Protein Kinase C May Not Involved in the Initiation of Decidualization at Pseudopregnant Rats

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Protein kinase C (PKC) isoforms have previously been found to be associated with the modulation of decidualization in pseudopregnant and pregnant rats. This study further investigates the effect of diphenhydramine on the expressions of PKC isoforms during trauma-stimulated decidualization. After trauma-stimulation, decidualization was inhibited by co-treatment with 1 mg diphenhydramine per rat. Levels of PKC α and PKC ζ proteins were down-modulated at hour 4 and 8, then returned to base level at hour 72. Moreover, pretreatment with the PKC inhibitors staurosporine and H7 blocked the inhibitory effect of diphenhydramine or 12-o-tetrodecanoyl-phobol-13-acetate (TPA). The inhibitory effect of other anti-decidualization agents, however, was not reversed by staurosporine. When pretreated with staurosporine only, decidualization was not influenced. Therefore, we suggested that PKCs may be uninvolved in the initiation decidualization in pseudopregnant rats.

Key words: protein kinase C, decidualization, diphenhydramine

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Introduction

Decidualization induced by the transformation of sensitized endometrium can be initiated by a variety of stimuli. It has been proposed that histamine and prostaglandins are released in the uterus in response to these stimuli [1]. Histamine, secreted from the mass cells in the uterus, is believed to stimulate conversion of stromal cells to decidual cells, which consequently grow to form deciduomata [2]. The histamine antagonists instilled into the uterine lumen prevent the formation of deciduomata and reduce the number of implantations in rats [3,4]. Therefore, it is suggest that histamine is responsible for initiating the decidual reaction, although the essential role of histamine remains paradoxical [5].

Histamine is a potent activator of phospholipase C [6], leading to polyphosphoinositide hydrolysis and subsequent increase in intracellular diacyglycerol. Diacylglcerol is well known to enhance protein kinase C (PKC) activity and cell growth. In response to extracellular signals such as growth factors, hormones, and neurotransmitters [7,8], PKC may play an essential role in trans-membrane signal transduction. It is also found to induce many cellular responses including cell proliferation, differentiation, gene expression and tumor promotion [9]. To date, twelve isoenzymes of PKC (α, β I, β II, γ, δ, ε, ζ, η, τ, ι, λ and μ) have been identified [10-13]. These isoenzymes exhibit differences in function and localization [14-17]. However, in decidualization the various expressions of PKCs in response to histamine are not known.

Our recent studies have found that the expression of PKC is involved in the cell growth of decidual cells [18,19]. The PKC α protein is highly localized on the membrane region in the proliferation period, and PKC ζ and PKC δ greatly increase, both on the membrane and in cytosolic regions. Thus, these findings suggested that PKCs may play an important role in decidualization.

In this study, we investigated the effect of a histamine antagonist diphenhydramine on the expressions of PKC isoforms in the initiation of decidualization. The results show that the initiation of decidualization blocked by diphenhydramine was correlated with PKCs activation and down-modulation. The inhibitory effect of diphenhydramine was reversed by PKC inhibitor staurosporine, suggesting that PKCs may negatively modulate the initiation of decidualization.

Methods

Chemicals

Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), ethylenediamine-tetraacetic acid (EDTA), sucrose, ethyleneglycol bis(α-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), leupeptin, soybean trypsin inhibitor type I-S, β-mercaptoethanol, Triton X-100 and $MgCl_2$ were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal treatment

