CHAPTER-IV

MATERIALS AND METHODS

I. Experiments in vivo

(1) Production of hypertension

Experimental model of hypertension produced was renal DOCA/salt model. Male albino rats, weighing 200-300 g were used for preparing renal DOCA/salt model. Under ether anaesthesia the capsule of the left kidney was removed carefully without disturbing the supra-renal gland and the kidney was exposed. The renal pedicle was ligated and the left kidney was cut off. The incision was closed by Michelclip. To prevent infection mercurochrome was applied locally and ampicillin (20 mg) was administered (i.m.) for 5 days. Along with unilateral nephrectomy mineralocorticoid-deoxycorticosterone acetate (DOCA) was given subcutaneously 10 mg/kg weekly for 6 weeks. 1% saline was given ad libitum.

(2) Recording of blood pressure and heart rate

Blood pressure and heart rate were recorded directly through carotid artery by Statham pressure transducer (P 23 AA) on calibrated Twin-Viso Recorder.

Normotensive and hypertensive rats were anaesthetized with sodium pentobarbitone (40 mg/kg i.p.).

Tracheostomy was done. Jugular vein and carotid artery were exposed and polyethylene cannula was inserted into the jugular vein. Carotid artery was connected to Statham pressure transducer (filled with heparinized saline 100 i.u./ml) recording on calibrated recorder.

(3) Chronic treatment with drugs

Rats were mainly divided into four groups - (a) normotensive control rats, (b) normotensive-indapamide treated rats, (c) hypertensive rats, and (d) hypertensive-indapamide treated rats.

(i) Indapamide was dissolved in 0.1 N NaOH solution. It was administered orally 10 mg/kg in a volume of up to 0.4 ml for 10 days from 10 mg/ml solution.

(ii) Indomethacin (dissolved in 4.2% $NaHCO_3$) was given i.p. 8 mg/kg for 14 days (Cangiano et al., 1981).

(iii) Verapamil dissolved in distilled water was administered orally 30 mg/kg for 15 days (Aguas and Nickerson, 1983).

(iv) Hydrallazine was also given orally (dissolved in distilled water) 80 mg/kg for 15 days (Charles et al., 1980).

(4) Cardiovascular reactivity to different agonists

After mounting of rat for blood pressure, 15 min were allowed for stabilization of blood pressure and then basal blood pressure and basal heart rate were recorded.

At stable pressure, pressor responses to intravenous administration of various agonists were recorded.

Pressor response and heart rate to the same dose of agonist in normotensive and hypertensive rats were compared. Also change in pressor response and heart rate to the same dose of agonist were compared before and after chronic treatment with various drugs and increase or decrease in pressor response was considered as change in reactivity.

(i) Reactivity to NA, ADR and PE

After stable blood pressure, three dose-related pressor responses to NA (0.5, 1,2 ug/kg), ADR (0.5,1,2 ug/kg), or PE (0.5, 1,2 ug/kg) were recorded and at peak pressor effect, the heart rate with each dose was also recorded. 5-10 min were allowed between 2 doses of agonist for the blood pressure to return to normal levels. Further increase in dose of the agonist did not produce further increase in blood pressure. Change in pressure was considered as reactivity to agonist.

(ii) Reactivity to TYR and ANG

Pressor responses and heart rate with TYR (100,200 ug/kg) or ANG (25,50, 100 ng/kg) were recorded. The time interval between 2 doses were 15-20 min to prevent tachyphylaxis.

II. Experiments in vitro

Isolated organ bath studies

Isolated preparations used were thoracic aorta, portal vein and non-vascular preparation, vas deferens from normotensive rats. Rats (250-350 g) of either sex were sacrificed by a blow on the head and bled to death by cutting the neck vessels. The abdomen was opened and aorta, portal vein and vas deferens (from male) were removed and mounted in the organ bath.

(1) Aortic strip

Helically cut aortic strips were prepared from aorta and mounted in organ bath of 50 ml capacity as described by Furchgott and Bhadrakom (1953). The bathing medium contained modified Krebs solution of the following composition (mM): NaCl, 95; KCl, 4.69; KH_2PO_4, 1.2; $MgSO_4,7H_2O$, 0.11; $CaCl_2$, 2.5; $NaHCO_3$, 15 and dextrose 10.1. The solution in the organ bath was maintained at 37 $\pm$ 0.5°C and gassed with oxygen and CO_2 (at very reduced rate of 1/20 of oxygen) so as to maintain pH between 7 and 8.

During 2 hr of stabilization period, the tissue was washed every 15 min. Contractile responses to cumulative addition of various agonists (till no further contraction developed) were recorded isotonically on smoked kymograph paper using frontal writing lever (X 12 and 2 g of tension). Contractile response to each dose of agonist was recorded for 2 min.

