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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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※　腺甘酸環化脢在腎臟水份再吸收作用中扮演的角色
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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 －017 －
執行期間：88 年 7 月 1 日至 89 年 8 月 31 日

計畫主持人：林庭慧
共同主持人：

本成果報告包括以下應繳交之附件：
- □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：中山醫學院生命科學係

中 華 民 國 89 年 10 月 25 日

行政院國家科學委員會專題研究計畫成果報告

國科會專題研究計畫成果報告撰寫格式說明

Preparation of NSC Project Reports

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一、中文摘要

關鍵詞： 血管增壓素，前列腺激素，腺甘酸，腺甘酸環化酶

血管增壓素(Vasopressin)，又稱抗利尿激素(Antidiuretics)，在人體脫水時產生。它的主要功能是促進水份之再吸收作用及血管收縮。而前列腺激素E2 (Prostaglandin E2)，可抗拮血管增壓素之作用，促進血管平滑肌之擴張，使血壓下降。此外，前列腺激素E2可以藉由抑制腎小管之再吸收作用而引發腎臟利尿作用(Diuresis)。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), 此細胞之重要第二訊息傳導物質, 以被證實牽涉其中。但許多文獻報告之結果並不一致, 主要原因是腎臟內部構造相當複雜且在組織準備方法上有所不同所致。腎臟集尿管 (Collecting ducts)是腎臟中，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，值得加以研究。

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導。此二者之受體都很複雜且有不同亞型 (subtypes)。V2 receptors 及 EP3 receptors 可能分別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。腺甘酸環化酶在近年來，陸續有不少 isozymes 被發現，各種 isozymes 分布在不同組織，且受到細胞內其他因子，如蛋白質激酶 (protein kinase C)，鈣離子 (Ca+2) 之調控。我對於腎臟中, 存在何種腺甘酸環化酶及此酵素如何調節由血管增壓素及前列腺激素E2引發之訊息傳導 (Signal Transduction) 感到興趣。利用 RT-PCR，我們偵測到腺甘酸環化酶第六亞型是老鼠腎臟及腎臟集尿管細胞株 (M-1 cell line) 中，最主要之腺甘酸環化酶。而第二亞型血管增壓素受體，則是分布在腎臟集尿管細胞株 (M-1 cell line)中，最主要的血管增壓素受體。至於前列腺激素 E2 受體之分布情形，更為複雜。以我們目前的結果顯示，EP3 beta form 是老鼠腎臟中最主要之 EP3 receptor。為了進一步瞭解腺甘酸環化酶，血管增壓素及前列腺激素 E2 之間的互動關係，我們將利用 in situ PCR 的技術，來偵測腺甘酸環化酶，血管增壓素受體及前列腺激素 E2 受體在腎臟集尿管的分布情形。此外，將利用 co-transfection 之方法，將 V2 receptors 及 EP3 receptors 此二種不同受體分別與不同腺甘酸環化酶 isozymes 送入真核細胞表現系統，以瞭解此二種受體與何種腺甘酸環化酶 isozymes 結合，來調控細胞內環腺甘酸之含量。藉由瞭解血管增壓素及前列腺激素 E2 之間的訊息傳導，及此二者與腺甘酸環化酶之間的相互關係，腺甘酸環化酶在腎臟生理功能上扮演之角色，將可得到進一步瞭解。

Abstract

Keywords : Vasopressin, Prostaglandin E2, cAMP, Adenylyl Cyclase

Vasopressin (AVP), also known as antidiuretic hormone (ADH), acts on the kidney to promote water retention and cause vasoconstriction. On the other hand, PGE2, the most important PG in the kidney, is well known as a potent antagonist of the AVP and causes vasodilation. It also inhibits water reabsorption from kidney cells into the blood and causes diuresis. The water reabsorption mechanism in the kidney is regulated by AVP via a cAMP-dependent pathway. The effect of AVP and PGE2 on cell cAMP content has been a subject of intensive investigation. However, the observations are contradictory and no definitive mechanisms have been proved yet. These different results may be due to the complex nephron heterogeneity and difference in tissue preparation.

