Mechanisms of Amphotericin B-Induced Decrease in Glomerular Filtration Rate in Rats

RAMZI SABRA* AND ROBERT A. BRANCH

Center for Clinical Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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Mechanisms responsible for the reductions in renal blood flow (RBF) and glomerular filtration rate (GFR) in response to acute infusions of amphotericin B were investigated in vivo in rats. The influence of salt status and the roles of adenosine, cyclic AMP, and calcium influx were examined. Amphotericin B was infused into the renal artery in seven groups of rats at 0.025 mg/kg of body weight per min for 15 min. RBF and GFR were measured over 15 min before, during, and after the infusion. Control rats were maintained on a normal salt diet; a second group of rats received a salt-depleted diet, and a third group received a high-salt intake. Four other groups were kept on a normal diet and received theophylline (0.5 μmol/kg/min into the renal artery, intra-arterially [i.a.]), dibutyl cyclic AMP (85 μg/min, i.a.), the 5'-nucleotidase inhibitor adenosine α,β-methylene diphosphate (4 mg/kg, intramuscularly), or diltiazem (20 μg/kg/min, i.a.). Control rats had a prompt 50% decrease in RBF in response to amphotericin B. This was sustained over the 15-min infusion period and was accompanied by a decrease in creatinine clearance (CLCR) (from 0.83 ± 0.08 to 0.40 ± 0.09 ml/min; P < 0.05). On stopping the infusion, RBF returned quickly to baseline but CLCR continued to decrease further (to 0.35 ± 0.07 ml/min; P < 0.05). Salt loading, theophylline, and diltiazem administration prevented the decreases in both RBF and CLCR. Both RBF and CLCR responses in the remaining groups were not significantly different from those in controls. The results of this study reveal a protective effect of salt loading and theophylline against amphotericin B nephrotoxicity in the rat but deny a role for adenosine in mediating these effects. They further suggest that theophylline inhibits the acute responses by a mechanism unrelated to either adenosine receptor blockade or phosphodiesterase inhibition and that calcium influx into the cells is probably responsible for the acute changes in RBF and GFR in response to amphotericin B.

Amphotericin B infusions induce acute reductions in renal blood flow (RBF) and glomerular filtration rate (GFR) in both rats and dogs (4, 9, 11), due mostly to elevation of renovascular resistance (21). The mechanisms underlying these acute hemodynamic effects have not been completely elucidated. Studies with dogs have suggested that activation of tubuloglomerular feedback (TGF) plays a role in these changes, since both TGF and amphotericin B-induced decreases in GFR are inhibited by the same pharmacologic interventions (salt loading, furosemide, or theophylline administration) (1, 7, 8, 24, 31). Unfortunately, these studies were conducted with different species (dogs for amphotericin B and rats for TGF). It is not clear whether both species behave similarly with respect to both TGF and amphotericin B. Therefore, it is important to examine this question since, if true, it would lend greater support to the hypothesis.

In chronic models of nephrotoxicity in the rat, salt loading attenuates the decreases in GFR induced by amphotericin B, while salt depletion potentiates them (16, 28). In addition, there are hemodynamic changes similar to those observed after acute infusions of the drug (28). At present, the effect of salt status on the acute renal response of the rat to amphotericin B is unknown, but it is possible that the acute hemodynamic effects of amphotericin B may contribute to the deterioration in renal function observed in chronic models. In order to examine this possibility and to further study the role of TGF, we decided to assess the influence of salt status on the acute responses to amphotericin B in rats. Theophylline inhibits the GFR-lowering effects of both TGF activation in rats (17, 18) and amphotericin B in dogs (9), but its effect on the amphotericin B response in rats is not known. In the case of TGF, the low concentrations of theophylline used suggested that it was acting through adenosine receptor blockade, but its mechanism of action in the latter case is not clear. Theophylline is known to act by at least two other mechanisms: phosphodiesterase inhibition, which raises intracellular cyclic AMP (cAMP) levels, and induction of changes in intracellular calcium levels (19). Any one of these mechanisms may account for its observed inhibition of the responses to amphotericin B. In the present study, we initially established the inhibitory effect of theophylline against the renal effects of amphotericin B in rats. We then made use of three different drugs in order to differentiate between possible alternative mechanisms of action of theophylline. Dibutylryl cAMP (DB-cAMP) was infused to mimic the rise in intracellular cAMP induced by theophylline (26). Adenosine α,β-methylene diphosphate (AMP-CP) is an ADP analog which inhibits 5'-nucleotidase. This enzyme is responsible for conversion of AMP to adenosine, inosine, and hypoxanthine. Thus, AMP-CP will inhibit the production of adenosine from AMP (29) and will provide an alternative means for assessing the role of adenosine. Finally, we examined the effect of the calcium channel blocker diltiazem on the responses to amphotericin B.

