行政院國家科學委員會補助專題研究計畫成果報告

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計畫主持人：楊玲玲

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行政院國家科學委員會專題研究計畫成果報告

安胎中藥--黃芩成份及作用機轉

Tocolytic Effects and Action Mechanism of Chinese Herbs-Scutellariae Radix and its Principle Constituents

計畫編號：NSC89-2314-B-038-037

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一、中文摘要

黃芩(Huang Qin) 唇形科(Labiatae) 植物 *Scutellaria baicalensis* Georgi 之乾燥根，為中藥安胎方劑常配伍之中藥材。本研究以現代藥理學之技術，進行其安胎作用之評估及相關機制之探討。發現黃芩水抽取物高劑量時對正常及懷孕初期(第 10 天) 離體雌性大白鼠子宮平滑肌收縮有意義抑制作用，IC_{50} 分別為 6.12 ±0.95 mg /ml, 4.98 ± 1.12 mg/ml,由以上結果證實，黃芩具有安胎活性物質。由黃芩抽得之成分 Oroxylin A 對懷孕初期(第 10 天)離體子宮平滑肌之收縮抑制作用，IC_{50} 分別為 50.38 + 4.91 μM，證實此 flavonoid 類成分具有安胎活性。

黃芩成分對誘縮劑 Acetylcholine (ACh)，Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)，Oxytocin，誘發離體子宮平滑肌收縮皆有抑制作用，並發現黃芩主成分- Oroxylin A 經由活化鈣依賴性-鉀離子通道通透性造成子宮鬆弛。綜合以上結果，傳統安胎中藥-黃芩確實具有安胎作用，其活性成分為 Oroxylin A。

關鍵詞：黃芩、安胎、活性成分、oroxylin A、鈣依賴性-鉀離子通道

Abstract

OBJECTIVE: This study is focused on the the tocolytic effect of Oroxylin A isolated from the Chinese herb *Scutellaria baicalensis* on rat uterus smooth muscle and investigate its pharmacological activities.

STUDY DESIGN: Uterine strips from Wistar rat at mid-pregnancy (day 10) were used for isometric tension recording.

RESULTS: In isometric tension experiments Oroxylin A potently relaxated pregnant uterine strips precontracted by acetylcholine (10^{-6} M), $PGF_{2\alpha}$ (10^{-7} M) and oxytocin (10^{-3} U/ml) and the IC_{50} of Oroxylin A on each contricter is 31.47 ± 4.21, 29.15 ± 2.23 and 11.12 ± 0.54 μM, respectively. In KCl (56.3 mM) precontracted uterins smooth muscle, Oroxylin A was unable to cause relaxation. Pharmacologyical studies showed that relaxative effect of Oroxylin A was significantly attenuated by tetraethylammonium (TEA; 1 and 10 mM), 4-aminopyridine (4-AP; 5 mM) and glipizide (30 μM). In contrast, NOS inhibitor (LNNA; 10^{-3} M), β-receptor blocker (propranolol;10^{-5} M), cyclooxyngase inhibitor (indomethacin; 60 M), had no effect on its relaxation .These results indicated involution of potassium channel special calcium potassium channel in Oroxylin A induced uterine smooth muscle relaxation.

CONCLUSION: In this study we demonstrated that Oroxylin A-mediated relaxation in rat uterus smooth muscle might be through attributable to the opening uterine calcium-dependent potassium channels and adenosine triphosphate potassium channel-activation. Therefore this specific potassium activity to inhibition the spontaneous contractile activity of rat uterine smooth muscle. In vitro of above results Oroxylin A is a potential tocolytic pro-drug.

