Direct Vasoconstriction as a Possible Cause for Amphotericin B–induced Nephrotoxicity in Rats

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Abstract

In anesthetized rats we tested the hypothesis that amphotericin B (AmB) reduces glomerular filtration rate (GFR) by activating the tubuloglomerular feedback (TGF) mechanism. Infusion of 1 mg/kg AmB over 50 min was followed by a reduction in kidney GFR (from 0.47 ± 0.03 to 0.39 ± 0.02 ml/min per 100 g body wt during the second hour after infusion; $P < 0.05$) and by an increase in urine flow and urinary chloride excretion. Single-nephron GFR (SNGFR) measured in proximal (TGF interrupted) or distal tubules (TGF intact) decreased to a similar degree from 33.4 ± 1.8 and 30.6 ± 1.2 nl/min in the control period to 19.7 ± 1.9 and 21.2 ± 1.6 nl/min during the second hour after AmB infusion ($P < 0.05$). Distal chloride concentrations and TGF responses to changes in loop of Henle flow rate were not significantly altered by AmB. AmB at 10^{-5} M reduced the diameter of isolated perfused afferent arterioles from rabbit kidneys. In isometrically contracting rings of rabbit aorta and renal artery in vitro AmB produced endothelium-independent constriction, with half-maximal contraction (EC_{50}) being achieved by 1.8×10^{-6} and 2.6×10^{-6} M in intact vessels and 1.3×10^{-6} and 1.7×10^{-6} M in endothelium-denuded vessels respectively. Tension development did not occur in Ca-free media or in the presence of Ca channel blockers. Pretreatment with ouabain or Bay K 8644 potentiated the effect of AmB. The vasoconstrictive effect of AmB was counteracted by aminophylline and atrial natriuretic peptide. We conclude that the AmB-induced reduction in GFR is not caused by TGF activation and that AmB has a direct vasoconstrictor effect that is probably initiated by depolarization-induced opening of Ca channels. This effect may be an important component of the nephrotoxic actions of AmB. (*J. Clin. Invest.* 1991. 87:2097–2107.) Key words: afferent arteriole • aminophylline • micropuncture • rabbit aorta • tubuloglomerular feedback • verapamil

Introduction

The polyene antibiotic amphotericin B (AmB)[1] is used extensively in the treatment of systemic fungal infections. Soon after

1. *Abbreviations used in this paper:* AmB, amphotericin B; BW, body weight; PSS, physiological salt solution; TGF, tubuloglomerular feedback.

its introduction as a therapeutic agent in 1956, it was recognized that a high incidence of nephrotoxic side effects can substantially limit its clinical usefulness (1, 2). In addition to signs of tubular dysfunction such as a distal tubular acidification defect (3–5), AmB administration often produces a reduction in renal blood flow and glomerular filtration rate (GFR) (6–8). The absence of marked structural alterations (9) and the dissociation between azotemia and morphologic changes (10) have suggested a functional pathogenesis of AmB-induced renal failure.

It has been proposed that AmB increases renal vascular resistance by activating the tubuloglomerular feedback (TGF) mechanism (11). Increases in NaCl concentration at the macula densa augment the tone of glomerular arterioles, specifically at preglomerular sites (12). Since AmB can alter ion fluxes in both artificial and biological membranes (13, 14), it seemed possible that AmB may increase salt concentrations at the macula densa and thus produce TGF-dependent vasoconstriction. Apparent support for this proposal came from observations that interventions known to block or attenuate the TGF mechanism ameliorate AmB-induced nephrotoxicity. These maneuvers include salt loading (11, 15) and pretreatment with furosemide (11, 16), aminophylline (17–19), or verapamil (8, 20).

The first aim of the present study was to directly test the role of the TGF mechanism in AmB-induced reductions of GFR. The following specific questions were addressed: (*a*) Is the fall in single-nephron GFR (SNGFR) caused by AmB dependent upon delivery of tubular fluid to the macula densa; (*b*) does AmB generate a signal for vascular constriction by increasing distal NaCl concentration; and (*c*) is the sensitivity of the TGF response to a known tubular signal abnormal?

These studies did not support the contention that the TGF mechanism contributes importantly to AmB-induced renal vasoconstriction. We therefore performed a second series of experiments in which we investigated a possible direct vasoconstrictor action of AmB in perfused afferent arterioles and isometrically contracting rings of rabbit aorta and renal artery. Our results show that AmB can produce vasoconstriction in vitro and thus are compatible with the notion that the renovascular effects of AmB may result at least in part from a direct effect of AmB on vascular smooth muscle cells.

Methods

Effect of AmB on renal function in rats

Studies were done in 41 male Sprague-Dawley rats (250–350 g) anesthetized by an intraperitoneal injection of 120 mg/kg thiobutabarbital (Inactin, Byk-Gulden, Constance, FRG). Catheters were placed in the left femoral artery for blood pressure monitoring and collection of blood samples, and in the left jugular vein for infusions of fluids and drugs. Catheters were also inserted into the trachea and ureters. Rats were placed on a heated table that maintains core temperature at 37°C. Upon completion of surgery, a waiting period of 1 h was allowed for equilibration. During the first hour of the experiment control measurements were made. AmB (Fungizone, lot no. 43730, E. R. Squibb &

Sons, Inc., Princeton, NJ) was then given as an intravenous infusion of 1 mg/kg over 50 min. AmB was freshly prepared for each experiment as an aqueous 5 mg/ml solution. For infusion the amount required (0.05–0.07 ml) was diluted in the infusion volume of 0.5 ml saline. All solutions were visually clear with no evidence of precipitation.

Inulin recovery. The effect of AmB on inulin recovery was examined in 13 rats receiving an infusion of 0.9% NaCl at the relatively high rate of 1 ml/100 g of body wt (BW) h^{-1} in order to increase urine flow rate. The left kidney was placed in a lucite cup and the kidney was covered with mineral oil. To determine inulin recovery, between 20- and 40-nl droplets of a stained Ringer solution containing fresh [^{3}H]-inulin (ICN Radiochemicals, Irvine, CA; purity > 95%) at a concentration of 50 μCi/ml were aspirated into mineral oil–filled micropuncture pipettes (tip diam 5 μm). The tip of the pipette was sealed with a small drop of stained castor oil. The pipette content was injected into proximal tubules over 1–3 min. Urine from both kidneys was collected separately in tared tubes in 30-min intervals. Radioactivity in urine and in triplicates of the injectate were counted in a liquid scintillation counter. A total of 9 control and 30 post-AmB microinjections were performed. Injections were not accepted as satisfactory if there was indication of upstream or unusually slow flow or of extraluminal deposition of some of the injectate. Microinjections were performed before AmB administration, at the end of the infusion period, and at hourly intervals thereafter for up to 4 h.

Clearance experiments. This group consisted of 11 rats given AmB and 5 control rats receiving vehicle alone (0.82 mg/kg Na deoxycholate). The left kidney was placed in a lucite cup and the left ureter was cannulated. Rats were infused with 4 g% albumin in 0.9% NaCl at a rate of 0.6 ml/100 g BW h^{-1} for 45 min during surgery followed by 1 g% albumin in saline at the same rate for the rest of the experiment. ^{3}H-labeled inulin was given as a bolus (4 μCi) followed by an infusion of 4.5 μCi/100 g BW h^{-1}. After 30 min of equilibration, a control period of 60 min was begun in which 35 μl of blood was collected at 30-min intervals. AmB infusion was then started and additional blood samples were taken every 45 min. Urine was collected for 20-min periods throughout the experiment. [^{3}H]Inulin was determined in plasma and urine samples by liquid scintillation counting.

Single-nephron studies. Micropuncture studies were performed in five rats infused with 0.9% NaCl at a rate of 0.6 ml/100 g BW h^{-1}. No albumin was given in order to maintain the animals in a hydropenic state which may enhance the susceptibility to the vascular effects of AmB (11). The left kidney was prepared as above. After completion of surgery and a waiting period of 45 min [^{3}H]inulin was given as a bolus (40 μCi) followed by an infusion of 45 μCi/100 g BW h^{-1}.

