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MYOCARDIAL SYNTHESIS OF PROSTAGLANDIN-LIKE SUBSTANCES AND CORONARY REACTIONS TO CARDIOSTIMULATION AND TO HYPOXIA

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1 Continuous recording of cardiac contractions and coronary flow from isolated perfused hearts of rats permitted the study of coronary reactions to: (a) cardiostimulation induced by single doses or slow infusions of noradrenaline, $CaCl_2$, glucagon or electrically induced tachycardia; (b) short interruptions of coronary inflow (hypoxia).

2 Except during tachycardia the heart rate was kept constant at 210 beats/min by electrical pacing.

3 Metabolic coronary vasodilatation (MCD) resulting from cardiac hyperactivity induced by noradrenaline, Ca^{2+}, tachycardia or glucagon was inhibited by administration of prostaglandin E_2. Reactive hyperaemia response to hypoxia was unaffected by prostaglandin administration.

4 Inhibition of MCD could also be obtained by prolonged infusion with arachidonic acid (1.6×10^{-7} M), presumably by its conversion into prostaglandin-like substance since arachidonic acid failed to block MCD in hearts from rats pretreated with non-steroidal anti-inflammatory drugs (indomethacin, naproxen, phenylbutazone).

5 Reactive hyperaemia was unaffected either by arachidonic acid or by blockade of the synthesis of prostaglandin-like substances by anti-inflammatory drugs.

6 Since prostaglandin synthetase inhibition does not prevent but may enhance MCD, we do not advocate prostaglandin-like substances as agents directly responsible for the coronary vasodilatation that follows cardiac hyperactivity.

7 We postulate that cardiac overproduction of prostaglandins may lead to a failure in the adaptive coronary flow response to cardiac hyperactivity (coronary insufficiency?).

Introduction

The metabolically induced coronary dilatation (MCD) obtained in mammalian isolated perfused hearts in response to an increase in heart rate and/or positive inotropic action (Sunahara & Talesnik, 1974), has been explained as a consequence of the activation of cardiac adenylate cyclase; the latter would trigger a vasodilator process resulting in the increased compensatory coronary flow accompanying cardiac hyperactivity (Sen, Sunahara & Talesnik, 1976). The exogenous administration of prostaglandin E_1 (PGE_1) or E_2 inhibits the MCD response to cardiostimulation and it has also been shown that this inhibition correlates with a diminution in the cardiac levels of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) induced by cardiostimulation (Sen *et al.*, 1976). In addition, a distinction between MCD and 'reactive hyperaemia' responses was pharmacologically substantiated (Sen, Sunahara & Talesnik, 1977). Nevertheless, it is unlikely that circulating prostaglandin-like substances (PGL) are involved in the regulation of cardiovascular function because a powerful prostaglandin-dehydrogenase in the lungs prevents the heart from receiving effective concentrations of PGL (Horton, 1976). Furthermore, it is accepted that PGL are not stored in tissues but rather biosynthesized *de novo* when a suitable stimulus activates the prostaglandin-synthetase system and this concept applies in full to the heart (Needleman, 1976). *In situ* biosynthesis occurs in the myocardium (Limas & Cohn, 1973) and it is widely accepted that non-steroidal anti-inflammatory agents are effective blockers of PGL synthesis (Flower & Vane, 1974a).

In this paper we describe results on MCD and reactive hyperaemia obtained in isolated perfused hearts of rats treated with different anti-inflammatory agents. The PGL synthesis and metabolism that normally occurs in the heart (Gudbjarnason, 1975) are processes in which the formation of PGL depends

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upon the availability of the essential unsaturated fatty acid, arachidonic acid (AA), which in the cell is incorporated into the phospholipids of the cell membrane (van Dorp, 1976). In the present experiments, we administered AA in the perfusate and found that it stimulated the production of endogenous PGL. The subsequent coronary reactions to cardiostimulation or to hypoxia quite closely resembled the changes obtained when exogenous PGE_2 was administered under similar experimental conditions (Sen *et al.*, 1976).

Methods

Male Sprague-Dawley rats, mean body weight 263 ± 14.7 g (s.e. mean) obtained from Canadian Biobreeding Laboratories were used. Hearts were prepared for perfusion, at 50 mmHg, according to the Langendorff method with Krebs-Henseleit-bicarbonate solution, gassed with 95% O_2 and 5% CO_2, modified to contain half the normal Ca^{2+} (Zachariah, 1961) and insulin 2 units/l (Bleehen & Fisher, 1954; Weissler, Atschuld, Gibb, Pollack & Kruger, 1973). Continuous recordings of coronary flow and heart activity were obtained by differential pressure and force-displacement transducers, respectively, as already described (Sen *et al.*, 1976). Spontaneous rhythm was suppressed by clamping the interventricular septum, and the heart was paced at 210 beats/min through dipolar electrodes with square wave pulses and a current about 20% above threshold. Drugs in single bolus (0.1 ml) were administered with a special injector (Sen *et al.*, 1976) attached to the aortic cannula so that the rate of injection was self-controlled by the actual coronary flow; slow infusions were applied through a side arm of the injector at a rate of 0.1 to 0.2 ml/min. Lastly, perfusion with constant concentrations of drugs was carried out by adding them to the reservoir in which the perfusate was stored. The data signals obtained from the transducers were suitably amplified and sent to the computer centre; the analog signals converted into digital information were stored and later retrieved by our laboratory for further analysis of results. Several programmes have been developed for analyzing the data acquired from the separate transducers. There are two data acquisition phases, control and trial period. During the control period, values for coronary flow, heart rate and force of contraction were obtained and subsequently displayed. During the trial period, pertinent time and integrated signal information was recorded and in turn displayed at the end of the trial. All calculated data for both periods as well as the raw values were stored on magnetic tapes. Proper calibration factors for each channel were entered into the system and the experiments were analyzed. The increases in coronary flow and in force of contraction were assessed by integrating the areas under the recordings from the beginning of the increase until the values had returned half way from maximum to control level.

At the end of each experiment the heart was removed, blotted and weighed. These data were sent to the computer centre and the changes in coronary flow were calculated in ml min^{-1} g^{-1} of tissue. A comparative analysis of paired and unpaired data by Student's t test was made before and during the administration of different drugs.

Drugs

The following drugs were used: noradrenaline bitartrate monohydrate (Levophed, Winthrop); heparin and insulin (Connaught Laboratories); glucagon (Eli Lilly and Co.); prostaglandin E_2 (Upjohn); arachidonic acid (Sigma Chemical Co.); indomethacin (Merck Frosst Laboratories); naproxen (Syntex Laboratories Inc.) and phenylbutazone (Ciba-Geigy Canada Ltd.).

