CHAPTER 3

MATERIALS AND METHODS

1. Alkaloid isolation

1.1 General

The purity of samples was checked by TLC 20x20 cm Merck precoated aluminum plates (silica gel 60 F254) using the solvent system methanol:chloroform (5:95 v/v), and Dragendroff's reagent was used for detection. Column chromatography was performed using open columns packed with Merck silica gel 60. ^{1}H NMR and ^{13}C NMR spectra were recorded on NMR 500 MHz model UNITY INOVA Varain spectrometer.

1.2 Plant material preparation

The fresh leaves of *C. papaya* L. (50 kg) were obtained from a papaya plantation, Huai-Yot District, Trang Province, Thailand, in January 2002. The plant material was identified by a botanist of Botany Section, Department of Biology, Prince of Songkla University. They were cleaned with distilled water. Only green leaves, which did not have scars or spots of disease, were selected for further extraction. Some large veins of the leaves were removed. The leaves were then air-dried at room temperature overnight, and further dried in hot air oven at 50 °C until dryness. The dried leaves were pulverized to coarse powder using an electric blender. The powder (7.5 kg) was then used for alkaloid extraction according to the solvent extraction technique by Cordell, (1981).

1.3 Extraction Procedure

The extraction procedures of the crude alkaloid from *C. papaya* L. leaves are as follows. 1) The papaya leave powder (7.5 kg) was moistened with concentrate NH_4OH solution. 2) It was then macerated in absolute methanol for 3 days at room temperature. The methanol extract was then removed, stored desiccated at room temperature protected from light. 3) The extraction was repeated for 7 times or until no trace of alkaloid was detected using Dragendroff 's reagent. 4) The whole methanolic extracts were pooled, and evaporated

at 50 °C under reduced pressure in Rotary Evaporator. 5) The extract (black viscous, oil-like mixture) was dissolved in 1,300 ml of 2% sulfuric acid in water, filtered and then extracted with 2,600 ml of hexane to remove fat materials. 6) The acid fraction was adjusted to pH 8-9 using concentrate NH_4OH solution and further extracted with excess chloroform. 7) The chloroform fractions were then pooled and evaporated at 50 °C under reduced pressure until dryness. 8) The crude alkaloid extract obtained from step 7 was dissolved in 2% sulfuric acid in distilled water and the extraction procedure from step 6 and step 7 was repeated. The crude alkaloid 21.3 g (0.028%) were obtained and stored in vacuum dessicator at room temperature protected from light until use.

1.4 Isolation of carpaine from *C. papaya*

TLC was used as an initial method for determining purity of extracts. Samples were spotted onto silica gel 60 F254, developed in methanol:chloroform (5:95 v/v), and plates were then exposed to Dragendroff's reagent to visualize organic compounds. TLC of the crude alkaloid showed 4 Dragendorff's reagent positive spots. Prior to use, the column was initially filled with stationary phase (silica gel 60 300 g.), then equilibrated with mobile phase. The column was considered in equilibrium when only chloroform eluted from the tail end. At this point, a crude extract of leaves (15 g) of *C. papaya* was dissolved in 10-15 ml of chloroform and loaded into the column, which was chromatographed with chloroform/acetone (40:60 v/v) as eluant, collecting 50 ml fractions. The fractions were monitored by TLC (silica gel, same solvent) and plates were then exposed to Dragendroff's reagent to visualize the spots. Since the amount of alkaloid material expected was low, the fractions were concentrated by evaporation prior to TLC. The chloroform/acetone (40:60 v/v) eluate was divided into two pools: A (fractions 14–81, R_f = 0.36) and B (fractions 82–99, R_f = 0.11). Pool A was crystallized with chloroform to give crystalline of carpaine. The yield was 1.3292 grams (0.1194%) of loaded crude alkaloid, finally purified on the recrystallization with chloroform. For further confirmation of the identity, the product was analyzed by ^{1}H and ^{13}C NMR by comparing the spectroscopic data with these reported in the literature (Tang, 1979). The spectrum was shown in the Appendix C and D. Moreover, this product was identified the melting point by capillary method. Pool B was concentrated to give yellow-brown viscous oil

and designated unknown 1. Moreover, the methanol/chloroform (5:95 and 20:80 v/v) eluate from the same column was concentrated to give a yellow-brown viscous oil and designated unknown 2. A further chromatography of crude alkaloid with methanol elution gave a yellow crystalline unknown 3. However, these minor alkaloids, which less amounts of yield were not studied by NMR.

