ELSEVIER

European Journal of Pharmacology 443 (2002) 13–18

ejp

www.elsevier.com/locate/ejphar

Up-regulation of Ca^{2+} channels in vas deferens after chronic treatment of newborn rats with nifedipine

Luciana Ferreira Verde, Simone S.L. Lafayette, Afonso Caricati-Neto, Neide H. Jurkiewicz, Aron Jurkiewicz*

Department of Pharmacology, Federal University of São Paulo, São Paulo 04034-970, Brazil

Received 27 February 2002; received in revised form 3 April 2002; accepted 5 April 2002

Abstract

Radioligand binding and contraction techniques were used to verify if L-type Ca^{2+} channels are modified in rat vas deferens after treatment with the blocker nifedipine (15 μg), injected at 7, 14, 21 and 28 days after birth. Vas deferens tissue was used 10, 30 and 90 days after the last injection, to verify if modifications are persistent. Binding studies with cell membranes, using [^{3}H]isradipine, showed an increase of the density (B_{max}) of Ca^{2+} channels by more than 60%, after 10 and 30 days, without changes of affinity (K_d). Maximal contractions (E_{max}) of KCl, were increased by 106% and 37%, respectively, after 10 and 30 days, without changes of apparent affinity (pD_2). After 90 days, the values of B_{max}, K_d, E_{max} and pD_2 were not different from the controls. Differences were also not found for rats injected when adult. It is concluded that treatment of newborn, but not of adult, rats with nifedipine produced a long-lasting, though reversible, up-regulation of L-type Ca^{2+} channels.

Keywords: Ca^{2+} channel; Nifedipine; Smooth muscle; Contraction; Binding; Vas deferens, rat

1. Introduction

Chronic use of receptor-specific agonists or antagonists can lead to decreases or increases of ligand binding densities, as a result of ''down-regulation'' or ''up-regulation'' (Hollenberg, 1985; Morgan et al., 1999), respectively. For instance, there is evidence that Ca^{2+} channel number and function can be regulated by chronic drug treatment, based on experiments with heart, brain, vascular smooth muscle and vas deferens (Nishiyama et al., 1986; Gengo et al., 1988; Skattebøl et al., 1989; Ferrante and Triggle, 1990; Castillo et al., 1992; Cingolani et al., 1996). It was shown that chronic treatment with Ca^{2+} channel blockers produces alterations in smooth muscle in response to drugs. In addition, it has been advanced that treatment should be made in newborn animals, during a critical period when the preparations would be more susceptible to this regulatory influence (Csaba et al., 1995). However, one could argue that there is still some uncertainty about the optimal period after birth for this regulation to occur, since changes have also been observed after chronic treatment with Ca^{2+} channel blockers in adult animals (Gengo et al., 1988; Morgan et al., 1999). Furthermore, it is still unclear whether or not the resulting changes are permanent.

The dihydropyridine, nifedipine, is a selective Ca^{2+} channel blocker of L-type voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channels). Our aim was to verify if L-type Ca^{2+} channels in the smooth muscle of the rat vas deferens are modified after inhibition of these channels in vivo with nifedipine. Another objective was to see if these modifications would occur equally on treatment of young and adult rats. Finally, we determined whether or not these alterations are permanent. Thus, we used newborn rats, 10, 30 and 90 days after treatment with nifedipine, as well as adult animals. We looked for changes of the parameters, affinity (K_d) and B_{max}, in receptor binding studies with the labeled dihydropyridine [^{3}H]isradipine, and the parameters, E_{max} and pD_2, in contraction experiments with the depolarising agent, KCl, whose effects are mainly due to Ca^{2+} influx through L-type Ca^{2+} channels.

* Corresponding author. Tel.: +55-11-5576-4449; fax: +55-11-5576-4569.

E-mail address: aron.farm@epm.br (A. Jurkiewicz).

0014-2999/02/$ - see front matter
PII: S0014-2999(02)01584-4

2. Material and methods

2.1. Animals

Male Wistar rats from our own colony, BAW-2, were treated with nifedipine injections, as described below. After withdrawal of treatment, the rats were killed by an overdose of ether. The vasa deferentia were removed, cleaned of surrounding tissues and of luminal secretion, and washed with a nutrient solution with the following composition (mM): 136.0 NaCl, 5.6 KCl, 1.8 $CaCl_2$, 0.36 NaH_2PO_4, 15.0 $NaHCO_3$ and 5.5 dextrose (Picarelli et al., 1962), to obtain membrane preparations, or for contraction experiments.