All reagents used were of analytical grade and drug solutions freshly prepared. Doses are expressed in terms of their respective base or acid. Stock solutions of PGE_2 (2 mg/ml) and AA (5 mg/ml) were made in 95% ethanol, according to the procedure described by Kulkarni, Roberts & Needleman (1976). Fresh 10 fold dilutions of AA were made daily in 2 mM sodium carbonate solution. Further dilutions of PGE_2 and sodium arachidonate were made in Krebs solution. Noradrenaline (NA) was prepared in 0.9% w/v NaCl solution (saline) with added ascorbic acid (20 mg/l; 1.14×10^{-4} M; Samuelsson & Wennmalm, 1971).

Results

Influence of arachidonic acid on coronary dilator response to cardiostimulation or to hypoxia

Cardiac hyperactivity induced by different single doses of NA or Ca^{2+} resulted in MCD responses whose magnitude depended on the attained inotropic action. Sudden increases in heart rate from the basic pacing of 210 beats/min to 270 or 300 beats/min for 30 s, were also followed by heart rate-dependent increases in coronary flow; the latter coronary reaction is also included under the term MCD (Sen *et al.*, 1977). Reactive hyperaemia, induced by brief interruption of the inflow, was also tested and an example of the results obtained in these experiments is shown in Figure 1. Addition of 50 μg/l AA to the perfusate (1.6×10^{-7} M) produced, after 30 to 40 min of AA administration, a marked inhibition of the MCD responses to NA, Ca^{2+} or tachycardia but reactive

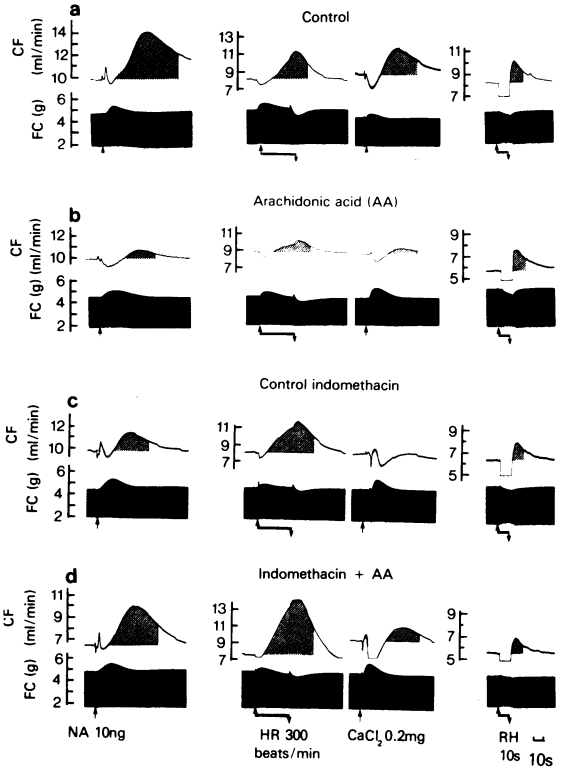

Figure 1 Influence of arachidonic acid (AA) on coronary reactions in hearts from normal and indomethacin-treated rats paced at 210 beats/min. Cardiostimulation was induced with noradrenaline (NA) or calcium (Ca^{2+}) at ↑. Doses are indicated at the bottom of the figure. Tachycardia 300 beats/min was applied for 30 s at ↑—↓ and reactive hyperaemia (RH) by 10 s of coronary occlusion at ↑—↓. Hatching under the recordings of coronary flow indicate the areas measured until 50% recovery of normal levels. Calibrations: coronary flow (CF) in ml/min and force of contraction (FC) in g. (a) Control reactions in hearts from normal rats; (b) reactions obtained in the same hearts as (a) after 30–45 min AA administration (50 μg/l); (c) control reactions in hearts from indomethacin-treated rats; (d) reactions obtained in the same hearts as (c) during AA administration.

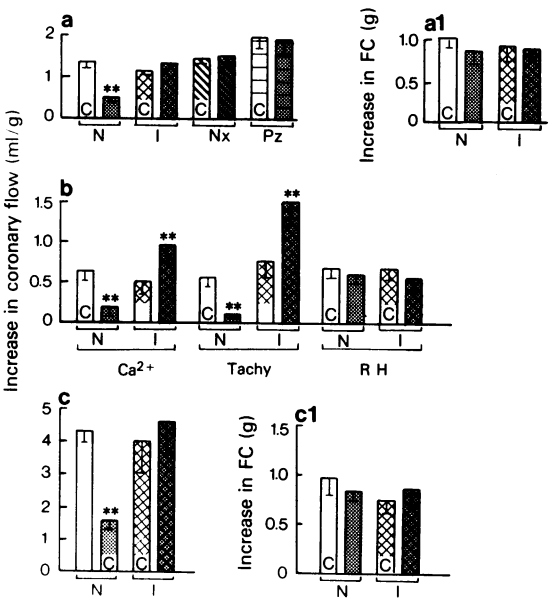

Figure 2 Effect of arachidonic acid (AA) and anti-inflammatory drugs on coronary reactions to cardiac hyperactivity (a) Increases in coronary flow (MCD) were obtained by administering a single dose of noradrenaline (NA, 0.01 μg) to hearts from normal (N; $n = 7$), indomethacin (I; $n = 7$) and naproxen-treated rats (Nx; $n = 5$). Phenylbutazone-treated hearts (Pz; $n = 5$) received NA 0.015 μg. (a1) Increases in force of contraction (FC) due to NA were not different between N, I, Nx or Pz-treated hearts. (b) MCD due to $CaCl_2$ 0.1 mg (Ca^{2+}) in N ($n = 5$) and in I ($n = 7$)-treated hearts; tachycardia 270 beats/min for 30 s (Tachy) in N ($n = 5$) and in I ($n = 8$)-treated hearts. Reactive hyperaemia (RH) responses to 10 s coronary occlusion are compared in N ($n = 4$) and in I ($n = 4$)-treated hearts. (c) MCD induced by slow infusion of glucagon (0.5 μg/min for 2 min) in N ($n = 6$) and I ($n = 6$)-treated hearts. (c1) The increases in force of contraction (FC), induced by glucagon, were not different in N ($n = 6$) and in I ($n = 6$)-treated hearts. C = controls; stippled columns (with or without hatching) = AA 50 μg/l; I = indomethacin (cross-hatched columns); Nx = naproxen (hatched columns) and Pz = phenylbutazone (horizontally hatched columns). **Significantly different from C, $P < 0.001$.

hyperaemia remained unaltered; these results are illustrated in Figure 1 and paired analysis of the results are shown in the bar graph of Figure 2. The basic coronary flow during the control period was 7.2 ± 0.25 ml min^{-1} g^{-1} and during the AA administration the flow was 6.8 ± 0.31 ml min^{-1} g^{-1}. This slight diminution can be accounted for by the duration of the experiment. The unavoidable deterioration of the preparation, after 2 h of perfusion, has been shown to be a regular limitation of the Langendorff preparation (Aronson & Serlick, 1976). Control experiments, in which no AA was added to the perfusate, were carried out to investigate the influence of time of perfusion on the MCD and reactive hyperaemia responses. It was found that the coronary reactions remained fairly constant if AA was not administered (these data are not included). Another type of control experiment, that provides at the same time additional interesting information, is presented in Table 1. In this Table, it is shown that the MCD

response was inhibited only when AA was infused and this effect was readily reversed if AA ceased to be supplied to the heart, even though this followed a prolonged period of perfusion. Furthermore, if the heart was challenged during the initial stages of AA administration (e.g. the first 15 to 20 min) the MCD response was indistinguishable from the normal reaction.

