Table II. Urinary excretion of M_2 and M_3 in dogs after intravenous or intramuscular injection of CTX (20 mg/kg)

Time after injection (h)	Intravenous injection		Intramuscular injection	
	M_2(%)	M_3 (%)	M_2 (%)	M_3 (%)
0–1	0.57±0.03	0.61±0.15	0.16±0.04	0.29±0.04
1–2	1.47±0.09	1.65±0.08	0.81±0.07	1.25±0.17
2–4	1.59±0.30	1.55±0.58	0.85±0.11	0.94±0.04
4–6	0.43±0.10	0.58±0.13	0.38±0.08	0.50±0.11
6–24	n.d.	n.d.	n.d.	n.d.
Total	4.00±0.34	4.63±0.40	2.35±0.33	4.38±0.57

Each value represents the mean of six dogs with standard error.
n.d.: not detectable.

Therefore, CTX disposition appears to obey linear pharmacokinetics. On the other hand, theoretical curves for plasma levels of CTX and desacetyl-CTX after intramuscular injection of CTX, obtained with the use of the pharmacokinetic parameters after intravenous administration, did not fit well with actual values, suggesting that intramuscular CTX, unlike intravenous CTX, does not show a simple first order kinetic pattern. The kinetic difference between the two routes of administration seems unrelated to the production of desacetyl-CTX, M_2, and M_3, because there was no significant difference between the urinary excretion of the three metabolites after intramuscular and intravenous administration of CTX.

(3) When CTX and CET were injected intramuscularly, they were detected in high concentrations in the blood, revealing that both drugs rapidly diffused from the injection site into the blood.

(4) When CET was injected intramuscularly, a large quantitity of desacetyl-CET was detected in the urine. Desacetyl-CET was produced to a much greater extent when CET was injected intramuscularly than when injected intravenously. These results were consistent with the higher AUC ratio of desacetyl-CET over unchanged CET after intramuscular than after the intravenous injection of CET. On the other hand, the urinary pharmacokinetics of CTX were almost the same when the drug was injected intramuscularly or intravenously.

(5) CTX metabolites other than desacetyl-CTX (M_2 and M_3) after intravenous and intramuscular injection were detected in trace amounts in the urine but were not detectable in the plasma. There was no significant difference for M_2 and M_3 between the two injection routes.

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Salutary Effects of Two Verapamil Analogs in Traumatic Shock[5]

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Abstract: We studied the effects of two verapamil analogs, anipamil and ronipamil, in traumatic shock. Noble-Collip drum trauma produced a shock state characterized by an eight-fold increase in plasma cathepsin D activity, a 13-fold increase in the rate of plasma myocardial depressant factor (MDF) accumulation, and a survival time of 1.9 ± 0.1 hours. Neither verapamil analog had any significant effect on attenuating the shock-induced rise in plasma cathepsin D activity. However, both anipamil and ronipamil ($p < 0.01$) significantly blunted the rate of MDF accumulation in the plasma. In addition, these agents significantly inhibited proteolysis *in vitro*. Both analogs significantly prolonged survival time to 3.1 ± 0.6 h at 0.25 mg/kg ($p < 0.05$) and to 4.4 ± 0.3 h at 1.0 mg/kg ($p < 0.001$). Anipamil appears to provide a more potent protection in this shock model; however, both verapamil derivatives possess promising anti-shock potential.

Agents that inhibit calcium influx are beneficial in protecting hypoxic and ischemic cells (1, 2, 3) and are useful in the treatment of hypertension (4, 5) as well as the hypodynamic

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state of shock (6, 7, 8, 9). A variety of calcium entry blockers have been reported to selectively improve tissue perfusion (10, 11), preserve mitochondrial function (12), protect the ischemic myocardium (2), hypoxic hepatocytes (13) and ischemic kidneys (2). Verapamil has been reported to improve survival in hemorrhagic shock with associated reductions in the severity of myocardial lesions and small bowel infarction (6, 14). More recently, we reported beneficial effects for nitrendipine in hemorrhagic shock, including significantly blunted plasma cathepsin D, free amino-nitrogen, and myocardial depressant factor (MDF) activities (7). Both nitrendipine (8) and nimodipine (9) were shown to improve survival and attenuate MDF accumulation in traumatic shock. In addition, the detrimental actions of many potential mediators of shock and ischemia are antagonized by calcium entry blockade, e.g., thromboxane (15, 16), leukotrienes (17) and angiotensin II (18).

