Vitamin D receptors in heart: Effects on atrial natriuretic factor

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Abstract. We report that receptors for vitamin D exist in distinct regions of the heart in female and male mice, predominantly in the right atrium where most of the cardial atrial natriuretic peptide (ANF) is produced. Tritiated 1,25-dihydroxyvitamin D_3 (1,25-D_3, vitamin D, soltriol) and ANF are colocalized in nuclei and cytoplasm respectively in identical cardiomyocytes. Changes of ANF tissue and blood levels under dietary deficiency and treatment with 1,25-D_3 suggest direct genomic actions of vitamin D on myoendocrine cells of the atrium for the regulation of ANF manufacture and secretion. These results were obtained by combining thaw-mount autoradiography with immunocytochemistry using tritiated 1,25-D_3 and an antibody against rat ANF. This antibody was also used in a radioimmunoassay to determine atrial natriuretic factor in plasma, atria and ventricles of normal or vitamin D-deficient mice.
Key words. Vitamin D receptor; heart; atrial natriuretic factor; mouse; autoradiography; soltriol.

Maintaining Ca^{2+}-homoeostasis is the best known function of vitamin D in vertebrates including man[1,2]. During the last decade new target tissues and functions were discovered[3] which are not obviously related to calcium metabolism but indicate a primary role in the regulation of endocrine glands, nervous system, immune system, reproductive organs, and cardiovascular system[3-10]. Since both, the steroid hormone 1,25-D_3 and the peptide hormone ANF are regulators of ion homoeostasis we investigated the interaction of the two hormones in the heart, where most of the ANF is produced[11].

Materials and methods

Animals and histochemical procedures: Female (n = 4) and male (n = 3) C 57 BL 6J mice, 10 weeks old, were fed a normal diet and kept under fluorescent light (14 h light/10 h dark). Animals were s.c. injected with 0.4 µg/100 g b.wt [26,27-^{3}H]-1,25-D_3 (spec. act. 160 Ci/mmol; Dupont Boston) dissolved in physiological saline/14% ethanol. As competition controls two animals, one female and one male, were injected with 4 µg 1,25-D_3 (gift from Hoffmann La-Roche) 30 min prior to the injection of ^{3}H-1,25-D_3. Four hours post injection of ^{3}H-1,25-D_3 the animals were anesthetized and hearts prepared for thaw-mount autoradiography by thaw-mounting 4-µm frozen sections onto nuclear emulsion (NTB 3, Eastman Kodak, Rochester) coated slides[12,13]. After 7-month exposure the sections were photographically processed and stained with methylgreen-pyronin[13] or fixed in 4% paraformaldehyde (40 s) rinsed in PBS-1 (0.1 M: pH 7.0), rinsed in PBS, developed in Kodak D 19 (diluted 1:1 with PBS-1; 1 min) and photographically fixed in 15% (w/v) sodium thiosulfate (1 min) and rinsed with PBS-2 (0.1 M; pH 7.6). Endogenous peroxidase was inhibited by an incubation in PBS-2 containing 3% H_2O_2 (5 min). After several rinses in PBS, autoradiograms were incubated for 30 min in 5% normal sheep serum in PBS-2 containing 0.2% Triton-X-100 followed by an incubation with rabbit-antibody against rat-ANF[24] [diluted 1:1500 with PBS-2] or with normal rabbit serum (control) for 14 h at 8 °C. Sections were incubated for 2 h at room temperature with a second peroxidase-coupled antirabbit antibody (#3212-0231, Organon Teknika, West Chester, PA) diluted 1:300 with PBS-2 and stained with 75 mg 3'3'-diaminobezidine in 100 ml Tris-HCl buffer (0.05 M; pH 7.6) containing 2.3 µl H_2O_2 for 6 min. After several rinses in Tris-HCl buffer autoradiograms were air-dried and counterstained with 0.05% (w/v) methylgreen in distilled water (5–10 s), rinsed in water, air-dried and coverslipped with Entellan (Hoechst).

