Regulation of cAMP-Dependent Protein Kinase Subunit Expression in CATH.a and SH-SY5Y Cells

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ABSTRACT
Increasing evidence supports a role for adaptations in the cAMP pathway in mediating aspects of neural plasticity. These adaptations include altered levels of the catalytic (C) and regulatory (R) subunits of cAMP-dependent protein kinase (PKA) in specific neuronal cell types. In an effort to understand the mechanisms underlying this regulation of PKA, the effects of perturbing the cAMP pathway on PKA expression were examined in the locus ceruleus-like CATH.a cell line and the human neuroblastoma SH-SY5Y cell line. Exposure of CATH.a and SH-SY5Y cells to forskolin, a direct activator of adenylyl cyclase, resulted in a time-dependent decrease in levels of immunoreactivity of C and the two types of R (RI and RII). This decrease in PKA subunit immunoreactivity was not attenuated by pretreatment of the cells with the protein synthesis inhibitor cycloheximide. Moreover, exposure of the cell lines to forskolin had no effect on levels of mRNA for these PKA subunits over a wide time course. In contrast, treatment of cells with a cAMP antagonist (Rp-8-bromo-cAMPS) dramatically increased levels of PKA subunit immunoreactivity, particularly that of RI. No change in RI mRNA levels, however, was observed under these conditions. The PKA catalytic inhibitor H-89 did not attenuate the forskolin-induced down-regulation. The PKA subunit down-regulation was blocked, however, by treatment of the cells with Leu-Leu-Leu or lactacystin, inhibitors of proteasomes that are implicated in the regulated proteolysis of specific cellular proteins. Together, these findings demonstrate that regulation of PKA subunit expression by forskolin or a cAMP antagonist occurs primarily through post-transcriptional mechanisms and suggests the involvement of proteasome-mediated degradation in these phenomena.

A large number of G protein-coupled receptors are known to elicit diverse physiological effects in the brain via regulation of the cAMP pathway. The most important mechanism of action of cAMP is the activation of cAMP-dependent protein kinase (PKA), which is known to phosphorylate a wide array of neuronal phosphoproteins.

The inactive holoenzyme of PKA consists of two regulatory (R) and two catalytic (C) subunits. On activation by cAMP binding to the R subunits, the holoenzyme dissociates into an R dimer and two free, active C subunits that can then phosphorylate target proteins. Two types of PKA (PKA I and PKA II) are distinguished by their R subunits, RI and RII. The molecular complexity of PKA increased with the cloning of multiple isoforms of these subunits, including four R subunit genes (RIα, RIβ, RIα and RIIβ) and three C subunit genes (Ca, Cβ, and Cγ) (reviewed in McKnight et al., 1988; Cadd and McKnight, 1989). Two splice variants of C subunits, Ca2 (Thomis et al., 1992) and Cβ2 (Wiemann et al., 1991), have more recently been cloned. In all cases, the α forms of the PKA subunits are ubiquitously expressed, whereas the β forms display a more tissue-specific expression with the highest levels in brain.

The importance of PKA in synaptic plasticity has been demonstrated in cellular models of learning and memory. In Aplysia, PKA-mediated phosphorylation has been implicated in both short- and long-term facilitation of the sensory motor neuron synapses (reviewed in Byrne and Kandel, 1996). Alterations in total levels of PKA subunits also have been documented under these conditions (Chain et al., 1995). Yin and Tully (1996) have shown some similar processes to be involved in the formation of long-term memory in Drosophila. In mammals, a role for PKA has been demonstrated in the formation of long-term potentiation in hippocampus as well as in the consolidation of long-term memory (Abel et al., 1997).

The cAMP pathway is also known to contribute to the neural plasticity responsible for drug addiction (Nestler and Aghajanian, 1997). Chronic exposure of rats to morphine (or

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ABBREVIATIONS: C, catalytic; R, regulatory; PKA, cAMP-dependent protein kinase; RI, regulatory type I; RII, regulatory type II; Rp-8Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer; LLL, Z-Leu-Leu-Leu-H; SSC, standard saline citrate; CRF, corticotrophin-releasing factor; PGE1, prostaglandin E1.
other drugs of abuse) up-regulates the cAMP pathway in specific brain regions known to mediate aspects of addiction. This up-regulation includes increased levels of PKA C and R subunits, which have been related directly both to drug-induced changes in the electrophysiological properties of neurons in these brain regions and to specific behavioral features of addiction (Self et al., 1998; Nestler and Aghajanian, 1997; White et al., 1998). A similar up-regulation of PKA function has been implicated in other brain regions as a mediator of the long-term actions of antidepressant treatments (Duman et al., 1997).