At the start of AA infusion, coronary vasodilatation was obtained in 11 out of 19 experiments. The magnitude of the vasodilatation varied; the peak increase was 1.6 ± 0.43 ml min^{-1} g^{-1} over the basic coronary flow. The duration of this increase was from 20 to 30 min after which the flow stabilized in the normal range notwithstanding the presence of AA in the perfusate. In two experiments, vasodilatation continued for over 60 min and therefore they were discarded; but even in these experiments discontinuation of the AA administration caused the coronary flow to return towards control values. In a few experiments, AA was increased to 100 μg/l, but the degree of coronary dilatation was not greater than with 50 μg/l. These irregularities prevented us from studying systematically the influence of different AA concentrations in the perfusate and the blocking effect on MCD reactions, without distracting from the main objective sought with these experiments.

Influence of non-steroidal anti-inflammatory agents and arachidonic acid on coronary reactions to cardiostimulation or hypoxia

Indomethacin (5 mg/l; 1.4×10^{-5} M) or naproxen (25 mg/l; 1×10^{-4} M) were administered by slow infusion or at constant concentration in a series of experiments in which we tested the coronary reactions as previously described for indomethacin and aspirin (Talesnik & Sunahara, 1973). The immediate reaction of the coronaries was a transient but slight vasodilatation that subsided in about 5 to 10 min. The MCD responses to challenging doses of NA after indomethacin were markedly enhanced as previously reported and the acute infusion of the drug did not prevent the inhibition of MCD by the addition of AA to the perfusate. Furthermore, in some experiments, we tested the acute administration of indomethacin after the MCD was markedly inhibited by AA and, as illustrated in Table 2, the inhibition of MCD was not reversed by acute indomethacin. Failure to reverse the block of MCD with AA could also be seen with acute naproxen administration, as illustrated by the examples shown in Table 3.

However, blockade of MCD by AA could be prevented regularly if the rats were pretreated with indomethacin for at least 12 to 24 h before the experiment

Table 1 Recovery of coronary dilatation inhibition after arachidonic acid (AA) withdrawal from the perfusate

		ΔCF (ml)		
Exp no.	*NA (μg)*	*Control*	*During AA*	*After AA withdrawal*
66 A	0.05 (s.i.)	8.0	6.7	8.3
83	0.1 (s.i.)	3.7	1.5	2.9
37	0.02 (b)	1.24	0.25	1.45

AA was perfused for about 40 min and the hearts were again challenged with noradrenaline (NA). The administration of NA was carried out by slow infusion (s.i.) or by a bolus dose (b). The NA was applied again about 5 to 7 min after withdrawal of AA from the perfusate. CF = coronary flow (increments).

Table 2 Persistent coronary dilatation inhibition by arachidonic acid (AA) during acute indomethacin administration

		ΔCF (ml)		
Exp no.	*NA (μg)*	*Control*	*During AA*	*During AA + Ind*
38	0.02	1.54	0.56	0.12
39	0.02	5.30		3.80
77	0.025	2.0	1.1	0.47

Single doses of noradrenaline (NA) administered during perfusion with AA 50 μg/l (1.6×10^{-7} M) produced diminished MCD in comparison with the controls; the addition of indomethacin (Ind 5 mg/l; 1.4×10^{-5} M) to the perfusate with AA did not reverse the inhibited MCD, even after 30 to 40 min of indomethacin administration. CF = coronary flow (increments).

on the isolated heart. Thus, we decided to continue this study by treating the animals with anti-inflammatory agents routinely for about 5 days before the experiments on the isolated hearts were carried out.

Daily treatment of rats with indomethacin (5 mg/kg i.p.), naproxen (25 mg/kg i.p.) or phenylbutazone (40 mg/kg i.p.) produced a remarkable change in the coronary flow reaction when cardiostimulation was applied in the presence of AA. As illustrated in Figure 2, the MCD induced by NA, tachycardia or Ca^{2+} was markedly inhibited by AA, but it failed to produce inhibition of MCD in hearts from rats treated by any of the mentioned anti-inflammatory agents. The basic coronary flow in paced hearts of rats chronically treated with anti-inflammatory agents was similar to that of control hearts obtained from animals injected daily with placebo for the same period of time. In Table 4, a summary of these data is presented.

Although there is a tendency in hearts from animals treated with indomethacin to have reduced basal coronary flow, this value was not significantly different from the controls. Furthermore, the MCD responses to NA showed a trend to be enhanced when AA was given to indomethacin-treated rats. But the MCD in response to Ca^{2+} or to tachycardia was greatly enhanced when AA was in the perfusate of indomethacin-treated hearts (Figure 2). Figure 2 also shows that chronic treatment with naproxen or phenylbutazone produced abolition of the AA effect on MCD induced by cardiostimulation and that AA whether administered to control or to indomethacin-treated rats did not alter the inotropic action of NA. Likewise, neither naproxen nor phenylbutazone produced any changes in the inotropic action elicited by NA or Ca^{2+} (not shown). It is also worth mentioning here that reactive hyperaemia was not affected by acute or chronic administration of indomethacin, or of AA in indomethacin-treated rats (Figure 2).

Effects of prostaglandin E_2 or arachidonic acid on coronary reaction to sustained cardiostimulation

The studies that led to the working hypothesis that MCD could be explained as a result of a cyclic AMP triggering action on a vasodilatory mechanism, were carried out by challenging the heart with a single bolus of NA or Ca^{2+}. It appeared to us that this hypothesis would be strengthened if we could demonstrate that sustained stimulation of the heart would also lead to an MCD that lasted for prolonged periods of time. As a first step we determined whether MCD responses due to sustained cardiac stimulation could be inhibited by PGE_2. Figure 3 is a good example of this type of experiment. It was unnecessary to show that inotropic effects elicited by slow infusions of NA are concentration-dependent and that the resulting MCD corresponded with these increases

Table 3 Coronary dilatation (MCD) inhibition produced by arachidonic acid (AA) during naproxen administration

		ΔCF (ml)	
Exp no.	*NA (μg)*	*During naproxen*	*During naproxen + AA*
88	0.0125	2.02	1.26
87	0.025	1.43	0.73
83	0.05	2.01	0.88

Naproxen was perfused at a concentration of 25 mg/l (1×10^{-4} M) for about 30 min before noradrenaline (NA) in a bolus dose was administered. AA 50 μg/l (1.6×10^{-7} M) was added to the perfusate with naproxen and the perfusion continued for about 30 to 40 min before the hearts were again challenged with NA. CF = coronary flow (increments).

