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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※ 腎臟腺甘酸環化脢的基因表現及其與腎G蛋白質受體之間的互動探討
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計畫類別：■個別型計畫 □整合型計畫

計畫編號：NSC 89－2320 －B －040 －066 －

執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：林庭慧

共同主持人：

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學 生命科學系

中 華 民 國 90 年 10 月 31 日

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一、中文摘要

血管增壓素及前列腺激素 E2 如何互相作用及抗衡，以維持腎臟正常的水份再吸收功能，曾被廣泛研究。環腺甘酸 (cAMP), 以被證實牽涉其中。集尿管是腎臟發生水份再吸收作用相當頻繁的一段區域。在這段區域中，血管增壓素及前列腺激素 E2 如何互相傳達彼此訊息，及其與環腺甘酸 (cAMP) 之間如何互動，並不是很清楚。血管增壓素及前列腺激素 E2 都是經由結合其在細胞膜上之受體，來引發一連串訊息傳遞。V2 receptors 及 EP3 receptors 已被證實是血管增壓素及前列腺激素 E2 在腎臟集尿管中最主要之受體亞型。此二種亞型之受體，都是屬於 G 蛋白質連結之受體。V2 受體已被證實連結到 Gs 蛋白質，而 EP3 受體已被證實連結到 Gi 蛋白質。藉由所連結之 G 蛋白質的不同，可以調節細胞內環腺甘酸之含量。大部分的研究都著重在此二受體所結合之 G 蛋白質上，但忽略了腺甘酸環化酶 (Adenylyl Cyclases), 此催化環腺甘酸形成之酵素，對於細胞內環腺甘酸之含量，亦伴演了舉足輕重之調控角色。利用 RT-PCR，我們偵測到腺甘酸環化酶第六亞型(AC VI)是老鼠腎臟及腎臟集尿管細胞株 (M-1 cells)中，最主要之腺甘酸環化酶。此外，第三、五、九亞型之腺甘酸環化酶亞型亦存在於(M-1 cells)中。在血管增壓素受體，第二亞型(V2 receptor)，可在 M-1 cell line 中被偵測到，而第一亞型(V1a receptor)則完全偵測不到。至於前列腺激素 E2 受體 (PGE2 receptor)之分布情形，更為複雜。以我們目前的結果顯示，EP3 及 EP4 是老鼠腎臟集尿管細胞株 (M-1 cells)中最主要之 PGE2 receptors。在 M-1 cell line 中，加入 [Arg]-vasopressin 此血管增壓素之刺激劑，可偵測環腺甘酸之量約上升為原來細胞內環腺甘酸之 58% 左右。利用轉染(transfection)方式，將不同亞型之腺甘酸環化酶分別大量表現在 M-1 cell line 中，我們發現，第一亞型之腺甘酸環化酶大量表現之 M-1 cell line， 其血管增壓素刺激所產生之環腺甘酸略顯下降。而第三亞型之腺甘酸環化酶大量表現之 M-1 cell line，其血管增壓素刺激所產生之環腺甘酸上升約可至兩倍左右。

關鍵詞：血管增壓素、前列腺激素 E2、環腺甘酸

Abstract

Both AVP and PGE2 exert their physiological functions through binding to their receptors on the cell membranes. V2 receptors and EP3 receptors are the major receptors found in the collecting ducts which are the major segments in the kidney undergoing water reabsorption. Both receptors are G protein coupled receptors. V2 receptors are linked to Gsα protein while the EP3 receptors are linked to Gi protein. A lot of attention has been paid to the G protein in modulating the cAMP level inside the cell. However, not much work has been done in investigating which adenylyl cyclase (AC) isozymes are coupled to these receptors. ACs catalyze the formation of cAMP from ATP. In the recent years, the control of cAMP content in the cell has become more complex by the finding of several types of ACs with different regulatory properties.

