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Treatment of acute myocardial ischaemia with a selective antagonist of thromboxane receptors (BM 13.177)

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1 In order to elucidate the role of endogenous thromboxane A_2 in myocardial ischaemia, cats were subjected to 5 h of permanent occlusion of the left anterior descending coronary artery (LAD) and treated with the thromboxane receptor antagonist BM 13.177 (5 mg $kg^{-1}h^{-1}$, i.v.).

2 In comparison with vehicle-treated LAD-occluded cats, BM 13.177 significantly attenuated the loss of creatine phosphokinase-specific activity from the ischaemic myocardium and antagonized the ischaemia-induced rise in the ST-segment of the electrocardiogram.

3 BM 13.177 at the dose used did not reduce plasma thromboxane levels or ischaemia-induced platelet aggregate formation but considerably antagonized thromboxane-dependent platelet secretion *ex vivo*.

4 The study demonstrates some beneficial effects of selective blockade of thromboxane receptors on biochemical and electrophysiological parameters of acute myocardial ischaemia.

Introduction

Increased local thromboxane formation in ischaemic myocardium has been considered to be a major pathophysiological event, contributing to tissue damage in the course of myocardial ischaemia (Coker *et al.*, 1981; Parratt & Coker, 1985). Several actions of thromboxane A_2 (TXA_2) are involved in these processes. These include: regional vasoconstriction which causes a further reduction in nutritional blood flow to the ischaemic myocardium, stimulation of platelet aggregation and degranulation as well as augmentation of polymorphnuclear cell adhesiveness (Spagnuolo *et al.*, 1980), facilitation of margination and migration of these cells to injured sites in the locality, such as the borderline of a developing infarct (Engler *et al.*, 1983).

In addition, TXA_2 might stimulate its own formation and release by stimulating more platelets to aggregate and synthesize TXA_2, thus forming a positive feedback loop. One possible way of interrupting this vicious cycle is to block thromboxane biosynthesis. Beneficial effects of thromboxane synthetase inhibitors in acute myocardial ischaemia are well documented and include agents such as imidazole (Smith *et al.*, 1980), dazoxiben (Burke *et al.*, 1983a, Thiemermann *et al.*, 1984), dazmegrel (Parratt & Coker, 1985) and OKY-1581 (Lefer *et al.*, 1982). These compounds, in addition to inhibiting selectively deleterious thromboxane formation, might also increase formation of potentially beneficial prostaglandin endoperoxide-derived products, such as prostaglandin I_2 (PGI_2, Aiken *et al.*, 1981). There is evidence for inhibition of 12-HPETE formation by some of these agents (Smith *et al.*, 1985), i.e. prevention of formation of a potent coronary vasoconstrictor (Trachte *et al.*, 1979). The thromboxane synthetase inhibitor dazoxiben was found to block thromboxane receptors (Smith *et al.*, 1981), while the thromboxane receptor antagonist pinane TXA_2 (PTA_2) additionally inhibits thromboxane biosynthesis (Nicolaou *et al.*, 1979). Thus, the results of these investigations convincingly demonstrate protective actions of thromboxane synthetase inhibitors in experimental myocardial ischaemia, but do not provide direct evidence for the fact that it is solely the inhibition of thromboxane formation which accounts for their beneficial effects.

TXA_2 actions on platelets and the vessel wall are receptor-mediated. Therefore, specific antagonism of TXA_2 receptors appears to be a useful approach to analyse the significance of endogenous TXA_2 in the pathophysiology of acute myocardial ischaemia. The only study presented so far, in which a thromboxane receptor antagonist was used, failed to demonstrate its

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beneficial actions on biochemical indices of acute myocardial ischaemia (Burke *et al.*, 1983b). 4-[-(Benzenesulphonamido)-ethyl]-phenoxyacetic acid is a new thromboxane receptor antagonist (Patscheke & Stegmeier, 1984, Stegmeier *et al.*, 1984) that has been found *in vitro* to block vascular and platelet thromboxane receptors at concentrations which did not affect thromboxane biosynthesis (Patscheke & Stegmeier, 1984; Latta *et al.*, 1985). Thus, this compound appeared to be a useful tool to analyse specifically the role of endogenous thromboxane in the pathophysiology of the developing myocardial infarction.

