Pflügers Arch – Eur J Physiol (1996) 431:791–793

SHORT COMMUNICATION

Jean-François Quignard · Eric Grazzini
Gilles Guillon · Marie-Cécile Harricane
Joël Nargeot · Sylvain Richard

Absence of calcium channels in neonatal rat aortic myocytes

Received: 4 December 1995 / Received after revision: 29 December 1995 / Accepted: 15 January 1996

Abstract We have investigated whole-cell Ba^{2+} currents through Ca^{2+} channels (I_{Ba}) in single myocytes freshly isolated from the aortic media of neonatal (1-day-old) and adult (12-week-old) rats. In neonatal myocytes, I_{Ba} was undetectable even in presence of the dihydropyridine (DHP) agonist Bay K 8644. Binding of [^{3}H]Nitrendipine on crude plasma membrane preparation of media confirmed the absence of DHP-receptors. By contrast, a robust DHP-sensitive 'L-type' I_{Ba} was recorded in adults which was consistent with the presence of specific [^{3}H]Nitrendipine binding sites. In conclusion, neonatal aortic myocytes do not express any Ca^{2+} channels. The acquisition of L-type Ca^{2+} channels may be related to cell differentiation and acquisition of contractility during postnatal development.

Key-words: Vascular smooth muscle, Calcium signalling, Development, L-type Calcium Channels, Nitrendipine binding sites.

Introduction

Voltage-gated Ca^{2+} channels are a major pathway for Ca^{2+} influx (I_{Ca}) and play a predominant role in the generation of vascular smooth muscle tone [7]. A slowly-inactivating dihydropyridine (DHP)-sensitive L-type I_{Ca} can be recorded in freshly isolated aortic myocytes of adult rats [8]. However, two additional I_{Ca} with distinct electrophysiological, pharmacological and regulatory profiles appear in primary cultured myocytes suggesting that dedifferentiation *in vitro* modulates the nature and properties of Ca^{2+} channels [8]. The myocytes in normal arteries of mature animals are differentiated, contractile and remarkably quiescent. In contrast, proliferation of myocytes occurs during development (neonatal myocytes are not yet differentiated) and in relation to pathology such as atherogenesis and restenosis after angioplasty (dedifferentiation of mature myocytes) [6]. Changes of I_{Ca} during postnatal development have been documented in cardiac and skeletal myocytes [2,5] but there is no information available for vascular myocytes of neonatal mammals. In the present study, we have investigated Ca^{2+}-channel currents in freshly isolated aortic myocytes of neonatal rats and, for comparison, in myocytes of adult animals.

J.-F. Quignard • M.-C. Harricane • J. Nargeot • S. Richard (✉)
CRBM, CNRS, UPR 9008, INSERM U249, Route de Mende, BP 5051, F-34033 Montpellier Cedex, France
E. Grazzini • G. Guillon
CCIPE, INSERM U 401, Rue de la Cardonille, F-34094 Montpellier Cedex 05, France

Materials and methods

Electrophysiology: The neonatal (1-day-old) and adult (12-week-old) aortic myocytes were enzymatically (papain) isolated from medias of Wistar Kyoto rats (n=20 for new-borns and for adults) [8]. Whole-cell recordings were performed at 20-22°C during 4 hours after the dispersion in conditions optimised to isolate Ca^{2+}-channel currents [8]. The recording pipettes were filled with (mM): CsOH 130, EGTA 10, HEPES 25, (Mg)ATP 3, (Na)GTP 0.5, glucose 10, succinic acid 5, aspartic acid 5. The bathing solution contained (mM): CsOH 120 , $Ba(OH)_2$ 20 , HEPES 10, 4-aminopyridine 5 , glucose 10. For both solutions pH was adjusted to 7.3 with CH_3SO_3H; osmolarity: 310-340 mOsm. The voltage-clamp circuit and the multiple microcapillary perfusion system for application of the DHP agonist (+/-) Bay K 8644 were described previously [8]. Capacitive transient and linear leakage currents were subtracted using a 4 subpulse (P/-4) to resolve small inward currents. Results are expressed as mean ±SD. Statistical comparisons between groups of values were performed using Student's unpaired *t*-test with $p < 0.05$ considered significant.

