# CHAPTER II
# MATERIALS AND METHODS

## ANIMAL PREPARATION

Male Wistar rats were supplied by the National Laboratory Animal Center of Salaya Campus, Mahidol University. The animals were maintained on normal rat food and tap water ad libitum. They were housed five per cage in stainless steel bottom cages in a well-ventilated room in which the temperature was 28-32°C The automatic lighting schedule provided 14 hour light : 10 hour dark cycle at the Experimental Animal Center, Faculty of Medicine, Chulalongkorn University.

## CHEMICAL SUBSTANCES

The list of chemical substances used in this study were summarized as follows:-

| CHEMICALS | COMPANY |
|---|---|
| Adrenomedullin (AM) | Support by Dr. Hideyuki Niimi, The National Cardiovascular Center of Japan. |
| Bradykinin | Sigma, USA. |
| Dimethyl sulfoxide | Sigma, USA. |
| Diethyl ether | Merck, USA. |
| Fluorescein isothiocyanate - dextran | Sigma, USA. |
| Glibenclamide | Sigma, USA. |
| Heparin | Leo, Denmark. |

| | |
|---|---|
| Indomethacin | Sigma, USA. |
| Kit for cAMP analysis | Amersham, UK. |
| $N^G$ - nitro - L - arginine (L - NNA) | Support by Dr. Hideyuki Niimi, The National Cardiovascular Center of Japan. |
| Normal saline | Hospital Products Public Co. Ltd., Thailand. |
| Pentobarbiturate sodium (Nembutal ®) | Sanofi, Thailand |
| Sodium nitroprusside | Merck, USA. |
| Trichloroacetic acid | Reidel - de Hach, Germany |
| Triton X - 100 | Merck, USA. |
| Krebs - Henseleit bicarbonate buffer | |
| Bovine serum albumin | Sigma, USA. |
| Calcium chloride ($CaCl_2$) | Riedel - de Hach, Germany |
| Glucose ($C_6H_{12}O_6$) | Riedel - de Hach, Germany |
| Magnesium sulphate ($MgSO_4$) | Riedel - de Hach, Germany |
| Potassium chloride (KCl) | Riedel - de Hach, Germany |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Riedel - de Hach, Germany |
| Sodium bicarbonate ($NaHCO_3$) | Riedel - de Hach, Germany |
| Sodium chloride (NaCl) | Merck, USA. |

**Krebs-Henseleit bicarbonate buffer :**

| | | |
|---|---:|---|
| NaCl | 118.00 | nM |
| KCl | 4.70 | nM |
| $CaCl_2$ | 2.52 | nM |
| $MgSO_4$ | 1.66 | nM |
| $NaHCO_3$ | 24.88 | nM |
| $KH_2PO_4$ | 1.18 | nM |
| $C_6H_{12}O_6$ | 5.85 | nM |
| Bovine serum albumin | 2.00 | g/100 ml |

**High $K^+$ Krebs-Henseleit bicarbonate buffer :**

| | | |
|---|---:|---|
| NaCl | 80.00 | nM |
| KCl | 39.70 | nM |
| $CaCl_2$ | 2.52 | nM |
| $MgSO_4$ | 1.66 | nM |
| $NaHCO_3$ | 24.88 | nM |
| $KH_2PO_4$ | 1.18 | nM |
| $C_6H_{12}O_6$ | 5.85 | nM |
| Bovine serum albumin | 2.00 | g/100 ml |

## METHODS

The ultimate goal of this investigation is to approach the most physiological relevance of adrenomedullin (AM) especially on the cardiovascular system. Therefore, the three different experimental models were used in this study. First, the whole body model was used to examine the biological actions of AM on systemic blood pressure and also the heart rate. Second, the dorsal skinfold chamber technique was used in order to eventually conclude the effect of AM on peripheral circulation. Third, the main protocol of isolated perfused rat heart model was used in order to assess the biological responses of both cardiac performance and coronary circulation to AM. In addition, the possible mechanism(s) of the AM on coronary circulation was also employed in this third protocol. By using different inhibitors, the mode of actions of AM on coronary vessel were able to identified. The followings are the details of each experimental protocol:-