Timed fluid collections were made in distal and end-proximal segments of the same nephron, identified by dye injections from a 5-μm tipped pipette that was connected to a manometer and was also used to control intratubular pressure during the collections. Collection times were between 2.5 and 4.5 min. In general, three sample pairs were collected during the control period, two pairs in the second half of the AmB infusion period and additional pairs in the first and second hour after AmB infusion. Plasma and urine samples were collected as described for clearance experiments. Tubular fluid volume was measured in a constant bore capillary. A portion of the sample was used for Cl determinations with an electrometric microtitrator (World Precision Instruments, Inc., New Haven, CT). The volume of the remaining sample was again measured and transferred into a scintillation vial for radioactivity counting. Urinary Cl was measured with a digital chloridometer (Haake Buchler Instruments, Inc., Saddle Brook, NJ).

Sensitivity of TGF mechanism. In four rats, TGF sensitivity was studied by measuring stop flow pressure (P_{SF}) responses to changes in loop of Henle perfusion rate before, during, and after AmB administration. Surgery and fluid replacement protocols were identical to that in the micropuncture group. As described previously (21), P_{SF} was measured with a servo-null pressure device (World Precision Instruments) during perfusion of loops of Henle at 0, 10, 20, and 45 nl/min with a Ringer solution containing (in mM): 136 NaCl, 4 NaHCO$_3$, 4 KCl, 2 CaCl$_2$, 7.5 urea, and 100 mg/100 ml FD&C Green. Measurements of

P_{SF} responses were made during the control period, during the last 20 min of the AmB infusion period, and during the first 2 h after AmB infusion. The relationship between P_{SF} and flow rate (21) was evaluated by determining feedback curve parameters (k, an exponential constant; $V_{1/2}$, the flow at which the response is half-maximum; P_{SFmax}, the maximum decrease of P_{SF}) from the experimental data using a quasi-Newton iterative least squares curve-fitting procedure (Systat, Evanston, IL).

Effect of peritubular infusion on P_{SF}. To test the effect of AmB on renal microvessels in vivo without the complicating effects of systemic administration, P_{SF} was measured in six nephrons in two rats during peritubular infusions of AmB. The loop of Henle was not perfused in these experiments. Rats were prepared for micropuncture as described above. P_{SF} was measured before, during, and after infusion of 10^{-4} M AmB in Ringer solution at 30 nl/min into prominent peritubular capillaries adjacent to the nephron under study. In additional experiments we tested the effect of peritubular infusion of a Ringer solution containing solvent alone (10^{-4} M Na deoxycholate).

AmB serum level. In four rats 250-μl blood samples were taken at the end of the AmB infusion, and 30 and 90 min thereafter for the determination of AmB levels. Serum was separated and stored frozen and protected from light until being sent for AmB determinations. Serum AmB levels were assessed by the National Reference Laboratory, Inc., Kirksville, MO, using a standard bioassay method that measures biologically active levels of AmB.

Effect of AmB on isolated vessels of rabbits

Afferent arterioles. Afferent arterioles of rabbit kidneys were dissected from slices of left kidneys of New Zealand white rabbits (800–1,500 g). Dissection was done at 4°C under a stereo dissection microscope (Bausch and Lomb, Inc., Rochester, NY, ×10–70). After selecting an afferent arteriole from the outer cortex tubular fragments were carefully removed using sharpened forceps. Care was taken not to stretch the vessels or to touch the vascular structures at the glomerular hilum. After dissection the interlobular artery was cut with a razor blade piece about 100 μm proximal and 100–200 μm distal to the arteriolar branchpoint. The dissected specimens consisting of a segment of interlobular artery, the afferent arteriole, the glomerulus, and a fragment of the efferent arteriole were transferred to a thermoregulated bath placed on the stage of an inverted microscope (model IMT-2, Olympus Corp. of America, New Hyde Park, NY). The afferent arteriole was cannulated by advancing a perfusion pipette through the lumen of the interlobular artery using concentric pipettes mounted on a moveable track system identical to that used for tubule perfusions (22). The perfusate was driven by a hydraulic pressure of 110 cm H$_2$O. After establishing vessel perfusion the bath temperature was elevated to 38°C. Fresh bath medium was supplied at a rate of 0.2 ml/min and removed by carefully collecting the accumulating bath fluid in 5-min intervals. In most vessels spontaneous contractions with varying intensity were observed in the warmup period which subsided during the waiting period of 30–40 min. Before studies were begun the specimen was inspected for cellular integrity using differential interference contrast optics (Olympus Corp. model IMT-2-DIC) at ×600 and ×1,000.

Dissection and bath media were prepared from Dulbecco's modified Eagle's medium (DME mixture F 12, Sigma Chemical Co., St. Louis, MO) with the addition of 1.2 g/l of NaHCO$_3$. Before use this solution was aerated with 95% O$_2$/5% CO$_2$ for 45 min and its pH was adjusted to 7.4. When used as dissection medium 3% fetal calf serum was added (Gibco Laboratories, Grand Island, NY). When used as bath medium 5 g/100 ml human serum albumin (Sigma Chemical Co.) was added instead. Vessel perfusion fluid was a modified Krebs-Ringer-HCO$_3$ buffer containing (in mM): 115 NaCl, 25 NaHCO$_3$, 0.96 NaH$_2$PO$_4$, 0.24 Na$_2$HPO$_4$, 5 KCl, 1.2 MgSO$_4$, 2 CaCl$_2$, 5.5 glucose, and 1 g/100 ml bovine serum albumin. The buffer was bubbled with 95% O$_2$/5% CO$_2$ and pH adjusted to 7.4. After a waiting period of about 30 min control measurements were made. AmB (5 × 10^{-5} M) was then added to the bath and effects were observed for 10 min. In some vessels AmB was given together with 10^{-5} M nifedipine.

Experiments were videotaped at ×600 or ×1,000 using differential interference contrast optics. Using an image analysis system (CUE 2, Olympus Corp.) vascular diameter changes were measured on the videoimages at 1, 3, 5, and 10 min at two sites of the vessel representing locations of maximum and minimum responsiveness to AmB.

Isolated rings of aorta and renal artery. Blood vessels were harvested from the same rabbits used as donors for the arteriolar perfusion experiments. Pieces of the abdominal aorta and right renal artery were excised, transferred into physiological salt solution (PSS), cleaned of fat and connective tissue, and cut into 3-mm segments. Connected to model FT .03 force transducers (Grass Instrument Co., Quincy, MA) vessel rings were suspended in glass chambers containing 40 ml PSS, and a passive force of 3,000 mg for the abdominal aorta and 1,500 mg for the renal artery was applied. Each chamber contained two strips, one intact and one with the endothelium removed by gentle rubbing. Vessels were allowed to equilibrate for 2 h in PSS at 37°C. The composition of the bathing medium was as follows (mM): NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 1.6, $NaHCO_3$ 14.9, dextrose 5.5, and CaNa-EDTA 0.03 (pH 7.2–7.4). After the equilibration period, all vessels were exposed twice to a depolarizing solution (100 mM KCl) which was used as reference point, to norepinephrine at 10^{-7} M and to 10^{-6} M acetylcholine to assure removal of the endothelium in the denuded vessels.

Drugs used were as follows: AmB (Squibb) dissolved in distilled water at a maximum concentration of 5×10^{-3} M and kept refrigerated and protected from light for a maximum of 1 wk; verapamil (Sigma Chemical Co.) dissolved in 100% alcohol (the final concentration of alcohol in the bath did not exceed 0.1%); BAY K 8644 dissolved in 100% alcohol with final bath concentrations not exceeding 0.1% (courtesy of Bayer AG, Wuppertal, FRG); ouabain (Sigma Chemical Co.); aminophylline (Sigma Chemical Co.); atrial natriuretic peptide (Sigma Chemical Co.); phentolamine (CIBA Pharmaceutical Co., Summit, NJ); and Na deoxycholate (Sigma Chemical Co.).

Statistical analysis. All results are expressed as means±SE. Statistical analysis was performed using either a single-factor analysis of variance (ANOVA) with repeated measures or a two-factor ANOVA with repeated measures on the second factor. Post-hoc comparisons using the Newman-Keuls multiple range test were used to supplement the ANOVA when necessary. Inulin recovery results were assessed using paired t-tests with missing data, as discussed in Snedecor and Cochran (23). The decision for statistical significance was made at $P < 0.05$.

Results

Effect of AmB on renal function in rats

Inulin recovery. The recovery of microinjected inulin in the urine averaged 104.2±3.1% ($n = 6$) in control and 100.2±1.7% ($n = 9$) during AmB administration. A modest reduction to 91.7±2.1% ($n = 9$; $P < 0.05$) and 94.0±2.5% ($n = 9$; $P < 0.05$) was noted in the injected kidneys in the second and third hour after AmB infusion. However, since at the same time recovery was negligible in the contralateral kidneys, the importance of this finding is questionable. Inulin recovery was 95.8±2.8% ($n = 9$; NS) in the fourth hour after AmB infusion.