Table 4 Basic coronary flow (ml min^{-1} g^{-1}) in hearts of control rats and in rats treated with anti-inflammatory agents

CI	*I*	*CN_x*	*N_x*	*CP_z*	*P_z*
7.0 ± 0.28	6.6 ± 0.1	7.55 ± 0.32	7.10 ± 0.43	7.55 ± 0.32	7.21 ± 0.37
$n = 8$	$n = 19$	$n = 4$	$n = 4$	$n = 4$	$n = 4$

Data collected from hearts of controls (C) and daily i.p. injected rats. I = indomethacin (5 mg/kg); N_x = naproxen (25 mg/kg); P_z = phenylbutazone (40 mg/kg).

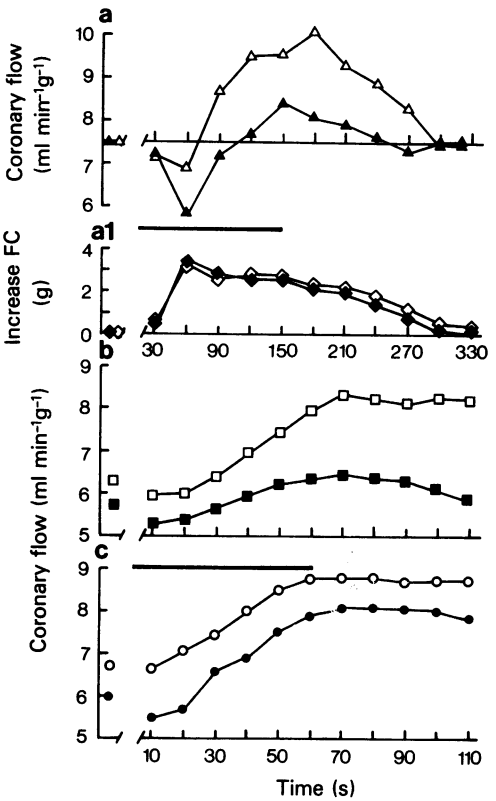

Figure 3 Influence of prostaglandin E_2 (PGE_2) or arachidonic acid (AA) on coronary flow (MCD) due to slow infusions of noradrenaline (NA). The concentrations and the duration of NA infusions were not the same in the different series shown in this graph. Thus, separate time scales are used for (a) and for (b) and (c). (a) NA 0.3 μg/min for 2.5 min (horizontal bar). Mean values of MCD ($n = 10$) before (△) and during PGE_2, 10 μg/l (▲). Paired analysis showed that the differences were significant ($P < 0.01$ or 0.001) from times 60 s to 270 s, after starting NA. (a1) Mean inotropic action induced by NA was the same before (◇) and during PGE_2 (◆). (b) NA 0.1 μg/min for 1 min (horizontal bar). Mean values of MCD ($n = 7$) were significantly higher ($P < 0.005$) in normal hearts before (□) than during AA, 50 μg/l (■) when compared at times 60 to 110 s after starting NA. (c) NA 0.1 μg/min for 1 min (horizontal bar) infused to hearts from indomethacin-treated rats ($n = 6$). MCD responses were similar before (○) and during AA (●).

in cardiac contractility. As expected, the inotropic action preceded the MCD and the peak increase in the recorded force of contraction was almost coincidental with a transient and discrete diminution of coronary flow. Since this reduction in coronary flow was blocked by phentolamine (results not included in the present paper) we assume that we are dealing with a coronary vasoconstrictor phase due to a direct α-adrenoceptor agonist action of NA. The inotropic action persisted during the period of slow infusion and the force of contraction slowly returned to the control level when the NA administration was stopped. The MCD developed after the inotropic effect and its maximum was obtained about 2 min after starting the NA infusion. The coronary response continued for another minute before it started to decline towards the pre-NA values. It can be seen in Figure 3 that about 5 min after starting the 2.5 min slow infusion of NA, the force of contraction and the coronary flow recovered to their normal values. After the heart was challenged several times and consistent MCD responses were obtained, PGE_2 was added to the perfusate (10 μg/l; 2.8×10^{-8} M). At first, coronary vasodilatation was produced but in about 30 min it subsided and the coronary flow stabilized at pre-PGE_2 levels. About 45 min after starting PGE_2 administration, the challenging of the heart by a slow infusion of NA, at the same concentration as before of PGE_2, produced similar inotropic responses but the MCD was markedly depressed, as can be seen in Figure 3. The initial diminution of coronary flow, ascribed to α-adrenoceptor stimulation, was enhanced in intensity as well as in duration and the onset of MCD was further delayed from the start of NA administration, by about 110 s. The peak MCD was obtained almost at the end of the infusion of NA and the flow returned to normal levels immediately after stopping the NA, although the inotropic action was as marked as before treating the hearts with PGE_2. Furthermore, the maximal MCD obtained was only a fraction (about 50%) of the value recorded before treating the hearts with PGE_2.

To facilitate the description of the effects obtained when AA was added to the perfusate, the results obtained in this experimental series were plotted on the same graph (Figure 3b and c). The important finding stressed in Figure 3b is the blockade that AA produced on MCD due to sustained cardiostimula-

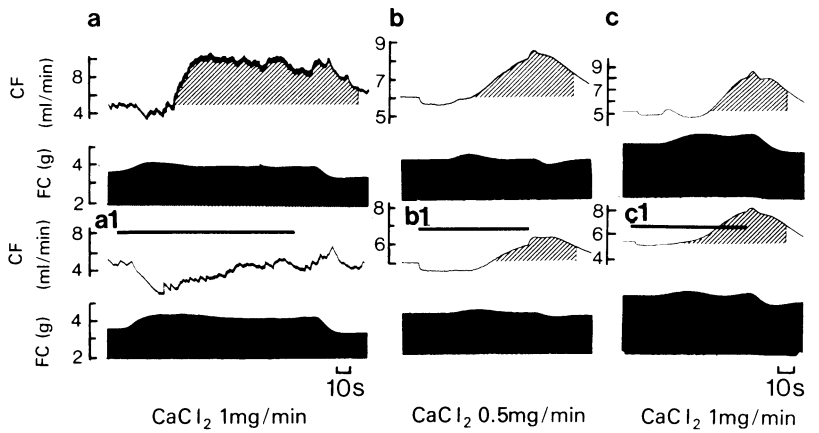

Figure 4 Influence of prostaglandin E_2 (PGE_2) or arachidonic acid (AA) on coronary flow (MCD) induced by slow infusions of calcium in normal and in naproxen-treated hearts. (a) MCD due to $CaCl_2$ (1 mg/min for 2 min) (horizontal bar) before the infusion of PGE_2. (a1) $CaCl_2$ administration during PGE_2 (10 μg/l) failed to induce MCD. (b) MCD due to $CaCl_2$ (0.5 mg/min for 1 min) (horizontal bar) before AA administration. (b1) MCD due to $CaCl_2$ was inhibited by AA administration (50 μg/l). (c) The infusion of $CaCl_2$ (1 mg/min for 1 min) to a heart of a naproxen-treated rat produced an MCD that in (c1) is shown not to be affected when AA (50 μg/l) was administered with the perfusate. Hatched areas under recordings of coronary flow indicate volumes measured until 50% recovery to normal levels. Calibration of coronary flow (CF) in ml/min and force of contraction (FC) in g.

tion by NA. Obviously, this is homologous to the blockade induced by PGE_2. Likewise the administration of AA did not affect the inotropic action of NA; AA by itself induced, in some cases, an increase in basal force of contraction, but this effect did not last for more than 10 to 15 min nor was it correlated with the initial, but transient, increase in coronary flow that appeared in the majority of the experiments. The increase in flow subsided to normal long before the new challenging infusion of NA was applied.