Despite the demonstrated importance of PKA in several forms of neuronal plasticity, little is known about the mechanisms controlling PKA expression in neural cells. Because several of the treatments that alter PKA levels after chronic exposure can regulate cAMP formation acutely, one possibility is that changes in PKA subunit expression represent homeostatic responses to repeated perturbations of the cAMP pathway. In this study, two continuous neural-derived cell lines were used as model systems to examine the mechanisms underlying the effect of cAMP pathway perturbations on PKA expression.

CATH.a cells are derived from brainstem tumors of transgenic mice in which the tyrosine hydroxylase promoter directs SV40 T-antigen expression (Suri et al., 1993). SH-SY5Y cells, a subclone of the human neuroblastoma cell line SK-N-SH (Ross et al., 1983), are known to contain several receptors linked to the cAMP pathway (Yu et al., 1988). We report regulation of PKA subunits in response to cAMP pathway perturbations in these neuronal cell lines, and show that this regulation likely results from post-transcriptional mechanisms.

Methods

Cell culture. CATH.a cells (Suri et al., 1993) were cultured at 37°C, 5% CO₂ in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 8% horse serum and 4% fetal bovine serum (GIBCO BRL). SH-SY5Y cells were grown at 37°C, 5% CO₂ in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with 10% fetal bovine serum. At 80% confluence, cells were treated with 5 μM forskolin or 1,9-dideoxysk forskolin (Sigma Chemical, St. Louis, MO) in ethanol, 100 μg/ml cycloheximide (Sigma) and 200 μM Rp-8Br-cAMPS (Biolog Life Science Institute, La Jolla, CA) in water, or 0.1 mM laetrile (Calbiochem), 1 mM p-nitrobenzamidine, 1 μM pepstatin), sonicated, incubated on ice for 30 min and centrifuged at low speed, and the supernatant was retained. EMSA extracts were shown to contain virtually all of the PKA subunit immunoreactivity of the total homogenate. Protein assays were performed according to the method of Bradford. Equal amounts of protein (20–30 μg) were loaded onto 8% SDS-polyacrylamide gels, electrophoresed and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Nitrocellulose blots were blocked in PBS (10 mM sodium phosphate, pH 7.4, 0.9% NaCl) supplemented with 0.1% Tween-20 and 5% (for anti-R) or 2% (for all other antibodies) nonfat dry milk and incubated with primary antibody. Dilutions for primary antibodies are as follows: C (Santa Cruz, Santa Cruz, CA) at 1:10,000; RI (Transduction Labs, Lexington, KY) at 1:1000; RII (Santa Cruz) at 1:1000; actin (Sigma) at 1:2000; G₀ (Upstate Biotechnology, Lake Placid, NY) at 1:5000; and G₁ (Upstate Biotechnology) at 1:5000. Primary antibodies were detected using peroxidase-conjugated secondary antibodies (Vector Labs, Burlingame, CA) and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Blots were exposed to a phosphor screen and analyzed using Molecular Imager (BioRad, Hercules, CA). The linear range of the chemiluminescence response is extended by use of the Molecular Imager instead of film. Signals detected fell within this linear range as determined through use of a protein standard curve. Equal loading and transfer of proteins were confirmed by amido black staining.

Preparation of RNA and Northern analysis. Cells were washed with Dulbecco's PBS and RNA was prepared using RNAqueous (Ambion, Austin, TX). RNA concentrations were measured by absorbance. Equal aliquots of total RNA (10 μg) were electrophoresed in formaldehyde/12% agarose gels, transferred by capillary blotting to reinforced nylon membranes and UV cross-linked to the membranes. Antisense riboprobe labeled with [α-32P]UTP using T7 or SP6 RNA polymerase (Boehringer-Mannheim, Indianapolis, IN) were used for Ca, Rβ and cyclophilin, whereas cDNA probes labeled with [α-32P]UTP using a random prime kit (GIBCO BRL) were used for Cβ and Rß. Hybridizations were performed at 65°C with ribo-probes or at 42°C with cDNA probes for 18 hr in buffer containing 20 mM Tris·HCl, pH 7.5, 1× Denhardt's, 0.1% SDS, 4× SSC (1×, 150 mM NaCl, 15 mM sodium citrate), 50% deionized formamide, 0.2% dextran sulfate and 200 μg/ml denatured salmon sperm DNA. The membranes were washed sequentially at the incubation temperature (or slightly higher) in 2× SSC/0.1% SDS, 0.5× SSC/0.1% SDS and 0.1× SSC/0.1% SDS, exposed to a phosphor screen and analyzed using the Molecular Imager (BioRad). PKA subunit isofrom ribo-probe vectors (Cadd and McKnight, 1989) were obtained from Dr. G. Stanford McKnight (University of Washington, Seattle, WA) and full-length rat cyclophilin was provided by Dr. Steven E. Hyman (NIMH).