Clearance experiments. The effect of AmB on inulin clearance (C_{In}) was measured in 11 rats. In view of only minimal inulin loss, C_{In} is taken as a measure of GFR. In the time control rats infused with the deoxycholate vehicle, C_{In} declined slightly over 3 h, but the change was not significant (Fig. 1 *a*). In the AmB-infused rats, C_{In} decreased in the second half of the AmB infusion period in half of the treated rats, but the overall reduction of GFR during this period did not reach statistical significance (Fig. 1 *a*). A significant reduction of C_{In} was found in the period beginning 40 min after terminating AmB infusion. Mean GFR in that period was 0.39±0.02 ml/100 g BW
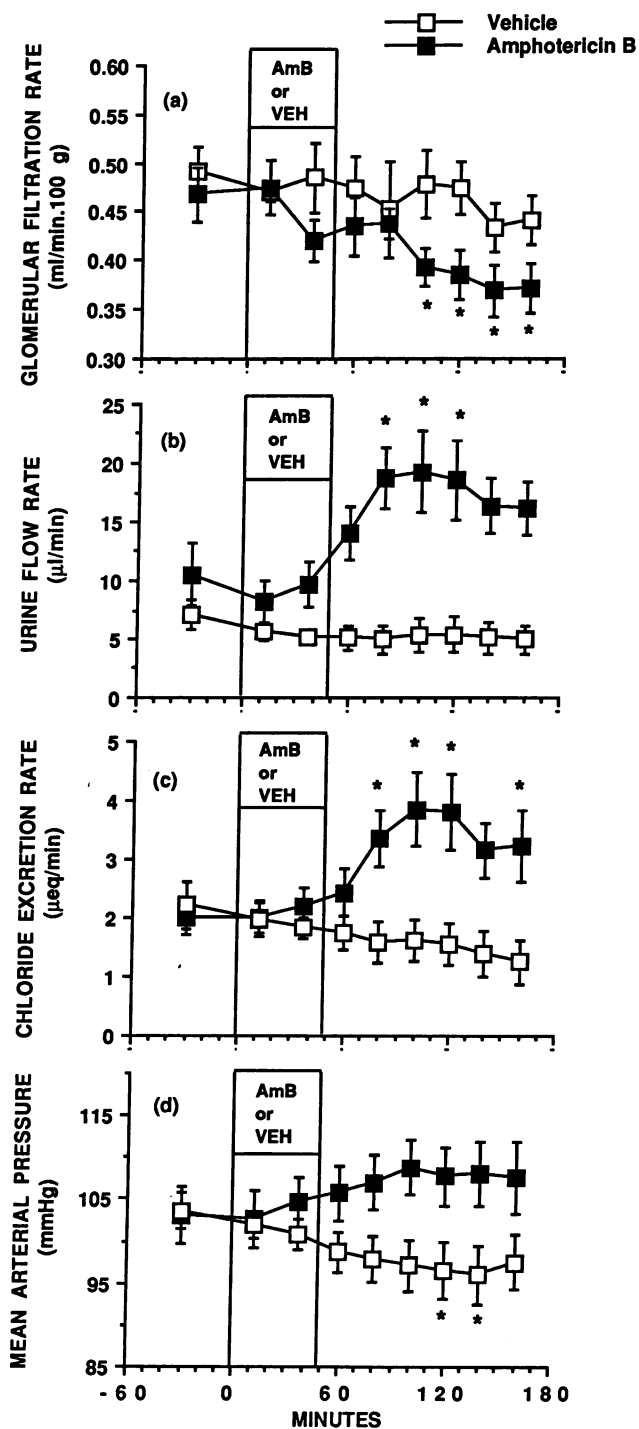

Figure 1. Effect of AmB (■, $n = 11$) or vehicle (□, $n = 5$) infusion on (*a*) glomerular filtration rate, (*b*) urine flow rate, (*c*) chloride excretion, and (*d*) mean arterial pressure in anesthetized rats. *$P < 0.05$ vs. control.

per min, significantly lower than the mean of 0.47±0.03 ml/100 g BW per min in the control period ($P < 0.05$, ANOVA). Significant reductions in C_{In} were also seen in all later collection periods. In response to AmB infusion urine flow rate rose from a control value of 10.5±3.3 μl/min to a peak of 19.3±3.0 μl/min 40 min after the end of the infusion ($P < 0.05$) (Fig. 1 *b*). Diuresis was associated with a modest decrease in urinary Cl

Table I. Summary of Micropuncture Results

	Control	During (%Δ)	After 1st h (%Δ)	After 2nd h (%Δ)
Glomerular filtration rate				
(*ml/100 g BW · min*)	0.44±0.01	0.34±0.03 (23%)*	0.39±0.03 (11%)	0.35±0.03 (20%)*
Proximal SNGFR (*nl/min*)	33.4±1.8	19.7±1.9 (41%)*	30.4±2.4 (9%)	19.7±1.6 (41%)*
Distal SNGFR (*nl/min*)	30.6±1.2‡	21.2±1.6 (31%)*	26.9±2.0 (12%)‡	18.7±0.4 (39%)*
ΔSNGFR (*nl/min*)	3.0±1.1	−3.3±2.3	3.5±2.1	1.0±1.2
Distal flow rate (*nl/min*)	6.8±1.0	4.9±1.0	7.1±1.4	4.9±0.6
Distal [Cl] (*meq/liter*)	49.1±13.4	66.5±10.1	61.5±4.1	49.9±4.9
Proximal-TF/P	2.1±0.3	2.5±0.3	1.8±0.2	1.8±0.2
Distal-TF/P	5.3±0.9	5.2±0.8	4.5±1.3	4.4±0.7
P-%H_2O absorption	48.8±5.5	56.4±5.8	43.0±6.5	38.8±6.9
D-%H_2O absorption	79.1±2.4	79.2±3.0	72.8±4.9	74.4±2.9
n (rats/tubules)	5/15	4/4	5/10	3/6

Abbreviations: P, proximal; D, distal; TF/P, tubular fluid/plasma inulin concentration. %H_2O absorption: proximal = $(1-1/TF/P) \times 100$; distal = (distal SNGFR − distal V̇/distal SNGFR) × 100. * $P < 0.05$ vs control; ‡ $P < 0.05$ distal vs. proximal.

concentration (control from 265.5±31.8 to 201.5±16.5 mM at the 40-minute postinfusion time point; $P < 0.05$). The resultant urinary Cl excretion rate increased significantly in the AmB-treated group from 2.0±0.27 μmol/min in the control period to 3.8±0.63 μmol/min at 40-min after infusion ($P < 0.05$, Fig. 1 *c*). No significant changes in Cl excretion were observed in the control rats.

In time control rats infused with vehicle, mean arterial pressure (MAP) decreased from 103±2.2 to 96±3.5 mm Hg ($P < 0.05$, Fig. 1 *d*). In contrast, MAP did not significantly change with time in the AmB-treated rats. MAP at 40 min after terminating AmB infusion reached 109±3.2 mm Hg.

Single-nephron studies. The results of this experimental series are summarized in Table I. In the control period, mean SNGFR measured in distal tubular segments of 30.6±1.2 nl/min was significantly lower than SNGFR in paired proximal segments of 33.4±1.8 nl/min ($P < 0.05$). Mean values for proximal and distal SNGFR in the second half of the AmB infusion period were 19.7±1.9 and 21.2±1.6 nl/min, respectively. Mean SNGFR values during the first hour after terminating AmB infusion recovered and were not significantly different from control values. However, in the period 60–120 min after AmB infusion proximal SNGFR averaged 19.7±1.6 nl/min and mean distal SNGFR was 18.7±0.4 nl/min. C_{In} was 0.44±0.01 ml/100 g BW per min in the control period, 0.34±0.03 during AmB infusion, 0.39±0.03 in the first hour, and 0.35±0.03 ml/100 g BW min in the second hour after AmB infusion ($P < 0.05$ for all comparisons with control).

Distal Cl concentrations tended to increase during and in the first hour after AmB infusion from a control value of 49.1±13.4 to 66.5±10.1 and 61.5±4.1 mM without reaching the 5% significance level. Distal Cl concentration was 49.9±4.9 mM in the 60–120-min postinfusion period at the time where both whole kidney and single nephron filtration rate were significantly depressed (Table I). Mean arterial pressure averaged 107±4.1, 108±4.1, 104±3.2, and 105±3.7 mm Hg before, during, and in the first and second hour after AmB, respectively.