2. Drug and Chemicals

Cromakalim, diethylstillbestrol (DES), dimethylsulfoxide(DMSO), forskolin, glibenclamide, L-arginine, N^{w}-nitro-L-arginine (L-NOARG), methylene blue (MB), oxytocin, papaverine, Rp-adensine 3' , 5' -cyclic monophosphothioate, triethylamine salt (Rp-cAMPS), sodium nitroprusside (SNP), tetraethylammonium (TEA), tataric acid and (±)-verapamil hydrochloride were purchased from Sigma Chemical Company (St. Louis, U.S.A.). All drugs, except DES, L-arginine and SNP were prepared as stock solutions (cromakalim 10^{-2} M, forskolin $2x10^{-2}$ M, glibenclamile 10^{-2} M, L-NOARG 10^{-1} M, MB 10^{-1} M, papaverine10^{-2} M, Rp-cAMPS 10^{-2} M, TEA 10^{-1} M, and verapamil10^{-3} M). DES was dissolved in olive oil to give a concentration of 1 mg/ml for intraperitoneal injection. Carpaine was dissolved in 2% tataric acid to give a stock solution of 10^{-1} M for use in all experiments. The final concentration of tataric acid in the organ bath was less than 0.2% and did not affect contraction or relaxation. Papaya leaf's crude alkaloid was dissolved in DMSO to give a stock solution (200 mg./ml). Stock solution of forskolin ($2x10^{-2}$ M) was prepared by dissolving forskolin in DMSO. The final concentration of DMSO in the organ bath was lass than 0.5% and did not affect contraction or relaxation. Glibenclamide was dissolved in 1 ml NaOH (0.1 mM) and 4 ml glucose (50 g/l) to make a stock solution. Cromakalim was dissolved in 70% ethanol. Aliquot stock solution was kept frozen and appropriate dilution of the stock solution with working solution was freshly prepared and used on the day of each experiment. SNP and L-arginine were dissolved daily in working solution. Because NO is highly unstable when exposed to daylight or ordinary laboratory light, all experiments involving the use of SNP and L-arginine were done under the protection from light. Other drugs were dissolved in distilled water. NaCl and $NaHCO_3$ were purchased from Merck. KCl, $CaCl_2$ and Glucose were purchased from Carlo Erba. These

common salts were used to prepare physiological solution, (see Appendix I), which was freshly prepared for each day of experiment.

3. Experimental Animals

All experiments were carried out using virgin female Wistar rats (200-300 g.) which were supplied from Division of Animal House, Faculty of Science, Prince of Songkla University. They were housed in air-conditioned room (24-26°C) with a 12 hours light/dark cycle. Rats were injected intraperitoneally with diethystillbestrol (DES) dissolved in olive oil (100 μg/ml) 24 hours before the experiment

4. Experimental Protocol

The rats were euthanased by cervical dislocation, uterine horns removed and placed in Jalon-Ringer solution. Each horn was cut into a segment of approximately 0.5 cm. in length. The uterus segment was then mounted in 5 ml organ-bath chamber filled with Jalon-Ringer solution. This medium was gassed continuosly with 95 % O_2, 5% CO_2 and maintained at 37 °C throughout the experiments. The uterus was set up under initial resting tension of 2 g. Changes in uterine tension were recorded isometrically with a force displacement transducer (Grass Instrument CO-, Quincy, Mass, U.S.A.) connected to a Grass Model 79 D polygraph. Before the commencement of each experiment, the uterus preparation was equilibrated for at least 1 hour and the Jalon-Ringer solution in the organ bath was changed periodically with fresh Jalon-Ringer solution. In all experiments, a uterus segment from the other horn was also set up as a time control preparation to compare the effect of time to the drug effect and/or vehicle. This uterus was prepared and used in the same manner as the drug treated uterus, but the relevant vehicle of the drug was added to the organ bath in stead of drug solution. Each experiment was repeated in at least 6-10 replicates.

5. Experimental Procedures

5.1 The determination of optimal KCl concentration in depolarizing solution

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After the equilibration period, the uterus was induced to contract by changing the solution in the organ bath to either of depolarizing solutions containing 10,

15, 20, 25, 30, 36.3, 46.3, 56.3, 66.3 or 76.3 mM of KCl. The effect of each depolarizing solution was allowed to reach maximum and the contraction was observed further for 10 minutes period. The uterus was then washed three times with fresh Jalon-Ringer solution before the other depolarizing solution was replaced. Changes in uterine tension were recorded and concentration-response curves were constructed. From the result of this experiment, a depolarizing solution containing 25 and 56.3 mM KCl was chosen for use in the following experiments.

