行政院國家科學委員會專題研究計畫　成果報告

不同亞型多巴胺受器對於血管張力素刺激皮質醛酮分泌調節的機轉研究

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-190-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院內科

計畫主持人：吳寬墩

計畫參與人員：張弘偉

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94 年 6 月 6 日

九十三年度國科會計畫期末報告

研究主題：第二型與第四型多巴胺受器對血管張力素刺激腎上腺皮質細胞分泌皮質酮的基轉，探討不同 PKC 酵素扮演的角色

中文摘要

背景：皮質酮的分泌已被證明受到多巴胺抑制，我們研究群之前已證明腎上腺皮質表現第二型與第四型多巴胺受器，而且它們對皮質酮的調控似乎扮演相反的角色，這兩種多巴胺受器均屬第二類多巴胺受器，它們的訊息傳導途徑可能不同，且尚未被了解。藉由人類腎上腺皮質癌細胞株 NCI-H295R 可受血管張力素刺激分泌皮質酮，且同時表現第二型與第四型多巴胺受器，我們以此細胞模型來進一步探討不同 PKC 亞型在第二型與第四型多巴胺受器對血管張力素刺激腎上腺皮質細胞分泌皮質酮的角色。

結果：血管張力素可刺激腎上腺皮質細胞表現皮質酮合成脢〈CYP11B2〉之 mRNA，此反應可被第四型多巴胺受器增強，但反而會被第二型多巴胺受器減弱。雖然血管張力素同時活化 PKC α / β II、PKC ε 、以及 PKC μ 。PD168,077 活化第四型多巴胺受器會選擇性加強血管張力素刺激的 PKC ε （Ser729）磷酸化，以及 PKC ε 轉位到細胞膜；以特定抑制 PKC ε 的多生肽抑制 PKC ε 顯著減弱血管張力素刺激之腎上腺皮質細胞表現皮質酮合成脢之 mRNA 以及皮質酮的分泌。另一方面，bromocriptine 活化第二型多巴胺受器會選擇性減弱血管張力素刺激的 PKC μ（Ser916）磷酸化，且抑制 PKC μ 轉位到細胞膜。雖然 PKC α / β II 抑制劑 chelerythrine 亦可減弱血管張力素刺激之腎上腺皮質細胞表現皮質酮合成脢之 mRNA 以及皮質酮的分泌，非選擇性 PKC α / β II 及 PKC μ 的抑制劑 Gö6976 可進一步壓抑血管張力素刺激之腎上腺皮質細胞表現皮質酮合成脢之 mRNA 以及皮質酮的分泌。

結論：我們的研究證明第二型與第四型多巴胺受器對血管張力素刺激腎上腺皮質細胞分泌皮質酮與表現皮質酮合成脢之 mRNA 的調控中扮演相反的角色，第四型多巴胺受器選擇性加強血管張力素刺激的 PKC ε 活化，而第二型多巴胺受器則可能是選擇性減弱血管張力素刺激的 PKC μ 活化，進而調控血管張力素刺激腎上腺皮質細胞分泌皮質酮與皮質酮合成脢之 mRNA 的表現。

英文摘要

Background (or purpose): There are evidences that aldosterone secretion is subjected to a dopaminergic inhibitory mechanism. Our previous study has demonstrated that both human D2 and D4 receptors can modulate aldosterone secretion, but in opposite direction. Belonging to the D2-like family, the signal pathways of these two dopamine receptors are not understood and may be different.

Methods: The human adrenocarcinoma (NCI-H295R) cells, which were demonstrated to express both D2 and D4 receptor proteins, were used to explore the roles of different PKC isoenzymes in dopaminergic regulation of aldosterone synthesis and secretion.

Results: The elevation of aldosterone synthase (CYP11B2) mRNA by angiotensin II (AII, 1μM) can be enhanced by D4 receptor, but attenuated by D2 receptor. Although AII activated PKCα/βII, PKCε and PKCμ, D4 agonist, PD168, 077, selectively enhanced AII-induced PKCε activation by specific phosphorylation on Ser729 and its translocation to cell membrane. Inhibition of PKCε by PKCε-specific inhibitory peptide significantly attenuated AII-induced elevation of CYP11B2 mRNA and aldosterone secretion. D2 agonist, bromocriptine, selectively attenuated AII-induced PKCμ activation by specific phosphorylation on Ser916. The AII-induced response was also attenuated by conventional PKC inhibitor, chlerythrine, and a further

attenuation was observed by adding Gö6976, a conventional PKC and PKCμ inhibitor.