Various agonists used were noradrenaline (NA), KCl, angiontensin (ANG), $CaCl_2$, 5-hydroxytryptamine (5-HT) and tyramine (TYR).

$CaCl_2$ responses were elicited by first stabilizing the aortic strip in calcium-free medium and exposing it to the depolarising solution of the following composition (mM): KNO_3, 80; $NaCl_2$, 20; $NaHCO_3$, 15; dextrose, 0.11 for 1 hr then $CaCl_2$ was added cumulatively.

Indapamide (10 mg/ml) was prepared in alkaline solution (0.1 N NaOH) and added into the bath maximum up to a volume of 0.06 ml which did not modify pH more than 8 and did not produce any effect on responses.

The dose of indapamide for in vitro study was selected roughly on the basis of following assumptions.

Steady state concentration of indapamide is reported to be 0.3 umol/l and it gets concentrated 10 times more

in vascular smooth muscle (Gross, 1977); additionally in animals 10 times higher dose may be required to produce the same effect. Thus 30 uM (3.0×10^{-5}M) dose was selected.

(i) Effect of indapamide on contractile responses to various agonists

After tissue stabilization, cumulative concentration response curves of NA (0.78×10^{-10}M to 7.8×10^{-6}M), KCl (10.0×10^{-3}M to 6.4×10^{-1}M), $CaCl_2$ (3.3×10^{-4}M to 8.5×10^{-2}M), TYR (7.18×10^{-7}M to 73.5×10^{-5}M), ANG (1.9×10^{-8}M to 2.48×10^{-6}M) or 5-HT (2.58×10^{-7}M to 3.0×10^{-5}M) were elicited; then the preparation was incubated in the medium containing indapamide (3.0×10^{-5}M or 3.0×10^{-4}M) for 1 hr. Responses to the agonists were repeated in the presence of indapamide. Separate sets of experiments were set up for each agonist.

(ii) Effect of indapamide on contractile responses to KCl in the presence of verapamil

Responses to KCl (5.0×10^{-3}M to 6.4×10^{-1}M) were elicited before and after incubating the preparation in a medium containing verapamil (1.1×10^{-6}M) for 1 hr. After washout the tissue was again exposed to medium containing verapamil and indapamide for 1 hr; then the responses to KCl were repeated in the presence of indapamide and verapamil.

(iii) Effect of indapamide on responses to NA in the presence of indomethacin

Responses to NA (1.56×10^{-10}M to 2.56×10^{-6}M) were elicited before and after incubating the preparation in the medium containing indomethacin for 1 hr. After washout the tissue was again exposed to medium containing indapamide (3.0×10^{-4}M) and indomethacin, then the responses to NA were repeated in the presence of indapamide and indomethacin.

(2) Portal vein

Portal vein was cleaned and the longitudinal muscle was mounted in a 25 ml organ bath containing modified Krebs solution of the following composition (mM): NaCl, 118; KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.8; $NaHCO_3$, 21 and dextrose, 11.1. The solution in the bath was maintained at 37 $\pm$ 0.5°C and gassed with oxygen. The tissue was allowed to stabilize for 1 hr during which the bathing medium was changed every 15 min. Cumulative dose-response curves of different agonists (NA, KCl, $CaCl_2$ and ANG) were recorded isotonically on smoked kymograph paper by frontal writing lever (X 10 and 1 g of load). Contractile responses to each dose of agonist were recorded for 1 min. The maximal height of contraction was determined by cumulative administration of increasing

concentrations of agonists until no further contraction was developed. After this the muscle preparation was incubated in the medium containing indapamide (3.0×10^{-5}M) for 1 hr. Cumulative concentration related responses to agonist were repeated.

Normal rhythmic movements of portal vein were recorded before addition of any agonist and the effect of indapamide on the same was studied.

(i) Effect of indapamide on contractile responses to various agonists

After tissue stabilization, cumulative contractile response curves of NA (3.13×10^{-8}M to 8.06×10^{-6}M), KCl (5.0×10^{-3}M to 4.0×10^{-2}M), ANG (9.7×10^{-10}M to 3.1×10^{-8}M) or $CaCl_2$ (1.7×10^{-4}M to 8.5×10^{-2}M) were elicited. The preparations were then incubated in the medium containing indapamide (3.0×10^{-5}M) for 1 hr. Responses to the agonist were repeated in the presence of indapamide. Separate sets of experiments were set up for each agonist.