Results

Regulation of PKA subunit expression by activators of adenyl cyclase in CATH.a and SH-SY5Y cells. The effect of perturbation of the cAMP system on PKA subunit expression was examined in CATH.a and SH-SY5Y cells by treating cells with forskolin, a diterpene that directly activates adenyl cyclase. Immunoreactivity of PKA subunits was examined using polyclonal antibodies that recognized both the α and β isoforms of each subunit. Immunoblot analysis for C revealed a 20- to 41-kDa band in human SH-SY5Y cells and a 39- to 40-kDa band in CATH.a cells. In SH-SY5Y cells, this C immunoreactivity was decreased significantly in response to forskolin but not its derivative, 1,9-dideoxysk forskolin, which does not activate adenyl cyclase (fig. 1). Similar results were obtained in experiments using either CATH.a or differentiated SH-SY5Y cell (data not shown).

We next examined the time course of the effect of forskolin on C as well as on RI and RII. In both cell lines, immunoblot analysis of RI and RII, respectively, revealed bands of 49 to 53 kDa and 51 to 54 kDa (fig. 2). Forskolin induced a time-dependent down-regulation of all three PKA subunits in CATH.a (fig. 2A), SH-SY5Y (fig. 2B) and differentiated SH-SY5Y (fig. 2C) cell lines. In all cases, the down-regulation of RI was most pronounced and most rapid, followed by C and then RII. RI immunoreactivity was reduced by 50% after only

Preparation of tissue and Western blot analysis. Cells were washed with Dulbecco's PBS (GIBCO BRL), lysed in EMSA buffer (20 mM HEPES, 0.4M NaCl, 20% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 10 μg/ml leupeptin, 0.1 mM 3-aminobenzamidine, 1 μg/ml pepstatin), sonicated, incubated on ice for 30 min and centrifuged at 8000 g, and the supernatant was retained. EMSA extracts were shown to contain virtually all of the PKA subunit immunoreactivity of the total homogenate. Protein assays were performed according to the method of Bradford. Equal amounts of protein (20–30 μg) were loaded onto 8% SDS-polyacrylamide gels, electrophoresed and transferred to nitrocellulose (Schleicher & Schuell, Keene, NH). Nitrocellulose blots were blocked in PBS (10 mM sodium phosphate, pH 7.4, 0.9% NaCl) supplemented with 0.1% Tween-20 and 5% (for anti-R) or 2% (for all other antibodies) nonfat dry milk and incubated with primary antibody. Dilutions for primary antibodies are as follows: C (Santa Cruz, Santa Cruz, CA) at 1:10,000; RI (Transduction Labs, Lexington, KY) at 1:1000; RII (Santa Cruz) at 1:1000; actin (Sigma) at 1:2000; G₀ (Upstate Biotechnology, Lake Placid, NY) at 1:5000; and G₁ (Upstate Biotechnology) at 1:5000. Primary antibodies were detected using peroxidase-conjugated secondary antibodies (Vector Labs, Burlingame, CA) and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Blots were exposed to a phosphor screen and analyzed using Molecular Imager (BioRad, Hercules, CA). The linear range of the chemiluminescence response is extended by use of the Molecular Imager instead of film. Signals detected fell within this linear range as determined through use of a protein standard curve. Equal loading and transfer of proteins were confirmed by amido black staining.