2.2. Treatment with nifedipine

For all types of treatment (newborn or adult rats), nifedipine was suspended in 5% aqueous solution with Tween 80, protected from light and administered subcutaneously. The respective control group received the corresponding volume of control solution without nifedipine (5% aqueous solution of Tween 80). Male newborn rats were treated with injections of nifedipine (15 μg), at 7, 14, 21 and 28 days after birth. Another group of newborn rats was injected with the control solution. The animals were killed 10, 30 or 90 days after the last nifedipine or control injection. In another group, 4-month-old male rats received daily subcutaneous injections of nifedipine (33.3 mg/kg/day) for 15 days. Control rats were injected with a similar solution without nifedipine. The animals were killed between the first and fifth days after the last injection.

2.3. Membrane preparations

The vas deferens membrane was prepared as described Castillo et al. (1992) and modified by Noël et al. (1998). For membrane preparations, about 48 (for 40-day-old rats), 24 (for 60-day-old rats), or 15 vasa deferentia (for 120-day-old rats) were used. The vasa deferentia were cut into small segments and homogenised at 4 °C in 25 volumes (v/w) 0.25 M sucrose buffered to pH 7.4 with 5 mM Tris–HCl containing 2 mM of dithiothreitol and 0.2 mM of phenylmethylsulfonyl fluoride, using an Ultraturrax homogeniser at 20,500 rpm for 1 min. This operation was repeated twice for 30 s and followed by 10 up-and-

