Effect of Calcium Channel Blockers on Serotonin Uptake (43043)

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Abstract. **We previously demonstrated that verapamil inhibits serotonin uptake by bovine pulmonary arterial endothelial cells by a mechanism not involving alterations in calcium fluxes. In this study, we determine whether verapamil inhibition of serotonin uptake occurs in other pulmonary cell types (bovine pulmonary artery smooth muscle cells), in cells from other organs and species (rat epididymal endothelial cells), and in intact organs (isolated rat lungs). We also compare the effects of verapamil with those of nifedipine and diltiazem. At concentrations of 10^{-6} *M* or greater, verapamil is an inhibitor of serotonin uptake by cultured cells and isolated lungs. Nifedipine and diltiazem are weak inhibitors of serotonin uptake by cultured bovine cells only at suprapharmacologic doses and have no effect on serotonin uptake by isolated lungs. Surprisingly, nifedipine stimulates serotonin uptake by rat epididymal endothelial cells. We conclude that inhibition of serotonin uptake by verapamil is a generalized phenomenon, occurring in a variety of cell types, in intact organs, and in different species that does not occur consistently with other calcium channel blockers.** [P.S.E.B.M. 1990, Vol 193]

In previous studies, we have demonstrated that serotonin (5-hydroxytryptamine [5-HT]) uptake by bovine pulmonary artery endothelial cells in culture occurs by both high affinity and diffusion processes (1), and that exposure of these cells to hypoxia or anoxia for 24 to 48 hr results in a several-fold stimulation of the high affinity 5-HT uptake (2). We have also shown previously that the high affinity serotonin uptake by endothelial cells and its stimulation by anoxia are competitively inhibited by verapamil without alterations in calcium fluxes (3). This latter observation relating to verapamil suggests a novel action unrelated to calcium blockade and raises the possibility that verapamil occupies a receptor or transporter site on the endothelial cell similar to that of 5-HT.

In the present investigation, we asked whether this action of verapamil was unique to bovine endothelial cells or whether it occurred in smooth muscle cells or endothelial cells of the rat. We also sought to determine whether verapamil inhibition of serotonin uptake occurred in an intact organ. We selected the isolated rat lung because our earlier studies were on cultured pulmonary artery endothelial cells (3) and the lungs avidly remove serotonin from the circulation (4). Finally, in order to determine whether other drugs classified as calcium channel blockers might also inhibit 5-HT uptake, we compared the effects of verapamil to those of nifedipine and diltiazem.

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Materials and Methods

Cell Culture. A modification of the method of Ross (5) was used for the isolation and culture of smooth muscle cells. Bovine pulmonary arteries were rinsed twice with Dulbecco's phosphate-buffered saline at 37°C containing antibiotics (300 units/ml penicillin G potassium, 300 μg/ml streptomycin sulfate, and 1.25 μg/ml Amphotericin B) and slit longitudinally on a sterile petri dish. The luminal side of the vessel was scraped with a scapel to remove endothelial cells and the abluminal side was peeled to remove connective tissue. The medial layer was peeled away and cut into 1-cm^2 strips. These explants were then placed intimal side down in a Falcon 6-well plate for 10 min. Three milliliters of RPMI 1640 containing 10% fetal bovine serum were then added to each well and the explants were incubated in a CO_2 intubator for 7 days without disturbance. Pulmonary arterial smooth muscle cells (PASMC) migrated out from the explant after 1 week in culture. The vessel explants were removed from the wells and cells were fed every 2–3 days until they

reached confluence. Cells were stored in liquid N_2 for subcultures. First to third passaged cells after the explant were used in these experiments. Smooth muscle cells were characterized by morphology and fluorescent antimuscle actin staining of first passage cells with antibody that was a gift from Dr. Ira Herman of Tufts University School of Medicine.

Bovine pulmonary arterial endothelial cells (BPAEC), used for comparison with PASMC, were cultured as described previously (6). Rat epididymal endothelial cells (REEC) were a gift from Dr. John Castellot of Harvard University School of Medicine. Endothelial cells (EC) were cultured utilizing the same conditions and medium as for PASMC. Tenth to twelfth passaged cells were used in the study.