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differentiated SH-SY5Y cells treated with PGE1, a hormone known to activate adenylyl cyclase in these cells (Iredale and Duman, 1997), in a 4-hr treatment with CRF, a hormone known to activate immunoreactivity of C, RI and RII was down-regulated after exposure in CATH.a and differentiated SH-SY5Y cells, respectively (fig. 2A). This recovery was not observed, however, in undifferentiated SH-SY5Y cells, where the down-regulation was greater and less pronounced with RI immunoreactivity measured at 50% of control at 24 to 48 hr (fig. 2B). The effect of forskolin was not generalized to other cellular proteins in that no loss of immunoreactivity of actin, G1α or G1β, was observed in CATH.a cells (fig. 3A), SH-SY5Y cells (fig. 3B) or differentiated SH-SY5Y cells (fig. 3C).

In addition, we examined whether the effects of activation of adenyl cyclase by forskolin could be seen using receptor-linked systems in each of the cell lines. In CATH.a cells, immunoreactivity of C, RI and RII was down-regulated after a 4-hr treatment with CRF, a hormone known to activate adenyl cyclase in these cells (Iredale and Duman, 1997), in a manner similar to the effect seen with forskolin (C: 74.3 ± 5.0% of vehicle, P = .0089 compared with vehicle; RI: 68.6 ± 6.5%, P = .0137; RII: 84.5 ± 2.4%, P = .0034). Likewise, in differentiated SH-SY5Y cells treated with PGE1, a hormone that stimulates adenyl cyclase in these cells (Yu et al., 1988), PKA subunit immunoreactivity was down-regulated (C: 79.6 ± 3.4%, P = .0003; RI: 60.3 ± 5.7%, P = .0023; RII: 83.3 ± 7.0%, P = .0054).

**Lack of effect of cycloheximide on forskolin-induced down-regulation of PKA subunit expression.** As a first effort to understand the mechanism underlying the forskolin-induced down-regulation of PKA subunit expression, the effect of a protein synthesis inhibitor, cycloheximide, was examined. Cells were pretreated with cycloheximide for 1 hr and subsequently treated with forskolin for 4 hr. These conditions have been shown to effectively inhibit protein synthesis in these and other cell lines (see Iredale and Duman, 1997). Cycloheximide had little or no effect either on basal levels of C immunoreactivity or on the forskolin-induced down-regulation of this subunit (fig. 4). Similar results were obtained for RI immunoreactivity. After 18 hr of exposure, cycloheximide alone significantly reduced levels of the PKA subunits (data not shown).

**Lack of regulation of PKA subunit mRNA expression by forskolin in CATH.a and SH-SY5Y cells.** Next, we determined whether the forskolin-induced reductions in levels of PKA subunit immunoreactivity are associated with changes in subunit expression at the mRNA level. Northern blots of total RNA from CATH.a and SH-SY5Y cell lines were analyzed with radiolabeled riboprobes for Cα and Rβ and a radiolabeled cDNA probe for Cβ. A single mRNA species was revealed for each of these subunits (Cα ~2.4 kb, Cβ ~4.3 kb, Rβ ~2.7 kb), consistent with previous reports (McKnight et al., 1988; Solberg et al., 1991). Time course studies revealed that exposure of CATH.a (fig. 5A), SH-SY5Y (fig. 5B), or differentiated SH-SY5Y (fig. 5C) cells to forskolin had no significant effect on the levels of Cα, Cβ or Rβ mRNA, standardized to cyclophilin mRNA, between 1 and 48 hr of treatment.

**Effect of inhibitors of PKA on PKA subunit expression.** To gain further insight into the regulation of PKA expression by perturbations of the cAMP pathway, levels of PKA subunit immunoreactivity were examined after cells were exposed to Rp-8Br-cAMPS, a cell-permeable cAMP antagonist (Gjertsen et al., 1995). This compound binds to the R subunits and prevents endogenous cAMP from activating the PKA holoenzyme. Rp-8Br-cAMPS has a significantly higher affinity for RI than for RII. After Rp-8Br-cAMPS treatment, RI immunoreactivity was dramatically increased to 170% of control in CATH.a cells (fig. 6C) and to 330% of vehicle in SH-SY5Y cells (fig. 6D). This Rp-8Br-cAMPS-induced up-regulation of RI was also observed in the presence of forskolin (fig. 6D,C). In SH-SY5Y cells, levels of C and RII immunoreactivity were also up-regulated to 150% of vehicle; however, concomitant treatment with forskolin and Rp-8Br-cAMPS returned C and RII to basal levels of immunoreactivity (fig. 6, B and F). In CATH.a cells, in which the effect of Rp-8Br-cAMPS exposure on RI is smaller, Rp-8Br-cAMPS had no apparent effect on levels of C or RII in the basal or forskolin-treated state (fig. 6, A and E).

