cAMP-dependent Protein Kinase Induces cAMP-response Element-binding Protein Phosphorylation via an Intracellular Calcium Release/ERK-dependent Pathway in Striatal Neurons*

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Activation of the cAMP-dependent protein kinase A (PKA) pathway may induce cAMP-response element-binding protein (CREB) phosphorylation either directly or via cross-talk mechanisms with other signal transduction pathways. In this study, we have investigated in striatal primary cultures the mechanism by which activation of the cAMP/PKA-dependent pathway leads to CREB phosphorylation via the extracellular signal-regulated kinase (ERK)-dependent pathway. We have found that PKA-induced CREB phosphorylation and CREB-dependent transcription are mediated by calcium (Ca²⁺) release from intracellular stores and are blocked by inhibitors of the protein kinase C and ERK pathways. This mechanism appears to be mediated by the small G-protein Rap1, whose activation appears to be primed by PKA-induced Ca²⁺ release but not further induced by direct or indirect PKA-or protein kinase C-dependent phosphorylation. These results suggest that, in striatal neurons, intracellular Ca²⁺ release, Rap1, and ERK pathway play a crucial role in the PKA-induced CREB phosphorylation and CREB-dependent transcription.

The dopaminergic striatal system is the main target of the antipsychotic agents used in the treatment of schizophrenia (1) and of the psychostimulant drugs cocaine and amphetamines (2). The prolonged administration of these drugs increases the synaptic availability of dopamine and induces many dopamine-dependent adaptive responses culminating in the transcription of striatal cAMP-response element (CRE)¹-dependent genes, such as the immediate early gene c-fos and the neuropeptides dynorphin, substance P, and enkephalins.

CREB phosphorylation, initially thought to be mediated exclusively by the cAMP/protein kinase A (PKA) pathway (3), is also induced by Ca²⁺-dependent signal transduction pathways. Two members of the Ca²⁺/calmodulin-dependent kinase family (CaMK), CaMKII (4) and CaMKIV (5, 6), are activated by Ca²⁺ entry through an L-type voltage-sensitive Ca²⁺ channel or glutamate N-methyl-D-aspartic acid (NMDA) receptors (7) and induce CREB phosphorylation. Moreover, Ca²⁺ influx via L-type voltage-sensitive Ca²⁺ channel or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors and the release of Ca²⁺ from intracellular stores, elicited by the stimulation of growth factors receptors, activate the extracellular signal-related protein kinase (ERK)/mitogen-activated protein kinase pathway and induce CREB phosphorylation via the ribosomal S6 kinase 2 in PC12 cells (8, 9), in primary neuronal cultures (10, 11), and in brain slices (12).

The ERK pathway plays a pivotal role in stimulus-dependent gene regulation in the central nervous system, because pharmacological manipulations of the ERK pathway functionality affect the synaptic plasticity mechanisms supposed to underlie learning and memory (13). The cascade responsible of the ERK pathway activation requires the stimulus-dependent recruitment of the small G protein Ras, which in turn activates the Raf and MEK kinases. Although Ca²⁺-dependent activation of Ras has been demonstrated in several experimental models (14), in the central nervous system an alternative route to ERK activation via the cAMP/PKA- or Ca²⁺-dependent pathway has been recently characterized. This route implies the participation of the small G protein Ras-related Rap1 and the downstream kinase B-Raf (15, 16).

The importance of the Rap1/B-Raf system in the cross-talk between the cAMP/PKA- and ERK-dependent pathways has received further attention by the recent identification of two novel families of Rap1GEF activated by direct binding to Ca²⁺ and cAMP (17). However, these results were obtained mainly in platelets and PC12 cells, rather than in primary neural cells, which might display distinct signal transduction pathways.

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¹ The abbreviations used are: CRE, cAMP-response element; PKA, protein kinase A; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; CaMK, CaMKII/calmodulin-dependent kinases; NMDA, N-methyl-D-aspartic acid; RSK, ribosomal S6 kinase; H89, N-[2-p-bromocinnamamylaminomethyl]-5-isouquinolinelsulfonamide; 8-CPT, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate sodium salt; PMA, phorbol 12-myristate 13-acetate; BAPTA-AM, 1,2-bis-(o-aminophenoxy)-ethane-N,N,N,N′-tetraacetic acid; fura-2/AM, 1-(2-(o-carboxyoxazol-2-yl)-6-aminobenzoyl-furan-5-oxyl)-2-(2′-amines-5′-methylphenoxy)-ethane N,N′,N″,N‴-tetraacetic acid pentacetoxymethyl ester; PD98059, 2′-amino-3′-methylflavon; G6976, [12(2-cyanoethyl)-6,12,13-tetrahydro-13-methyl-