[^{3}H]-Nitrendipine binding to membrane preparations : Aortic medias from adult (20 animals per experiment) and neonatal (25-35 animals per experiment) rats were dissected at 4°C and resuspended in an ice-cold isotonic medium (Sucrose 250 mM, Tris HCl pH 7.4 10 mM, $MgCl_2$ 3 mM, EDTA 1mM, PMSF 0.1 mM) and homogenised using a Douce potter. After centrifugation (15 min, 4000 rpm, 4°C), the pellet was resuspended in the same (but sucrose-free) hypotonic medium and kept at

4°C for 1 hour. The homogenate was then centrifuged twice (15 min, 4,000 rpm, 4°C). The pellet was resuspended in the hypotonic medium and used immediately for measuring Nitrendipine (Dupont, Les Ullis, France) binding site as described previously [1]. Briefly, crude plasma membranes (16 µg protein per assay) were incubated 1 hour at 25°C in 200 µl of an incubation medium containing : [^{3}H]-Nitrendipine 0.1 to 1 nM, Tris-HCL pH 7.4 10 mM, $MgCl_2$ 3 mM, soybean trypsin inhibitor 0.05 mg/ml, bacitracine 0.2 mg/ml, bovine serum albumin 1 mg/ml. Non- specific binding was determined in the same condition by adding 1 µM of unlabelled nifedipine in the assay. Reaction was stopped by adding 3 ml of an ice cold filtration medium (Tris-HCl pH 7.4 10 mM, $MgCl_2$ 3 mM) and filtering immediately through GF/C Whatman glass fiber filters. Filters were then washed out 3 times with the same filtration solution and radioactivity was measured by liquid spectrophotometry.

Results

In order to compare densities, rather than amplitudes, of current, cell surface of neonatal and adult myocytes was estimated by measuring their capacitance. Averaged values were 20 ±2 pF (n=22) for the neonatal and 30 ±7 pF (n=45) for the adult myocytes. Therefore, the myocytes isolated from neonatal rats were significantly smaller than those obtained from adult animals ($p < 0.001$). We show that in freshly isolated neonatal cells (n=22), no IBa was detected even in the presence of the DHP agonist Bay K 8644 (Fig. 1A,C). It is possible that, in some cells, currents of less than 5 pA (density < 0.3 pA/pF) were not detected. However, such level would reflect a very small number of functional Ca^{2+} channels. It should be noted that large TEA-sensitive K^+ currents could be recorded in these cells (data not shown) indicating the presence of functional voltage-gated channels. By contrast to the absence of Ca^{2+}-channel current at the neonatal stage, DHP-sensitive L-type IBa were recorded in aortic myocytes isolated from adult animals (Fig. 1B,C), the averaged current amplitude being 70 ±45 pA (n=45) for a step depolarisation at +10 mV from a holding potential of -100 mV. Current density was 3.1 ±1.5 pA/pF (n=45). Application of 1 µM Bay K 8644, which is a potent DHP agonist of L-type Ca^{2+} channels, markedly increased this current (Fig. 1C).

Since the difference in the density of IBa between neonatal and adult myocytes could reflect enzymatic digestion or inactivation of the Ca^{2+} channels during cell dissociation, we further investigated whether DHP- binding sites were present on intact aortas. We show that crude plasma membrane from medias of adult rats exhibited specific Nitrendipine-binding (Fig. 2). Binding was dose-dependent and saturable. Scatchard plot of the dose-response curve was linear suggesting the presence of a single class of high-affinity DHP-receptors (dissociation constant Kd = 1.7 ±0.3 nM, 3 distinct determinations). The maximal binding capacity (Bmax) was 66 ±8 fmoles of [^{3}H]-Nitrendipine specifically bound per mg of protein. When similar experiments were performed using membranes derived