Both AVP and PGE2 exert their physiological functions through binding to their receptors on the cell membranes. Different subtypes of AVP and PGE2

receptors have been reported. The collecting ducts are the major segments in the kidney which undergo water reabsorption. V2 receptors and EP3 receptors are the major receptors found in the collecting ducts. According to previous reports, both V2 receptors and EP3 receptors are G protein coupled receptors. V2 receptors are linked to Gs□protein while the EP3 receptors are linked to Gi protein. A lot of attention has 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. V2 receptor was detected as the major AVP receptor in M-1 cells. Further *in situ* PCR will be used to investigate the co-localization of V2 receptor and AC VI in the collecting duct. Co-transfection of V2 receptor and AC VI into CHO-K1 cells will provide the coupling efficiency between V2 receptor and this specific AC. For EP3 receptors, EP3 beta form seems to be the major isoform in the rat kidney. Whether this isoform is the major EP3 receptor in M-1 cell and the collecting duct will be verified by RT-PCR. The co-localization of EP3 receptors with AC isozymes and V2 receptor in the collecting duct will be tested by *in situ* PCR. By understanding the cellular signaling of AVP and PGE2 and their coupling efficiency to ACs, the relevance of ACs to the renal physiological functions, such as water reabsorption in collecting ducts, will be clarified.

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 probably are the main types of receptors for AVP and PGE2 in the collecting ducts. The collecting ducts is 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、結果與討論、

Results

Using RT-PCR, we have identified ACIV and ACVI as the major AC isozymes found in the rat kidney. Primers designed from ACI to ACVIII isozymes were listed in table 1. In fig. 1, ACI, ACIV and AC VI could be amplified in the total rat kidney with fragments around 200 bp. AC IV and AC VI could also be amplified using another set of primers listed in table 2. This further confirmed that these two isozymes are the major AC in the rat kidney (as shown in fig. 2). AC V, which has been reported by Chabardes et al., (1996) as the major AC isozyme in the rat kidney, was not detected in our PCR condition from total kidney. However, AC V was detected in the M-1 cell although its expression level was much lower than AC VI (as shown in fig. 3). Thus, AC VI appears to be the major AC isozyme in both rat kidney and the M-1 cell.

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 3. As indicated in fig. 4, V2 receptor seems to be the major AVP receptor amplified in M-1 cells, while V1a receptor also could be detected weakly. This data consist with that V2 receptor is the major AVP receptor in the kidney collecting ducts.

Primers designed to identify EP3 receptors were shown in table 4. EP3 receptors have been reported as the major PGE2 receptors in the kidney collecting ducts. Three variants of EP3 receptors with different carboxyl termini have been reported in both human and mouse kidney (Regan et al., 1994; Irie et al., 1993). In our preliminary data, EP3 beta form seems to be the major isoform amplified in the rat kidney cell, while EP3 alpha form could be weakly detected, with EP3 gamma form totally undetectable (as shown in fig. 5). Further identification of these three EP3 receptors in the collecting ducts and M-1 cell will be performed soon.

Using RT-PCR, we have identified that V2 receptor was the major AVP receptor in M-1 cells. In order to investigate the coupling efficiency between AC VI and V2 receptor, we need to have full length of V2 receptor gene. Using the cDNA prepared from the rat kidney, the coding region of V2 receptor was amplified using PCR. The primers used to amplify the full length V2 receptor gene was shown in table 3. We have tried different Taq DNA polymerase and found Pfx Taq DNA polymerae from Gibco BRL could successfully amplify the V2 receptor gene (as shown in fig. 6). The amplified PCR fragment showed a band around 1100 bp, which is consistent with the estimated size of V2 receptor gene. Further subcloning of this PCR fragment into a pcDNA vector

will be performed using an Eukaryotic TOPO TA cloning kit.