Methods

Operative procedures

Adult cats, weighing between 2.6 and 3.4 kg, were anaesthetized with sodium pentobarbitone, 30 mg kg^{-1} i.v. All animals were tracheotomized and positive-pressure ventilation was instituted by means of a small animal respirator (Schuler, HSE, Freiburg, F.R.G.). After thoracotomy, the left anterior descending coronary artery (LAD) was prepared and a 4-0 silk ligature placed beneath the vessel 12–14 mm distal from its origin. Myocardial ischaemia (OP) was induced by permanently ligating the vessel. Cats were either subjected to LAD occlusion or a sham-operation procedure (SOP) consisting of all surgical procedures except ligation of the LAD. A catheter was placed in the right femoral vein for infusion of substances, another placed in the right atrium via the right jugular vein for blood sampling. A third catheter, placed in the left femoral artery, was connected with a Statham P23Db pressure transducer to determine mean arterial blood pressure. The heart rate was computed from the systolic pressure curves by a Hellige Recomed measuring unit (Hellige, Freiburg, F.R.G.). Changes in the ST-segment were detected by a standard lead electrocardiogram (ECG). The pressure rate product was used for calculation of myocardial oxygen consumption (see Schrör *et al.*, 1981 for details).

Sampling and analysis of blood

Samples of right atrial blood (2 ml) were collected into polyethylene tubes, containing disodium-edetate (EDTA) (0.1 mol l^{-1}) and indomethacin (0.03 mmol l^{-1}) in order to avoid *ex vivo* thromboxane formation. The cells were separated by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was subjected to radioimmunoassay of TXB_2 using specific and highly selective TXB_2 antibodies prepared in our laboratory (see Gallenkämper *et al.*, 1984, for details). The separation of free and bound activity was performed with the double-antibody technique.

Another 0.3 ml sample of right atrial blood was taken with a plastic syringe and a 100 µl aliquot immediately transferred into a plastic tube, containing 40 µl (200 iu) of heparin (Serva, Heidelberg, F.R.G.). The mixture was gently shaken and a 20 µl aliquot transferred into test tubes (Thromboplus R, Sarstedt, Frankfurt, F.R.G.). The free platelet count was determined in these samples by interference phase contrast microscopy.

Platelet adenosine 5'-triphosphate (ATP) secretion was measured in whole blood using a Lumi-aggregometer (Chronolog, Coulter, Krefeld, F.R.G.). One millilitre of right atrial blood was taken into citrate (1:10; v:v) and 1 min later stimulated *ex vivo* at 37°C by addition of collagen 2 µg ml^{-1} (Hormon-Chemie, München, F.R.G.). The samples were stirred at 1,200 r.p.m. ATP secretion was measured by a luminescence technique following the recommendations of the manufacturer. Synthetic ATP (Sigma, St. Louis, U.S.A.) was used as a standard.

Sampling and analysis of cardiac tissue

After 5 h the hearts were excised, rinsed in ice-cold 0.9% w/v NaCl solution (saline) and placed into cold saline. The free left ventricular wall of the heart was divided into normal and ischaemic regions (about 400 mg each) by inspection of the myocardial surface. Transmural samples of the severely ischaemic anterior myocardium (MI) and of the normal posterior left ventricular myocardium (NMI) were excised, blotted and weighed. Anatomically equivalent areas were excised from sham-operated animals. The tissue samples were homogenized in 0.125 M sucrose (1:20, w:v), containing EDTA 25 mmol l^{-1} and mercaptoethanol 0.1 mmol l^{-1}, for determination of myocardial creatine phosphokinase (CK) (assay kit Boehringer, Mannheim, F.R.G.) following the procedures described by Rosalki (1967). The protein content was measured by the method of Lowry *et al.* (1951).

