

The effects of second messenger cAMP and its relative components on the contraction of uterine smooth muscle of rat

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Abstract. – OBJECTIVE: To investigate the effect of second messenger pathways on the uterine smooth muscle contraction and their associated mechanisms, and compare the evaluation methods.

MATERIALS AND METHODS: Preparation of uterine smooth muscle strips from healthy pregnant 18-21 d SD and non-pregnant rats. When the contraction of muscle strips was stable, we conducted gradient administration: PDE4 inhibitors (Z90), prostaglandin PGE2, adenylate cyclase inhibitor (SQ 22,530), cAMP analogs (dbcAMP) and AMPK agonists (AICAR), solvent dimethyl sulfoxide (DMSO) as controlled. Gradient administration of acetylcholine (ACh) and oxytocin (oxytocin) induced the contraction of muscle strips. The tension transducer and biological information collecting system were applied to record the changes, including duration, dilation tension, contraction tension, peak height, and mean tension, before and after different administration. Principal components analysis was adopted to evaluate the five changes.

RESULTS: SQ 22,530, DMSO, cAMP alone had no significant effect on the contraction of uterine smooth muscle; Z90 can inhibit the spontaneous contraction of pregnant uterine smooth muscle strips; dbcAMP and AICAR can antagonize acetylcholine and oxytocin-induced the contraction of pregnant uterine smooth muscle strips. Z90, SQ 22,530 + Z90, dbcAMP, AICAR can inhibit the uter-

ine contraction peak, diastolic amplitude, average muscle tone and contraction duration of the pregnant uterine smooth muscle in a concentration-dependent manners. At the same time, we compared the parameters, which reflect the contraction of uterine smooth muscle, and conduct main components analysis to determine the effect of the drugs.

CONCLUSIONS: The second messenger cAMP and its related components ATP, 5'-AMP, AC, PDE, PKA, and AMPK can affect the uterine smooth muscle contraction via related signaling pathway in rats, and principal components analysis can be adopted to evaluate the smooth muscle relaxant.

Key Words

cAMP, ATP, 5'-AMP, AC, PDE, PKA, AMPK, Rat, Uterine smooth muscle, Contraction.

List of Abbreviations

ACh: Acetylcholine; AC: adenyl cyclase; ATP: adenosine triphosphate; AMPK: adenosine 5'-monophosphate (AMP)-activated protein kinase; cAMP: Cyclic Adenosine monophosphate; dbcAMP: Dibutyryl-cAMP; DMSO: Dimethyl sulfoxide; Oxy: Oxytocin; PDE: phosphodiesterase; PGE2: prostaglandin E2; PTL: Preterm labor.

Introduction

Preterm labor (PTL) is the important cause of perinatal morbidity and mortality. Currently, there are several classes of tocolytics available in clinical trial¹, but none of them are ideal. Over the past decades, a large number of investment hoped to reduce complications of premature birth², improve predicting of preterm birth³, and reduce premature birth⁴, but the situation is still unchanged. So, better tocolytics and better understanding of the mechanism, which initiates labor, remains urgent⁵. cAMP, the second messenger in cellular signal transduction, plays an important role in smooth muscle contraction. Previous studies showed that cAMP can maintain homeostasis by the regulation of adenylyl cyclase (AC) and phosphodiesterase (PDE). cAMP is produced by AC from adenosine triphosphate (ATP) and hydrolysed by PDE. The ATP→cAMP→5'-AMP axon and its related components may affect the contraction of uterine smooth muscle. Under most circumstances, the increased cAMP level decreased the muscle tension while the decreased cAMP level increases the muscle tension. The potential mechanism may include: (1) hyperpolarization of membrane potential⁶; (2) regulation of the intracellular Ca²⁺ level⁷; (3) phosphorylation of proteins related to muscle contraction⁸. Since there were few direct researches about the influence of the second messenger cAMP and its related components on the contraction of uterine smooth muscle, at the same time the theory or hypothesis mentioned above may be need justifications. So, we explored those experiments to make a direct verification of their functions.

Materials and Methods

Animals

Normal female SD rat, pregnant (18-21 d) and non-pregnant (200-220 g) were provided by the animal center of Zhejiang University. The experimental protocols were strictly according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Research Ethics Committee of Zhejiang University.

Agents

Incubated solution for rat uterine smooth muscle [NaCl 9.0, KCl 0.42, CaCl₂ 0.03, NaHCO₃ 0.5, glucose 0.5 (g/L)], Dimethyl sulfoxide (DM-SO), PDE4 inhibitor (Z90), PGE₂ (82475, Fluka,

Sigma-Aldrich, St. Louis, MO, USA) adenylyl cyclase inhibitor (SQ22,530, Sigma-Aldrich, St. Louis, MO, USA), cAMP (018K1409, Sigma-Aldrich, St. Louis, MO, USA), cAMP analogue Dibutyryl-cAMP (dbcAMP, 2B/86309, Tocris Bioscience, Avonmouth, Bristol, UK), AMPK agonist AICAR (096K47036, Sigma-Aldrich, St. Louis, MO, USA), diethylstilbestrol (041101, Shanghai Tongyong Co. Ltd., Shanghai, China), acetylcholine (A2661, Sigma-Aldrich, St. Louis, MO, USA), Oxytocin (0703/9-1, Fengyuan Medicine Co. Ltd., Hefei, Anhui, China).

Instruments

Water bath, JZJ01 tension transducer, RM6240 system of biological information collection (Chengdu Instrument Factory, Chengdu, Sichuan, China).

Preparation of Uterine Strips, Recording and Evaluation of the Contraction of Uterine Smooth Muscle

24 h before the experiment started, estrus or proestrus rats were made by subcutaneous injection of diethylstilbestrol 0.5 mg/kg of adult virgin female SD rats for induction of the hypersensitivity of the uterus. Cervical dislocation euthanasia was performed, and isolated uteruses were obtained. Full-thickness uterine strips were made and immediately put into pre-cooling (4°C) incubated solution saturated by 95% O₂ and 5% CO₂. Uterine strips measuring 2 cm long × 1 cm wide were prepared and burdened with a 1 g isometric force in a 32°C bath with incubated solution for about 30 min. It was ready when the pregnant uterine strips began to contract spontaneously and rhythmically or when the non-pregnant ones became stabilized. The traditional parameters for evaluating the contraction of uterine smooth muscle were respectively the contractile force, frequency, and area under the curve. In our experiment, we used five parameters: duration (s), dilation tension (g), contraction tension (g), peak height (g), and mean tension (g). As shown in Figure 1, we can define the contraction tension and dilation tension, and their difference – peak height. The quotient of those two tensions is the mean tension. We can also easily define the area under the curve and contraction duration, and the reciprocal of the contraction duration was frequency. Thus, duration (s), dilation tension (g), contraction tension (g), peak height (g), and mean tension (g) can provide more details about the effect on dilation or contraction, more than the contractile force.

Pregnant Uterine Strips

The bath volume was maintained at 20 ml, the work concentration of drugs was 200 times of the final concentration.