TGF sensitivity. Stop flow pressure (P_{SF}) at zero flow was not different between control tubules and tubules studied during and immediately after AmB infusion (Fig. 2). However, P_{SF} at zero flow decreased significantly in the second hour after

termination of AmB infusion presumably indicating a fall in glomerular capillary pressure despite the interruption of distal flow. P_{SF} responses to changes in loop flow were comparable in all periods as indicated by similar maximum reductions of P_{SF} and similar values for the feedback curve parameters $V_{1/2}$ and k (Table II). Arterial pressure was 121±3.2, 119±2.8, 118±5, and 115±5.2 mm Hg before, during, and in the first and second hour after AmB, respectively.

Effect of AmB on P_{SF}. The possibility of a direct and acute vasoconstrictor action of AmB was tested in vivo by assessing the effect of peritubular AmB infusion on P_{SF} at zero loop perfusion. Infusion of a Ringer solution containing 10^{-4} M AmB into peritubular capillaries decreased P_{SF} acutely from 46.2±0.9 to 40.6±1.2 mm Hg. This decrease in P_{SF} was transient, returning to a steady mean value of 44.3±1.3 mm Hg within 1 min. An original recording of such an experiment is shown in Fig. 3. Peritubular infusion of Ringer solution containing 10^{-4} M Na deoxycholate had no effect on P_{SF} ($n = 4$).

Serum AmB level. Mean serum AmB concentration at the end of AmB infusion was 5.0±0.6 μM. 30 and 90 min later serum AmB levels averaged 3.6±0.2 and 3.9±0.3 μM, respectively.
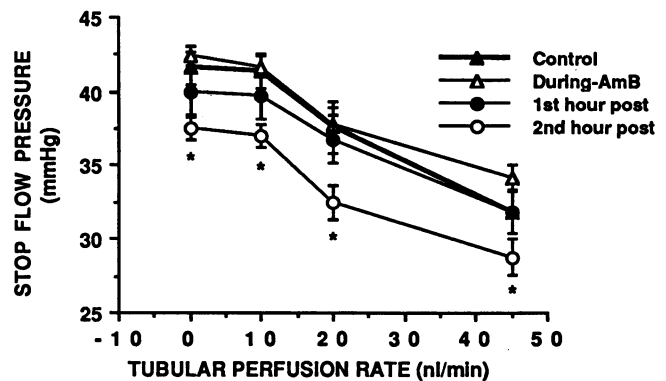

Figure 2. Tubuloglomerular feedback response of stop flow pressure to changes in loop of Henle perfusion rate in control (▲), during AmB infusion (△), and in the first and second hour after AmB infusion (● and ○, respectively). *$P < 0.05$ vs. control.

Table II. Tubuloglomerular Feedback Parameters before, during, and after AmB Infusion

	Control	During	After 1st h	After 2nd h
$\Delta P_{SF\text{-}max}$ (mm Hg)	9.7±0.8	8.2±0.7	8.1±0.8	8.7±0.9
$\Delta P_{SF\text{-}max}$ (%)	23.8±2.1	19.4±1.7	20.3±2.1	23.2±2.4
$\dot{V}_{1/2}$ (nl/min)	21.2±0.88	19.2±1.06	25.0±3.3	19.1±1.19
K (nl/min)$^{-1}$	0.32±0.12	0.26±0.08	0.14±0.05	0.32±0.16
n (rats/tubules)	4/14	4/10	4/15	4/9

Parameters: P_{SF} = stop flow pressure; $\Delta P_{SF\text{-}max}$ = maximum decrease in stop flow pressure, $\dot{V}_{1/2}$ = perfusion rate at which the decrease in P_{SF} is half maximum, K = exponential constant defined as: $[4 \times f'(\dot{V}_{1/2})]/\Delta P_{SF\text{-}max}$, with $f'(\dot{V}_{1/2})$ referring to the slope of the curve at the midpoint.

Effect of AmB on isolated vessels of rabbits

Afferent arterioles. AmB at 10^{-5} M significantly reduced the diameter of perfused afferent arterioles ($n = 7$). The reduction in vessel diameter waned somewhat at exposure times longer than 3 min, but vessels remained constricted compared to control. The vessel response to AmB was usually inhomogeneous as can be seen in the example shown in Fig. 4. The most consistently responsive part of the arteriole was the segment at the entrance into the glomerulus. Other responsive foci were located at irregular distances from the glomerulus. Fig. 5 shows individual and mean vessel diameter changes caused by AmB in a more (*top*) and in a less responsive segment (*bottom*). Mean diameter of the responsive segments in control was 12.6±1.2 μm ($n = 7$), decreasing to 6.3±1.5 μm at 3 min ($P < 0.01$) and to 8.6±1.4 μm at 10 min of AmB exposure ($P < 0.01$). After removal of AmB vessel diameters returned to control within 10–20 min. A significant reduction in vessel diameter was also seen in the nonsphincter segment in which mean diameter decreased from 17.4±1.44 to 14.5±1.15 ($P < 0.05$) and 14.6±1.4 μm ($P < 0.05$) at 3 and 10 min of AmB exposure. Nifedipine (10^{-5} M) fully inhibited the contractile response of AmB in both sphincterlike segments (diameter in micrometers: 10.7±1.5 in control, 12.3±2.1 at 3 min, and 11.2±1.07 at 10 min; $n = 4$) and nonsphincter segments (diameter in micrometers: 15.6±1.66 in control, 15.7±1.6 at 3 min, and 15.8±1.6 at 10 min; $n = 4$).

Aorta and renal artery. 38 experiments on rings of rabbit aorta were performed to assess contractile responses to increasing concentrations of AmB. Since preliminary studies indicated that prior exposure of vessels to AmB might augment the response to subsequent AmB administration each concentration was tested in four to eight rings exposed to a single drug concentration only. In Fig. 6 data are expressed as percentage of the vasoconstrictor effect of 100 mM KCl which in terms of force development was 2,524±102 mg in the intact vessels and 1,637±114 mg in the vessels which had their endothelium removed. AmB produced isometric contractions in a concentration-dependent fashion with most of the effect occurring between 10^{-6} and 10^{-5} M (Fig. 6 *a*). Maximum force generation was comparable to that produced by 100 mM KCl. The contractile effect of AmB was not significantly different between intact and endothelium-denuded vessels although tension development tended to be consistently larger in the absence of the endothelium. The AmB concentration producing half maximal contraction (EC_{50}) was 1.8×10^{-6} M in intact vessels and 1.3×10^{-6} M in the denuded vessels. A vasoconstrictor effect of AmB was also seen in isolated rings of the renal artery (Fig. 6 *b*). Force generation in response to 100 mM KCl in these vessels was 1,852±153 mg in intact vessels and 1,745±146 mg in denuded vessels. EC_{50} of AmB-induced contraction was 2.6 $\times 10^{-6}$ in intact vessels and 1.7×10^{-6} in denuded rings. Vessels exposed to the vehicle deoxycholate did not generate any force even at a relatively high dose of 5×10^{-4}.

The time course of the contractile response to AmB was slow with $t_{1/2}$, the time to reach half-maximal contraction to 6 $\times 10^{-6}$ M AmB, being 19.3±3.2 min in intact and 18.6±2.2 min in denuded vessels (Fig. 7 *a*). Likewise, a time span of 45 min elapsed to achieve half-maximal relaxation after removal of the drug. In renal arteries the time course of changes in tension following addition or removal of AmB was similarly sluggish and comparable to that found in the aorta (Fig. 7 *b*).

Role of calcium and calcium channels in AmB-induced contractions. Addition of AmB (10 μM) to aortic rings suspended in calcium-free solutions (0 $CaCl_2$ + 1 mM EGTA) was essentially without effect on tension (Fig. 8). When 1.6 mM $CaCl_2$ was added in the presence of AmB there was a force generation with a time course much faster than seen in nonpreincubated vessels. Pretreatment with verapamil (1 μM for 30 min) significantly blunted the vasoconstrictor effect of 10 μM AmB in both intact and endothelium-denuded vessels (Fig. 9 *a*). Verapamil added to aortic rings during AmB-induced contraction (10 μM) caused a reduction in the force generated with an EC_{50} of 2 $\times 10^{-7}$ M (Fig. 9 *b*). Similar results were obtained in denuded vessels.