Animals and ANF determinations: Five-week-old male C57 BL 6J mice (Jackson Laboratories) were divided into five groups. One group (18 males) was reared under normal diet and light conditions for 9 weeks. Four groups (10 males each) were kept under UVB-free light and on a vitamin D-free diet (ICN Biochemicals, Cleveland, OH) for 9 weeks. Then all groups received daily injections (5 µl/g b.wt) s.c. for five days of isotonic saline containing 14% ethanol. Three of the vitamin D-deficient groups received with their solvent injection: a) 0.1; b) 20.8; c) 4160 fg 1,25-D_3/g b.wt, respectively.

Four hours after the last injection, animals were injected with 1 unit/g b.wt heparin (Elkins-Sinn, INC, Cherry Hill, NJ) and 20 min later decapitated. Blood samples of 2 animals were pooled and collected in chilled Eppendorf tubes containing per ml 1 mg EDTA (Sigma), 34.3 ng Pepstatin A (Sigma) and 1.44 µg Phenylmethylsulfonyl Fluoride (Sigma). Tubes were shaked vigorously and centrifuged at 4 °C and 3000 × g for 10 min. Plasma was decanted and the samples were frozen in liquid nitrogen and stored at − 80 °C. Ventricle and left and right atria were dissected immediately after decapitation and each single tissue was frozen in liquid nitrogen and stored at − 80 °C. ANF was extracted from the tissues and determined for each tissue by radioimmunoassy (RIA) using ANF antibody (#17/12) according to Gutkowska et

