Arg3.1/Arc mRNA Induction by Ca^{2+} and cAMP Requires Protein Kinase A and Mitogen-Activated Protein Kinase/Extracellular Regulated Kinase Activation

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Long-term potentiation (LTP) is a cellular model for persistent synaptic plasticity in the mammalian brain. Like several forms of memory, long-lasting LTP requires cAMP-mediated activation of protein kinase A (PKA) and is dependent on gene transcription. Consequently, activity-dependent genes such as *c-fos* that contain cAMP response elements (CREs) in their 5' regulatory region have been studied intensely. More recently, *arg3.1/arc* became of interest, because after synaptic stimulation, *arg3.1/arc* mRNA is rapidly induced and distributed to dendritic processes and may be locally translated there to facilitate synapse-specific modifications. However, to date nothing is known about the signaling mechanisms involved in the induc-

tion of this gene. Here we report that *arg3.1/arc* is robustly induced with LTP stimulation even at intensities that are not sufficient to activate *c-fos* expression. Unlike *c-fos*, the 5' regulatory region of *arg3.1/arc* does not contain a CRE consensus sequence and *arg3.1/arc* is unresponsive to cAMP in NIH3T3 and Neuro2a cells. However, in PC12 cells and primary cultures of hippocampal neurons, *arg3.1/arc* can be induced by cAMP and calcium. This induction requires the activity of PKA and mitogen-activated protein kinase, suggesting a neuron-specific pathway for the activation of *arg3.1/arc* expression.

Key words: plasticity; long-term memory; hippocampus; LTP; gene induction; *Arg3.1*; *Arc*

Like long-term memory, enduring forms of synaptic plasticity including long-term potentiation (LTP) (Bliss and Collingridge, 1993) require alterations in the molecular composition and structure of neurons and are dependent on mRNA and protein synthesis (Goelet et al., 1986; Curran and Morgan, 1987; Sheng and Greenberg, 1990). Plasticity therefore might be achieved by activity-dependent changes in the expression of immediate early genes (IEGs) (Kuhl, 2000). These encode transcription factors (Greenberg et al., 1986; Morgan et al., 1987; Saffen et al., 1988) as well as a set of proteins that have the potential to transduce synaptic activity directly into immediate changes of neural function (Nedivi et al., 1993; Qian et al., 1993; Yamagata et al., 1993; Frey et al., 1996; Kauselmann et al., 1999; Konietzko et al., 1999). A particular role might be played by *arg3.1/arc*, because this gene is thus far unique among IEGs in that its mRNA is rapidly distributed throughout the dendritic arbor of stimulated neurons (Link et al., 1995; Lyford et al., 1995). *Arg3.1/arc*, also named BAD-1, was originally identified in differential screens for seizure-stimulated hippocampal mRNAs (Qian et al., 1993; Link

et al., 1995; Lyford et al., 1995). However, *arg3.1/arc* is also robustly induced after LTP induction in the absence of seizures (Link et al., 1995; Lyford et al., 1995), and *arg3.1/arc* mRNA and protein specifically localize to the field of stimulated synapses (Steward et al., 1998). Moreover, induction of *arg3.1/arc* in the hippocampus after an exploration paradigm has been demonstrated to occur specifically in neurons engaged in the spatial encoding process (Guzowski et al., 1999), and blocking of *arg3.1/arc* expression in antisense experiments results in a loss of LTP and impaired hippocampal learning (Guzowski et al., 2000). Although these observations strongly suggest that *arg3.1/arc* expression is required for synaptic plasticity, nothing is known about the signal transduction pathways that lead to the induction of this gene.

Activity-dependent changes in neuronal function are mediated to a large degree by elevations in intracellular calcium levels. In neurons, calcium ions can stimulate the production of cAMP and the activation of protein kinase A (PKA) (Ginty et al., 1991; Xia et al., 1991; Cali et al., 1994). LTP can be initiated by calcium influx through glutamate-gated NMDA channels (Lynch et al., 1983; Malenka et al., 1992), whereas the late phase of LTP (L-LTP) can be initiated by cAMP, requires the activation of PKA, and is dependent on gene transcription (Frey et al., 1993; Huang et al., 1994; Abel et al., 1997). In addition, accumulating evidence suggests a pivotal role for mitogen-activated protein kinase [(MAPK) also known as extracellular regulated kinase (ERK)] in enduring forms of synaptic plasticity (Impey et al., 1998; Orban et al., 1999). In *Aplysia*, stimuli that induce long-term facilitation, including cAMP, increase MAPK activity and nuclear localization (Martin et al., 1997). Similarly, MAPK activity is essential for hippocampal LTP (English and Sweatt, 1996, 1997) and learning in the intact animal (Atkins et al., 1998;

Received April 4, 2001; revised May 4, 2001; accepted May 8, 2001.

