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Ontogenic appearance of Ca^{2+} channels characterized as binding sites for nitrendipine during development of nervous, skeletal and cardiac muscle systems in the rat

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The appearance of specific receptors for the Ca^{2+} channel antagonist nitrendipine has been followed during the fetal and post-natal development of rat brain without cerebellum, cerebellum, skeletal muscle and cardiac muscle. The number of nitrendipine receptors is low at the fetal stage and increases drastically during post-natal development of brain, cerebellum, skeletal muscle and cardiac muscle. The time course of this increase is different for each type of tissue studied. No significant change in receptor ligand dissociation constant (K_d) can be detected over the development period studied. The results are discussed in relation with the known properties of the differentiation process in the four types of excitable tissues studied.

Ontogenesis *Nitrendipine-receptor* *Ca^{2+} channel* *Excitable tissue*

1. INTRODUCTION

Voltage-dependent Na^+ and Ca^{2+} channels are very important structural elements of the surface membrane in a wide variety of excitable cells [1–4]. They are involved in both the generation of action potentials and in the excitation-contraction or excitation-secretion coupling process in heart and smooth muscle on one hand or in central neurons and secretary systems on the other [1–5].

The purpose of this work is to study with the radiolabeled nitrendipine, which is one of the most potent calcium channel blockers described until now [5], the ontogenesis of the Ca^{2+} channel in brain, cerebellum, skeletal muscle and cardiac muscle during the fetal and post-natal development of the rat.

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2. EXPERIMENTAL

2.1. *Tissue preparation*

Fetal and post-natal Wistar albino rats were used throughout this work. Pregnant rats were killed at various times from 19–20 days of gestation by decapitation and fetal rats taken out. Post-natal rats were raised in litters of 5 and killed at various times from 1–60 days post-natal. From each animal, brain without cerebellum, cerebellum, leg skeletal muscles and heart were dissected, weighed and washed in ice-cold 20 mM Tris–HCl buffer containing 0.25 M sucrose and 1 mM EDTA (pH 7.4) (TSE buffer). For skeletal muscles and hearts, homogenization was performed at 4°C in 10 vols of TSE buffer using a polytron apparatus (Brinckman instruments) at setting 5 with three 5-s bursts. For brains and cerebella, homogenization was performed using a Teflon Potter-Elvehjem in 10 vols of TSE buffer at 4°C. Homogenates were washed twice by centrifugation at $1000 \times g$ for 10 min followed by resuspension of the pellet in fresh TSE

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buffer to a concentration of 100 mg original tissue weight/ml TSE buffer. Subsequently the homogenates were filtered through four layers of cheesecloth.

2.2. *Binding assays*

[^{3}H]Nitrendipine binding assays were carried out as follows: 0.5–1 mg homogenate protein was equilibrated in 1 ml of the standard incubation medium containing 20 mM Tris–Cl, 50 mM choline chloride and 10^{-4} M of the protease inhibitor, phenylmethylsulfonyl fluoride (pH 7.4) at 4°C in the absence (total binding) and in the presence (non specific binding) of 1 μM unlabeled nitrendipine. After incubations for 40 min at 4°C binding was stopped by filtering in duplicate, aliquots of 400 μl of the incubation medium through Whatman GF/B glass fiber filters under reduced pressure. Filters were rapidly washed twice (<15 s) with 5 ml of an ice-cold solution containing 200 mM choline chloride and 20 mM Tris–Cl buffer (pH 7.4). Aliquots of 100 μl of the incubation mixture were taken for measurement of the concentration of [^{3}H]nitrendipine present. All experiments were carried out under dim light because of the light sensitivity of the dihydropyridine derivative. Protein content was determined as in [6] using bovine serum albumin as standard.