Evaluation

The cats were allowed to recover for 30 min after the end of the operative procedures. Then, the LAD was occluded at time 0 for a total time of 5 h. Infusion of BM 13.177 (Boehringer, Mannheim, F.R.G.) was started at time 30 min at a rate of 5 mg kg^{-1} h^{-1} and continued until the end of the observation period. Since BM 13.177 at high concentrations might also inhibit thromboxane biosynthesis, a dose of BM 13.177 was chosen which did not exert any thromboxane synthetase inhibitory activity. In 2 pilot experiments, it was found that an infusion of 5 mg kg^{-1} h^{-1} i.v. did not result in any significant depres-

sion of thromboxane formation in cats, but caused a nearly complete suppression of platelet ATP secretion. In these cats the plasma TXB_2 level was 398 and 462 pg ml^{-1} at 30 min and 453 and 431 pg ml^{-1} at 5 h; these values were not different from vehicle controls (Gallenkämper *et al.*, 1984, see Table 2). The platelet ATP secretion was reduced from 97% and 76% at 30 min to 5% and 8% of control at 5 h. Cats not receiving BM 13.177 were treated with the vehicle (isotonic saline solution) at the same infusion rate. Measurements of blood pressure, heart rate, platelet count and ECG were performed at time 0 (i.e. immediately prior to LAD occlusion or sham-operation, respectively), at time 20 min, 40 min, 60 min and at 1 h intervals thereafter until the end of the observation period, i.e. time 5 h. The plasma TXB_2 levels were determined at times 0, 30 min, 90 min, 3 h and 5 h. The platelet count and plasma TXB_2 were additionally measured immediately after introducing anaesthesia, i.e. prior to any surgery.

Statistics

All values in the text are expressed as the mean ± s.e.mean of n observations. Statistical analysis was performed by means of Student's two-tailed t test. P levels of <0.05 were considered significant.

Results

General haemodynamics and ECG

Initially, the mean arterial blood pressures, heart rates and the computed pressure-rate products of all groups of cats were not different from each other. As illustrated in Figure 1, no time-dependent alterations in blood pressure occurred in sham-operated, vehicle-treated cats. LAD occlusion resulted in a significant decrease in blood pressure, by 17% (vehicle-treated) and 14% (BM 13.177-treated) of control (time 0), at 20 min ($P<0.05$). There was no further decrease in the vehicle-treated OP-cats during the remainder of the experiment, whereas the blood pressure in BM 13.177-treated OP animals tended to rise after 2 h. The differences in mean arterial blood pressure between OP-vehicle and OP-BM 13.177-treated cats became significant at 3 to 5 h ($P<0.05$). There was no change in this parameter in 2 cats undergoing a sham-operation and treated with BM 13.177 at the same infusion rate (not shown).

As summarized in Table 1, no significant changes in heart rate were observed in any group of animals, although a tendency for a reduction was observed in the OP-BM 13.177-treated group as compared with OP-vehicle-treated group ($0.1<P>0.05$). There was also no difference in the computed pressure-rate product between the several groups at any time in the experiment (not shown).

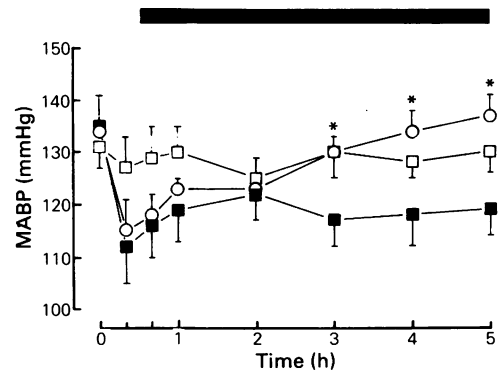

Figure 1 Mean arterial blood pressure (MABP) in cats, subjected to left anterior descending coronary artery (LAD)-occlusion (OP) and treatment with vehicle (■) or BM 13.177 (○) as compared with vehicle-treated sham-operated cats (□). The solid bar shows the infusion period. Each point represents the mean of 5–7 observations; vertical lines indicate s.e.mean. $*P<0.05$ (BM 13.177-treated vs. vehicle-treated OP cats at same time point).

