Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Receptor mRNA in the Rat Adrenal Gland: Localization by In Situ Hybridization and Identification of Splice Variants

Hiroyuki Nogi, Hitoshi Hashimoto, Takashi Fujita, Nami Hagihara, Toshio Matsuda and Akemichi Baba*

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan

Received July 3, 1997; Accepted August 19, 1997

ABSTRACT—The distribution of mRNA for pituitary adenylate cyclase-activating polypeptide receptor (PACAP-R) was examined by in situ hybridization in rat adrenal gland. In the adrenal medulla, PACAP-R mRNA was expressed in almost all chromaffin cells without any significant expression in the cortical region. Using the reverse transcription-polymerase chain reaction, we analyzed the mRNA expression of PACAP-R splice variant forms in the adrenal gland. The predominant forms observed were the variant having a 28-amino acid insert in the third intracellular loop (termed PACAP-R-hopl). As PACAP is localized in the noradrenaline secreting cells of the adrenal chromaffin cells, and stimulates catecholamine release, the present results suggest that PACAP may serve as a paracrine or autocrine regulatory factor for the chromaffin cells through PACAP-R.

Keywords: PACAP (pituitary adenylate cyclase-activating polypeptide)/VIP (vasoactive intestinal polypeptide) receptor mRNA expression, In situ hybridization, Adrenal gland

Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuropeptide isolated from ovine hypothalamus, exists in two amidated forms, PACAP-38 and PACAP-27, sharing the same N-terminal 27 amino acids (1). PACAP-27 has an amino acid sequence identity of 68% with vasoactive intestinal polypeptide (VIP) and of 37% with secretin, indicating that PACAP belongs to the VIP/secretin/glucagon family (1). Both PACAP and VIP are distributed widely throughout mammalian body, and they may function as neurotransmitters and/or modulators in the peripheral and central nervous systems (1, 2).

The cDNAs encoding the PACAP receptor (PACAP-R) (3) and two subtypes of VIP receptors, VIP1 receptor (VIP1-R) (4) and VIP2 receptor (VIP2-R) (5), have been recently cloned (6). The recombinant VIP1 and VIP2 receptors interact with both PACAP and VIP almost equally (4–6). On the other hand, although PACAP-R also binds both PACAP and VIP, it binds VIP with 100-1,000 times less affinity (3, 6).

The distribution patterns of the three receptors, PACAP-R, VIP1-R and VIP2-R, are markedly different from each other (3–7). Although the precise roles of PACAP and VIP in the central and peripheral nervous systems have not been fully understood, pharmacological studies on these peptides elucidated their effects on the sympathoadrenal system: PACAP stimulates neurite outgrowth of PC-12 cells (8) and stimulates secretion of catecholamine in adrenal chromaffin cells (9, 10). To elucidate the mechanism of PACAP actions in the adrenal gland, it is essential to specify the subtypes of the receptors expressed in this tissue. In the present study, we examined the expression of mRNAs for PACAP-R, VIP1-R and VIP2-R in the rat adrenal gland by in situ hybridization.

Alternative splicing of the region encoding the third intracellular loop (11) and the N-terminal extracellular region (12) of the PACAP-R generates variant forms with differential signal transduction properties. Therefore, we analyzed the expression of PACAP-R splice variant forms in the adrenal gland by the reverse transcription-polymerase chain reaction (RT-PCR).

In situ hybridization was carried out as described elsewhere (7, 13). The sections of adrenal gland (13-μm-thick) obtained from adult male Wistar rats (250–300 g body weight) were fixed on poly-l-lysine-coated slides and hybridized. A 1,814-bp insert of pCAP7F, a functional
cDNA encoding rat PACAP-R (3), and a 1,604-bp Xhol-SacI cDNA fragment spanning the entire coding sequence of the rat VIP1-R (clone pV19, a generous gift from Dr. S. Nagata) (4) were subcloned into pBluescript II KS(+) . A 1,101-bp PstI cDNA fragment spanning almost the entire coding sequence of the rat VIP2-R subcloned into pBluescript II SK(−) (5) was kindly supplied by Dr. T.B. Usdin. 35S-Labeled antisense RNAs were prepared by in vitro transcription using T7 RNA polymerase (Stratagene) and [α-35S]CTP (Du Pont NEN, Boston, MA, USA) to a specific activity of 1.0 × 10⁹ cpm/μg. Unlabeled antisense RNAs were synthesized similarly with unlabeled nucleotides. These cRNAs, which were alkaline hydrolyzed, were used as probes at the concentration of 2 × 10⁶ cpm/ml in the presence or absence of a 100-fold excess of unlabeled cRNA. After hybridization, the slides were treated at 37°C for 30 min with 20 μg/ml of RNase A and washed at 60°C for 1 hr with 0.1 × standard saline citrate (SSC) buffer containing 10 mM β-mercaptoethanol. The slides were dipped in NTB2 emulsion (Eastman Kodak Co., Rochester, NY, USA) diluted 1:1 with distilled water and exposed for 8 weeks. After developing, the sections were counterstained with cresyl violet. The specificity of in situ hybridization was confirmed by the incubation of adjacent sections with labeled probe in the presence of 100-fold excess cold probe.

Total RNAs were isolated from adult male Wistar rat adrenal gland and whole brain (8 weeks) and PC12 cells. RT-PCR was performed as described with several modifications (14). Briefly, the following primers were used: F8, 5'-CTG GCA TGT GGG ACA ATA TC-3'; R9, 5'-AGA TAG CCC TCA GCA TGA AG-3'; F24, 5'-CTT TAC ACT GCT GGT GGA GA-3'; R31, 5'-AGT CTT TCC CTC TTG CTG AC-3'. PCR was performed using Taq DNA polymerase according to the following schedule: 40 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec and extension at 72°C for 1 min. The PCR products were resolved by 1.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer, and the gel was then stained with ethidium bromide. The numbers of amplification and the amounts of cDNAs used for the reaction were optimized for quantitation of RNAs in preliminary experiments. The cDNA fragments was ascertained by DNA sequencing or restriction enzyme mapping. The β-actin housekeeping gene was simultaneously reverse transcribed and amplified as the internal reference standard to control for variations in product abundances.

