeks of age, and that I-1 levels had almost reached adult levels at one week before birth in the neostriatum. It has been suggested that I-1 is, phylogenetically and ontogenetically, an earlier occurring phosphatase inhibitor and that DARPP-32 is more specialized in distribution and function [24]. In the renal cortex both DARPP-32 and I-1 increased slightly during development. In the renal medulla the increase was more pronounced for both

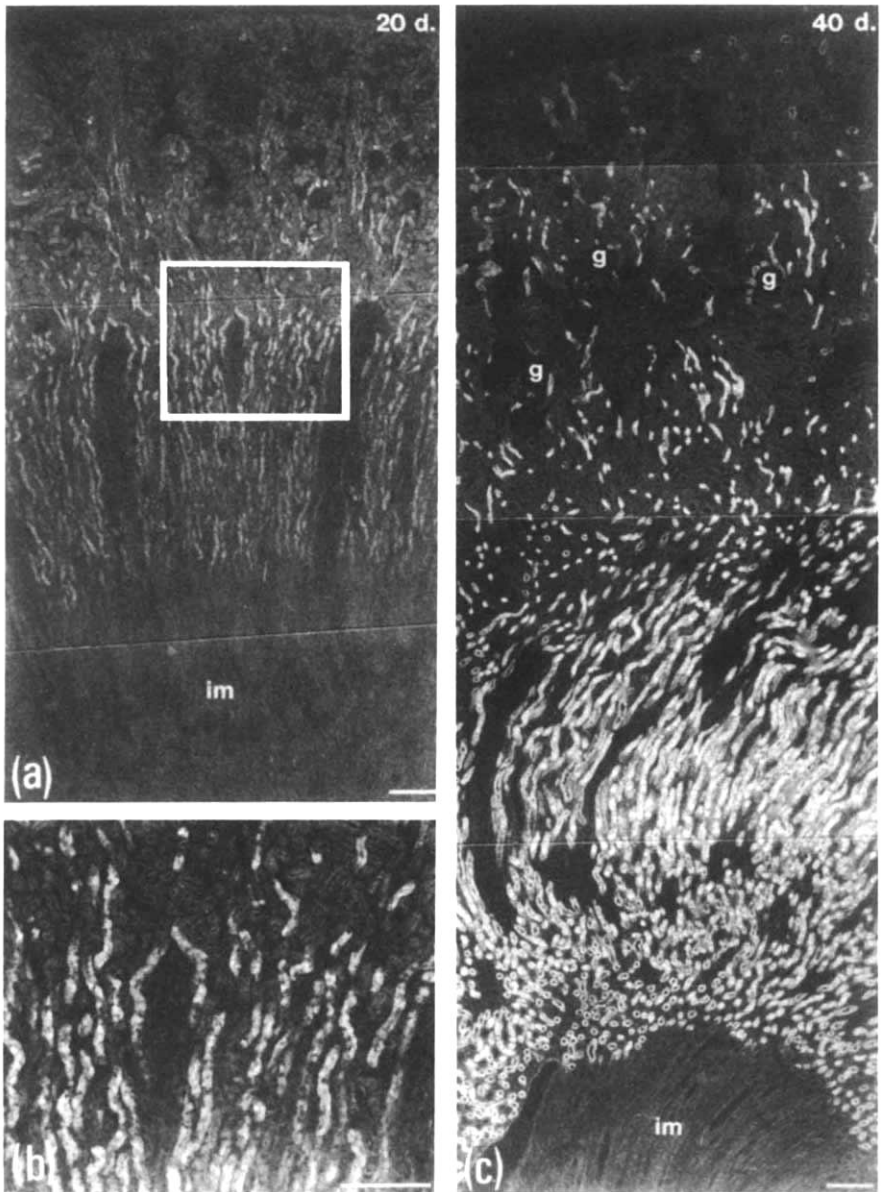

Fig. 5. *Immunofluorescence photomicrographs of sections of rat kidney on postnatal days 20 (a,b) and 40 (c) after incubation with monoclonal antibodies against DARPP-32.* Rectangle in (a) represents higher magnification seen in (b). On postnatal day 20 DARPP-32-LI can be seen in mTAL cells in the outer medulla (a, b). On day 40 strong DARPP-32-LI is distributed in mTAL cells in the outer medulla with medullary rays extending into the cortex. Weak DARPP-32-LI is present in the proximal tubules in the cortex at both ages. No DARPP-32-LI can be seen in the glomeruli (g) or inner medulla (im). Bars = 100 μm.

proteins, but particularly for DARPP-32. Despite the semiquantitative nature of these determinations, the relative abundances and the large increase in both I-1 and DARPP-32 in the renal medulla were clearly observed. Many tubular transport functions develop between PN10 and PN40 [32]. This study suggests that DARPP-32 postnatally is a marker for differentiated dopaminoceptive cells in the kidney. The role of the high concentration of DARPP-32 in adult mTAL remain to be clarified.

Immunohistochemistry demonstrated that DARPP-32-LI was found early during renal development and that it reached its final adult distribution at PN20. During the intermediate period the localization of DARPP-32-LI differed partly from that in the adult. Similar observations have been made in the brain [33]. In both brain and kidney, DARPP-32 may have a function differing from that in the adult during development. The nature of this proposed developmental role remains unclear. In the brain DARPP-32 appearance preceded tyrosine hydroxylase by approximately two days [33], indicating that the appearance of DARPP-32 was independent of dopaminergic input. This might not be the case in the kidney since here the appearance of DARPP-32 coincides with the appearance of AADC in the proximal tubules, as reported in previous studies from our laboratory [34]. Dopamine content has been studied from postnatal age of three days and was seen to increase to day 20 [34]. A tentative conclusion could be that at this time, preceding birth by a few days, the dopamine system starts to play a role in the kidney. The low content of DARPP-32 in the PCT at PN10 could possibly explain the previously reported impaired dopamine inhibition of Na^+,K^+-ATPase activity in this segment at this time [9, 10].