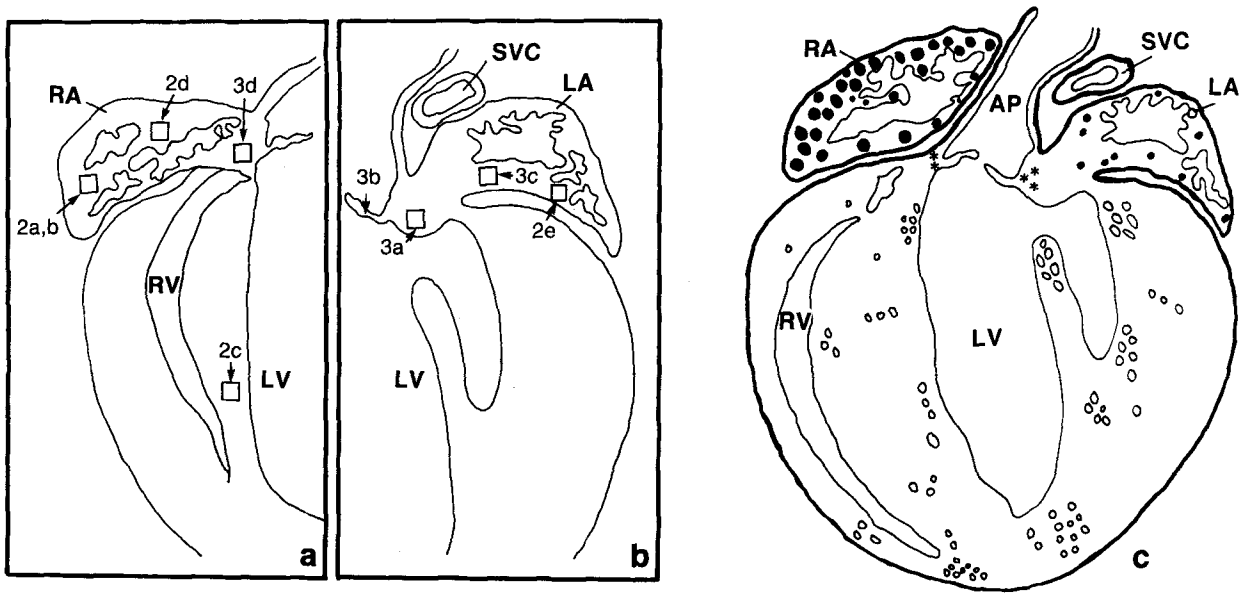


Figure 1. Schematic of right (*a*) and left (*b*) atrium and ventricle showing the regions from which figures 2a–e, 3a–d are taken. (*c*) shows a schematic of a horizontal section through the atria and ventricles summerizing the results on the topographical location of the specific nuclear binding of [26, 27-^{3}H]-1,25-D_3 and atrial natriuretic factor (ANF). Labelled ANF-positive cardiomyocytes small dot, 3–5% 1,25-D_3; big dot, > 90% of 1,25-D_3. Star, ANF-negative cells with nuclear receptors sites for 1,25-D_3. Circle, ANF-positive cardiomyocytes of the conductive system in the ventricles without 1,25-D_3 binding. Aorta pulmonalis, AP; atrium right RA, left LA; ventricle right RV, left LV; left superior vena cava, SVC.

al.[14,15]. From each plasma sample different dilutions ranging from 1:1 up to 1:10 were prepared to show the reliability of the assay and assayed directly by RIA[14,15].

Results and discussion

After exposure of the autoradiograms and photographic processing only or in combination with immunocytochemistry, high affinity binding sites (receptors) for 1,25-D_3 are visible as concentrations of silver grains over target cell nuclei (figs 2 and 3). No nuclear concentration of radioactivity in the nuclei of target cells is seen in autoradiograms prepared from mice injected with unlabelled 1,25-D_3 prior to the tracer-injection showing specificity of the identified binding sites (fig. 2b).

