Alternative Splicing in the N-terminal Extracellular Domain of the Pituitary Adenylate Cyclase-activating Polypeptide (PACAP) Receptor Modulates Receptor Selectivity and Relative Potencies of PACAP-27 and PACAP-38 in Phospholipase C Activation*

(Received for publication, April 16, 1996)

Colette Pantaloni, Philippe Brabet, Benoit Bilanges, Aline Dumuis, Souheir Houssami, Dietmar Spengler*, J oël Bockaert, and Laurent J ounnot†

From CNRS-UPR 9023, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, F-34094 Montpellier Cedex 05, France and § M ulecular Neuroendocrinology, Max Planck Institute of Psychiatry, Kraepelinstrasse, D-80804 Munich, Federal Republic of Germany

Pituitary adenylate cyclase-activating polypeptide (PACAP)27 and PACAP-38 are neuropeptides of the vasoactive intestinal peptide/secretin/glucagon family. We previously described alternative splicing of the region encoding the third intracellular loop of the PACAP receptor generating six isoforms with differential signal transduction properties (Spengler, D., Waebener, C., Pantaloni, C., Holsboer, F., Bockaert, J., Seeburg, P. H., and Jounnot, L. (1993) Nature 365, 170–175). In addition, we demonstrated that the potencies of the two forms of PACAP are similar for adenylate cyclase stimulation, whereas PACAP-38 is more potent than PACAP-27 in phospholipase C activation. In the present work, we document the existence of a new splice variant of the PACAP receptor that was characterized by a 21-amino-acid deletion in the N-terminal extracellular domain. We demonstrate that this domain modulates receptor selectivity with respect to PACAP-27 and -38 binding and controls the relative potencies of the two agonists in phospholipase C stimulation.

The abbreviations used are: PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; AC, adenylate cyclase; PLC, phospholipase C; RT-PCR, reverse transcriptase-polymerase chain reaction.

* This work was supported by Boehringer-Ingelheim. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. 33-67-14-29-32; Fax: 33-67-54-24-32; E-mail: jounnot@ccipe.montp.inserm.fr.
‡ Recipient of an Australian NHMRC/INSERM exchange fellowship.
§ The abbreviations used are: PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; AC, adenylate cyclase; PLC, phospholipase C; RT-PCR, reverse transcriptase-polymerase chain reaction.
Alternative Splicing of the PACAP Receptor

**Experimental Procedures**

Screening of Mouse Genomic and Human cDNA Libraries—A genomic library (a generous gift from Dr. Philippe Soriano, Seattle) was constructed by partial digestion of genomic DNA from 129Sv mouse strain with MboI and subcloning of the digested fragments into pBluescript II (Stratagene). We screened 1.9 × 10^9 clones with a random primed probe derived from the rat PACAP-V1-R cDNA and isolated seven positives clones, which were excised with the mouse PACAP-V1-R gene.

for high affinity calcitonin binding (26). In addition, experiments with chimeric receptors suggested a role for the N-terminal extracellular domain of the calcitonin receptor in calcitonin binding (27). Similarly, it was demonstrated that the extracellular N-terminal domain of PACAP/VIP1-R is critical for VIP binding (28, 29). Moreover, mutation of seven residues conserved among the PACAP-VIP-secretin-glucagon receptor family (cysteine 63, aspartate 68, cysteine 72, tryptophan 73, cysteine 86, glycine 109) completely abolished VIP binding (30, 31). This domain (residues 63–109) is therefore likely to interact with VIP. The authors have not assessed whether PACAP binding is also altered by the same mutations, and it is therefore not known whether the same domain also interacts with PACAP. Regarding the PACAP-V1-R, Cao and co-workers (32) demonstrated that the N-terminal extracellular and the first transmembrane domains are sufficient to confer high affinity PACAP binding. Interestingly, the PACAP-V1-R displays a 21-aa sequence in the N-terminal extracellular domain, which has no homologous domain in any other member of the VIP-secretin-glucagon receptor family. The present study established the existence of a new splice variant missing the above mentioned 21 aa and described its functional properties regarding binding and activation of AC and PLC.

**Fig. 1.** Alternative splicing of exons 5 and 6 of the mouse PACAP-V1-R gene. A, partial structure of the mouse PACAP-V1-R gene. Appropriate fragments from genomic clones were isolated, subcloned, and sequenced as described under "Experimental Procedures." B, partial alignment of the PACAP-VIP-secretin-glucagon receptor family indicated amino acid conservation except for the 21-aa domain encoded by the mouse PACAP-V1-R gene.