Keywords: Scutellariae Radix, tocolytic effect, active compound, oxytoxin, Ach, $PGF_{2\alpha}$, K^+, Ca^{2+} channel, NO, cyclooxygenase

1. Introduction

Preterm birth is a leading cause of neonatal morbidity and mortality in the word [1]and etiological studies indicated that preterm birth can be subdivided into three categories including idiopathicpreterm labor, premature rupture of membranes and medically premature labor[2]. Treatment of high risk population of pregnant woman with tocolytic agents is a strategy in preventing the occurrence of preterm birth. Several previous studies demonstrated that tocolytic agents such as ritodrine effectively prevent the occurrence of preterm birth through inhibiting uterus smooth muscle contraction. However, some deleterious side effects also have been found including hypokalemia, hyperamylasemia, toxic hepatitis and pulmonary edema[3,4]. Therefore, development of new effective tocolytic agents is an important research topic.

Nitric oxide (NO) has been shown to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication and host defense，NOS present in the endotholium (eNOS) is constitutive and its activation is Ca^{2+} dependent[5,6]. Continuous release of NO by eNOS plays a role in keeping the vascular smooth muscles in an active state of vasodilatation[7]. Previous studies demonstrated that elevation of NO production was detected in the early stage of gestation, but not in the term and delivery. Therefore NO is an important regulator in the process of gestation[8-10].

Membrane potential of smooth muscle cells is a major determinant of uterine tone and activities of potassium channels are one of regulators of membrane potential [11]. Activation of these potassium channels increases of potassium efflux, thereby producing hypolarization of membrane of smooth muscle cells[12]. There are several potassium channels have been identified in the uterine smooth muscle including calcium-dependent potassium channels, adenosine triphosphate potassium channel , voltage-dependent potassium channel, large Ca^{2+} -dependent potassium channel, and small-conductance potassium channel[13]. Therefore, activation of potassium channels appeared to be an important mechanism of uterine relaxation in response to several stimuli.

Chinese herbs- *Scutellaria baicalensis* have been used widely in treatment of several diseases such as inflammation , hypertension, suppressive dermatitis, diarrhea , pyrogenic infections.[14] Oroxylin A was an active component and isolated from the root of *Scutellaria baicalensis.* Previous studies demonstrated that Oroxylin A performed the inhibitory activity on 12-lipoxygenase and cyclooxygenase 2 [15] and inhibit NO production in lipopolysaccharide activated RAW 264.7 cells[16] . However, effect of Oroxylin A on the uterine is still unknown. The tocolytic Oroxylin A was demonstrated in the present study.

2. Materials and Methods

Tissue bath experiment Female Wistar rats (250-350 g) with or without pregnancy were from Animal Center of National Science Council (Taipei, Taiwan). The female wistar rats were estrogenized by injecting 5 mg/kg of estradiol benzoate. After 24 hours, the uterine rings were excised and placed in Locke solution of the following composition (mM): NaCl 154, KCl 5.63, $NaHCO_3$ 1.79, $CaCl_2 \cdot 2H_2O$ 2.55, Glucose 5.55. Uterine rings were cut into 1.5 cm segments, and the fetuses and placentas were gently removed. Then, the uterine rings were positioned for isometric tension recording and placed in organ chambers containing 12 mL of Locke buffer

bubbled with 95 % oxygen and 5% carbon dioxide in air, and maintained at 37℃ Locke solution (pH 7.4). The passive tension was gradually increased to the optimal level of 1 g over an equilibration period of at least 30 minutes. The contraction was recorded by force displacement transducers (Kent Scientific Corporation, USA) on an MP100 workstation software (Biopac Systems Inc. USA) in PC. The responses induced were expressed as a percentage of the maximum relaxation by papaverine (PPV; 10^{-3}M) which was added at the end of each experiment.