5.2 Determination of optimum concentration of oxytocin

The uterine horn was equilibrated for 1 hour in Lock-Ringer solution under resting tension of 2 g. and maintained at 37 °C. The uterus was induced to contract using oxytocin (0.001-10 mU/ml) in a cumulative concentration manner. The responses of the uterus to oxytocin were recorded and the concentration-response curve was then constructed. The concentration of oxytocin (1mU/ml) which produced uterine contraction approximately 70-80% of the maximum contraction was selected for use in the next experiments.

5.3 The effect of carpaine on the role of Ca^{2+} channels in isolated rat uterus.

5.3.1 Effect of carpaine or crude alkaloid on uterine contraction induced by depolarizing solution

This experiment was performed using K^{+}-depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After the equilibration period, the uterus was induced to contract by changing the solution in the organ bath to depolarizing (56.3 mM KCl). When the contraction plateau was reached, cumulative concentrations of carpaine or crude alkaloid were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of carpaine or crude alkaloid. The time control treatment was performed in parallel to that of carpaine or crude alkaloid treatment. The total volume of drug added was kept as small as possible with a maximum allowance volume of 1 ml. Changes in uterine tension were recorded and the log concentration-response curves were then constructed.

5.3.2 Determination of optimal concentration of verapamil

The uterine horns were bathed for 1 hour in Jalon-Ringer solution with resting tension of 2 g. After then, the solution was replaced by Ca^{2+}-free high K^+ solution (60 mM, see Appendix I) and the uterus was washed with this solution for at least 3 times in 10-minute intervals and then the uterus was induced to contract by adding 10^{-3} M of $CaCl_2$. When the plateau contraction was reached, cumulative concentrations of verapamil were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of verapamil. The time control treatment was performed in parallel to that of the verapamil treatment. Changes in uterine tension were recorded and the log concentration-response curves were then constructed. The concentration of verapamil (10^{-7} M) which produced uterine relaxation to approximately 80-90% of maximum contraction was selected to use in the next experiment.

5.3.3 Effect of carpaine or verapamil on uterine contraction induced by $CaCl_2$ in K^+-depolarized uterus

The uterine horns were bathed for 1 hour in Jalon-Ringer solution with resting tension of 2 g. The solution was then replaced by Ca^{2+}-free high K^+ solution (60 mM KCl) and the uterus was washed with this solution for at least 3 times in 10-minute intervals until the baseline tension was achieved. Cumulative concentrations of $CaCl_2$ (10^{-6}-$3x10^{-2}$ M) were then added. After the uterine contraction reached a maximum, the uterus was then washed using Ca^{2+}-free high K^+ solution for many times. After uterine tension returned to the baseline position, the uterus was then preincubated with either carpaine ($3x10^{-5}$ M) or verapamil (10^{-9} M) for 20 minutes followed by cumulative concentration of $CaCl_2$ (10^{-6}-$3x10^{-2}$ M). This experiments were repeated but the concentration of carpaine or verapamil was changed to 10^{-4} or $3x10^{-4}$ M for carpaine or 10^{-8} or 10^{-7} M for veapamil respectively. The uterine tension in response to each concentration of $CaCl_2$ in the present or absence of carpaine or verapamil was recorded and the concentration response curves were constructed.

5.3.4 Effect of carpaine or verapamil on uterine contraction induced by oxytocin

A uterine horn was incubated in Lock-Ringer solution with resting tension of 2 g. for 1 hour at 37°C. The uterus was precontracted submaximally by addition of oxytocin

(1mU/ml). After the rhythmic contraction was stable, carpaine (10^{-6}-$3x10^{-3}$ M) or verapamil (10^{-9}-10^{-4} M) were then added cumulatively to the organ bath. The uterine tension in response to each concentration of carpaine or verapamil was allowed to develop maximally before adding the next drug concentration. The time control treatment was also performed similarly to that of carpaine or verapamil treatment and both treatments were performed in parallel. Change in force and frequency of contraction was recorded. The force of contraction was the value of the maximum change of the uterine tension in response to each concentration of carpaine or verapamil. The frequency of rhythmic contraction was calculated by averaging the number of contraction within 10 minutes. The log concentration-response curves of force or frequency of contraction were then constructed.

