CHAPTER - 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Pieces of spleen, aorta, trachea and heart were obtained from healthy goats of either sex (10-20 kg) from Baroda Slaughter House immediately after killing. The tissues were collected in a thermos flask containing carbogenated ice-cold Krebs-Henseleit solution of the following composition (mM): NaCl (118.0); $NaHCO_3$ (25.0); $CaCl_2$ (2.52); $MgSO_4,7H_2O$ (0.58); NaH_2PO_4 (1.1); KCl (5.4) and dextrose (11.0). The pH of the solution was 7.4.

3.1. Pharmacological Experiments

3.1.1. Splenic strip

For all the experiments, strips of 25-30 mm length and 2-3 mm width were prepared from spleens of goats (10-20 kg), as described by Innes (1962) for cat spleen. Each strip was suspended in an organ bath containing 35 ml of Krebs-Henseleit solution maintained at $37^{\circ} \pm 0.5^{\circ}$ C and bubbled with 95% oxygen and 5% carbon dioxide. Isotonic contractions at 0.5 g tension were recorded on a smoked paper by frontal writing lever which gave 10-fold maghification. Unless otherwise specified responses to agonists were obtained by the cumulative addition of increasing concentrations of agonists. Contact time for each agonist dose was 2.5 minutes. The antagonists were kept in the bath for 10 minutes before adding agonists and remained in the bath during the record of the actions of agonists. In order to bring the isotonic lever back to the baseline after eliciting a maximum response, an extra load of 0.5 g had to be applied for 15-30 minutes. Before addition of any drug, the tissue was equilibrated for 2 hours. A rest period of 60 minutes was allowed between cumulative responses. During the equilibrium and the rest periods the tissue was washed every 15 minutes.

To study the relaxant effect of ISO, KCl was used as a spasmogen. 1.36×10^{-2} M KCl produced a steady submaximal spasm (70 ± 8.01% of NA maximal response).

In a set of experiments, responses to agonists were first elicited in Krebs-Henseleit solution containing normal Ca^{++} concentration (2.52×10^{-6} M). This was followed by repetition of responses in the presence of Ca^{++}-free medium containing disodium ethylenediamine tetra-acetic acid (EDTA, 1.10×10^{-5}M).

In desensitization experiments (Gaddum, 1953), control single responses to naphazoline (2.44×10^{-7} M), NA (2.40×10^{-8} M) and tyramine (5.66×10^{-5} M) were obtained and then a high concentration of either naphazoline (8.83×10^{-3} M) or NA (1.83×10^{-4} M) or tyramine (0.83×10^{-3} M) was added and allowed to act for 60 minutes (the solution containing the high concentration of the agonist was changed every 20 minutes three times). At the end of 60 minutes, repeated washes with normal Krebs-Henseleit solution were given till the baseline was obtained. Then the responses to naphazoline (2.44×10^{-7} M), NA (2.40×10^{-8} M) and tyramine (5.66×10^{-5} M) were repeated.

3.1.2. Aortic strip

The aortic strip was prepared and mounted in an organ bath of 35 ml capacity according to the technique of Furchgott and Bhadrakom (1953) described for rabbit aortic strip. The bathing medium (Krebs-Henseleit solution) was bubbled with 95% oxygen and 5% carbon dioxide. Cumulative concentration-response curves of the agonists in the presence and absence of blockers were recorded on a smoked drum by a frontal writing isotonic lever under 2.5 g tension and 10-fold magnification. Contact time for all the agonists was 2.5 min and that for the blockers was 10 min. Before addition of any drug, the tissue was equilibrated for 2 hours. A rest period of 60 minutes was allowed between

cummulative responses. During the equilibrium and the rest periods the tissue was washed every 15 minutes.

3.1.3. Tracheal Chain

The tracheal chain was prepared and mounted as per the method of Akcasu (1959) for the guinea-pig tracheal chain. On each side of the trachea, two vertical cuts were made 3-5 mm lateral to the central smooth muscle of the trachea and then each ring was separated. The 3-4 cm long chain was prepared by joining the cartilaginous parts of the rings and suspended in Krebs-Henseleit solution at $37^{\circ} \pm 0.5^{\circ}$ C. The solution was bubbled with 95% oxygen and 5% carbon dioxide. The responses were recorded on a smoked paper with an isotonic frontal writing lever under 0.5 g tension and 10-fold magnification. Unless otherwise specified, responses to stimulant agonists were obtained by the cumulative addition of their increasing concentrations.

For eliciting relaxant responses to ISO, a spasm was obtained by exposure of the tissue to either pilocarpine (3.70×10^{-6} M) or potassium chloride (1.36×10^{-2} M). 3.70×10^{-6} M pilocarpine which produced a steady submaximal spasm ($83 \pm 6.91\%$ of the maximal spasm) was used for this set of experiments. In agreement with the literature (Nickerson and Collier, 1975), phenoxybenzamine (3.27×10^{-6} M) reversed the pilocarpine-induced spasm presumably because of its anticholinergic action. Therefore, while studying the influence of phenoxybenzamine, KCl (1.36×10^{-2} M) was used to obtain a steady submaximal spasm. The magnitude of the spasm produced with 1.36×10^{-2} M KCl was similar to that produced with 3.70×10^{-6} M pilocarpine. The spasm with pilocarpine and KCl was fully developed in 7-8 minutes. The relaxant drugs were added at the height of spasm.

