0031-3998/91/2903-0278$03.00/0
PEDIATRIC RESEARCH

Vol. 29, No. 3, 1991
Printed in U.S.A.

Differential Ontogeny of *in Vitro* Vascular Responses to Three Categories of Calcium Channel Antagonists in Rats[1]

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ABSTRACT. We examined the ontogeny of relaxation responses to three categories of calcium channel antagonists, represented by verapamil, diltiazem, and nifedipine, for both potential-operated (KCl-mediated) and receptor-operated channels [norepinephrine (NE)-mediated] in rat thoracic aorta. Aortic rings from 2- to 3-d, 1-wk, and 12-wk-old Sprague Dawley rats were mounted in an organ bath, bathed in Krebs' solution, and connected to a force-displacement transducer to measure isometric tension. Endothelium intact vessels at optimal passive force were exposed to a single ED_{50} of isotonic KCl or NE, equilibrium contraction was measured, then vessels were washed and exposed for 30 min to 1 μM verapamil, 1 μM diltiazem, or 0.1 μM nifedipine, followed by another dose of KCl or NE. Verapamil and diltiazem demonstrated significant ($p < 0.05$) age-related increases in effectiveness for blocking KCl-mediated contraction [(% reduction of control contraction ± SEM) (Verapamil: 2–3 d, 67.7 ± 4.2; 1 wk, 72.5 ± 1.8; 12 wk, 89.5 ± 1.0. Diltiazem: 2–3 d, 64.6 ± 2.9; 1 wk, 73.5 ± 3.0; 12 wk, 83.1 ± 1.8]. Nifedipine was equally effective at all ages: 2–3 d, 85.6 ± 1.3; 1 wk, 90.0 ± 1.6; and 12 wk, 91.3 ± 1.4. Verapamil and diltiazem also showed significant age-related increases in effectiveness for blocking NE-mediated contraction (Verapamil: 2–3 d, 6.2 ± 3.9; 1 wk, 28.0 ± 4.8; 12 wk, 44.1 ± 6.0. Diltiazem: 2–3 d, 8.0 ± 3.1; 1 wk, 20.5 ± 3.9; 12 wk, 46.5 ± 4.8). Again, nifedipine was equally effective at all ages: 2–3 d, 42.0 ± 6.8; 1 wk, 35.8 ± 3.9; and 12 wk, 37.5 ± 3.2. In summary, for the categories of calcium channel antagonists that interact at the phenylalkylamine (verapamil) and benzothiazepine (diltiazem) binding sites, there were age-related increases in effectiveness for blocking both potential-operated and receptor-operated channels. However, for nifedipine, which binds to the 1,4-dihydropyridine binding site, no maturational change was observed. These results suggest that the ontogeny of calcium channel antagonists' function may vary depending on the site of binding within the calcium channel. (*Pediatr Res* 29: 278–281, 1991)

Abbreviations

NE, norepinephrine
POC, potential-operated channels
ROC, receptor-operated channels
B_{max}, receptor density
K_d, receptor affinity

Selective calcium channel antagonists bind to specific sites in the calcium channel. Radioligand binding studies (1–3) indicate three discrete, allosterically linked binding sites for the chemical categories phenylalkylamine, benzothiazepine, and 1,4-dihydropyridine. Selective calcium channel antagonists are represented by the three prototype calcium channel antagonists verapamil, diltiazem, and nifedipine, respectively. Receptor characteristics such as K_d and B_{max} have been shown to be affected by a myriad of factors, including ontogeny (4–7). However, some ontogenic studies have clearly indicated that receptor binding by calcium channel antagonists may be temporally unrelated to calcium channel function. For instance, Renaud *et al.* (5) demonstrated that nitrendipine (*i.e.* dihydropyridine) receptors exist in 3-d-old embryonic chick hearts but calcium channels, if present, are not physiologically functional.