**Fig. 2.** Ethidium bromide-stained gels of RT-PCR experiments with total RNA purified from different brain regions and tissues. Following incubation with (RT+) or without (RT-) reverse transcriptase, cDNAs were amplified by PCR using primers flanking the sequence encoding the 21-aa domain. 15 μl of each PCR reaction were loaded on a 2% agarose gel, electrophoresed, and stained with ethidium bromide. Olf, olfactory bulb; fCx, frontal cortex; pCx, posterior cortex; Hip, hippocampus; Hyp-Thal, hypothalamus/thalamus; BrSt, brain stem; CB, cerebellum; Pit, anterior pituitary gland; Ad, adrenal gland.
formed with Statview software.

Total Inositol Phosphate Measurement—Transfected cells were incubated at 4 °C with 100 µm [125I]PACAP-27 in the presence of the indicated concentrations of unlabeled PACAP-27 and -38. Data are expressed as percent of [125I]PACAP-27 bound to each splice variant in the absence of unlabeled competitor. Data are representative of seven to nine independent experiments performed in triplicate, which were analyzed with the EBDA-Ligand software.

RESULTS

Mouse PACAP1-R Gene Isolation and Characterization—Using the rat PACAP1-R cDNA as a probe, we isolated several genomic clones encoding the mouse PACAP1-R gene. It is composed of at least 18 exons and spans more than 50 kilobases.

Alternative Splicing of the PACAP Receptor

**TABLE I**

|                | PACAP-38          | PACAP-27          |
|----------------|-------------------|-------------------|
| [125I]PACAP-27 | 3.3 ± 0.6         | 16 ± 2.3*         |
| displacement   | (n = 9)           | (n = 7)           |
| Ki, nM         | 0.03 ± 0.03       | 0.33 ± 0.08       |
| (n = 5)        | (n = 6)           |
| Stimulation of | 0.30 ± 0.03       | 0.63 ± 0.03       |
| cAMP-regulated | (n = 9)           | (n = 7)           |
| luciferase     | 0.22 ± 0.03       | 0.33 ± 0.08       |
| activity (EC50) | (n = 6)           | (n = 6)           |
| Stimilation of  | 21 ± 3            | 212 ± 26±         |
| total IP       | (n = 6)           | (n = 6)           |
| production     | 10 ± 1            | 26 ± 3            |
| (EC50; nM)     | (n = 6)           | (n = 6)           |

* Significantly different from Ki value for PACAP-38 binding on PACAP1-R s (p < 0.002) and for PACAP-27 binding on PACAP1-R (p < 0.001).

**Fig. 3.** Comparison of the ligand binding properties of PACAP1-R s versus PACAP1-R vs. Intact transiently transfected cells were incubated at 4 °C with 100 µm [125I]PACAP-27 in the presence of the indicated concentrations of unlabeled PACAP-27 and -38. Data are expressed as percent of [125I]PACAP-27 bound to each splice variant in the absence of unlabeled competitor. Data are representative of seven to nine independent experiments performed in triplicate, which were analyzed with the EBDA-Ligand software.

**Fig. 4.** Binding of PACAP-(6–38) at PACAP1-R s versus PACAP1-R vs. Binding experiments were performed as described in Fig. 2. Binding of PACAP-(6–38) was not impaired by deletion of the 21-aa domain. PACAP1-R s, K, = 97 ± 3 nM (n = 3); PACAP1-R vs, K, = 56 ± 9 nM (n = 3).

Analysis of the exon-intron structure indicated that two separate exons encode a 21-aa sequence that is specific to PACAP1-R as compared with other members of the VIP-secreting glucagon receptor family of G protein-coupled receptors (Fig. 1). In addition, splicing out of these exons would keep the reading frame and generate a shorter form of PACAP1-R missing the 21-aa in the N-terminal extracellular domain.

Existence and Distribution of the New PACAP1-R Splice Variant—To test whether such a splice variant may exist, we designed oligonucleotides flanking the 21-aa domain. PACAP1-R s, K, = 97 ± 3 nM (n = 3); PACAP1-R vs, K, = 56 ± 9 nM (n = 3).