3. RESULTS

3.1. *Central nervous system*

[^{3}H]Nitrendipine has been used as a biochemical marker of the differentiation of the nitrendipine-sensitive Ca^{2+} channel in different excitable tissues of rat. The inset of fig.1A shows representative Scatchard plots for the specific binding of [^{3}H]-nitrendipine to its receptors in rat brain without cerebellum. Dissociation constants (K_d) and maximal number of [^{3}H]nitrendipine binding sites (B_{max}) are 0.48 nM and 42 fmol/mg protein and 0.44 nM and 54 fmol/mg protein in brain homogenates from 11 days and 35 days post-natal, respectively. Scatchard plots are linear at all stages of development demonstrating that [^{3}H]nitrendipine binds to a single class of sites in the range of concentrations used (0.1–10 nM). The main panel of fig.1A presents K_d and B_{max} values during development from the fetal to adult stage. K_d values remained stable at 0.42 ± 0.03 nM throughout the

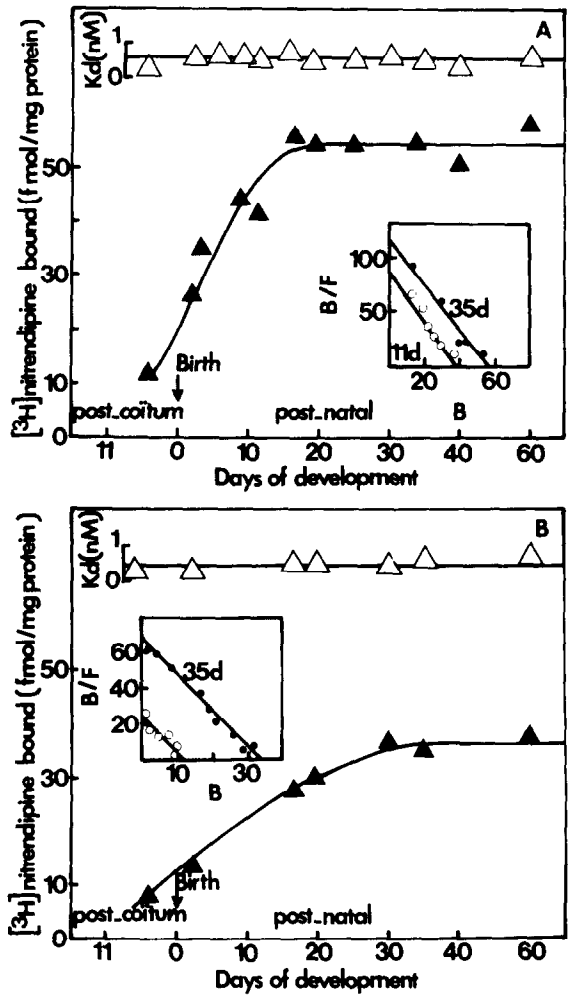

Fig.1. Ontogenesis of the specific [^{3}H]nitrendipine binding component in rat brain (A) and cerebellum (B). Insets: Scatchard plots of the specific [^{3}H]nitrendipine binding on homogenates of brain (A) and cerebellum (B). B is bound [^{3}H]nitrendipine expressed in fmol/mg protein and B/F is bound over free [^{3}H]nitrendipine expressed in nM. Main panels: evolution of the maximum [^{3}H]nitrendipine binding capacity as a function of time in days (▲) for brain (A) and cerebellum (B). K_d values (△) are obtained at each stage of development from Scatchard analysis. Specific binding compared to total binding is between 45 and 60%.

developmental process. The maximal number of [^{3}H]nitrendipine binding sites (B_{max}) increased regularly during development from 17 days post-coitum (12 fmol/mg protein) to 15 days post-natal (to reach a plateau value at 56 fmol/mg protein):

B_{max} values remained constant between 15 days post-natal and the adult stage at a value of 56 ± 3 fmol/mg protein.

The ontogenesis of the [^{3}H]nitrendipine binding component of the Ca^{2+} channel in rat cerebellum is shown in fig.1B. The inset in fig.1B shows representative Scatchard plots for the specific binding of [^{3}H]nitrendipine to its receptor in rat cerebella at two stages of development. K_d and B_{max} values are 0.42 nM and 12 fmol/mg protein and 0.53 nM and 34 fmol/mg protein at 2 days post-natal and 35 days post-natal, respectively. Scatchard plots are linear indicating that [^{3}H]nitrendipine binds to a single class of site in the range of concentrations used (0.1–10 nM). The main panel of fig.1B shows the variation of B_{max} values at different stages of development and the corresponding K_d values. Values of the K_d remained constant at all stages studied at a value of 0.46 ± 0.06 nM. B_{max} values regularly increased during development from 8 fmol/mg protein at 17 days-coitum to reach a plateau value at 30 days post-natal. B_{max} values remained stable at a value of 30 ± 4 fmol/mg protein between 30 and 60 days post-natal.