Virgin Sprague-Dawley rats (National Science Council Animal Center, Taipei, Taiwan, R.O.C.) weighing 180-300g were maintained in a temperature-controlled room (at 24 ℃) and illuminated for 12 hours daily (lights on from 5 AM to 5 PM). Vaginal smears were obtained daily and only those animals showing two consecutive 4-day cycles were used. Pseudopregnancy was induced by vagino-cervical vibration with a glass rod for 1 min on the morning of estrus [20]. Uterine decidualization was induced by twice scratching the endometrium on the morning of day 4 of pseudopregnancy [18]. One uterine horn was used for experiment. The lumen was injected (0.1 ml) with

an anti-decidualized agent or/and PKC inhibitors (staurosporine and H7). These variously consisted of diphenhydramine (1mg), promethazine (0.5 mg), 12-o-tetrodecanoyl-phobol-13-acetate - TPA, a PKC activator - (100 ng) and 20 nmole epinephrine. The other horn was injected with saline as control group. On hours 0, 4, 8, 24, 48 and 72 after scratching stimulation both horns were removed, weighed, and quickly frozen on dry ice for tissue extraction.

Preparation of cytosolic and particulate fractions

All procedures were performed at 4℃. The uterine tissues were slit open and the endometrial tissues were removed from the myometrium by gentle scraping with a glass slide. Both treated- and control-samples were homogenized by thirty strokes using a Konetes homogenizer at a ratio of 1 g tissue/10 ml homogenization buffer A. The homogenization buffer A consisted of 20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.3 M sucrose, 2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor type I-S and 50 mM β-mercaptoethanol at pH 7.5. These homogenates were centrifuged at 100,000g for 1 hour at 4℃. The supernatant was stored at -70 ℃ for the cytosolic fraction and the pellet was suspended in buffer B (20 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 2 mM PMSF, 10 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor type I-S, 50 mM β-mercaptoethanol and 0.1% Triton X-100 at pH 7.5) and homogenized with thirty strokes. A 30 min exposure to 0.1% Triton X-100 at 0oC has been demonstrated to extract PKC from tissue suspensions of rat brain, liver, and kidney [21]. This procedure was modified and the homogenates were incubated at 4℃ in a rotating mixer for 1 hour. Centrifugation (100,000 g, 1 hour) was performed to remove any non-solubilized material and the supernatant was then referred to as the particulate fraction and stored at -70℃. The enzyme was stable for at least 1 month.

Electrophoresis and Western Blot

Tissue extract samples were prepared as described above. The protein content was determined using the Bio-Tad assay with bovine serum albumin as a standard. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli [22] using 10% polyacrylamide gels. Equal amounts of protein were loaded. After samples were electrophoresed at 140 V for 3.5 hours, the gels were equilibrated for 15 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (v/v) methanol, as described by Brockenbrough et al. [23]. Electrophoresed proteins were transferred to nitrocellulose paper (Amersham, Hybond-C Extra Supported, 0.45 Micro) using Hoefer Scientific Instruments Transphor Units at 100 mA for 14 hours. Nitrocellulose papers were incubated at room temperature for 2 hours in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 and 3% (v/v) fetal bovine serum. Monoclonal antibodies to PKC isoforms (Transduction Laboratories) were diluted 1:200 in antibody binding buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 and 1% (v/v) fetal bovine serum. Incubations were performed at room temperature for 3.5 hours. The immunoblots were washed three times in 50 ml blotting buffer for 10 min and then immersed in the second antibody solution containing alkaline phosphatase goat anti-rabbit IgG (Promega) for 1 hour and diluted 1000-fold in binding buffer. The filters were then washed three times in blotting buffer for 10 min. Color development was presented in a 20 ml mixture consisting of 7 mg nitro blue tetrazolium, 5 mg 5-bromo-4-chloro-3- indolyl-phosphate, 100 mM NaCl and 5 mM $MgCl_2$ in 100 mM Tris-HCl, pH 9.5. The band density was

quantified by densitometer (Alphalmager 2000, Alpha Innotech Corporation).

Histology examination

After removal of the uterus, small portions were immediately fixed in 10% formalin and stained with hematoxylin and eosin. For the mitotic index, at least 10000-15000 nuclei were scored for each time point.