Measurement of Serotonin Uptake by Cells. Serotonin uptake was measured as described previously (1). The PASMC or EC monolayer was rinsed twice with phosphate-buffered saline containing 15 m*M* dextrose (pH 7.4) and was incubated for 30 min in this solution containing 10^{-4} *M* iproniazid to block monamine oxidase activity. [5-*hydroxy*-G-^{3}H]Tryptamine creatinine sulfate (sp act, 28 Ci/n*M*; New England Nuclear, Boston, MA) ([^{3}H]5-HT) was added from a stock solution containing ascorbic acid (10 μg/ml) and EDTA (10 μg/ml) as antioxidants. The uptake of 5-HT (1.5×10^{-8} *M*) was saturated in 30 min for PASMC and in 45–60 min for EC. Therefore, incubations were carried out for 10 min for bovine PASMC (BPASMC) and 30 min for EC. Under these conditions, radioactivity taken up by cells was no more than 1% of that in the medium. After the incubation, medium was removed and monolayers were washed three times with ice-cold phosphate-buffered saline plus 15 m*M* dextrose. Smooth muscle cells were scraped off the dishes and suspended in 1 ml of 0.02 *M* potassium phosphate buffer (pH 8.3) by sonication. A 0.5-ml aliquot was dissolved in 10 ml of Aquasol II and the radioactivity was counted in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Irvine, CA). Cell protein was measured according to the method of Lowry *et al.* (7). Uptake of serotonin was expressed as nmol/mg protein/10 min for PASMC and per 30 min for EC.

Lung Isolation and Perfusion. Male Sprague-Dawley rats (200–250 g) were obtained from Charles River Laboratories (Wilmington, MA) and kept in standard cages with free access to food and water until the day of sacrifice. Following anesthesia (pentobarbital, 30 mg/kg ip), rats underwent tracheostomy and median sternotomy. The pulmonary artery and left ventricle were cannulated and the heart and lungs were removed en bloc as described previously (8). Lungs were ventilated with 95% air-5% CO_2 using a rodent ventilator (Harvard Apparatus, Natick, MA) at 64 breaths/min. Peak inspiratory pressure was 9 cm H_2O and end-expiratory pressure was 2.5 cm H_2O. Lungs were perfused at a rate of 0.04 ml/g body wt/min with Krebs-Henseleit solution (pH 7.4) containing 3% bovine serum albumin while mean pulmonary arterial pressure was monitored. The pH in effluent perfusate was measured at the start of each experiment and was maintained between 7.33 and 7.43, and the lung was weighed continuously using a force-displacement transducer (FT 0.03; Grass Instruments, Quincy, MA). At the end of each experiment, a single bolus of cardiogreen dye was injected intra-arterially to confirm even distribution of perfusate. Lungs were discarded if mean pulmonary arterial pressure or lung weight increased more than 3 mm Hg or 500 mg, respectively, during the course of the experiment, or if dye distribution was uneven. After completion of the experiment, lungs were dissected from the heart, weighed wet, allowed to dry in a 60°C oven for 48 hr, and reweighed for determination of wet to dry weight ratios.

Serotonin Uptake by Lung. Following a 20-min equilibration period, [^{3}H]5-HT (1.5×10^{-7} *M*) in 0.1 ml of saline was infused intra-arterially for 2 min and lung effluent was collected in a fraction collector (Gilson Instruments) for 3 min at 15 fractions/min. Single vials were counted in a liquid scintillation counter for determination of the effluent curve. Percentage of uptake of serotonin was calculated as total effluent counts subtracted from total counts in the injectate divided by total counts in injectate.