The purposes of this study, therefore, were first, to examine the effect of salt status on the acute responses to amphotericin B in rats and compare them with the chronic situation and with those in other species and second, to further investigate the mechanism of action of the drug by dissecting the mechanism of protection provided by theo-

* Corresponding author.
phylline. The results from both would, in addition, provide some insight into the contribution of TGF to the response.

MATERIALS AND METHODS

Preparation. Experiments were performed on male Sprague- Dawley rats weighing 300 to 350 g. The model used in these studies was the following: rats were anesthetized with pentobarbital (50 mg/kg of body weight intraperitoneally), and a tracheal cannula was inserted to maintain an adequate airway. The right jugular vein was catheterized with PE-60 tubing, which served for administration of plasma and drugs and for withdrawal of blood. Next, the abdomen was opened through a midline incision, and the right kidney was excised after the vessels to and from it were ligated. The left carotid artery was cannulated with PE-90 tubing filled with heparin. The abdominal aorta was ligated at a site just proximal to its bifurcation, and a PE-160 catheter was introduced proximally in the upstream direction. The carotid and aortic catheters were connected by means of an extracorporeal system made up of silastic tubing having a flow probe within it and filled with heparinized saline. The flow probe was connected to a Statham Blood Flowmeter (SP 2202) and served for continuous recording of RBF. The tubing also had several portholes for monitoring of pressure and administration of drugs. After tying off all arteries arising from the aorta between the site of cannulation and the left renal artery, 1,500 U of heparin was administered intravenously. This was followed by ligating the aorta at a site between the left renal artery and the superior mesenteric artery. This maneuver allowed the blood to flow from the carotid artery through the extracorporeal system into the renal artery, while keeping the perfusion of the intestines intact. The resulting system allowed for continuous monitoring of RBF and renal perfusion pressure (RPP) (with a Gould P 23 XL pressure transducer connected to a Grass model 79 D polygraph recorder), administration of drug directly into the renal artery, and obtaining arterial blood samples when necessary. Finally, the left ureter was catheterized with PE-10 tubing for collection of urine.

Protocol. Rat plasma was administered after opening the abdomen at a rate of 10 ml/kg/h for 45 min followed by 1.5 ml/kg/h for the remainder of the experiment to replace fluid losses. After surgery was completed, rats were allowed to stabilize for 45 to 60 min. The experiment consisted of three observation periods of 15 min each, during which RBF and RPP were continuously monitored and urine samples were collected. Period 1 was the baseline period and was followed by a 15-min infusion of amphotericin B at 0.025 mg/kg/min into the extracorporeal shunt (intra-arterially i.a.), after which there was the final (postinfusion) period. Plasma samples were collected during period 1 and at the end of period 3. Concentrations of creatinine in urine and plasma were measured with a Beckman creatinine autoanalyzer (Beckmann Instruments Inc., Fullerton, Calif.), and creatinine clearance (CLCR) was calculated during each period by using the formula UCR V/P CR, where UCR and P CR are the concentrations of creatinine in urine and plasma, respectively, and V is urine flow rate. Renal resistance for each period was calculated by averaging the individual resistance values during each 1-min interval during that period.

