The adrenal: A new target organ of the calcitropic hormone 1,25-dihydroxyvitamin D$_3$

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Summary. Target cells for 1,25-dihydroxyvitamin D$_3$ were demonstrated in the adrenal medulla by frozen-section autoradiography. The appearance of these target cells was age-dependent in neonatal mice. Immunocytochemical staining for phenylethanolamine-N-methyltransferase revealed that both epinephrine and non-epinephrine cells concentrate 1,25-dihydroxyvitamin D$_3$ in their nuclei. In contrast, immunocytochemical staining for “vitamin D-dependent calcium-binding protein” (D-CaBP) demonstrated that D-CaBP immunoreactivity is localized in only a small percentage of adrenomedullary cells, in mice and rats. Comparison of PNMT and D-CaBP immunoreactivities in sequential sections showed that epinephrine-producing cells do not contain D-CaBP. These results indicate that adrenal medullary cells have receptors for 1,25-dihydroxyvitamin D$_3$ and that 1,25-dihydroxyvitamin D$_3$ may directly affect certain functions of these endocrine cells.

Key words: 1,25-dihydroxyvitamin D$_3$ – Adrenal medulla – Ontogenetic development – Phenylethanolamine-N-methyltransferase – Vitamin D-dependent calcium-binding protein – Mouse, rat

1,25-dihydroxyvitamin D$_3$ is a hormone well known for its role in calcium homeostasis. In recent years, however, it has become apparent that 1,25-dihydroxyvitamin D$_3$ is a hormone with many diverse target cells and tissues (Stumpf et al. 1979, 1982; Norman et al. 1982), some of which have no apparent role directly related to the calcitropic effects of this hormone. 1,25-dihydroxyvitamin D$_3$ has been shown to influence such diverse functions as insulin secretion (Clark et al. 1981; Ishida et al. 1983), and differentiation of skin (Hosomi et al. 1983) and leukemia cells (Abe et al. 1981). The presence of both receptors for 1,25-dihydroxyvitamin D$_3$ and vitamin D-dependent calcium-binding protein (D-CaBP) has been been demonstrated, not only in the intestine, but also other target organs such as skin (Stumpf et al. 1979, 1984; Simpson and DeLuca 1980; Laouari et al. 1980), pancreatic islets (Clark et al. 1980; Roth et al. 1982; Sonnenberg et al. 1984), and kidney (Stumpf et al. 1980; Simpson et al. 1980; Narbaitz et al. 1981; Rhoten and Christakos 1981; Kendrick et al. 1984). In these tissues, D-CaBP is thought to be a molecular marker of 1,25-dihydroxyvitamin D$_3$ action. Homogenates of the chicken adrenal gland have been found to contain low levels of D-CaBP immunoreactivity and the immunoreactive material exhibits vitamin D-dependence (Christakos et al. 1979), but it is not known whether D-CaBP immunoreactivity is present in adrenocortical or adrenomedullary cells. Interrelationships between 1,25-dihydroxyvitamin D$_3$ and either the medulla or the cortex are conceivable since adrenocortical hormones and 1,25-dihydroxyvitamin D$_3$ interact at several target sites (Manolagas et al. 1979; Mascallo et al. 1982), and adrenal medullary hormones influence mineral homeostasis (Body et al. 1983). In the present studies 1,25-dihydroxyvitamin D$_3$ target cells were demonstrated by an autoradiographic technique, which has been used to show nuclear uptake of steroids (Stumpf and Sar 1975). The identity of these target cells was elucidated by immunocytochemical staining for phenylethanolamine-N-methyltransferase (PNMT) and for D-CaBP.

Materials and methods

Chemicals and antisera

$^3$H-1,25-dihydroxyvitamin D$_3$ with a specific activity of 160 Ci/mmol was synthesized (Napoli et al. 1980) and purified with high performance liquid chromatography. Photographic emulsion (NTB-3) and other photographic chemicals were from Kodak. Diaminobenzidine and hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). Antiserum to PNMT was produced in rabbits and characterized as previously described (Park et al. 1982; Teitelman et al. 1979). Chick intestinal D-CaBP was purified (Bishop et al. 1983) and antibody to the purified D-CaBP was raised in rabbits. Sheep-antirabbit serum (P4) was obtained from Antibodies, Inc. (Davis, CA) and peroxidase-antiperoxidase complex was from Pel Freez (Rogers, AR).

Animals

The mice were born of mothers that had been fed a vitamin D-deficient diet (Suda et al. 1970) from weaning through mating, gestation and lactation. Suckling mice used

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for autoradiographic experiments were one to two, ten, fifteen, and twenty-two days of age (n = 3 per age). The tissues taken for immunocytochemical studies of D-CaBP were collected from mature rats and mice which were fed vitamin D replete diets (n = 10 animals).