If the rats were pretreated for several days with indomethacin, the MCD response induced by slow infusion of NA remained unaltered when AA was added to the perfusate. Thus, with slow infusions we obtained similar results as with bolus doses of NA. Sustained cardiostimulation was also induced in a few experiments ($n = 4$) in which Ca^{2+} was slowly infused for one or more minutes. PGE_2 produced, as shown in Figure 4a, a marked inhibition of MCD that was replaced by an enhanced vasoconstrictor phase, typical of the action of Ca^{2+} on vascular smooth muscle. In other experiments, the slow infusion of Ca^{2+} was carried out before and during administration of AA to hearts of rats untreated ($n = 4$) and naproxen pretreated for 5 days ($n = 4$). As shown in Figure 4b,, AA perfusion also caused a diminution of MCD response to Ca^{2+} while AA in naproxen-treated hearts (Figure 4c) failed to block MCD. No attempts were made to present statistical evaluation of these data because of the restricted number of experiments, because of their consistent results and, particularly, because they confirmed what had already been studied with single doses of $CaCl_2$ and shown in detail in Figure 2.

We felt, nevertheless, that glucagon would be an interesting cardiostimulating agent to include in these studies as its actions have not been included in our previous publications. Slow infusions of glucagon appeared to be the most suitable method for its administration. We confirmed that concentration-dependent inotropic actions obtained by 2 min infusion produced the slowest developing MCD responses found so far in our experiments, and they lasted for a prolonged period of time. These characteristics of the glucagon-induced MCD follow the pattern of the inotropic action, which continued long after stopping the infusion.

It is interesting to note again the parallelism that exists between the induction of cardiostimulation and the development of MCD. If we take into consideration the increase in cardiac activity due to inotropic effects, this relationship becomes quite evident (Table 5). The time of onset of cardiostimulation after starting a slow infusion of Ca^{2+}, NA or glucagon and the time to obtain the peak inotropic action, followed by the times of onset and peak MCD response, can be compared in Table 5: the conclusion that can be drawn from this table is that the shorter the time of onset of inotropic action (Ca^{2+}), the shorter the starting time for MCD. On the other hand, the longer

the delay in reaching the peak value of inotropic action (glucagon) the longer the time to reach peak MCD and, therefore, the longer the duration of the increased coronary flow.

The quantification of results obtained with glucagon are expressed in Figure 2, in which it can be seen that the large MCD response to glucagon infusion was greatly reduced (about 65%) during AA administration. In these experiments it was also demonstrated that if AA infusions were given to hearts from indomethacin-treated rats, the MCD reactions to glucagon remained unaltered. Figure 2 shows that the inotropic effect of glucagon remained unaltered whether AA or indomethacin plus AA were administered. These results also show that the inhibition of MCD is unrelated to changes that AA may have induced in cardiac contractility.

Effect of non-steroidal anti-inflammatory agents on the reactive hyperaemia induced by coronary occlusion

The subject of reactive hyperaemia and its relation to the synthesis of PGL is under continuous revision because contradictory results have been reported (see Discussion). We reinvestigated this problem in spontaneously beating isolated perfused hearts because previously we had demonstrated that separate mechanisms are apparently involved in the production of MCD and in reactive hyperaemia (Sen, *et al.*, 1977).

The results of the experiments on reactive hyperaemia are summarized in Figure 5 in which it can be seen that the longer the period of occlusion the larger the reactive hyperaemic response. Treatment of rats with indomethacin, naproxen or phenylbutazone produced no significant differences in basic coronary flow, or in spontaneous heart rate, of the isolated perfused heart, as indicated in Table 6. Nevertheless in Figure 5 it can be seen that, by pretreating the animals with anti-inflammatory agents, there is an enhancement of reactive hyperaemia. This effect became more pronounced during the longer (15 and 20 s) coronary occlusion periods and was statistically significant for naproxen and phenylbutazone. A similar tendency can be seen with indomethacin. However, the large s.e. mean prevented us from obtaining a statistically significant difference between the control and the indomethacin-treated hearts.

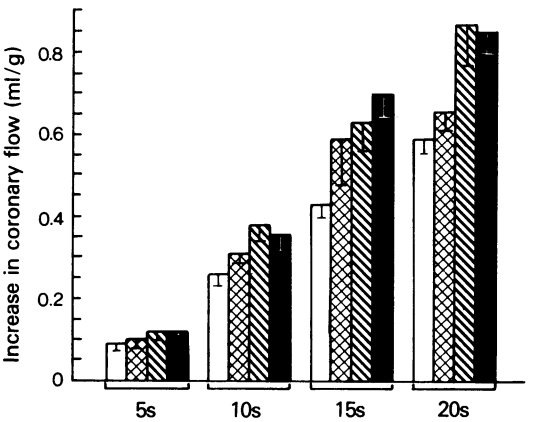

Figure 5 Reactive hyperaemia responses (mean) induced by interruption of coronary perfusion for the periods indicated by 5, 10, 15 and 20 s; vertical lines show s.e. mean; untreated controls (open columns); indomethacin (cross-hatched columns); naproxen (hatched columns) and phenylbutazone (solid columns) treated hearts. The 15 and 20 s reactive hyperaemia responses were significantly higher ($P < 0.05$) in naproxen- and phenylbutazone-treated hearts compared to respective controls. Basal coronary flows and heart rates for these hearts are indicated in Table 6.

Discussion

It is agreed that the coronary vasodilatation, following cardiac hyperactivity induced by a number of unrelated cardiostimulating agents, is mostly dependent on the increased metabolism which correlates with the degree of cardiostimulation (Gregg & Fisher, 1963; Parratt, 1969; Rowe, 1974). It is also agreed that the increased metabolism and cardiac oxygen

Table 5 Time for onset and peak inotropic and coronary dilatation (MCD) responses to slow infusions of Ca^{2+}, noradrenaline (NA) and glucagon

	Inotropic action		*MCD*	
Drugs infused	*Onset*	*Peak*	*Onset*	*Peak*
$CaCl_2$ 0.5 mg/min	13.0 ± 1.6	26.0 ± 2.0	27.0 ± 2.1	62.5 ± 1.04
NA 0.1 μg/min	20.0 ± 1.2	44.2 ± 3.4	42.0 ± 1.04	82.2 ± 2.0
Glucagon 0.5 μg/min	44.8 ± 2.8	116.0 ± 11.0	72.6 ± 6.0	178.5 ± 7.14

The number of cases ($n = 5$) were taken at random from different series of experiments, and the rate of slow infusion was constant (0.2 ml/min). Onset and peak values are expressed in seconds ± s.e.

consumption correlate with cardiac mechanical function (Theisohn, Friedrich, Justus, Güttler & Klaus, 1977). The mechanism responsible for the diminution in coronary resistance in response to increased cardiac metabolism remains unknown. Although the adenosine hypothesis of Berne (1975) is continuously under critical evaluation (Moir & Downs, 1972) the consensus is that the coronary vasodilatation could be due to the action on the vasculature of certain unidentified metabolite(s) produced during the cardiac hyperactivity (Broadley, 1976).