### I.  The whole body model: to study the effect of AM on systemic blood pressure

Adult male Wistar rats (n=7) weighing 250-300 g were used is this study.  Twelve hours before the start of the experiment, food was withdrawn with no limitation on water supply.  Rats were anesthetized for the duration of experiments with intraperitoneal injection (i.p.) of 45 mg/kg body weight of sodium pentobarbital.  Additional doses of anesthetics were given as required to maintain surgical anesthesia based on testing of corneal reflex and response to tail pinch.  A tracheostomy was performed to facilitate the respiration.  The mean arterial blood pressure and heart rate were monitored by a right common carotid artery catheter (PE-50) connected to a Statham

pressure transducer (Nikhon model TP-300T) and a polygraph (Nikhon RM 6000, Nikon Khoden, Japan). Catheter was also positioned in an external jugular vein for bolus injection of rat AM (Peptide Institute, Osaka), 1 nmol/kg body weight (Kitamura et al., 1993; Ishiyama et al., 1993).

The rats were left for 45 min after surgery to allow for equilibration. Then rat AM dissolved in saline was injected intravenously. Mean arterial blood pressure and heart rate were measured before (0 min) and 30 sec, 1, 5, 10, 30 and 60 min after the AM administration.
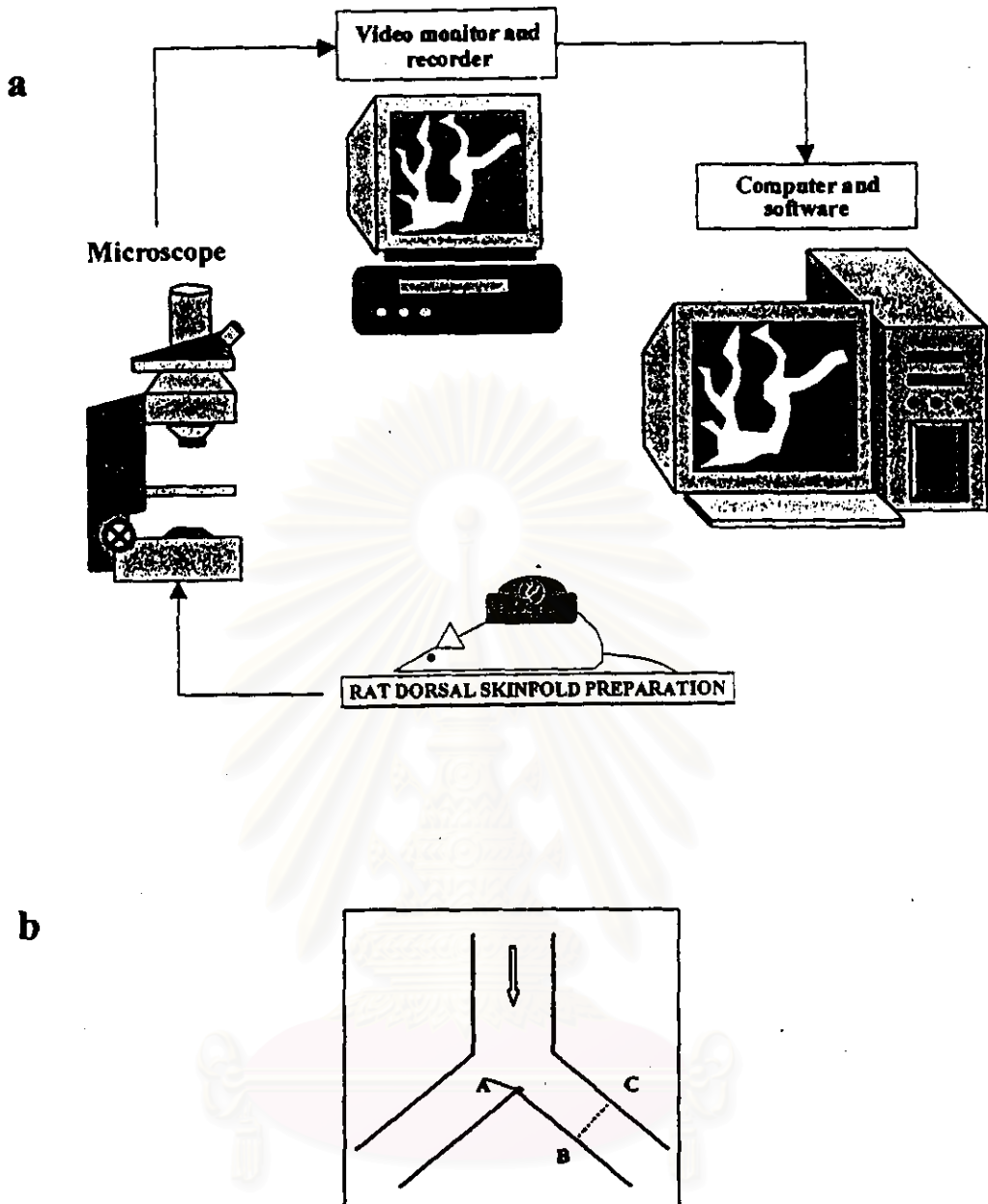
## II. The dorsal skinfold chamber model: to study the effects of AM on rat skin microcirculation