Effects of Verapamil, Nifedipine, and Diltiazem on Serotonin Uptake by Lung and Cultured Cells. To test the effect of calcium channel blockers on 5-HT uptake in isolated lungs, we added verapamil or diltiazem dissolved in saline, or nifedipine dissolved in dimethyl sulfoxide (1%) to the perfusate containing [^{3}H]5-HT. For each isolated lung, infusions of [^{3}H]5-HT were repeated every 15 min for a total of three injections. The first infusion was performed without a calcium channel blocker, to serve as a control. For the next two infusions, calcium channel blockers were added at perfusate concentrations of 10^{-5} *M* and 10^{-3} *M*, respectively. To determine the threshold concentration for inhibition of serotonin uptake by verapamil, concentrations of 10^{-7} and 10^{-6} *M* verapamil were also used in some experiments. Effluent radioactivity curves were performed following each [^{3}H]5-HT injection, as described above. To control for the effects of time and repetition of injections, a separate group of lungs received three repeated [^{3}H]5-HT infusions every 15 min in the absence of calcium channel blockers. In cultured cell systems, calcium channel blockers were added 20 min prior to [^{3}H]5-HT in the same range of concentrations as used for isolated lungs. Control solutions containing dimethyl sulfoxide (1%) had no effect on serotonin uptake.

Statistics. Cell culture experiments were repeated at least three times. Differences between control and

treated groups were analyzed for statistical significance using Student's t test or one-way analysis of variance for comparison of multiple group means. When F ratios indicated statistically significant differences, differences between individual group means were assessed using the Student-Newman-Keuls test (9). Data are expressed as mean ± SE, and differences were considered significant when $P < 0.05$.

Results

Cell Culture Studies. Table I shows the effects of nifedipine, diltiazem, and verapamil in various concentrations on serotonin uptake by BPAEC, PASMC, and REEC. Uptakes of 5-HT by BPAEC and PASMC were similar in magnitude, but that of REEC was considerably higher when compared with the other cells. Nifedipine and diltiazem brought about a slight but statistically significant reduction in serotonin uptake at concentrations of 10^{-5} and 10^{-3} M in BPAEC and at concentrations of 10^{-5}, 10^{-4}, and 10^{-3} M in BPASMC. For REEC, nifedipine had a stimulatory effect on serotonin uptake at concentrations of 10^{-4} and 10^{-5} M, and there was a slight inhibition of serotonin uptake by diltiazem at 10^{-4} M. In contrast, concentrations of verapamil that were comparable to the lowest concentrations of nifedipine and diltiazem used brought about a marked reduction in serotonin uptake for all cell types. Figure 1 shows that the threshold concentration for inhibition of serotonin uptake by verapamil in REEC occurs at approximately 10^{-6} M, similar to that for BPAEC and BPASMC, as reported previously (3, 10).

Isolated Lung Studies. Mean pulmonary arterial perfusion pressures, lung wet weights, dry weights, and wet to dry weight ratios did not differ among groups of lungs (Table II). Table III shows the effect of increasing concentrations of calcium channel blockers on fractional serotonin uptake by the isolated rat lungs. The first uptake measurement in each experiment was done without a calcium channel blocker and served as a control. As anticipated, uptake did not differ among the groups. For the second and third uptake measurements, calcium channel blocker concentrations of 10^{-5} and 10^{-3} M, respectively, were added to the perfusate. There was a slight reduction in serotonin uptake in the control group of lungs during the third uptake experiment compared with the second. Nifedipine and diltiazem had no effect on serotonin uptake at either concentration compared with the respective control measurements. Verapamil, on the other hand, significantly reduced serotonin uptake at both perfusate concentrations compared with controls. Figure 2 shows that the threshold concentration for inhibition of serotonin uptake by verapamil in isolated lungs was similar to that for REEC, between 10^{-7} and 10^{-5} M.

Table I. Effects of Calcium Channel Blockers on Serotonin Uptake by Endothelial and Smooth Muscle Cells

	5-HT uptake (pmol/mg protein)[a]		
	BPAEC	PASMC	REEC
Control	0.44 ± 0.03[b]	0.52 ± 0.03[c]	5.32 ± 0.63[c]
Nifedipine			
10^{-5} M	0.32 ± 0.01[c]	0.39 ± 0.01[c]	8.15 ± 0.72[c]
10^{-4} M	0.39 ± 0.01	0.35 ± 0.01[c]	8.97 ± 0.45[c]
10^{-3} M	0.31 ± 0.03[c]	0.27 ± 0.01[c]	
Diltiazem			
10^{-5} M	0.35 ± 0.01[c]	0.42 ± 0.03[c]	4.93 ± 0.32
10^{-4} M	0.38 ± 0.01	0.36 ± 0.02[c]	3.80 ± 0.33[c]
10^{-3} M	0.20 ± 0.01[c]	0.19 ± 0.01[c]	
Verapamil			
5×10^{-5} M	0.16 ± 0.01[c]	0.11 ± 0.01[c]	1.05 ± 0.08[c]

[a] Endothelial cells, 30-min uptake; smooth muscle cells, 10-min uptake.
[b] Mean ± SE ($n = 4$).
[c] Significantly different from control, $P < 0.05$.