Conclusion: These data indicate that D4 and D2 receptors have opposed regulatory effects on AII-stimulated aldosterone secretion and CYP11B2 mRNA expression by activation of different PKC isoenzymes, The effect of D4 receptor was attributed to enhance AII-induced PKCε activation and that of D2 receptor probably via the activation of PKCμ.

Results

D2 and D4 receptors expression on NCI-H295R cells.

In the previous study, we have demonstrated mRNA expression of human D2 and D4 receptors in H295R cells. Immunobloting with D2-receptor antibody showed 100-kDa-, 68-kDa-, and 50-kDa-sized bands as described in the manufacture's instruction in both human adrenal gland and H295R cells (fig 1a). The 50-kDa-sized protein is compatible with the size of D2 receptor. The D4 receptor-specific antibody revealed a single band around 50 kDa in both human adrenal cortical tissue and H295R cells (fig 1b). The signals were abolished when blotting with preimmunized serum or by pretreating the anti-D4 serum with D4 receptor-specific immunizing peptide (fig 1b).

AII-stimulated aldosterone secretion depends on an increase of CYP11B2 transcription

The basal level of aldosterone in the supernatant of 24 hour-cultured NCI-H295R cells was very low, approximately 0.15±0.01 pg/ml-μg protein. Elevation of aldosterone level was significantly as soon as 4 hours after adding AII. A dose-dependent increase of aldosterone level by AII was observed. The aldosterone concentration in the culture supernatant 24 hours after AII 1μM stimulation was 33.89±2.02 pg/ml-μg protein (fig 2a).

The basal level of CYP11B2 mRNA was much lower than CYP11B1 mRNA level (data not shown). Addition of AII significantly increased CYP11B2 mRNA level as early as at 2 hours, but no effect on CYP11B1 transcription. . The peak effect of AII on CYP11B2 transcription was observed at 8 hours (fig2c).

The increase of CYP11B2 mRNA level was completely abolished after treating the cells with actinomycin D 10 μg/ml (fig3a), which was accompanied by a decrease of aldosterone levels to the basal level without AII treatment (Fig 3b). The inhibitory effect of actinomycin D was also dose-dependent. The results suggest that the increase of aldosterone level in the in the supernatant by AII is largely dependent on the increase of CYP11B2 transcription.

Effects of D2 and D4 receptors on AII stimulated aldosterone through modulations of CYP11B2 transcription

As shown in our previous study, D2 receptor agonist, bromocriptine, attenuated the increase of AII-stimulated aldosterone secretion. A significant inhibitory effect of bromocrptine was observed as low as 10^{-8} M (fig 4a). The inhibitory effect of D2 receptor on the regulation of aldosterone secretion was further confirmed by a significant elevation of aldosterone level when a selective D2-antagonist, raclopride, was added to the cells treated with 1μM of AII and DA (Fig 4a). The enhancing effect of raclopride was significant with a level of 10^{-8} M (Fig 4a). On the contrary, D4

receptor-specific agonist (PD168, 077) enhanced, and D4 antagonist (L745, 870) attenuated the AII-stimulating effect on aldosterone secretion (Fig 4b).

Bromocriptine 1μM effectively attenuated AII-stimulated CYP11B2 expression at all 2, 4, 8, and 24 hours (fig 5a). On the other hand, raclopride enhanced the stimulatory effect of AII on the transcription of CYP11B2 (fig 5a). D4 agonist, PD168, 077 1μM, significantly enhanced, and D4 antagonist, L745, 870, attenuated the mRNA levels of CYP11B2 induced by AII (fig 5b).

D2 and D4 selectively influenced the activities of PKC isoenzymes induced by AII

Immunobloting with phospho-PKC specific antibodies demonstrated that AII increased the phosphorylation of PKCα/βII, PKCε（Ser 729）, PKCμ （Ser 744/748）and PKCμ（Ser 916）(fig 6a and 6b). The phosphorylation of Thr 638/641, PKCδ（Thr 505）, PKCδ（Ser 643）, PKCθ（Thr 538）, and PKCζ/λ（Thr 410/403）was, however, not altered by AII. Bromocriptine 1μM did not change basal and AII-stimulated phosphorylation of PKCα and PKCε, but attenuated PKCμ（Ser 916）phosphorylation induced by AII (fig 6a). In contrast, D2 antagonist, raclopride, further increased the phosphorylation of PKCμ（Ser 916）induced by AII, but exerted no effect on PKCα and PKCε. (fig 6a).

