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Pituitary adenylate cyclase-activating polypeptide (PACAP) is an upstream regulator of prodynorphin mRNA expression in neurons

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ABSTRACT

Although dynorphins are widely involved in the control of not only nociceptive neurotransmission but also a variety of brain functions such as memory and emotion, no natural regulator for inducing the mRNA expression of prodynorphin (Pdyn), a precursor protein of dynorphins, is known. Using primary cultures of rat cortical neurons, we found that pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon neuropeptide family, markedly induces *Pdyn* mRNA expression. PACAP was much more effective than VIP, indicating a major role for PAC1 in the PACAP-induced *Pdyn* mRNA expression. The increase in *Pdyn* mRNA expression was independent of *de novo* protein synthesis. Administration of forskolin, an activator for adenylate cyclase/protein kinase A (PKA), but not TPA, an activator for protein kinase C (PKC), induced *Pdyn* mRNA expression, suggesting a major role for PKA. The involvement of PKA was supported by the inhibition of PACAP-induced *Pdyn* mRNA expression upon addition of H89, an inhibitor for PKA. The PACAP-induced potentiation of NMDA-R was involved in the mRNA expression of *Bdnf* or *c-fos* but not *Pdyn*. These results suggest PACAP to be an upstream regulator for inducing *Pdyn* mRNA expression through PKA.

Prodynorphin (Pdyn) is a precursor protein that generates endogenous opioid neuropeptides, including dynorphin A (Dyn A), dynorphin B (Dyn B), and big dynorphin (Big Dyn), consisting of Dyn A and Dyn B sequences [26]. Although dynorphins interact with κ-opioid receptors and exert analgesic effects [7], their prolonged administration induces hyperalgesia through *N*-methyl-D-aspartate receptors (NMDA-R) [13,28]. The expression of *Pdyn* mRNA and its protein products was found to be up-regulated in the spinal cord in animal models of inflammatory and neuropathic pain [9,18,24,31,32]. However, a natural regulator for inducing *Pdyn* mRNA is still unclear. On the other hand, chronic nociceptive responses are markedly reduced in mice lacking PAC1, a specific receptor for pituitary adenylate cyclase-activating polypeptide (PACAP) [11,25]. PACAP has been isolated from ovine hypothalamus extracts on the basis of its ability to stimulate the formation of cAMP in rat pituitary cells [19] and is widely expressed in the central nervous system [29], being involved in the control of pleiotropic physiological functions such as pain, synaptic plasticity, and emotional control. In the dorsal root ganglion (DRG), PACAP is expressed in small to medium-sized neurons projecting to superficial layers of the dorsal horn of the spinal cord, where PAC1 is expressed, and up-regulated after nerve transection [10]. Considering that the expression of PACAP is increased in DRG and that of dynorphins in the spinal dorsal horn, it seems possible that PACAP is a candidate regulator for stimulating *Pdyn* mRNA expression. In order to know the relationship between Pdyn and PACAP, in this study, we therefore investigated whether PACAP induces the *Pdyn* mRNA expression in neurons or not.

Primary cultures of rat cortical neurons were prepared from the cerebral cortex of Sprague–Dawley rats as described previously [6]. The cerebral cortex was isolated from the brain at embryonic day 17. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum (FCS), 100 U/mL of penicillin and 100 μg/mL of streptomycin. After 3 days, the medium was replaced with DMEM not containing FCS. Each experiment was performed after 5 days in culture. The proportion of neurons expressing PAC1 was estimated at about 60% by immunostaining the cortical neurons with anti-PAC1 antibody. Most of the PAC1-positive cells were positive for MAP2 (data not shown).