**Different from EC50 value for stimulation of luciferase activity by PACAP-38 through PACAP1-R vs (p < 0.004).**

**Different from EC50 value for stimulation of total IP production by PACAP-38 through PACAP1-R vs (p < 0.0006).**

**Different from EC50 value for stimulation of luciferase activity by PACAP-27 through PACAP1-R vs (p < 0.002) and by PACAP-27 through PACAP1-R vs (p < 0.004).**

**Different from EC50 value for stimulation of total IP production by PACAP-27 through PACAP1-R vs (p < 0.002) and for PACAP-27 binding on PACAP1-R (p < 0.001).**

**Different from EC50 value for stimulation of total IP production by PACAP-38 through PACAP1-R s (p < 0.0008) and by PACAP-27 through PACAP1-R vs (p < 0.0006).**
21-aa domain (data not shown). This demonstrates the existence of a previously uncharacterized splice variant of the N-terminal extracellular domain of the PACAP1-R.

Isolation of Human PACAP1-R cDNA—To isolate a full-length cDNA encoding the new splice variant and to characterize its functional properties, we screened a fetal brain CDNA library. We chose a human library for use of the isolated clones in future pharmacological studies. We isolated seven independent clones encoding the human PACAP1-R. Most of these were partial clones, and several contained unspliced introns. Clone C19 contained the entire coding region and was the human homologue of the rat PACAP1-R s (18) (94% homology). This sequence was identical to the sequence published by Ogi and co-workers (36) except in the 5′ region. These authors found a long repeat in the 5′-coding and non-coding regions that has no homologous sequence in the rat PACAP1-R. The sequence we found did not display this repeated sequence and was strictly homologous to the PACAP1-R sequences already published. The clone described by Ogi and co-workers (36) was therefore likely to result from rearrangement in the 5′ region of their cDNA. The other clones we isolated were partial and corresponded to human homologues of the rat hip, hop1, and hop2 splice variants. This demonstrated that alternative splicing of the region encoding the third intracellular loop of PACAP1-R also occurred in human, at least in fetal brain. In addition, clone C24 started at codon encoding N60 and was otherwise identical to clone C19 in the coding region but for the deletion of the sequence encoding V89 to S109 in the putative extracellular N-terminal domain. Two additional clones displayed the same deletion. These results indicated that alternative splicing of the region encoding V89-S109 also occurred in human fetal brain. Using PCR and restriction enzymes, we reconstructed the human PACAP1-R hip and hop1 splice variants as well as the new variant. Since this latter variant was shorter than the variant referred as "s" (short), we named it "vs" for "very short" (Fig. 1). All reconstructed clones were entirely sequenced on both strands (data not shown) and subsequently subcloned into pRK5, a cytomegalovirus-based expression vector.

FIG. 5. Stimulation of cAMP production by PACAP-27 and PACAP-38. Cells were cotransfected with pMCM16-Luc, a cAMP-regulated reporter luciferase construct. Data are representative of five to six independent experiments performed in triplicate, which were analyzed with a four-parameter equation with the Kaleidagraph software.

FIG. 6. Stimulation of total inositol phosphate production by PACAP-27 and PACAP-38. Data are representative of six independent experiments performed in triplicate, which were analyzed with a four-parameter equation with the Kaleidagraph software.

Functional Characterization—We transiently transfected LLC PK1 cells by electroporation and performed binding experiments and second messenger measurements on intact cells to characterize the functional properties of PACAP1-R vs as compared with those of PACAP1-R s. We used a dose of each plasmid, which resulted in expression of approximately 5000 receptors per cell. Scatchard plots on intact transfected cells were determined for each experiment to verify that both variants were expressed at similar densities at the cell surface.

Saturation experiments with [125I]PACAP-27 indicated that the dissociation constant (Kd) was affected only 2-fold by the presence of the 21-aa domain (PACAP1-R s, 0.31 ± 0.03 nm (n = 8); PACAP1-R vs, 0.16 ± 0.01 nm (n = 8), p < 0.0007). Competition experiments with non-iodinated peptides indicated that both variants displayed similar affinity for PACAP-38, whereas PACAP-27 binding was impaired by the 21-aa domain (Table I and Fig. 3).

Since the 21-aa domain is strongly acidic and since the C-terminal extension of PACAP-38 is rich in basic residues, we hypothesized a direct interaction between these domains. To test this model, we measured the displacement of [125I]PACAP-27 by PACAP-(6–38) at each splice variant on intact cells. Binding of PACAP-(6–38) was not decreased by the absence of the 21-aa domain (Fig. 4).

