PC12 Cells as a Model to Study the Neurotrophic Activities of PACAP

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TRANSDUCTION PATHWAYS ACTIVATED BY PACAP IN PC12 CELLS

PACAP was first reported to increase cAMP and inositol phosphate production in PC12 cells.[1] Cyclic AMP production in turn activates genes whose promoters bear an AP-1– or a CREB-binding motif.[2] Stimulation of PAC1-R also induces a calcium influx current that depends upon activation of both the phospholipase C and adenylate cyclase pathways.[3] The PACAP-induced stimulation of adenylate cyclase in PC12 cells leads to an increase in neuroendocrine gene expression, including activation of transcription of the TH gene[4] and reporter genes driven by neuropeptide promoters.[5] Stimulation of PKC by PACAP itself has been reported to induce PACAP gene expression.[6]

TRANSDUCTION PATHWAYS INVOLVED IN PC12 CELL DIFFERENTIATION

Besides its effect on peptide synthesis and TH activity, PACAP also promotes neurite outgrowth and inhibits proliferation of PC12 cells cultured in the presence of serum[1,7] (unpublished observations). Since cyclic AMP promotes PC12 cell differentiation,[8] it would first have been assumed that the neurotrophic effect of PACAP is simply mediated through the classical cAMP/protein kinase A signaling pathway. Two obervations suggest that the situation is more complex. First, inhibition of PKA by H89 does not block the neuritogenic effect of PACAP. Second, pre-treatment of PC12 cells with PMA blocks the ability of PACAP to cause neurite outgrowth.[9] We have observed that PMA not only inhibits neurite outgrowth, but also induces neurite retraction when added 48 h after PACAP. This observation and the fact that PKC inhibitors failed to block the effect of PACAP[7] (unpublished observations) suggest that PKC is not involved in PACAP-stimulated neurite outgrowth per se, but that PKC activation can exert a dominant neurite-collapsing activity in PC12 cells. Another re-

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port demonstrates that PACAP-stimulated neurite outgrowth was abolished by the cAMP antagonist, RpcAMPS, and we have observed (unpublished data) that inhibition of adenylate cyclase with 2′,5′-dideoxyadenosine strongly reduced the ability of PACAP to induce PC12 cell differentiation. It has traditionally been assumed that PKA is an obligate downstream effector for cAMP, but evidence is accumulating for the existence of a direct coupling to the Ras superfamily signaling pathway.[10]

Two apparently contradictory data sets apply to a full molecular description of the signaling pathways used for PACAP-induced neuritogenesis in PC12 cells. There is general agreement that PACAP activates the MAP kinases ERK1 and -2 and, further, that activation of ERK1/2 is required for PACAP-induced neuritogenesis, because the MEK inhibitors U0126 or PD98059, according to several reports, block both PACAP activation of ERK1/2 and neurite outgrowth initiated by PACAP.[9,11–14] Thus it would seem most likely that PACAP causes neuritogenesis by elevating cyclic AMP, which then activates ERK1/2 through a PKA-dependent process.[11,12] However, contradicting this, there is ample evidence that both PACAP-stimulated activation (phosphorylation) of ERK1/2 and PACAP-induced neuritogenesis are not dependent on activation of PKA[9,13] (unpublished observations).

Activation of two distinct small G proteins, Ras and Rap-1, link the stimulation of both the EGF and NGF receptors to the MAPK cascade (ERK1/2 phosphorylation) by activating c-Raf and B-Raf, respectively.[14,15] Inhibition of Ras is reported not to interfere with the neuritogenic effect of PACAP.[9] Therefore, the possible roles of both PKA-dependent[11,12] and PKA-independent[16,17] activation of Rap-1 in the control of ERK phosphorylation in PC12 cells are under investigation[18] (unpublished data). Both PACAP and NGF induce a rapid and long-lasting stimulation of ERK, but promote neurite outgrowth with different kinetics—PACAP acts faster than NGF by several days. It may be that sustained ERK stimulation arising from several convergent signaling pathways can drive neuritogenesis, and that this allows some signal redundancy that accounts for disparate pharmacological observations when different neurotrophins and culture conditions (serum factors; cell line variation) are employed.

PC12 CELLS AND EFFECTS OF PACAP ON NEURONAL DEVELOPMENT

As reported with cortical neuron precursors,[19] PACAP decreases the proportion of mitotic PC12 cells when cultured in the presence of serum and induces neurite outgrowth[1] (unpublished observations). The similarities between the neuroprotective effects of PACAP on immature cerebellar granule neurons and PC12 cells cultured in defined medium should also be pointed out (FIGS. 1 and 2).[20] PC12 cells cultured in complete medium with serum began to die apoptotically when serum was removed, within about 24 h of serum withdrawal (FIG. 1). Addition of PACAP 24 h after serum withdrawal strongly reduced PC12 cell death within the next two days (FIGS. 1 and 2). It has also been demonstrated that PACAP induces ERK1 and -2 phosphorylation through a PKA-independent mechanism in both PC12 and granule cells.[9,21] It remains to be investigated whether the downstream effectors activated by PACAP remain identical; these preliminary observations, however, suggest that