We have previously (8) demonstrated the ontogeny of the concentration-dependent response in thoracic aortic rings among 2-d-, 1-wk-, and 12-wk-old rats for both potential-mediated (KCl-induced) and receptor-mediated (NE-induced) contraction. Thus, calcium channel function is present from 2 d of age with perhaps increasing physiologic function during development. However, many aspects of calcium channel function during ontogeny are unknown. We therefore designed the present study to examine one aspect of calcium channel function, *viz.* the ontogeny of responses to three categories of calcium channel antagonists for KCl- and NE-mediated contraction in rat aorta. The objectives were 3-fold: to determine if there were *1*) age-related differences in response to selective calcium channel antagonists; *2*) differential effects among the three categories of calcium channel antagonists; and *3*) differential calcium channel antagonists' effects on POC *versus* ROC.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were sedated with an intramuscular injection of 25 mg/kg ketamine (Vetalar; Parke-Davis, Morris

Received July 12, 1990; accepted October 31, 1990.
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Supported by the U.S. Army Health Services Command, The Research Corporation of the University of Hawaii Leahi Trust, and the Research Board, Kapiolani Medical Center. L.K.K. is the recipient of an American Heart Association, Hawaii Affiliate, Fellowship award.

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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Plains, NJ) and killed with an intraperitoneal injection of 250 mg/kg thiopental sodium (Pentothal; Abbott Laboratories, Irving, TX). Newborns were killed with an intraperitoneal injection of 1 g/kg thiopental. Thoracic aortas were removed from 2- to 3-d- and 6- to 7-d (hereafter referred to as 1-wk)-old rats of either sex and 12-wk-old male rats [respective animal weights: 7.8 ± 0.01 g (n = 40); 13.3 ± 0.3 g (n = 53); and 391.1 ± 5.3 g (n = 57)]. Vessels were placed in cold Krebs' solution (containing in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, di-sodium EDTA 0.025, and glucose 11), cleaned of connective tissue, and cut into rings of 4-mm width. Rings were mounted on two fine stainless steel wires that were passed through the lumen and connected to a Grass FT.03 force transducer (Grass Instrument Co., Quincy, MA) coupled to a Gould 2600S pen recorder (Gould Inc., Cleveland, OH) for continuous measurement of isometric force. Aortas were suspended in an organ bath with 25 mL of Krebs' solution (37°C; pH 7.4), gassed with 95% O_2-5% CO_2, and equilibrated for 60 min with a bath change every 15 min. Vessels were stretched to the optimal point on their length tension curve determined by equilibrium tension developed to a ED_{50} of NE (8, 9). Optimal passive forces were: 2–3 d, 0.32 ± 0.01 g; 1 wk, 0.38 ± 0.01 g; and 12 wk, 3.2 ± 0.03 g. Endothelial function was assessed by observing relaxation upon addition of 10 μM acetylcholine to rings preconstricted with a ED_{50} of NE. All experiments were performed with endothelium-intact vessels.

This study was approved by the Animal Care and Use Committee, Tripler Army Medical Center. Procedures on the rats were in accordance with National Institutes of Health policies and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985).

Preliminary experiments. Before assessing the effects of calcium channel antagonists on single-dose KCl or NE contractions, it was established that control contractions to both KCl and NE were reproducible (time controls). Vessels at optimal passive force were exposed to a single ED_{50} of isotonic KCl or NE and the equilibrium response allowed to develop. Vessels were then washed until optimal passive force was reattained and stabilized. After 30 min in control Krebs', the equilibrium response (R_2) as a percentage of the initial equilibrium response (R_1) was calculated ($R_2/R_1 \times 100\%$): KCl: 2–3 d, 110 ± 5 (n = 14); 1 wk, 99 ± 3 (n = 5); 12 wk, 111 (n = 2); NE: 2–3 d, 109 ± 3 (n = 9); 1 wk, 104 ± 6 (n = 6); 12 wk, 112 ± 4 (n = 6).