1. DMSO: to exclude the intervention of DMSO, each concentration of DMSO was tested alone to fresh new strip.
2. Z90 alone: the contractions of strips were observed in the work concentration of Z90 from 1.0×10^{-7} M to 1.0×10^{-2} M (final concentration from 1.0×10^{-9} M to 1.0×10^{-4} M). Z90 was prepared with the method of gradual dilution (n=6). 1.0×10^{-2} M of Z90 was dissolved in 100% dimethyl sulfoxide (DMSO), 1.0×10^{-3} M in 50% dimethyl sulfoxide (DMSO), 1.0×10^{-4} M in 10% dimethyl sulfoxide (DMSO), and lower concentration in water bath.
3. PGE2 (prostaglandin E2): new trips were observed under PGE2 work concentration 3×10^{-4} M (final concentration 3×10^{-6} M), (n=6).
4. SQ22, 530 alone: the contractions of 2 strips were observed in the cumulative work concentration of SQ from 3.0×10^{-7} to 3.0×10^{-2} M (final concentration from 3.0×10^{-9} to 3.0×10^{-4} M), (n=6).
5. cAMP: the contractions of 6 strips were observed in the cumulative work concentration of cAMP from 1.0×10^{-7} to 1.0×10^{-2} M (final concentration from 1.0×10^{-9} to 1.0×10^{-4} M), (n=6).
6. cAMP analogue dbcAMP: the contractions of 5 strips were observed in the cumulative work concentration of cAMP analogue from 1.0×10^{-7} to 1.0×10^{-2} M (final concentration from 1.0×10^{-9} to 1.0×10^{-4} M), (n=5).
7. AMP-activated protein kinase (AMPK) agonist AICAR: the contractions of strips were observed in the cumulative work concentration of AMPK agonist from 1.0×10^{-7} to 1.0×10^{-2} M (final concentration from 1.0×10^{-9} to 1.0×10^{-4} M), (n=9);
8. PGE2 and Z90 combined: new strips were observed under PGE2 work concentration 3×10^{-4} M (final concentration 3×10^{-6} M) and then plus Z90 (work concentration from 1.0×10^{-7} M to 1.0×10^{-2} M, final concentration from 1.0×10^{-9} M to 1.0×10^{-4} M), (n=6);
9. SQ22,530 and Z90 combined: new trips were observed under work concentration of SQ22,530 was 3×10^{-4} M (final concentration 3×10^{-6} M) and then plus Z90 (work concentration from 1.0×10^{-7} M to 1.0×10^{-2} M, final concentration from 1.0×10^{-9} to 1.0×10^{-4} M), (n=6);
10. Z90 and cAMP analogue combined: new trips were observed under Z90 work concentration 3.5×10^{-2} M (final concentration 3.5×10^{-4} M) and then plus cAMP analogue (work concentration from 1.0×10^{-7} M to 1.0×10^{-2} M, final concentration from 1.0×10^{-9} M to 1.0×10^{-4} M), (n=5).
11. Z90 and AMPK agonist combined: new trips were observed under Z90 work concentration 3.5×10^{-2} M (final concentration 3.5×10^{-4} M) and then plus AMPK agonist (work concentration from 1.0×10^{-7} M to 1.0×10^{-2} M, final concentration from 1.0×10^{-9} M to 1.0×10^{-4} M), (n=5).

Non-Pregnant Uterine Strips

1. Acetylcholine alone: 6 new strips were observed under acetylcholine final concentration 39 μ M, 78 μ M, and 156 μ M. Each contraction of the strip was recorded for 10 min (n=6).
2. Acetylcholine after pre-incubation of cAMP analogue: 6 new strips were pre-incubated by cAMP analogue (1×10^{-3} M) for 15 min, and observed under acetylcholine final concentration 39 μ M, 78 μ M, and 156 μ M. Each contraction of the strip was recorded for 10 min (n=6).
3. Acetylcholine after pre-incubation of AMPK agonist: 6 new strips were pre-incubated by AMPK agonist (1×10^{-3} M) for 15 min, and observed under acetylcholine final concentration 39 μ M, 78 μ M, and 156 μ M. Each contraction of the strip was recorded for 10 min (n=6).
4. Oxytocin alone: 6 new strips were observed under final oxytocin concentration 39 μ M, 78 μ M, and 156 μ M. Each contraction of the strip was recorded for 10 min (n=6).
5. Oxytocin after pre-incubation of cAMP analogue: 6 new strips were pre-incubated by cAMP analogue (1×10^{-3} M) for 15 min, and observed under final oxytocin concentration 0.0025 U/ml, 0.005 U/ml, 0.01 U/ml, and 0.02 U/ml. Each contraction of the strip was recorded for 10 min (n=6).
6. Oxytocin after pre-incubation of AMPK agonist: 6 new strips were pre-incubated by AMPK agonist (1×10^{-3} M) for 15 min, and observed under final oxytocin concentration 0.0025 U/ml, 0.005 U/ml, 0.01 U/ml, and 0.02 U/ml. Each contraction of the strip was recorded for 10 min (n=6).

Statistical Analysis

The Dunn's Multiple Comparison Test was carried out as a post-hoc test for ANOVA to compare the means of different groups, with $p < 0.05$ being considered as significant. Results were reported as means $\pm$ SD. We drew on main components analysis of SPSS15 (SPSS Inc., Chicago, IL, USA) to analyze the data collected when no drug or only one drug takes effect, including blank control, Z90 alone, DMSO alone, SQ alone, cAMP alone, dbcAMP alone and AICAR alone. Two main components were factors of contraction of uterine smooth muscle: component 1 contains peak height, contraction tension and means tension; component 2 contains duration and dilation tension.

Results

The Effect of cAMP and Related Drugs on the Contraction of Pregnant Uterine Smooth Muscle

DMSO did not Affect the Contraction of Pregnant Uterine Smooth Muscle

From each parameter, pregnant uterine strips had no response to DMSO in any concentration. So the influence of dimethyl sulfoxide (DMSO) could be excluded.

Z90 Suppresses the Contraction of Pregnant Uterine Smooth Muscle

Our results with gradient administration of Z90 showed that low concentration (lower than 1.0×10^{-6} M) decreased the frequency, dilation tension, contraction tension, peak height, and mean tension, but the differences were not statistically significant. High concentration of Z90 showed significant concentration-dependent inhibition. The sensitive concentration of Z90 was higher than 10^{-6} M. Based on the data of Z90, we had four trend lines in Figure 1B, and calculated each 50% value as ED_{50} : 8.04×10^{-2} M, 1.77×10^{-2} M, 1.75×10^{-2} M, 2.27×10^{-2} M, the average was 3.46×10^{-2} M.

SQ22530 did not Affect the Contraction of Pregnant Uterine Smooth Muscle

According to Figure 1C, SQ22,530 in the concentration of 10^{-9} M- 10^{-4} M had no effect on the contraction of pregnant uterine smooth muscle. Under conditions of a higher concentration, there was a decreasing trend similar with Z90, but without statistically significance.

cAMP did not Affect the Contraction of Pregnant Uterine Smooth Muscle

To explore the effect of cAMP, we used cAMP directly in the bath. According to Figure 1E, cAMP out of cells at the concentration of 10^{-9} M- 10^{-4} M had no significant effect on the contraction of pregnant uterine smooth muscle. It may imply an increased contraction in higher concentration, but no data for it was so far available.

dbcAMP Suppresses Contraction of Pregnant Uterine Smooth Muscle

To confirm the effect of cAMP in cells and out of cells, we used the cAMP analogue dbcAMP, which can penetrate into cells. The results suggested that dbcAMP prolongs the contraction duration, but decreased the contraction tension, dilation tension, peak height and mean tension in a concentration-dependent manner.