Cortical neurons were stimulated with 100 nM PACAP, cultured for specified periods, and collected to prepare total RNA

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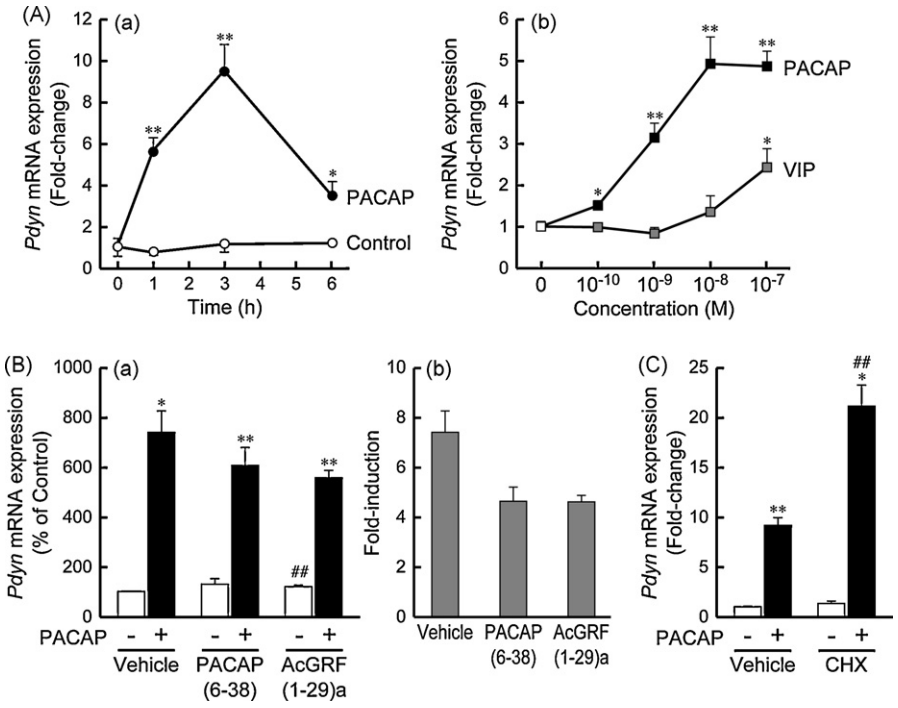

Fig. 1. Effect of PACAP on *Pdyn* mRNA expression in cultured rat cortical neurons. A: (a) At 5 days in culture, cortical neurons were treated with 100 nM PACAP, and total RNA was extracted at the indicated times (h). The quantity of *Pdyn* mRNA was measured using quantitative RT-PCR. Mean ± S.E. $n = 3$–4. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control at the same time. (b) At 5 days in culture, neurons were treated with different concentrations of PACAP (black) or VIP (gray) for 3 h, and total RNA was extracted. The quantity of *Pdyn* mRNA was measured by quantitative RT-PCR. Mean ± S.E. $n = 3$. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control. B: A PAC1 antagonist, PACAP(6–38) (5 μM), or a VPAC1/VPAC2 antagonist, [Ac-Tyr1, D-Phe2] GRF (1–29) amide (AcGRF(1–29)a, 5 μM), was added to cultured neurons 10 min before the treatment with 100 nM PACAP. Total RNA was extracted 3 h after the treatment with PACAP, and the quantity of *Pdyn* mRNA was measured by quantitative RT-PCR. (a) Data was shown as a percent of control (in the absence of PACAP and antagonists). Mean ± S.E. $n = 3$–4. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control. $^{\#\#}p < 0.01$ versus vehicle-treated sample with PACAP. (b) Data was shown as a fold-induction (compared to the sample without PACAP but with each antagonist or vehicle). Mean ± S.E. $n = 3$–4. C: Ten minutes before the treatment with PACAP, cells were pretreated with cycloheximide (CHX, 10 μg/mL). After the treatment with PACAP for 3 h, total RNA was extracted, and the quantity of *Pdyn* mRNA was measured by quantitative RT-PCR. Mean ± S.E. $n = 3$. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control. $^{\#\#}p < 0.01$ versus vehicle.