Because KCl-induced contractions are dependent on extracellular calcium influx, virtually complete blockage of contraction can be obtained with calcium channel antagonists. Residual contraction is due to the hypertonicity induced by cumulative addition of KCl to obtain the concentration response curve (10). A KCl dose-response curve in the absence (control) and presence of 0.01 μM, 0.1 μM, and 1 μM verapamil is shown in Figure 1. Very similar results using verapamil in aortas from adult male Wistar rats were reported by Koch *et al.* (11). These authors also found that both 0.1 and 1 μM nifedipine equally inhibited KCl-induced contraction. For diltiazem, inhibition of intracellular calcium release during NE-induced contraction has been reported (12) for concentrations above 1 μM. Based on our results and such information in the literature (11, 12), doses of calcium channel antagonists selected for this study were 1 μM verapamil, 1 μM diltiazem, and 0.1 μM nifedipine. These doses blocked approximately 90% of a single isotonic ED_{50} of KCl in aortas from 12-wk-old rats.

Experimental protocol. After a ED_{50} of either isotonic KCl (NaCl replaced with equimolar KCl) or NE, equilibrium control sustained contraction was allowed to develop. Preparations were washed until optimal passive force was reattained and stabilized. Preparations were then exposed for 30 min to the selected calcium channel antagonist, after which the ED_{50} of isotonic KCl or NE was repeated.

Drugs. The following drugs were used: NE, acetylcholine, verapamil, diltiazem (Sigma Chemical Co., St. Louis, MO), and nifedipine (Pfizer, New York, NY). NE, acetylcholine, verapamil, and diltiazem stock solutions (concentration not <0.5 mg/mL) were made in distilled water and stored at −20°C. Nifedipine was dissolved in 100% ethanol and stored at −80°C. Aliquots were freshly diluted daily in distilled water. Each dose was administered in 100-μL aliquots, and drug concentrations are expressed as final molar concentration in the bath. Experiments with nifedipine were performed in a darkened room.

Statistical analysis. Two-way analysis of variance with Duncan's multiple range post hoc tests (13) were performed to determine differences among ages and calcium channel antagonists, with $p < 0.05$ considered significant. Data are expressed as means ± SEM.

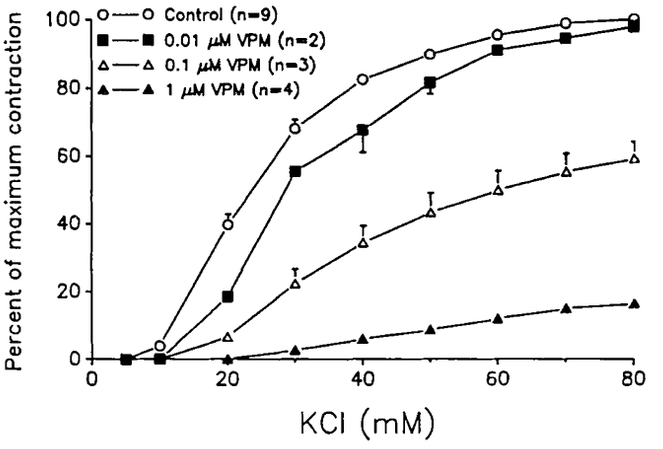

Fig. 1. Effect of verapamil (*VPM*) or KCl-induced contraction in 12-wk rat thoracic aortic rings. Values are means ± SEM; n = number of aortic rings.

Table 1. *Equilibrium control contraction in rat thoracic aorta**

	2–3 d	1 wk	12 wk
KCl	0.25 ± 0.03 (21) [50 mM]	0.41 ± 0.02 (23) [40 mM]	2.43 ± 0.13 (26) [20–25 mM]
NE [0.03 μM]	0.22 ± 0.02 (19)	0.38 ± 0.02 (30)	2.94 ± 0.11 (31)†

* Units are mean g ± SEM; () = number of aortic rings; [] = dose administered.

† Significant difference between KCl and NE at 12 wk ($p < 0.05$).

RESULTS

Because the amount of induced tone may affect relaxation (14), within each age group we attempted to maintain a relatively constant single-dose control contraction to KCl and NE. Shown in Table 1 are the control equilibrium contractions with the doses of KCl and NE used. For all ages, both isotonic KCl concentrations (2–3 d, 50 mM; 1 wk, 40 mM; 12 wk, 20–25 mM) and NE concentration (0.03 μM) approximated the ED_{50} values obtained in our previous study (8).