Male Wistar rats (n=5) weighing between 150-200 g were used in this study. Twelve hours before the start of the experiment, solid food was withdrawn with no limitation on water supply. The animals were anesthetized by i.p. injection of 45 mg/kg body weight of pentobarbital sodium. The tracheostomy was performed, fine polyethylene catheters were inserted into the jugular vein and the carotid artery for injection of fluorescent marker, 5% fluorescein isothiocyanate-dextran 150, and for arterial pressure recording, respectively. The entire back of the animals was shaven. The aluminium chamber frames were implanted so as to sandwich the extended double layer of the skin (Figure 2.1). One layer was completely removed in a circular area of 15 mm in diameter and a remaining layer, consisting of epidermis, subcutaneous tissue, and a thin striated skin muscle was covered with a cover slip incorporated in one of the frames. In order to assess the direct microcirculatory effect of topical AM application, AM at a concentration of $10^{-7}$ M (Niimi et al., 1996) was topically applied to the skin

**Figure 2.1**  Dorsal skinfold chamber preparation

**Figure 2.2**    a: Dorsal skinfold chamber model with intravital fluorescent microscopy and instruments used for direct studies of the acute change in the skin microvessels diameter. b: Schematic of arteriole showed the reference point A and the defined point B and C. The diameter of arteriole was measured as the length of B-C.

muscle within an observation window. Its application was achieved by delivering 0.5 ml/min of AM directly onto the skin muscle. The diameter of the second and the third order arterioles was assessed from the fluorescent videoimages obtained before and at 1 min, 3 min, 5 min, 10 min and 20 min after the application. The measurement was made with a computer – assisted image analyzer as the instruments used was shown in Figure 2.2(a). The diameter of each arteriole was determined by using the computer program "Global Lab Image". The diameter of arteriole was assessed by the software indicated by number of pixels. Then the software could convert number of pixels to micrometer. Figure 2.2(b) shows measurement of distance between two points (B to C) by using the position of the reference point in order to define points A to B.

## III. The isolated perfused rat heart model: to study the effect of AM on cardiac performance and coronary circulation.