Regarding signal transduction properties of the vs variant, potencies in stimulation of cAMP-stimulated luciferase activity by PACAP-27 and -38 were not significantly different for PACAP1-R s and differed only 2-fold for PACAP1-R s (Table I and Fig. 5). On the other hand, total inositol phosphate measurements indicated that PACAP-27 and -38 were closely potent at PACAP1-R vs whereas, as previously shown, PACAP-38 was one order of magnitude more potent than PACAP-27 at PACAP1-R s (Table I and Fig. 6).

DISCUSSION

The partial structure of the mouse PACAP1-R gene presented in this study is in agreement with recently reported data (37). Interestingly, the exon-intron structure of the PACAP1-R gene is conserved as compared with that of other
members of the VIP-secretin-glucagon receptor family except for two regions: (i) the third intracellular loop for which two
cassettes named hip and hop are possibly inserted (18) and (ii)
a 21-aa domain in the N-terminal extracellular region, which is
also encoded by two exons. We previously demonstrated alter-
cassettes named hip and hop are possibly inserted (18) and (ii)
for two regions: (i) the third intracellular loop for which two
receptor at another site, the conformation and accessibility of
more likely interacts with the extracellular domains of the
which is not modified by the presence of the 21-aa domain.
that binding of PACAP-(6–38) was not affected by deletion of
one can predict that deletion of the 21-aa domain should
the receptor as deduced from the high affinity binding of
which is shared with PACAP-27 and is the major determinant
the inhibition of PACAP binding and AC activation, and (ii) the C-terminal
due to the presence of the 21-aa domain. Concurrent alter-
the N-terminal domain of PACAP-1-R was restricted to pituitary
and adrenal glands and hypothalamus/thalamus, which sug-
splicing properties by alternative splicing of extracellular
and intracellular domains. Further experiments at the single
cell level, however, will be necessary to correlate expression of
the different PACAP1-R splice variants with functional param-
eters such as intracellular calcium concentration.

Acknowledgments—We gratefully acknowledge the generous gifts by
Drs. Philippe Soriano and Peter J. Flor of the mouse genomic library
and the human brain cDNA library, respectively. We are thankful to
Drs. Annie Varrault and J-éan-Philippe Pin for critical reading of
the manuscript.