Seven groups of rats were studied. Each group consisted of seven rats except for the salt-loaded and diltiazem-treated groups, in which six rats were used. The control group consisted of rats maintained on a normal-salt diet. Another group of rats (−NaCl rats) was maintained on a low-salt diet (Na composition, <0.05%; Ralston Purina Co., St. Louis, Mo.) and tap water for at least 1 week prior to the day of the experiment. A third group was kept in a high-salt status (+NaCl rats) by subcutaneous injection of 10 mg of deoxycorticosterone acetate 1 week prior to the day of the experiment, followed by maintenance on a normal-salt diet and 1.0% saline drinking water. This manipulation of the diets has been shown to result 1 week later in Na depletion and Na loading as reflected by the urinary Na excretion rate (18). In addition, +NaCl rats received an infusion of 0.9% saline at a rate of 0.1 ml/min to maintain volume expansion. This infusion was started at the time of surgery and maintained throughout the experiment and has been previously used to inhibit TGF activity. The remaining groups of rats received a normal-salt intake and one of the following interventions: (i) theophylline (5 μmol/kg/min for 10 min, and then 0.5 μmol/kg/min into the extracorporeal system i.a.), started 30 min prior to amphotericin B, and continued throughout the experiment). This dose has been shown to inhibit responses to exogenous adenosine in previous studies with rats (4, 17) as well as pilot experiments in this model. It also is similar to the dose that inhibits TGF activation (6) and amphotericin B-induced fall in RBF in rats (4); (ii) AMP-CP (4 mg/kg intramuscularly 2 h prior to amphotericin B). This dose has previously been shown to cause a maximal inhibition of 5′-nucleotidase activity 2 h after intramuscular injection (15); (iii) DB-cAMP (85 μg/kg/min i.a., started 30 min prior to amphotericin B and continued throughout the experiment). This dose has previously been shown to induce biological effects in the kidney, without significantly altering the baseline RBF (14); and (iv) diltiazem (20 μg/kg/min, i.a., started 30 min prior to amphotericin B and continued throughout the experiment). This dose was found, in pilot experiments, to be the highest tolerated dose without changing baseline renal vascular resistance.

Statistical analysis. Values are presented as means ± standard error of the mean. Changes in RBF within each group were compared with baseline by one way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. Comparison of the RBF response between control and experimental groups was done by repeated measures ANOVA. CLCR was compared within and between groups by two-way ANOVA, one factor being treatment group and the other being the experimental period. The paired Student’s t test was then used for comparison of individual time points within each group. A P value of <0.05 was considered statistically significant.

RESULTS

Baseline RBF, RPP, and calculated renal resistance values in each of the study groups are presented in Table 1. The only difference from control in baseline RBF was in the salt-loaded group, in which it was significantly higher. In addition, RPP was significantly lower than control in both the theophylline and diltiazem groups. Since both RBF and RPP were essentially stable during each period except for the first 1 to 2 min of the second and third periods, we were able to calculate values for average total renal resistance (R T) for each period. Calculation of baseline RT revealed significant differences only between the control and theophylline groups.

The urinary sodium excretion rates during the baseline period were calculated in the control, −NaCl, and +NaCl groups to evaluate the adequacy of the dietary manipulation. The control and −NaCl groups had similar values of 0.07 ±
0.02 and 0.09 ± 0.03 μeq/min, respectively, while +NaCl rats had a substantially higher rate of 1.52 ± 0.44 μeq/min (P = 0.004).

In the control group, i.a. infusion of amphotericin B induced a rapid fall in RBF, which reached 50% of the baseline value at 2 min and was maintained at approximately that level throughout the duration of the infusion (Fig. 1). Upon stopping amphotericin B, there was a similarly rapid return of RBF toward the baseline level. Measurement of CLCR during these three periods revealed a decrease during the amphotericin B infusion (from 0.83 ± 0.08 to 0.40 ± 0.09 ml/min; P < 0.05) (Table 2). Despite the return of RBF to baseline in the postinfusion period, there was a progressive fall in CLCR (to 0.35 ± 0.07 ml/min; P < 0.05).

In the salt-loaded group, both RBF and GFR responses were significantly inhibited. There was no significant change in RBF over the first 11 min of the drug infusion in this group of rats, but during the last 3 min, there was a progressive reduction in RBF (Fig. 1). Since the baseline RBF was higher in +NaCl rats, this drop (25%) is much smaller percent decrease than that in the control group (50%). ANOVA revealed a significant difference in the RBF response over time compared with that in the control group (P < 0.0001). Baseline CLCR was significantly higher in this group compared with that in the rest (P < 0.02) but did not change significantly over the three experimental periods (Table 2). In contrast the RBF response in the salt-depleted group was almost identical to that of the control group (Fig. 1), with repeated measures ANOVA showing no significant differences between the two. The decrease in CLCR also followed a similar pattern (Table 2).