**Autoradiographic experiments**

$^3$H-1,25-dihydroxyvitamin D$_3$ was dissolved in 70% ethanol-saline and intraperitoneal injections were given to mice in doses of 40 μCi/100 g body weight (100 ng/100 g). In newborn mice backflow at the injection site was prevented by placing petroleum jelly on the injection site. Two and one half hours after injection of $^3$H-1,25-dihydroxyvitamin D$_3$ the mouse pups were killed, adrenals and kidneys dissected, and a blood sample collected. The tissue samples were frozen onto tissue holders, and 4 μm frozen sections thaw-mounted onto slides coated with photographic emulsion (Stumpf and Sar 1975). The autoradiograms were exposed one to twelve months at -20 °C, then photographically processed and stained immunocytochemically or with methyl green-pyronin. The kidneys served as positive controls for the autoradiographic studies, since we have previously characterized the specific uptake of $^3$H-1,25-dihydroxyvitamin D$_3$ by the kidney (Stumpf et al. 1979, 1980; Narbaitz et al. 1981). Radioactivity levels in the plasma of suckling mice were 0.35 ± 0.08 μCi/ml, which represents 2–3 pmol/ml if all of the radioactivity is unaltered hormone. A nuclear to cytoplasmic ratio of silver grains ≥ 3:1 was required for a cell to be considered to have “nuclear concentration”. When tissue silver grain levels are low we have found this approach to give results which are similar to the Poisson statistic (p, 0.05).

**Immunocytochemical studies**

Autoradiograms used for immunohistochemistry were fixed in 4% paraformaldehyde for 2–3 min before photographic processing. Tissue samples used for immunohistochemistry were fixed for 24 h in 4% paraformaldehyde, embedded in paraffin and 6 μm sections prepared. The antiserum for immunostaining was diluted 1:1500 (D-CaBP) and 1:2000 (PNMT). Specificity controls for D-CaBP included omission of the primary anti-serum and precipitation of anti-D-CaBP with purified D-CaBP. Working strength antisera precipitated with purified D-CaBP at concentrations of 0.1 μg/ml yielded reduced immunostaining, while antisera precipitated with D-CaBP at concentrations > 1.0 μg/ml resulted in no detectable immunoreactivity in the tissues studied.

**Results**

In the present study nuclear concentration and retention of radioactivity was found in cortical tubules of mouse kidney at all ages. Similar concentration of $^3$H-1,25-dihydroxyvitamin D$_3$ was noted in autoradiograms of adrenal taken from the same animal and mounted on the same slides as the kidney. Nuclear concentration of $^3$H-1,25-dihydroxyvitamin D$_3$ was first seen in a few cells of the adrenal from one-day-old mice (Fig. 1A). Cells with labeled nuclei were predominantly found in the forming adrenal medulla; occasionally, labeled cells were also found in the adjacent cortical region. At this age catecholamine cells are still migrating through the cortex to the medulla and labeled cells in the cortex may represent migrating cells. In the adrenal of ten-day-old mice the medullary cells are centrally located (Miller 1926; Millar and Unsicker 1981) and in autoradiograms at ten, fifteen and twenty-two days only cells of the adrenal medulla showed nuclear concentration of $^3$H-1,25-dihydroxyvitamin D$_3$ (Fig. 1B, C). Most, but not all adrenal medullary cells had a significant concentration of radioactivity in their nuclei. Autoradiograms of the mouse adrenal were immunostained with an antibody to PNMT to identify the epinephrine containing cells. The PNMT-immunoreactive (iPNMT) cells showed a nuclear concentration of $^3$H-1,25-dihydroxyvitamin D$_3$. In addition, many of the non-immunoreactive cells also concentrated $^3$H-1,25-dihydroxyvitamin D$_3$ in their nuclei (Fig. 1C). A percentage of both iPNMT cells (25%) and non-iPNMT cells (30%) had no significant accumulation of radioactivity (n = 290 iPNMT cells, and n = 60 non-iPNMT cells). The nuclear uptake and retention of $^3$H-1,25-dihydroxyvitamin D$_3$ by cells of the

![Fig. 1 A–C. Autoradiograms of mouse adrenal 2½ h after injection of $^3$H-1,25-dihydroxyvitamin D$_3$. Adrenal from a one-day-old mouse (A) shows nuclear concentration of radioactivity in a few cells (arrowheads). Adrenal from a ten-day-old mouse (B) shows nuclear concentration of radioactivity in cells of the medulla (M), but not the cortex (C). At this age catecholamine cells have assumed their central position. Broken line approximates limits of medulla in A and B. Immunoreactive phenylethanolamine-N-methyl-transferase (iPNMT) is revealed in cells of the medulla from a fifteen-day-old mouse (C). Cells which contain iPNMT (p) show nuclear concentration of radioactivity in cells of the medulla (M), but not the cortex (C). This age catecholamine cells have assumed their central position.](image-url)