During early nephrogenesis the ureteral bud (seen at arrows

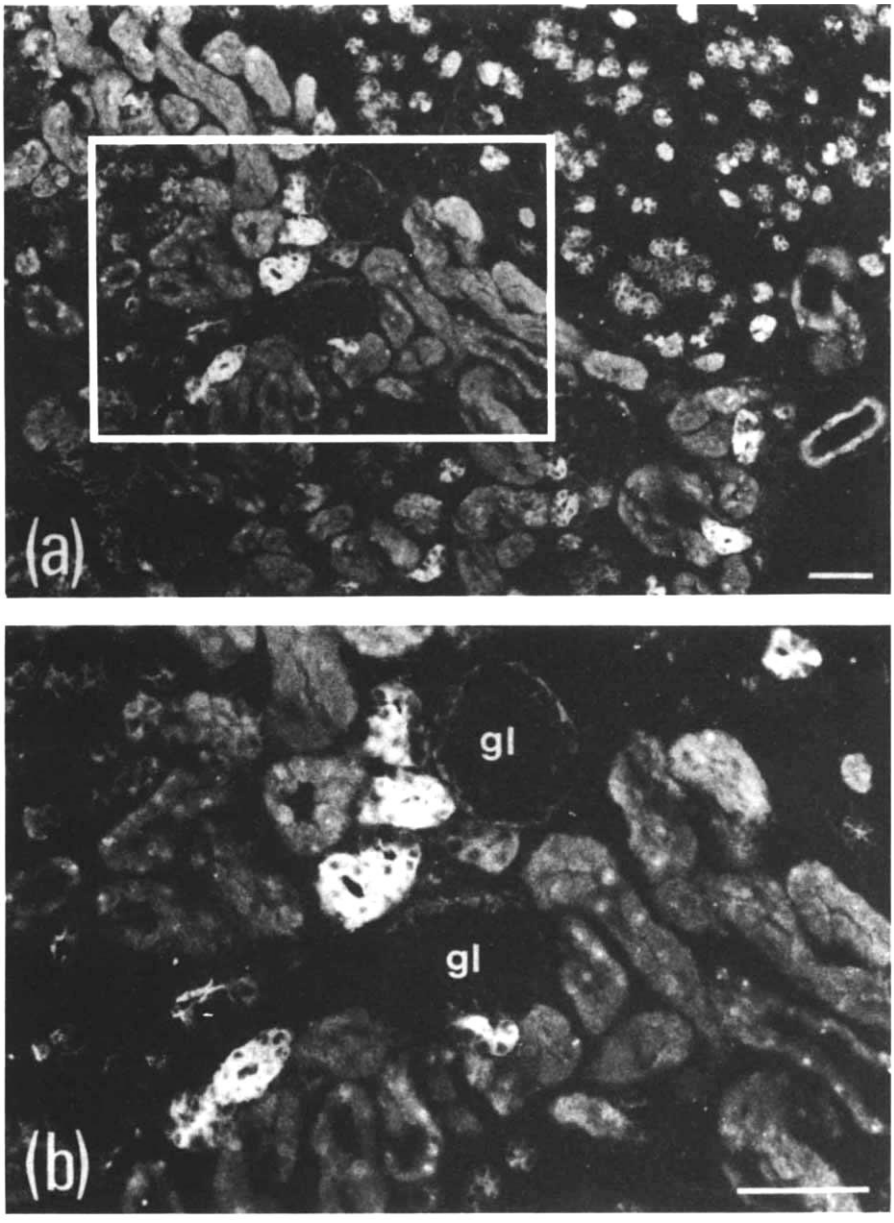

Fig. 6. *Immunofluorescence photomicrographs of sections of rat kidney within 24 hours of birth after incubation with rabbit polyclonal antiserum to inhibitor-1.* No immunoreactivity is found in ureteral buds. Immunoreactivity is found in some tubule cells in the inner part of the cortex. The majority of all glomeruli are not immunoreactive. **(b)** Represents higher magnification as indicated by rectangle in **(a)**. Bars = 100 μm.

in Figs. 2 a, b, and 3a) grows into a cortical layer of undifferentiated mesenchymal cells. These are induced to proliferate and differentiate into the first tubular structure, the S-shaped body (S in Fig. 2a), which further develops into the glomerular and tubular epithelia. The ureteral bud divides and gives rise to new nephrons in a centrifugal pattern all through tubulogenesis from prenatal day 11 to postnatal days 7 to 8 in the rat kidney [35]. The specific localization of DARPP-32-LI in the ureteral bud and the developing structures suggest that DARPP-32 could be involved in the process of induction and/or early differentiation. Interestingly, I-1 was not localized to the ureteral bud, indicating a specific role for the dopamine/DARPP-32 system during nephrogenesis. Dopamine has been shown to influence the developmental state of the cells in the central nervous system [12, 11, 36], possibly by modulating gene transcription [13–15]. The role of dopamine for induction/differentiation in the mammalian kidney is an interesting subject for further studies.

Acknowledgments

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