Results

The Effect of Diphenhydramine on the Development of the Deciduomata in Rat Uterus

As shown in previous reports, the rat uterine horn undergoes a large increase in weight (Fig. 1 and 2). The increased weight in the control group is attributed to growth of the deciduomata as well as hypertrophy of the myometrial cells, while the increased weight in the diphenhydramine-treated group is attributed only to hypertrophy of the myometrial cells. The rate of the decidual cell mitosis in the saline-treated group was increased at 24 hours (data not shown), and reached a maximum (0.0051%) at 48-72 hours, while the decidual cell mitosis in the diphenhydramine-treated group was not observed.

The Effect of Diphenhydramine on the Expression of PKC Isoforms in Decidualization

Because the two isoforms (α and ζ) of PKC were significantly correlated with the initiation of the decidualization in pseudopregnant rat [18], we then examined the effect of diphenhydramine on the expression of these isoforms in the early stage of decidualization. These data reveal that PKCα content in the cytosolic fraction of the deciduomata of the saline-treated group on hours 48-72 decreased 40%-52% as compared to hour 0 (Fig. 2A and 2B). PKCα content increased 50%-60% in the particulate fraction on hours 48-72 as compared to hour 0 (Fig. 3A and 3B). This phenomenon was designated as PKC activation and preceded the increase in cell mitosis, which was maximal at 48-72 hours.

The diphenhydramine-treated group PKCα content in both the cytosolic and particulate fractions of the endometrium decreased 50%-60% at hours 4-8 as compared to hour 0 (Fig. 2A, 2B, 3A and 3B), and then reversed at hour 72 to the basal level. This event was also designated as PKC activation and down-modulation [24], but prevented cell mitosis and decidualization. In the saline-treated group, PKCζ content increased 80%-150% in both the cytosolic and particulate fractions of the deciduomata of on hours 48-72 as compared to hour 0 (Fig. 2A, 3A, 2C and 3C). In the diphenhydramine-treated group PKCζ content also decreased 50%-70% in the cytosolic and particulate fractions of the endometrium of the on hours 4-8 as compared to hour 0 (Fig. 2A, 3A, 2C and 3C), and then reversed at hour 72 to the basal level .