Adult male Wistar rats (n=5 for each experiment) weighing 250-300 g were used in this study. Twenty four hours before the start of the experiment, food was withdrawn with no limitation on water supply. To prevent thrombosis of the coronary arteries in the remainder of the experiment, the animals were pre-injected with approximately 500 USP-units of heparin per 100 g body weight, intraperitoneally (i.p.), one hour before the surgery to inhibit blood coagulation. Experimental rats were anesthetized for the duration of experiments by intraperitoneal administration of 45 mg/kg of sodium pentobarbital. Additional doses of anesthetics were given as required to maintain surgical anesthesia. After tracheostomy, the ventilation was assured by using positive pressure ventilator (rodent ventilator model 683, Harvard Apparatus, USA)

The skin was incised by a longitudinal cut from the middle of the abdomen up to the throat. Then the abdomen was opened up to the diaphragm. The diaphragm was cut off following the anterior part of the inferior thoracic aperture. The thorax was cut open on the left and right side following to bone-cartilage-border on a line parallel to the sternum starting at the diaphragm and proceeding as far cranial as to the first rib. The complete anterior thoracic wall was turned upwards over the animal's head and fixed in this position.

The pericardium was removed then free the ascending aorta of any connective tissue and separate from the pulmonary artery. In order to prevent large amounts of blood from pouring out of the opened aorta and impeding the view on the surgical area, the inferior vena cava was clamped with a vessel clamp. The insertion of the aortic cannula was facilitated and flooding of the surgical area with blood was reduced by sprinkling the heart with cold physiological saline (4 to 8°C) so that the heart stop beating or slow down.

The pulmonary artery was incised so that the right ventricle would not be overstretched by continuing blood flow during the time when the aortic cannula has been inserted but the heart has not been removed from the thorax. The aortic cannula was filled with perfusate [Krebs-Henseleit bicarbonate buffer containing (in mM) 118 NaCl, 4.70 KCl, 2.52 $CaCl_2$, 1.66 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 $KH_2PO_4$, 5.85 $C_6H_{12}O_6$ and bovine serum albumin 2 g/100 ml; the perfusate was oxygenated with a gas mixture of 95% $O_2$ 5% $CO_2$, pH 7.4] and was connected to the heat exchanger. The ascending aorta was incised and the cannula was inserted. At this moment a slight perfusion of the coronary arteries commenced, any blood in the

coronary arteries would be washed out and the re-supply of oxygen to the heart begins.

The heart, which was now attached to the cannula, was slightly elevated and cut out. The cannula with the attached heart was removed and was connected to the isolated heart apparatus (Size 3, Type 830, Hugo Sachs Elektronik, Germany). Any tissues that might still stick to the heart, such as fat tissue, lung pieces or vessel roots, were removed.