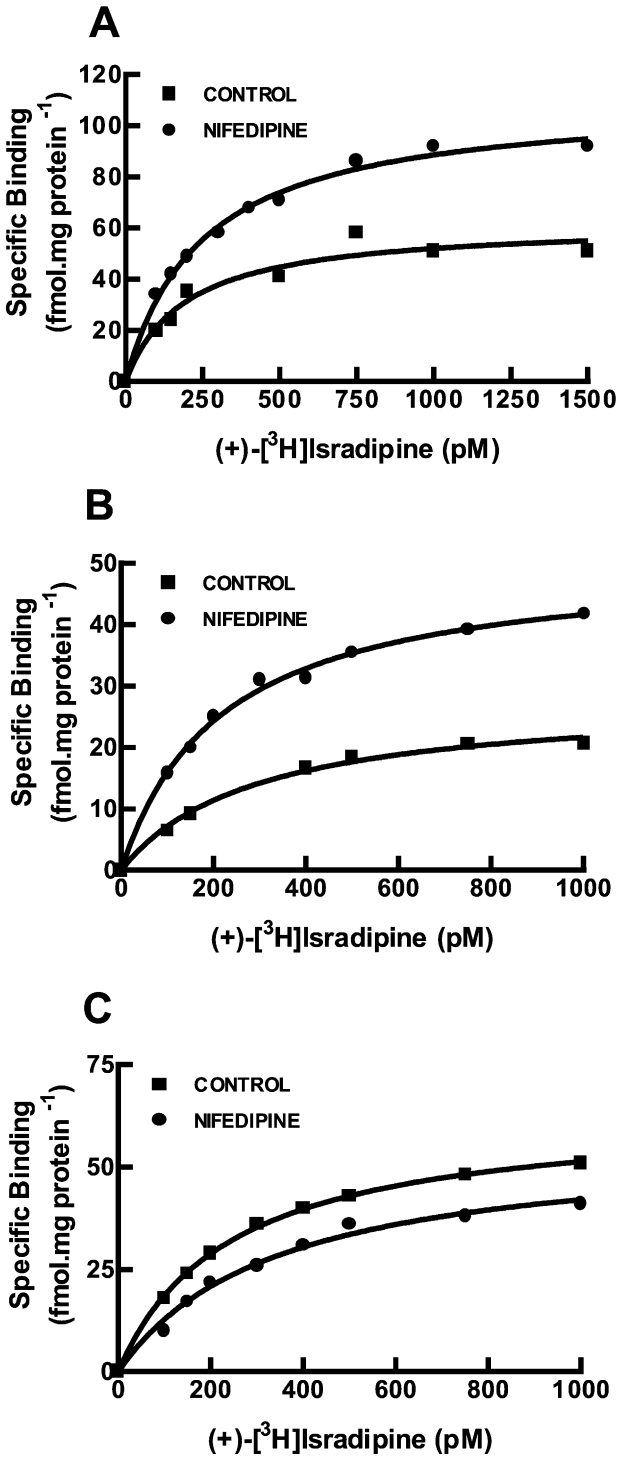

Fig. 1. Typical experiments showing specific binding of (+)-[^{3}H]isradipine in membrane preparations of vas deferens: (A) Saturation paired curves showing one out of six similar curves in triplicate, obtained 10 days either after withdrawal of nifedipine (●), or after withdrawal of control injections (■). The K_d and B_{max} values on the Scatchard plot were respectively 249 pM and 111 fmol mg protein^{-1} (treated group) and 228 pM and 67 fmol mg protein^{-1} (control group). The rats were about 40-days-old at this time. (B) Similar saturation experiment, showing one out of at least four curves in triplicate, 30 days after either withdrawal of nifedipine (●), or withdrawal of control injections (■). The K_d and B_{max} values on the Scatchard plot were respectively 217 pM and 51 fmol mg protein^{-1} (treated group) and 290 pM and 28 fmol mg protein^{-1} (control group). The rats were about 60 days old at this time. (C) Similar saturation experiment, showing one out of at least three curves in triplicate, 90 days after either withdrawal of nifedipine (●), or withdrawal of control injections (■). The K_d and B_{max} values on the Scatchard plot were respectively 282 pM and 38 fmol mg protein^{-1} (treated group) and 212 pM and 49 fmol mg protein^{-1} (control group). The rats were 120 days old at this time.

down strokes in a glass Potter homogeniser. After filtration under vacuum through four layers of gauze, the homogenate was centrifuged at 108,000 × *g* for 1 h. The final pellet was then resuspended in 7 volumes (w/v) of the same buffer without dithiothreitol and stored at −70 °C for subsequent use in the binding assay. The protein concentration was determined by the method of Lowry et al. (1951).

2.4. Binding assay

Saturation binding studies were done as described by Castillo et al. (1989, 1992). Aliquots of the membrane suspension (final concentration 200 μg protein ml^{-1}) were incubated with 100–1500 pM of (+)-[^{3}H]isradipine for 90 min at 37 °C, under sodium light. Incubations were terminated by dilution of the samples with 5 ml ice-cold buffer, followed by rapid filtration in a Millipore System no. xx2702550, through Whatman GF/C glass fiber filters and washing of the filters with 3 × 5 ml buffer. After this procedure, the filters were dried at 80 °C for 15–20 min and added to a scintillation cocktail for radioactivity measurement with a Packard Tri-carb 1600 TR liquid scintillation counter. Specific receptor binding of (+)-[^{3}H]isradipine was defined as the difference between total binding and that obtained in the presence of 1 μM non-labeled (±)-isradipine.

2.5. Contractile responses

The vasa deferentia were mounted in 10 ml organ chambers, in aerated nutrient solution, at 30 °C, for contraction experiments, as previously described (Jurkiewicz et al., 1969). Isotonic contractions were recorded by means of kymographs, with levers giving 9-fold amplification, under 0.5-g load (for 40-day-old rats) or 6-fold amplification and 1-g load (for the other rats). Experiments were initiated after an equilibration period of 30 min. Under these conditions, cumulative concentration–response curves for KCl were made as described (Van Rossum, 1963) with the nutrient solution described above for washout thereafter. Each preparation was used for two or three concentration–response curves of KCl. An interval of about 30 min was allowed between consecutive curves.

2.6. Analysis of parameters

Saturation data were analysed by linear regression, using a computer program (Graphpad Prism), and represented graphically as the relation of bound (B) to the ratio bound/free (B/F) ligand (Scatchard analysis). The parameters dissociation constant (K_d), defined as the concentration of ligand required to occupy 50% of the binding sites, and maximal density of specific binding sites (B_{max}), were determined from the corresponding graphs.