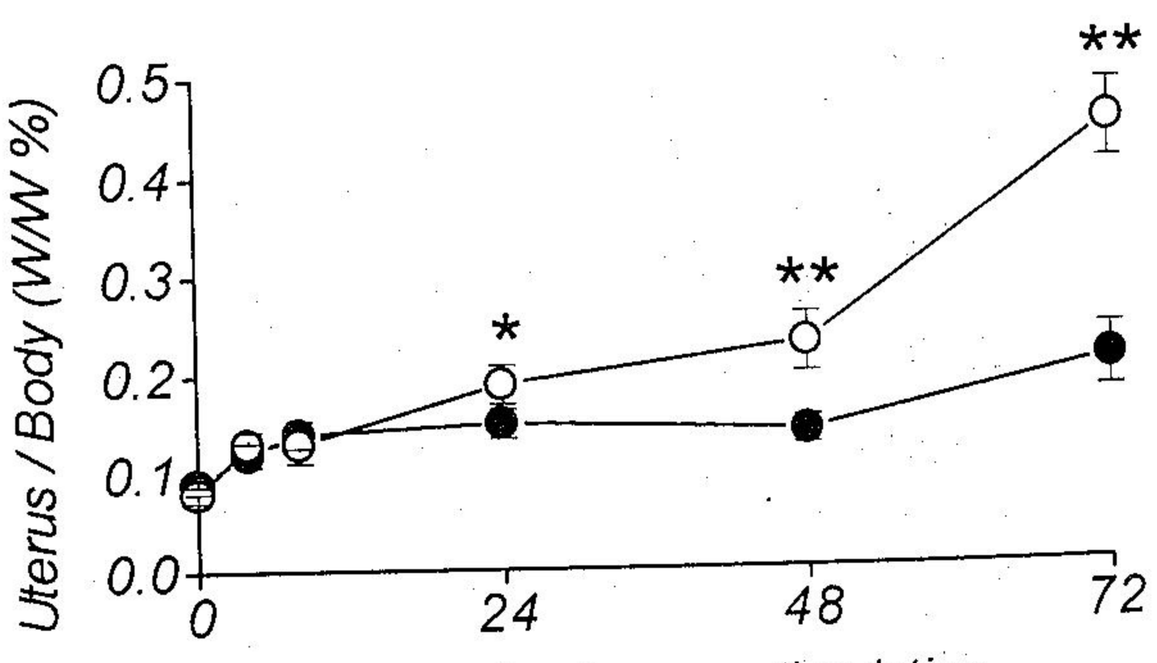

Fig. 1 Change in uterine horn weight after induction of decidualization by uterine trauma stimulation. Values expressed are the mean±S.D. for 3-5 measurements. —o—: the saline-treated uterine horn; — • —: the 1 mg/0.1ml diphenhydramine-treated uterine horn. *, P<0.05 as comparing to diphenhydramine-treated group. **, P<0.01 as comparing to diphenhydramine-treated group.