Oroxylin A in uterine smooth muscle precontracted by Ach, $PGF_{2\alpha}$ and oxytocin. In each experiment on the isolated uterine strip, a control response to ACh (10^{-6} M bath concentration), $PGF_{2\alpha}$ (10^{-7} M bath concentration) and oxytocin (10^{-3} U/ml bath concentration) was obtained, and the bath was then washed three times prior to incubation of the tissue with of the test compound. The latter were always tested on different uterine strips. The Oroxylin A to be tested for antagonist activity was then added to the bath and left in contact with the tissue for 10 minutes. ACh, $PGF_{2\alpha}$ and Oxytocin (at the concentrations mentioned above) was then added to the bath and the contractions were recorded. After 5 minutes the bath was washed three times and the control response to the regained. The volume of drug solution added to the 12-ml tissue bath never excessed 0.1 ml. Concentration-response experiment with ACh, $PGF_{2\alpha}$ and oxytocin on the isolated uterus. Rat uterine strips were incubated for 10 minutes with Oroxylin A at a concentration of 1×10^{-4} M.

Relaxative effect of Oroxylin A on contractile response of uterus to oxytocin-in Ca^{2+}-free solution. After obtaining the control response to acetylcholine in physiological solution, the strips were incubated for 60 minutes in a Ca^{2+}-depleted buffer containing 3 mM EDTA and then for a further 10 minutes in Ca^{2+}-free medium with a uterine horn was equilibrated for 30 min in Locke solution under a resting tension. The solution was then replaced by Ca^{2+}-free solution containing 3 mM EDTA and incubation was continued for 30 min. Sustained contractile responses to oxytocin (10^{-3} U/ml) were obtained and cumulative amounts of Oroxylin A were added.

Experimental procedure K^{+}-depolarized uterus. The effects of Oroxylin A (10^{-7}~10^{-4} M), were also assayed on tonic contraction induced by KCl (56.3 mM). The drug was added at increasing and cumulative doses. Each dose was left until its effect was stable (approximately 10 min). One hundred percent relaxation was obtained when the baseline was reached.

Pharmacological study In each experiment on the isolated uterine strip were stimulated with oxytocin (10^{-3} U/ml bath concentration) to induced contraction and allowed to equilibrate for a 15-minute period before control contractile performance was assessed. Oroxylin A (10^{-7}~10^{-4} M) was then added to the bath cumulatively at 15-minute intervals, and the resultant contractile activity was measured for each period. The strips were washed and allowed to return to Oroxylin A control contractions, after which time they were incubated in the presence of either tetraethylammonium (TEA 1 and 10 mM), 4-aminopyridine (4AP 5 mM), glipizide (30 μ M), prooranolol (β -adrenoceptors antagonist) 10^{-5} M, indomethacin (COX antagonist) 60 μM and N-nitro-L-arginine (L-NNA was NOS inhibitor) 10^{-3} M alone for 15 minutes. Oroxylin A was reintroduced into the bath as before and the concentration-effect values were similarly obtained.

Drugs and chemicals Oxytocin, acetylcholine (ACh), (S)-(-) propranolol hydrochloride, DMSO (Dimethyl Sulfoxide), papaverine HCl, Tetraethylammonium (TEA), 4-aminopyridine (4-AP), glipizide, and N-nitro-L-arginine (L-NNA) were obtained from Sigma Chemical Co. (St. Louis, MO). estradiol benzoate (China Chemical & Pharmaceutical Co., Taiwan) and Prostaglandine F2 α (PGF2 α) were purchased from Pharmaceutical Company (Japan). The stock solutions of all drugs were diluted to the desired concentrations with physiological salt solution. Oroxylin A was isolated from the root of *Scutellaria baicalensis* (Labiatae) and the structure was shown in Fig 1. The stock concentration of Oroxylin A is 4 mg/ml in DMSO.

Statistic analysis: The results are expressed as the means ± S.E.M.or more preparations (n) obtained from different animals. Relaxation was expressed as a percentage of the maximum tension obtained by addition of the agonist. Statistical significance of differences between the means was assessed using Student's t-test for unpaired data. P values of less than 0.05 were considered to represent significant differences.

3. Results

Effects of Oroxylin A on spontaneous uterine contraction in mid-stage of pregnant rats.