y-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole]; APV, D-(−)-2-amino-5-phosphonopentanoic acid; GST-ral-BBD, GST fusion protein of the minimal Rap1-binding domain of ralGDS; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; TBS, Tris-buffered saline; TBSM, TBS containing 4% nonfat dry milk; MOPS, 3-(N-morpholino)propanesulfonic acid; P-CREB, phosphorylated Ser¹³³ CREB; P-ERK2, phosphorylated ERK2; GST, glutathione S-transferase.
Furthermore, the exact mechanism by which the cAMP/PKA-dependent pathway induces ERK activation and the role, if any, of Rap1 in CREB phosphorylation and CRE-dependent transcription have not yet been investigated.

In an attempt to address these questions, in this study we have investigated the mechanisms involved in cAMP/PKA-dependent ERK activation, CREB phosphorylation, and CRE-dependent gene transcription in striatal neurons.

**EXPERIMENTAL PROCEDURES**

**Materials**—The antibodies directed against phosphorylated Ser\textsuperscript{133} CREB (P-CREB) and phosphorylation state-independent CREB were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The antibodies directed against dually phosphorylated (Thr\textsuperscript{202} and Tyr\textsuperscript{204}) and phosphorylation state-independent ERK were from New England Biolabs (Beverly, MA); those against Rap1, B-Raf, and the protein-tyrosine kinase PYK2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Dopamine, sulpiride, SCH 23390, SKF-38393, D-[(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole] (Go6976), and thapsigargin were from Alexis Corp. (San Diego, CA). Dexamethasone, sulpiride, SCH 23390, SKF-38393, and the PKA inhibitor H89 (20 μM). Total cell lysate extracts were then analyzed by Western blot with antibodies against dually phosphorylated (Thr\textsuperscript{202} and Tyr\textsuperscript{204}) ERKs (P-ERK2, upper panel) or against phosphorylation state-independent ERKs (ERK2, lower panel), to normalize for the total amount of ERKs. Data are reported as dually phosphorylated/phosphorylation state-independent ERK immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4).

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in Fig. 9. For each experimental condition, fluorescence responses were taken in at least three fields on each coverslip.

**Pull-down Assay for the Determination of Rap1 Activation**—The pull-down assay for the determination of Rap1 activation was carried out as previously reported (19). Briefly, the GST fusion protein of the minimal Rap1-binding domain of rolGDS (rol-RBD) was induced in *Escherichia coli* (strain BL21DE3) by isopropyl-1-thio-β-p-galactopyranoside, and bacterial lysates were prepared as described (19). The GST fusion protein was immobilized by incubating the bacterial lysate for 1 h at 4 °C with glutathione-Sepharose pre-equilibrated in pull-down buffer (50 mM Tris buffer, pH 7.5, 200 mM NaCl, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 1% Nonidet P-40, 2 μg/ml aprotinin, 1 μg/ml leupeptin). Sepharose beads were washed three times to remove excess GST fusion protein. After stimulation with agonists for 10 min, the cells were rinsed twice with stimulation buffer and then chilled immediately in pull-down buffer, and then cell lysate was cleared by centrifugation at 10,000 × g for 10 min at 4 °C. Approximately 5% of the total cell lysate was analyzed to normalize for the total amount of Rap1. The remaining supernatant (about 500 μg of proteins) was incubated with the GST-Sepharose beads (50 μl of a 1:1 slurry containing about 10 μg of immobilized GST fusion protein) for 2 h to allow for the association of activated Rap1 with the effector-GST fusion protein. Samples were washed twice with pull-down buffer, resuspended in Laemmli sample buffer and applied to SDS-polyacrylamide gels. Proteins on gels were then visualized using specific antibodies in a 1:1000 dilution by immunoblot analysis (see below).