5.4 The effect of carpaine on the role of K^+ channels in isolated rat uterus.

5.4.1 Experiments on preparations contracted by potassium chloride (KCl)

5.4.1.1. Effect of cromakalim on KCl-elicited contraction

This experiment was performed using K^+-depolarized uterus. The uterine horns were bathed in Jalon-Ringer solution with resting tension of 2 g. for 1 hour maintained at 37°C. After equilibration period, the uterine contraction was induced by changing the solution in the bath to the 25 mM K^+ -depolarizing solution. This addition caused a monophasic contraction. When the rhythmic contraction was stable, cromakalim (10^{-8} - $3x10^{-5}$ M) were then added cumulatively to the organ bath. After the uterine relaxation has reached a maximum, the uterus was washed with Jalon-Ringer solution for many times. The experiment was then repeated but the concentration of depolarizing solution was changed to 56.3 mM KCl. This addition caused a biphasic contraction. When the tonic contraction plateau was reached, cumulative concentration of cromakalim (10^{-8} - $3x10^{-4}$ M) was added to the organ bath. The time control treatment was performed similarly to that of cromakalim treatment and both treatments were performed in parallel. Change in force and frequency of contraction was recorded. The force of contraction was the value of the maximum change of the uterine tension in response to each concentration of cromakalim. The frequency of rhythmic contraction was calculated by averaging the number of contraction within 10 minutes. The log concentration-response curves of force or frequency of contraction were

then constructed. Cromakalim relaxed the contractions induced by 25 mM K^+ preferably to those induced by 56.3 mM K^+. The concentration of KCl (25 mM) in the depolarizing solution was selected for use in the next experiment.

5.4.1.2 Determination of optimum concentration of TEA

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After equilibration period, the uterine contraction was induced by changing the solution in the bath to a depolarizing solution (KCl 25 mM). When the phasic contraction plateau was reached, cumulative concentrations of cromakalim (10^{-8}-$3x10^{-5}$ M) were added to the organ bath. After the uterus has reached a maximum relaxation, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding cromakalim, either a single concentration of TEA (10^{-5}, 10^{-4} and 10^{-3} M) was added for 15 minutes. The time control treatment was performed similarly to that of TEA treatment and both of the treatments were done in parallel. The effect of each concentration of cromakalim in the presence or absence of TEA was recorded and the concentration-response curves were then constructed. The concentration of TEA (10^{-3} M) which produced 70-80% inhibition of maximum effect of cromakalim was selected for use in next experiments.

5.4.1.3. Effect of TEA on uterine relaxation induced by carpaine or cromakalim

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After equilibration period, the uterine contraction was induced by changing the solution in the bath to a depolarizing solution (KCl 25 mM). When the phasic contraction plateau was reached, cumulative concentrations of carpaine (10^{-6}-$3x10^{-3}$ M) or cromakalim (10^{-8}-$3x10^{-5}$ M) were added to the organ bath. After the uterus relaxation has reached a maximum, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding carpaine or cromakalim, the uterus was preincubated with TEA (10^{-3} M) for 15 minutes. The time control treatment was performed similarly to that of TEA treatment and both treatments were done in parallel. Only one carpaine or cromakalim was used in each tissue. The effect of each concentration

cromakalim in the presence or absence of TEA was recorded and the concentration-response curves were then constructed.

5.4.1.4. Effect of glibenclamide on uterine relaxation induced by carpaine or cromakalim

The uterus was immersed in Jalon-Ringer solution. After equilibration, an isometric contraction was elicited by changing the solution in the organ bath to a concentration of KCl (25 mM). When the contraction plateau was reached opener was added cumulatively: cromakalim (10^{-8}-$3x10^{-5}$ M), carpaine (10^{-6}-$3x10^{-3}$ M). Subsequent concentrations were administered only when the response to previous concentration was stable. In experiments using cromakalim and carpaine, the procedure was repeated following 15 minutes incubation with a single concentration of glibenclamide (10^{-7}, 10^{-6}, 10^{-5} M). Only one carpaine or cromakalim was used in each tissue. The time control treatment was performed similarly to that of glibenclamide treatment was done in parallel. Any relaxant effect of the glibenclamide was received from apparent effect of the K^+ channel blocker. This was only necessary with high concentrations of glibenclamide (10^{-5} M). The effect of each concentration cromakalim or carpaine in the presence or absence of glibenclamide was recorded and the concentration-response curves were then constructed.