For contraction studies, the maximal effect (E_{max}), and the apparent affinity (pD_2) of KCl, were determined (Jurkiewicz et al., 1977). The pD_2 is defined as the negative logarithm of the concentration of agonist which elicited a half-maximal effect.

2.7. Statistical analysis

The significance of differences between controls and rats treated with nifedipine was evaluated with Student's *t*-test and results were considered as significant if $P < 0.05$.

2.8. Drugs

Nifedipine (Bayer, Brazil), (+)-[^{3}H]isradipine (Amersham, USA), (±)-isradipine (RBI, EUA), and KCl (Merck, Brazil) were used. All the chemicals were American Chemical Society (ACS) certified reagent grade.

3. Results

3.1. Specific binding of [^{3}H]isradipine

The specific binding of [^{3}H]isradipine to membrane preparations of rat vas deferens was linear up to 200 μg protein ml^{-1} and typically represented more than 50% of total binding, as previously described by Castillo et al. (1992). Scatchard analysis showed an increase of more than 60% of B_{max} of [^{3}H]isradipine in vas deferens excised 10 and 30 days after withdrawal of treatment with nifedipine (Fig. 1A and B, and Table 1). However, no significant increase in the values of B_{max} for [^{3}H]isradipine could be detected after 90 days (Fig. 1C). No significant differences

Table 1
Values for B_{max} and K_d for [^{3}H] isradipine binding to crude membrane preparations of vas deferens from nifedipine-treated rats and respective controls

Groups	10 days[a]		30 days[a]		90 days[a]	
	B_{max} (fmol mg^{-1})	K_d (pM)	B_{max} (fmol mg^{-1})	K_d (pM)	B_{max} (fmol mg^{-1})	K_d (pM)
Control	55 ± 10 (6)	217 ± 11 (6)	29 ± 2 (4)	252 ± 23 (4)	46 ± 18 (3)	308 ± 65 (3)
Nifedipine	89 ± 14[b] (6)	222 ± 18 (6)	69 ± 8[b] (5)	214 ± 8 (5)	29 ± 5 (5)	275 ± 35 (5)

Values are means ± S.E.M. Number of experiments in parenthesis.

[a] Rats were injected subcutaneously with nifedipine (15 μg) or vehicle (control) at days 7, 14, 21 and 28 after birth. The vas deferens was used 10, 30 or 90 days after the last injection.

[b] Significantly different from the respective control value.