**Immunoblot Analysis**—Stimulated cells were rinsed twice with ice-cold phosphate-buffered saline and then lysed in 400 μl of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Nonidet P-40, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml pepstatin, and 2 μg/ml leupeptin). Cell lysates were centrifuged at 16,000 × g for 5 min at 4 °C, and the supernatant was incubated for 15 h at 4 °C with primary antibodies and then with 15 μl of protein A-agarose for 1 h. Immunoprecipitates were washed three times with lysis buffer and resuspended either in SDS-sample buffer for immunoblot experiments or in kinase buffer for immunocomplex protein kinase assay (see below). Western blot analysis was carried out either on 20 μg of nuclear extracts (for phosphorylated CREB, phosphorylation state-independent CREB, and p90RSK), 20 μg of total cell lysate (for phosphorylated ERK and phosphorylation state-independent ERK), or immunoprecipitates for PYK2. Proteins resolved by 12.5% (PCREB, CREB, and PERK) or 7% (PYK2 and p90RSK) SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose membranes by tank blotting overnight at 4 °C. The membranes were rinsed in Tris-buffered saline (TBS) composition 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20), incubated for 1 h in TBS containing 4% nonfat dry milk (TBSM), and then incubated overnight at 4 °C with primary antibodies in TBSM. After three 15-min washes in TBS, the membranes were incubated in TBSM for 1 h at room temperature with horseradish peroxidase-conjugated goat polyclonal anti-rabbit IgG for rabbit polyclonal primary antibodies, or with horseradish peroxidase-conjugated goat polyclonal anti-mouse monoclonal primary antibodies. The chemiluminescent signals were detected using the Super signal peroxidase-conjugated goat polyclonal anti-rabbit IgG for rabbit poly-Software. The band intensity was quantified with NIH Image software. The chemiluminescent signals were detected using the Super signal peroxidase-conjugated goat polyclonal anti-rabbit IgG for rabbit poly-

RESULTS

**Activation of the cAMP/PKA Pathway Induces CREB Phosphorylation in Striatal Neurons**—The striatum is the brain area with the most abundant dopaminergic innervation, and striatal neurons in primary cultures express all of the dopaminergic receptor subtypes. We first sought to determine which receptor subtypes and signal transduction pathways are involved in CREB phosphorylation induced by the neurotransmitter dopamine in cultured striatal neurons. Dopaminergic regulation of Ser133 CREB phosphorylation was investigated by using selective agonists and antagonists specific for the D1-like and D2-like dopaminergic receptor subtypes. Dopamine-induced increase in Ser133 CREB phosphorylation (2.1 ± 0.2-fold basal level, n = 4) was mimicked by the D1-like receptor agonist SKF38393, not affected by the D2-like receptor antagonist sulpiride, and blocked by the D1-like antagonist SCH 23390 (Fig. 1A). To confirm the involvement of cAMP and to rule out any possible G protein-dependent activation of cAMP-independent signal transduction pathways, CREB phosphorylation was stimulated by agents capable of increasing cAMP formation downstream of dopamine receptors. The membrane-permeable cAMP analogue 8-CPT (200 μM) and the direct activator of adenylcyclase forskolin (20 μM) induced a significant increase (3.2 ± 0.5-fold basal level, n = 4) of CREB phosphorylation, similar to that induced by 100 μM dopamine.

The recent characterization of a family of cAMP-binding proteins that directly activates the Ras-like Rap1 protein (21), raises the possibility that the forskolin-induced CREB phosphorylation might be mediated by a cAMP-dependent but PKA-independent mechanism; this mechanism seems unlikely under the conditions, since forskolin-induced CREB phosphorylation was blocked by the PKA inhibitors KT5720 and H89 (Fig. 1B).

**PKA-induced ERK Activation and CREB Phosphorylation Require PKC Activity**—In different experimental models (16, 22), the activation of the cAMP/PKA-dependent pathway leads to activation of the ERK pathway. To assess whether this
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Cross-talk occurs in cultured striatal neurons, we performed a set of experiments using a phosphospecific antibody directed against the dually phosphorylated ERK isoforms ERK1 and ERK2. We found that DA and forskolin stimulated predominantly the phosphorylation of the ERK p42 isoform ERK2 (FSK: 4.5 ± 0.8-fold over basal level, n = 4; DA: 3.2 ± 0.5-fold over basal level, n = 4), and this effect was PKA-dependent, being reduced by the PKA inhibitor H89 (Fig. 2). PKA-induced activation of the ERK pathway was an intermediate step in PKA-induced CREB phosphorylation, since the ERK kinase (MEK) inhibitor PD98059 strongly blocked CREB phosphorylation induced by forskolin and dopamine (Fig. 3). In previous in vitro kinase assays, we found that, under our experimental conditions, the effect of PD98059 was specific for the ERK pathway and did not interfere with the activity of other kinases such as PKA and CaMKs (data not shown).