R.W. and P.W. were supported by the Graduiertenkolleg Grant GRK255 to D.K. This research was supported by Deutsche Forschungsgemeinschaft Grants SFB444 and FOR296 to D.K. We thank Drs. U. Müller and C. Weissmann for library AB-1, and Drs. N. Irwin and L. Benowitz for PC12 cells.

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Berman et al., 1998; Blum et al., 1999), and both PKA and MAPK promote the expression of genes required for the formation of long-term memory (Martin and Kandel, 1996; Impey et al., 1998). An important mediator of such transcriptional changes is the transcription factor cAMP response element-binding (CREB) protein (Dash et al., 1990; Bourtschuladze et al., 1994; Frank and Greenberg, 1994; Yin et al., 1994; Bartsch et al., 1995; Yin et al., 1995; Guzowski and McGaugh, 1997). Therefore the objectives of our studies were to define the roles of the PKA and MAPK pathways in the activation of arg3.1/arc expression.

MATERIALS AND METHODS

Animal preparation and chronic electrophysiology. Adult male Sprague Dawley rats (250–300 gm) were used. Kainic acid (10 mg/kg) was administered by intraperitoneal injection, and animals were killed 1 hr after seizure onset. Control animals were injected with identical volumes of isotonic saline (PBS). Implantation of a monopolar stimulating electrode in the perforant path and a monopolar recording electrode in the hilus of the dentate gyrus was as described (Konietzko et al., 1999). The animals recovered for at least 7 d before being transferred to a chronic recording cage for the actual experiment involving standard *in vivo* electrophysiological techniques (Staubli and Scafidi, 1997). During the experimental sessions, the current intensity was adjusted (20–100 μ A, pulse width 150 μ sec, biphasic) to produce a baseline response with a minimal population spike of 0.5–1 mV. Test pulses were delivered at 0.07 Hz for at least 20 min to establish stable baseline potentials, after which either high-frequency stimulation (HFS) or low-frequency stimulation (LFS) was initiated randomly. The 12-train HFS paradigm consisted of 12 trains of 20 msec each at 500 Hz, delivered 10 sec apart (Cole et al., 1989). The 50-train HFS paradigm consisted of five 25-msec-long trains at 400 Hz, delivered 1 sec apart and repeated 10 times with a 1 min interval between repetitions (Abraham et al., 1993; Konietzko et al., 1999). The same number of pulses was delivered for the LFS treatment but at a rate of 0.2 Hz. Potentials were then monitored at 0.07 Hz for 1 hr, after which the rats were decapitated; the brains were removed, frozen on dry ice, and stored at -75°C .