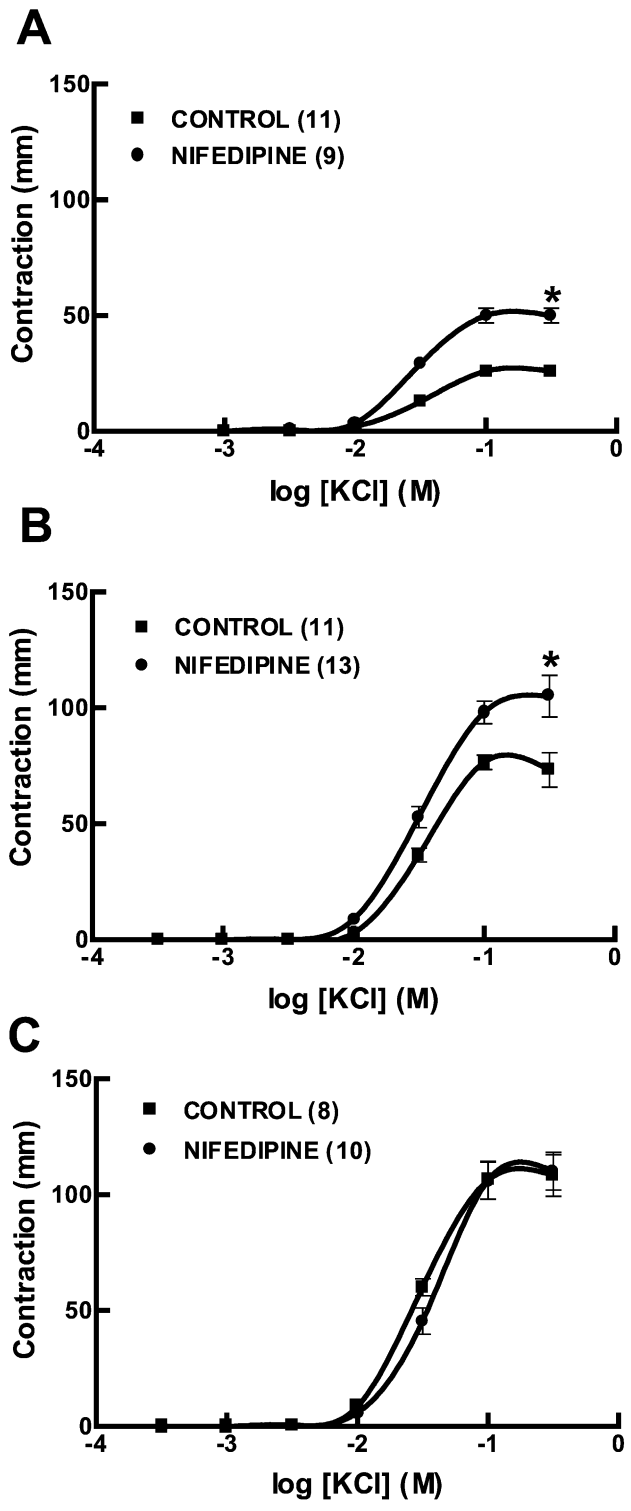

Fig. 2. Mean concentration–response curves for KCl in rat vas deferens 10 days (A), 30 days (B) and 90 days (C) after withdrawal of nifedipine. The curves after the respective withdrawal of control injections are also shown. Points represent means ± S.E.M. Number of experiments in parenthesis. * $P < 0.05$.

were detected for K_d values. The mean values of B_{max} and K_d for [^{3}H]isradipine are shown in Table 1.

3.2. Contractions induced by KCl

Fig. 2 and Table 2 show that treatment of young rats with nifedipine induced increases of the contractile response (E_{max}) to KCl, which persisted after (A) 10 and (B) 30 days. The values of E_{max} were increased by 106% and 37%, respectively. On the contrary, in vasa deferentia obtained 90 days after the last injection, the values of E_{max} of KCl were not influenced by nifedipine (C). Table 2 also shows that pD_2 values of KCl were not significantly different from controls in any of the treated groups. Furthermore, E_{max} and pD_2 values for KCl for rats treated for 15 days with nifedipine when adult, were not significantly different from the controls (Fig. 3 and Table 3).

4. Discussion

Our binding studies with a selective ligand of the dihydropyridine receptor coupled to L-type Ca^{2+} channels ([^{3}H]isradipine) showed that in vivo treatment with nifedipine produced up-regulation (increase of B_{max}) of these channels that persisted at least 30 days after the end of treatment with the Ca^{2+} channel blocker. In addition, functional studies using a depolarising agent which induces Ca^{2+} influx mainly through L-type Ca^{2+} channels (KCl) revealed that the treatment with nifedipine also induced an increase of the contractile response (E_{max}) of isolated vas deferens, confirming the up-regulation of L-type Ca^{2} channels. The fact that concentration–response curves for KCl were not potentiated when the animals were injected when adult, agrees with the hypothesis that there is a period during animal development in which Ca^{2+} channels are more susceptible to regulatory influences (Csaba et al., 1995). These authors had postulated that in this critical period of development, the contact with endogenous ligands determines the characteristics of binding capacity and responsiveness of the cell of the healthy adult (Csaba, 1981, 1986). In the present experiments, this up-regulation was not permanent, as indicated by our finding that no increase of B_{max} for [^{3}H]isradipine and of E_{max} for KCl could be detected 90 days after the end of treatment.

The regulation of the number and function of Ca^{2+} channels is a well-described phenomenon (Ferrante and Triggle, 1990). For instance, in rabbit heart, treatment of adult animals with nifedipine for 25 days induces an increase of dihydropyridine binding sites, without changes in aorta (Cingolani et al., 1996). A similar up-regulation was observed in rat heart (Morgan et al., 1999). A number of controversial results after chronic treatment have been described for many tissues. For example, there are reports that the effect of chronic treatment with nifedipine, although influencing Ca^{2+} channels, produces the opposite result,