3.2. *Skeletal muscle and cardiac muscle*

The ontogenesis of the nitrendipine binding component of the Ca^{2+} channel in rat leg skeletal muscles is shown in fig.2A. Scatchard plots are shown in inset A at two stages of development. K_d and B_{max} values are 1.6 nM and 250 fmol/mg protein, and 1.9 nM and 400 fmol/mg protein at 6 days post-natal and 60 days post-natal, respectively. Variation of B_{max} and K_d values at different stages of development are presented in the main panel of fig.2A. Scatchard plots of the specific [^{3}H]nitrendipine binding component are linear in the range of concentrations used (0.1–10 nM). B_{max} values increased regularly between 17 days post-coitum (95 fmol/mg protein) and 15 days post-natal. Then B_{max} values remained constant between 15 days post-natal and the adult stage at a value of 416 ± 20 fmol/mg protein.

The same type of data are presented for the cardiac muscle in fig.2B. Representative Scatchard plots are shown in inset B for two stages of development. K_d and B_{max} values are 0.4 nM and 75 fmol/mg protein and 0.6 nM and 175 fmol/mg protein at 4 days post-natal and 40 days post-natal, respectively. The variation of B_{max} values during

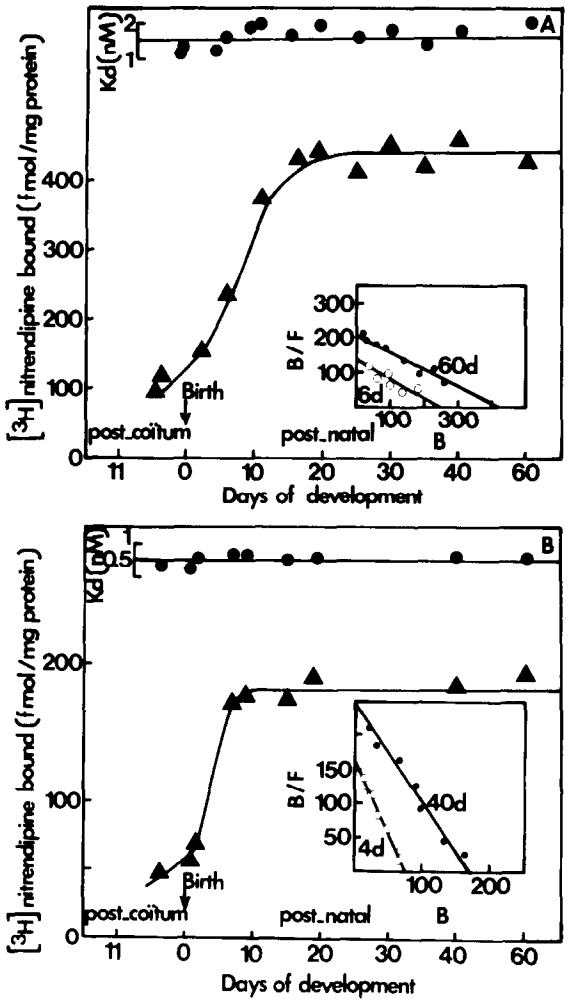

Fig.2. Ontogenesis of the [^{3}H]nitrendipine binding component in rat skeletal muscles (A) and heart (B). Insets: Scatchard plots of specific [^{3}H]nitrendipine binding on homogenates of skeletal muscles (A) and heart (B). B is bound [^{3}H]nitrendipine expressed in fmol/mg protein and B/F is bound over free [^{3}H]nitrendipine expressed in nM. Main panels: A and B show the evolution of the maximum [^{3}H]nitrendipine binding capacity as a function of time in days (▲) for skeletal muscles (A) and heart (B). K_d values (●) are obtained at each stage of development from Scatchard analysis. Specific binding compared to total binding is between 45 and 55%.

cardiac development and their corresponding K_d values are presented in main panel of fig.2B. Scatchard plots of the specific [^{3}H]nitrendipine binding component are linear in the range of concentrations used (0.1–10 nM). Values for the K_d remained constant at all stages studied at a value of

0.53 ± 0.03 nM. Only B_{max} varied during development from 50 fmol/mg protein at 17 days post-coitum to 176 ± 15 fmol/mg protein between 7–9 and 60 days post-natal.