Anipamil and ronipamil (Knoll AG, Ludwigshafen, W. Germany) are new calcium entry blockers derived from verapamil (19, 20). In the present study we have investigated the actions of anipamil and ronipamil in a standardized model of traumatic shock. It was our purpose to determine their effectiveness as therapeutic agents in circulatory shock and to investigate the mechanism of any beneficial effect.

Methods

Male Sprague-Dawley rats weighing between 230–290 g were utilized in this study. The rats were maintained on a 12 h light/12 h dark cycle with free access to food and water. All animals were anesthetized with sodium pentobarbital, 40 mg/kg, i.p., prior to all experimental procedures. Traumatic shock was induced in anesthetized rats by whole body trauma in a Noble-Collip drum apparatus (21). Prior to the induction of trauma, a polyethylene catheter (PE-50) filled with heparinized 0.9 % NaCl was inserted into the right carotid artery. Pre-trauma heart rate (HR) and mean arterial blood pressure (MABP) were recorded on a Grass Model 7 oscillographic recorder using Statham P23AC pressure transducers. Baseline values were recorded, and a 1.0 ml arterial blood sample was allowed to bleed into iced tubes containing 100 μl of 3.8 % sodium citrate for the subsequent analysis of cathepsin D activity. After the blood was replaced by an equal volume of heparinized 0.9 % NaCl, the right carotid catheter was sealed, and the neck incision was closed prior to the onset of trauma.

Anesthetized rats were subjected to 475 revolutions in a Noble-Collip drum at 45 rpm resulting in approximately 90 % mortality 2.5 h after trauma. Immediately after trauma, a polyethylene cannula (PE 250) was inserted into the trachea to maintain a patent airway. MABP and HR were recorded (time 0), and the left external jugular vein was catheterized for drug administration, and the intravenous injection of anipamil (1,7-Bis-(3-methoxyphenyl)-3-methylaza-7-cyano-nonadecane-hydrochloride monohydrate) or ronipamil (13-cyano-13-phenyl-17-aza-17-methyl-19-phenyl-nonadecane-hydrochloride) was given at doses of either 0.25 or 1 mg/kg 10 to 15 minutes after the completion of trauma.

Experimental animals were randomly divided into the following four groups: a) Sham trauma + anipamil – 6 rats; b) Trauma + vehicle (30 % ethanol) – 10 rats; c) trauma + anipamil, low dose (0.25 mg/kg) – seven rats; d) trauma + anipamil, high dose (1.0 mg/kg) – eight rats. Additional groups of 7 to 9 rats were also used in the ronipamil groups. All sham trauma rats were anesthetized and subjected to the same surgical manipulations and blood sampling, but were not traumatized. Drug treated rats received either 0.25 mg/kg or 1.0 mg/kg anipamil or ronipamil at time 0. All animals were given an equal volume (0.2 ml) of either the drug or its vehicle (30 % ethanol). At five hours post-trauma or when the MABP declined to 45 mm Hg, experimental observation was terminated and blood samples were collected for determination of final plasma cathepsin D activity and myocardial depressant factor (MDF) activity.

Plasma Analysis

Blood samples were centrifuged at 2500 x g for 15 min at 2 °C. The plasma was collected, and the final samples were assayed for cathepsin D and MDF activities. Plasma cathepsin D activity was determined by the method of Anson (22) and was used as an index of lysosomal labilization. Plasma MDF was measured according to the method of Barenholz et al. (23) with the chromatographic modifications of Yamada and Pettit (24). The elution of the active region of the paper chromatogram was followed by its spectrophotometric analysis.

In Vitro Preparations

Cat pancreatic homogenates were prepared as previously described (25) for the study of possible direct anti-proteolytic actions of anipamil and ronipamil. We utilized the generation of free amino-nitrogen groups measured by the ninhydrin method (26) as our index of proteolysis. The units are expressed as μmoles serine x 10^{-2}/mg protein for six separate pancreatic homogenates.

Statistics

Intergroup comparisons were performed using the analysis of variance (ANOVA) and the Newman-Keuls multiple comparison test. The paired "t" test was used to analyze cathepsin D activity, and was confirmed by analysis of variance (ANOVA). All values are means ± SEM.