In light of the robust effect of Rp-8Br-cAMPS exposure on RI immunoreactivity, total RNA harvested from CATH.a cells treated with vehicle, forskolin or Rp-8Br-cAMPS was analyzed for Rβ mRNA by Northern blotting. As shown in figure 7, exposure of CATH.a cells to Rp-8Br-cAMPS for 3, 6 or 12 hr had no significant effect on Rβ mRNA levels.

**Concluding remarks.** To better interpret the findings of the Rp-8Br-cAMPS experiments, we chose to examine the effects of H-89, a selective PKA inhibitor that has been shown to interact directly with C (Engh et al., 1996), on forskolin-induced regulation of PKA subunit expression. In differentiated SH-SY5Y cells, H-89 was totally ineffective at attenuating the forskolin-induced down-regulation of the PKA subunits (C: forskolin, 73.2 ± 9.9% of vehicle, P = .0093, compared with vehicle, forskolin H-89, 75.3 ± 10.6%, P = .0153; RI: forskolin, 60.1 ± 3.6%, P = .0087, forskolin H-89, 71.5 ± 5.6%, P = .0059; RII: forskolin, 83.3 ± 5.6%, P = .0020, forskolin H-89, 83.9 ± 4.3%, P = .0009).
Effect of proteasome inhibitors on PKA subunit expression. Proteasomes play a role in the proteolytic degradation of specific cellular proteins (see Discussion). Given our evidence of rapid reductions in PKA subunit levels in response to forskolin exposure, which occurs in the absence of equivalent changes in subunit mRNA levels, we were interested in the possible involvement of proteasomes in the regulation of PKA subunit levels. To test this possibility, we examined the effect of a proteasome inhibitor, LLL, on levels of RI immunoreactivity in CATH.a cells (fig. 8A) or differentiated SH-SY5Y cells (fig. 8B). We focused on RI because this was the most dramatically regulated subunit (see fig. 2). In both cell lines, levels of RI immunoreactivity were significantly increased as a result of LLL treatment alone. In addition, the forskolin-induced down-regulation of RI levels was completely blocked in the presence of this proteasome inhibitor.

To further investigate the role of the proteasome in PKA subunit regulation, we examined the effect of lactacystin, a more specific inhibitor of proteasome-mediated proteolysis. As shown in table 1, exposure of differentiated SH-SY5Y cells to lactacystin completely attenuated the forskolin-stimulated down-regulation of C, RI and RII immunoreactivity.

**Discussion**

The major objective of the present study was to further our understanding of the molecular mechanisms by which perturbations of the cAMP pathway regulate PKA subunit expression in neural cells. Indeed, the cAMP-induced down-
regulation of PKA subunits described here contrasts with the findings reported for non-neuronal systems. In Sertoli cells, an extensively studied model system for PKA regulation (reviewed in Skålhegg and Taskén, 1997), activation of the cAMP pathway elevates PKA subunit protein levels along with a 2- to 4-fold increase in RIo, RIIα and Ca mRNA and a 50-fold increase in RIIβ mRNA. This up-regulation of mRNA levels involved both increased transcription and increased mRNA stability. In mouse epithelial cells (Lange-Carter and Malkinson, 1991), elevated levels of cAMP resulted in a similar increase in RIIβ mRNA but a decrease in mRNA for RIo and no change in that for RIIα. Earlier studies have suggested cAMP stimulated proteolytic degradation of C, but not R, subunits in rat small intestine (Alhanaty and Shaltiel, 1979), porcine epithelial cells (Hemmings, 1986), hepatocytes (Houge et al., 1990), GH3 pituitary tumor cells (Richardson et al., 1990) and a thyroid follicular cell line (Armstrong et al., 1995). This study demonstrates concurrent down-regulation of C, RI and RII subunit proteins by cAMP in two neuronal cell lines.

Using these two neuronal cell lines as model systems, we have shown that forskolin caused the down-regulation of PKA subunit immunoreactivity in a time-dependent manner. Although forskolin produces many effects in addition to activation of adenyl cyclase, the observed down-regulation of PKA subunits appears to result from elevations in cAMP levels because 1,9-dideoxys forskolin, which does not activate adenyl cyclase but exerts many of the other effects of forskolin (Laurenza et al., 1989), does not alter PKA subunit levels. In addition, the forskolin-induced down-regulation of PKA subunits can be mimicked using receptor-linked systems: CRF in CATH.a cells and PGE1 in differentiated SH-SY5Y cells. However, the forskolin-induced down-regulation of PKA subunit immunoreactivity was not associated with changes in subunit mRNA levels and did not require new protein synthesis. These findings suggest that the decreases in immunoreactivity were due to post-transcriptional or even post-translational mechanisms.