Using RT-PCR, we have investigated the distribution of AC isozymes, V2 receptors and EP3 receptors in the rat kidney and M-1 cells. AC VI was the major AC isozyme found in both rat kidney and M-1 cells, while AC III, V, IX were also detected in M-1 cells. V2 receptor was detected as the major AVP receptor in M-1 cells, while V1a receptor was not detected. For PGE2 receptors, EP3 and EP4 were found in M-1 cells. The cAMP level in M-1 cells transfected with pCEP4 was increased 58.1 % after the activation of V2 receptor. In AC-I transfected M-1 cells, 13.05 % of the cAMP level was inhibited after AVP treatment. In AC III and AC VI-transfected M-1 cells, the cAMP level after AVP treatment was increased 206% and 73.75%, respectively.

Keywords: Vasopressin, PGE2, cAMP

二、緣由與目的

The present study is designed to understand the interactive mechanism between AVP and PGE2 in balancing water

reabsorption in kidney. AVP and PGE2 exhibited opposite physiological functions in kidney. Both AVP and PGE2 exerted their physiological functions through binding to their receptors on the cell membrane. V2 receptors and EP3 receptors are the main types of receptors for AVP and PGE2 in the collecting ducts. The collecting ducts are the major segment in kidney, which undergoes intensive water reabsorption. Both V2 receptors and EP3 receptors are G protein coupled receptors and their coupling efficiency to AC isozymes have never been reported. Investigation of the co-localization of V2 receptors and EP3 receptors in CCD segments and their coupling efficiency to ACs will provide more information to understand the opposite physiological functions of AVP and PGE2. By understanding the mechanism of AVP and PGE2 -evoked cAMP signaling pathway, the physiological roles of ACs in kidney will be elucidated.

三、Results (結果)

Effect of reverse transcriptase on V2 receptor mRNAs amplification in the kidney and M-1 Cells.

With specifically designed full-length V2 receptor primers as shown in table 1, a clear single band around 1100 bp was expressed in the kidney and M-1 cells using RT-PCR (fig.1, lane 1 and 2). When the PCR reaction was performed in the absence of reverse transcriptase, this 1100 bp band was not seen.(fig.1, lane 4) This indicated that the 1100 bp band originated from mRNA, not from contamination of genomic DNA. A beta-actin fragment around 680 bp was amplified as a positive control in the RT-PCR reaction (fig.1, lane3).

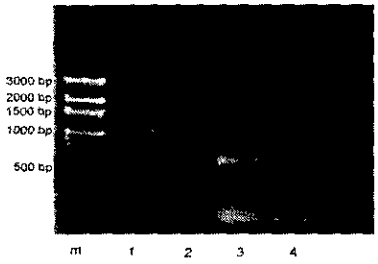

Figure 1. V2 receptor can be amplified in the kidney and M-1 cell using RT-PCR.

Distribution of V2, but not V1a receptors mRNAs in the M-1 cells.

RT-PCR was also used to identify the major AVP receptors in M-1 cell. Two sets of primers to detect V1a and V2 receptors were shown in table 1. As indicated in fig. 2, both the V1a and V2 receptors could be amplified in the kidney (fig.2, lane 1 and 2), however, only V2 receptor, but not V1a receptor could be detected in M-1 cells (fig.2, lane 4 and 5). This data consist with that V2 receptor is the major AVP receptor in the kidney collecting ducts.

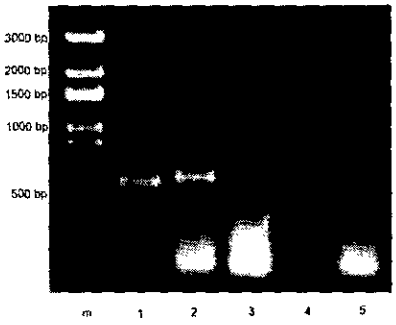

Figure2. Both V1a and V2 receptors can be amplified in the kidney; only V2 receptor can be detected in M-1 cells.

Detection of ACIII, ACV, ACVI and AC IX as the major AC isozymes in M-1 cells.