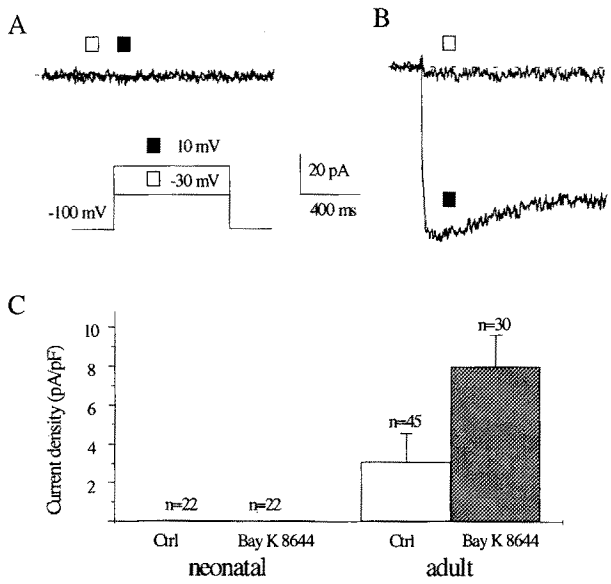

Figure 1: Ca^{2+}-channel currents in freshly isolated neonatal and adult rat aortic myocytes. Test pulses to -30 mV (•) and +10 mV (•) applied from a holding potential of -100 mV (see inset). **(A)** Absence of IBa in a neonatal myocyte. **(B)** Typical L-type IBa recorded in an adult myocyte. **(C)** Mean (±SD) densities of IBa recorded at +10 mV in the absence (Ctrl) and presence of 1µM Bay K 8644 in neonatal and adult myocytes.

from the media of neonatal rats, only very few specific binding sites were detected.

Determinations of maximal binding capacity and dissociation constant were hampered by the limited number of receptors. For a concentration of 0.8 nM [^{3}H]-Nitrendipine, the specific binding was 4 ±3 fmoles/mg protein (3 distinct determinations). For comparison, this value represented only 5 % of the similar determination performed on plasma membrane from adult medias.

Discussion

The major finding of this study is the lack of voltage-gated Ca^{2+}-channel currents in freshly isolated aortic myocytes of neonatal rats (1 day *post-partum).* Ligand binding of [^{3}H]-Nitrendipine on membrane preparation of neonatal medias confirmed the quasi-absence of DHP receptors. Our results, consistent with the deficiency of contractile response of neonatal rabbit aortas to the depolarising agent KCl [3], suggest that the voltage-gated ICa does not provide a major pathway for Ca^{2+} influx in the aortic cells at the time of birth. By contrast, the presence of robust L-type ICa and specific Nitrendipine-binding sites in adult aortic myocytes suggests a post-natal appearance of DHP-sensitive L-type Ca^{2+} channels. The binding affinity of the single class of high affinity sites (Kd = 1.7 ±0.3 nM) evidenced here is similar to that determined for vascular smooth muscles