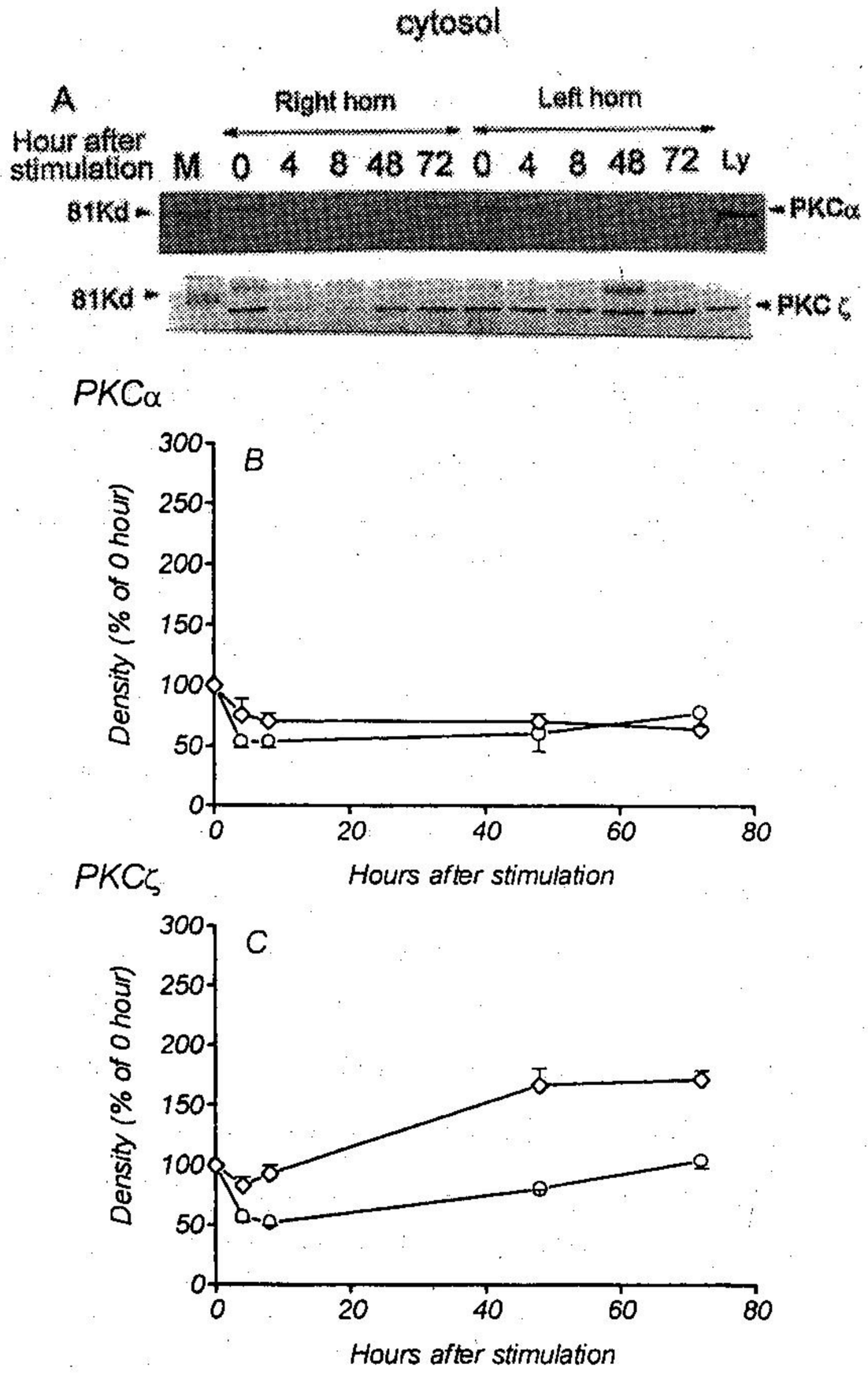

Fig. 2 Immunoblot analysis of PKC α and ζ isoenzymes in the cytosolic fraction. Uterine cytosolic fraction was prepared as described in " Materials and Methods". Aliquots of the uterus homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isoenzyme-specific antibodies. Samples in each lane were from individual rats (A). The quantitative data of PKC α of the cytosolic fraction from all experimental animals are presented (B). The quantitative data of PKC ζ of the cytosolic fraction from all experimental animals are presented (C). On hours 0, 4, 8, 48 and 72, the samples were the homogenates of the endometrium or the deciduomata. The levels of PKC isoforms on hour 0 were defined as 100%. Values expressed were the mean for 3-5 measurements. $-o-$: Right horn, 1mg/0.1 ml diphenhydramine-treated group; $-\diamond-$: Left horn, saline-treated group.