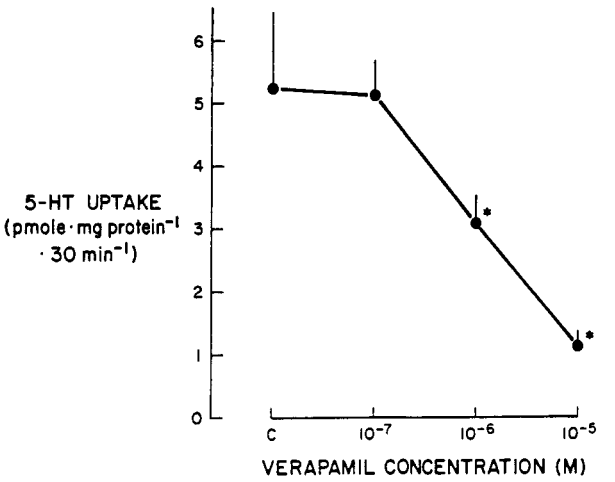

Figure 1. Effect of increasing concentrations of verapamil is shown on 5-HT uptake by rat epididymal endothelial cells in culture. $n = 4$ at each concentration. Asterisk indicates $P < 0.05$ compared with control.

Discussion

Our study demonstrates that verapamil is an effective inhibitor of serotonin uptake not only in BPAEC, as we reported previously (3), but also in BPASMC, REEC, and isolated rat lungs. Thus, the inhibitory effect of verapamil on serotonin uptake is not species dependent, is not confined to endothelial cells, and is not a phenomenon confined to cell culture. These results also extend previous observations by other investigators that verapamil inhibits high affinity serotonin uptake by rat brain synaptosomes (11), isolated rabbit aorta (12), and rat brain and human platelets (13). Using Scatchard and Lineweaver-Burk analyses, the latter investigators concluded, as we have previously (3), that verapamil is a competitive inhibitor of serotonin uptake.

The above findings suggest that verapamil occupies

Table II. Mean Perfusion Pressures, Wet and Dry Lung Weights, and Wet to Dry Weight Ratios in Isolated Rat Lungs

Group	*n*	Mean pulmonary arterial pressure (mm Hg)	Wet lung weight (g)	Dry lung weight (g)	Wet/dry
Control	4	6.9 ± 0.7	1.37 ± 0.16	0.20 ± 0.3	6.9 ± 0.7
Verapamil	5	7.2 ± 0.4	1.39 ± 0.22	0.19 ± 0.2	7.1 ± 0.4
Nifedipine	4	6.9 ± 0.4	1.20 ± 0.12	0.18 ± 0.2	6.9 ± 0.4
Diltiazem	3	6.8 ± 0.3	1.15 ± 0.19	0.17 ± 0.2	6.8 ± 0.7

Table III. Effect of Calcium Channel Blockers on Fractional 5-HT Uptake (%) by Rat Lungs

Measurement	% Uptake of 5-HT		
	1 (K-H)[a]	2 (10^{-5})[b]	3 (10^{-3})[b]
Control (4)[c]	54.0 ± 1.7	49.5 ± 1.6	43.1 ± 4.8
Nifedipine (4)	54.2 ± 2.7	50.5 ± 1.7	41.1 ± 3.0
Diltiazem (3)	49.2 ± 3.1	50.9 ± 2.2	41.9 ± 4.2
Verapamil (3–5)	54.4 ± 2.3	35.8 ± 4.7[d]	29.7 ± 1.6[d]

[a] Krebs-Henseleit perfusate with 3% bovine serum albumin.
[b] Concentration of calcium channel blocker.
[c] Numbers in parenthesis, number of lungs.
[d] $P < 0.05$ compared with control.