In most preparations rhythmic contractile activity was observed to occur spontaneously, shortly after mounting the uterine strips. In Fig 2, the spontaneous contractions were reproducible during the period of 4-5 h, and Oroxylin A treatment showed the dose-dependent relaxation on spontaneous contraction in uterus. Comparing with the clinical drug ritodrine, IC_{50} value of Oroxylin A on inhibition of uterine contraction is far less than that of ritodrine. DMSO, even at the concentration of 0. 1 % v/v, had no effects on precontracted basal tone (data not shown).

Attenuation of acetylcholine, $PGF_{2\sim}$ and oxytocin-induced uterine contraction by Oroxylin A. Addition of acetylcholine (10^{-6} M), $PGF_{2\alpha}$ (10^{-7} M) and oxytocin (10^{-3} U/ml) to the uterine segments incubated in Lockes solution induced rhythmic contractile response of stable intensity and duration. Cumulative concentrations (10^{-7}~10^{-4} M) addition of Oroxylin A into the reaction diminished the stimulators induced uterine contractions in a concentration-dependent manner (Fig 3). Oroxylin A showed the similar inhibition activity on acetylcholine (10^{-6} M), $PGF_{2\alpha}$ (10^{-7} M) and oxytocin (10^{-3} U/ml) induced contraction as described in the Table 1. The IC_{50} values of Oroxylin A on acetylcholine, $PGF_{2\alpha}$ and oxytocin-induced contractions are 31.47 ± 4.21, 29.15 ± 2.23 and 11.12 ± 0.54 μM, respectively. It is indicated that Oroxylin A was more sensitive in inhibiting oxytocine induced uterine contraction.

Ca^{2+} was not involved in Oroxylin A induced uterine relaxation. Many different cellular processes have evolved to regulate Ca^{2+} mediated signal transduction pathways including Ca^{2+} influx, Ca^{2+} efflux and Ca^{2+} sequestration pathways[17]. Ginsburing LT et al. reported that in vitro increases of intracellular Ca^{2+} concentration showed the obvious contractive response in rate uterus[18]. In order to demonstrate the possible role of Ca^{2+} played in Oroxylin A induced relaxation in uterus, EDTA, a Ca^{2+}/Mg^{2+} chelator, was used in the present study to remove the extracellular Ca^{2+} and the response of Oroxylin A on oxytocin induced contraction was measured. The data appeared that oxytocin induced sustained contraction in a Ca^{2+}-free condition, and cumulative addition of Oroxylin A from 10^{-7} to 10^{-4} M showed the similar relaxative pattern as the results presented (Fig 4). It is suggested that abolishment of extacelluar Ca^{2+} did not affect the relaxative response of Oroxylin A. We predicted that Ca^{2+} ion might not be an essential factor in modulation of Oroxylin A-induced relaxation.

Inhibition of Oroxylin A induced relaxation by KCl. Membrane potential in modulation of the uterine contraction has been proposed and activation of potassium channels have been demonstrated in the modulation of uterine relaxaion in previous studies[19]. Therefore, study whether activation of potassium channels is involved in Oroxylin A induced relaxation in uterus was performed here. The results showed that adding 56.3 mM KCl into the reaction buffer induced uterine contraction and Oroxylin A treatment appeared no significant relaxation, even at the concentration 100 μM (Fig 5). IC 50 value of Oroxylin A on relaxation in KCl-treated uterus is 94.43 ± 12.96 μM which is far higher than that in oxytocin treated uterus (IC_{50}=12.96 ± 1.12 μM). It is indicated that alternation of membrane potential is involved in the process of Oroxylin A induced uterine relaxation.