5.4.2 Experiments on preparations contracted by oxytocin

5.4.2.1 Determination of optimum concentration of TEA

A uterine horn was incubated in Lock-Ringer solution with resting tension of 2 g. for 1 hour at 37°C. The uterus was precontracted submaximally by addition of oxytocin (1mU/ml). After the rhythmic contraction was stable, cumulative concentrations of cromakalim (10^{-8}-$3x10^{-5}$ M) or its vehicle were added to the organ bath. The uterine tension in response to each concentration of cromakalim was allowed to develop maximally before the addition of the concentration. After the uterus has reached a maximum relaxation, the uterus was washed using Lock-Ringer solution for many times. The experiment was then repeated but before adding cromakalim, either a single concentration of TEA (10^{-5}, 10^{-4} or 10^{-3} M) was added for 15 minutes. The time control treatment was performed similarly to that of TEA treatment and both of the treatments were done in parallel. The effect of each concentration

cromakalim in the presence or absence of TEA was recorded and the concentration-response curves were then constructed. The concentration of TEA (10^{-3} M) which produced 70-80% inhibition of maximum effect of cromakalim was selected for used in next experiments.

5.4.2.2 Effect of TEA on uterine relaxation induced by carpaine or cromakalim

The uterus was immersed in Lock-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After equilibration period, the uterine contraction was induced by oxytocin (1mU/ml). When the phasic contraction plateau was reached, cumulative concentrations of carpaine (10^{-6}-$3x10^{-3}$ M) or cromakalim (10^{-8}-$3x10^{-5}$ M) were added to the organ bath. After the uterus has reached a maximum relaxation, the uterus was washed using Lock-Ringer solution for many times. The experiment was then repeated but before adding carpaine or cromakalim, the uterus was preincubated with TEA (10^{-3} M) for 15 minutes. The time control treatment was performed similarly to that of TEA treatment and both of the treatment was done in parallel. Only one carpaine or cromakalim was used in each tissue. The effect of each concentration cromakalim or carpaine in the presence or absence of TEA was recorded and the concentration-response curves were then constructed.

5.4.2.3 Effect of glibenclamide on uterine relaxation induced by carpaine or cromakalim

After equilibration, an isometric contraction was elicited to a concentration of oxytocin (1 mU/ml) which gave 70-80% of the maximum response and at the plateau of contraction, cromakalim (10^{-8}-$3x10^{-5}$ M) or carpaine (10^{-6}-$3x10^{-3}$ M) was added cumulatively to the organ bath. Subsequent concentrations were administered only when the response to previous concentration was stable. In experiments using cromakalim and carpaine, the procedure was repeated following 15 minutes incubation with a single concentration of glibenclamide (10^{-7}, 10^{-6}, 10^{-5} M). Only one carpaine or cromakalim was used in each tissue. The time control treatment performed similarly to that of glibenclamide treatment was done in parallel. Any relaxant effect of the glibenclamide was subtracted from apparent effect of the K^+ channel blocker. This was only necessary with high concentrations of glibenclamide (10^{-5}

M). The effect of each concentration cromakalim or carpaine in the presence or absence of glibenclamide was recorded and the concentration-response curves were then constructed.