Results

The time course of MABP changes for the four groups of rats in the anipamil study is illustrated in Figure 1. Anipamil (1 mg/kg) had no significant effect on MABP at any time over the five hour experimental period when given to sham-operated control rats (137 ± 8 vs 123 ± 9 mm Hg, initial vs final, respectively). However, trauma significantly reduced MABP by 31 ± 3 % ($p < 0.001$). In rats receiving only the vehicle, MABP recovered slightly over the first 30 min and then declined steadily to 45 mm Hg over the next 1 to 2 hours. In contrast, rats treated with anipamil maintained significantly higher ($p < 0.01$) MABP from 60 minutes to the end of the experiment when compared to shock rats given only the vehicle. At 150 minutes after trauma, only 10 % (1/10) of the untreated, traumatized rats survived, the survivor having a MABP of only 45 mm Hg. In contrast, 71 % (5/7) of the low dose (0.25 mg/kg) drug-treated rats survived at least 150 min with a MABP of 87 ± 10 mm Hg. Similarly, all traumatized rats (8/8) given 1 mg/kg of anipamil survived 150 minutes with a MABP of 90 ± 6 mm Hg. Furthermore, at 5 hours 63 % (5/8) of the rats given this dose of anipamil survived with a MABP of 75 ± 11 mm Hg compared to only 29 % (2/7) of the low dose group.

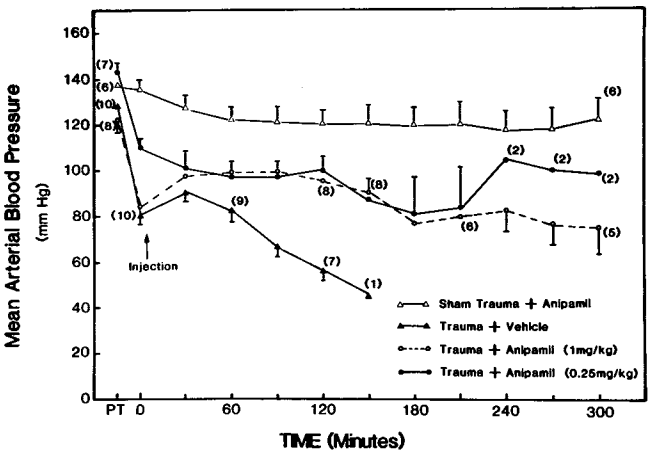

Fig. 1 This figure illustrates the time course of the changes in mean arterial blood pressure (MABP) for the four experimental groups of rats. Values are means ± SEM. The numbers in parentheses indicate the number of rats surviving at that time. Traumatic shock was induced between PT and Time 0. PT = Pre Trauma. ↑ = drug injection.

These data indicate that anipamil treatment results in a dose-dependent improvement in both the survival and hemodynamic status of rats in traumatic shock.

Administration of anipamil to sham traumatized rats had no significant effect on heart rate over the five hour observation period at any dose tested. Drum trauma induced a significant reduction in heart rate which was not consistently altered by anipamil. Therefore, it appears that anipamil maintains MABP in shock rats by some mechanism independent of changes in heart rate.

All sham trauma rats given anipamil survived the entire five hour observation period. The mean survival times for the experimental groups are illustrated in Figure 2. The trauma protocol resulted in a severe shock state characterized by a survival time of 1.9 ± 0.1 hours in untreated rats. Anipamil significantly improved the mean survival time at both 0.25 mg/kg (3.1 ± 0.6 h, $p < 0.05$), and at 1.0 mg/kg (4.4 ± 0.3 h, $p < 0.001$), indicating a dose-dependent prolongation of survival time.

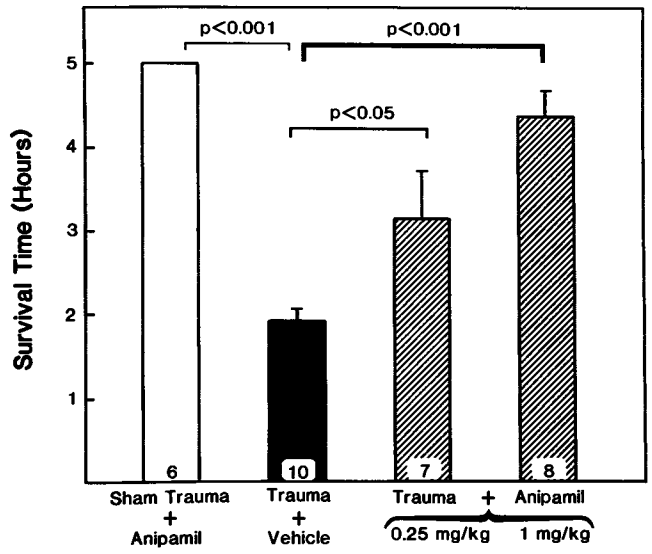

Fig. 2 This graph illustrates the mean survival time in hours following Noble-Collip drum trauma. Bar heights represent mean values ± SEM. Numbers within the bars represent the number of animals in each group. Anipamil significantly improved survival at both treatment doses.