 Pretreatment with theophylline resulted in a higher baseline RBF and a lower renal resistance (Table 1). Theophylline significantly inhibited the reduction in RBF induced by amphotericin B (Fig. 2). When compared with the control group there was a significant effect of theophylline treatment on the RBF response for the experimental period. In addition, CLCR remained at baseline values throughout the experimental period (Table 2).

In order to analyze the mechanism by which theophylline prevented the effects of amphotericin B on the kidney, either DB-cAMP or AMP-CP was administered to rats. Neither drug substantially modified the fall in RBF (Fig. 2). In the DB-cAMP-treated group, the vasoconstrictor response dur-

### Table 1. Effects on rats of treatment with amphotericin B at 0.025 mg/kg/min for 15 min i.a.

<table>
<thead>
<tr>
<th>Rat group</th>
<th>RBF (ml/min)</th>
<th>RPP (mm Hg)</th>
<th>R_T (mm Hg/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.1 ± 0.4</td>
<td>115 ± 5</td>
<td>12.8 ± 0.4</td>
</tr>
<tr>
<td>−NaCl</td>
<td>8.0 ± 0.6</td>
<td>113 ± 3</td>
<td>15.0 ± 1.8</td>
</tr>
<tr>
<td>+NaCl</td>
<td>12.0 ± 1.2*</td>
<td>124 ± 5</td>
<td>11.1 ± 1.7</td>
</tr>
<tr>
<td>Theophylline</td>
<td>10.5 ± 0.5</td>
<td>96 ± 5*</td>
<td>9.1 ± 0.3*</td>
</tr>
<tr>
<td>AMP-CP</td>
<td>8.3 ± 0.7</td>
<td>118 ± 4</td>
<td>15.3 ± 1.5</td>
</tr>
<tr>
<td>DB-cAMP</td>
<td>8.8 ± 0.8</td>
<td>110 ± 3</td>
<td>13.0 ± 1.2</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>8.1 ± 0.6</td>
<td>97 ± 1*</td>
<td>12.2 ± 0.5</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with control group.

### Table 2. Creatinine clearance during each experimental period in the seven groups of rats

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Baseline CLCR (ml/min)</th>
<th>Amphotericin B</th>
<th>Post-ampothericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>0.83 ± 0.08</td>
<td>0.40 ± 0.09*</td>
<td>0.35 ± 0.07*</td>
</tr>
<tr>
<td>−NaCl (5)</td>
<td>0.74 ± 0.15</td>
<td>0.56 ± 0.18*</td>
<td>0.40 ± 0.05*</td>
</tr>
<tr>
<td>+NaCl (7)</td>
<td>1.13 ± 0.18*</td>
<td>0.96 ± 0.17*</td>
<td>1.34 ± 0.25*</td>
</tr>
<tr>
<td>Theophylline (6)</td>
<td>0.76 ± 0.19</td>
<td>0.66 ± 0.18</td>
<td>0.74 ± 0.28*</td>
</tr>
<tr>
<td>AMP-CP (7)</td>
<td>0.69 ± 0.05</td>
<td>0.52 ± 0.03*</td>
<td>0.37 ± 0.07*</td>
</tr>
<tr>
<td>DB-cAMP (6)</td>
<td>0.76 ± 0.09</td>
<td>0.50 ± 0.13*</td>
<td>0.21 ± 0.10*</td>
</tr>
<tr>
<td>Diltiazem (6)</td>
<td>0.65 ± 0.07</td>
<td>0.60 ± 0.02*</td>
<td>0.60 ± 0.08*</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with baseline.

* P < 0.05 compared with same period in control group.
ing the infusion period was not significantly different from that of the control, but the RBF during the postinfusion period remained significantly below baseline and was less than that in the control group. However, in this group of rats, there was a significant decrease in renal perfusion pressure during the postinfusion period compared with baseline (baseline, 110 ± 3 versus postinfusion, 95 ± 5 mm Hg). Calculation of renal resistance showed that there was no significant difference from baseline (baseline, 13.0 ± 1.2 versus post-infusion, 14.5 ± 0.13 mm Hg/min/ml). Thus, the results were essentially similar to the control group. Calculation of CLCR in these two groups revealed significant decreases over the two consecutive periods (Table 2), and these changes were similar to the control group.

