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Regulation of PC12 Cell Differentiation by cAMP Signaling to ERK Independent of PKA: Do All the Connections Add Up?

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Adenosine 3',5'-monophosphate (cyclic AMP, also abbreviated cAMP) is a universal second messenger that mediates various physiological events in the mammalian central nervous system after the activation of G_s-coupled G protein-coupled receptors (GPCRs), such as the pituitary adenylate cyclase-activating polypeptide (PACAP)-activated PAC1 receptor. The primary effector for cAMP is cAMP-dependent protein kinase (PKA), a heterotetramer consisting of two regulatory and two catalytic subunits. After dissociation, the catalytic subunits phosphorylate multiple substrates affecting cell behavior and genetic programming. Historically, PKA was thought to be the only effector directly activated after the elevation of cAMP, and hence cAMP and its direct target PKA were defined as the canonical cAMP signaling cassette. However, work in various systems, including PC12 cells, suggests that there are PKA-independent, and thus noncanonical, cAMP signaling cassettes (cAMP to X). One of the open questions is whether any of these provide a mechanism for the activation of extracellular signal-regulated kinases [ERKs, members of the mitogen-activated protein kinase (MAPK) family], major downstream effectors on which several cAMP-initiated processes, including neuritogenesis (the extension of neurites) in PC12 cells, apparently converge.

A major advance in identifying potential noncanonical signaling effectors came with the identification of a guanine nucleotide exchange factor (GEF) for the small guanosine triphosphatase (GTPase) Rap. This GEF was named EPAC, for exchange protein directly activated by cAMP, and bound cAMP in a manner independent of PKA (1, 2). The development of a specific agonist for EPAC, 8-CPT-2Me-cAMP, facilitated the study of this noncanonical cAMP signaling pathway (3). Several groups have since used this compound or its derivatives to probe the involvement of EPAC in various physiological and signaling processes, including β -adrenergic receptor-mediated calcium release in cardiac myocytes (4), leukocyte adhesion and chemotaxis (5), anti-inflammatory effects of cytokines in endothelial cells (6), activation of R-Ras and H-Ras (7, 8), modulation of Ca²⁺-regulated potassium channels through p38 MAPK (9), and activation of ERK (8, 10–12). However, it must be pointed out that EPAC remains only a candidate for noncanonical cAMP signaling to ERK in PC12 and neuronal cells: Direct application of EPAC-specific agonists fails to activate ERK in either PC12 cells (3, 10, 13) or primary locus ceruleus neurons (10). It

might be involved in ERK activation indirectly, because EPAC agonist treatment in some cases lifts the inhibition of GPCR-stimulated ERK activation after treatment with adenylate cyclase inhibitors (10).

PC12 cells are especially amenable to the mechanistic study of noncanonical cAMP signaling in cAMP-dependent neuritogenesis (14). PC12 cells adopt a neuronal-like morphology when treated with nerve growth factor (NGF) or with cAMP-elevating agents, such as forskolin or PACAP (15–17). The extension of neurites is one hallmark of the neuronal phenotype, along with cessation of proliferation; expression of differentiation-specific genes, such as those encoding transin and sodium-channel proteins; and production of specific neurotransmitters (18). Based on pharmacological analysis, PACAP-mediated neuritogenesis is independent of PKA (not blocked by H89, a specific PKA inhibitor) but is dependent on ERK (blocked by UO126, a specific inhibitor of MEK, the kinase upstream of ERK that is required for ERK activation) (17, 19) (Fig. 1). Although the PAC1 receptor couples to both G_q and G_s G proteins, PACAP-mediated neuritogenesis in PC12 cells is believed to proceed through cAMP because cAMP analogs or cAMP elevation with forskolin mimic the effects of PACAP (16, 20–22). In addition, neuritogenesis induced by PACAP is blocked by the cAMP antagonist dideoxyadenosine (22), and there is currently no evidence that direct activation of G_q -mediated signaling mediates differentiation in PC12 cells.

EPAC, as one known target of cAMP, is a candidate for PACAP-mediated PKA-independent neuritogenesis. Although the EPAC-selective agonist 8-pCPT-2-O-Me-cAMP elicited some neurite extension, neurites were longer when 8-pCPT-2-O-Me-cAMP was combined with a selective PKA agonist (23). Although this synergy with PKA casts some doubt on the idea that EPAC is the sole direct mediator of PACAP-mediated neuritogenesis, other experiments connect EPAC to PACAP-mediated neuritogenesis. The small GTPase Rit is required for PACAP-mediated neuritogenesis in PC6 cells, a subline of PC12 cells (24). Rit was activated by PACAP in PC6 cells in a cAMP-dependent but PKA-independent manner and was activated by the EPAC-selective agonist 8-CPT-2-Me-cAMP, suggesting a noncanonical signaling cassette cAMP-EPAC-Rit (24). Although it is tempting to propose this cAMP-EPAC-Rit signaling cassette as the noncanonical pathway mediating neuritogenesis, there still remains a crucial unconnected component: ERK. PACAP activation of ERK is well documented as essential for PACAP-mediated neuritogenesis in PC12 cells (17, 19). However, knockdown of Rit did not prevent PACAP activation of ERK in PC6 cells (24), yet it did diminish (though not abolish) PACAP-mediated neurite formation. This suggests that although the noncanonical signaling cassette cAMP-EPAC-Rit is present, it is not the only cAMP-dependent signal that contributes to PACAP-mediated neuritogenesis in PC12 cells.