In order to further confirm the AC isozymes in M-1 cells. We designed degenerate primers, as shown in table 1, to amplify all subtypes of ACs in M-1 cells. A PCR fragment around 243 bp was amplified from the primer set designated as ACXf-ACEr (fig.3, lane3). The 243 bp PCR products obtained by the degenerate

primers were subcloned to pCR™II vector (*Invitrogen*). Sequence analysis detected AC III, V. VI, IX, but not other AC isozymes in M-1 cells. AC VI was shown to be the major AC isoform in M-1 cells, constituting 72% of all the isolated clones. AC V (20%), AC IX (5%) and AC III (1%) followed AC VI in the number of isolated clones (table 2). Thus, AC VI appears to be the major AC isozyme in M-1 cells.

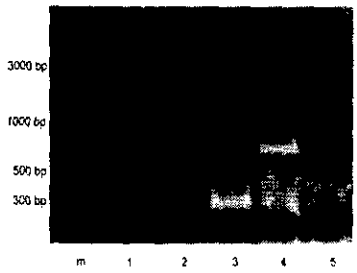

Fig. 3. Degenerate primers : ACXf (forward) and AC Er (reverse) were designed to amplify all subtypes of AC in M-1 cells by RT-PCR. The sequences of each set of primers were listed in table 1. A PCR product (243 bp) was amplified in M-1 cells using primers as seen in lane 3. Beta-actin was used as a positive control in lane 4. No RT as a negative control was shown in lane 5.

Confirmation of the expression of EP3 receptors mRNA in M-1 cells.

Primers designed to identify EP receptors were listed in table 1. Four distinct EP receptors have been identified in different tissues (Breyer et al., 2000). EP3 receptors have been reported as the major PGE2 receptors in the kidney collecting ducts (Breyer et al., 2000). Using primers listed in table 1, RT-PCR demonstrated EP1, EP3 and EP4 as the major EP receptors expressed in rat kidney (fig.4, lane1, 3, 4). On the other hand, only EP3 and EP4 are expressed in M-1 cells (fig.4, lane 8 and 9). A 700 bp fragment was amplified in M-1 cells when the EP1 specific primers were used in the RT-PCR reaction (fig.4, lane 6). However, the size of this PCR product was different from 336 bp as expected.

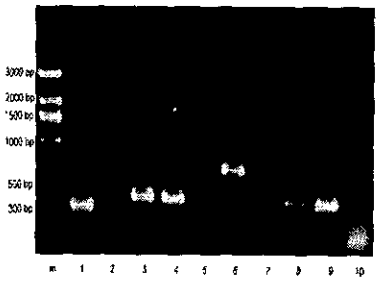

Fig. 4. PGE2 receptor (EP1 to EP4) in kidney and M-1 cells were detected using PCR according to the primers described in table 1.

AC I activity was inhibited; AC III and AC VI activity was enhanced, after V2 receptor activation in M-1 cells.

As shown in fig 5, when the M-1 cells transfected with pCEP4 vector was stimulated with AVP (10^{-8}M), around 58 % of cAMP level was increased, compared with the AVP untreated one. On the other hand, in pCEP4-ACI transfected M-1cells, the cAMP level following AVP activation was lowered than the AVP untreated one. Around 13.05 % of cAMP level was inhibited. This indicated the AC I activity was inhibited by V2 receptor activation. In pCEP4-ACVI transfected M-1 cells, the cAMP level was increased 73.75 % after AVP stimulation. This increase percentage is very close to that of pCEP4 transfected M-1 cells. This suggests not much change of AC activity was observed in pCEP-AC VI transfected M-1cells. Very interestingly, in pCEP4-AC III transfected M-1 cells, 206 % of cAMP level was increased after AVP treatment, which is 2 fold higher than that of pCEP4 transfected M-1 cells.