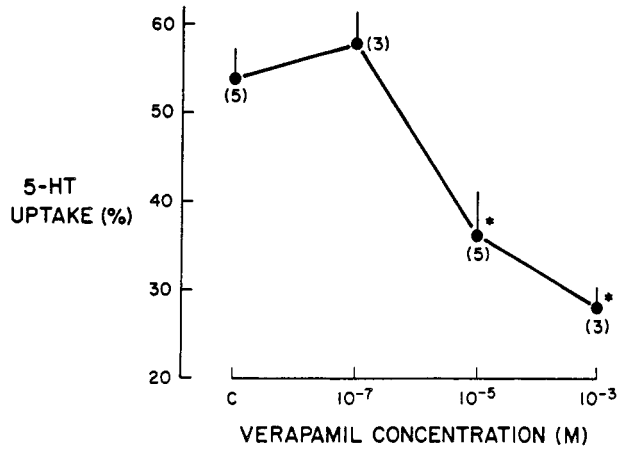

Figure 2. Effect of increasing perfusated verapamil concentrations is shown on 5-HT uptake by isolated rat lungs. Number in parentheses is number of lungs. Asterisk indicates $P < 0.05$ compared with control.

a receptor or transporter site not only on the surface of cultured endothelial cells, but also on vascular cells in general, that is very similar to that of serotonin. The verapamil concentration that inhibits serotonin uptake by 50% (IC_{50}) was 10^{-6} *M* in our previous study (3), compatible with our current findings. This IC_{50} corresponds to plasma levels reported clinically of 10^{-6} to 10^{-7} *M* (14) and raises the possibility that certain actions of verapamil heretofore ascribed to blockade of calcium channels may actually be due to inhibition of serotonin uptake.

Nifedipine and diltiazem also inhibited serotonin uptake in BPAEC and PASMC, but their inhibitory effect was small in comparison to that of verapamil and there was no dose-response relationship. These observations suggest a nonspecific effect occurring only at very high, probably toxic concentrations. The possibility that alterations in calcium fluxes were associated with this inhibition was not investigated in the current study. Surprisingly, nifedipine stimulated serotonin uptake in REEC. This finding, coupled with the observation that serotonin uptake was 10-fold greater in REEC than in either of the cells of bovine origin, suggests that there may be important organ- or species-related differences in the serotonin transporter, but these differences do not apply to verapamil.

In our previous investigation (3), we demonstrated that inhibition of serotonin uptake by verapamil occurred without alteration of calcium channel fluxes. The much diminished inhibitory effect of nifedipine and diltiazem on serotonin uptake compared with that of verapamil is compatible with the hypothesis that verapamil inhibition of serotonin uptake is not dependent upon calcium channel inhibition. Rehavi *et al.* (13) also observed that verapamil inhibition of serotonin uptake by rat brain synaptosomes and human platelets was not dependent upon calcium ion concentration.

In our isolated lung studies, slight reductions in uptake occurred during the second and third uptake measurements of each experiment during perfusion with nifedipine and diltiazem. However, the same reductions occurred during repeated uptake measurements in controls. These reductions were probably related to development of mild edema in the lungs and a small amount of residual radioactivity in the perfusate at the beginning of the second and third collections that slightly increased the total amount of radioactivity in the collected perfusate. Notably, significant reductions in serotonin uptake compared with timed controls occurred only during perfusion with verapamil.

The threshold concentration of verapamil for inhibition of serotonin uptake by isolated rat lungs in the present study was approximately 10^{-6} *M*, a level that is comparable to the threshold concentration of verapamil that inhibits hypoxic pulmonary vasoconstriction in

isolated rat lungs (15). Our previous studies with endothelial cells detected no alteration of calcium influx or efflux in endothelial cells exposed to verapamil concentrations as high as 5×10^{-5} *M*. Thus, the inhibitory effect of verapamil on hypoxic vasoconstriction is unlikely to be mediated by a mechanism involving calcium channel blockade of the endothelium. The verapamil effect on hypoxic pulmonary vessels is more likely mediated via inhibition of voltage-dependent calcium channels in smooth muscle (16). It is unknown at the present time what role, if any, verapamil inhibition of serotonin uptake may play in the hypoxic response. Nifedipine (17) has also been shown to inhibit hypoxic pulmonary vasoconstriction in a variety of species, consistent with the involvement of calcium channel fluxes in the mediation of hypoxic pulmonary vasoconstriction.