Table 2
Values for E_{max} and pD_2 for contractions induced by KCl in rat vas deferens 10, 30 and 90 days after withdrawal of nifedipine (i.e., 40-, 60- and 120-day-old rats) and respective controls

Groups	10 days[a]		30 days[a]		90 days[a]	
	E_{max} (mm)	pD_2	E_{max} (mm)	pD_2	E_{max} (mm)	pD_2
Control	24.3 ± 1.5 (11)	1.45 ± 0.06 (11)	76.5 ± 3.5 (11)	1.47 ± 0.03 (11)	108.3 ± 8.9 (8)	1.34 ± 0.08 (8)
Nifedipine	50.0 ± 3.2[b] (9)	1.51 ± 0.03 (9)	105.0 ± 9.0[b] (13)	1.48 ± 0.03 (13)	110.2 ± 8.1 (10)	1.35 ± 0.05 (10)

Values are means ± S.E.M. Number of experiments in parenthesis.
[a] Rats were injected subcutaneously with nifedipine (15 μg) or vehicle (control) at days 7, 14, 21 and 28 after birth. The vas deferens was used 10, 30 or 90 days after the last injection.
[b] Significantly different from the respective control value.

namely a decrease of the contractile response to drugs in uterus of newborn rats when tested 5 weeks after treatment with nifedipine 24 h after birth (Csaba et al., 1995). In experiments with heart and brain, the chronic treatment of adult rats with intravenous nifedipine for 20 days resulted in a decrease of dihydropyridine binding (Gengo et al., 1988). However, chronic nifedipine treatment for 14 days has been reported not to change [^{3}H]nitrendipine binding sites in the rat heart (Nishiyama et al., 1986). Chronic treatment with nifedipine of PC12 cells in culture also causes an increase in the density of dihydropyridine binding sites, while treatment with the Ca^{2+} channel agonist, BayK 8644, causes down-regulation (Skattebøl et al., 1989). These findings suggest that tissue, age of animals, dose, and duration of treatment are important factors for the regulation of these binding sites.

It is known that receptor activation can induce either an increase or decrease of ligand binding, involving changes in receptor number, affinity or both parameters (Hollenberg, 1985). This regulation of receptors can be homologous or heterologous. In homologous regulation, a ligand regulates its own receptor, as shown here for the treatment with nifedipine. In heterologous regulation, other factors are known to influence or regulate Ca^{2+} channels in vas deferens. This is the case of innervation and male sex hormones such as testosterone, since denervation and castration can produce a decrease of about 60% and 85% in the density of dihydropyridine receptors, respectively (Castillo et al., 1992; Jurkiewicz et al., 1994; Lafayette, 1997). In other studies with vas deferens, the expression and function of Ca^{2+} channels appear to be increased in hypertensive rats (Caricati-Neto et al., 1992).

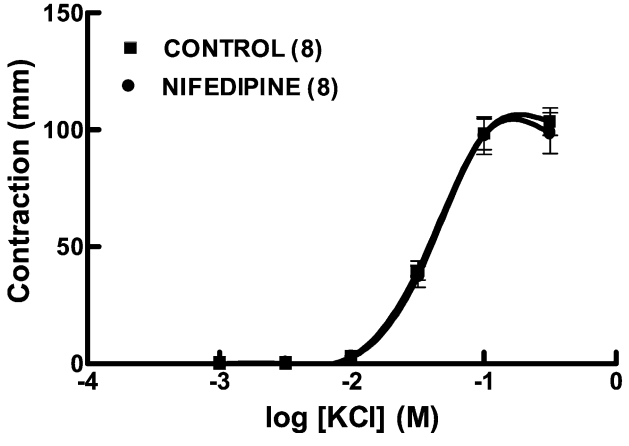

Fig. 3. Mean concentration–response curves for KCl in vas deferens of adult (4-month-old) rat after treatment with nifedipine. The curve for the treatment with control injections is also shown. Points represent means ± S.E.M. Number of experiments in parenthesis.