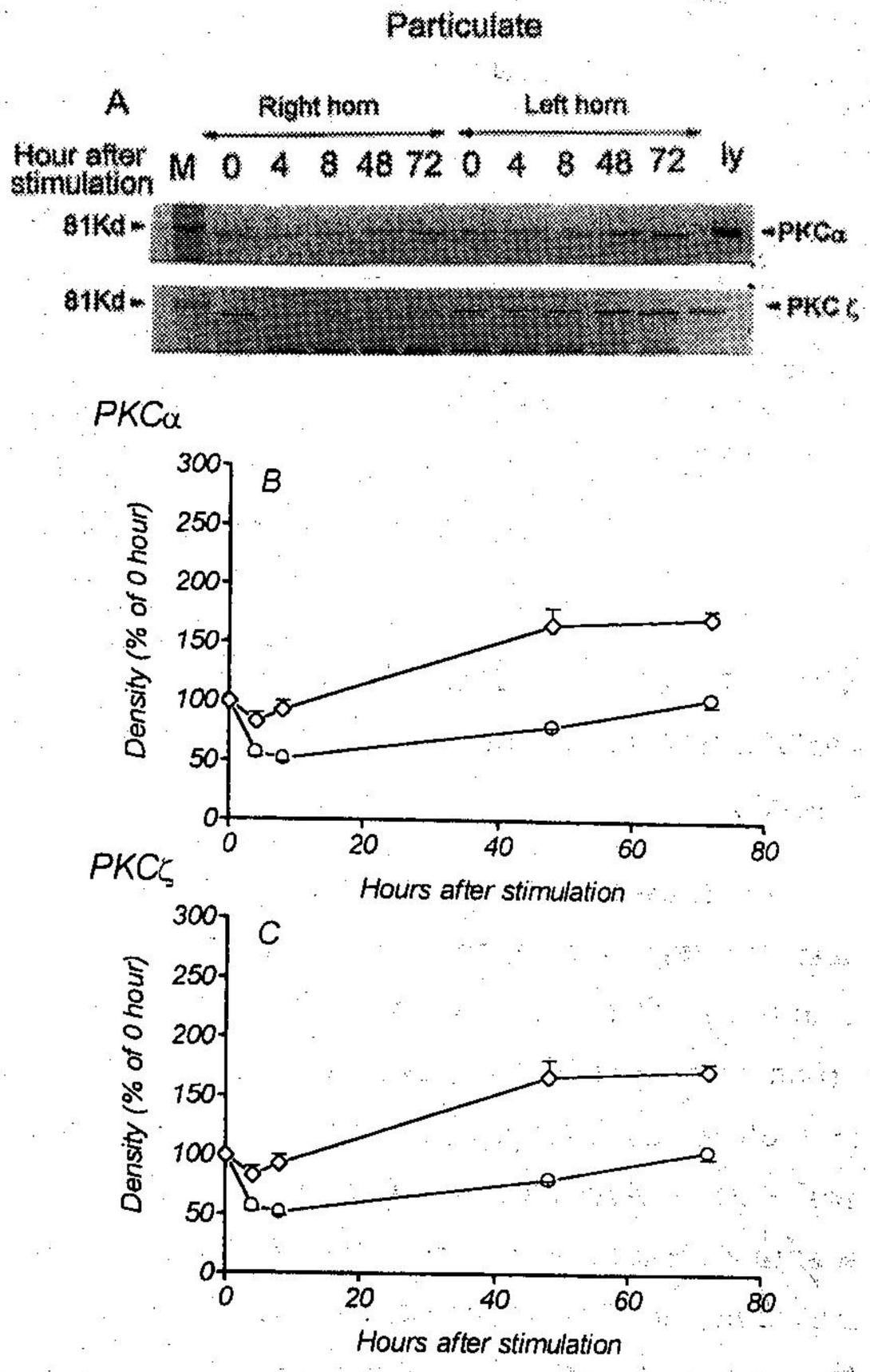

Fig. 3 Immunoblot analysis of PKC α and ζ isoenzymes in the particulate fraction. Uterine particulate fraction was prepared as described in "Materials and Methods". Aliquots of the uterus homogenates were separated on denaturing polyacrylamide gels and transferred to nitrocellulose paper. The blots were stained with PKC isoenzyme-specific antibodies. Samples in each lane were from individual rats (A). The quantitative data of PKC α of the particulate fraction from all experimental animals are presented (B). The quantitative data of PKC ζ of the particulate fraction from all experimental animals are presented (C). On hours 0, 4, 8, 48 and 72, the samples were the homogenates of the endometrium or the deciduomata. The levels of PKC isoforms on hour 0 were defined as 100%. Values expressed were the mean for 3-5 measurements. $-o-$: Right horn, 1mg/0.1 ml diphenhydramine-treated group; $-\diamond-$: Left horn, saline-treated group.

The Response of the Anti-Decidualized Agents Modified by PKC Inhibitors in Decidualization

To verify that PKC activation existed in the endometrium of the diphenhydramine-treated group, the strauma-stimulated horn of the uterus was co-treated with PKC inhibitors (staurosporine and H7) and diphenhydramine. The result showed that the impairment of decidualization by diphenhydramine was inhibited by staurosporine and H7. Of note, the inhibition by staurosporine was found to be dose dependent (Fig. 4). Other anti-decidualized agents such as promethazine, TPA and epinephrine also impaired the decidualization. Except for TPA, they were not inhibited by staurosporine (Fig. 5).