The beneficial actions of anipamil on survival and maintenance of MABP are probably not mediated by a lysosomal stabilizing action. Table I summarizes the effects of the experimental protocols on plasma cathepsin D activity. Drum trauma induced a 8-fold increase ($p < 0.01$) in plasma cathepsin D activity, indicative of the characteristic lysosomal labilization in this shock model. Neither dose of anipamil had any significant effect on attenuating this rise in plasma cathepsin D activity. The magnitude of the final plasma cathepsin D activities for the drug treated rats was not significantly different from that of untreated shock rats.

Table I. Effects of anipamil on plasma cathepsin D activity in rats subjected to sham trauma or to traumatic shock.

Group	Plasma Cathepsin D activity (U/ml) Initial	Final
Sham Trauma + Anipamil (5)	0.7 ± 0.2	1.3 ± 0.3
Trauma + Vehicle (9)	1.1 ± 0.2	8.9 ± 2.0*
Trauma + Anipamil (0.25 mg/kg) (6)	0.5 ± 0.2	6.2 ± 1.0*
Trauma + Anipamil (1 mg/kg) (6)	1.1 ± 0.3	10.4 ± 2.1*

*Significantly different from the corresponding control value at $p < 0.01$.
() number of samples
Mean ± SEM

In contrast, the rate of MDF accumulation in the plasma was markedly curtailed by drug treatment. Figure 3 illustrates the dose-dependent effect of anipamil on MDF accumulation in the plasma. Untreated traumatized rats produced and accumulated MDF at a rate of 169 ± 18 U/h, which was significantly greater than the rate for the sham operated control rats ($p < 0.001$). At 0.25 mg/kg, anipamil significantly attenuated the rate of MDF accumulation when compared to the vehicle. Drug treatment at 1 mg/kg reduced the rate of accumulation of MDF in the plasma by approximately 70 % of that for rats receiving only vehicle ($p < 0.01$).

Although anipamil does not appear to stabilize lysosomes *in vivo*, it does reduce the accumulation of MDF. In order to elucidate a possible mechanism by which anipamil could attenuate plasma MDF accumulation, we studied the direct effects of the drug on the rate of proteolysis in pancreatic homogenates. Anipamil (20 μg/ml) significantly retarded the rate of proteolysis in homogenates incubated for 30 minutes when compared to the vehicle, 0.74 ± 0.09 vs 1.12 ± 0.07 μmoles serine x 10^{-2}/mg protein ($p < 0.001$), respectively. This direct antiproteolytic action of anipamil may explain part of its anti-MDF effect.

Ronipamil at 0.25 to 1.0 mg/kg exerted no significant hemodynamic effect in non-traumatized rats. However, ronipamil, at a dose of 1 mg/kg given 15 minutes post-trauma, significantly prolonged survival time ($p < 0.02$), although the effect was not as striking as with the same dose of anipamil. Table II summarizes these results. Ronipamil also failed to stabilize lysosomal membranes during traumatic shock.

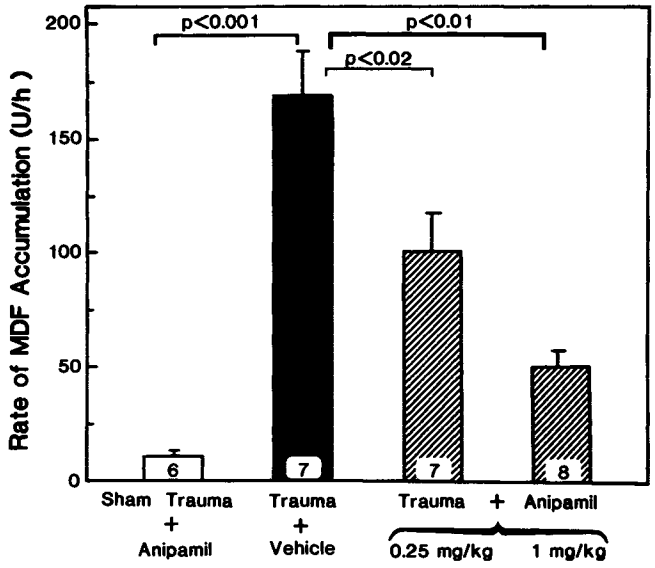

Fig. 3 This graph represents the rate of accumulation of myocardial depressant factor (MDF) in the plasma for the four experimental groups. Bar heights represent means ± SEM. The numbers in the bars represent the number of samples assayed. Anipamil treatment produced a significant dose-dependent reduction in the rate of MDF accumulation in the plasma.