In the adrenal gland, PACAP-R was expressed intensely in the adrenal medulla, while no significant expression of PACAP-R was detected in the cortex (Fig. 1: a and e). In the medulla, almost all chromaffin cells were intensely labeled (Fig. 1e). No significant labeling was detected in small cells with dark nuclei. These cells were considered to be the sustentacular cells and satellite cells. No significant hybridization of PACAP-R was observed in parallel cold-excess experiments (Fig. 1b). No significant expression of VIP1-R or VIP2-R mRNA was observed in the adrenal gland (Fig. 1: c and d), although the parallel in situ hybridization experiments in the rat brain showed the expression of VIP1-R and VIP2-R mRNAs, the distribution patterns of which were identical to those previously described (4, 5). However, the possibility that

![Fig. 1. Dark- (a–d) and bright-field (e) photomicrographs showing the distribution of PACAP receptor (PACAP-R) (a, b, e), VIP receptor (c) and VIP2 receptor (d) mRNAs in the rat adrenal gland. As controls, adjacent sections were hybridized in the presence of a 100-fold excess of unlabeled cRNA (b). In the adrenal medulla, PACAP-R mRNA was expressed in almost all chromaffin cells without any significant expression in the cortical region. Neither VIP1-R mRNA nor VIP2-R mRNA was detected in the adrenal cortex or medulla. An arrow in panel a indicates the adrenal medulla. C, adrenal cortex; M, adrenal medulla. Bars: 500 μm (d, common to a, b and c) and 30 μm (e).](image-url)
VIP1-R and/or VIP2-R mRNAs exist at a lower level than the sensitivity of in situ hybridization in this study and that they are expressed in an inducible manner in the adrenal gland cannot be excluded.

To study the expression of PACAP-R splice variant forms of the third intracellular loop, we performed RT-PCR with RNA obtained from the adrenal gland, whole brain and PC-12 cells (Fig. 2). In the adrenal gland, the predominant form observed was PACAP-R-hop1 (lane 2 in Fig. 2c). PACAP-R-hop2 (lane 6 in Fig. 2c) was also seen at lower levels. PACAP-R-s (the shortest form, without insert) was observed very weakly (lane 2 in Fig. 2c). As PACAP-R-hop gave the same band as PACAP-R-hop1 and -hop2 upon agarose gel electrophoresis, we isolated and subcloned this band into pBluescript and then sequenced it. Among 10 independent clones analyzed, all clones contained the hop sequence (8 clones, PACAP-R-hop1; 2 clones, PACAP-R-hop2), and PACAP-R-hop could not be found. PC-12 cells, catecholamine-secreting rat pheochromocytoma cells, and superior cervical ganglion (SCG), a sympathetic ganglion, displayed the expression pattern of variant forms similar to that of the adrenal gland. In contrast, the brain expressed PACAP-R-s and PACAP-R-hop almost equally.
Concerning the alternative splicing in the N-terminal extracellular domain of the PACAP-R, a 21-amino-acid sequence encoded by two exons can be deleted (PACAP-R-ns). However, PACAP-R-ns was not detectable in the adrenal gland, PC-12 cells, brain and SCG (Fig. 2b).

VIP increases the catecholamine secretion from the adrenal medullary cells; however, this response is observed at micromolar levels of VIP, which are extremely high in terms of specific peptide actions (9, 10). Although PACAP elicits biological actions similar to those of VIP, its potency is 100- to 1,000-fold higher than that of VIP, suggesting that the effects of PACAP are mediated by PACAP-R, but not by VIP1-R or VIP2-R (9, 10). The potent effects of PACAP compared with VIP on the adenylate cyclase and phospholipase C, whereas the hip variants potently activate both cAMP and inositol phosphates in cultured PC12 cells and promotes neurite outgrowth.

In accordance with this view, PACAP-R mRNA was expressed intensely in virtually all chromaffin cells, but VIP1-R mRNA or VIP2-R mRNA were not detectable in the adrenal gland. This regional distribution of PACAP-R mRNA agrees with that determined by autoradiographic studies using 125I-labeled PACAP (9).

Immunohistochemical studies indicated that PACAP is contained in the noradrenaline secreting cells of the adrenal chromaffin cells (15). The present results indicating the expression of PACAP-R in almost all chromaffin cells suggests coexpression of PACAP and PACAP-R in noradrenaline secreting cells. Recently we have demonstrated that PACAP-R, but not VIP1-R or VIP2-R, is expressed intensely in virtually all principal neurons of the rat SCG and that PACAP is colocaized in half of the principal neurons (13). Thus, PACAP may function at least in part as a autocrine or paracrine regulatory factor for the sympathoadrenal cells.

PACAP has been reported to stimulate the production of both cAMP and inositol phosphates in cultured adrenal medullary cells (10). Among the splice variants of the third intracellular loop of the PACAP-R, PACAP-R-s as well as the hop variants potently activate both adenylylate cyclase and phospholipase C, whereas the hip variant does not stimulate phospholipase C (11). In accordance with these observations, the present results confirmed that the hop variants (PACAP-R-hop1 and -hop2) are predominant forms in the adrenal gland. Interestingly, the hop insert encodes a consensus motif for phosphorylation by protein kinase C (11). Whether protein kinase C modulates PACAP-R function through a phosphorylation at the hop insert remains to be determined.

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