Effect of ouabain and BAY K 8644 on AmB-induced contractions. In aortic rings, pretreatment with ouabain (1 μM for 30 min) potentiated as well as accelerated the tension generated by
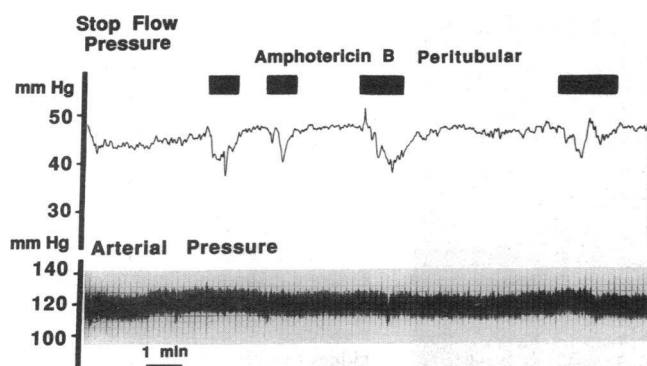

Figure 3. Original recording showing the effect of peritubular application of AmB on stop flow pressure (*upper tracing*). Periods of peritubular AmB infusion are indicated by block bars. Lower tracing shows arterial blood pressure.
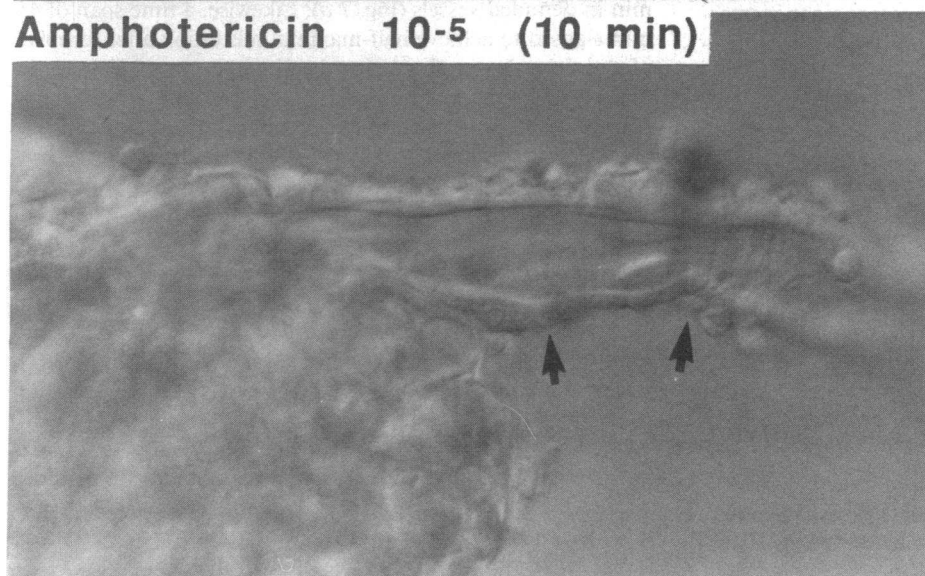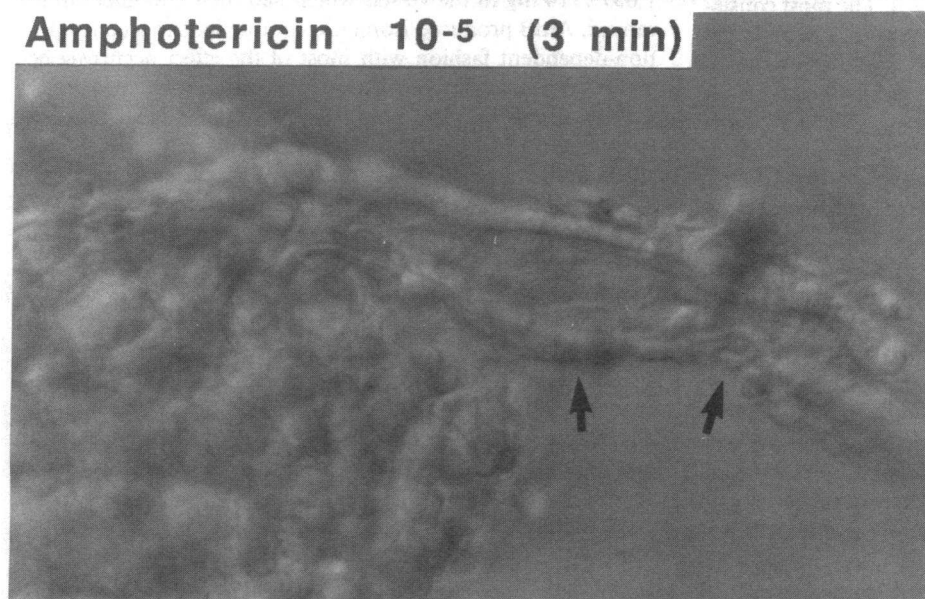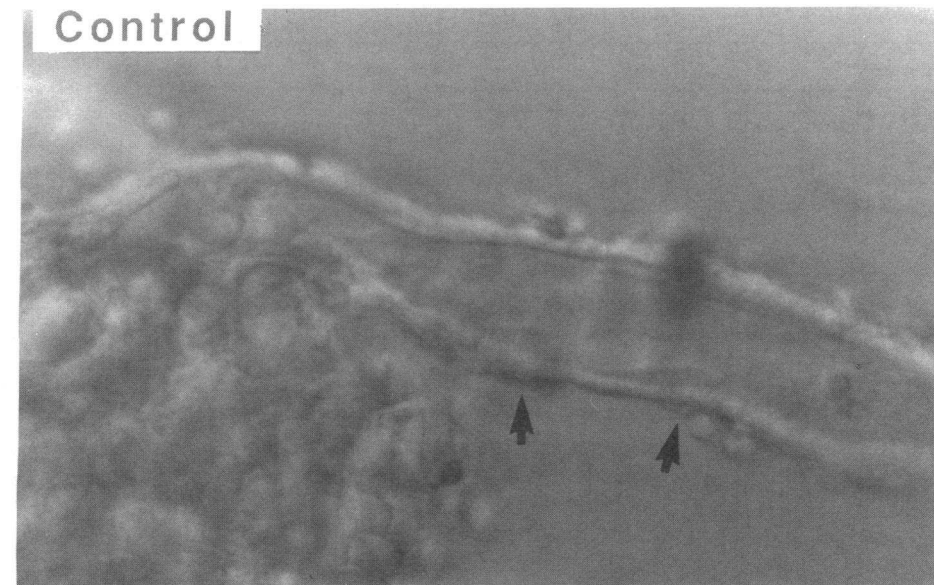
Figure 4. Microphotographs showing the effect of AmB on the diameter of a perfused afferent arteriole from rabbit kidney (×600). Arrows indicate sites at which diameter measurements were made. Note contractility differences in the more responsive (*right arrows*) and less responsive segments (*left arrows*).
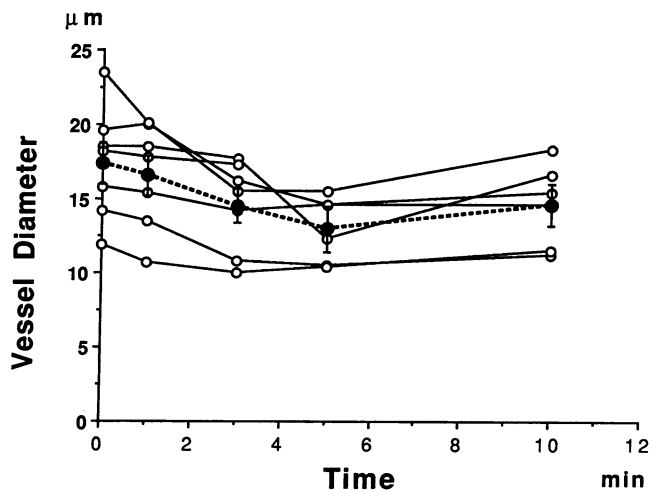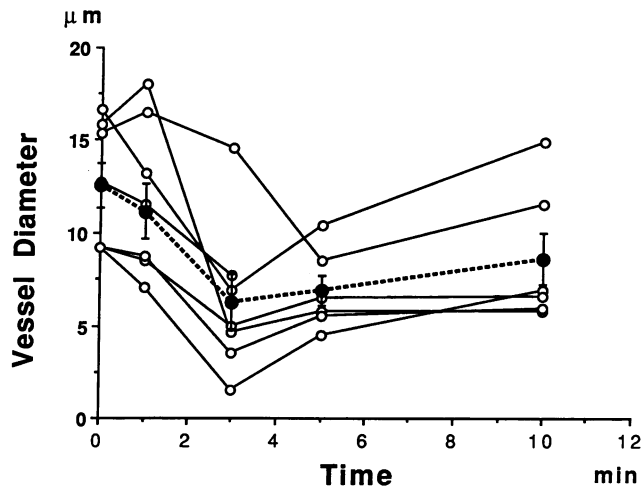
Figure 5. AmB-induced changes in the diameter of afferent arterioles in more (*top*) and less responsive segments of the vessel (*bottom*). Open symbols and solid lines indicate individual vessel responses; closed symbols and broken lines indicate diameter changes.