The D4 agonist, PD168, 077, did not alter the phosphorylation of PKCα and

PKCμ, but enhanced AII-stimulated phosphorylation of PKCε（Ser729）(fig 6b), which was attenuated when 1 μM D4 antagonist, L745, 870, was added, instead. (fig 6b).

Effect of D2 and D4 receptor on AII-stimulated PKC translocation

Immunobloting with antibodies for total forms of PKCα, PKCβII, and PKCε showed significant translocation of all the four PKC isoenzymes to the membrane fraction with a reciprocal decrease of their cytosol distributions. Bromocriptine selectively inhibited, and raclopride enhanced AII-induced translocation of PKCμ to the membrane (fig 7a). PD168, 077 selectively augmented AII-induced PKCε translocation to the membrane, and L745, 870 significantly attenuated PKCε translocation (fig 7b).

PKCε specific peptide inhibited AII-induced aldosterone secretion and CYP11B2 expression

The aldosterone and CYP11B2 mRNA levels induced by AII were not altered by saponin 15mg/ml. Administration of PKCε-specific inhibitory peptide blocked PKCε translocation and reduced AII-induced aldosterone secretion by approximate 25%. The AII-induced aldosterone secretion was not changed when the control peptide was added (fig 8). A similar effect of the PKCε specific peptide on CYP11B2 mRNA expression was also observed, with a reduction by 50% of the CYP11B2 mRNA (Fig

8)

Phosphorylation of PKCα and PKCμ on AII-stimulated aldosterone secretion and CYP11B2 mRNA expression.

The conventional PKC inhibitor, chelerythrine chloride, dose-dependently inhibited AII-stimulated aldosterone secretion (data not shown). The inhibitory effect of chelerythrine chloride was observed as low as 10^{-9}M and reached the plateau effect at a concentration greater than 10^{-7}M. The effect was accompanied by a decrease of CYP11B2 mRNA expression. Treatment with 1 μM Gö6976, a PKCμ to, also attenuated AII-stimulated aldosterone secretion, and CYP11B2 mRNA expression (data not shown). The PKCμ inhibitor, Gö6976 10^{-6}M, augmented the inhibitory effect of Chelerythrine 10^{-6}M on AII 1μM-stimulated aldosterone secretion, and the AII 1μM-stimulated CYP11B2 mRNA expression (fig 9).

DRD2 and DRD4 expression in NCI-H295R cells
and human adrenal gland

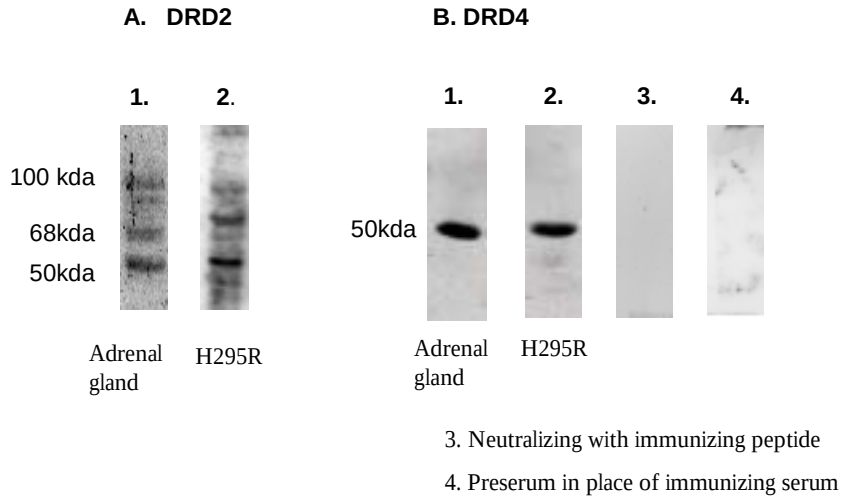
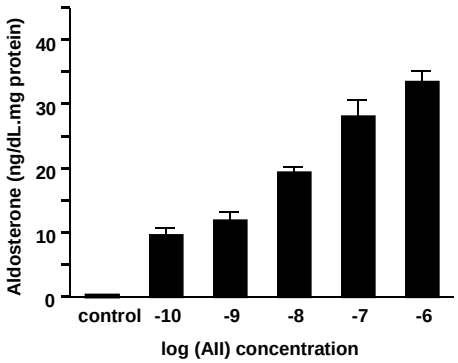
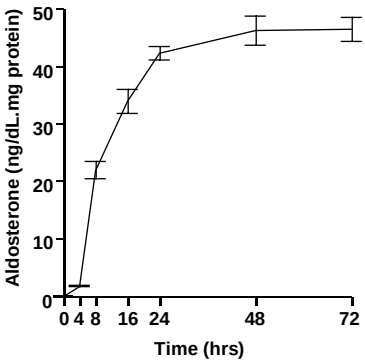