There is actually a common property shared by the different agents used in our experiments to induce cardiostimulation: the activation of cardiac adenylate cyclase. We demonstrated in the mammalian isolated perfused heart that the increased coronary flow resulting from cardiac hyperactivity correlates with the activation of adenylate cyclase and we postulated that the increased cyclic AMP level in the heart triggered a vasodilatory mechanism that adapted the coronary flow to the increased oxygen demands (Sen *et al.*, 1976; Sen *et al.*, 1977). The increased myocardial contractility, heart rate and activation of adenylate cyclase due to NA (Robison, Butcher, Øye, Morgan & Sutherland, 1965) Ca^{2+} (Sen *et al.*, 1976), tachycardia (Sen *et al.*, 1977; Rényi-Vàmos, 1977) or glucagon (Bussuttil, Paddock, Fisher & George, 1976), are abundantly documented. We included glucagon in the present experiments because it has repeatedly been shown that its administration produces an increase in cardiac work, oxygen consumption and activation of the adenylate cyclase enzyme (Nayler, McInnes, Chipperfield, Carson & Daile, 1970) and because the increase in coronary flow after glucagon administration appears as a consequence of the metabolic effects due to the increased myocardial contractility (Von Tarnow, Gethmann, Patschke, Weymar & Eberlein, 1975).

The mechanism responsible for the diminution in coronary resistance in response to increased cardiac metabolism, although it correlates with the levels of cardiac cyclic AMP, remains unknown (Sen *et al.*, 1977). In the present paper, we give data confirming that MCD can be induced by NA, Ca^{2+}, tachycardia and also by glucagon. We also confirmed that the sustained administration of PGE_2 produces inhibition of the prolonged MCD due to slow infusions of NA, Ca^{2+} or glucagon, in a similar manner to the previously reported observations in which single doses of cardiostimulating agents were applied (Sunahara & Talesnik, 1974).

While these observations may be of importance in establishing the cardiovascular role of prostaglandin-like substances (PGL) the physiological and pharmacological implications would be valuable only if it can be demonstrated that endogenous PGL, synthesized in the heart, produce similar effects. It is unlikely that circulating PGL is involved in the regulation of cardiovascular function because the circulating PGL levels are very low (Willman, 1971) due to the powerful 15-hydroxy-prostaglandin dehydrogenase in the lungs, which prevents the heart from receiving effective concentrations of PGL that may be released from organs into their venous or lymphatic drainage (Horton, 1976). *In situ* biosynthesis (and perhaps degradation) may occur in the microsomal fraction of the myocardium in which the presence of a prostaglandin synthetase has been demonstrated (Limas & Cohn, 1973). Another possible source of PGL locally synthesized within the heart is the coronary arteries (Kalsner, 1976; Kulkarni *et al.*, 1976; Moncada, Gryglewski, Bunting & Vane, 1976).

In our experiments, AA administered with the perfusate produced vasodilation and increased coronary flow in conformity with the results reported by, among others, Isakson, Raz, Denny, Wyche & Needleman (1977a) and Hintze & Kaley (1977). The coronary vasodilatation obtained under our experimental conditions was of rather short duration, although the AA administration was continued for well over 60 min. Vasodilatation due to AA also occurs in the resistance vessels of the systemic circulation leading to a lowering of blood pressure (Larsson & Änggärd, 1973; Deby, Barac & Bacq, 1974). It may be that the immediate coronary action of AA is due to the transformation of AA into PGL, most probably

Table 6 Basic coronary flow and heart rate in rats treated with anti-inflammatory agents

	Control ($n = 4$)	*Indomethacin* ($n = 4$)	*Naproxen* ($n = 5$)	*Phenylbutazone* ($n = 4$)
Coronary flow (ml min^{-1} g^{-1})	7.4 ± 0.48	8.3 ± 0.5	7.6 ± 0.62	8.4 ± 0.74
Heart rate (beats/min)	283 ± 21	270 ± 6	263 ± 17	291 ± 5

Hearts were allowed to beat spontaneously; the slight differences among the groups were non-significant.

PGE_2 (van Dorp, 1976; Isakson *et al.*, 1977a) although several other unsaturated fatty acids have been shown to increase coronary flow in Langendorff perfused hearts and in the 'intact' coronary circulation of the dog. The dilatory efficacy was found to be much greater, nevertheless, for AA and linolenic acid than for oleic and linoleic acids (Mentz, Blass & Förster, 1976). The fact is that the administration of AA into the perfusate of an isolated heart leads to the synthesis of PGL that may appear in the coronary venous effluent (Junstad & Wennmalm, 1973; Block, Poole & Vane, 1974; Gudbjarnason, 1975; Needleman, 1976) as an overflow of the biologically active compounds synthesized in the cellular compartment where prostaglandin synthetase is constantly available and in which the substrate (AA) is the rate-limiting step for prostaglandin synthesis (Needleman, 1976). More recently it has been reported that AA metabolism in the rabbit heart leads to the formation of PGE_2 and to a new PGL whose identification is still under investigation (Isakson, Raz, Denny, Pure & Needleman, 1977b).

Vasodilatation due to AA has also been reported by Needleman (1976) who explains its transient action to 'rapid washout' of the newly synthesized PGL produced by the heart. Although at the moment we do not have a better explanation to offer, Needleman's suggestion is not particularly convincing since AA, the availability of which is the rate-limiting step for prostaglandin synthesis, was continuously offered as a substrate; on the other hand we have already shown, and confirmed in the present paper, that prolonged exogenous administration of PGE_2 also produced a coronary vasodilatation that subsided after some minutes of infusion (Sen *et al.*, 1977).

Arguments favouring the conversion of AA into PGE_2 are further supported by numerous experiments demonstrating that the prostaglandin-synthetase may be inhibited by administration of non-steroidal anti-inflammatory drugs (Flower & Vane, 1974a; Flower, 1974). From our results a direct vasodilator action of AA on the rat coronary resistance vessels cannot be ruled out since indomethacin, naproxen or phenylbutazone were unable to prevent completely the vasodilatation when the fatty acid was administered; one may argue, nevertheless, that full inhibition of prostaglandin-synthetase was not achieved with the concentrations of anti-inflammatory agents used. This last assumption could also be applied to most studies in which these drugs have been used to block the synthesis of prostaglandins, including the recent publications (Kulkarni *et al.*, 1976; Dusting, Moncada & Vane, 1977) in which it is suggested that other metabolites produced by prostaglandin synthetase from AA are responsible for coronary vasodilatation. The synthesis of these newly described vascular smooth muscle dilating substances is also inhibited when indomethacin is added to the system containing prostaglandin synthetase (Isakson *et al.*, 1977b).