AICAR Suppresses the Contraction of Pregnant Uterine Smooth Muscle

To confirm the effect of cAMP Hydrolyzate, 5'-AMP, we used AMPK agonist AICAR, which can activate AMPK and mimic 5'-AMP function. Those results suggested that AICAR increased the contraction duration, decreased the contraction tension, dilation tension, peak height and mean tension in a concentration-dependent manner.

The Combined Effect of Z90 and cAMP Related Drugs on the Contraction of Pregnant Uterine Smooth Muscle

According to the results shown in Figure 1, 3.0×10^{-6} M PGE_2 increased the contraction of pregnant uterine smooth muscle. All parameters went down in value, with an especially significant decreased seen in dilation tension and mean tension. After pre-incubation with 3.0×10^{-6} M PGE_2 , the threshold of response of the uterine strips to Z90 was higher. This showed an antagonistic effect between PGE_2 and Z90. The antagonistic effect after pre-incubation did not last long. It showed a fast decrease during the first hour and then slowed down. Pre-incubation of PGE_2 increased the mean dilation tension and mean contraction tension. Since PGE_2 increased the dilation and contraction in the approximately same amplitude, the peak height did not change significantly. Mean tension increased with statistical significance; as we compared the effects of Z90 and PGE_2 when their concentration was 10^{-6} M, the result demonstrated that PGE_2 significantly increased the basic tension, contraction

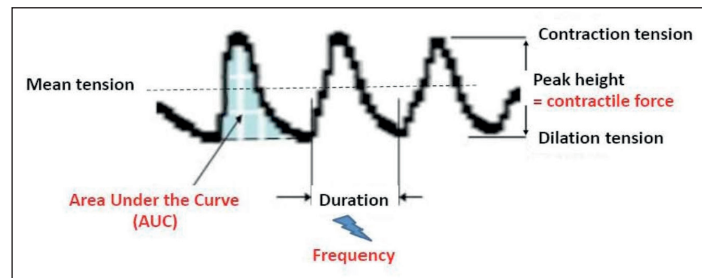


Figure 1. Parameters reflected contraction of the uterine smooth muscle. The traditional parameters for evaluating the contraction of the uterine smooth muscle are peak height, frequency and area under the curve. In our experiment, we used five parameters: duration (s), dilation tension (g), contraction tension (g), peak height (g), and mean tension (g). We can easily define the contraction tension and dilation tension, and their difference – peak height. We can also easily define the area under the curve and contraction duration. The quotient of this two is mean tension. The reciprocal of contraction duration is frequency. Thus, duration (s), dilation tension (g), contraction tension (g), peak height (g), and mean tension (g) contain more information than traditional parameters do.

and frequency of the uterine muscle. According to Figure 2 and Figure 3, Z90 may affect the uterine muscle above the concentration of 10^{-6} M, while SQ22,530 in the concentration of 10^{-9} M- 10^{-4} M had no effect on the contraction of pregnant uterine smooth muscle. In higher concentration, there was a decreasing trend like Z90, but without statistical significance. After the pre-incubation of 3.0×10^{-6} M SQ22,530, those uterine strips responded to Z90 in a similar trend but more robustly: the threshold decreased, the curves moved to the

left, the mean duration prolonged. Means of dilation tension, contraction tension, peak height and mean tension all decreased more obviously than duration. The test curve moved to the left when SQ combined with Z90 from Z90 alone. These results revealed the synergistic effect of SQ22,530 and Z90. According to Figure 4, Z90 pre-incubation had little impact on the contraction, but the contraction stopped after the cAMP analogue or AMPK agonist was added, stronger than dbcAMP and AICAR alone. The test curves also moved left.

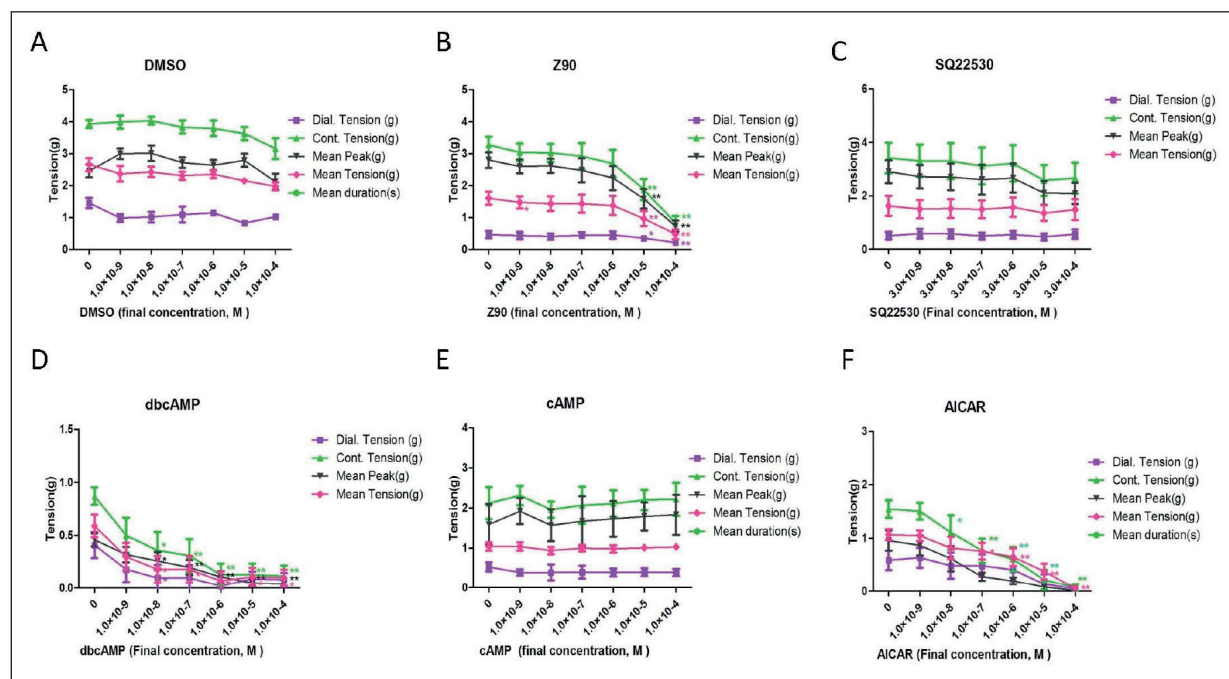


Figure 2. The action of gradient administration of DMSO (A), Z90 (B), SQ 22530 (C), dbcAMP (D), cAMP (E) and AICAR (F) on the contraction parameters of pregnant uterine strips. The parameters in this figure are: dilation tension (g), contraction tension (g), peak height (g), and mean tension (g). ANOVA shows that there is at least one different group in each parameter. ($p < 0.05$); t -test (normal, paired, single tailed) shows there are significant differences within the “0” group. (* $p < 0.05$, ** $p < 0.01$).