Fig 1: D2 and D4 receptors expression on human adrenal gland and NCI-H295R cells. A. Western blotting with commercial available D2 receptor antibody revealed three bands. The 50kDa-sized band was compatible with the size of human D2 receptor. B. Western blotting with D4 receptor antibody showed a single band approximately 50kDa, which was compatible with the size of human D4 receptor.

A



B



C

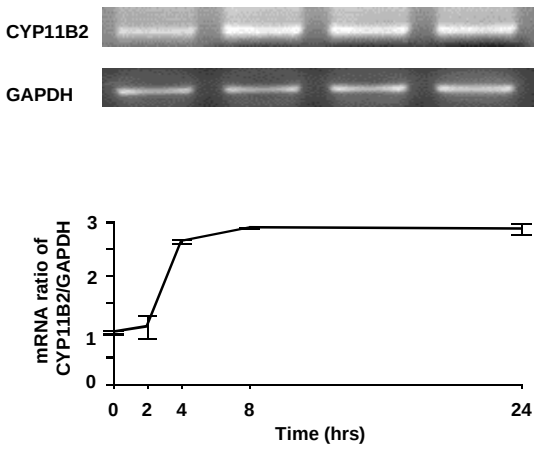


Fig 2: AII stimulated aldosterone secretion and CYP11B2 mRNA expression of NCI-H295R cells. A. Dose-dependent stimulation of aldosterone secretion 24 hours after AII treatment. B. Time-dependent accumulation of aldosterone in culture supernatant after AII 10^{-6}M treatment. C. Semi-quantification of CYP11B2 mRNA by RT-PCR was normalized with simultaneously amplified GAPDH mRNA.

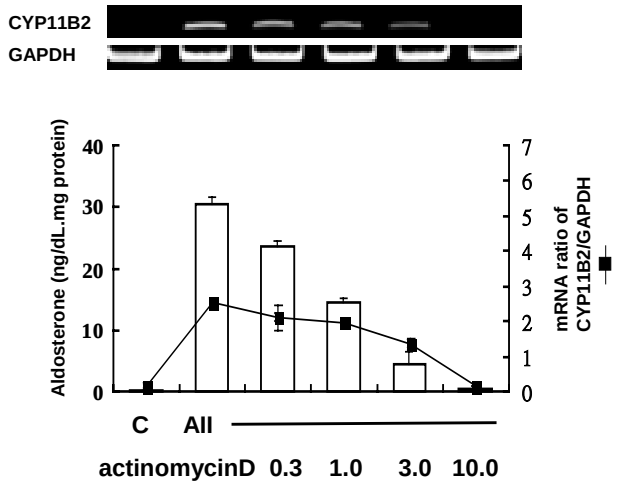


Fig. 3: Transcription inhibitor, actimomycin D, inhibited AII-stimulated CYP11B2 transcription as well as aldosterone secretion in the culture supernatant. Right scale: RT-PCR of CYP11B2 mRNA 4 hours after treatment is shown by mean±SD. Left scale: Aldosterone levels in the culture supernatant 24 hours after treatment are shown by mean±SD. All data was from quadraplicate results in 3 independent experiments.