The down-regulation we observed in CATH.a and SH-SY5Y cells in response to elevations of cAMP occurred at different rates for the different subunits. Although the ratio of PKA I and PKA II holoenzymes varies across different cell lines and different tissues (Otten and McKnight, 1989), RII appears to have higher affinity than RI for C. This has been demonstrated by the overexpression of individual subunits in heterologous expression systems (McKnight et al., 1988; Otten and McKnight, 1989; Amieux et al., 1997). The overexpression of RII results in the elimination of detectable levels of PKA I and a dramatic increase in the levels of PKA II. The overexpression of C produces a compensatory increase in PKA I without affecting RII, whereas the overexpression of RI has no demonstrable effect on either PKA I or PKA II. These results suggest that PKA II forms preferentially and that PKA I forms only when C is present at a level in excess of that of RII. Hence, in the absence of elevated cAMP, RII exists with C as a holoenzyme and is thereby protected from degradation, whereas RI is more rapidly degraded (Amieux et al., 1997). The CAMP-mediated dissociation of the holoenzyme makes the now free subunits susceptible to proteolytic degradation (Steinberg and Agard, 1981). The association of PKA subunits with other cellular proteins may also protect the subunits from degradation. Both free RII and the RII within PKA II have been shown to interact with members of a family of A-kinase anchoring proteins known as AKAPs (Rubin, 1994). Through AKAPs, RII can remain tethered to cellular structures even after the cAMP-stimulated dissociation of the holoenzyme, possibly protecting the RII subunits from proteolytic degradation. Similarly, the binding of C to a protein kinase inhibitor (PKI), while blocking its catalytic function, may also protect it from degradation (Olsen and Uhler, 1991). Thus, the increased stability of C and particularly of RII, compared with that of RI, reported here may be explained by the contributions of binding proteins such as PKI and AKAPs.

The down-regulation of PKA subunits in CATH.a and SH-SY5Y cell lines was not accompanied by alterations in the levels of other cellular or signal transduction proteins. The lack of change in G protein immunoreactivity was somewhat surprising based on previous reports. In differentiated SH-SY5Y cells, for example, Ammer and Schulz (1993) reported an increase in the levels of several G protein subunits in response to chronic administration of morphine, which acutely inhibits adenyl cyclase. In addition, altered levels of G protein subunits have been observed in vivo and in vitro in response to morphine and several other treatments known to perturb the cAMP pathway (see Nestler and Aghajanian, 1997). Nonetheless, our present results in CATH.a and SH-SY5Y cell lines demonstrate that the forskolin effect is specific to PKA subunits and not generalized to other cellular proteins.

Our findings in SH-SY5Y cells demonstrate that blocking CAMP-mediated subunit dissociation using the CAMP antagonist Rp-8Br-cAMPS significantly increased levels of C, RI and RII immunoreactivity and attenuated the forskolin-induced decrease in levels of these subunits. The 300% increase in RI was twice that seen for RII and can be attributed to the preferential stabilization of PKA I due to the greater affinity of Rp-8Br-cAMPS for RI compared with RII (Gjertsen et al., 1995). Rp-8Br-cAMPS potently antagonizes cAMP at PKA I by binding to RI without dissociating the holoenzyme. In
Fig. 5. Expression of the mRNAs for PKA subunits in CATH.a and SH-SY5Y cells after exposure to forskolin. The time courses of Cα, Cβ and Rβ mRNA expression after exposure of CATH.a cells (A), SH-SY5Y cells (B) or differentiated SH-SY5Y cells (C) to 5 μM forskolin were examined by Northern blot analysis as described in Methods. Results are standardized to cyclophilin mRNA levels. Data shown are the mean ± S.D. of four independent determinations.

Fig. 6. Expression of PKA subunits in CATH.a and SH-SY5Y cells after exposure to the cAMP antagonist Rp-8Br-cAMPS. CATH.a (A, C, E) or SH-SY5Y (B, D, F) cells were treated with vehicle (V) or 5 μM forskolin (F) in the presence of 200 μM Rp-8Br-cAMPS (R) or vehicle for 12 hr and harvested as described in Methods. Western blots for C (A, B), RI (C, D) and RII (E, F) were performed as described. Data shown are the mean ± S.D. of five or six independent determinations.