The ST-segment changes were depicted in Figure 2. By 20 min after LAD occlusion a considerable elevation of the ST-segment had occurred that persisted for the entire 5 h period in vehicle-treated animals ($P<0.01$). There was apparently the same ST-segment elevation at 20 min (i.e. prior to any treatment) in both groups of cats subjected to LAD occlusion ($P>0.05$). However, soon after starting BM 13.177 infusion, the ST-segment diminished and was significantly reduced compared with that in vehicle-

Table 1 Heart rate in cats treated with BM 13.177 or vehicle (Veh) and subjected to 5 h of permanent coronary artery ligation (OP) or sham-operation (SOP)

Time (min)	*Heart rate (beats min^{-1})*		
	SOP ($n=5$)	*OP-Veh* ($n=8$)	*OP-BM 13.177* ($n=6$)
0	186 ± 9	196 ± 5	193 ± 10
20	187 ± 9	174 ± 8	171 ± 3
40	180 ± 8	176 ± 5	167 ± 3
60	184 ± 6	172 ± 4	171 ± 2
120	176 ± 8	170 ± 4	176 ± 5
180	174 ± 8	170 ± 5	172 ± 5
240	170 ± 6	175 ± 6	165 ± 2
300	176 ± 8	171 ± 6	163 ± 3

Each value in the mean ± s.e.mean of n animals. No significant alterations were obtained by comparing the several groups of animals at the same times.

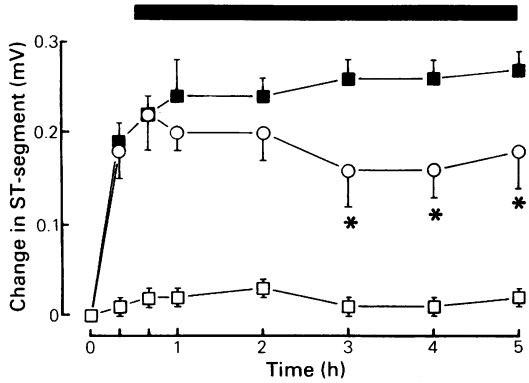

Figure 2 Changes in the ST-segment in cats, subjected to LAD-occlusion (OP) and treatment with vehicle (■) or BM 13.177 (○) as compared with sham-operated, vehicle-treated cats (□). The solid bar shows the infusion period. Each point represents the mean of 5–7 observations; vertical lines indicate s.e.mean. $*P<0.05$ (BM 13.177-treated vs. vehicle-treated OP cats at same time point).

treated cats at 3 to 5 h ($P<0.05$), although it was still significantly elevated in comparison with that of vehicle-treated sham-operated cats ($P<0.05$).

Initially, the mean free platelet number in right atrial blood ranged from 465,000 to 513,000 platelets μl^{-1} (100%) in the different groups of cats. This platelet count was uniformly decreased, by 20%, in all groups of animals during the operative procedures. After LAD occlusion, there was another 15–20% drop in free platelet count which then remained unchanged in both vehicle- and BM 13.177-treated animals (Figure 3). Thus, the free platelet count after LAD occlusion was significantly reduced in comparison with sham-operated cats from time 20 min to 5 h ($P<0.05$) and unchanged by BM 13.177 treatment.

Platelet ATP secretion ex vivo

Figure 4 shows the data on collagen-induced platelet ATP secretion *ex vivo*. In comparison with control at time 0 (100%), there was a slight reduction in platelet ATP secretion during the course of the experiment in both sham-operated and LAD-occluded vehicle-treated cats. However, no significant differences between these two groups of animals were obtained at any time in the study ($P>0.05$). In contrast, LAD-occluded cats treated with BM 13.177 exhibited a considerably reduced ATP release which was significantly lower than that in vehicle-treated LAD-occluded animals at 3 and 5 h ($P<0.01$), amounting to only 20 and 11% of control, respectively.