Finally, treatment with diltiazem caused a significant reduction in baseline RPP but not in baseline resistance (Table 1). The RBF response to amphotericin B in this group was similar to that observed in the theophylline-treated group (Fig. 3). Diltiazem was also successful in preventing the decrease in GFR (Table 2).

**DISCUSSION**

The results of this study confirm that the decreases in RBF and GFR associated with acute administration of amphotericin B in rats are inhibited by salt loading and theophylline administration. They further suggest that these changes are related to activation of calcium fluxes into cells. They, however, do not support a role for adenosine in mediating these changes.

Examining the effects of changing the salt status on the urinary sodium excretion rate revealed that, despite manipulation of the diet, the control and salt-depleted rats had similarly low values. This suggested that the model that was used introduced significant stress to render the kidney salt retaining. In contrast, the salt-loaded group had elevated sodium excretion rates compared with those of the former groups, at values previously reported to be associated with inhibition of TGF (6). The control and salt-depleted group had similar RBF and GFR responses, while the salt-loaded group had significantly attenuated responses. In the last group, the RBF started to decrease linearly as of 8 min into the infusion and became similar to that in the control group at 12 to 15 min. A possible explanation for this effect is that a pool of a mediator which protects the kidney during salt loading is being continuously depleted during the drug infusion or, conversely, a mediator whose production or action is being inhibited by salt loading is accumulating. The results are consistent with the hypothesis that the acute hemodynamic effects of amphotericin B contribute to chronic nephrotoxicity. This conclusion is based on two points: (i) the hemodynamic effects are similar in the acute and chronic situation, and (ii) salt loading protects against both acute and chronic renal effects of amphotericin B. The results are also consistent with the hypothesis that amphotericin B induces the acute changes by activating TGF, since TGF-induced fall in GFR is also prevented by salt loading (1). However, alternative explanations, exist. Given the slightly reduced renal resistance in the +NaCl group, it could be argued that the vasculature became unresponsive to constriction. A similar argument can be used to explain the protective effects of theophylline in this study, such that it would be acting as a functional antagonist of amphotericin B. Prior studies, however, have revealed that vasodilation per se, with hydralazine (4) or nitroprusside (11) does not alter the vasoconstrictor response to amphotericin B. Irrespective of the mechanisms involved, this finding may have relevant implications clinically in that recent findings in clinical trials (3, 10, 14) have revealed a protective effect of salt loading against amphotericin B nephrotoxicity.

This study also evaluated the various mechanisms that might contribute to the changes in renal response to amphotericin B induced by theophylline. Previous studies have shown that theophylline inhibits the RBF and GFR responses to amphotericin B infusions in dogs (9). In rats, it has an inhibitory effect on the RBF response (11), but the GFR response has not been studied. In this study, we were able to confirm an inhibitory effect of theophylline on the fall in both RBF and CLCR.

Here, it is necessary to emphasize the need for conducting these experiments in rats. As can be seen from the results with salt loading, it is now clear that this measure inhibits the acute renal effects of amphotericin B in rats and dogs, its chronic effects in rats and in humans, and its acute and chronic effects in rats. This finding suggests that the mechanisms of action of the drug in the three species is similar. The similar results obtained by using theophylline with rats and dogs support this. Therefore, it is now possible to investigate the mechanisms of action of this agent in one species (rats, since they are the easiest to work with) and be more confident in extrapolating the results to others, notably, humans. Furthermore, as mentioned in the Introduction, the role of TGF was based on studies conducted in different species which may differ not only in their response to amphotericin B but also in their intrarenal regulatory homeostatic mechanisms such as TGF.