At 2–3 d and 1 wk, we used male and female rats. No significant differences were noted between males and females in percent reduction with any of the three calcium channel antagonists for either KCl- or NE-induced contraction at either age. For example, percent reduction with verapamil for KCl was: 2–3 d, female, 66 (n = 2); male, 68 ± 13 (n = 5); 1 wk, female, 71 ± 6 (n = 4); male 74 ± 4 (n = 4). Percent reduction with verapamil for NE was: 2–3 d, female, 6 ± 10 (n = 3); male, 6 ± 11 (n = 3); 1 wk, female, 28 ± 18 (n = 8); male, 29 ± 13 (n = 5); $p > 0.05$.

Effect of verapamil, diltiazem, and nifedipine on KCl-induced contraction (Fig. 2). Both verapamil and diltiazem demonstrated significant age-related increases in effectiveness in blocking KCl-induced contractions. With verapamil, percent reduction of con-

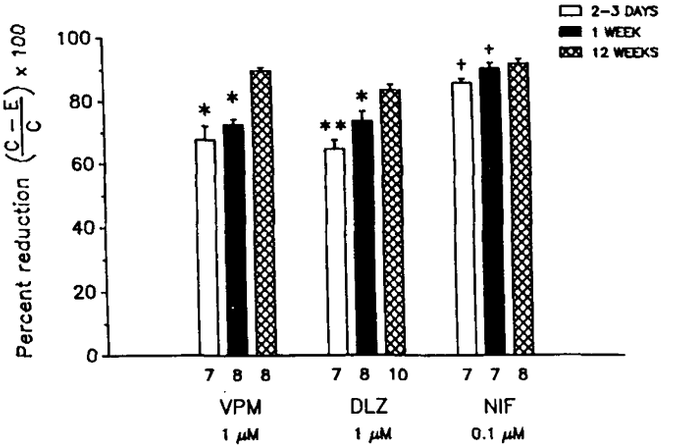

Fig. 2. Effect of verapamil (*VPM*), diltiazem (*DLZ*), and nifedipine (*NIF*) on KCl-induced contraction in 2- to 3-d, 1-wk, and 12-wk rat thoracic aortic rings. Values are means ± SEM; *numbers under bars* = number of rats. *, Significantly less than 12 wk, same calcium channel antagonists; **, significantly less than both 1 wk and 12 wk, same calcium channel antagonists; +, significantly greater than verapamil and diltiazem, same age (all $p < 0.05$).

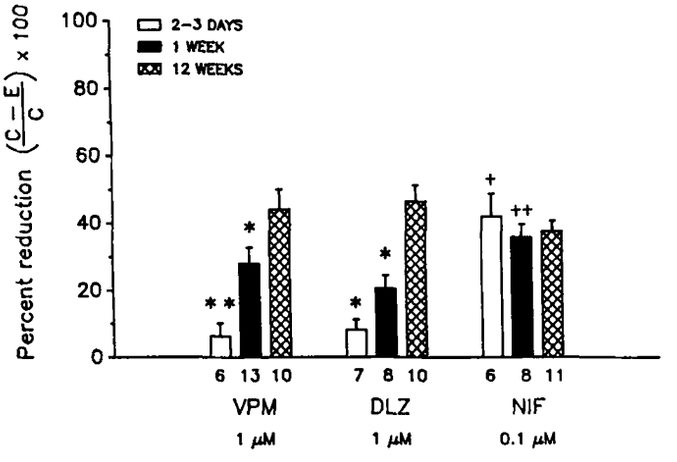

Fig. 3. Effect of verapamil (*VPM*), diltiazem (*DLZ*), and nifedipine (*NIF*) on NE-induced contraction in 2- to 3-d, 1-wk, and 12-wk rat thoracic aortic rings. Values are means ± SEM; *numbers under bars* = number of rats. *, Significantly less than 12 wk, same calcium channel antagonists; **, significantly less than both 1 wk and 12 wk, same calcium channel antagonists; +, significantly greater than verapamil and diltiazem, same age; ++, significantly greater than diltiazem, same age (all $p < 0.05$).