Thromboxane formation

Table 2 summarizes the plasma levels of immunoreactive TXB_2. Before surgery these plasma levels were very low and did not vary between the several groups of animals. However, cardiac surgery resulted in a 2–3 fold increase of plasma TXB_2. LAD occlusion was associated with an additional 2–4 fold increase ($P<0.05$), the plasma TXB_2 concentration being 6–7 fold higher in LAD-occluded animals than in the sham-operated group ($P<0.01$). There was no fur-

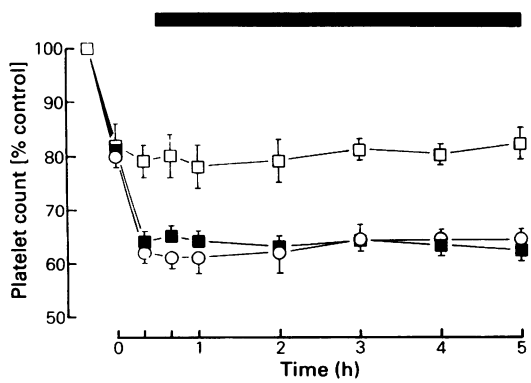

Figure 3 Changes in the free platelet count in right atrial blood in cats, subjected to LAD-occlusion (OP) and treatment with vehicle (■) or BM 13.177 (○) as compared with sham-operated, vehicle-treated cats (□). The solid bar indicates the infusion period. Each point represents the mean of 5–7 observations; vertical lines show s.e.mean.

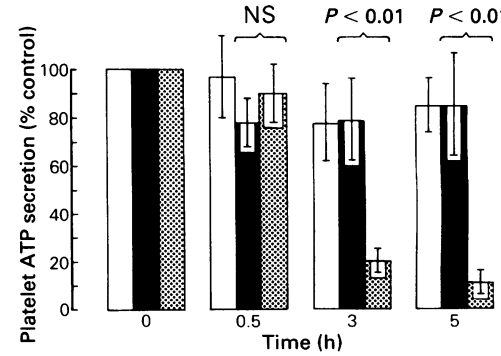

Figure 4 Platelet ATP secretion by collagen *ex vivo* in cats subjected to LAD-occlusion and treatment with vehicle (solid columns) or BM 13.177 (stippled columns) as compared with sham-operated, vehicle-treated controls (open columns). The data are mean of 5–6 observations; vertical lines show s.e.mean. Note the significant reduction of ATP secretion in BM 13.177-treated cats at 3 and 5 h, compared with vehicle-treated controls.

Table 2 Plasma thromboxane, assayed as immuno reactive thromboxane B_2 (TXB_2), in cats subjected to sham-operation (SOP), LAD-occlusion and vehicle (Veh) treatment or LAD-occlusion and treatment with BM 13.177

Time (min)	*Plasma-TXB$_2$* (pg ml^{-1})		
	SOP ($n = 5$)	*OP-Veh* ($n = 8$)	*OP-BM 13.177* ($n = 6$)
Prior to surgery	37 ± 18	49 ± 21	56 ± 13
0	66 ± 16	164 ± 38	108 ± 26
30	59 ± 14	397 ± 122	429 ± 116
180	71 ± 23	432 ± 96	456 ± 128
300	57 ± 19	334 ± 99	414 ± 102

Each value in the mean ± s.e.mean of n animals.
No significant differences were obtained between OP-Veh and OP-BM 13.177 at any time of the experiment.

ther change in plasma TXB_2 during the remainder of the experiment and no specific action by BM 13.177 on this parameter.

Myocardial creatine phosphokinase specific activity

Figure 5 depicts the alterations in myocardial CK specific activity. The MI area of LAD-occluded cats treated with vehicle exhibited a 41% decrease in CK specific activity at 5 h in comparison with non-ischaemic myocardium (NMI) from the same hearts, while this reduction in BM 13.177-treated cats was only 20%. Thus, the loss of CK-specific activity in the ischaemic myocardium of BM 13.177-treated cats was significantly less than that of vehicle-treated OP cats ($P < 0.05$). There were no changes in CK-specific activities in the NMI areas of left ventricular myocardium induced by BM 13.177 as compared to either vehicle-treated LAD-occluded cats or cats subjected to sham operation ($P > 0.05$).