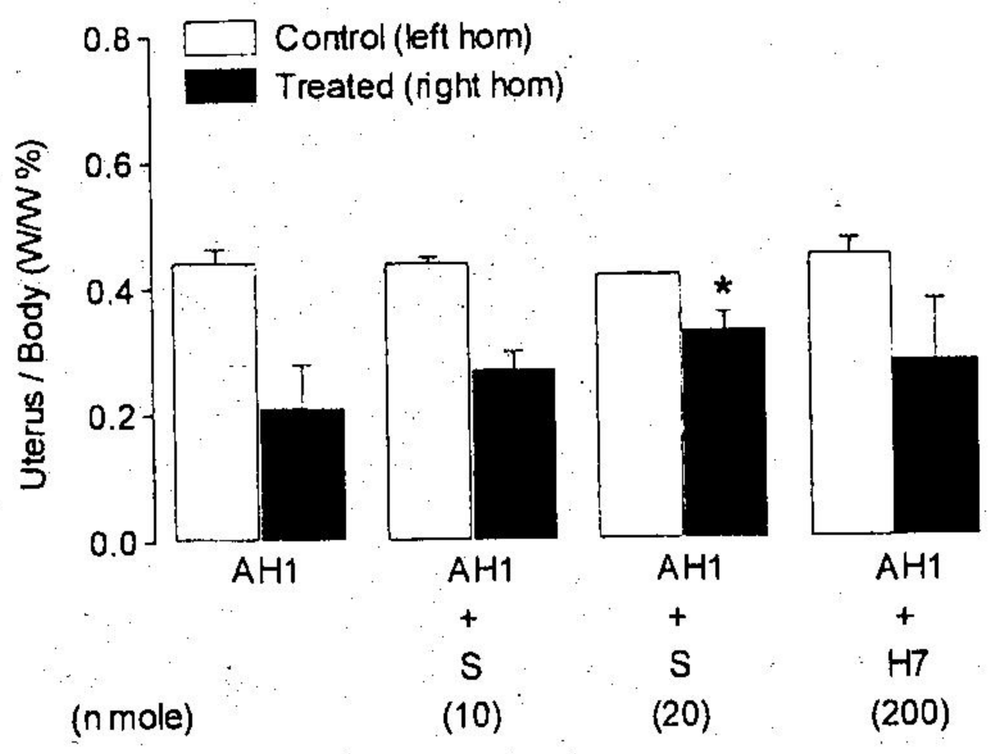

Fig. 4 Change in uterine horn weight on hour 72 after induction of decidualization by uterine trauma stimulation, or combined with 1mg diphenhydramine (AH1) and PKC inhibitors (S, staurosporine). Values expressed are the mean ±S.D. for 3-5 measurements. *, P<0.05 as comparing to diphenhydramine treated only.

Discussion

Results of this study confirm that PKC isoforms may be involved in the modulation of ducidual cell growth. In agreement with our previous data [18,19] we here found that the expression of PKCα in the saline-treated group was increased and localized in the particulate fraction from hour 48 to hour 72 of decidual cell proliferation. Further, PKCζ simultaneously increased in the cytosolic and particulate fractions.

PKCα and PKCζ were found to be down-regulated after diphenhydramine treatment, and the initiation of decidualization was consequently blocked. When co-treated with PKC-inhibitors staurosporine or H7, the effect of diphenhydramine on decidualization was reversed.

We suggest that the inhibition of decidualization by diphenhydramine may be mediated through PKC activation and down-regulation. Feyles et al [25] also demonstrated that the activation of PKC by TPA inhibited the initiation of decidualization. Since both TPA and diphenhydramine caused the high activation of PKC and