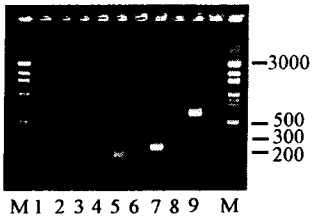

Fig 1. Detection of specific AC isozymes expressed in the rat kidney by RT-PCR.
Lane1: No reverse transcriptase as a negative control in the RT-PCR reaction.
Lane 2: A PCR fragment of AC I (189 bp) was amplified in the rat kidney.
Lane 3: AC II, no detection.
Lane 4: AC III, no detection.
Lane 5: A PCR fragment of AC IV (171 bp) was amplified in the rat kidney.
Lane 6: AC V, no detection.
Lane 7: A PCR fragment of AC VI (230 bp) was amplified in the rat kidney.
Lane 8: AC VIII, no detection.
Lane 9: A beta-actin fragment around 680 bp as a positive control in the RT-PCR reaction.

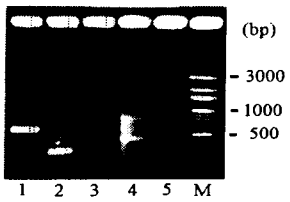

Fig 2. Detection of Specific AC isozymes expressed in the rat kidney by RT_PCR
(using primer set II)

Lane 1: A beta-actin fragment around 680 bp as a positive control.
Lane 2: AC IV was expressed in the rat kidney.
Lane 3: AC V was not detected in the rat kidney.
Lane 4: AC VI was expressed strongly in the rat kidney.
Lane 5: AC IX was not detected in the rat kidney.

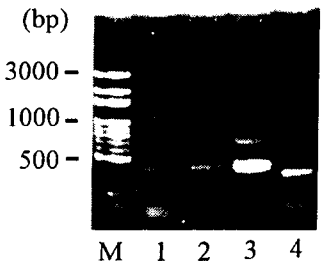

Fig 3. Detection of specific AC isozymes expressed in the M-1 cell line by RT-PCR.
Lane1: AC IV was expressed in the M-1 cell line.
Lane 2: AC V was expressed in the M-1 cell line.
Lane 3: AC VI was expressed strongly in the M-1 cell line.
Lane 4: AC IX was expressed in the M-1 cell line.

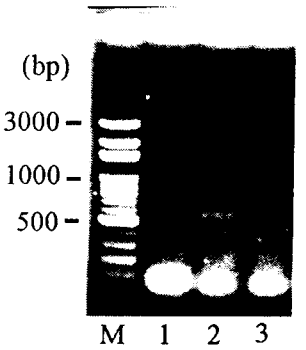

Fig. 5 Detection of EP3 receptor isoforms in the rat kidney by RT-PCR.
Lane 1: EP3 alpha form receptor was weakly detectable.
Lane 2: EP3 beta form receptor , the major EP3 receptor amplified in the rat Kidney.
Lane 3: EP3 gamma form receptor was hardly detectable.

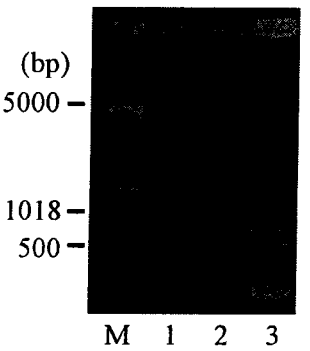

Fig 6. RT-PCR cloning of V2 receptor.
Lane 1: A PCR fragment around 1100 bp, an estimated size of the V2 receptor, was amplified by RT-PCR from the rat kidney.
Lane 2: No reverse transcriptase as a negative control in the RT-PCR reaction.
Lane 3: A beta-actin fragment around 680 bp as a positive control in the RT-PCR reaction.