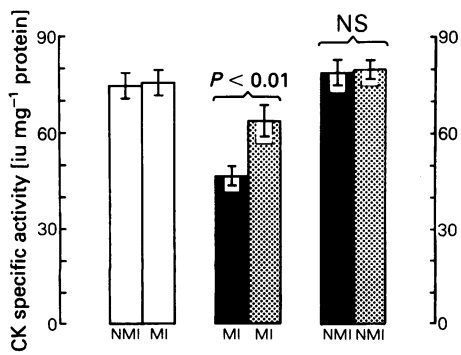

Figure 5 Creatine phosphokinase (CK)-specific activity in left ventricular ischaemic (MI) and non-ischaemic myocardium (NMI) of cats subjected to 5 h of permanent LAD occlusion and treatment with vehicle (solid columns) or BM 13.177 (stippled columns) as compared with sham-operated, vehicle-treated cats (open columns). The data are means of 5–7 observations; vertical lines shown s.e.mean.

Discussion

Shimamoto *et al.*, (1979) were the first to demonstrate that administration of a thromboxane generating system to intact animals results in severe myocardial ischaemia. This was confirmed in subsequent investigations and led to the overall impression that elevated endogenous thromboxane in the course of regional ischaemia is detrimental to the damaged myocardial tissue (see Walinsky *et al.*, 1984). Since the endogenous level of TXA_2 is entirely determined by biosynthesis, it was impressive but not entirely surprising to find that inhibition of thromboxane biosynthesis resulted in improved preservation of ischaemic myocardium.

On the other hand, beneficial actions of selective inhibitors of thromboxane formation *in vivo* might not be attributable to reduced thromboxane formation because of the non-specificity of many of these agents (see Introduction). Furthermore, the large increase in tissue free fatty acids during myocardial ischaemia will allow the formation of numerous other fatty acid peroxidation products including free oxygen-centered radicals (Victor *et al.*, 1984). Inhibition of thromboxane biosynthesis might modify the formation of those products, leading, for example, to an increase in prostacyclin formation (Aiken *et al.*, 1981). Thus, the role of endogenous thromboxane in myocardial ischaemia cannot be assessed from only studying synthetase inhibitors.

The present study demonstrates the beneficial effects of a thromboxane receptor antagonist on myocardial ischaemia in the absence of any inhibition of thromboxane biosynthesis. These beneficial actions include a significant inhibition of the loss of intracellular CK, strongly suggesting a reduced extent and severity of myocardial cell damage (Humphrey *et al.*, 1984). This biochemical finding was corroborated by the attenuation of the ischaemia-induced rise in the ST segment. This is, as far as we know, the first demonstration of cardioprotective actions resulting from a selective blockade of thromboxane receptors. Burke *et al.* (1983b), while showing an antagonism of ischaemia-induced increase in the ST-segment in animals treated with the thromboxane-receptor antagonist 13-azaprostanoic acid, failed to demonstrate biochemical evidence for improved myocardial

preservation, as seen from an unchanged CK-loss in These experiments. However, Parratt & Coker (1985) have recently shown antiarrhythmic actions of the thromboxane receptor antagonist AH 23848 in dogs, provided the animals were pretreated with the compound prior to LAD occlusion.

In the present study, the efficacy of thromboxane-receptor blockade was assessed *ex vivo* by measuring low collagen-induced platelet ATP secretion. This reaction is known to require TXA_2 (Charo *et al.*, 1977) and is prevented in the same model by thromboxane synthetase inhibitors (Thiemermann & Schrör, 1984). Furthermore, BM 13.177 has been found to antagonize platelet reactions that are dependent upon the mobilization of endogenous arachidonic acid or, alternatively, induced by thromboxane mimetics in the absence of significant alterations in thromboxane biosynthesis (Patscheke & Stegmeier, 1984). These and other data (Stegmeier *et al.*, 1984) clearly demonstrate the potency of BM 13.177 in blocking platelet thromboxane receptors *in vivo* at doses that do not alter endogenous thromboxane concentrations.