The retrograde perfusion is performed by the Langendorff technique, with perfusion pressure (PP) of 90 mmHg. From the apparatus, the coronary blood flow (CBF) were monitored continuously by using electromagnetic flowmeter. Mean coronary PP was computed from the signal obtained from a small catheter that was located above the aortic valve and connected to the Isotec pressure transducer. The whole apparatus was enclosed in a thermostatic chamber at 37.5°C. A cannulated fluid-filled balloon connected to the Isotec pressure transducer by a rigid catheter was inserted into the left ventricle via a left atrial incision. Intraventricular volume was held constant, thus left ventricular developed pressure, i.e., the maximal positive derivative ($+dP/dT_{max}$), reflected the contractile state of the myocardium, the maximal negative derivative ($-dP/dT_{max}$), reflected left ventricular relaxation. Heart rate, $+dP/dT_{max}$, and $-dP/dT_{max}$ were computed from the left ventricle signal. The ratio ($-dP/dT_{max}$)/($+dP/dT_{max}$) was also calculated to indicate whether the myocardial rate of contraction or relaxation were more influenced by AM. Electrocardiogram (EKG) was recorded by suction EKG electrodes. All these signals are displayed on the screen of computer set during the experiment as shown in Figure 2.3 The evaluated data are displayed in the right side of the screen and are stored in a data file on the hard disk which
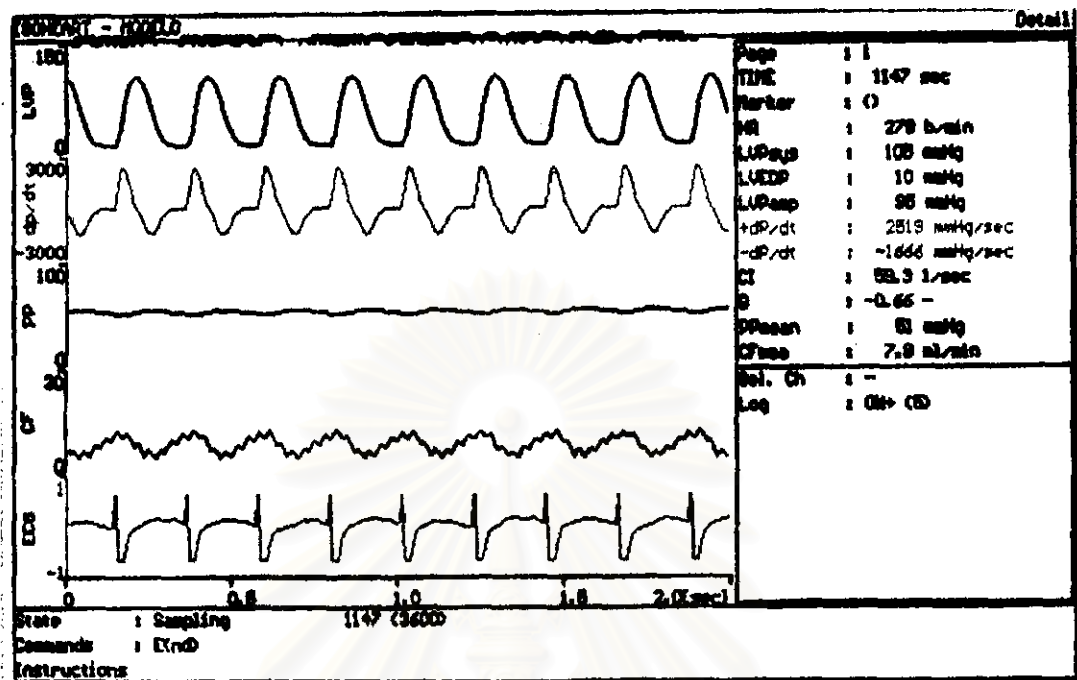
**Figure 2.3** The screen of the computer set displaying signals of the cardiac parameters during the experiment. The screen is continuously updated, the time axis for the detail screen is 2 seconds. The left side of the screen consists of the graphic display. The right side reproduces the measured values in numerial form which are also stored in a data file on the harddisk.
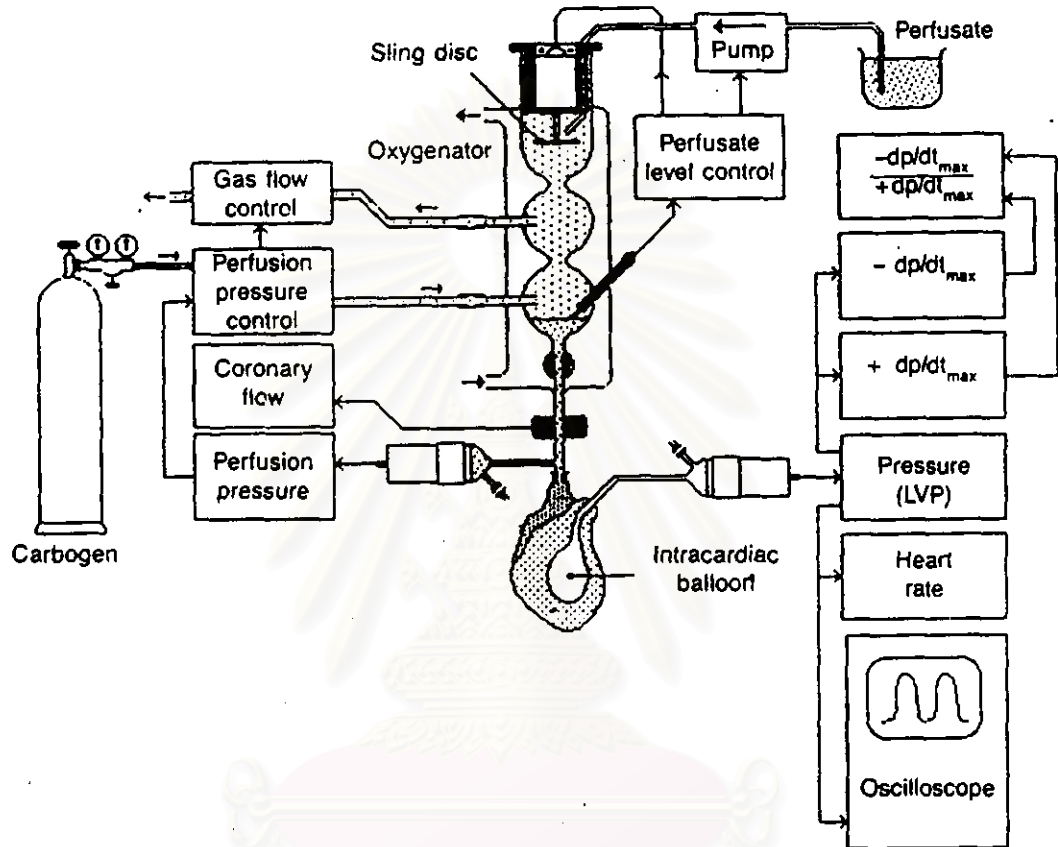
can be analyzed later. The experimental set-up and block diagram of the measuring devices used on the isolated perfused rat heart according to LANGENDORFF is shown in Figure 2.4. To examine the effects of AM on cardiac performance and coronary circulation, various doses of AM were injected into the heart via an aortic cannulation. The minimal dose that causes coronary vasodilation was 20 µg and was chosen for this experiment.