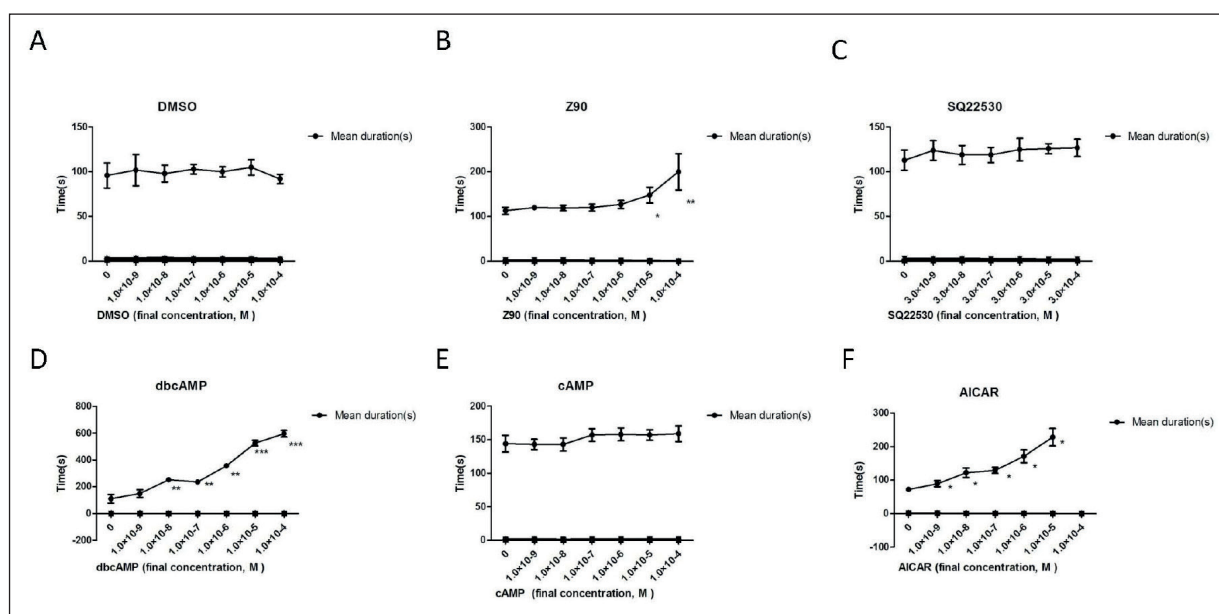


Figure 3. The effect of gradient administration of DMSO (A), Z90 (B), SQ 22,530 (C), dbcAMP (D), cAMP (E) and AICAR (F) on the contraction duration of pregnant uterine strips. *t*-test (normal, paired, single tailed) shows there are significant differences within the “0” group. (**p*<0.05, ***p*<0.01).

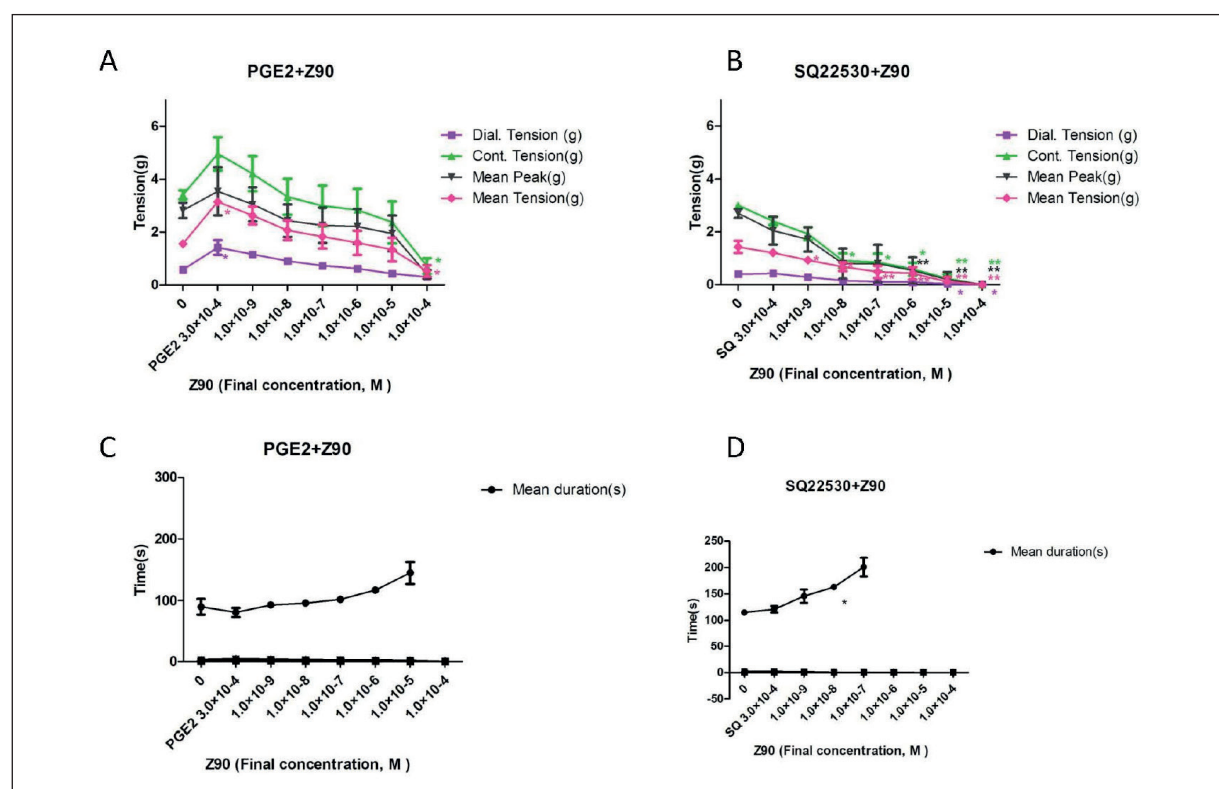


Figure 4. The effect of combination of PGE2 and Z90 (A, C), SQ 22,530 and Z90 (B, D) on the contraction of pregnant uterine strips. A/B: parameters included in this Figure are dilation tension (g), contraction tension (g), peak height (g), and mean tension (g); C/D: parameter in this Figure is contraction duration (s). ANOVA shows no difference between each concentration in every perimeter. */*: *t*-test (normal, paired, single tailed) shows there are significant differences within the “0” group. (**p*<0.05, ***p*<0.01).

Those results also suggested a synergistic effect between Z90 and cAMP analogue, or AMPK agonist.

cAMP Analogue and AMPK Agonist Inhibited the Contraction of Non-Pregnant Uterine Smooth Muscle

To further confirm the effect of cAMP analogue and AMPK agonist, we used dbcAMP and AICAR in non-pregnant uterine smooth muscle. Oxytocin took effect via oxytocin receptors, which also coupled with G-protein and influenced the calcium release⁹. Acetylcholine influenced the intracellular Na⁺ level through the acetylcholine M receptor. Figure 5A-C showed that the cAMP analogue and AMPK agonist were antagonistic to the effect of acetylcholine, and both effects were concentration-dependent. The antagonistic effects were more apparent in contraction tension than peak height. Figure 5D-F showed that cAMP analogue and AMPK agonist were antagonistic to

the effect of oxytocin, and both effects were also concentration-dependent. The antagonistic effects were more apparent in peak height. Those results indicated that both cAMP analogue and AMPK agonist could inhibit the contraction of uterine smooth muscle induced by acetylcholine and oxytocin in a concentration-dependent manner.