Since in the hippocampus the ERK pathway could be activated by PKC (22), we investigated the role of this kinase in cAMP/PKA-induced CREB phosphorylation. The PKC inhibitor bisindoleylmaleimide I strongly reduced DA- and FSK-induced ERK2 and CREB phosphorylation (Fig. 4A); the same results were obtained using two other specific PKC inhibitors, chelerythrine chloride and Gö6976 (Fig. 4B). In vitro kinase assays demonstrated that, under our experimental conditions, PKC inhibitors blocked PKC activity but did not interfere with nuclear PKA activity (Fig. 4C). To further confirm a causal relationship between PKC activation and ERK2/CREB phosphorylation, we investigated the functional consequence of short and long term treatment of striatal cells with the direct PKC activator PMA on ERK and CREB phosphorylation. When cells were stimulated for 10 min with 1 μM PMA to activate PKC, the MEK inhibitor PD98059 blocked both PMA-induced ERK2 and CREB phosphorylation (Fig. 5A). Accordingly, when cells were treated for 18 h with 10 nM PMA to desensitize PKC, forskolin-induced ERK2 and CREB phosphorylation were significantly reduced, as compared with untreated cells (Fig. 5B). Taken together, these experiments strongly suggest that PKA causes CREB phosphorylation via a PKC/ERK-dependent pathway.

**PKA-induced CREB Phosphorylation and CRE-dependent Gene Transcription Require Intracellular Ca^{2+}**—Previous studies have shown that neuronal Ca^{2+} represents a link between the ERK- and PKA-dependent pathways (16). These observations prompted us to investigate the Ca^{2+} requirement in PKA-induced CREB phosphorylation mediated by ERK in striatal neurons; a set of experiments was performed using the cell-permeable intracellular Ca^{2+} chelator BAPTA-AM. When neurons were preincubated with BAPTA-AM (50 μM) for 30 min, both ERK2 (Fig. 6A) and CREB phosphorylation (Fig. 6B) induced by forskolin were significantly reduced. Having established the requirement of Ca^{2+} in PKA-mediated CREB phosphorylation, we then analyzed the possible untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM). CREB phosphorylation in nuclear extracts was analyzed as described in the legend to Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR) or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). * p < 0.05 compared with the correspondent stimulus without inhibitor. B, cells were preincubated for 30 min with the PKC inhibitors chelerythrine chloride (CLRT, 1 μM) or Gö6976 (5 μM) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM). CREB phosphorylation in nuclear extracts was analyzed as described in the legend to Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR) or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). * p < 0.05 compared with forskolin without inhibitor.

![Fig. 4. Forskolin- and dopamine-induced CREB phosphorylation is blocked by PKC inhibitors.](image)

A, cells were preincubated for 30 min with the PKC inhibitor bisindoleylmaleimide I (BIS, 10 μM) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM) or with dopamine (DA, 100 μM). P-CREB and P-ERK2 phosphorylation was analyzed as described in the legends of Figs. 1 and 2, respectively. Data are reported as P-ERK2/ERK2 immunoreactivity (IR) or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). * p < 0.05 compared with the correspondent stimulus without inhibitor. B, cells were preincubated for 30 min with the PKC inhibitors chelerythrine chloride (CLRT, 1 μM) or Gö6976 (5 μM) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM). CREB phosphorylation in nuclear extracts was analyzed as described in the legend to Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR) or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). * p < 0.05 compared with forskolin without inhibitor.
involvement of L-type Ca^{2+} channels and NMDA receptors in this mechanism. Both L-type Ca^{2+} channels and NMDA receptors have been previously shown to be involved in CREB phosphorylation and CRE-dependent gene expression (7). Preincubation of striatal cells with the competitive NMDA antagonist APV or the noncompetitive NMDA antagonist MK-801 did not interfere with forskolin-induced CREB phosphorylation (Fig. 7A). This finding clearly demonstrates that in striatal neurons NMDA receptors are not responsible for the intracellular Ca^{2+} elevation required in forskolin-induced CREB phosphorylation.

**FIG. 5.** Activation of PKC induces ERK2 and CREB phosphorylation. A, cells were preincubated for 30 min with the MEK inhibitor PD98059 (50 μM) and then left untreated (−) or stimulated (+) for 10 min with PMA (1 μM). P-CREB and P-ERK2 phosphorylation was analyzed as described in the legends of Figs. 1 and 2, respectively. Data are reported as P-ERK2/ERK2 or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal is reported in the bar graph (n = 4). *, p < 0.05 compared with forskolin in PMA-untreated cells.