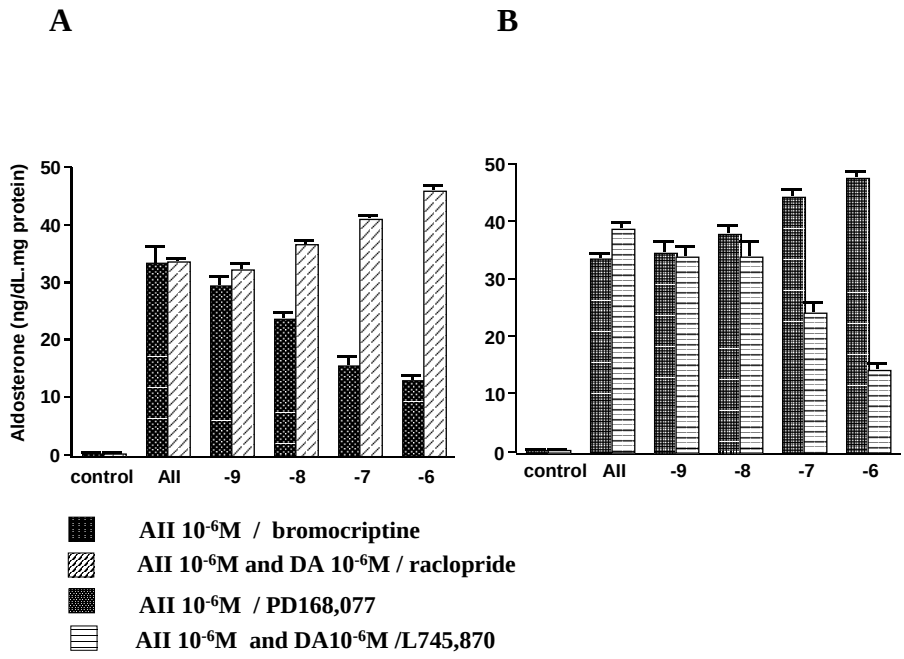


Fig. 4: D2 and D4 receptors differently regulated AII-stimulated aldosterone secretion of NCI-H295R cells. A: D2 agonist, bromocriptine10^{-9} to 10^{-6}M, dose-dependently inhibited AII-stimulated aldosterone secretion. On the other hand, D2 antagonist, raclopride 10^{-9} to 10^{-6}M, dose-dependently augmented AII 10^{-6}M and DA10^{-6}M co-stimulated aldosterone secretion. B: D4 agonist, PD168, 077 10^{-9} to 10^{-6}M, dose-dependently accentuated AII-stimulated aldosterone secretion. Conversely, D4 antagonist, L745, 870 10^{-9} to 10^{-6}M, dose-dependently attenuated AII 10^{-6}M and DA 10^{-6}M co-stimulated aldosterone secretion.

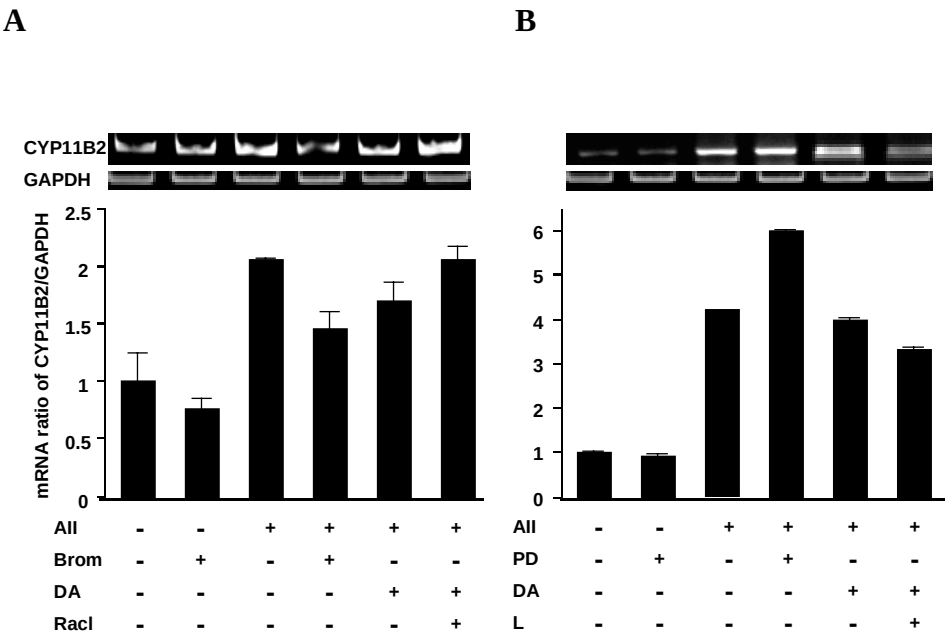
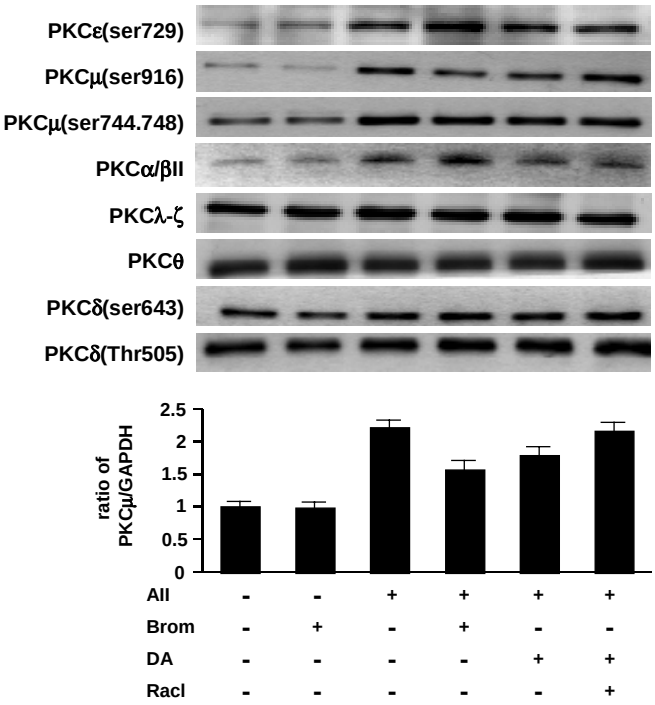