BM 13.177, while antagonizing platelet secretion including the release of platelet derived vasoconstrictors such as 5-hydroxytryptamine (5-HT; Patscheke & Stegmeier, 1984; Latta *et al.*, 1985), did not break up ischaemia-induced platelet aggregates. It has been reported that treatment of LAD-occluded cats with the thromboxane synthetase inhibitor dazoxiben did not reduce platelet aggregate formation while prostacyclin did (Thiemermann & Schrör, 1984). These findings and those in this study suggest that the formation of reversible platelet aggregates *in vivo* is not associated with platelet-derived thromboxane release, i.e. mediated by other platelet aggregating agents, and that thromboxane released locally from the ischaemic myocardium may be pathologically important. The demonstration of protective actions of the thromboxane synthetase inhibitor OKY-1581 in a platelet-free globally perfused ischaemic heart preparation (Lefer *et al.*, 1982) would tend to support the conclusion of platelet-independent thromboxane generation by the injured myocardium. Certainly, the similarities, in both the extent and direction of anti-platelet actions by thromboxane synthetase inhibitors and this thromboxane receptor antagonist, would suggest that a major part of the beneficial actions of these synthetase inhibitors in myocardial ischaemia *in vivo*, indeed, results from prevention of thromboxane formation and not from effects on other pathways of arachidonic acid metabolism.

An unexpected finding was the difference in arterial blood pressure between BM 13.177- and vehicle-treated LAD-occluded cats, namely an increase in blood pressure in BM 13.177-treated OP animals after 2 h of ischaemia and a tendency for heart rate to decline, whereas vehicle-treated cats exhibited no changes. This could be due to improved myocardial performance by the agent. However, no significant changes in blood pressure were found with pinane thromboxane A_2 (Schrör *et al.*, 1981) or dazoxiben (Burke *et al.*, 1983a), which act beneficially in a similar cat model of acute myocardial ischaemia. Alternatively, BM 13.177 might act as a vasoconstrictor. However, a direct coronary vasoconstriction by BM 13.177 was obtained in *in vitro* only at concentrations of BM 13.177 that considerably inhibited platelet thromboxane formation (Verheggen & Schrör, 1985). No decreased thromboxane formation was obtained in the present study. Moreover, two sham-operated cats treated with BM 13.177 at the same dose as the LAD-occluded animals failed to show any change in blood pressure during the entire observation period. BM 13.177 has been shown to enhance thromboxane formation *in vitro* at concentrations that do not affect its biosynthesis (Verheggen & Schrör, 1985). Similar conclusions can be drawn from a recent study, by O'Keefe *et al.* (1985), in lung tissue using the thromboxane receptor antagonist SQ 29,548. Since BM 13.177 (Verheggen & Schrör, 1985), like 13-aza-prostanoic acid (Burke *et al.*, 1983b), appeared to be less effective on vascular thromboxane receptors than on those of the platelets, the inhibition of thromboxane-mediated platelet secretion is not necessarily linked to prevention of vasoconstrictor thromboxane actions. Further investigations are needed to evaluate the mechanism(s) involved. Certainly, these considerations do not rule out the possibility that BM 13.177 acts as a spasmolytic agent in coronary arteries of the ischaemic myocardium. Thus both the inhibition of platelet aggregation and prevention of vasospastic mediator release (5-HT) would result in improved coronary perfusion.

In summary, our data indicate that selective blockade of thromboxane receptors protects the cat myocardium *in vivo* from ischaemic injury, as evidenced by the large inhibition of CK loss and prevention of ischaemia-induced rises in the ST-segment, without affecting the concomittant fall in platelet count or rise in plasma thromboxane. This suggests that TXA_2, possibly of non-platelet origin, contributes to the pathophysiological sequelae of the ischaemic process in the heart.

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