In a subset of striatal neurons, PKA-induced phosphorylation of the L-type voltage-sensitive Ca^{2+} channel increases the peak current through these channels, thereby augmenting the amount of Ca^{2+} entering the cell (23). We therefore investigated whether extracellular Ca^{2+} was required for PKA-induced CREB phosphorylation in striatal neurons. To our surprise, we found that neither the extracellular Ca^{2+} chelator EGTA nor the specific L-type voltage-dependent Ca^{2+} channel blocker nifedipine interfered with CREB phosphorylation induced by forskolin (Fig. 7B). To test whether an increase in intracellular Ca^{2+} could induce CREB phosphorylation, we analyzed the effect of the SERCA pump inhibitor thapsigargin. As expected from the previous experiments, thapsigargin induced an increase (2.8 ± 0.3-fold basal level, n = 4) of CREB phosphorylation (Fig. 7B). Interestingly, we found that neither nifedipine nor MK-801 interfered with forskolin-induced ERK2 phosphorylation (Fig. 7C), strongly suggesting, although without proving definitively, that ERK2 activation mediates PKA-dependent CREB phosphorylation.

To investigate whether intracellular Ca^{2+} was also required for the cAMP/PKA-induced gene transcription, we measured the mRNA levels of the immediate early gene c-fos, which contains in its promoter a CRE sequence. Treatment of striatal neurons for 30 min with 20 μM forskolin induced a 2.3 ± 0.3-fold increase (n = 4) of c-fos mRNA over basal levels; when striatal neurons were preincubated for 30 min with BAPTA-AM (50 μM) to chelate intracellular Ca^{2+}, or with the MEK inhibitor PD98059 to block the ERK pathway, forskolin-induced increase of c-fos mRNA was prevented (Fig. 8).

**PKA Activation Releases Intracellular Ca^{2+}**—Our experiments with BAPTA-AM and thapsigargin indicate that PKA activation might induce the release of intracellular Ca^{2+} from Ca^{2+}-store organelles in striatal neurons. To test this hypothesis, intracellular Ca^{2+} measurements in striatal neurons were performed using the fluorescent Ca^{2+} probe fura-2/AM. The Ca^{2+} ionophore A23187 (5 μM), which causes a large Ca^{2+} entry, was first tested as a positive control (Fig. 9, open

**FIG. 6.** Forskolin-induced ERK2 and CREB phosphorylation requires intracellular Ca^{2+}. Cells were preincubated for 30 min with BAPTA-AM (50 μM) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM). P-CREB and P-ERK2 phosphorylation was analyzed as described in the legends of Figs. 1 and 2, respectively. Data are reported as P-ERK2/ERK2 or P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). *, p < 0.05 compared with forskolin in BAPTA-untreated cells.
cells were preincubated for 15 min with the extracellular Ca\(^{2+}\) chelator BAPTA-AM (50 \(\mu\)M) or with the MEK inhibitor PD98059 (50 \(\mu\)M) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 \(\mu\)M). P-CREB phosphorylation was analyzed as described in the legend to Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). *, p < 0.05 compared with forskolin without inhibitor.

**FIG. 7.** Forskolin-induced CREB and ERK2 phosphorylation is not mediated by Ca\(^{2+}\) channels or NMDA receptors. A, cells were preincubated for 15 min with the competitive NMDA receptor antagonist APV (100 \(\mu\)M) or with the noncompetitive NMDA receptor antagonist MK-801 (10 \(\mu\)M) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 \(\mu\)M). P-CREB phosphorylation was analyzed as described in the legend to Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). B, cells were preincubated for 15 min with the extracellular Ca\(^{2+}\) channel blocker nifedipine (NIF, 10 \(\mu\)M) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 \(\mu\)M) or with the SERCA pump inhibitor thapsigargin (TPG, 200 \(nM\)). P-CREB phosphorylation was analyzed as described in the legend of Fig. 1. Data are reported as P-CREB/CREB immunoreactivity (IR), and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). C, cells were preincubated for 15 min with the voltage-sensitive Ca\(^{2+}\) channel blocker nifedipine (NIF, 10 \(\mu\)M) or with noncompetitive NMDA receptor antagonist MK-801 (10 \(\mu\)M) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 \(\mu\)M). P-ERK2 phosphorylation was analyzed as described in the legend to Fig. 2. Data are reported as P-ERK2/ERK2 immunoreactivity (IR), and the average fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4).