Table II. Effect of ronipamil on the sequelae of traumatic shock in rats.

Measurement	Traumatic Shock + Vehicle (7)	Traumatic Shock + Ronipamil (1 mg/kg) (9)	Statistical Significance
Survival time (h)	1.8 ± 0.4	3.1 ± 0.3	$p < 0.02$
Final Plasma Cathepsin D activity (Units/ml)	6.6 ± 0.7	8.3 ± 0.9	NS
Rate of Plasma MDF Accumulation (Units/h)	149 ± 27	74 ± 11	$p < 0.01$

All values are means ± SEM.

Plasma cathepsin D activity increased markedly in both groups of traumatized rats to about the same extent. However, ronipamil treatment markedly attenuated the plasma accumulation of MDF activity ($p < 0.01$). Thus, ronipamil appears to be somewhat less potent than anipamil in traumatic shock in the rat. This is also consistent with the effect of ronipamil on proteolysis in cat pancreatic homogenates. Ronipamil (20 μg/mg) significantly retarded the rate of proteolysis compared to the vehicle, 0.91 ± 0.1 vs 1.16 ± 0.09 μmoles serine x 10^{-2}/mg protein ($p < 0.02$). However, the anti-proteolytic effect of ronipamil was not as marked as that of anipamil.

Discussion

Calcium entry inhibitors have been shown to have beneficial actions in at least two different types of circulatory shock (7, 8). Several aspects of the biological action of these inhibitors may participate in this anti-shock action: a) selective vasodilation of critical vascular beds (i. e., splanchnic, coronary, etc.) (5, 10); b) prevention of mitochondrial Ca^{++} overload (2, 12); c) inhibition of Ca^{++} activated phospholipases (l) and subsequent damage to cell membrane systems including lysosomal membranes (7, 27); and d) inhibition of Ca^{++} dependent proteases which may participate in MDF formation (7, 8, 9). Agents which block calcium influx into cells may exhibit beneficial actions to varying degrees depending upon their primary site of action. In addition, calcium entry blockers antagonize the vascular actions of a number of potential mediators in circulatory shock and in some cases attenuate both their production and vascular actions (e. g., thromboxane A_2 and peptide leukotrienes) (16).

The data presented in this study provide strong evidence that anipamil improves the hemodynamic status and survival of rats in traumatic shock. All non-treated rats died within 2.5 hours, whereas the anipamil treated rats survived 4.4 ± 0.3 h with 63 % (5/8) surviving at least 5 hours in this lethal shock model. Our findings are also in agreement with previously reported data showing improvement in survival with anipamil pretreatment in hemorrhagic shock and scalding injury (19). Anipamil does not appear to stabilize lysosomal membranes *in vivo* in traumatic shock, similar to the lack of membrane stabilizing effect of nitrendipine in this same model. Nevertheless, Balogh and Kovach (20) have reported significant cytoprotective effects of anipamil and ronipamil in the heart, liver and kidneys of rats in hemorrhagic shock which may contribute to their beneficial effect in trauma. The significant antiproteolytic effect of anipamil and ronipamil in pancreatic homogenates is consistent with that of the dihydropyridines, nitrendipine and nimodipine (7, 9). This action could explain the significant reduction in MDF accumulation following anipamil treatment. Anitproteolytic agents are known to attenuate the production of this cardiotoxic peptide (28). Furthermore, this anti-MDF effect may contribute to some of the previously reported cardioprotective actions of anipamil in hemorrhagic shock (19, 20).

Agents that improve splanchnic blood flow are also effective in attenuating plasma MDF accumulation. We were unable to measure splanchnic blood flow in the rats in these experiments; however, in a previous study employing cats (7), nitrendipine treatment maintained superior mesenteric artery flow (SMAF) at values not significantly different from control during the postoligemic phase. Anipamil and ronipamil may have similar beneficial actions on SMAF in traumatic shock.

In summary we report a number of beneficial effects for anipamil and ronipamil in traumatic shock. These beneficial effects include: a) prolonged survival; b) a direct antiproteolytic action; and c) a reduced rate of accumulation of MDF. The effects on survival time and rate of MDF accumulation are dose-dependent with noteworthy effects at 1 mg/kg. Our data are in agreement with those of other investigators (19, 20) and represent protective effects. However, additional experiments are necessary to further elucidate the complete anti-shock actions of these interesting compounds. Nevertheless, anipamil and ronipamil appear to have significant potential for use in ischemic and hypoxic states including circulatory shock, hepatic ischemia, and perhaps myocardial ischemia.

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