Compare the Parameter to Evaluate the Contraction of Uterine Smooth Muscle and the Value for their Combination

Firstly, by combining the definition with statistics, we attempted to evaluate the role of each parameter in an empirical way. From the perspective of coefficient of variation (CV), the individual differences in the ascending order were frequency (0.23), dilation tension (0.30), contraction tension (0.36), peak height (0.38), and mean tension (0.59). When unhandled, the most stable indicator for the evaluation of pregnant rat uterine

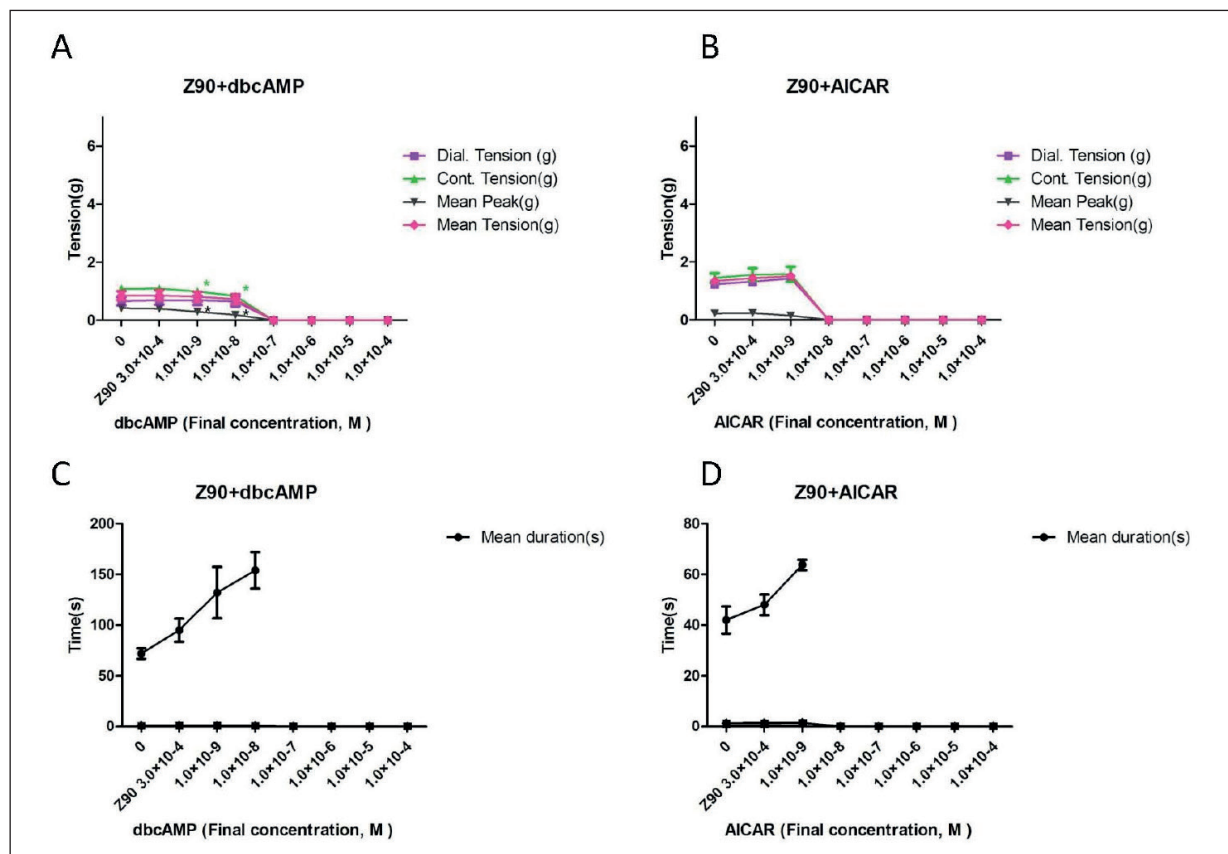


Figure 5. The combined effect of Z90 and dbcAMP, Z90 and AICAR on the contraction of pregnant uterine strips. **A/B:** parameters included in this Figure are dilation tension (g), contraction tension (g), peak height (g), and mean tension (g); **C/D:** parameter in this Figure is contraction duration (s). ANOVA shows no difference between each concentration in every parameter. */**: *t*-test (normal, paired, single tailed) shows there are significant differences within the "0" group. (**p*<0.05, ***p*<0.01).

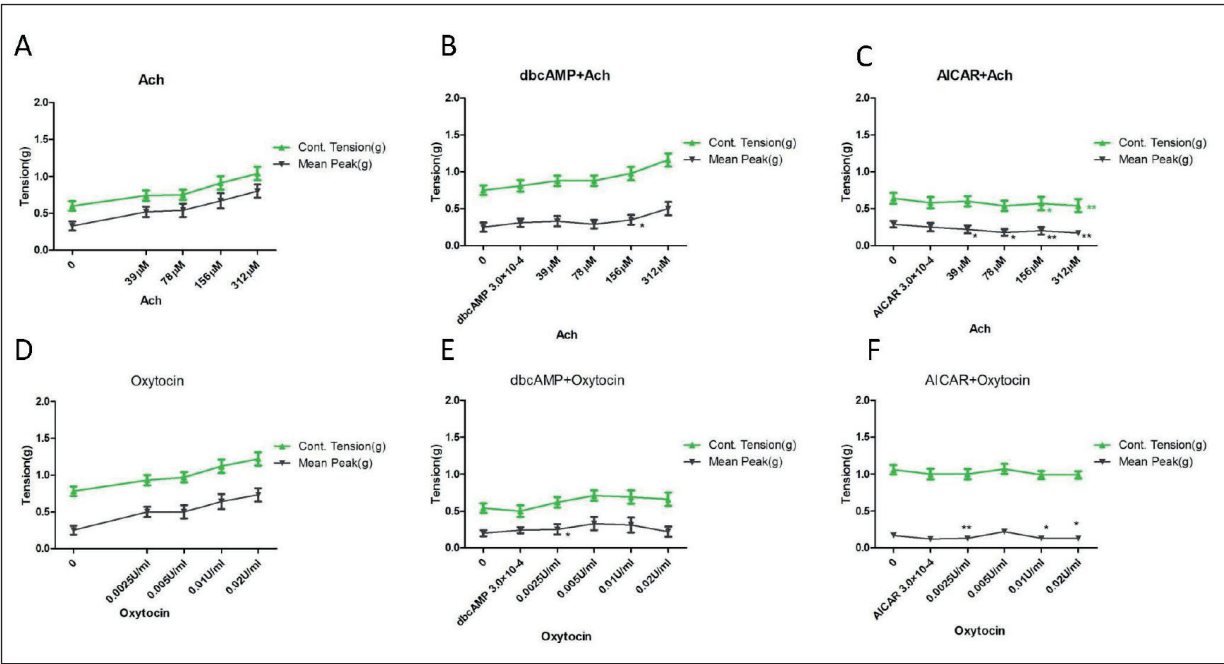


Figure 6. **A**, Effect of oxytocin alone, cAMP analogue + oxytocin and AMPK+oxytocin on the non-pregnant uterine contraction. **B**, Effect of acetylcholine alone, cAMP analogue+acetylcholine and AMPK agonist+ acetylcholine. Parameters in those figures are contraction tension and peak height. **/: *t*-test (normal, two samples with homogeneity of variance, single tailed) shows there are significant differences within the oxytocin or acetylcholine alone group. (* $p < 0.05$, ** $p < 0.01$).