In a preliminary communication, Wennmalm (1977) showed that AA, when infused into the brachial artery in humans after indomethacin, produced only about 50% of the increase in forearm blood flow as compared to the increase before indomethacin. The author suggested that the vasodilator capacity of AA was due only in part to its conversion into PGL.

The results of the experiments favouring the local synthesis of prostaglandins do not provide an answer as to the location of the synthetase responsible for the appearance of PGL detected in the venous effluent of the coronary circulation (Minkes, Douglas & Needleman, 1973; Block *et al.*, 1974). The speed at which PGL appears when AA is injected into the coronaries suggests that the formation of endoperoxide intermediates occurs in the course of prostaglandin synthesis near the site where the availability of AA to the enzyme is higher. The coronary artery wall itself has been shown *in vitro* (Kulkarni *et al.*, 1976; Dusting *et al.*, 1977; Isakson *et al.*, 1977a) and *in vivo* (Hintze & Kaley, 1977) to be a likely candidate for the location of prostaglandin-synthetase. Moreover, endothelial cells synthesize and release prostaglandins from AA (Weksler, Marcus & Jaffe, 1977) and in all these instances indomethacin, or other non-steroidal anti-inflammatory agents block the conversion of AA to prostaglandins (Flower, 1975).

At the concentration of AA used in our experiments, coronary vasodilatation was recorded in almost all cases; nevertheless, we did not test whether higher concentrations would have been more effective. We meet the greatest difficulty in trying to explain the production of coronary vasodilatation by AA in hearts acutely or chronically treated with indomethacin, naproxen or phenylbutazone, since it does not conform with other reports (Needleman, 1976; Hintze & Kaley, 1977), particularly when indomethacin had been administered to inhibit cardiac prostaglandin-synthetase in isolated perfused or intact hearts (Minkes *et al.*, 1973). It may well be that we are dealing with a complex type of reaction to unsaturated fatty acids, since linolenic and oleic acids may also cause 'direct' relaxation of coronary vessels, unaffected by indomethacin, as demonstrated *in vitro* by Kulkarni *et al.* (1976). However, the latter point is also controversial since, in Langendorff perfused hearts of guinea-pigs, coronary vasodilatation induced by oleic, linoleic, linolenic as well as AA has been reported by Mentz *et al.* (1976); the vasodilatation due to these fatty acids (except oleic acid) was depressed, but not abolished, by indomethacin. Thus, unsaturated fatty acid may relax coronary vascular smooth muscle by direct or indirect mechanisms unrelated to prostaglandin synthesis. The possibility of species differences, methods of study, time and mode of indometh-

acin administration and the origin of the coronary vessel under study may add to the complexities of this problem. The source of vessels for the *in vitro* preparations are the large arteries while the behaviour of the resistance vessels can only be examined in the intact organ.

There are aspects of our results that are somewhat in conflict with certain data found in the literature. We established, for instance, that AA administration was effective in blocking MCD not only in control hearts but also if the perfusate contained indomethacin. However, if AA was given to hearts that had been treated with indomethacin for 24 h or more, then AA became ineffective. The latter may be interpreted as due to 'competitive-irreversible' inhibition of the prostaglandin-synthetase by indomethacin (Lands, LeTellier, Rome & Vanderhoek, 1974; Flower, 1974). The failure of indomethacin to block AA conversion into PGL when given acutely with the perfusate could be explained by the slow intracellular penetration of indomethacin, as demonstrated by Raz, Stern & Kenig-Wakshal (1973). According to these authors, when the enzyme system is localized intracellularly (microsome-bound) it would not be easily affected by indomethacin; however, prostaglandin synthesis would be inhibited when the synthetase complex is cell membrane bound in which case the presence of the anti-inflammatory agent in the extracellular fluid would have easy access to inhibit the synthetase. We think that inhibition of prostaglandin formation would occur if enough time is allowed for indomethacin to reach an adequate concentration within the myocardial cell. These arguments may also be helpful in explaining the fact that if AA inhibition of MCD had already developed, neither indomethacin nor naproxen were able to reverse the blockade of MCD. This might explain the obvious discrepancy with the reports, in which prostaglandin release from isolated hearts could be abolished by the acute administration of indomethacin (Minkes *et al.*, 1973; Block *et al.*, 1974).

Although there is not a simple, nor a single, explanation for this discrepancy, we would like to emphasize that perhaps we are dealing with different processes. In our experiments, the intramyocardial cell synthesis of PGL would be activated by the exogenous administration of its substrate, AA, while the release of PGL from the heart, although it may be an indication of *de novo* synthesis of PGL (Piper & Vane, 1971) could also indicate that a severe disturbance had affected the myocardium as a consequence of which the release of prostaglandin would take place; this opinion led Block *et al.* (1974) to suggest... 'that a local prostaglandin release does not contribute to the maintenance of coronary flow or the genesis of the anoxic-induced vasodilator response' because the prerequisite for prostaglandin release in the heart is some form of cellular distortion produced for instance by traumatic stimuli, anoxia or embolization (Block *et al.*, 1974). This opinion is supported by the observation that experimentally induced myocardial ischaemia is followed by the increase in prostaglandin release into the venous effluent of the coronaries in dogs (Berger, Zaret, Speroff, Cohen & Wolfson, 1976; Kraemer, Phernetton & Folts, 1976) and in humans with myocardial alterations induced by myocardial ischaemia (Berger, Zaret, Speroff, Cohen & Wolfson, 1977). Therefore, prostaglandin release in these severe pathological circumstances does not necessarily establish a causal relationship since prostaglandins may arise from myocardial cells or directly from the coronary vasculature as it becomes involved in the ischaemic reaction. The inhibition produced by indomethacin clearly indicates that the released prostaglandin is newly formed and that the synthetase responsible for its formation is probably localized in the vascular tissue itself where the enzyme inhibitor is obviously found at its highest concentration. This would also explain the enhancement of MCD obtained when aspirin-like substances were perfused through hearts from normal rats. It must also be considered that exogenously administered AA may be metabolized to prostaglandins somewhat differently from endogenously released arachidonate although AA could easily be incorporated into the phospholipids of the intact heart (Isakson *et al.*, 1977a). Thus, the explanation originally given by us for the enhancement (Talesnik & Sunahara, 1973) is incomplete in as much as inhibition of the prostaglandin-synthetase located in the myocardial cell is concerned.