Fig. 5: D2 receptor attenuated AII-stimulated CYP11B2 mRNA expression of NCI-H295R cells, and D4 receptor accentuated AII-stimulated CYP11B2 mRNA expression of NCI-H295R cells. Total mRNA was collected 2, 4, 8, and 24 after treatment RT-PCR of CYP11B2 mRNA was normalized with simultaneously amplified GAPDH mRNA of each sample. Value of CYP11B2 mRNA 4 hours after treatment were shown as ratio of GAPDH. Data was shown by mean±SD from quadraplicate results in 3 independent experiment.

A



B

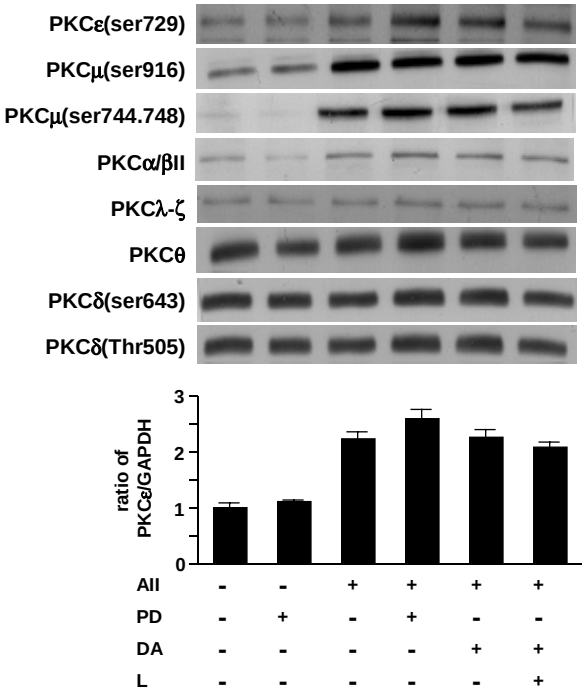
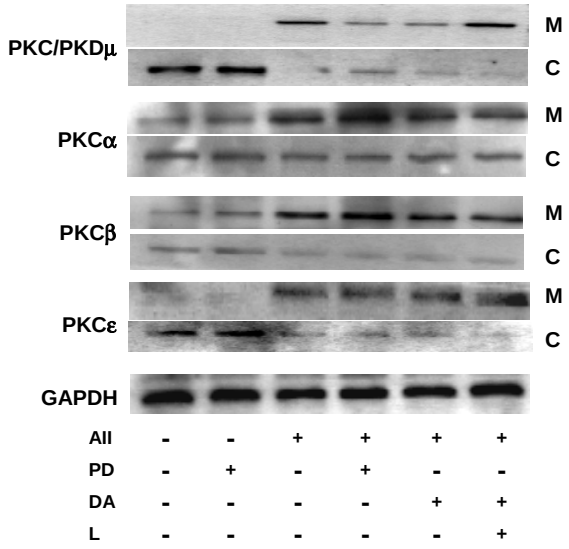


Fig. 6: AII activated phospho-PKC α / β II, ε and μ expression. A: D2 receptor selectively inhibited AII-activated phopho-PKC μ at Ser916. B: D4 receptor selectively enhanced AII-activated phopho-PKC ε .All samples were collected 5 minutes after treatment. Phospho-PKCs were detected by antibodies, which were specific to target phosphorylated sites on each PKC isoforms. All data was at least triplicates.