The further experiments were divided into 5 protocols in order to study the cardiac and coronary effects of AM and the mechanism of action of AM on coronary circulation.

After the aortic cannulation, hearts were allowed to stabilize for about 60 min with the warm oxygenated perfusate. Then, AM at the dose of 20 µg was injected into the heart. The cardiac parameters were recorded before and 30 sec, 1,3,5,10 and 20 min after the bolus injection of AM.

## A : The effect of AM on ventricular cAMP accumulation

For measurement of ventricular cAMP accumulation, following the equilibration period of 20 min hearts were injected with vehicle or AM (20 µg). Three minutes after injection, atrial tissue was removed, and the ventricles were immediately frozen with liquid nitrogen and stored at -80°C until assayed. Tissue samples were homogenized with 6% trichloroacetic acid at 4°C to give a 10% (wt/vol) homogenate, followed by centrifugation at 2000 g for 15 min. Then supernatants were collected and washed with 5 vol of water-saturated diethyl ether four times. The extracts were lyophilized and processed for the measurement of cAMP content with an enzyme immunoassay kit (Biotrak, Amersham Life Science).

**Figure 2.4** The experimental set-up and block diagram of the measuring devices used on the isolated perfused rat heart according to LANGENDORFF.

The assay is based on the competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP for a limited number of binding sites on a cAMP specific antibody. With fixed amounts of antibody and peroxidase-labelled cAMP, the amount of peroxidase-labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand. The peroxidase ligand that is bound to the antibody is immobilised onto polystyrene microtitre wells pre-coated with second antibody. Thus any unbound ligand can be removed from the well by a simple washing procedure. The amount of peroxidase labelled cAMP bound to the antibody is determined by addition of a tetramethylbenzidine. The reaction is stopped by addition of an acid solution, and the resultant colour read at 450 nm in a microtitre plate spectrophotometer.