Cell culture, stimulation, and inhibition experiments. NIH3T3 and Neuro2a cells were grown in DMEM (Life Technologies, Gaithersburg, MD) with 10% fetal calf serum (Life Technologies), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. PC12 cells were plated on poly-L-lysine-coated (molecular weight > 300,000; Sigma, St. Louis, MO) Petri dishes and were grown in DMEM with 10% horse serum (Boehringer Mannheim, Mannheim, Germany), 5% fetal calf serum (Life Technologies), 100 IU/ml penicillin, and 100 μ g/ml streptomycin. All experiments with permanent cell lines were conducted in 60 mm dishes at a cell density of 50–60% confluency. Primary hippocampal neuronal cultures were prepared from 18-d-old Wistar rat embryos as described previously (Papa et al., 1995). Dissociated cells were plated on poly-L-lysine-coated 35 mm dishes in Eagle's MEM with L-alanyl-L-glutamine (Life Technologies), enriched with 0.6% glucose, 5% fetal calf serum, and 5% horse serum, at a density of 1.2×10^6 cells per well. Two days after plating, the medium was changed to one containing 10% horse serum, 20 μ g/ml 5'-fluoro-2-deoxyuridine, and 50 μ g/ml uridine to stop the proliferation of glial cells. The neuronal cultures were used for experiments at 2 weeks in culture, with no further medium changes. NIH3T3 cells were serum stimulated as described previously for Rat6 cells (Link et al., 1995). Forskolin stimulation of NIH3T3, Neuro2a, PC12 cells, and primary neuronal cultures was performed by adding forskolin (RBI, Natick, MA, or Tocris) dissolved in DMSO to a final concentration of 50 μ M. PC12 cells were depolarized by adding KCl to a final concentration of 60 mM. In an alternative protocol, KCl was washed out and replaced by non-KCl-containing medium; however, this did not influence mRNA expression and time course in comparison to continuous exposure to KCl (data not shown). In the case of the alternative protocol, 6 hr before the start of the experiment the medium was changed to exclude stimulation caused by starvation and the addition of serum. The following inhibitors were added to the medium 30 min before stimulation: cycloheximide (Sigma; dissolved in ethanol), final concentration of 10 μ g/ml; nifedipine (RBI; dissolved in DMSO), final concentration of 10 μ M; H-89 (Biomol; dissolved in DMSO), final concentration of 20 μ M; and PD098059 (RBI; dissolved in DMSO), final concentration of 50 μ M. Actinomycin D (Sigma; dissolved in DMSO) was added 15 min before stimulation to a final concentration of 3 μ g/ml.

Generation of stably transfected cell lines. PC12 cells were transfected with the arg3.1/arc promoter constructs [pALΔ1737 + 3' untranslated region (UTR), pALΔ838 + 3'UTR, or pALΔ177 + 3'UTR] and an expression plasmid carrying a neomycin resistance gene under control of a PGK-1 promoter at a ratio of 10:1 using Lipofectamine reagent (Life Technologies). Selection was performed by addition of 500 μ g/ml G418 (Life Technologies) to the culture medium. All G418-resistant clones of each culture dish were pooled.

Cloning of genomic DNA and plasmid constructions. A 21 kb genomic fragment was isolated from a λ phage genomic library (AB-1) prepared from [129/Sv (ev)] embryonic stem cells. Two *Xho*I fragments were subcloned into pZErO-1 (Invitrogen, San Diego). The first subclone, pArg-1, contained 10 kb of arg3.1/arc 5' untranslated and the whole 3.5 kb transcribed region including the poly(A)⁺ site. The second subclone, pArg-2, contained 6.5 kb immediately downstream of the pArg-1 sequence. From pArg-1 two smaller fragments were subcloned: a 4.3 kb *Hind*III-*Xho*I fragment, containing 838 bp upstream and 3.5 kb downstream of the transcription start site, was inserted into pZErO-1 to obtain pArgGene. A *Stu*I fragment, containing 1737 bp upstream and 477 bp downstream of the transcription start site, was inserted into the *Eco*RV site of pZErO-1 to yield pArgProm. The luciferase reporter pGL2-Basic Vector (Promega, Madison, WI) was modified in the multiple cloning region by the insertion of a *Not*I and a *Spe*I site between the *Xho*I and *Bgl*II sites and the insertion of a *Nsi*I site between the *Xba*I and *Mlu*I sites. To generate pALΔ1737, a blunted *Nsi*I-*Sac*II fragment from pArgProm, extending from position -1737 to +250 and carrying part of the pZErO-1 polylinker at its 5' end, was inserted into the blunted *Hind*III site of the modified pGL2-Basic Vector. pALΔ838 was obtained by restriction of pALΔ1737 with *Hind*III, releasing a fragment from position -1737 to -839, and subsequent religation. pALΔ177 was constructed in two steps: in a first step a *Xho*I-*Bss*HIII fragment of pALΔ1737, containing the sequence from position -1737 to -221, was released, and the resulting plasmid was blunted and religated. In a second step, a deletion, comprising position -220 to -178, was introduced using the double-stranded nested deletion kit (Pharmacia) and the *Nsi*I site as the protected site and the *Nhe*I site as the nonprotected site. To generate pALΔ1737 + 3'UTR, the *Xho*I site was removed from the modified pGL2-Basic Vector multiple cloning region of pALΔ1737 by *Spe*I and *Nhe*I restriction and religation of the blunted ends. The arg3.1/arc 3'UTR, contained in the *Eco*47III-*Xba*I fragment of pArgGene, was inserted with blunted ends between the *Clal*-*Pfl*MI sites of the modified pALΔ1737, thereby replacing the SV40 3'UTR. In addition a 0.5 kb *Xho*I-*Nhe*I fragment from pArg-2, containing the genomic sequence immediately downstream of the *Xho*I site in the *Eco*47III-*Xba*I fragment of pArgGene, was inserted into the *Xho*I-*Sall* sites after blunting the *Nhe*I and *Sall* ends. pALΔ838 + 3'UTR was obtained by removing position -1737 to -839 from pALΔ1737 + 3'UTR after a *Mlu*I-*Hind*III restriction and blunt-end ligation. To yield pALΔ177 + 3'UTR, the *Xba*I-*Eco*RI fragment of pALΔ1737 + 3'UTR was exchanged against the *Xba*I-*Eco*RI fragment of pALΔ177.