5.5 The effect of carpaine on the role of the cAMP-dependent relaxation of uterine smooth muscle

5.5.1 Determination of optimum concentration of Rp-cAMPS

A uterine horn was incubated in Jalon-Ringer solution with resting tension of 2 g. for 1 hour at 37 °C. The solution was then replaced by K^+-free depolarizing solution (KCl 56.3 mM) and the uteri were washed with this solution for at least 3 times in 10-minute intervals until the baseline tension was achieved. The uterus was induced to contract by adding 56.3 mM of KCl. Cumulative concentrations of papaverine (10^{-7}-10^{-4} M) and forskolin (10^{-7}-$3x10^{-5}$ M) were added to the organ bath when the tonic response of KCl (56.3 M)-induced contraction was stable (10-15 minutes). The response to each concentration was allowed to reach maximum relaxation. A 100% relaxation was obtained when the base line of the recording was reached. The modification of the relaxing effect by Rp-cAMPS was studied. The effect of papaverine or forskolin was assayed in the presence of Rp-cAMPS ($3x10^{-6}$, 10^{-5} and $3x10^{-5}$ M). Thus, the Rp-cAMPS were added to the bath 30 minutes before KCl. At this time Rp-cAMPS produced its maximum effect (Péres-Vallina et al., 1997). In each preparation, only one concentration response curve was assayed. In the same experimental conditions, DMSO, the vehicle of forskolin was added the organ bath. The final concentration of DMSO in the organ bath did not modify the KCl-induced tonic contractions (Figure 51). The effect of each concentration papaverine or forskolin in the presence or absence of Rp-cAMPS was recorded and the concentration-response curves were then constructed. The concentration of Rp-cAMPS ($3x10^{-5}$ M) which produced 70-80% inhibition of maximum effect of papaverine or forskolin was selected for used in next experiments.

5.5.2 Effect of Rp-cAMPS on uterine relaxation induced by carpaine, papaverine or forskolin

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. The solution was then replaced by K^+-free depolarizing solution (KCl 56.3 mM) and the uteri were washed with this solution for at least 3 times in 10-minute

intervals until the baseline tension was achieved. The uterus was induced to contract by adding 56.3 mM of KCl. Cumulative concentrations of carpaine (10^{-6}-$3x10^{-3}$ M) or its vehicle, papaverine (10^{-7}-10^{-4} M) and forskolin (10^{-7}-$3x10^{-5}$ M) were added to the organ bath when the tonic response of KCl (56.3 M)-induced contraction was stable. A 100% relaxation was obtained when the base line of the recording was reached. Only one concentration-response curve was performed for each uterine horn. In the same experimental conditions, the effect of carpaine or its vehicle, papaverine or forskolin was assayed in the presence of Rp-cAMPS ($3x10^{-5}$ M). The Rp-cAMPS was added to the bath 30 minutes before KCl, followed by cumulative concentration of these drugs. The uterine tension in response to each concentration of carpaine, papaverine or forskolin in presence or absence of Rp-cAMPS ($3x10^{-5}$ M) was recorded and the concentration response curves were constructed.

5.5.3 Effect of Rp-cAMPS on uterine relaxation induced by carpaine

In the same experimental conditions, cumulative concentration-response curves for carpaine (10^{-6}-$3x10^{-3}$ M) were made when the tonic response of KCl (56.3 mM)-induced contraction had become stable. The response to each concentration was allowed to reach maximum of relaxation. The modification of the carpaine effect by the protein kinase A inhibitors (Rp-cAMPS) was assayed. Rp-cAMPS ($3x10^{-6}$, 10^{-5} and $3x10^{-5}$ M) were added 30 min before KCl induced a stable contraction, followed by cumulative concentration of carpaine (10^{-6}-$3x10^{-3}$ M). Only one concentration-response curve was performed for each uterine horn. The time control treatment was performed similarly to that of carpaine treatment. This was only necessary with high concentrations of Rp-cAMPS ($3x10^{-5}$ M). The effect of each concentration carpaine in the presence or absence of Rp-cAMPS was recorded and the concentration-response curves were then constructed.

5.6 The effect of carpaine on the role of NO-induced relaxation in isolated rat uterus

5.6.1 Effect of SNP or L-arginine on uterine contraction induced by depolarizing solution

This experiment was performed using K^+-depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g.

After the equilibration period, the uterus was induced to contract by changing the solution in the bath to depolarizing solution (56.3 mM KCl). When the contraction plateau was reached, cumulative concentrations of SNP (10^{-6}-10^{-3} M) or L-arginine (10^{-6}-$3x10^{-3}$ M) were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of SNP or L-arginine. The time control treatment was performed in parallel to that of SNP or L-arginine treatment. Changes in uterine tension were recorded and the log concentration-response curves were then constructed.

5.6.2 Effect of NOS substrate on uterine contraction induced by depolarizing solution

5.6.2.1 Determination of optimum concentration of L-NOARG

This experiment was performed using K^+-depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After the equilibration period, the uterus was contracted by changing the solution in the bath to depolarizing (56.3 mM KCl). When the contraction plateau was reached, cumulative concentrations of L-arginine (10^{-6}-$3x10^{-3}$ M) were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of L-arginine. The time control treatment was performed in parallel to that of L-arginine treatment. After the uterus reached a maximum relaxation, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding L-arginine, a single concentration of L-NOARG (10^{-4}, $3x10^{-4}$ or 10^{-3} M) was added for 15 minutes. The time control treatment was performed similarly to that of TEA treatment and both of the treatments were done in parallel. The effect of each concentration L-arginine in the presence or absence of L-NOARG was recorded and the concentration-response curves were then constructed. The concentration of L-NOARG (10^{-3} M) which produced inhibition of maximum effect of L-arginine was selected for used in the next experiments.