for measuring the expression of Pdyn mRNA. Quantitative RT-PCR was performed using Mx3000p (Stratagene), according to methods described previously [6]. For an internal control, *Gapdh* cDNA was amplified, and the levels of each transcript were normalized to *Gapdh*. Primer sequences for measuring mRNA were: for *Pdyn*, 5′-TTGTGTTCCCTGTGTGCAGT-3′ and 5′-AGTGCCCA GTAGCTCAGATT-3′; for *c-fos*, 5′-GTTTCAACGCGGACTACGAG-3′ and 5′-AGCGTATCTGTCAGCTCCCT-3′; for *Bdnf*, 5′-CCACCAGGTGA GAAGAGTGATGACC-3′ and 5′-GCCCATTCACGCTCTCCA-3′; and for *Gapdh*, 5′-ATCGTGGAAGGGCTCATGAC-3′ and 5′-TAGCCCAGG ATGCCCTTTAGT-3′.

All values represent the mean ± S.E. of results from a number of separate experiments performed in duplicate, as indicated in the corresponding figures. Statistical analyses were performed using Student's *t*-test followed by the *F*-test with the probability level for significance set to $p < 0.05$.

To investigate whether PACAP induces the expression of *Pdyn* mRNA in neurons, we examined the changes in *Pdyn* mRNA levels in primary cultures of rat cortical neurons stimulated with PACAP, using the quantitative RT-PCR method. As shown in Fig. 1A(a), the mRNA expression increased 1 h after the treatment of cultured neurons with 100 nM PACAP, peaked at 3 h, and then decreased 6 h later, indicating that the expression of *Pdyn* mRNA is transiently induced by a direct effect of PACAP.

We next investigated the responses of *Pdyn* mRNA expression to the administration of PACAP or VIP and compared them. As shown in Fig. 1A(b), the mRNA level began to increase at 100 pM PACAP, peaking at 10 nM PACAP, whereas it took 100 nM for VIP to have any effect, the difference corresponding to that in the affinity for PAC1 [29]. These results indicate that PACAP induces *Pdyn* mRNA expression by acting on PAC1 in culture, though, at higher concentrations of PACAP, VPAC1/VPAC2 could also be involved.

PACAP receptors are classified into two types based on affinity for PACAP and VIP [4,29]. PAC1, a type I receptor, exhibits high affinity for PACAP, and much lower affinity for VIP. VPAC1 and VPAC2, type II receptors, possess similar affinity for PACAP and VIP. To determine which receptors are involved in the increase in *Pdyn* mRNA expression induced by PACAP at 100 nM, we added PACAP(6–38) and [Ac-Tyr1, D-Phe2] GRF(1–29) amide (AcGRF(1–29)a), antagonists for PAC1 and VPAC1/VPAC2, respectively, in excess amounts to the medium (final concentration; 5 μM). Although the basal level of *Pdyn* mRNA expression slightly increased upon the treatment of cultured cells with PACAP(6–38) or AcGRF(1–29)a (Fig. 1B(a)), PACAP-induced *Pdyn* mRNA expression was partially repressed by pretreatment with these antagonists (Fig. 1B(b); Fold-induction; Vehicle, 7.41 ± 0.856; PACAP(6–38), 4.62 ± 0.559; and AcGRF(1–29)a, 4.61 ± 0.254). The antagonistic effect of PACAP(6–38) on the PACAP-induced *Pdyn* mRNA expression seemed to be relatively low. Inhibitory effect of PACAP(6–38) on the PACAP-induced *c-fos* and *Bdnf* mRNA expression were also observed (Supplementary Fig. 1). It has been suggested that PACAP(6–38) acts as a VPAC2 agonist besides its antagonistic effect on PAC1 [29]. The low antagonistic effect of PACAP(6–38) on the PACAP-induced *Pdyn* mRNA expression might be due to such a side-effect of PACAP(6–38).

In the rat *Pdyn* promoter, the binding site for activator protein-1 (AP-1), an inducible transcription factor, is found [20]. Therefore, to investigate whether or not the PACAP-induced *Pdyn* mRNA expression is dependent on *de novo* protein synthesis of such an inducible transcription factor, we examined the effect of cyclohex-