trol contraction at 2–3 d was 67.7 ± 4.2%, at 1 wk 72.5 ± 1.8%, and at 12 wk 89.5 ± 1.0%. Similarly, with diltiazem the percent reduction at 2–3 d was 64.6 ± 2.9%, at 1 wk 73.5 ± 3.0%, and at 12 wk 83.1 ± 1.8%. There was a significant increase in percent reduction between 2 and 3 d and 1 wk for diltiazem but not for verapamil and between 1 and 12 wk for both antagonists ($p < 0.05$). On the other hand, nifedipine was equally effective at all ages (2–3 d, 85.6 ± 1.3%; 1 wk, 90.0 ± 1.6%; 12 wk, 91.3 ± 1.4% reduction, $p > 0.05$). In addition, nifedipine was significantly more effective than both verapamil and diltiazem in reducing contraction at 2–3 d and 1 wk ($p < 0.05$).

Effect of verapamil, diltiazem, and nifedipine on NE-induced contraction (Fig. 3). Similar to their effect on KCl-induced contraction, both verapamil and diltiazem demonstrated significant age-related increases in effectiveness in blocking NE-induced contractions. For verapamil, the percent reduction at 2–3 d was 6.2 ± 3.9%, at 1 wk 28.0 ± 4.8%, and at 12 wk 44.1 ± 6.0%. With diltiazem, the percent reduction at 2–3 d was 8.0 ± 3.1%, at 1 wk 20.5 ± 3.9%, and at 12 wk 46.5 ± 4.8%. There was a significant increase in percent reduction between 2 and 3 d and 1 wk for verapamil but not diltiazem, and between 1 and 12 w for both antagonists ($p < 0.05$). In contrast, nifedipine was again equally effective at all ages (2–3 d, 42.0 ± 6.8%; 1 wk, 35.8 ± 3.9%; 12 wk, 37.5 ± 3.2% reduction). Nifedipine was significantly more effective than both verapamil and diltiazem in reducing contraction at 2–3 d, and significantly more effective than diltiazem at 1 wk in reducing contraction ($p < 0.05$).

DISCUSSION

Results herein contribute new and important information regarding the ontogeny of functional responses to calcium channel antagonists. Much of previous work on the influence of age on calcium channel antagonist effects in rat aorta has been on aging (15, 16), not ontogeny. Moreover, previous work on ontogeny of calcium channel antagonists has employed radioligand binding studies to examine changes in calcium K_d and B_{max}. Studies on perinatal development of ^{3}H-nitrendipine (dihydropyridine) binding in several rat tissues indicate that increases in B_{max} occur without changes in K_d (4). Increases in B_{max} during embryonic development in chick heart (5) and skeletal muscle (6), with an immediate rise in K_d at hatching, have been reported. Although these studies give an indication that changes in binding site characteristics occur during ontogeny, they have not provided insight into the functional physiologic relevance of such changes, particularly in light of studies indicating that receptor binding and calcium channel function may be temporally unrelated (5). However, Hyman *et al.* (17) examined both dihydropyridine binding site changes and function in the neonatal period and found age-related increases in B_{max} in isolated cells from rabbit stomach antrum, which is highly dependent on extracellular calcium to support contraction, and an age-related increase in contraction in isolated strips from antrum. These authors found no age-related increase in B_{max} in the fundus, which utilizes intracellular calcium stores.

Present results demonstrate an increase in sensitivity to two categories of calcium channel antagonists during the same time period in rats when contractile responses to KCl and NE are rapidly developing (8). In contrast, results employing a third category of calcium channel antagonists, represented by nifedipine, indicate a different ontogeny than simply a generalized increase in calcium channel antagonists function, inasmuch as nifedipine was equally effective at 2–3 d through 12 wk.