5.6.2.2 Effect of L-NOARG on uterine relaxation induced by carpaine or L-arginine

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After equilibration period, the uterine contraction was

induced by changing the solution in the bath to depolarizing solution (56.3 mM KCl). When the tonic contraction plateau was reached, cumulative concentrations of carpaine (10^{-6}-$3x10^{-3}$ M) or L-arginine (10^{-6}-$3x10^{-3}$ M) were added to the organ bath. After the uterus reached a maximum relaxation, it was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding carpaine or L-arginine, the uterus was preincubated with L-NOARG (10^{-3} M) for 15 minutes. The time control treatment was performed similarly to that of L-NOARG treatment and both of treatment was done in parallel. The effect of each concentration of L-arginine or carpaine in the presence or absence of L-NORAG was recorded and the concentration-response curves were then constructed.

5.6.3 Effect of NO-donor on uterine contraction induced by depolarizing solution

5.6.3.1. Determination of optimum concentration of methylene blue

This experiment was performed using K^+-depolarized uterus. The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After the equilibration period, the uterus was contracted by changing the solution in the bath to depolarizing (56.3 mM KCl). When the contraction plateau was reached, cumulative concentrations of SNP (10^{-6}-10^{-3} M) were added to the organ bath. The relaxation was allowed to reach maximum before the addition of the next concentration of SNP. The time control treatment was performed in parallel to that of SNP treatment. After the uterus has reached a maximum relaxation, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding SNP, methylene blue at a concentration of either 10^{-7}, 10^{-6} or 10^{-5} M was added for 15 minutes. The time control treatment was performed similarly to that of MB treatment and both of the treatments were done in parallel. The effect of each concentration SNP in the presence or absence of MB was recorded and the concentration-response curves were then constructed. The concentration of MB (10^{-5} M) which produced inhibition of maximum effect of SNP was selected for used in next experiments.

5.6.3.2. Effect of L-NOARG on uterine relaxation induced by carpaine or SNP

The uterus was immersed in Jalon-Ringer solution and equilibrated for 1 hour under resting tension of 2 g. After equilibration period, the uterine contraction was induced by changing the solution in the bath to a depolarizing solution (56.3 mM KCl). When the tonic contraction plateau was reached, cumulative concentrations of carpaine (10^{-6}-$3x10^{-3}$ M) or SNP (10^{-6}-10^{-3} M) were added to the organ bath. After the uterus has reached a maximum relaxation, the uterus was washed using Jalon-Ringer solution for many times. The experiment was then repeated but before adding carpaine or SNP, the uterus was preincubated with L-NOARG (10^{-3} M) for 15 minutes. The time control treatment was performed similarly to that of MB treatment and both of the treatments were done in parallel. The effect of each concentration SNP or carpaine in the presence or absence of MB was recorded and the concentration-response curves were then constructed.

6. Data Analysis

Contractile responses of the uterus were determined as a change in isometric tension (in g.), that induced by each stimulant (oxytocin, $CaCl_2$ and depolarizing solution) or uterine relaxant or carpaine. All data were expressed as a mean $\pm$ standard error of the mean (Mean $\pm$ S.E.M) of percentage of the maximum contraction induced by each stimulant. Fifty percent of inhibition concentration (IC_{50}) was calculated graphically from a plot of log concentration vs. the maximum response (E_{max}) produced by each uterine relaxant in individual experiment. Statistical significance of differences between each group of mean was assessed by repeated measure analysis of variance (ANOVA) or independent samples *t*-test. In case that there was a significant difference among group of means, multiple comparison among means were then performed using Duncan's multiple range test or Dunnett's test. A significant difference will be determined when p is less than 0.05. The label on the side of each symbol in some figure with character a, b, c and d indicate the result of statistical analysis of means within the same treatment group (a $CaCl_2$ concentration, carpaine concentration etc.). Similar character means non-significant difference. Different characters mean significant difference among the comparing means at $p < 0.05$.