Application of forskolin (20 \(\mu\)M) to fura-2/AM-loaded neurons caused a significant long lasting (about 30 s) and slowly decreasing rise in intracellular Ca\(^{2+}\) levels (Fig. 9, full circles, n = 4). The amplitude, but not the shape, of the forskolin-induced Ca\(^{2+}\) response was greatly reduced by preincubating the cells with the PKA inhibitor H89 (20 \(\mu\)M; Fig. 9, open circles, n = 4). Finally, to study the role of extracellular Ca\(^{2+}\), fura-2/AM-loaded neurons were preincubated with the chelator of extracellular Ca\(^{2+}\) EGTA (5 mM) and then stimulated with forskolin. In the absence of extracellular Ca\(^{2+}\), forskolin still elicited a significant sharp, very short lasting (about 7 s) and fast decreasing rise in intracellular Ca\(^{2+}\) levels (Fig. 9, open circles). Taken together, these data indicate that, in addition to playing a role in regulating voltage-dependent Ca\(^{2+}\) channel function, PKA may also increase intracellular Ca\(^{2+}\) by releasing Ca\(^{2+}\) from intracellular stores.

**FIG. 8.** Forskolin-induced c-fos transcription is mediated by Ca\(^{2+}\) and ERK signaling pathways. Cells were preincubated for 30 min with the intracellular Ca\(^{2+}\) chelator BAPTA-AM (50 \(\mu\)M) or with the MEK inhibitor PD98059 (50 \(\mu\)M) and then left untreated (−) or stimulated (+) with forskolin (FSK, 20 \(\mu\)M) for 30 min. Total RNA was extracted from the cell lysates, separated by 1% gel agarose electrophoresis, and blotted to nylon membranes. The membrane was hybridized with a c-fos (upper panel) and an 18 S ribosomal RNA (18 S, lower panel) probe and then exposed to x-ray film for autoradiography. Data are reported as c-fos mRNA levels normalized to 18 S mRNA, and the average -fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). *, p < 0.05 compared with forskolin without inhibitor.

**FIG. 9.** PKA Stimulation Induces Rap1 and PYK2 Activation—In several experimental models, the family of small G proteins plays a key role in Ca\(^{2+}\)-induced activation of ERK. Recently, it has been reported that Rap1, a Ras-like small G protein that may activate the ERK pathway by interacting with the kinase B-Raf, is activated by membrane depolarization in a PKA-dependent manner (16) and by PKA via the ERK pathway (24). To test the possibility that Rap1 participates in the signaling pathway responsible for PKA-induced CREB phosphorylation, we decided to investigate the activation of Rap1 by means of a pull-down assay using the GST-railRBD fusion protein. Stimulation of striatal cells with 20 \(\mu\)M forskolin for 10 min induced a large increase in the amount of Rap1-GTP (Fig. 10A). We then investigated the effects of both kinase inhibitors and of agents capable of interfering with intracellular Ca\(^{2+}\) levels. Forskolin-induced Rap1 activation was blocked by the PKA inhibitor H89, but not by the specific PKC inhibitor bisindoleylmaleimide I. Intracellular Ca\(^{2+}\) release appears to be necessary and sufficient for Rap1 activation, because Rap1 activation was not observed in the presence of BAPTA-AM and was mimicked by elevation of intracellular Ca\(^{2+}\) induced by thapsigargin (Fig. 10A). These data indicate that forskolin mainly stimulates Rap1 activation in a PKA/Ca\(^{2+}\)-dependent but PKC-independent manner, suggesting that the role of PKC in PKA-dependent CREB phosphorylation is likely to be downstream of Rap1.

Finally, since in the central nervous system the concomitant elevation of intracellular Ca\(^{2+}\) and activation of PKC leads to tyrosine phosphorylation and activation of PYK2 (25), we investigated whether PYK2 was activated by PKA under our experimental conditions. As expected, PKA activation induced an increase of tyrosine phosphorylation of PYK2 immunoprecipitated from total cell lysate of striatal neurons (Fig. 10B).
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Fig. 9. Forskolin induces intracellular Ca\(^{2+}\) release. Fura-2/AM-loaded striatal neurons were preincubated for 30 min with EGTA (5 mM) or the PKA inhibitor H89 (10 μM) and then stimulated at 21°C at the time indicated by the arrows with forskolin (FSK, 20 μM) or the Ca\(^{2+}\) ionophore A23187 (5 μM). Fura-2 fluorescence (340/380-nm ratio) was measured every 2 s, and data were analyzed as described under “Experimental Procedures.” The figure shows averages obtained from a total of 15 cells in one representative experiment; the SEM (not shown) was less than 15%. Within each optic field, the number of cells that responded to the treatment was 5.2 ± 1.4 (n = 24 optic fields). A total of four independent experiments (four different sets of cultures) were performed and gave similar results. *, p < 0.05 compared with their respective basal (unstimulated) values.