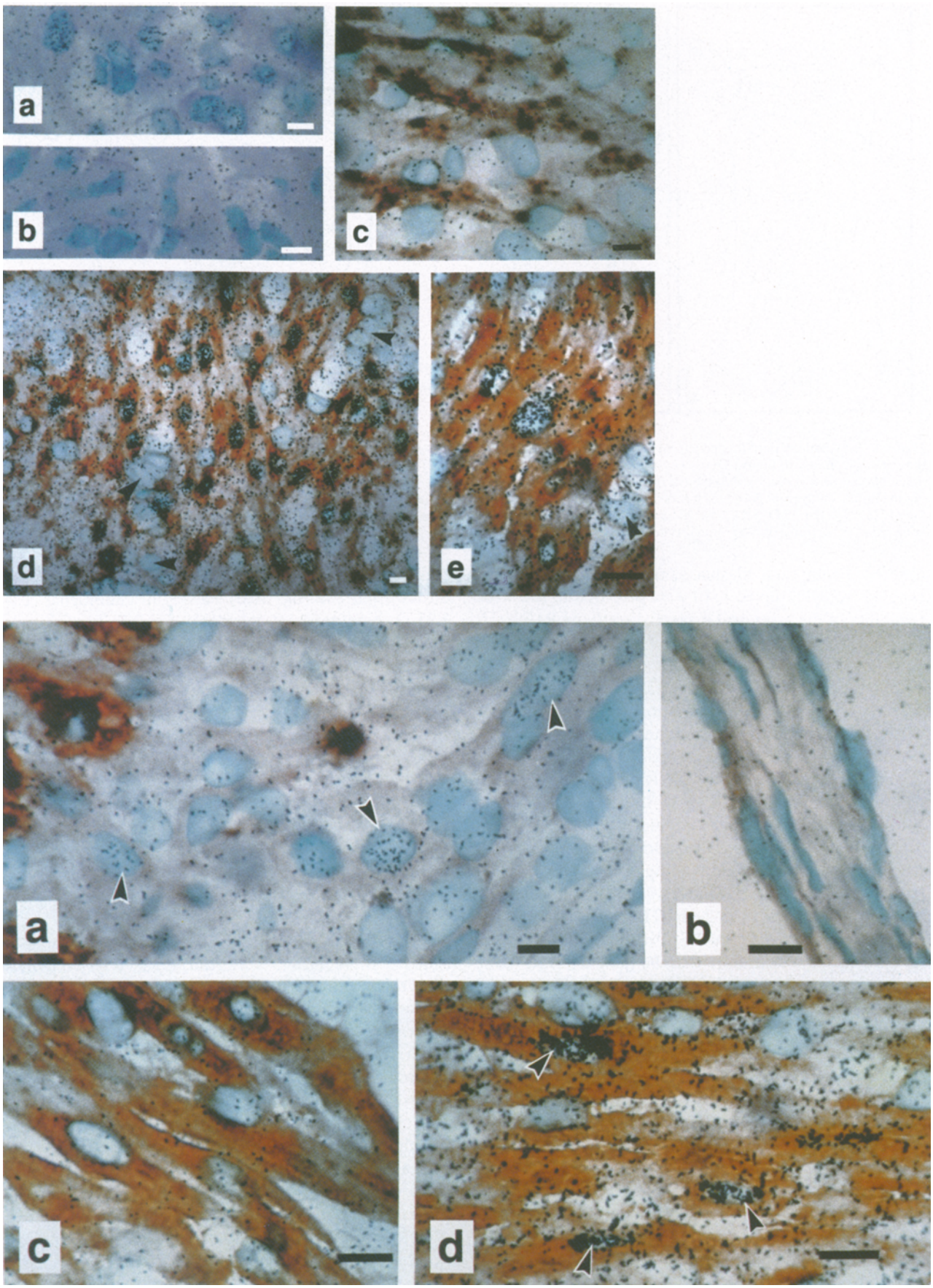
Nuclear receptors for ^{3}H-1,25-D_3 are found throughout the right atrium (figs 1b,c; 2a,b). The highest amounts of receptors are present in nuclei of cardiomyocytes in the right auricle in which more than 90% of the ANF-containing cells are labelled (figs 2d; 1b,c). The intensity of nuclear labelling declines toward the medial part of the right atrium, where approximately 55–60% of the muscle cells contain nuclear receptors for 1,25-D_3. Approximately 3–5% of the cells are labelled in the left auricle and atrium. In most regions of the left atrium the ^{3}H-1,25-D_3 labelled cardiomyocytes form small clusters of 2–4 cells with high amounts of nuclear 1,25-D_3 binding (figs 2e; 1a,c). The number of 1,25-D_3 target cells is lowest near the atrial septum in both atria. Some cells at the base of the valves, probably fibroblasts show nuclear 1,25-D_3 receptors but these cells as well as the cells of the valves do not contain ANF immunoreactive material (figs 3a,b; 1a,c). Cardiomyocytes near the valves are ANF positive but show nuclear 1,25-D_3-receptors only on the right site (figs 3c,d; 1a–c). In endothelial cells 1,25-D_3 binding is not detectable (fig. 2d). In both ventricles, nuclear receptors are generally absent with the exception of a few scattered cells that concentrate low amounts of radioactivity in their nuclei. While all cells with nuclear receptors for 1,25-D_3 in the right and left atrium are colocalized with ANF, no colocalization is seen in the ventricles (figs 2c, 1c). No differences in the distribution and labelling intensity of nuclear ^{3}H-1,25-D_3 binding and immunostaining of ANF are observed between male and female mice.

The results from these histochemical studies suggest that receptors for 1,25-D_3 in ANF-producing atrial cardiomyocytes are involved in the regulation of the secretory activity of these cells.

To evaluate this possible action of 1,25-D_3, we kept 40 male C57BLJ6 mice (5 weeks old) for 68 days on a vitamin D deficient diet in a container free of UVB-irradiation and 18 males on a regular diet and under normal fluorescent light.

The vitamin D-deficient mice were divided into four groups of 10 each. Three groups were injected s.c. daily for five days with increasing amounts of 1,25-D_3. The control animals received a daily solvent injection. From all animals we determined the ANF-content of plasma, right and left atria and ventricles.