III. Discussion

Our preliminary data indicated that AC VI is the major AC isozyme in both rat kidney and M-1 cell. AC IV was also detected in kidney, however, their distribution in M-1 cell line seems not so abundant as AC VI. Chabardes at el., (1996) have reported that ACV and AC VI are the two major AC isozymes along the renal tubules. Using their primer design (as listed in table2), we did not detect the existence of ACV in rat kidney. On the other hand, AC V was detected in M-1 cell. Their occurrence was much less than ACVI but equally distributed as AC IV and AC IX. This indicated ACVI is the major AC isozyme in the whole kidney including renal tubules, while ACV may confine to more specific renal regions. Besides these two Ca+2-inhibitable AC isozymes, ACI, ACIV and

ACIX were also found in the kidney and M-1 cell. Whether these AC isozymes play physiological roles in kidney remains unclear and is an interesting issue to pursue.

Our current objective is to investigate the distribution of AC isozymes in specific renal segments, such as the medullary collect duct and the cortical collecting duct. These two regions are the major renal portions, which process intensive water reabsorption. AVP has been known as antidiuretic hormone, which acts on the kidney to promote water retention. Among different AVP receptors, the presence of V2 receptors along the collecting duct has been reported (Terada et al., 1993). Our preliminary data also indicated the existence of V2 receptor in M-1 cell (as shown in fig.4). We are interesting to see if this G-protein coupled receptors exhibit good coupling efficiency with ACVI, the predominate AC isozyme identified in M-1 cell.

As a potent antagonist of the AVP, PGE2 inhibits water reabsorption from kidney cells into the blood. The complexity of PGE2 receptors has been reported elsewhere and each receptor couples to different signal transduction pathways. Among PGE2 receptors, the EP3 receptors were involved in inhibition of water reabsorption in kidney tubules (Garcia-Perez and Smith, 1984). Sugimoto (1992) have cloned the mouse EP3 receptor from a mouse mastocytoma P-815 library and found this receptor is a G-protein coupled receptor engaged in inhibition of AC. They later reported the second and third isoform of EP3 receptors with different carboxy-terminal tails produced through alternative splicing (Sugimoto et al., 1993; Irie et al., 1993). These three EP3 receptors, name as EP3 alpha, EP3 beta and EP3 gamma, show different efficiencies for activation of the coupling inhibitory Gi protein and inhibition of AC. Indeed, Irie at al., (1993) have reported that EP3gamma is coupled to both stimulation and inhibition of AC, but that EP3 alpha and EP3 beta are exclusively coupled to inhibition of AC. Regan et al., (1994) also cloned four human variants of the EP3 receptors. Three of the variants represent (EP3A, EP3B and EP3D) share the same primary amino acid sequences except for their carboxyl termini. The fourth variant (EP3A1) has a nucleotide coding sequence identical to EP3A but has a completely different 3' untranslated sequence. Only one of the human variants of the EP3 receptors corresponds to the mouse EP3 alpha receptor but the other three do not correspond to any of the mouse EP3 receptors. Not much information is available about the distribution of EP3 receptors in the rat kidney. In our preliminary data, we use the primers designed from the mouse EP3 alpha, beta and gamma to investigate the distribution of EP3 receptors in the rat kidney. As shown in fig. 5, EP3 beta seems to be the major EP3 receptor exist in the rat kidney, while alpha form could be weakly detected, with gamma form totally undetectable. Further study of the EP3 receptors will be performed in the renal collecting ducts and M-1 cell.

四、計畫成果自評

計畫成果自評部份，研究結果與原計畫相符，達成預期目標。已經可以建立集尿管細胞株(M-1 cell line)之細胞培養情形，並且抽取 RNA，成功的將此研究計畫中，所要探討之三種蛋白質，AVP receptors, EP3 receptors, and AC isoforms 在腎臟及集尿管細胞株之基因分佈情形用 RT-PCR 偵測出來。並且也找到了 amplify V2 receptors 的實驗情形。此外，也用 eukaryotic cell line system (CHO cells) 測試 beta-gal DNA transfection condition 我們測試各種 transfection reagents 在 CHO cells 及 M-1 cell line 之 transfection efficiency 情形。這些測試情形將提供未來實驗之參考，以利評估。

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