DNA sequencing and analysis. All plasmid constructs were confirmed by sequencing. The arg3.1/arc genomic sequences were determined from the plasmids pArgProm and pArgGene using multiple synthetic primers and the dideoxynucleotide chain termination method (Sanger et al., 1977) and an ABI Prism sequencer (GenBank accession number AF177701). Analysis of the nucleotide sequence was performed with the GCG software package (University of Wisconsin, Biotechnology Center, Madison, WI). Analysis of potential transcription factor binding sites was assisted by the MatInspector program (Quandt et al., 1995).

S1 analysis. S1 analysis was as described (Kuhl et al., 1987, 1992). The following arg3.1/arc antisense oligonucleotide, S1-arg3.1/arc, was synthesized and gel purified: CGC CGC TGA AGC TAG AGA GGC CCA GAG ACT GCG GCT GCG GGA GAA CTC GCT TGA GCT CTG CAC CGA AAC CGC CAC CAG CGG CTA TTT ATG TGC GCG GGG CCC GT. Forty-one nucleotides were derived from the 5' untranslated region to differentiate digested from undigested probe. The S1-arg3.1/arc probe (0.015 pmol) was end labeled with γ -³²P-ATP to a specific activity of $>2 \times 10^6$ cpm/ μ g using T4 kinase (Life Technologies). Total RNA (50 μ g) isolated from 60 min serum-stimulated NIH3T3 cells was used in the S1 nuclease digest. The S1 reaction product was analyzed on a 12% urea-PAGE. M13mp18 DNA was sequenced according to the manufacturer's instructions (USB 70770 sequencing kit; Amersham Pharmacia Biotech, Braunschweig, Germany) and served as a length marker.

RNA preparation, Northern blot analysis, and in situ hybridization. Cells

Figure 1. Comparison of *arg3.1/arc* and *c-fos* mRNA levels after kainic acid-induced seizures and LTP-producing stimulation. Coronal sections were assayed for *arg3.1/arc* and *c-fos* mRNA using *in situ* hybridization with antisense RNA probes. Representative autoradiographs of three independent experiments are shown. *A, B*, One hour after saline injection. *C, D*, One hour after kainic acid (10 mg/kg)-induced seizures. *E, F*, One hour after 50-train LFS of the perforant path in freely moving rats. *H, I, K, L*, One hour after 50-train HFS ($n = 5$). *N, O*, One hour after 12-train LFS. *Q, R*, One hour after 12-train HFS. *G, J, M, P, S*, Superimposed field potentials before and 1 hr after LFS or HFS, respectively. Calibration: 5 mV, 5 msec.

were lysed at the indicated times after stimulation. Total RNA was prepared using the RNeasy kit (Qiagen). mRNA was prepared from total RNA using the Oligotex mRNA kit and was additionally desalted with the RNeasy kit (Qiagen). Northern blot analysis, *in situ* hybridizations, and quantifications of autoradiographs were performed as described (Link et al., 1995). Probes were generated from full-length rat cDNAs: *arg3.1/arc* (Link et al., 1995), *c-fos* (Curran et al., 1987), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985).