These data reveal that the amount of ANF in the right atrium of vitamin D-deficient animals is significantly lower compared to controls and that 1.25-D_3 treatment at a concentration of 4.16 pg/g b.wt reduces the ANF content even further (fig. 4). The latter of which suggests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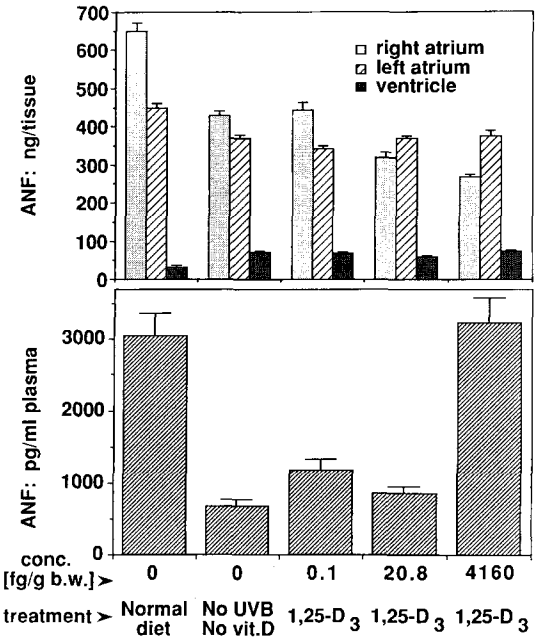

Figure 4. Changes in ANF content (+/− SEM) of the heart, in ventricles (n = 10), left atrium (n = 10) and right atrium (n = 10), expressed as total ANF per one ventricle or one atrium under normal, vitamin D deficient conditions, and after 1.25-D_3 injections at various concentrations (upper panel). Changes in plasma ANF (n = 5, normal control n = 9) under the same treatment conditions (lower panel). Note the drop in the ANF content of the right atrium and the increase in plasma ANF titer after treatment with 4160 fg/g b.wt in the vitamin D deficient mice. ANF plasma levels from normal reared control animals are not significantly different (Student t-test alpha-value 0.05, SAS, Statistical analysis system, Institute, Inc., Cary, NC) from all other groups.

that 1,25-D_3 influences the release of ANF even in vitamin D deficient mice. Plasma ANF levels are also decreased under vitamin D deficiency. Treatment with 1.25-D_3 (4.16 pg/g b.wt, fig. 4) increases ANF plasma levels significantly compared to vitamin D-deficiency. ANF plasma levels of animals reared under normal conditions are not significantly different from the other groups (fig. 4). Animals reared under normal conditions show high individual variations in plasma ANF which are not observed in animals kept under vitamin D deficiency. Similar to our study high individual variations of plasma ANF titers determined with the same method are also described for normal reared mice by Steinhelper et al.[16]. These individual variations may be due to the influence of other regulatory factors such as sex and adrenal steroids[17–19], neurotransmitters or circadian rhythms but may be diminished under vitamin D deficiency.

This decrease in ANF under vitamin D deficiency shows that the absence of UVB irradiation and vitamin D deficiency influences both the atrial ANF titer and the plasma ANF titer. The decline in the atrial ANF titer and the increase in plasma ANF titer after 1,25-D_3 application indicates that 1,25-D_3 stimulates ANF release in order to restore the normal ANF titer in the plasma. These observations are consistent with results obtained with vitamin D action on TSH secretion[20,21] and the localization of ^{3}H-1,25-D_3 in TSH producing pituicytes[9] as well as with the localization of vitamin D target cells in the pancreas in which 1,25-D_3 alters insulin secretion[22,23].

The obtained data indicate that the high amounts of nuclear receptors for 1,25-D_3 in the right atrium, where most of the ANF is produced[11,14], are involved in a regulation of ANF turnover, especially release. The regional and selective distribution of 1,25-D_3 in the atria as well as absence or very low levels in only a few cells in the ventricles, suggest that the steroid hormone is not involved directly in the intramuscular calcium regulation, as one might assume.

Vitamin D-deficiency causes hypertension in rats, and hypertension is cured and a relaxation of the heart muscle achieved by vitamin D administration[24]. It has been proposed that vitamin D causes relaxation by changing intramuscular calcium levels[24]. Our data suggest that 1,25-D_3 acts via the regulation of ANF metabolism and release. The latter is supported by the finding that ANF relaxes the heart muscle and causes hypotension by cardiac inhibition in rats[25] and coronary dilatation in dogs[26]. Since nuclear binding of 1,25-D_3 exists in the tunica media of the aorta pulmonalis (not shown) and a relaxation of blood vessels is described for ANF[27] a synergistic action of both hormones is possible.