Since Ca^{2+} channel blockers are used clinically for the management of various disorders, such as hypertension, in either adult or pediatric patients (Bartosh and Aronson, 1999), children hypoglycaemia (Eichmann et al., 1999), asphyxiated newborn infants (Levene et al., 1990), or in prevention of preterm delivery during pregnancy (Koks et al., 1998), one is tempted to speculate about the significance of our results for developing humans. Although, to our knowledge, there are no reports of long-lasting changes in calcium channels after treatment of infants, a number of results have been presented in relation to the development of tolerance and of the so-called withdrawal syndrome in adults (Martsevich et al., 1998). In patients with angina pectoris it was demonstrated that abrupt withdrawal of the Ca^{2+} channel blocker, verapamil, induces severe angina (Subramanian et al., 1983). This phenomenon could be a result of up-regulation of L-type Ca^{2+} channels induced by prolonged treatment with verapamil. The possibility cannot be excluded that up-regulation of L-type Ca^{2+} channels could increase Ca^{2+} influx through these channels, inducing vasospasm and angina pectoris, after drug withdrawal. However, a direct comparison of this and other related information with our results is not yet possible.

In conclusion, although more strict quantitative information is still lacking, such as the whole period of susceptibility to nifedipine, and the influence of up-regulation on the effect of other agonists besides KCl, we believe that the

Table 3
Values for E_{max} and pD_2 for contractions induced by KCl in vas deferens of adult rats treated with nifedipine and respective control

Group	E_{max} (mm)	pD_2
Control[a]	106.9 ± 5.4 (8)	1.28 ± 0.06 (8)
Nifedipine[a]	99.6 ± 8.7 (8)	1.30 ± 0.03 (8)

Values are means ± S.E.M. Number of experiments in parenthesis.
[a] Four-month-old rats were injected subcutaneously with nifedipine (33.3 mg/kg/day) or vehicle (control), during 15 days. The vas deferens was used 1–5 days after the last injection.

present results can contribute to a better understanding of Ca^{2+} channel regulation, not only from the pharmacodynamic point of view, but also as it concerns the extensive clinical use of Ca^{2+} channel antagonists.

Acknowledgements

Financial support for this work was provided by Fundaç ão de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Bartosh, S.M., Aronson, A.J., 1999. Childhood hypertension. Pediatr. Clin. North Am. 46, 235–252.

Caricati-Neto, A., Sette, M., Jurkiewicz, A., 1992. Increased density of α-adrenoceptor in vas deferens of spontaneously hypertensive rats (SHR), indicated by functional and receptor binding studies. Eur. J. Pharmacol. 218, 51–58.

Castillo, C.J.F., Fonteriz, R.I., Lopez, M.G., Rosenheck, K., Garcia, A.G., 1989. (+) PN 200-110 and ouabain binding sites in purified bovine adrenomedullary plasma membranes and chromafin cells. J. Neurochem. 53, 1442–1449.

Castillo, C.J.F., Lafayette, S., Caricati-Neto, A., Sette, M., Jurkiewicz, N.H., Garcia, A.G., Jurkiewicz, A., 1992. Low dihydropyridine receptor density in vasa deferentia of castrated rats. Br. J. Pharmacol. 105, 257–258.

Cingolani, G.E.C., Mosca, S.M., Moreyra, A.E., Cingolani, H.E., 1996. Chronic nifedipine treatment diminishes cardiac inotropic response to nifedipine: functional up-regulation of dihydropyridine receptors. J. Cardiovasc. Pharmacol. 27, 240–246.

Csaba, G., 1981. Ontogeny and Phylogeny of Hormone Receptors. Karger, Basel, pp. 1–172.

Csaba, G., 1986. Receptor ontology and hormonal imprinting. Experientia 42, 750–759.

Csaba, G., Kenéz, C., Karabélyos, C., Dobozy, O., 1995. Effect of nifedipine treatment (imprinting) of rat feti and newborns on the responsiveness of adult rat's uterus. Extension of the imprinting theory. Gen. Pharmacol. 26, 533–536.

Eichmann, D., Hufnagel, M., Quick, P., Santer, R., 1999. Treatment of hyperinsulinaemic hypoglycaemia with nifedipine. Eur. J. Pediatr. 158, 204–206.

Ferrante, J., Triggle, D.J., 1990. Drug- and disease-induced regulation of voltage-dependent calcium channels. Pharmacol. Rev. 42, 29–44.

Gengo, P., Skattebøl, A., Moran, J.F., Gallant, S., Hawthorn, M., Triggle, D.J., 1988. Regulation by chronic drug administration of neuronal and cardiac calcium channel, beta-adrenoceptor and muscarinic receptor levels. Biochem. Pharmacol. 37, 627–633.