contraction was the average contraction time (or frequency) and the average contraction tension, so these two indicators could be used as the base for uterine contractions in pregnant rats. These two indicators of the muscle contraction required higher experimental concentration that the vast majority of smooth muscle cells in the uterine muscle should contract at the same time for the formation of a single peak systolic with a good rhythm. However, the experiment shows that a variety of drug treatment may lead to unsynchronized cell contraction of the uterine smooth muscle, scattered single-peak changes and an unstable rhythm, which caused the two above mentioned indicators to be fluctuating and insensitive. Under such circumstances, the use of average muscle tension as an evaluation indicator, and the shortcomings can be eliminated. The fluctuation of average diastolic (valley) tension suggested the instability of the smooth muscle preload. To improve that, experimental devices can be used to increase the stability of smooth muscle preload and provide more accurate tension measurement. Mean tension (average contraction amplitude) was a function of single contraction peaks, which can exclude interference from the baseline drift, and the misleading of percentage analysis on the

amplitude of the peaks and valleys. In this way, it can evaluate smooth muscle contractility in a more direct and practical manner. To reveal the significance of various indicators, we collected and analyzed the information contained in the test results, an appropriate statistical tool – principal component analysis had been taken into consideration. We used SPSS15 (SPSS Inc., Chicago, IL, USA) to analyze all data collected, including blank control, Z90 alone, DMSO alone, SQ 22,530 alone, cAMP alone, dbcAMP alone and AICAR alone. The results were as follows: Table I: two main components were two factors

Table I. Component Matrix.

	Component	
	1	2
Mean Duration	-0.124	0.584
Dial. Tension	0.586	-0.674
Cont. Tension	0.973	0.208
Mean Peak	0.819	0.511
Mean Tension	0.971	-0.158
Cumulative %	58.410	22.479

Total cumulative % = 80.88%, which means that two principal components can explain 80.88% of all variations.

of contraction of the uterine smooth muscle, component 1 contained peak height, contraction tension and means tension; component 2 contained duration and dilation tension. We can describe the effect of each treatment on these two components. In Figure 7, spots in blue circles were effects of different contractions of Z90. They were all above the X-axis, which meant the prolongation of duration and the decrease of dilation tension were not concentration-dependent. When the concentration of Z90 increased, they moved towards left, which meant the concentration-dependent effects were contraction tension, peak height, and mean tension. The effects of PGE2 were mainly on component 1, which included contraction tension, peak height and mean tension. Effects of dbcAMP and AICAR, similar with Z90, were also mainly on component 1, which were contraction tension, peak height and mean tension. DMSO, SQ22,530 and cAMP had no obvious trend in PCA analysis. This analysis demonstrated that most treatment in our experiment took effect only on contraction tension, peak height and mean tension. Multiple variables with plenty of samples will undoubtedly provide a wealth of information for scientific research. However, if the analysis only focused on each indicator, the result may be isolated rather than integrated. On the other hand, blind reduction on parameters can easily cause a loss of information and lead to wrong conclusions. To avoid the situations discussed above, principal component analysis was a worth try.

Discussion

In our test, we revealed that cAMP and its related drugs may exert certain influences on the contraction of rat uterine smooth muscle. Both can inhibit the contraction by increasing cAMP, decreasing cAMP, or activating 5'-AMP. There are several PG receptors out of the cell membrane according to their agonists and blockers, which are EP1, EP2, EP3 and EP4¹⁰. Previous reports had pointed out that the contraction was induced by EP3¹¹ while dilation by EP2¹². The contraction before delivery was enhanced by increased expression of EP3¹³. Up to now, two independent mechanisms were found by which PG affected the uterine contraction: (1) increasing the level of intracellular Ca^{2+} and activate myosin light chain kinase. We believed the main effect was to increase the intracellular calcium level directly

via the calcium pool instead of decreasing the cAMP level or activating phospholipase C alone. (2) Strengthening gap junctions. The gap junctions provided low-resistance channel for electrical coupling, which promoted the coordination of the uterus contraction¹⁴. The antagonistic effect of Z90 on PGE2 was caused by decreasing the concentration of Ca^{2+} , or interfering the gap junctions. These could be controlled by inhibiting PDE4 and then increasing the second messenger cAMP. PDE4 is the main pathway to influence the level of cAMP. PDE4 inhibitor has an anti-inflammatory effect which decreases the release of inflammatory agents, increases the anti-inflammatory agents, inhibits expression of cyclooxygenase and promote synthesis of PGE2¹⁵. Our results demonstrated that cAMP in 10^{-9}M - 10^{-4}M cannot affect the contraction. Through research we have known the extracellular and intracellular pathway of cAMP¹⁶. For the extracellular pathway, cAMP can transfer the signal to cGMP, IP3/DG and intracellular cAMP via the extracellular receptor, cAR¹⁷. In the concentration of 10^{-9}M - 10^{-4}M in our experiment, there was low functional affinity for the extracellular receptor. That meant that cAMP cannot work via the extracellular pathway in this situation but only via the intracellular pathway. This implied that there were no autocrine or paracrine of cAMP on pregnant uterine smooth muscle. Dibutyryl-cAMP is a type of cAMP analogue, which can activate protein kinase A and C (while cAMP can only activate protein kinase A). Due to its lipophilic nature, dibutyryl-cAMP can move freely through the cell membrane, while cAMP cannot. So we used dibutyryl-cAMP to increase the concentration of intracellular cAMP. Figures 2 and 3 demonstrated that cAMP in cells increased the contraction duration, decreased contraction tension, dilation tension, peak height and mean tension with concentration-dependent manners. It can be the effect of activated PKA by dbcAMP. Then we had a question: why can't we use the AC inhibitor (decrease cAMP) to enhance the contraction? SQ 22,530 is a common tool agent, which effectively inhibits AC and decreases the level of cAMP. Theoretically, SQ 22,530 decreased the level of cAMP, and should take opposite effect of PDE inhibitor Z90. However, our experiment did not agree with it. This abnormal phenomenon was noticeable. Although the AC inhibitor (SQ 22,530) decreased cAMP, deactivated PKA and BKCa, lowered the permeability of potassium, enhanced membrane depolarization, it also