Effects of potassium channel blockers, tetraethylammonium (TEA), 4-aminopyridine (4AP), glipizide, on Oroxylin A-induced relaxation in uterine smooth muscle. Preincubation of rings from rats with the nonspecific potassium channels inhibitors tetraetylammonium (TEA; 1 and 10 mM) shifted the concentration-response curve of Oroxylin A to the right and attenuated the relaxative acivity induced by Oroxylin A (Fig 6). The concentrations causing 50% of the inhibition concentration were 66.03 ± 5.75 and 98.48 ± 13.87 μM in the presence of TEA 1 mM and TEA 10 mM, respectively. The result indicated that relaxation of Oroxylin A was inhibited by tetraetylammonium. As the same part of experiment, pretreatment of uterine with another potassium channels inhibitors including 4-aminopyridine and glipizide showed the significant inhibition on oroxylin A induced relaxation (Fig 6) and IC_{50} values are 93.28 ± 8.98 and 40.42 ±

7.75 μM, respectively (Table 2). Upon Comparing with the results of these potassium channels antagonists, tetraetylammonium appeared more effective than glipizide in attenuation of Oroxylin A induced uterine relaxation. Therefore, activation of potassium channels in the myomertrium during including Ca^{2+} dependent or ATP-dependent potassium channels is involved in the Oroxylin A induced uterine relaxation.

β-adrenergic receptor, cyclooxygenase and NO were not involved in Oroxylin A-induced relaxation. In order to demonstrate the mechanism of oroxylin A-induced uterine relaxation, pharmacological studies were performed to study the role of NO, prostaglandin and β-receptor were involved played in Oroxylin A-induced uterine relaxation. A cyclooxygenase inhibitor indomethacin, a NOS inhibitor L-NNA and a β-receptor blocker propranolol were added into the reaction followed by oroxylin A treatment. The results revealed that propranolol indomethacin, and LNNA showed no obvious effect on oroxylin-induced relaxation in oxytocin-precontracted uterines (Fig 7) and IC_{50} values are 15.53 $\pm$ 1.10 , 15.54 $\pm$ 1.03 and 13.06 $\pm$ 1.12 μM, respectively (Table 3).. We proposed that activation of cyclooxygenase activity, NO production and β-receptor might not be essential in the process of oroxylin A induced relaxation.

Comment

The results of the present study demonstrated that Oroxylin A, one of major components of Chinese herb Huang Qui, exerted significant relaxative effects on spontaneous and agonists-induced uterine contraction. Relaxative activity of Oroxylin A was attenuated in KCl-contracted uterus and blocked by addition of TEA and 4-AP into the reaction, however LNNA, propanolol or indomethacin did not show any effect on Oroxylin A induced relaxation. These data indicated that potassium channel activation was involved in Oroxylin A induced relaxation. These results provided more scientific evidences to support the traditional tocolytic effect of Huang Qui and demonstrated oroxylin A might be its active component and deserved further clinical study.

Cytoplasmic Ca^{2+} played an important role in the modulation of uterine contraction[20]. Previous studies demonstrated the cytoplasmic Ca^{2+} can be regulated by intracellular and extracellular Ca^{2+} pools[21], and agents induced influx of Ca^{2+} from extracelluar space or increased the intracellular Ca^{2+} by ER releasing cause the uterine contraction[21]. These studies indicated that Ca^{2+} is a critical mediator in uterine contraction. In this study, oroxylin A showed the similar relaxative pattern with of without Ca^{2+} in the reaction. These data indicated that Ca^{2+} ion might not be an essential factor in modulation of Oroxylin A-induced relaxation.

At lease three types of potassium ions currents have been described in rat myometium such as a fast transient current and two calcium dependent noninactivating currents[21]. In a single-channel recording experiment revealed that large conductance calcium dependent potassium channels are appeared in both pregnant and non pregnant myometrium[22], and its activation results in cell hyperpolarization and suppress the concentration of intracellular Ca^{2+}. Therefore, potassium channel opener is a strong uterine relaxant.