DISCUSSION

This study describes a novel mechanism by which activation of the cAMP/PKA-dependent pathway may stimulate CREB phosphorylation in striatal neurons (Fig. 11). Activation of PKA, induced by dopamine or forskolin, releases Ca\(^{2+}\) from the intracellular stores, which in turn activates the Rap1-B-Raf complex whose main target is the phosphorylation of ERK. Finally, the activation of ERK induces CREB phosphorylation, probably via the translocation of the ERK-RSK complex into the nucleus. The prevailing model dictates that an increase of cAMP induces CREB phosphorylation via a mechanism in which the PKA catalytic subunit translocates into the nucleus and directly phosphorylates CREB; in this mechanism, the localization of PKA may contribute to enhance cAMP-dependent CREB phosphorylation and CRE-dependent gene expression (20). However, several recent studies have demonstrated that, in addition to this direct PKA-dependent pathway, other signaling pathways, such as the ERK pathway (10), can induce CREB phosphorylation in a PKA-dependent manner. The participation of the ERK pathway appears to be very important for PKA-dependent transcription, because several neurotransmitter receptor systems (22) as well as depolarization-induced Ca\(^{2+}\) influx (16) have been shown to activate the ERK pathway via a PKA-mediated mechanism. This mechanism appears to have an important functional role, since a previous study in Aplysia has shown that long term facilitation involves PKA-dependent activation of ERKs (26). In agreement with these data, we have demonstrated that in striatal neurons the novel mechanism involving PKA-induced intracellular Ca\(^{2+}\) release participates in ERK and CREB phosphorylation and ultimately regulates the transcription of the immediate early gene c-fos.

Previous studies in various experimental models have shown that the functionality of intracellular Ca\(^{2+}\) stores could be modulated by PKA-dependent phosphorylation in vivo and in vitro. The neuronal type-I IP3 receptor, immunopurified from rat cerebellar membranes, can be phosphorylated by PKA in vitro (27), and activation of PKA induces phosphorylation of the type-I IP3 receptor in cerebellar slices (27) and in cultured cells (28, 29). Notably, a recent paper has described that, in cardiac tissue, PKA-dependent phosphorylation of ryanodine receptor/Ca\(^{2+}\) release channel regulates the channel open prob-

ability (30). These studies suggest that PKA-dependent phosphorylation could modulate the functionality of intracellular Ca\(^{2+}\) store organelles by increasing their sensitivity to physiological agonists or toward external Ca\(^{2+}\). Given the importance of the cross-talk between PKA- and Ca\(^{2+}\)-dependent pathways, the precise mechanism of PKA-mediated intracellular Ca\(^{2+}\) release will certainly become a focus of future studies in neuronal cell physiology.

In PC12 cells and primary cortical neurons, ERK activation induced by Ca\(^{2+}\) entry through voltage-sensitive channels or by the release of Ca\(^{2+}\) from intracellular stores is mediated by the small G protein Ras (14). Several factors, such as the route and magnitude of Ca\(^{2+}\) influx or the subcellular compartmentalization of protein kinases in the cell type under investigation, contribute to determine which signaling pathway upstream of ERK is activated. Several recent studies performed in various cell types have demonstrated that Ca\(^{2+}\), in addition to Ras, may also activate ERK via another route involving the Ras