Three alternative mechanisms of action have been attributed to theophylline. These include adenosine receptor blockade, phosphodiesterase inhibition, and changes in intracellular calcium levels (17). A recent study has suggested that adenosine receptor blockade is probably not responsible for modification by theophylline of the effects of amphotericin B. That study revealed that the adenosine receptor antagonist, 1,3-dipropyl-8-p-sulfophenylxanthine, a xanthine which lacks the other actions of theophylline, was unsuc-
cessful in antagonizing the RBF-lowering effects of amphotericin B (13). Using AMP-CP as an alternative pharmacological probe of the role of adenosine, we were also unable to substantially alter the response to amphotericin B. Conversely, there was a tendency for AMP-CP to potentiate the response. The dose of AMP-CP used here had been shown to inhibit the activity of 5′-nucleotidase in response to ischemia to the rat kidney by 80% at 2 h postinjection. These results, therefore, strongly argue against a role for adenosine in mediating the changes in RBF and GFR in response to amphotericin B. Since adenosine is considered a putative mediator for TGF (17, 18, 25), this result suggests that TGF is not involved in these responses. However, it should be noted that several other messengers have been proposed for the efferent limb of TGF, including arachidonic acid metabolites, angiotensin, and calcium (2, 15, 30).

Although the group treated with DB-cAMP tended to have an attenuated response during the first few minutes of the infusion, the overall response was not significantly better than that in the control group. In fact, during the latter stages of the experiment the RBF was lower. It is, therefore, unlikely that theophylline-induced phosphodiesterase inhibition was responsible for the modification by theophylline of the renal effects of amphotericin B.

Finally, and in order to examine the role of intracelluar calcium levels in mediating the effects of amphotericin B and their inhibition by theophylline, we used the voltage-dependent calcium channel blocker diltiazem. As the results demonstrate, diltiazem protected the kidney against the amphotericin B insult. These results with diltiazem are similar to those obtained by Tolins and Raji (27) who, using the structurally different calcium channel blocker verapamil, demonstrated a marked inhibition of the amphotericin B-induced decrease in RBF and a significant attenuation of that in GFR. Therefore, these two studies strongly suggest that voltage dependent calcium entry has a pivotal role in mediating the acute hemodynamic effects of amphotericin B.

Regarding the role of TGF in mediating the effects of amphotericin B, the results of this study are inconclusive. While the protection by salt loading is supportive of such a role, the evidence against involvement of adenosine is contradictory. The protection by diltiazem can be explained in many ways, one of which is inhibition of TGF (2), but there are also vasodilation and calcium channel blockade in the effector cells. Since submission of the manuscript, however, new findings have provided evidence against a relationship between the acute renal effects of amphotericin B and activation of TGF. A study by Sawaya et al. examined the role of TGF directly by using micropuncture techniques (22). The authors found no difference between single nephron GFR measured in proximal (TGF interrupted) or distal (TGF intact) tubules. Nor was there any effect of amphotericin B on distal chloride concentration or on TGF sensitivity. The drug, however, contracted isolated renal arteries and arterioles, an effect that was inhibited by removal of calcium from the bathing medium, by calcium channel blockade with verapamil, and by the presence of theophylline. We have also observed a similar response in cultured glomerular mesangial cells, in which amphotericin B induced a rise in cytosol-free calcium concentrations suggestive of contraction, which was inhibited by removal of extracellular calcium and by diltiazem (20). Thus, it is probable that the effects observed with diltiazem in the present study involve inhibition of calcium channel activity in the cells that are being contracted by amphotericin B. The in vitro inhibition of contraction by theophylline also points to a mechanisms unrelated to inhibition of TGF by the xanthine. Thus, it is also probable that amphotericin B acts directly on contractile cells, in vivo as it does in vitro, possibly by altering membrane permeability. Alternatively, it may induce these cells to release vasoactive agents which result in a contractile response.

The ability of both diltiazem and theophylline to prevent the RBF changes secondary to amphotericin B raise the possibility that the xanthine may be acting on calcium channels. The effects of xanthines on intracellular calcium levels vary according to the type of tissue used. In skeletal and cardiac muscle, these drugs increase calcium levels and potentiate contractions (19). In intestinal smooth muscle, they cause relaxation of muscle tone and decrease intracellular calcium levels, mainly because of inhibition of calcium entry and partly because of increased binding of calcium by intracellular proteins (12). It is possible that the protective effect of theophylline in the present study may relate to some degree of calcium channel blockade. Alternatively, the effects of the two drugs may be totally unrelated.

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