## B : Role of the endothelium in the coronary response to AM

The coronary vasodilating effect of AM was investigated without and with endothelial degradation by intracoronary infusion with 0.2 ml of 0.5% Triton X-100. The physical damage thus caused to the endothelium required several modifications of the experimental protocol. In preliminary experiments with beating heart, the infusion of Triton X-100 was followed by a decrease in myocardial performance. To avoid the variations in coronary tone associated with these metabolic changes, the series of experiments were carried out after cardiac arrest by high $K^+$ perfusate (in mM: 80.0 NaCl, 39.7 KCl, 2.5 $CaCl_2$, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 5.0 glucose, and bovine serum albumin 2 g/100ml). The role of the endothelium was therefore explored with nonbeating hearts. After the aortic cannulation, hearts were allowed to stabilize for about 20 min with the warm oxygenated high $K^+$ perfusate. A bolus injection of AM, 20 µg, was

administered into the heart. The coronary blood flow was measured before and after the injection of AM. The same protocol was performed in another group of rat except that AM was administered after the treatment with 0.2 ml of 0.5% Triton X-100.

Additional experiments were performed to assess the endothelial dysfunction by a bolus injection of 0.1 µg bradykinin (BK) without and with the treatment with Triton X-100. To check that the coronary smooth muscle is able to vasodilate in response to an endothelium-independent dilator, a bolus of 15 µg sodium nitroprusside (SNP) was injected at the end of the Triton X-100 treated group. The coronary blood flow was measured before and after the injection of BK and SNP.

**C : Role of the cyclooxygenase pathway in the coronary response to AM**

After the aortic cannulation, hearts were allowed to stabilize for about 20 min with the warm oxygenated perfusate. The coronary blood flow were recorded. Next, a continuous perfusion of indomethacin, an inhibitor of cyclooxygenase pathway, at an intracoronary concentration of 1.4 µM was started via an infusion pump (Harvard Apparatus model 22, USA). Because of indomethacin, a decrease in CBF was observed. At the steady state, about 40 min after the start of the perfusion, AM (20 µg) was administered while indomethacin perfusion was maintained, and the coronary blood flow were recorded before (0 min) and 30 sec, 1, 3, 5, 10, 20 min after the injection of AM.

**D : Role of the nitric oxide pathway in the coronary response to AM**

The same protocol as protocol C was applied, but $N^G$-nitro-L-arginine (L-NNA), an inhibitor of NO synthesis, was perfused instead of indomethacin to obtain an intracoronary concentration of 3.5 μM.

**E : Role of the ATP-sensitive $K^+$ channels on coronary response to AM**

In this group, glibenclamide, an inhibitor of the ATP-sensitive $K^+$ channels was perfused instead of indomethacin under the same conditions as in protocol C to obtain an intracoronary concentration of $6 \times 10^{-7}$ M.

Adrenomedullin was dissolved in normal saline solution, indomethacin was dissolved in $3 \times 10^{-2}$ M $NaHCO_3$, glibenclamide was dissolved in dimethyl sulfoxide (DMSO) and then dilute in distilled water. The DMSO concentration in the final solution was 0.0001%. Previous experiment showed that 0.01% DMSO did not significantly change coronary blood flow or myocardial performance in isolated heart model (Mouren et al., 1997). In all these experiments, indomethacin, L-NNA, and glibenclamide were infused using an infusion pump (Havard Apparatus model 22, USA) at a rate corresponding to 1% of the coronary blood flow.

## STATISTICAL ANALYSIS

All values that were recorded are present as mean ± standard error of the mean (SEM). Differences between paired or unpaired mean values were analyzed by Student's $t$-test (SPSS version 7.5 for Windows). A $P$-value less than 0.05 was considered to be statistically significant.