In some experiments, the tissues were preincubated with normetanephrine (2.73×10^{-5} M) for 10 min before eliciting responses to ISO.

Effect of lower bath temperature on responses to ISO was studied by incubating the tissue at the bath of temperature of $22 \pm 0.5^{\circ}$ C for 10 min and then adding ISO keeping the temperature low.

The tracheal chain was equilibrated for 2 hours before starting the drug additions. A rest period of 60 minutes was allowed between two cumulative responses. A wash was given to the tissue during the equilibrium and the rest periods every 15 minutes. Contact time for agonists was 2.5 min and that for antagonists was 15 min.

3.2. Biochemical Experiments

3.2.1. Uptake of NA

The control NA contents of goat aorta, heart and spleen were estimated by the method of Anton and Sayre (1962). For studying the neuronal uptake of NA ($uptake_1$), the tissue was incubated with 0.59×10^{-6} M, 1.77×10^{-6} M, 5.91×10^{-6} M, 1.18×10^{-5} M and 2.36×10^{-5} M concentration of NA for 1, 5 and 15 minutes. In some experiments, cocaine (1×10^{-5} M) was added 15 minutes before incubation with 5.91×10^{-6} M and 1.77×10^{-5} M of NA for 5 minutes.

3.2.2. Estimation of acetylcholinesterase (AChE) content of spleen

The method of Pilz (1965) was used to estimate the AChE enzyme in goat spleen. For reference purposes, estimations of the enzyme were also made in guinea-pig spleen, liver and intestine.

3.3. Drugs

The following drugs were used:

(-) noradrenaline (Fluka-AG, Buchs-SG); (-) adrenaline and atropine sulphate (C.H. Boehringer Sohn, Ingelheim); cocaine hydrochloride and mepyramine maleate (May & Baker Private Ltd., Dagenham); tyramine hydrochloride (Hoffman-La Roche & Co., Basle); phentolamine methanesulphonate and antazoline methanesulphonate (Ciba-Geigy Pharmaceutical Co., Basle); reserpine (Serpasil, Ciba of India Ltd., Bombay); naphazoline (Alembic Chemical Works Co. Ltd., Baroda); isoprenaline sulphate (Ward Blenkin Sope & Co. Ltd., London); acetylcholine chloride and potassium chloride (E. Merck, Darmstadt); physostigmine salicylate (Burroughs Wellcome & Co., London); hexamethonium chloride (Sarabhai Chemicals, Baroda); 5-hydroxytryptamine creatinine sulphate (Sandoz Ltd., Basle); cyproheptadine (Merck Sharp & Dohme, Rahway); xylocaine hydrochloride and phenoxybenzamine hydrochloride (Smith Kline & French Laboratories, Philadelphia); ethylenediamine tetra-acetic acid disodium salt (Pfizer Private Ltd., Bombay); histamine acid phosphate (BDH Chemicals Ltd., Poole); metiamide (Smith Kline & French Laboratories Ltd., Herts); (-) normetanephrine methanesulphonate (Sterling Winthrop Research Institute, Rensselaer); pilocarpine hydrochloride (C.H. Boehringer Sohn, Ingelheim) and propranolol hydrochloride (Imperial Chemical Industries Ltd., Cheshire).

Stock solutions (1 mg/ml) of Adr and NA were prepared in 0.1 N HCl containing sodium metabisulphite (0.1%). Stock solution (1 mg/ml) of ACh was prepared in 5% NaH_2PO_4 solution. Stock solutions of these drugs were used for preparing appropriate dilutions. Solutions of all other drugs were prepared fresh for each experiment. Drug doses are expressed as final molar (M) bath concentrations.

3.4. Analysis of Data

Each response was expressed as a percentage of control maximal response. The mean response for each dose was used by plotting dose-response curves. The EC_{50} value was computed from the concentration-response curve as the concentration which corresponded to 50% its own maximal response. The dose-ratio was given by the ratio of EC_{50} concentration of agonist in presence of blocker to that in its absence and was used as a measure of the leftward or rightward shifts of the dose-response curve.

pA_2 values were computed from pA plots constructed according to the method of Arunlakshana and Schild (1959). According to this method, the negative log of the molar concentration of the blocker is plotted on the abscissa and log (dose-ratio -1) is plotted on the ordinate. The intercept of the regression line with the abscissa at the zero level of ordinate gives pA_2 value. According to the hypothesis of competitive antagonism, the regression coefficient should be -1.

Statistical tests are those described by Snedecor (1957). $P < 0.05$ was considered to be significant.