Potassium channels are composed of diverse groups of membrane channels including voltage-dependent potassium channel, large Ca^{2+}-dependent potassium channel, and small-conductance potassium channel. Opening of potassium channels causes hyperpolarization of the plasma membrane by increasing potassium conductance and reduces cell excitability by shifting membrane potential away from the threshold for excitation[23]. To block potassium channels, we used two nonselective potassium channel blockers, tetraethylammonium (1 and 10 mM) and 4-aminopyridine (5 mM) and a selective adenosine triphosphate-dependent potassium channel blocker, glipizide (30 μM) [24,25]. Oroxylin A-induced relaxation in uterus was blocked completely by tetraethylammonium (1 mM and 10 mM) and 4-aminopyridine (5 mM), but glipizide only showed the inhibition at high concentration (50—100 μM) of Oroxylin A induced relaxation. These data indicated that involvement Ca^{2+} dependent potassium activation in Oroxylin A induced uterine relaxation, and higher concentration (50-100 μM) of Oroxylin A might be partially acted on ATP-dependent potassium channels.

Nitric oxide (NO) is a unique and ubiquitous biologic second messenger and several studies demonstrated that NO is an important regulatory molecule in uterine contractions during pregnancy. Action of NO in the uterus is believed to maintain myometrial quiescence during the period of pregnancy, and decreased the amount of NO in the uterine smooth muscle in the period near labor and delivery[26,27]. Therefore, NO is an essential factor in the process of pregnancy. In this study, pretreatment of uterus with NOS inhibitors L-NNA didn't suppress the relaxative activity of Oroxylin A in oxytocin precontracted uterine. This result indicated that modulation of NO production might not be involved in Oroxylin A induced uterine relaxation.

Prostaglandins have been reported to be a mediator in the regulation of contraction status during pregnancy and parturition (Kelly 1994). Prostaglandin synthesis is controlled by two rate-limiting enzyme, phospholipase A_2 and cyclooxygenase (COX) [28]. Two distinct isoforms of COX including COX-1 and COX-2 have been identified. Previous reports demonstrated that inhibition of COX was able to inhibit the abnormal contraction in uterus. However, Enkin et al. demonstrated that inhibition of COX by indomethacin caused the risk of neonatal cerebral haemorrhage and necrotizing enterocolitis[28]. Therefore, the role of COX played in uterus is under contradiction. In the present study, pretreatment of indomethacin did not show any effect on Oroxylin A induced relaxation. It is suggested that cyclooxygenase was not involved in oroxylin A induced relaxation.

Although Oroxylin A will most likely never be used clinically to achieve tocolysis , but the data presented here clearly demonstrate that Oroxylin A is able to relax rat pregnant myometrium through a mechanism involving the activation of potassium channels . The results showed that TEA, 4-AP and glipizise effectively block Oroxylin A induced uterine relaxation. This study demonstrated that the tocolytic activity might be through attributable to the opening uterine calcium-dependent potassium channels and partially acted on ATP-dependent potassium channels. . These result indicate that the plant examined possess direct uterine relaxation activity which could justify Oroxylin A usage in traditional herbal remedies used during prematurity. This preliminary has shown that the plant extracts should now be subjected to procedures in other to characterize the pharmacologically active principle, with a view to identifying a novel target site through which to achieve tocolysis.

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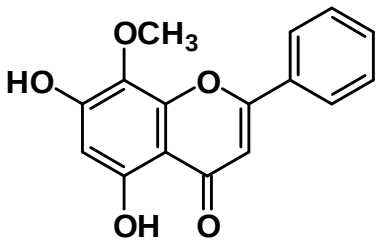

Fig 1. Oroxylin A isolated from *Scutellaria baicalensis*.