REFERENCES

1. Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uehara, A., Jiang, L.,
Culler, M. D., and Coy, D. H. (1989) Biochem. Biophys. Res. Commun. 164,
567–574.
2. Miyata, A., Jiang, L., Dahl, R. R., Kitada, C., Kubo, K., Fujino, M., Minamino,
N., and Arimura, A. (1990) Biochem. Biophys. Res. Commun. 170, 643–648.
3. Deutsch, P. J., and Sun, Y. (1992) J. Biol. Chem. 267, 5108–5113.
4. Pincus, D. W., DiCicco-Bloom, E. M., and Black, I. B. (1990) Nature 343,
564–567.
5. DiCicco-Bloom, E., and Deutsch, P. J. (1992) Regul. Pept. 37, 319–325.
6. Tatsuno, I., Somogyvari-Vigh, A., Mizuno, K., Gotschall, P. E., Hijikata, H.,
and Arimura, A. (1991) Endocrinology 129, 1797–1803.
7. Rawlings, S. R., Canny, B. J., and Leong, D. A. (1993) Endocrinology 132,
1447–1452.
8. Vigh, S., Arimura, A., Gotschall, P. E., Kitada, C., Somogyvari-Vigh, A., and
Childs, G. V. (1993) Peptides 14, 59–65.
9. Yada, T., Vigh, S., and Arimura, A. (1993) Peptides 14, 235–239.
10. Rawlings, S. R., Denaurex, N., and Schlegel, W. (1994) J. Biol. Chem. 269,
5680–5686.
11. Rawlings, S. R., Piuz, I., Schlegel, W., Bockaert, J., and Jurut, L. (1995)
Endocrinology 136, 2096–2098.
12. Frödin, M., Hannibal, J., Wulf, B. S., Gammeltoft, S., and Fahrenkrug, J.
(1995) Neuroscience 65, 599–608.
13. Watanabe, T., Masuo, Y., Masuda, Y., Matsuno, H., Suzuki, N., Ohtaki, T.,
Masuda, Y., Kitada, C., Tsuda, M., and Fujino, M. (1992) Biochem. Biophys. Res.
Commun. 192, 403–411.
14. Isokobe, K., Nakai, T., and Takawu, Y. (1993) Endocrinology 132, 1757–1765.
15. Piersch, R., and Wank, S. A. (1993) Proc. Natl. Acad. Sci. U.S.A. 90,
6345–6349.
16. Hashimoto, H., Ishihara, T., Shigemoto, R., Mori, K., and Nagata, S. (1993)
Neuron 11, 333–342.
17. Hosoya, M., Ono, H., Ogii, K., Masuda, Y., Miyamoto, Y., Ohtaki, T.,
Okazaki, H., Arimura, A., and Fujino, M. (1993) Biochem. Biophys. Res.
Commun. 194, 133–143.
18. Spengler, D., Waeder, C., Pantaloni, C., Holsboer, F., Bockaert, J., and Seeburg,
P. H., and Jurut, L. (1993) Nature 365, 170–175.
19. Svoboda, M., Tastenoy, M., Ciccarelli, E., Stievenart, M., and Christophe, J.
(1993) Biochem. Biophys. Res. Commun. 193, 881–886.
20. Jurut, L., Spengler, D., Pantaloni, C., Dumuis, A., Sebben, M., and Bockaert,
J. (1994) Semin. Cell Biol. 5, 263–272.
21. Arimura, A., and Shioda, S. (1995) Front. Neuroendocrinol. 16, 53–88.
22. Furlin, A., Scapagnini, U., and Canonico, P. L. (1995) Neuroendocrinology 61,
377–382.
23. Basille, M., Gonzalez, B. J., Desures, L., Demas, M., Fournier, A., and Vaudry,
H. (1995) J. Neurochem. 65, 1318–1324.
24. Dixon, R. A., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G.,
Frielle, T., Bidaloglu, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Muenter,
R. A., Slater, E. E., Sigl, J. S., Caron, M. G., Lefkowitz, R. J., and
Strader, C. D. (1986) Nature 321, 75–79.
25. Strader, C. D., Fong, T. M., Tota, R. M., Underwood, D., and Dixon, R. A.
(1994) Annu. Rev. Biochem. 63, 103–132.
26. Houssami, S., Findlay, D. M., Brady, C. L., Myers, D. E., Martin, T. J., and
Sebben, M. (1995) Biochemistry 34, 1050–1057.
27. Holtmann, M. H., Hadac, E. M., and Miller, L. J. (1995) J. Biol. Chem. 270,
14394–14398.
29. Vilardaga, J.-P., De Neef, P., De Padov, E., Bollien, A., Waelbroeck, M., and Robberecht, P. (1995) Biochem. Biophys. Res. Commun. 213, 885–891
30. Couvineau, A., Gaudin, P., Maoret, J.-J., Rouyer-Fessard, C., Nicole, P., and Laburthe, M. (1995) Biochem. Biophys. Res. Commun. 206, 246–252
31. Gaudin, P., Couvineau, A., Maoret, J.-J., Rouyer-Fessard, C., and Laburthe, M. (1995) Biochem. Biophys. Res. Commun. 211, 901–908
32. Cao, Y.-J., Gimpl, G., and Fahrenholz, F. (1995) Biochem. Biophys. Res. Commun. 212, 673–680
33. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J., and Goeddel, D. V. (1990) Cell 61, 361–370
34. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
35. Coton, P., Ibarondo, J., Devilliers, G., Balestre, M.-N., Duvoid, A., and Guillon, G. (1992) Am. J. Physiol. 263, E1054-E1062
36. Ogi, K., Miyamoto, Y., Masuda, Y., Habata, Y., Hosoya, M., Ohtaki, T., Masuo, Y., Onda, H., and Fujino, M. (1993) Biochem. Biophys. Res. Commun. 196, 1511–1521
37. Aino, H., Hashimoto, H., Ogawa, N., Nishino, A., Yamamoto, K., Nogi, H., Nagata, S., and Baba, A. (1995) Gene 164, 301–304
38. Gourlet, P., Woussen-Colle, M.-C., Robberecht, P., De Neef, P., Cauvin, A., Vandermeers-Piret, M.-C., Vandermeers, A., and Christophe, J. (1991) Eur. J. Biochem. 193, 535–543
39. Robberecht, P., Gourlet, P., De Neef, P., Woussen-Colle, M.-C., Vandermeers, A., Vandermeers-Piret, M.-C., and Christophe, J. (1992) Eur. J. Biochem. 207, 239–246
40. Hou, X., Vandermeers, A., Gourlet, P., Vandermeers-Piret, M.-C., and Robberecht, P. (1994) Neuropharmacology 33, 1189–1195
41. Arimura, A., Somogyvari-Vigh, A., Miyata, A., Mizuno, K., Coy, D. H., and Kitada, C. (1991) Endocrinology 129, 2787–2789