A further combination of a general calcium homoeostatic regulation of 1,25-D_3 with ANF effects is also likely, since ANF is functioning as a regulator of the physiological balance of sodium and other ions including calcium[28]. The peptide hormone ANF and the steroid hormone 1,25-D_3 have at least three target organs in common that are involved in osmoregulation and regulation of blood pressure. These include adrenals, kidneys, and heart[3,4,11].

Additionally, vitamin D may be involved in the regulation of glutamate metabolism. Glutamate is beneficial for the mechanical function of the ischemic and hypoxic heart[29], and increased glutamate uptake is found in

◀ Figure 2. Autoradiograms showing nuclear concentrations of ^{3}H-1,25-D_3 related silver grains over many cardiomyocytes of the right atrium [*a*]. Competition controls from mice, which had received unlabelled 1,25-D_3 prior to the tracer, show no nuclear silver grain concentration over cardiomocytes of the right atrium [*b*]. In ANF positive cardiomyocytes in the left and right ventricles binding sites for ^{3}H-1,25-D_3 are absent [*c*]. In the posterior right atrium more than 90% of the ANF-positive cardiomyocytes exhibit nuclear 1,25-D_3 binding sites [*d*]. Only a few cell clusters (2–4 ANF-positive cells) in the left atrium concentrate 1,25-D_3 in their nuclei [*e*]. In both atria 1,25-D_3 binding is not detectable in endothelial cells [arrowheads, *d–e*]. Bar, 15 µm.

Figure 3. Autoradiograms prepared similar to those of fig. 2. A few ANF-negative, single 1,25-D_3 target cells are located at the base of each valve [*a*] but not in the valve as it is shown for the left atrium [*a,b*]. ANF-positive cardiomyocytes which are connected to the base of the valve show no 1,25-D_3 binding in the left atrium [*c*] while most of the right atrium are 1,25-D_3 targets [*d*, arrowhead]. Bar, 15 µm.

hearts of patients who suffer from coronary diseases[30]. Vitamin D stimulates enzymes like glutamate-transaminases that are involved in glutamate metabolism in the skin[31]. Such a possible mechanism in the heart needs further investigation. Together our data from combined histochemical and physiological studies indicate that 1,25-D_3 has direct genomic actions on cardiomyocytes predominantly in the right atrium that result in changes of manufacture and secretion of ANF. Other simultaneous effects on glutamate turnover, ionic concentrations, and conductivity are likely. Simultaneous cooperative or antagonistic genomic actions of sex and adrenal steroids[4,17–19,32,33] on ANF turnover must be considered and viewed interactively with those of vitamin D. As in other tissues, the role of vitamin D – soltriol is likely to adjust cardiovascular and other vital functions to the seasonal changes to optimize survival and reproduction[4,6].

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Reevaluation of hydropathy profiles of voltage-gated ionic channels

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Abstract. A reevaluation of the secondary structure of Na, Ca and K channel proteins led to the following results. Only three segments (S1, S5 and S6) of each repeat are sufficiently hydrophobic to be predicted as transmembrane helices, if a window of 19 amino acids is used. Some of the S2 and S3 segments show higher hydrophobic values when calculated with the window of 9 amino acids and can be predicted as short helices. S4 segments are strongly hydrophilic and cannot be predicted as transmembrane helices. Some of the S2, S3 and S4 segments have an amphipathic character; however, these helices do not span a membrane. A model is proposed where 12 hydrophobic transmembrane helices surround 12 shorter helices, forming a hydrophilic pore. In addition, a unique pattern for S4 segments of voltage-gated channel proteins is defined.
Key words. Voltage-gated ionic channels; Na, Ca, K channel proteins; hydropathy profiles; secondary structure; sequence pattern of segment S4.