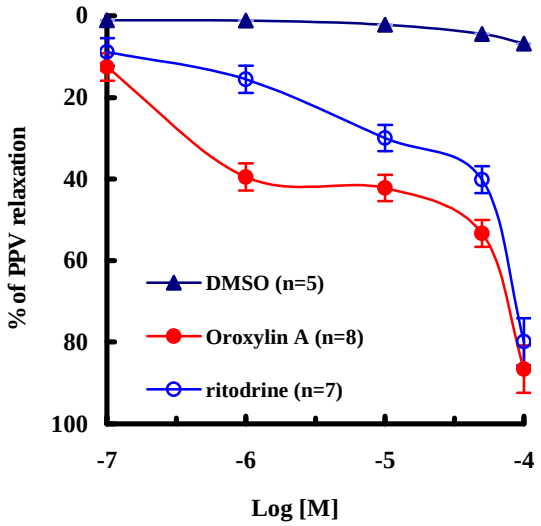

Fig 2. Concentration-effect curves of Oroxylin A and ritodorine on rat isolated uterus of 10 gestational days. Vertical bars represent S.E.M. and n is the number of experiments. *p＜0.05,**p＜0.01, indicate significant difference from the respective control.

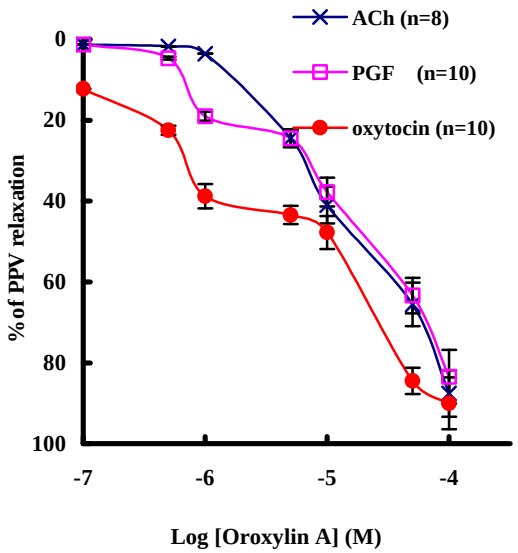

Fig 3. Dose-dependent inhibition of Oroxylin A on the acetylcholine 10^{-6} M , PGF2α and oxytocin preconstricted uterus. Each value was described as mean ± S.E.M. of at least three independent experiments. n is the number of the segments of uterus used in this study. papaverine HCl (PPV；10^{-3} M) added at the end of each experiment was described as 100% of relaxation.

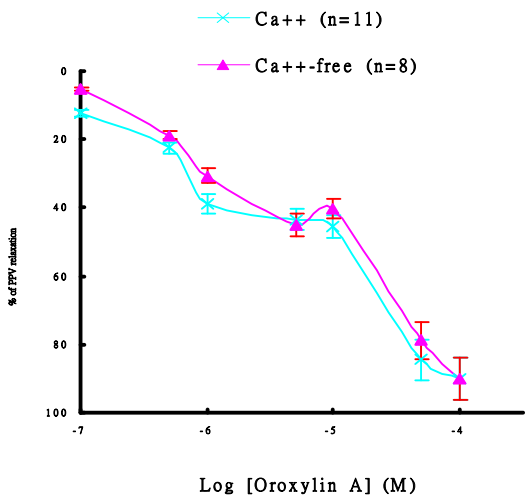

Fig 4. Relaxative effect of Baicalin on oxytocin-induced uterine contraction in Ca^{2+}-free solution. Each point was described as mean ± S.E.M. of at least three independent experiments and n is the number uterine segments used in this study. Papaverine HCl (PPV ; 10^{-3} M) added at the end of each experiment was described as 100% of relaxation.*p < 0.05,**p < 0.01, indicate significant difference from the respective control.