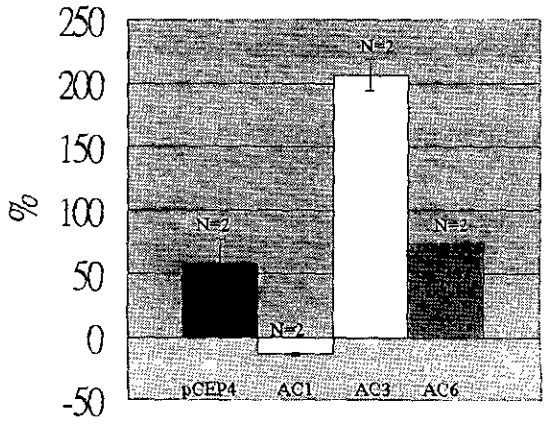

Fig. 5 The percentage of cAMP change in M-1 cells transfected with pCEP4, pCEP-ACI, pCEP4-ACIII and pCEP4-AC VI plasmid DNA after V2 receptor activation.

Table 1. Primers use to detect AVP receptors, AC isozymes, and EP3 receptors

AVP receptor	Sequences	Size
V1a	5'-TAGTGCCATTGTTCACCTCG-3'	**598 bp**
	5'-ATGAGTTTCCCGCGAGGCTCC-3'	
V2	5'-CCATGGTTCTGCAAATCGGG-3'	**625 bp**
	5'-TAGGTCATCATCAACCACCCCA-3'	
V2 (full length)		
5'-TGTTAGGTCATCATCAACCACCCCATC-3'		**1139bp**
5'-GGAGGGTGTATCCTTCATCAAAGAGGA-3		
AC	Sequences	Size **243 bp**
ACXf		
5'-GCTCTAGACCATCGGTAGCACCTACATGGC-3'		
ACEr		
5'-GCGAATTCACTGTATTCCCCAGATGTCATA-3'		

PGE2 receptors	Sequences	Size
EP1-F	5'-CGCAGGGTTCACGCACACGA-3	**336 bp**
EP1-R	5'-CACTGTGCCGGGAACATACGC-3'	
EP2-F	5'-AGGACTTCGATGGCAGAGGAGAC-3'	**401bp**
EP2-R	5'-CAGCCCCTTACACTTCTCCAATG-3'	
EP3-F	5'-CCGGGCACGTGGTGCTTCAT-3'	**437 bp**
EP3-R	5'-TAGCAGCAGATAAACCCAGG-3'	
EP4-F	5'-TTCCGCTCGTGGTGCGAGTGTTC-3'	**423 bp**
EP4-R	5'-GAGGTGGTGTCTGCTTGGGTCAG -3	

四、討論 (Discussion)

Our preliminary data indicated that AC V, VI, IX and ACIII are the major AC isozymes in M-1 cells. Chabardes at el., (1996) have reported that ACV and AC VI are the two major AC isozymes along the renal tubules. Besides these two Ca+2-inhibitable AC isozymes, ACIX, a novel AC recently identified by Premont et al., (1996) was found in M-1 cells. AC III, an AC isozyme inhibited by calcium through calmodulin kinase II, was also detected in M-1 cells. Among different AVP receptors, the presence of V2 receptor along the collecting ducts has been reported (Terada et al., 1991). Our preliminary data indicated the existence of V2, but not V1a receptor in M-1 cells. Among different AC isozymes, AC I was hardly detected in the kidney and M-1 cells. Our preliminary indicated the AC activity in AC I-transfected M-1 cells was inhibited after V2 receptor activation Some endogenous factors in M-1 cells might be activated after V2 receptor stimulation and involved in AC I inhibition. In AC III-transfected M-1 cells, the cAMP level was increased around 206 % after AVP treatment. On the other hand, in AC VI-transfected M-1 cells, the cAMP level was increased around 73 % after AVP treatment. This increase percentage is very close to that of the pCEP4-transfected M-1 cells (58 %). From our preliminary data, the coupling efficiency between V2 receptor and AC III isozymes seems superior to ACI and AC VI isozymes in M-1 cells.

五、參考文獻 (References)

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