Fig. 10. Forskolin activates Rap1 and PYK2. A, cells were preincubated for 30 min with BAPTA-AM (BAPTA, 50 μM), the PKC inhibitor bisindoleylmaleimide I (BIS, 10 μM), and the PKA inhibitor H89 (20 μM) and then left untreated (−) or stimulated (+) for 10 min with forskolin (FSK, 20 μM) or thapsigargin (TPG, 200 nM). Preincubation with these agents did not modify basal Rap1-GTP levels (not shown). Total cell lysate (0.5 mg) was processed for the pull-down assay of Rap1 using the GST-ral-RBD protein as described under “Experimental Procedures.” Western blot analysis was performed on the pulled down Rap1-GTP (upper panel) and on total Rap1 (lower panel) in a 5% (25-μg) extract of total cell lysate as a loading control (bottom panel). Data are reported as Rap1-GTP immunoreactivity, and the average ±fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). *, p < 0.05 compared with forskolin. B, cells were stimulated for 10 min with forskolin (FSK, 20 μM), and then PYK2 was immunoprecipitated from total cell lysate (1 mg). The immunoprecipitate was analyzed by Western blot with antiphosphotyrosine (WB P-Tyr, upper panel) or anti-PYK2 (WB PYK2, lower panel) antibodies as described under “Experimental Procedures.” Data are reported as Tyr(P) immunoreactivity (IR), and the average ±fold increase (mean ± S.E.) over basal level is reported in the bar graph (n = 4). *, p < 0.05 compared with basal level.
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homologue Rap1 and its effector Raf-like kinase B-Raf. The Rap1-B-Raf system participates in PKA-mediated ERK activation induced by growth factors in PC12 cells (14, 31, 32) and by β2 adrenergic receptor stimulation in HEK 293 cells (24). In agreement with these reports, we propose that in striatal neurons the Rap1-B-Raf system is an intermediate step in the activation of the ERK pathway triggered by PKA-induced intracellular Ca2+ release. This model is in contrast with what was found by other authors in PC12 cells (16), in which depolarization-induced Ca2+ entry activates the ERK pathway via a two-step mechanism involving first PKA and then Rap1. In a previous study (33) and in experiments not reported here, we found that, in cultured striatal neurons, Ca2+ entry induced by a Ca2+-ionophore or by depolarization inhibits cAMP formation, probably interacting with the Ca2+-inhibitable type V adenyl cyclase isoform, which is abundantly expressed in these neurons (18).

The complexity of Rap1 activation mechanisms in neuronal cells has been emphasized by the characterization of new families of Rap guanine nucleotide exchange factors activated by Ca2+ and diacylglycerol (34) or by cAMP and Ca2+ (17, 21). On the other hand, our findings demonstrate that, in agreement with another report (35), Rap1 activation is induced by PKA-dependent release of intracellular Ca2+ (35). The differences in the mechanism of Rap1 activation are probably due to the differential expression of adenyl cyclase isoforms and small G protein guanine nucleotide exchange factors in the cell types under investigation. Moreover, since Rap1 activation is not triggered by a PKA- or PKC-dependent phosphorylation, two mechanisms may be considered. First, Rap1 could be directly activated by the novel Rap1GEFs, which in turn are directly activated by Ca2+. Second, Rap1 could be activated indirectly via a multiple step mechanism involving the Ca2+-dependent stimulation of PYK2, which then interacts with Rap1GEF C3G (36). In agreement with this latter hypothesis, in the present study we have found that activation of the cAMP/PKA-dependent pathway leads to activation of the tyrosine kinase PYK2 (25). This is consistent with the observation that, in striatal brain slices, stimulation of D2 receptors induces tyrosine phosphorylation of PYK2 via a phospholipase C-mediated intracellular Ca2+ release (37). So far, it is still unclear whether in striatum activation of PYK2 is induced in CREB phosphorylation, because D2-dependent CREB phosphorylation was not blocked by the broad spectrum tyrosine kinase inhibitor genistein (37).

Although the experiments presented in this study show that in striatal neurons PKC inhibitors block PKA-induced CREB phosphorylation, no conclusion can be drawn about the mechanism by which the cAMP/PKA-dependent pathway activates PKC and at what level PKC modulates CREB phosphorylation. Having excluded a direct role of PKC in Rap1 activation in our model, it is reasonable to assume that PKC acts downstream of Rap1 and upstream of B-Raf. This hypothesis is supported by studies showing that PKC may play a facilitator role on the small G protein-dependent ERK pathway activation by enhancing Raf-1 or B-Raf functionality (38).

In conclusion, we have found that intracellular Ca2+ release induced by PKA leads to CREB phosphorylation via a PKC- and ERK-dependent mechanism, most likely involving Rap1 activation. Given the complexity of cross-talk between the cAMP/PKA and the ERK pathways, the plethora of signaling pathways converging on ERK, and the wide array of transcription factors activated by these signaling pathways, this and other studies (16, 23) raise fundamental questions concerning the role of Rap1 and the functional effects of its activation at the level of transcription factor activation and CRE-dependent gene expression.

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