$10~\mu M$ AmB (Fig. 10; $n = 6$). In some vessels in which ouabain caused some contraction AmB-induced tension was measured from that level of tension. $T_{1/2}$, the time to reach half-maximum force generation, was 9.7 ± 3.4 min compared to 21.9 ± 4.2 in control vessels ($P < 0.05$). As shown in Fig. 11 exposure of aortic rings to BAY K 8644 produced little constrictor effects. However, pretreatment with a substreshold concentration of AmB significantly potentiated the effect of BAY K 8644.
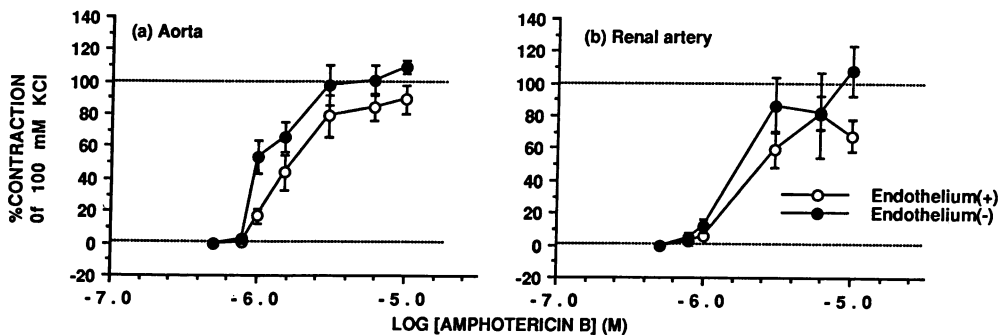
Effect of theophylline and ANP on AmB-induced contractions. As shown in Fig. 12, theophylline and ANP reduced the force generated by 10^{-5} M AmB. EC_{50}, the concentration to produce half-maximal responses, was 2.8×10^{-8} M with ANP and 1.5×10^{-4} M with aminophylline. Similar results were observed in the denuded vessels.

Effect of phentolamine. Alpha adrenergic blockade was associated with a significant, albeit modest reduction in the force generated by AmB in both intact and denuded vessels. Expressed as percent of contraction caused by 100 mM KCl, 1 μM phentolamine reduced the tension developed by 10 μM AmB from $73.6\pm10.3\%$ to $58.7\pm8.4\%$ in intact vessels ($n = 4$; $P < 0.05$) and from $115\pm9.8\%$ to $98.8\pm7.2\%$ in denuded vessels ($n = 4$, $P < 0.05$).

Discussion

AmB has proven to be a useful therapeutic agent for the treatment of systemic fungal infections. Its beneficial actions however are limited by the relatively frequent occurrence of nephrotoxic side effects, particularly reductions in GFR (6–8). Using animal models of AmB nephrotoxicity, attempts have been made to clarify the mechanisms responsible for AmB-induced changes in renal function. Results from such studies led to the suggestion that the hemodynamic alterations caused by AmB are secondary to a change in tubular NaCl absorption with subsequent activation of the tubuloglomerular feedback (TGF) mechanism (8, 11, 18, 19, 24). Since this proposal is based on observations in the whole kidney, and had not been directly tested at the single nephron level, we performed micropuncture studies to examine whether TGF has a causal role in AmB-induced changes of renal hemodynamics.

Consistent with the results of earlier studies the present experiments show that AmB infusion in rats induced both an acute and a persistent reduction in GFR (8, 9). The fact that the decrease of GFR was less pronounced than in an earlier study by Cheng et al. (9) may be related to the fact that our animals were maintained in a euvolemic state by the infusion of albumin-containing saline. Extracellular fluid volume has been shown to modulate the effect of AmB (11, 25). A greater fall of GFR was in fact seen in the hydropenic animals used for micropuncture. In these rats GFR fell by 23% during the infusion of AmB, a decrease similar to that observed in another recent micropuncture study (24). In the absence of consistent inulin leakage, the clearance of inulin can be taken as a measure of glomerular filtration rate. In contrast to the present results, Cheng et al. (9) have reported that AmB induced a marked and progressive reduction of inulin recovery. The reason for the

Figure 6. Effect of AmB on isometric contractions of (*a*) isolated rabbit aorta and (*b*) renal artery. Contractions are expressed in percent of the constrictor effect of 100 mM KCl. (o) Intact vessels; (•) endothelium-denuded vessels.
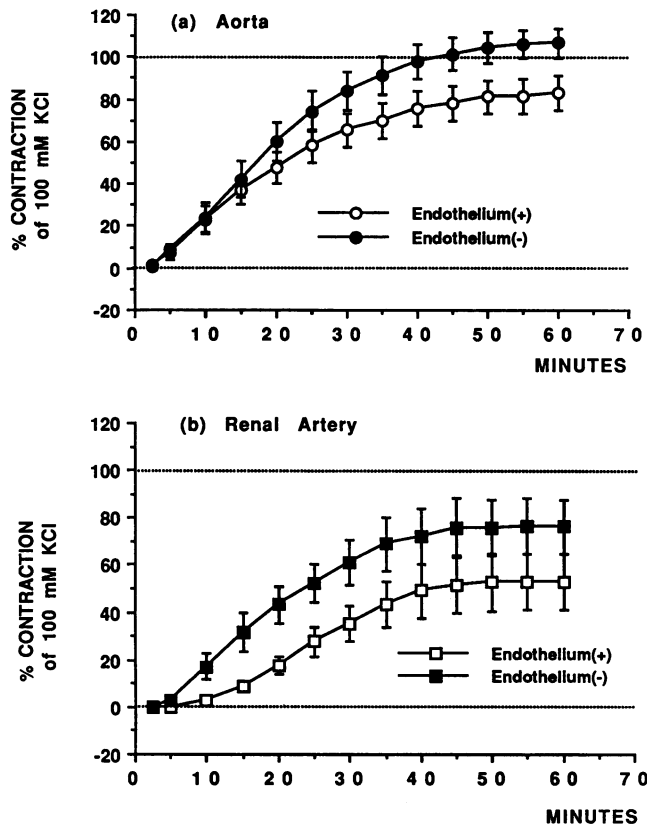
Figure 7. Time course of tension development in response to 6 μM of AmB in rings from (a) rabbit aorta and (b) renal artery in the presence or absence of a functional endothelium.

discrepancy between these results may lie in differences in the experimental protocol. Cheng et al. (9) performed their studies in mannitol diuresis and it is possible that the combination of