In addition to the inhibition of vascular smooth muscle synthetase, indomethacin by inhibiting phosphodiesterase (Flower, 1974) may augment the elevations in cyclic AMP induced by cardiac hyperactivity. It is a well established fact that such an increase in the cyclic AMP level in the vasculature may produce vasodilatation (Kukovetz & Pöch, 1970; Triner, Vulliemoz, Verosky, Habib & Nahas, 1972; Andersson, 1973; Namm & Leader, 1976).

We decided, in this paper, to limit our study to the modulatory influence that increased cardiac prostaglandin synthesis exerts on the coronary responses elicited by cardiac hyperactivity or to short periods of hypoxia. From our results it can be inferred that prolonged administration of AA, at about 5 to 10 times higher concentration than PGE_2, produced an unquestionable inhibition of MCD. The inhibitory action of AA on the adaptive coronary vasodilatation resulted irrespective of the hyperactivity inducing agent: tachycardia, NA, Ca^{2+} or glucagon. Another important finding is that the infusion of AA had to be maintained for more than 30 min, under our experimental conditions, for the inhibitory effect

to become evident. During this period, the coronary flow normalized if a vasodilator effect of AA had appeared, and the MCD responses remained unchanged. A similar picture was obtained for PGE_2 administration, confirming results of our previous studies (Sunahara & Talesnik, 1974; Sen *et al.*, 1976). The slowness of AA in producing blockade of MCD may be ascribed to the build-up of a suitable intramyocardial cell concentration that would activate the prostaglandin-synthetase and induce the subsequent production of prostaglandins; the intracellular increase of prostaglandins would inhibit the adenylate cyclase activation induced by cardiostimulating agents (Sen *et al.*, 1977). Since the local synthesis is followed by the further metabolism of prostaglandins to inactive derivatives in the cytoplasm, (Flower & Vane, 1974b) one could explain the need for the continuous supply of AA to keep the prostaglandin levels in the myocardial cell at a suitable concentration. Thus, withdrawal of AA from the perfusate resulted in the fast recovery of the original MCD response.

The results of our experiments indicate that AA inhibition of MCD is probably due to an increased production of a PGE_2-like substance, not only because it resembles the inhibition produced by prolonged administration of PGE_2, but because indomethacin and naproxen blocked the inhibitory action of AA. In a multi-enzyme system such as prostaglandin-synthetase, inhibition may be exerted at a number of sites and the available evidence suggests that indomethacin and naproxen inhibit the formation of the cyclic endoperoxide in the initial stages of the reaction (Flower & Vane, 1974a). In contrast, phenylbutazone inhibits the formation of PGE_2 by interfering with the breakdown of the endoperoxide (Flower & Vane, 1974b) since it selectively blocks its isomerase, thus perhaps increasing the production of $PGF_{2\alpha}$ (Stone, Mather & Gibson, 1975). Furthermore, indomethacin or naproxen, both of which prevent the formation of cyclic endoperoxides, do not block but may even enhance MCD responses, thus advocating against a role for prostaglandins, including those (prostacyclin) recently described by Kulkarni *et al.* (1976) and Dusting *et al.* (1977) as agents directly responsible for the compensatory vasodilatation that follows cardiac hyperactivity.

The administration of AA to hearts from rats chronically treated with anti-inflammatory agents resulted in enhanced MCD responses particularly when cardiostimulation was induced with Ca^{2+} or tachycardia; nevertheless, the tendency was also shown when single doses of NA or infusions of glucagon were used. We do not have enough information to permit us to elaborate explanations for this rather surprising but interesting observation.

In the present experiments, we confirmed that reactive hyperaemia, induced by a short interruption of the coronary flow, was not influenced by PGE_2 (Sen *et al.*, 1977) and that the induction of cardiac prostaglandin synthesis by AA administration does not affect the reactive hyperaemia response. We also found that the administration of indomethacin, naproxen or phenylbutazone does not inhibit reactive hyperaemia. On the contrary, enhanced responses to hypoxia were obtained particularly in hearts from naproxen or phenylbutazone-treated rats. At present, we lack information which would allow a sensible explanation for the enhancement of reactive hyperaemia in hearts of pretreated rats. This is a research area in which there are abundant discrepancies among different authors; thus, while some regard favourably the participation of prostaglandins in reactions to hypoxia (Alexander, Kent, Pisano, Keiser & Cooper, 1975), others deny such a role (Hinze & Kaley, 1977). We have decided to investigate further the behaviour of the cardiac prostaglandin-synthetase under several experimental conditions but these results, as well as their implications, deserve a separate discussion in a future paper.

The data presented in this paper support our contention that there are at least two types of coronary vasodilatation: one, in response to increased cardiac activity, would be modulated by endogenously synthesized prostaglandins while another, reactive hyperaemia, appears to be independent of prostaglandins. The excess cardiac synthesis of prostaglandins or lack of its metabolic degradation, could lead to a diminished adenylate cyclase activation and subsequent reduced levels of cyclic AMP necessary to trigger the mechanism responsible for MCD reactions (Sen *et al.*, 1976; Sen *et al.*, 1977). Thus, abnormally high levels of prostaglandin might be, at least in part, the underlying mechanism of coronary insufficiency reported in humans with normal coronary arteriogram (Neill, Judkins, Dhindsa, Metcalfe, Kassebaum & Kloster, 1972; Bamrah, Bahler and Rakita, 1974; Rocher, Fayard, Manin, Bens, Guermonprez, Vagner, Leclerc, Ourbak & Maurice, 1974; Lawson, Rosch & Rahimtoola, 1976; Rosenblatt & Selzer, 1977; Oliva & Breckinridge, 1977; Selzer, 1977). Since patients with 'normal' coronary arteries may show angina pectoris, with abnormal myocardial lactate metabolism, the diagnosis of 'small vessel disease' with changes in structure or dysfunction of structurally normal small arteries has been suggested (Schaper & Schaper, 1977); the lack of evidence nevertheless inclined other authors to revive the hypothesis of neurogenic vasospasm as a mechanism of coronary insufficiency (Yasue, Touyama, Shimamoto, Kato, Tanaka & Akiyama, 1974; Johnson & Detwiler, 1977; Hellstrom, 1977). We think that although metabolic factors in which prostaglandins may be involved, are often of great clinical significance in heart attacks (Marx, 1977), failure of the cor-

onaries to adapt to increased O_2 demands, should also be considered as a potential site for therapeutic tests (Talesnik & Sunahara, 1973). The modulation of the coronary responses by prostaglandins agrees with the concept of Silver & Smith (1975), in which prostaglandins are thought to act as intracellular messengers rather than local hormones or vasoactive substances acting on distant target cells. This concept does not exclude the role that prostaglandins might play in the local control of microvascular tone in other tissues or under particular circumstances (Messina, Weiner & Kaley, 1976; McGiff, Malik & Terragno, 1976) nor the important interactions involving platelet aggregation that may contribute to the thrombus formation on a damaged vascular area (Flower, 1975; Bunting, Gryglewski, Moncada & Vane, 1976; Moncada *et al.*, 1976; Moncada, Vane & Higgs, 1977).

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