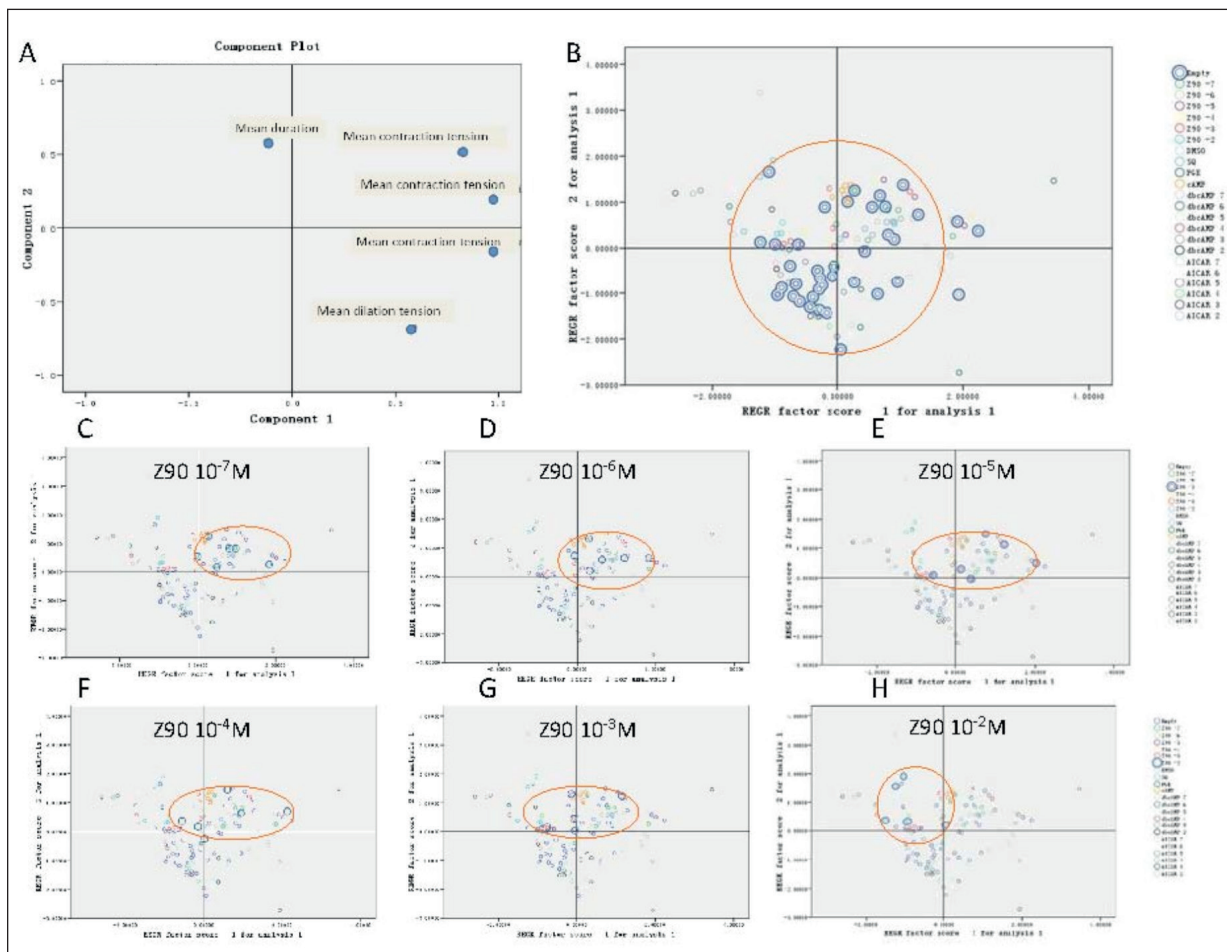
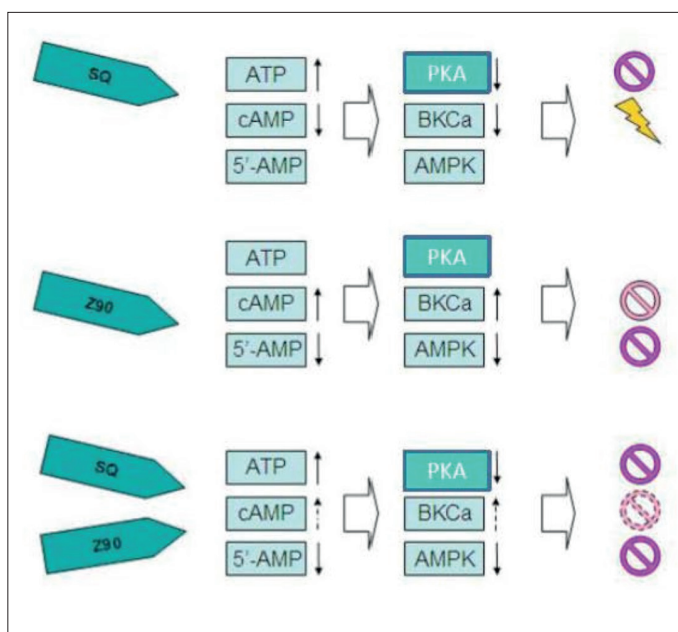


Figure 7. Principal component analysis. **A**, Component plot analysis schematic diagram. We use SPSS15 (SPSS Inc., Chicago, IL, USA) to analyze all data collected when no drug or only one drug alone takes effect including blank control, Z90 alone, DMSO alone, SQ alone, cAMP alone, dbcAMP alone and AICAR alone. Two main components are factors of contraction of the uterine smooth muscle. Component 1 contains peak height, contraction tension and means tension; Component 2 contains duration and dilation tension. We can describe the effect of each treatment on these two components. **B**, Spots in blue circles are blank controls, which distribute in a circle range from (0, 0). **C-H**: the effects of gradient concentration of Z90, spots in blue circles are different concentrations of Z90. They are all above the X-axis which means the prolongation of duration and the decrease of dilation tension are not concentration-dependent, but when the concentration of Z90 increases, they move towards left which means the concentration-dependent effects are contraction tension, peak height, and mean tension.

affects the level of ATP, and indirectly affects AMPK. These two different influences might offset each other. The previous statement that Z90 inhibits PDE4, increases cAMP and decreases Ca^{2+} , seems to be hasty and questionable. AMPK is a kind of energy sensor of eukaryotic cells, which is closely related to energy regulation¹⁸ and metabolism¹⁹ including gene transcription, membrane transporter activity, mitochondrial synthesis, glucose and glycogen metabolism, protein metabolism, and fat metabolism. We speculated that 5'-AMP and energy sensor AMPK may affect the smooth muscle contraction. AICAR, a type of AMPK agonist, which can penetrate the

cell membrane, plays a functional role similar to that of 5'-AMP. We used AICAR to simulate the change of 5'-AMP concentration. Previous research had revealed that the activity of AMPK is regulated by the AMP/ATP ratio. AMPK is activated when AMP/ATP increases (e.g. hypoxia, exercise, shock and low blood sugar). Activated AMPK will maintain the supply of energy for cell²⁰, inhibit the consumption and enhance the generation of ATP. AMPK is deactivated when the AMP/ATP ratio decreases, followed by the reduction of the glucose transporter and uptake²¹, decrease of generation of cGMP²², reduction of energy consumption and other effects. Thus, the

Figure 8. The deduced mechanism of effects of SQ and Z90. SQ increases the level of ATP, deactivates AMPK, lowers the consumption of energy and inhibits the contraction of smooth muscle; SQ may also decrease cAMP by blocking its source, deactivate PKA and BKCa, lower the permeability of potassium, enhance membrane depolarization and then increase the contraction activity. These two effects may offset each other. Z90 increases the level of cAMP, activates PKA and BKCa and then inhibits the contraction; Z90 may also block the source of 5'-AMP and deactivate AMPK. High concentration of Z90 deactivates most of AMPK and inhibits the contraction, which is reflected by sudden falls of parameters on our test curves. A combination of SQ and Z90 increases the ATP level, decreases the AMP level, decreases AMP/ATP and deactivates AMPK, and then the contraction is inhibited. Ban symbol indicates the inhibition of smooth muscle contraction, while lightning symbol indicates the enhancement of smooth muscle contraction.