Hollenberg, M.D., 1985. Examples of homospecific and heterospecific receptor regulation. TIPS 9, 242–245.

Jurkiewicz, A., Jurkiewicz, N.H., Barros, G.G., Valle, J.R., 1969. Relative responsiveness (ρ) of pharmacological receptor systems in the rat vas deferens. Pharmacology 2, 89–99.

Jurkiewicz, N.H., Jurkiewicz, A., Gomes, C.B., Aucélio, J.G., 1977. Pharmacodynamic analysis of the rat isolated vas deferens after transplantation to intestinal wall. J. Pharmacol. Exp. Ther. 203, 112–119.

Jurkiewicz, A., Lafayette, S.S., Nunes, S.H., Martini, L.C., Carmo, L.G., Wanderley, A.G., Jurkiewicz, N.H., 1994. Decreased density of binding sites for the Ca^{2+} channel antagonist [^{3}H]isradipine after denervation of vas deferens. Eur. J. Pharmacol. 256, 329–333.

Koks, C.A.M., Brolmann, H.A.M., de Kleine, M.J.K., Manger, P.A., 1998. A randomized comparison of nifedipine and ritrodine for suppression of preterm labor. Eur. J. Obstet. Gynecol. Reprod. Biol. 77, 171–176.

Lafayette, S.S.L., 1997. Papel modulador da inervação autonômica e do hormônio sexual masculino sobre os canais de cálcio voltagem-dependentes e sua importância no processo contrátil em ducto deferente de rato. Doctoral thesis, UNIFESP/EPM, São Paulo, Brasil.

Levene, M.I., Gibson, N.A., Fenton, A.C., Papathoma, E., Barnett, D., 1990. The use of a calcium-channel blocker, nicardipine, for severely asphyxiated newborn infants. Dev. Med. Child Neurol. 32, 567–574.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Martsevich, S.Y., Koutishenko, N., Metelitsa, V.I., 1998. Withdrawal effects of antianginal therapy: comparison of isosorbide dinitrate and nifedipine. Int. J. Cardiol. 64, 137–144.

Morgan, P.E., Aiello, E.A., de Cingolani, G.E.C., Mattiazzi, A.R., Cingolani, H.E., 1999. Chronic administration of nifedipine induces up-regulation of functional calcium channels in rat myocardium. J. Mol. Cell. Cardiol. 31, 1873–1883.

Nishiyama, T., Kobayashi, A., Haga, T., Yamazaki, N., 1986. Chronic treatment with nifedipine does not change the number of [^{3}H]nitrendipine and [^{3}H]dihydroalprenolol binding sites. Eur. J. Pharmacol. 121, 167–172.

Noël, F., Quintas, L.E.M., Freitas, E., Caricati-Neto, A., Lafayette, S.S.L., Wanderley, A.G., Jurkiewicz, A., 1998. Quantitative analysis of the high-affinity binding sites for [^{3}H] Ouabain in the rat vas deferens and their immunological identification as the α_2 isoform of Na^+/K^+ ATPase. Biochem. Pharmacol. 55, 1531–1535.

Picarelli, Z.P., Hyppolito, N., Valle, J.R., 1962. Synergistic effect of 5-hydroxytryptamine on the response of rat seminal vesicle to adrenaline and noradrenaline. Arch. Int. Pharmacodyn. 138, 354–363.

Skattebøl, A., Brown, A.M., Triggle, D.J., 1989. Homologous regulation of voltage-dependent calcium channels by 1,4-dihydropyridines. Biochem. Biophys. Res. Commun. 160, 929–936.

Subramanian, V.B., Bowles, M.J., Khurmi, N.S., Davies, A.B., O'Hara, M.J., Raftery, E.B., 1983. Calcium antagonist withdrawal syndrome: objective demonstration with frequency-modulated ambulatory ST-segment monitoring. Br. Med. J. 286, 520–521.

Van Rossum, J.M., 1963. Cumulative dose response curves: II. Techniques for making dose–response curves in isolated organs and the evaluation of drugs parameters. Arch. Int. Pharmacodyn. Ther. 143, 299–330.