Figure 9. (top) Effect of 30-min pretreatment with verapamil (1 μM) on vasoconstriction induced by 10 μM AmB. Circles: control vessels; squares: verapamil-treated vessels. Open symbols: intact rings; closed symbols: endothelium-denuded rings. *P < 0.05; §P < 0.001. (Bottom) Effect of verapamil on the tension generated by the presence of 10 μM AmB in intact (o) and endothelium-denuded rings of rabbit aorta (●).
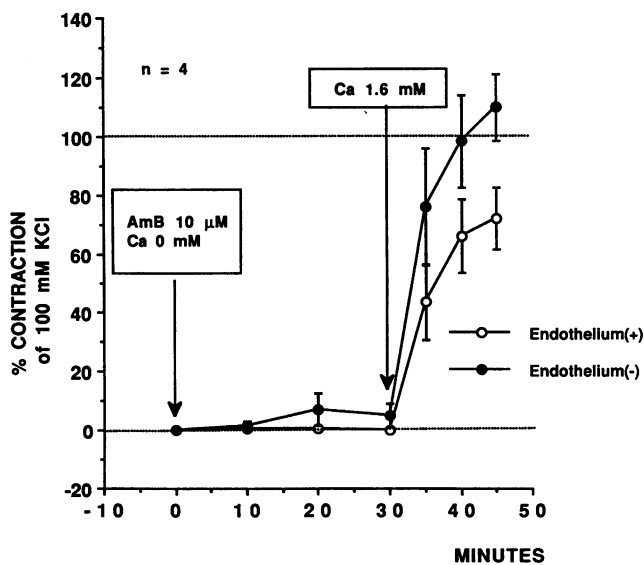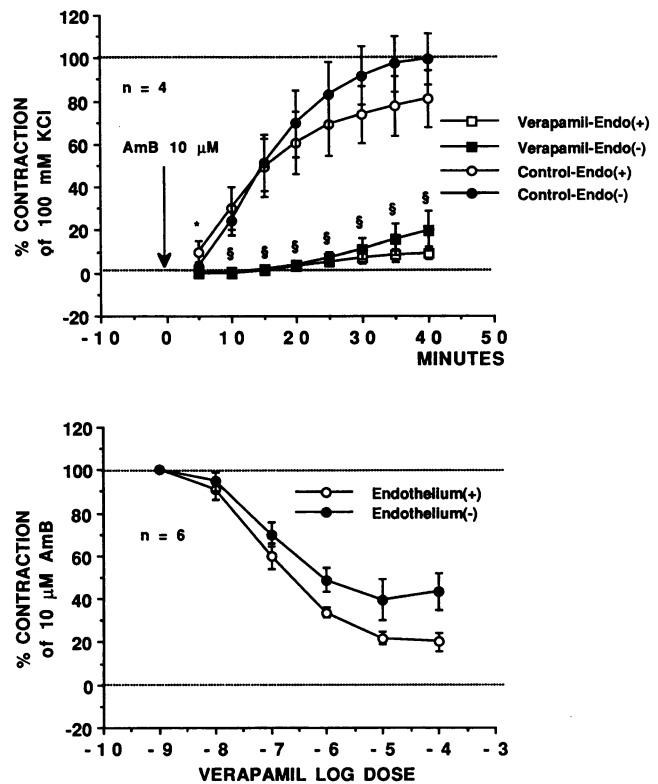

Figure 8. Effect of extracellular calcium concentration on AmB-induced vasoconstriction. Arrows indicate the time of AmB or calcium addition to the bath. (o) Intact vessels; (●) endothelium-denuded vessels.
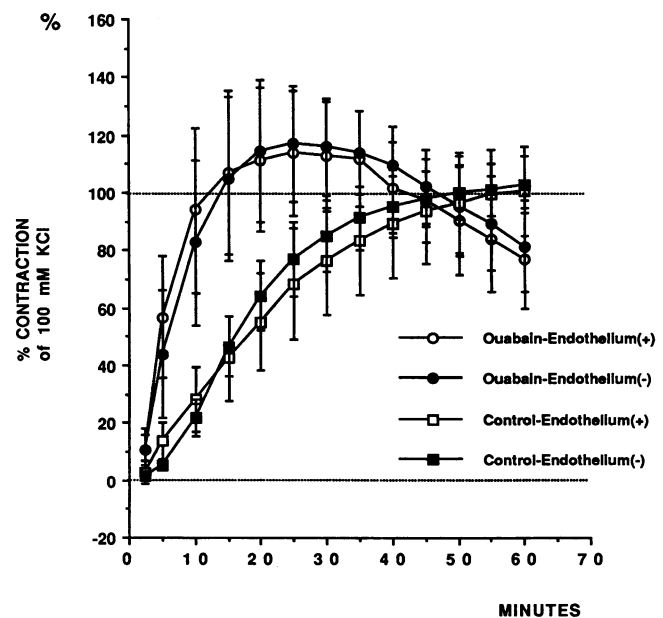

Figure 10. Effect of 30-min pretreatment with ouabain (1 μM) on vasoconstriction induced by 10 μM AmB. Squares: control vessels; circles: ouabain-treated vessels.
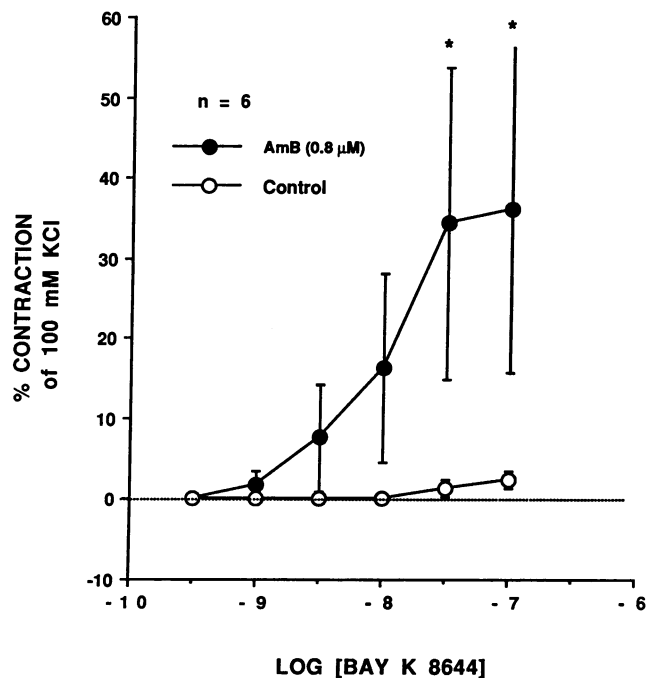
Figure 11. Relationship between BAY K 8644 concentration and contractile responses of rabbit aortic rings to subthreshold concentrations of AmB. (o) Control vessels; (●) vessels pretreated with AmB (0.8 μM). *$P < 0.05$.

mannitol and AmB augmented the permeabilizing effect of AmB. Some evidence suggests that AmB in combination with mannitol in man alters morphology to a greater extent than AmB alone (26). Erroneous overestimates of inulin leakage can be caused by a number of technical problems, such as absorption of low molecular weight breakdown products of labeled inulin, or unrecognized loss of injectate.

To examine a possible participation of the TGF system in the AmB-induced reduction of GFR we first determined the effect of AmB on SNGFR in the absence and presence of a macula densa signal. If AmB suppresses GFR by an increased NaCl delivery to the macula densa and subsequent TGF activation, blockade of the TGF signalling pathway should prevent the AmB-induced fall of GFR. Our results show that blockade
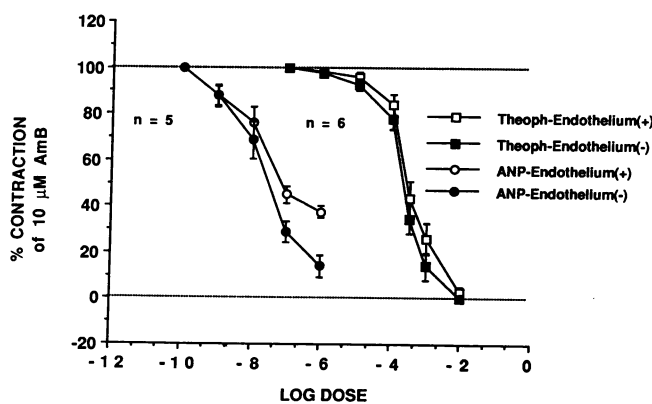

Figure 12. Effect of aminophylline (*squares, n = 6*), and atrial natriuretic peptide, ANP (*circles, n = 5*) on the vasoconstrictor effect of AmB (10 μM) in isolated rings of rabbit aorta.

of the macula densa signal by proximal fluid collections did not increase filtration rate during AmB administration. Low SNGFR in the absence of distal NaCl delivery has also been noted in another recent micropuncture study (24). Thus, distal fluid delivery does not appear to be critical for the maintenance of AmB-induced vasoconstriction. The absence of a demonstrable effect of blocking the TGF pathway on the AmB-induced fall in GFR is consistent with the finding that distal Cl concentration, the presumed signal in TGF-mediated vasoconstriction, did not increase significantly. These observations seem incompatible with the notion of NaCl-dependent TGF activation being primarily responsible for AmB-induced reductions in GFR. We also addressed the possibility that TGF-mediated AmB nephrotoxicity could result from an increase in TGF sensitivity. These studies showed that the characteristics of the feedback response to changes in loop perfusion rate, both $V_{1/2}$ and maximum responses, were not detectably altered by AmB. Thus, no measurable resetting of TGF sensitivity occurred that could have affected arteriolar tone in a way not predictable from the changes in distal Cl concentrations.