A



B

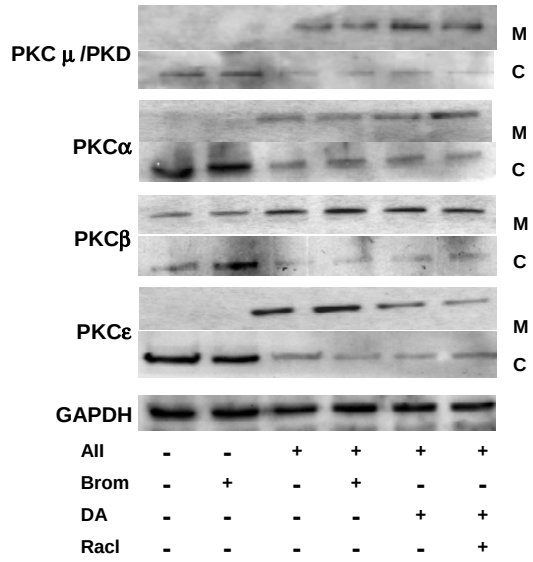


Fig. 7: AII activated phospho-PKC α, β II, ε and μ translocation. D2 receptor selectively attenuated AII-activated phopho-PKC μ. D4 receptor selectively enhanced AII-activated phopho-PKC ε. All samples were collected 5 minutes after treatment. All data was at least triplicated.

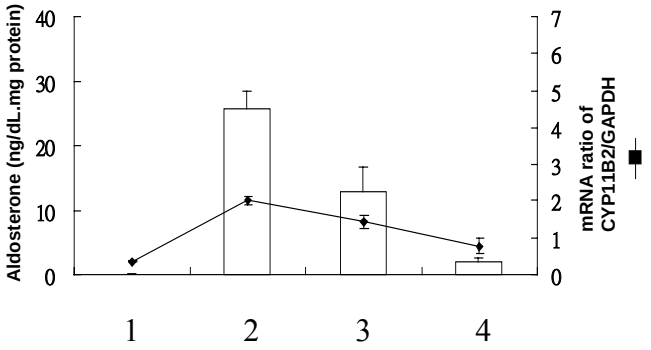


Fig. 8: PKC α / β II and PKC μ played the roles on AII-stimulated aldosterone secretion (left scale) and CYP11B2 mRNA expression (right scale). Lane 1 control, lane 2 AII 10^{-6}M, lane 3 AII and conventional PKC inhibitor chelerythrine 10^{-6}M, lane 4 AII and Go6976, an inhibitor for PKCα/ β II and PKC μ . Data was shown by mean± SD from quadruplicates results in 3 independent experiment.

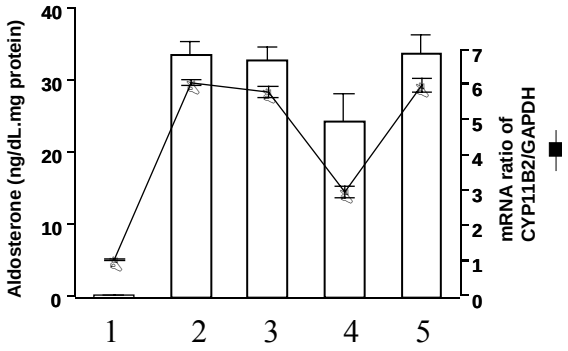


Fig 9: PKC ε specific inhibitory peptide inhibited AII-stimulated aldosterone secretion (left scale) and CYP11B2 mRNA expression (right scale). Lane 1 control, lane 2 AII 10-6M, Lane 3 AII and saponin 15 mg/ml, lane 4 AII, saponin and. PKC ε specific inhibitory peptide, lane 5 AII, saponin and control peptide Data was shown by mean±SD from quadruplicates results in 3 independent experiment.