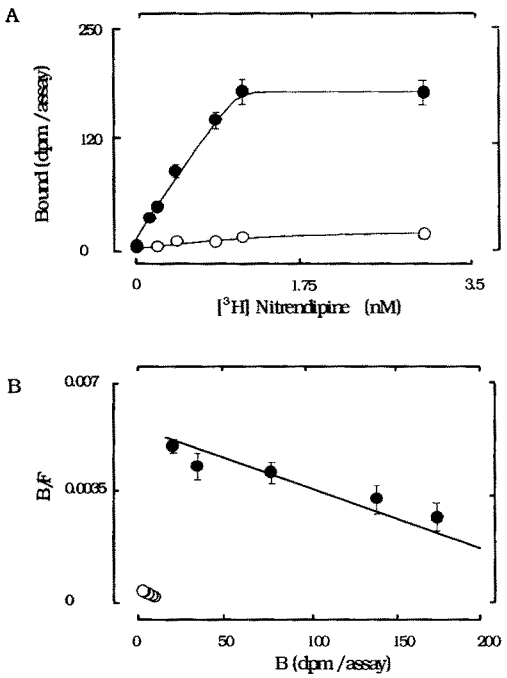

Figure 2: Binding of [^{3}H]-Nitrendipine to aorta plasma membrane preparations from neonatal and adult rats. Crude plasma membranes from the media of new-born (O) or adult (λ) aorta were prepared as described in METHODS. The radioactivity found associated to membrane was expressed in dpm per assay. **(A)** Specific binding (Bound), calculated as the difference between total and corresponding non-specific value, was plotted versus the concentration of the tritiated ligand used (Free). **(B)** Scatchard representation of the dose-dependent binding curve. Results are expressed as the mean ± SE of triplicate determinations from a single representative experiment. When no error bars are shown, they are comprised within the symbols.

[4]. The binding site density (Bmax : 66 ±8 fmol/mg protein) was also homogenous with that found in bovine, canine and rabbit aortas (20-80 fmol/mg protein) [4].

Voltage-gated L-type Ca^{2+}-channel currents play a major role in the excitation-contraction coupling of vascular cells [7]. Their presence in adult, but not in neonatal, aortic myocytes is in good agreement with the postnatal increase of the KCl-induced contraction observed in the rabbit aorta [3]. This is also consistent with the postnatal increase in peak ICa reported in cardiac cells [2] and with the increase of DHP-binding sites during development of nervous, skeletal and cardiac cells in rats [5]. However, we show that the timing is different because neurones and cardiac and skeletal myocytes exhibit substantial ICa and DHP-binding sites at the time of birth [2,5,7]. It is worth noting that the aortic myocytes in the new-born rat are by several criteria still relatively undifferentiated and lacking some of the contractile proteins that characterise the mature myocytes [6]. Therefore, our data strongly suggest that neonatal aortic myocytes lack L-type ICa in relation to their undifferentiated state. The acquisition of the L-type ICa probably parallels that of contractility during postnatal development.

ACKNOWLEDGEMENTS. We thank Drs P. Lory, S. Lemaire, M. Morris, G. Loirand and P. Pacaud for their critical reading of the manuscript. This work was supported by the *Fondation pour la Recherche Médicale* and la *Fondation de France.*

References

1. Aguilera G, Catt KJ (1986) Participation of voltage dependant calcium channels in the regulation of adrenal glomerulosa function by Angiotensin II and potassium. Endocrinology 118: 112-118.
2. Gomez JP, Potreau D, Branka JE, Raymond G (1994) Developmental change in Ca^{2+} currents from new-born rat cardiomyocytes in primary culture. Pflügers Arch. 428: 241-249.
3. Hayashi S, Toda N (1978) Age-related changes in the response of rabbit isolated aorta to vasoactive agents. Br. J. Pharmacol. 64: 229-237.
4. Janis RA, Triggle DJ (1984) 1,4-Dihydropyridine Ca^{2+} channel Antagonists and activators: a comparison of binding characteristics with pharmacology. Drug Development Research 4: 257-274.
5. Kazazoglou T, Schmid A, Renaud JF, Lazdunski M (1983) Ontogenic appearance of Ca^{2+} channels characterised as binding sites for nitrendipine during development of nervous, skeletal and cardiac muscle systems in the rat. FEBS Letters 164 (1): 75-79.
6. Kocher O, Gabbiani G (1986). Expression of actin mRNAs in rat aortic smooth muscle cells during development, experimental intimal thickening, and culture. Differentiation 32: 245-251.
7. Mc Donald TF, Pelzer S, Pelzer DJ (1994) Regulation and modulation of calcium channels in cardiac, skeletal and smooth muscle cells. Physiol. Reviews 74 (2): 365-507.
8. Neveu D, Quignard JF, Fernandez A, Richard S, Nargeot, J (1994) Differential β-adrenergic regulation and phenotypic modulation of voltage-gated calcium currents in rat aortic myocytes. J. Physiol. 479 (2): 171-182.