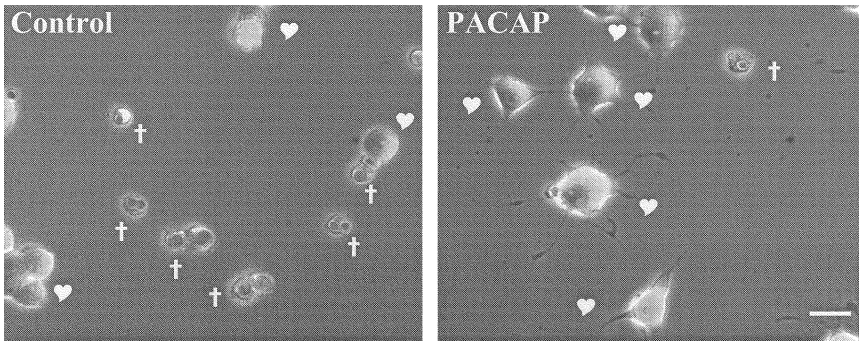

FIGURE 1. Microphotographs illustrating the effect of PACAP on survival and differentiation in PC12 cells. (**A**) Appearance of PC12 cells cultured in serum-free medium for 48 h. (**B**) Appearance of PC12 exposed to 10^{-7} M PACAP for 48 h. (*Cross symbol*) Dying cell labeled with propidium iodine. (*Heart symbol*) Healthy cells labeled with calcein. Scale bar = 15 μm.

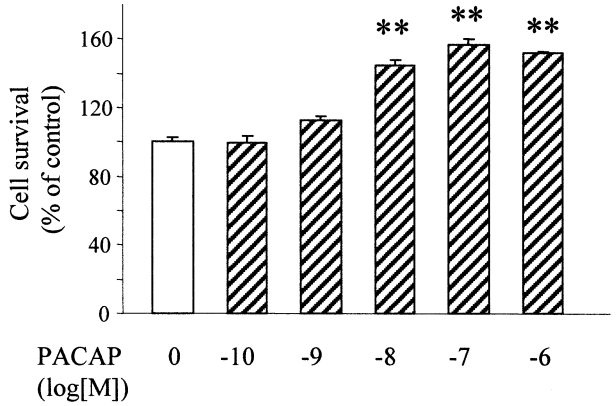

FIGURE 2. Effect of graded concentrations of PACAP on survival of PC12 cells cultured in serum-free medium for 48 h. Representative data of three independent experiments. ** $P < 0.001$.

PC12 cells could be a useful model to understand the role of PACAP during neurodevelopment.

PC12 CELLS AND NEUROPROTECTIVE ACTIONS OF PACAP

Apoptosis of PC12 cells can be induced by depletion of serum and NGF from the culture medium; in these conditions it has been reported that PACAP protects PC12 cells from apoptosis. However, PC12 cells have also been used to investigate the ability of PACAP to counteract the deleterious effect of neurotoxic agents. In particular, ceramide,[22] or the lipid peroxidation product 4-hydroxynonenal,[23] induces ap-

optosis that is inhibited by PACAP. Because these same neuroprotective effects of PACAP have been reported in primary neuronal cultures,[23,24] PC12 cells have become a valuable model for investigation of neuroprotective properties of PACAP.

PC12 CELLS AND THE ABILITY OF PACAP TO ACT AS AN ANTITUMOR AGENT

PACAP has been reported to either increase tumor cell proliferation or induce differentiation. For example, on the small-cell lung tumor cell line NCI-H345 or the pancreatic carcinoma AR4-2J cells, PACAP stimulates proliferation.[25,26] Overexression of PACAP may also be involved in some cancer development because the PAC1-R antagonist PACAP(6-38) reduces tumor growth in mice bearing PC-3 xenografts[27] or breast cancer cell xenografts.[28] On the other hand, PACAP inhibits proliferation of glioblastoma and colonic adenocarcinoma cells.[29,30] PACAP inhibits PC12 cell proliferation within 48 h, which is rapid compared to the antiproliferative action of NGF. Signaling molecules such as Ras[31] or CREB[32] have been reported to mediate the neurotrophic effects of NGF. With regard to PACAP, firm data are not available yet concerning the signaling components involved in cell-cycle and cell-survival regulation. This lack of information has encouraged several laboratories to attempt to identify genes and proteins involved in the neurotrophic effects of PACAP using high-output screening techniques[33] with PC12 cells as a model.

CONCLUSION

There is now clear evidence that PACAP exerts trophic effects on multiple cell types, but many questions remain unanswered regarding the molecular mechanisms involved in the effects of PACAP on proliferation, migration, differentiation, and apoptosis. We anticipate that PC12 cells will be useful in explicating the mechanisms of action of PACAP on key regulatory proteins of the cell cycle and components of apoptotic pathways, and accelerate progress in focusing on the mechanisms of PACAP function in various pathological conditions such as ischemia and cancer.

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