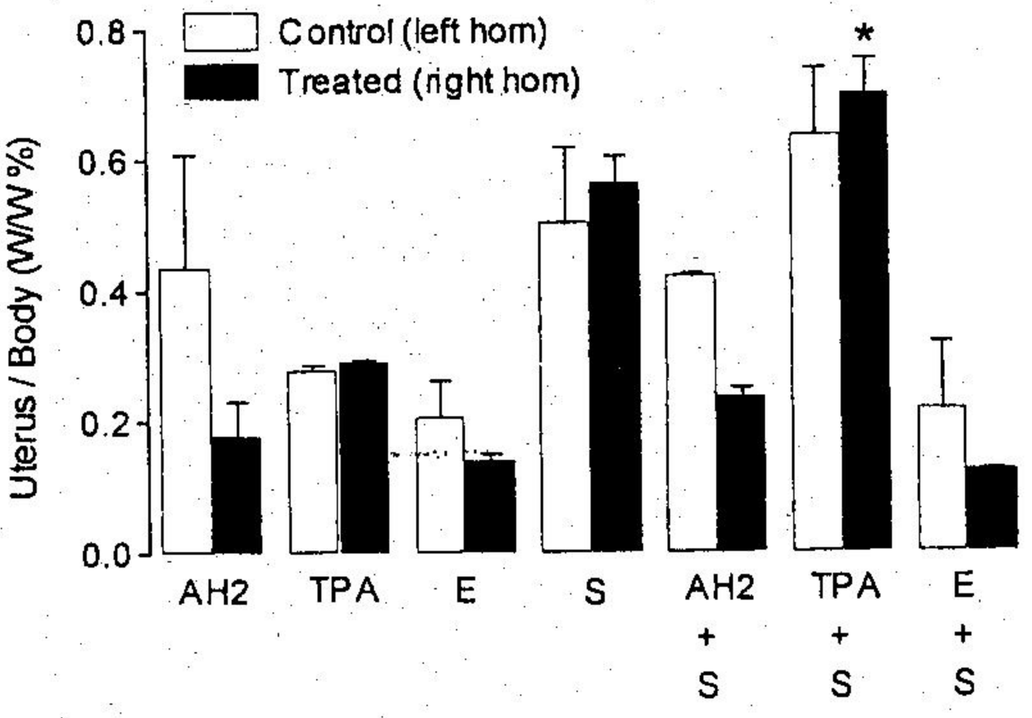

Fig. 5 Change in uterine horn weight on hour 72 after induction of decidualization by uterine trauma stimulation, or combined with anti-decidualized agents (0.5 mg AH2, promethazine; 100ng TPA, 12-o-tetrodecanoyl-phobol-13-acetate ; 400 ug E, epinephrine) and PKC inhibitor (20 nmol S, staurosporine). Values expressed are the mean ±S.D. for 3-5 measurements. *, P <0.05 as comparing to anti-decidualized agent treated only.

then down-regulation, this suggests that the inhibitory effect may be due to the depletion of PKC. Moreover, our data show that the development of decidual tissue was unaffected if only staurosporine was administered. Thus, we suggest that PKC may be uninvolved in the initiation of

decidualization, and it may in fact act as a negative modulator. Effects of the other anti-decidualization agents tested here were not reversed by staurosporine. The mechanism involved is unknown.

In summary, our data demonstrates that PKC activation and down-modulation by diphenhydramine is correlated with the inhibition of the initiation of decidualization. In the cell proliferation period of decidualization PKC was dramatically activated. Thus, it is suggested that PKCs may play dual distinct roles in decidualization.

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蛋白激酵素C可能沒有參與假孕鼠子宮蛻膜之誘發

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先前之研究發現假孕鼠與真懷孕鼠之子宮蛻膜形成與蛋白激酵素C異構體之表現有關，然而組織胺（Histamine）對蛋白激酵素之影響至今尚未清楚。本篇實驗乃應用抗組織胺藥物（Diphenhydramine）對於假孕鼠子宮蛻膜形成時蛋白激酵素C異構體之表現加以觀察。大白鼠施予刮劃刺激誘導後應用抗組織胺藥物注射，結果發現抗組織胺藥物組子宮蛻膜形成被抑制，蛋白激酵素C異構體α和ζ表現在蛻膜細胞質與微粒部份含量都降低。若同時給予蛋白激酵素C抑制劑（Staurosporine, H7）之處理，結果發現Diphenhydramine和TPA抑制子宮蛻膜形成有被抑制的現象，而其他抗蛻膜形成相關之藥物諸如Promethazine、Epinephrine等就無法恢復。另外單獨應用Staurosporine處理，不影響子宮蛻膜之形成。綜合以上結果，推論蛋白激酵素C可能不需要參與假孕鼠子宮蛻膜形成初期之誘發。

關鍵詞：蛋白激酵素C、子宮蛻膜形成、抗組織胺藥物

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