Multiple mechanisms may be invoked to explain these observations. For example, these developmental changes may result from alterations in binding site characteristics. Most ontogenic studies of calcium channel antagonists binding sites have been performed using calcium channel antagonists that bind to the dihydropyridine binding site. Binding sites for verapamil and diltiazem are in close proximity, more so than either of them are to the nifedipine (dihydropyridine) binding site, although all three are allosterically linked (18). In fact, Murphy *et al.* have proposed (19) that all nondihydropyridine calcium channel antagonists act at a single site that is allosterically linked to the dihydropyridine binding site. Later studies, however, have emphasized differences between verapamil and diltiazem binding sites (1, 2). No information is available as to the ontogeny of the verapamil and diltiazem binding sites and it is possible that they may differentiate and/or become distinct during ontogeny.

Ontogenic changes in calcium channel antagonists' function may also be due to other factors, such as differential access of calcium channel antagonists to their binding sites. It is of interest to note that both verapamil and diltiazem are classified as "use-" or "state-dependent", *i.e.* lipid penetration of these ionized drugs through the bilipid membrane is so low that entry to the calcium channel is dependent upon the state of the channel (*i.e.* open, resting, inactivated), which in turn depends on the membrane potential (20). Although access of neutral dihydropyridines

such as nifedipine is also voltage-dependent, such compounds, being lipophilic, may reach the calcium channel antagonist binding sites by penetration of the lipid bilayer as well. If access of verapamil and diltiazem to calcium channel antagonist binding sites is state-dependent, age-related alterations in this parameter would presumably affect verapamil and diltiazem similarly and to a greater extent than nifedipine. Thus, functional changes in calcium channel antagonists' effectiveness could occur without concomitant alterations in binding site characteristics.

There remains an ongoing debate as to whether POC and ROC are indeed distinct entities (10, 21). Chiu *et al.* (21) demonstrated in adult rat aorta that both POC and ROC exhibited the same sensitivity to inhibition by calcium channel antagonists (nifedipine and verapamil) of ^{45}Ca influxes and suggested that POC and ROC in rat aorta share similar structural characteristics, but are gated separately and distinctly by their respective activators. Results herein demonstrate a qualitatively similar pattern of calcium channel antagonist effects between contractions mediated through POC and ROC, supporting the concept that these channels share similar functional and possibly structural characteristics. In addition, our previous work (8), which examined summation effects of POC- and ROC-mediated contractile antagonists, supports the concept of separate and distinct gating mechanisms.

Finally, in addition to agonist effect at the cell membrane, contractile agonist sensitivity to calcium channel antagonists depends also on the extent to which that agonist mobilizes intracellular calcium (22). An expected finding was the reduced overall effectiveness of calcium channel antagonists during NE-induced contraction, which depends in part on intracellular Ca^{2+}, compared to KCl-induced tone at all ages. However, although an age-related change in the ratio of extra/intracellular calcium utilization could explain our results with verapamil and diltiazem, it does not explain the results with nifedipine.

In summary, our results demonstrate that in rat aorta between 2 d and 12 wks: *1*) there is a marked ontogeny of increasing responsiveness to the two categories of calcium channel antagonists, represented by verapamil and diltiazem, that are generally classified as use-dependent; *2*) the third category of calcium channel antagonists (*i.e.* nifedipine), however, demonstrates no age-related change in effectiveness; and *3*) this pattern is qualitatively similar for both KCl- and NE-induced contractions.

The clinical use of currently available calcium channel antagonists (18, 23) and development of new selective calcium channel antagonists with perhaps new therapeutic indications requires an awareness of basic developmental aspects of calcium channel function. Results herein strongly suggest that the ontogeny of calcium channel antagonist function may vary depending on the site of binding within the calcium channel.

Acknowledgments. The authors thank Dr. E. M. Weiss, Pfizer Laboratories, for the generous supply of nifedipine; Drs. R. A. Hebden and C. F. T. Uyehara for their critical reading of the manuscript; and C. F. T. Uyehara for help with statistical analysis.

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