III. Vas deferens

Male rats were dissected after bleeding to death and both the vasa deferentia were promptly removed, desheathed, cleaned and mounted in a 40 ml organ bath

containing Krebs Hukovic solution of the following composition (mM): NaCl, 1.12; KCl, 4.69; $CaCl_2$, 2.5; $MgSO_4$, $7H_2O$, 1.19; KH_2PO_4, 1.18; $NaHCO_3$, 25 and dextrose, 11.1.

The solution was maintained at 37 ± 0.5°C and gassed continuously with a mixture of oxygen and CO_2. The contractile responses of vas deferens were recorded on smoked kymograph paper by frontal writing lever (X 10 and 0.5 g of tension). The tissue was allowed to equilibrate for 30 min during which the bathing medium was changed every 10 min. Contact time for each was 30-40 sec and responses were repeated at 5-7 minute intervals.

Agonists used were NA, KCl, $CaCl_2$, PE, TYR, 5-HT and ANG. After completion of the dose response curve of agonist, the preparations were allowed to rest for 30 min and then incubated for 1 hr in the medium containing indapamide (3.0×10^{-5}M or 3.0×10^{-4}M). Responses to agonists were repeated in the presence of indapamide.

Indapamide was prepared in the alkaline solution. To study the effect of 3.0×10^{-5}M indapamide, it was added to the bath up to the volume of 0.05 ml from the concentrated solution which did not produce any effect on the responses. To study the effect of the higher

dose of indapamide (3.0×10^{-4}M), it was added in the bath up to the volume of 0.5 ml from the concentrated solution which made the pH more alkaline and affected the control responses. Therefore, responses were elicited in the presence of vehicle and then repeated in the presence of indapamide.

(i) Effect of indapamide on contractile responses to NA, KCl and $CaCl_2$

Paired preparations were set up to study the effects of two doses of indapamide on contractile responses to NA (9.39×10^{-8}M to 5.0×10^{-5}M), KCl (10.0×10^{-3}M to 12.8×10^{-1}M) or $CaCl_2$ (0.17×10^{-3}M to 8.4×10^{-2}M). The preparation was incubated in medium containing either indapamide 3.0×10^{-5}M or 3.0×10^{-4}M; then the responses to the agonist were repeated in the presence of indapamide. Separate sets of experiments were set up for each agonist.

(ii) Effect of indapamide on submaximal contraction of TYR, 5-HT and ANG

Maximal response to PE (9.0×10^{-5}M) was elicited; then submaximal responses to TYR (9.6×10^{-5}M), 5-HT (3.9×10^{-5}M) and ANG (6.4×10^{-7}M) were elicited. The muscle was incubated in the medium containing indapamide (3.0×10^{-5}M) for 1 hr and responses to the above agents were repeated in the presence of indapamide.

(iii) Effect of indapamide on responses to KCl in the presence of verapamil

Responses to KCl (20.0×10^{-3}M to 4.8×10^{-1}M) were elicited before and after incubating the preparation in a medium containing verapamil (1.0×10^{-6}M) for ½ hr. After washout, the preparation was again exposed to indapamide and verapamil for 1 hr and responses to KCl were repeated in the presence of indapamide and verapamil.

(iv) Effect of indapamide on responses to NA in the presence of indomethacin

Responses to NA (9.39×10^{-8}M to 5.0×10^{-5}M) were elicited before and after incubating the preparation in a medium containing indomethacin for 1 hr. After washout, the preparation was incubated in the medium containing indapamide plus indomethacin for 1 hr and the responses to NA were repeated in the presence of indapamide and indomethacin.

Statistical analysis

Student's 't' test was applied to determine the level of significance. $P < 0.05$ was considered as statistically significant.

Drugs used

Indapamide (USV Laboratories, New Yprk, USA), (-1)noradrenaline bitartrate (Sigma Chemical Co., St.Louis, USA), potassium chloride (Sarabhai Chemicals, Baroda, India), tyramine hydrochloride (Hoffman-La Roche and Co., Basle, Switzerland), angiotensin II (Ciba-Geigy Pharmaceuticals Co., Switzerland), 5-hydroxytryptamine creatinine sulphate (Sandoz Ltd., Basle, Switzerland), phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, USA), calcium chloride (Sarabhai Chemicals, Baroda, India), adrenaline (Sigma Chemical Co., St.Louis, USA), indomethacin (Merck Sharpe Dohme, USA), verapamil (Boeringer Knoll Ltd., Bombay, India), hydrallazine (Sigma Chemical Co., St.Louis, USA), pentobarbitone sodium (Sigma Chemical Co., St.Louis, USA), ampicillin (Sims Laboratories, Ahmedabad, India) and heparin (Gland Pharma Pvt.Ltd., Hyderabad, India).