contraction of the smooth muscle can be inhibited when AMPK is over-activated (similar with the effect of AICAR). When AMPK is deactivated (like the decrease of 5'-AMP), the contraction can be inhibited too. In conclusion, as the energy sensor and controller of ATP/AMP for cell, the activity of AMPK is well balanced in which condition the smooth muscle contracts appropriately. Either too much ATP or too much AMP can inhibit the contraction of the smooth muscle. A recent research shows that AMPK has phosphatase activity for Ser815 of MLCK. The phosphorylation has an effect of desensitization of MLCK; when MLCK is not sensitive to the calcium-triggered calmodulin activation, its ability to trigger the formation of myosin cross-bridge and to induce muscle contraction, is inhibited. cAMP is derived from ATP via AC while 5'-AMP from cAMP via PDE. The block of PDE induces dilation of uterine smooth muscle while block of AC cannot induce contraction. That meant the factor was not cAMP, but 5'-AMP. Block of PDE by Z90 can lead to the decrease of 5'-AMP, increase of cAMP and cAMP/5'-AMP. Block of AC by SQ 22,530 can lead to the decrease of cAMP, but may not cause immediate decrease of 5'-AMP. Block of both AC and PDE can lead to the decrease of 5'-AMP and cAMP which exhausts the upstream source of 5'-AMP. As 5'-AMP can regulate its downstream AMPK, it is also an intracellular messenger²³. Figure 8 concludes the deduced

mechanism of the effect of SQ and Z90. Based on these analyses, we can provide an explanation for the aforementioned abnormal phenomenon, that SQ alone will not make any difference, but when it is combined with Z90, it can produce a synergistic effect. There may be two reasons for that: first, AMPK may have stronger effects than BKCa; second, cAMP may increase when SQ combined with Z90. Now, we summarized the effect of cAMP and its components on rat uterine smooth muscle in Figure 9: PGE₂ takes effect via the EP₃ receptor which coupled with G-protein on cellular membrane²⁴, and that inhibits the adenylate cyclase and reduces the cAMP²⁵. Importantly, activates phospholipase and enhances the release of intracellular calcium²⁶. Oxytocin takes effect via the oxytocin receptors, which also coupled with G-protein and influences the calcium release. Acetylcholine influences the intracellular Na⁺ level through the acetylcholine M receptor. Z90 inhibits the PDE4 activity, increases the cAMP level in cell. SQ 22,530 is a common tool agent which effectively inhibits AC and decreases the level of cAMP. dbcAMP activates BKCa by the activation of PKA, making the K⁺ outflow and membrane hyperpolarization. The membrane potential then declined and smooth muscle contraction was inhibited. AICAR activated AMPK then lowered the consumption of energy and promoted decomposition of ATP rather than synthesis²⁷, so smooth muscle contraction was also inhibited.

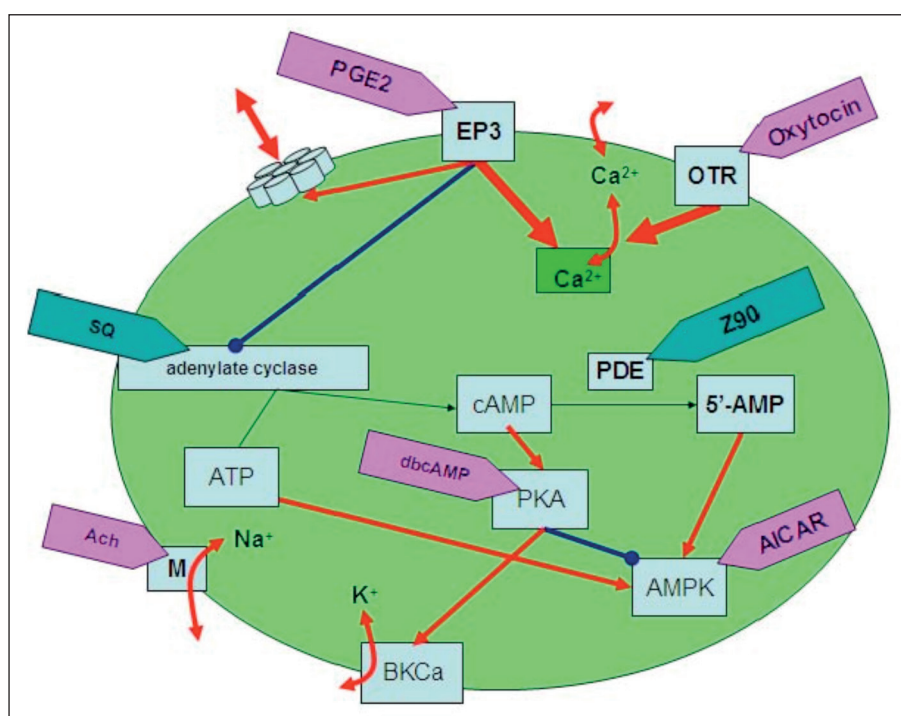


Figure 9. Possible molecular pathway of cAMP and its related components on contraction of uterine smooth muscle. PGE2 takes effect via the EP3 receptor, which coupled with G-protein on cellular membrane, that inhibits the adenylate cyclase and reduces the cAMP, and more importantly, activates phospholipase and enhances the release of intracellular calcium. Oxytocin takes effect via oxytocin receptors, which also coupled with G-protein and influences the calcium release. Acetylcholine influences the intracellular Na⁺ level through acetylcholine M receptor. Z90 inhibits PDE4, increases the cAMP level in cell. SQ 22,530 is a common tool agent which effectively inhibits AC and decreases the level of cAMP. dbcAMP activates BKCα by the activation of PKA, making the K⁺ outflow and membrane hyperpolarization. The membrane potential then declines and the smooth muscle contraction is inhibited. AICAR activates AMPK then lowers the consumption of energy and promotes decomposition of ATP rather than synthesis. Green oval: smooth muscle cell; light blue boxes: membrane proteins, channels, intracellular proteins and non-protein molecule; seven conjoined bodies: gap junctions. Purple head: agonist; gray and blue head: inhibitor; red head: enhancement or activation; blue round head: inhibition or inactivation; black head: transforming.

Conclusions

Our findings suggested that cAMP and its associated components mediated the myometrial relaxation, and the results depended on the balance of cAMP/5'-AMP and ATP. Recent reports suggested that PKA is not the sole mediator of cAMP-induced myometrial relaxation, and that prolonged prophylactic elevation of cAMP alone is unlikely to prevent PTL²⁸. With the contradictory finding in the experiment, the research tries to explore the still unknown signal pathway of the second messenger affecting the uterine smooth muscle contraction. The signaling pathway discovered and confirmed in the experiment may be a new explanation and proof on the physiological role of the cAMP and its related components, and also becomes the target of a new type of drug. Reasonable and effective inhibition of the uterine smooth muscle contraction is very im-

portant to the treatment of premature delivery in pregnant women and women with dysmenorrhea. The results of this experiment as a preliminary application basis on the clinical regulation of uterine smooth muscle contraction and diastolic can provide some suggestions for the prevention and treatment of preterm labor as well as the development of dysmenorrhea drugs.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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