Since our studies did not provide evidence for a major role of TGF in AmB-induced reductions of GFR, we examined the possibility that AmB administration may cause constriction of blood vessels by a direct effect on smooth muscle cells, an alternative that, to our knowledge, has not been studied previously. Since it is difficult to differentiate between direct and indirect vasomotor responses in the intact organism, we examined the vasoactive properties of AmB in vitro in both large and small vessels. Our studies show that AmB is a potent vasoconstrictor of preglomerular arterioles and both renal and nonrenal arteries. Previous indirect evidence is in agreement with the conclusion that vasoconstriction is not limited to the preglomerular vascular bed, as one would expect if the TGF mechanism or another strictly intrarenal mechanism were primarily responsible for the nephrotoxic actions of AmB. Experimental animals frequently show an increase of mean arterial pressure suggestive of an increase in systemic vascular resistance (7, 11, 19, 27). For example, Reiner et al. (27) have shown in studies in dogs that intravenous AmB administration increased not only renal vascular resistance, but also pulmonary and total systemic resistance. The fall in cardiac output reported in that study may explain the absence of more severe hypertension after AmB infusion (27).

Our studies indicate that AmB-induced vasoconstriction can be elicited in a setting where an influence of central and reflex-mediated effects can be excluded. It is conceivable, however, that the vasoactive effect of AmB may be dependent upon, or modified by, the local release of vasoactive autacoids. A slight, but consistent increase in the constrictor response to AmB was seen after mechanical removal of a functional endothelium, suggesting that the effect of AmB may be blunted by the release of endothelium-derived relaxing factor. Furthermore, AmB effects were slightly reduced by phentolamine, indicating a possible contribution of norepinephrine released from nerve endings within the vessel wall to AmB-induced vasoconstriction. Further studies are required to explore the effect of AmB on generation and release of locally active agents.

The results of the present experiments indicate that AmB-produced changes in renal function and in vascular tone follow a complex time course. Exposure to AmB produced an immediate effect, as seen in the constrictor response of isolated arterioles, in the stop flow pressure response during peritubular infu-

sion of AmB, and in the rapid onset of demonstrable reductions in SNGFR. This immediate reaction to AmB appears to decrease in magnitude despite continued presence of the drug. The response in large vessels, on the other hand, was slower in onset, requiring ~ 40–50 min for peak effects, and persistent for a longer time after withdrawal of the drug. This long-lasting action of AmB may be of particular importance for the development of nephrotoxic side effects. It is to be pointed out that although the rate of relaxation in vitro was faster (half-maximum relaxation of 45 min) than the recovery of GFR in vivo, these results are not necessarily in contradiction. When AmB is withdrawn from the bath in vitro, the decay is likely to reflect removal of AmB from the membrane pool. In the in vivo experiments, on the other hand, AmB levels in plasma are known to remain elevated for prolonged periods of time after termination of AmB infusion (28). The difference in the temporal pattern of AmB-induced vasoconstriction may indicate a different constrictor mechanism in small or large vessels. Since it is likely that incorporation of AmB into the membranes of smooth muscle cells is a prerequisite for its biological actions, it is also possible that the time course of entry and removal of the drug from the membrane may be different in vessels of varying calibers.

Our results furnish some insight into the causes for the delayed vasomotor effects of the drug. The abolition of constrictor effects in Ca-free solutions together with the inhibitory effect of the Ca channel blocker verapamil strongly indicates a dependence of AmB-induced vasoconstriction on Ca influx through voltage-dependent Ca channels. Earlier findings of Tolins et al. (8) had already shown that verapamil completely abolished the acute effect of AmB on renal blood flow. Recent evidence indicates that AmB can act as a calcium ionophore in sterol-containing liposomes (29). However, the concentration of AmB required to induce Ca entry into liposomes was substantially higher than AmB concentrations needed to induce vasoconstriction. Furthermore, one may not expect verapamil to block an ionophore effect of AmB, so that entry of Ca through pores formed by AmB may be unlikely. On the other hand, AmB incorporated into membranes generates channels that are preferentially permeable to univalent ions (13, 14, 30). Exposure to AmB is thus predicted to cause cell depolarization owing to increased sodium influx, an effect which has been demonstrated in skeletal muscle cells (31) and necturus gallbladder cells (32). Depolarization may be followed by increased calcium influx through voltage-gated Ca channels (33). Indirect evidence for a depolarizing effect of AmB in our studies is furnished by the observation that low concentrations of ouabain significantly accelerated the onset of AmB actions. We assume that the depolarizing effect of ouabain was additive to that of AmB in facilitating the entry of Ca (34). Furthermore, BAY K 8644 did not cause vasoconstriction in untreated rings of rabbit aorta as shown earlier (35), but produced constriction in the presence of subthreshold concentrations of AmB. It has been shown that facilitation of Ca entry by dihydropyridine agonists is voltage-dependent and can be elicited by slight cell depolarization (35–37), an effect that may have been produced by exposure to AmB. The validity of this likely mechanism of AmB action needs to be studied with direct methods.

The hypothesis of a causal role of the TGF system in AmB nephrotoxicity was based for the most part on the blunting of AmB-induced vasoconstriction that accompanies interventions known to block TGF-mediated vascular effects such as salt loading or the administration of furosemide, aminophylline or verapamil (8, 11, 18, 19). The present studies provide possible alternative explanations for some of these observations. Acute salt loading is associated with a reduction in sympathetic tone and plasma renin concentration as well as an increase in plasma levels of atrial natriuretic peptide. The resulting relative relaxation of the renal vasculature caused by volume expansion may reduce the vasoconstrictor potential of AmB, a possibility supported by previous studies showing that the combination of saralasin and low dose dopamine attenuated the renal constrictor effect of AmB (27) and by the present observation that the contractile response of isolated vessels to AmB was reduced by atrial natriuretic peptide. We believe that the attenuation of the acute effects of AmB on GFR in volume replaced compared to hydropenic rats may also reflect the modulatory influence of other vasoactive agents on the direct vascular actions of AmB. At the cellular level this modulation of AmB effects may be caused by relative cell hyperpolarization, a decrease in cytosolic Ca, and an increase in cellular cAMP and cGMP levels. Aminophylline has been shown both to reduce the nephrotoxic effect of AmB (18, 19) and to inhibit the TGF mechanism (17). Since in the present study aminophylline antagonized the constrictor action of AmB in isolated vessels, it appears that aminophylline may alter the vascular response to AmB by a direct effect rather than by inhibiting the TGF mechanism. The high concentrations of aminophylline required to blunt the vasomotor action of AmB suggest that aminophylline may interfere with contractile responses by increasing cyclic nucleotide levels following inhibition of phosphodiesterase. As suggested above, the protective effect of verapamil may result from the central role of voltage-activated Ca channels in AmB-induced vasoconstriction. Finally, there is experimental evidence to suggest that furosemide has direct effects on smooth muscle cells which may be related to its beneficial actions in AmB nephrotoxicity (11). Furosemide has been shown to counteract the effect of phenylephrine-induced contractile responses in rat aorta (38) and to decrease systemic vascular resistance in functionally anephric humans (39). We have not tested the influence of furosemide on the vasoconstrictive effect of AmB in isolated vessels of the rabbit because previous results suggest insensitivity of rabbit smooth muscle cells to furosemide (38). Although further work is needed to fully understand the protective effects of salt loading, aminophylline and in particular furosemide, our results provide solid experimental support for an alternative explanation of the renal hemodynamic actions of AmB that is independent of TGF activation. Another argument supporting the concept of TGF-mediation of AmB vasoconstriction has been the observation that the changes in the determinants of glomerular ultrafiltration caused by AmB administration were similar to those induced by the macula densa signal (12, 24). However, our studies show that AmB can induce vasoconstriction of afferent arterioles in vitro and a fall in SNGFR in vivo, both in the absence of a functional TGF system. Thus, the identity of the vascular effects of AmB and TGF cannot necessarily be taken as indicative of a causal link.

In conclusion, the results of the present studies do not support the proposal that the effect of AmB on GFR is caused by TGF activation subsequent to an elevation in distal NaCl delivery. In isolated perfused afferent arterioles and in rings of aorta and renal artery, AmB causes dose-dependent and endothelium-independent vasoconstriction probably by augmenting

Ca influx through voltage-gated Ca channels. Aminophylline and atrial natriuretic peptide attenuated the constrictor effect of AmB suggesting a role for cyclic nucleotides as intracellular antagonists of AmB activation. These observations are consistent with the hypothesis that a direct AmB-induced vasoconstriction may be an important factor in the reduction of GFR after AmB administration.

Acknowledgments

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