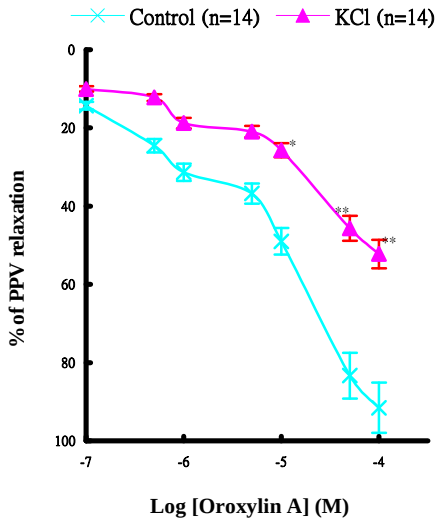

Fig 5. Relaxative effect of Oroxylin A on oxytocin (10^{-3} U/ml)-induced uterine contraction in KCl solution solution. Each point was described as mean ± S.E.M. of at least three independent experiments and n is the number uterine segments used in this study. apaverine HCl (PPV ; 10^{-3} M) added at the end of each experiment was described as 100% of relaxation.

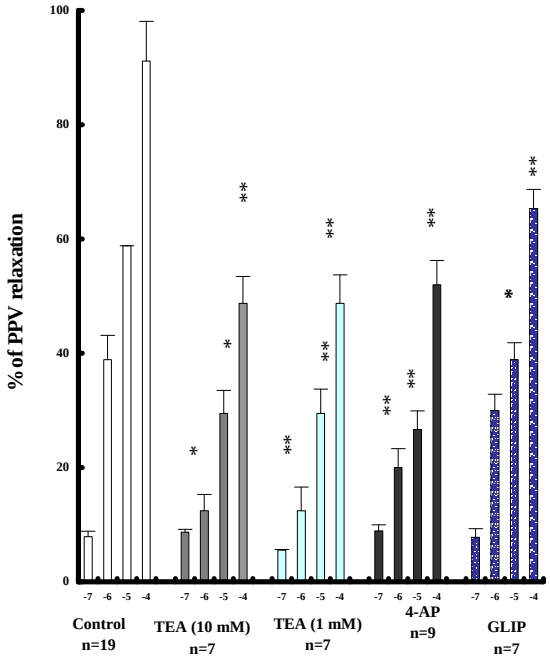

Fig 6. Effect of potassium channel inhibitor tetraethylammonium (TEA; 1 and 10 mM), 4-AP (4-aminopyridine) and Glipizide on Oroxylin A induced uterine relaxation from least three independent experiments and n is the number uterine segments used in this study. Papaverine HCl (PPV ; 10^{-3} M) add iment was described as 100% of relaxation. .*p < 0.05 and **p < 0.01 indicate significant difference from the respective control.

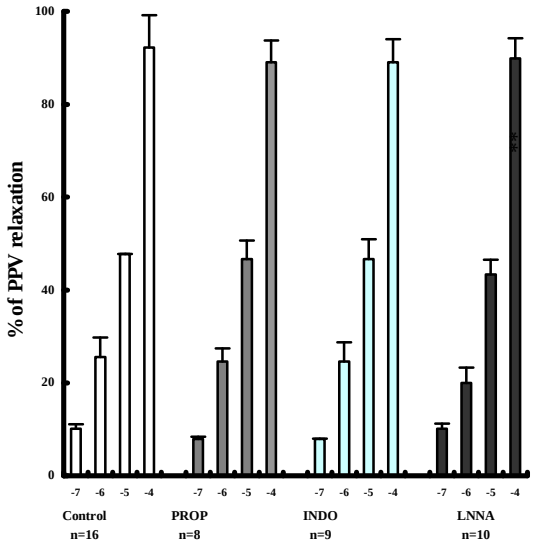

Fig 7. Effect of β-receptor blocker propranolol (PROP; 10^{-5} M), indomethacin (INDO; 60 μM) and LNNA (10^{-3} M) on Oroxylin A induced uterine relaxation from least three independent experiments and n is the number uterine segments used in this study. papaverine HCl (PPV ; 10^{-3} M) add experiment was described as 100% of relaxation.