4. DISCUSSION

Nitrendipine has been shown to block calcium current or calcium-mediated processes in a variety of excitable cells [3,7–9] and recently specific receptors for the Ca^{2+} channel antagonist nitrendipine have been identified in brain, skeletal muscle and heart from adult animals [10–12]. This study describes the ontogenesis of specific saturable high affinity [^{3}H]nitrendipine receptors in the rat brain, cerebellum, skeletal muscle and heart.

Specific receptors for the Ca^{2+} channel antagonist nitrendipine are present in very low amounts in rat brain without cerebellum and cerebellum at the fetal stage (17–20 days post-coitum) and increase regularly during post-natal life. A plateau level corresponding to the maximal number of nitrendipine receptors is reached 15 and 30 days after birth in brain and cerebellum, respectively. The dissociation constant (K_d) of the complex nitrendipine receptor does not vary during fetal and post-natal development. The maximal binding capacity (B_{max}) remains relatively low at the adult stage at a value of 56 and 30 fmol/mg protein in brain without cerebellum and cerebellum respectively, as compared to previously reported values [13] for the voltage-dependent Na^+ channel at the same stage of development (1.8–2 pmol/mg protein). A comparison between the results presented here and those previously published for the ontogenesis of the Na^+ channel [13] indicates that in brain the development of the nitrendipine-sensitive Ca^{2+} channel parallels the maturation of the voltage-dependent Na^+ channel. Moreover, post-natal increase of the nitrendipine receptor in brain is roughly parallel to that found for the receptors of several neurotransmitters like the dopamine receptor [14], GABA-receptor [15], benzodiazepine receptor [16] and histamine H_1 receptor [17].

The ontogenesis of the nitrendipine-sensitive Ca^{2+} channel in the cerebellum is different from that observed in the brain. The number of nitrendipine-receptors increase slowly between 17 days post-coitum and 4–5 weeks after birth. The post-natal development of the nitrendipine receptor in the rat cerebellum parallels the appearance of the voltage-sensitive Na^+ channel which also reaches a maximal level between 30 and 40 days after birth [13]. The ontogenesis of the nitrendipine receptor closely follows the ontogenesis of GABA receptors which are associated with granule cells [18].

The amount of nitrendipine receptors in skeletal muscle increases regularly during fetal and early post-natal life. A maximal number of receptors for nitrendipine is attained between 15 and 20 days after birth and remain constant to the adult stage (60 days post-natal). As for brain without cerebellum and cerebellum, the affinity of the nitrendipine receptor complex does not vary during development. However, the affinity of [^{3}H]nitrendipine for its receptor is slightly lower in skeletal muscle (1.9 ± 0.1 nM) than in central nervous system and cardiac muscle (0.4–0.5 nM). There is a close correlation between the chronology of the appearance of the nitrendipine receptor and the ontogenesis of the TTX-sensitive Na^+ channel in rat skeletal muscles [13]. In a previous work it has been shown that [^{3}H]nitrendipine receptors are preferentially localized in the transverse tubule membranes of rabbit and frog muscle [11]. Therefore, it is of interest to remark that the ontogenesis of rat skeletal muscle nitrendipine receptors closely follows the appearance and the differentiation of the T-tubule and sarcoplasmic reticulum systems in the rat skeletal muscle during fetal and post-natal development [19,20].

The rate of development of the nitrendipine-sensitive Ca^{2+} channel in the heart is different from that of other excitable tissues (a recent meeting abstract reveals the same observation for mouse heart [21]), with a more rapid rise in receptor density from 50 to 175 fmol/mg protein between 17 days post-coitum and 7 days of post-natal life. The maximal number of binding sites for nitrendipine is attained between 7 and 10 days post-natal. At this time the number of low affinity TTX-sensitive Na^+ channels is at the adult level [22]. In rat heart the presence of β receptors and of a catecholamine-mediated activation of adenylate cyclase have been demonstrated as early as at 13 days of embryonic development [23]. At that time nitrendipine-sensitive Ca^{2+} channels are undetectable (not shown) and, therefore, one would not expect a β-adrenergic activation of Ca^{2+} channels via

cAMP. Effectively at this stage of development the heart does not respond to catecholamines in terms of inotropic and chronotropic effects [23]. A marked increase in heart rate to catecholamine appears at about 18 days post-coitum when nitrendipine-sensitive Ca^{2+} channels are detectable and physiologically functional [24].

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