This investigation extends our previous observations that verapamil specifically and competitively inhibits serotonin uptake by bovine pulmonary artery endothelial and smooth muscle cells. The finding is reproducible in cultured cells from other organs and species and in intact lungs. Similar findings by other investigators suggest that verapamil binds to the serotonin transporter in a wide variety of cell types and species. Other calcium channel blockers have inconsistent effects on serotonin uptake by cells and intact lungs. Further investigation will be necessary to determine whether specific pharmacologic actions of verapamil can be ascribed to its inhibition of serotonin uptake.

1. Lee SL, Fanburg BL. Serotonin uptake by bovine pulmonary artery endothelial cells in culture. I. Characterization. Am J Physiol **250**:C761–C765, 1986.
2. Lee SL, Fanburg BL. Serotonin uptake by bovine pulmonary artery endothelial cells in culture. II. Stimulation by hypoxia. Am J Physiol **250**:C766–C770, 1986.
3. Lee SL, Long K, Ueda S, Fanburg BL. Verapamil inhibits serotonin uptake of endothelial cells in culture by a mechanism unrelated to Ca^{++} channel blockade. J Cell Physiol **32**:178–182, 1987.
4. Junod AF. Uptake, metabolism and efflux of ^{14}C-5-hydroxytryptamine in isolated perfused rat lungs. J Pharmacol Exp Ther **183**:341–355, 1972.
5. Ross R. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. J Cell Biol **50**:172–186, 1971.
6. Lee SL, Douglas WHJ, Lynn PS, Fanburg BL. Ultrastructural changes of bovine pulmonary artery endothelial cells radiated in vitro with a ^{137}C source. Tissue Cell **15**:193–204, 1983.
7. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:165–175, 1951.
8. Hill NS, Ou LC. The role of pulmonary vascular responses to chronic hypoxia in the development of chronic mountain sickness in rats. Respir Physiol **58**:171–185, 1984.
9. Steele RCD, Torrie JH. Principles and Procedures of Statistics. New York: McGraw-Hill, 1960.
10. Lee SL, Dunn J, Yu FS, Fanburg BL. Serotonin uptake and configurational change of bovine pulmonary artery smooth muscle cells in culture. J Cell Physiol **138**:145–153, 1989.
11. Brown NL, Sirugue O, Worcel M. The effects of some slow channel blocking drugs on high affinity serotonin uptake by rat brain synaptosomes. Eur J Pharmacol **123**:161–165, 1986.
12. Auguet M, Delaflotte S, Clostre R, DeFeudis FV. Verapamil as an apparent competitive antagonist of the serotonin receptor of rabbit isolated aorta. Gen Pharmacol **17**:133–135, 1986.
13. Rehavi M, Carmi R, Weizman A. Tricyclic antidepressants and calcium channel blockers: Interactions at the (−)-desmethoxyverapamil binding site and the serotonin transporter. Eur J Pharmacol **155**:1–9, 1988.
14. Dominic JA, Bourne DWA, Tan TG, Kirsten EB, McAllister RG Jr. The pharmacology of verapamil. III. Pharmacokinetics in normal subjects after intravenous administration. J Cardiovasc Pharmacol **3**:25–38, 1981.
15. McMurtry IF, Davison AB, Reeves JT, Grover RF. Inhibition of hypoxic pulmonary vasoconstriction by calcium antagonist in isolated rat lungs. Circ Res **38**:99–104, 1976.
16. Harder DR, Madden JA, Davison C. Hypoxic induction of Ca^{++}-dependent action potentials in small pulmonary arteries of the cat. J Appl Physiol **59**:1389–1393, 1985.
17. Kennedy T, Summer W. Inhibition of hypoxic pulmonary vasoconstriction by nifedipine. Am J Cardiol **50**:864–868, 1982.