* P ≤ .05; ** P ≤ .01; *** P ≤ .001, compared with vehicle control. †† P ≤ .01; ††† P ≤ .001, compared with Rp-8Br-cAMPS alone.
contrast, Rp-8Br-cAMPS induces significant dissociation of PKA II and in the presence of endogenous cAMP may contribute to the partial activation of PKA II (Jgertsen et al., 1995). In CATH.a cells, Rp-8Br-cAMPS protected RI from degradation (and presumably stabilized PKA I) as shown by the 150% increase in RI immunoreactivity. No effect was seen in CATH.a cells, however, for C and RII subunits. This may be due to the higher basal levels of RI and RII in CATH.a cells compared with SH-SY5Y cells. It is possible that a higher concentration of Rp-8Br-cAMPS would have effectively increased these subunits, comparable to that observed in SH-SY5Y cells. The lack of effect of Rp-8Br-cAMPS on RI mRNA levels further supports the role of post-transcriptional mechanisms in the cAMP-mediated regulation of PKA subunit expression. The hypothesis that Rp-8Br-cAMPS increased PKA subunit immunoreactivity by blocking cAMP-mediated subunit dissociation and not by blocking PKA phosphorylation is further supported by the lack of effect of the PKA inhibitor H-89. H-89 inhibits PKA activity by binding directly to C and preventing its phosphorylation of substrate proteins. This interaction does not, however, prevent either the subunit dissociation or the forskolin-stimulated down-regulation of PKA subunits. These results suggest that the decrease in levels of immunoreactivity of PKA subunits does not involve PKA-mediated phosphorylation.

We examined the role of a specific proteolytic pathway in mediating regulation of PKA subunit expression by use of LLL, a peptide aldehyde that has been shown to inhibit the ubiquitin-proteasome pathway in intact cells (Rock et al., 1994; Jensen et al., 1995) and lactacystin, a Streptomyces metabolite that specifically and selectively inhibits proteasomes (Fenteany et al., 1995). This pathway has been demonstrated to mediate regulated proteolysis and has been implicated in the degradation of a variety of signal transduction proteins (Ciechanover and Schwartz, 1994). In Aplysia neurones, where the role of PKA in synaptic plasticity is being studied, the ubiquitination and proteasome-mediated degradation of RI have been reported. This degradation was shown to be regulated by elevated cAMP levels in neurons but not in muscle (Chain et al., 1995). Furthermore, a neuron-specific ubiquitin C-terminal hydrolase has recently been shown to be essential for long-term facilitation in Aplysia (Hegde et al., 1997). Findings from the present study show that LLL increases levels of RI immunoreactivity in CATH.a and SH-SY5Y cells and reduces the ability of forskolin to down-regulate levels of this protein. Our results also show that lactacystin, a more specific proteasome inhibitor, completely blocks the forskolin-induced down-regulation of C, RI and RII in differentiated SH-SY5Y cells without elevating levels of subunit immunoreactivity above control levels. Together, these findings support the interesting possibility that alterations in PKA levels seen in these cell lines and in other mammalian systems (see the introduction), may occur via regulation of the ubiquitin-proteasome pathway. Because the ubiquitin-proteasome pathway is involved in the processing of many cellular proteins, these findings do not distinguish whether the inhibition of proteasome activity prevents the proteolytic processing of PKA subunits directly or of other proteins that may have protected the PKA subunits from degradation.

Together, these results highlight the importance of understanding the mechanism of PKA subunit regulation by cAMP. Our work in neural-derived cell lines establishes a foundation for future studies aimed at examining similar mechanisms in vivo. For example, the ability of Rp-8Br-cAMPS to increase levels of PKA subunits in neural-derived cell lines may shed light on the up-regulation of PKA observed in specific neuronal cell types in vivo after chronic exposure to drugs of abuse (see Nestler and Aghajanian,
1997; Self et al., 1998). If so, the present findings would suggest that this up-regulation may be achieved via post-transcriptional mechanisms, possibly regulated proteolysis, elicited by sustained inhibition of the CAMP pathway. In any event, this line of investigation will provide further insight into the role of PKA in neural plasticity.

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