Pharmacological studies place ERK as an essential component in both forskolin and PACAP-mediated neuritogenesis; therefore, it is important to understand how ERK is activated by cAMP in PC12 cells. Enserink *et al.* found that cAMP-mediated activation of ERK occurred independently of both Rap and EPAC in PC12 cells (3). This implies that EPAC is not upstream of ERK for PACAP-mediated neuritogenesis in PC12 cells. There are two Rap GEFs—EPAC and Crk SH3 domain guanine nucleotide exchanger (C3G)—and in

PC12 cells ERK activation is dependent on which Rap GEF is used (13). Specifically, stimulation of ERK by forskolin is dependent on PKA, Rap1, and C3G and independent of EPAC, and these findings are recapitulated in COS cells, which lack endogenous EPAC (13). PC12 cells contain very low levels of EPAC, and even overexpression of EPAC in PC12 cells does not lead to PKA-independent forskolin-mediated ERK activation (13), which is consistent with previous observations (3). Instead, forskolin uses a PKA-dependent C3G pathway to activate ERK in PC12 cells (13).

The critical link missing from these studies is the component responsible for cAMP-dependent PKA-independent activation of ERK in PC12 cells (3, 13, 16, 20). To summarize the existing data on PC12 cells: (i) Wang *et al.* show that ERK activation by forskolin is PKA-dependent (13); (ii) Enserink *et al.* suggest that EPAC is not involved in ERK activation by cAMP (3); and (iii) in PC6 cells, Shi *et al.* suggest that Rit is a PKA-independent effector of cAMP activated by EPAC, but Rit is not essential for ERK activation by PACAP (24) (Fig. 2). The experimental design of these studies was similar in that they used forskolin, 8-bromo-cAMP, or PACAP to elevate cAMP, but two points deserve to be mentioned. Enserink *et al.* (3) and Wang *et al.* (13) used 8-bromo-cAMP or forskolin as the cAMP stimulus rather than a G_s-coupled GPCR such as the PACAP PAC1 receptor. Cyclic AMP generation by forskolin, for example, may differ spatiotemporally from cAMP generation after GPCR activation. Second, the ERK activation assessed in these studies (3, 13, 24) followed serum starvation of the PC12 or PC6 cells; the signaling cascades engaged by cAMP may be different from those engaged by NGF or PACAP during differentiation in serum-containing media. Thus, it would be instructive to assess the involvement of EPAC in the activation of ERK after stimulation of the cell by either forskolin or a G_s-coupled receptor ligand, such as PACAP, when serum is present. The immediate challenge is to establish the signaling cascade that PACAP uses to mediate neuritogenesis in PC12 cells that require cAMP, is independent of PKA, and yet depends on ERK. Progress has been made in identifying two signaling molecules regulated by cAMP independently of PKA: EPAC and Rit. However, the connection of these molecules, if any, leading to ERK activation that contributes to cAMP-mediated neuritogenesis remains to be established. Furthermore, it appears that cAMP engages both canonical and noncanonical signaling pathways, which may in combination regulate different aspects of the overall cellular differentiation program, including neuritogenesis, growth arrest, and regulation of expression of differentiation-specific genes such as those encoding sodium-channel and transin proteins. Clearly, one cannot rule out a cAMP-initiated signaling cascade simply by ruling out the involvement of PKA. An entirely new manifold of cAMP-regulated downstream effectors in addition to PKA is coming into view in PC12 cell differentiation. Whether the two noncanonical cAMP cassettes that include EPAC and Rap1 or Rit represent noncanonical cAMP signal transduction pathways operating in neuronal differentiation and neuronal signaling in vivo remains to be seen. Verification of this hypothesis will require the definitive demonstration that ERK activation involves EPAC in neuronal cells, and the determination of whether EPAC or another noncanonical cAMP-initiated signaling pathway is involved in neuritogenesis in primary neurons as well as in their signal transduction stand-in, the PC12 cell.

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Fig. 1.

A putative pathway from cAMP to ERK in PACAP-mediated neurite outgrowth in PC12 cells. Pharmacological experiments show that PACAP acting through the PAC1 G protein-coupled receptor stimulates neurite outgrowth through a pathway that requires ERK but is independent of PKA. Some other aspects of PC12 cell differentiation stimulated by PKA are also ERK-dependent (Fig. 2) and therefore blocked by UO126. AC, adenylate cyclase

Fig. 2.

PACAP and cAMP-mediated activation of ERK leading to neuritogenesis. PACAP-mediated neurite outgrowth is cAMP initiated, PKA independent, and ERK dependent. EPAC-dependent, PKA-independent, and ERK-independent pathways, blue ovals; PKA-dependent activation of ERK, pink ovals.