RESULTS

Arg3.1/arc is robustly induced with plasticity-producing stimulation

Previous studies by Worley and coworkers (Lyford et al., 1995) and by us (Link et al., 1995) established that *arg3.1/arc* transcript

Figure 2. Depolarization regulates *arg3.1/arc* and *c-fos* mRNA levels in PC12 cells. Autoradiograph of Northern blot analysis of *arg3.1/arc* and *c-fos* transcripts. Four independent experiments were conducted ($n = 4$). Six micrograms of RNA isolated from PC12 cells were loaded per lane. The blot was hybridized to a probe specific for *arg3.1/arc* and a probe specific for *c-fos*. Hybridization to a probe specific for GAPDH was used as a loading control. Numbers below the lanes indicate the period of 60 mM KCl exposure in hours. Lane C/4, RNA isolated 4 hr after exposure to KCl in the presence of cycloheximide (CHX, 10 μ g/ml). Lane C, RNA isolated 4 hr after exposure to CHX only. Note that the increase in *arg3.1/arc* mRNA levels is maintained for 4 hr and not superinduced by CHX. In contrast, *c-fos* mRNA is only transiently induced but strongly superinduced in the presence of CHX.

levels are dramatically increased by single seizures induced by pentylenetetrazole or maximal electroconvulsive shock. Moreover, these studies demonstrated that *arg3.1/arc* can be induced in the absence of seizures by LTP-producing stimulation. A similar responsiveness to synaptic activity has been demonstrated for *c-fos*, a gene well characterized for the signal transduction pathways contributing to its induction in the nervous system (Curran and Morgan, 1987; Dragunow et al., 1989; Morgan and Curran, 1995). Here we examined the levels of *arg3.1/arc* transcripts after recurrent kainic acid (KA)-induced seizures and after two *in vivo* LTP-producing stimulation protocols of different strengths. For comparison we monitored *c-fos* mRNA levels in the same animals. *In situ* hybridization analysis demonstrated that *arg3.1/arc* and *c-fos* differ in their cellular and regional expression patterns (Fig. 1). Constitutive expression of *c-fos* was low (Fig. 1*A*), whereas *arg3.1/arc* was expressed at moderate levels in cortex and hippocampus (Fig. 1*B*). After KA-induced seizures, both mRNAs were strongly elevated 1 hr after the onset of seizure in cortex, hippocampus, and amygdala. Whereas *c-fos* transcripts were confined to somata, *arg3.1/arc* transcripts rapidly translocated to the dendrites of activated neurons of the dentate gyrus. Moreover, we found that *c-fos* but not *arg3.1/arc* transcripts were induced in diencephalon (Fig. 1*C,D*). These results demonstrate that *arg3.1/arc* and *c-fos* are expressed by distinct but overlapping populations of neurons. We next examined the responsiveness of *arg3.1/arc* and *c-fos* to LTP-producing stimulation. Long-term potentiation can be induced at synapses within the hippocampus by high-frequency orthodromic stimulation (Bliss and Collingridge, 1993). The dentate gyrus granule cells were synaptically stimulated by activating their major afferent projection from the entorhinal cortex using a chronically implanted stimulating electrode (Staubli and Scafidi, 1997). Stimulation of the perforant path at the intensity required to produce a population spike, when administered in 50 or 12 trains at low frequency, did not result in LTP or an increase in *arg3.1/arc* or *c-fos* mRNA levels (Fig. 1*E,F,K,L*). By contrast, when LTP was evoked in the granule

Figure 3. Regulation of *arg3.1/arc* and *c-fos* transcription by cAMP. Autoradiographs are of Northern blots. RNA amounts and labels are as in Figure 2. Cells were stimulated with the adenylyl cyclase activator forskolin (50 μ M). *A*, *B*, RNA isolated from PC12 cells ($n = 4$). Where indicated, cells in *B* were exposed to 3 mM of the transcription inhibitor actinomycin D (*ActD*), 1.25 and 4.25 hr before lysis, or 15 min before stimulation with forskolin, respectively. *C*, RNA isolated from NIH3T3 fibroblasts ($n = 2$). *D*, RNA isolated from Neuro2a neuroblastoma cells ($n = 2$). Note that *arg3.1/arc* is induced by forskolin in PC12 cells but not in NIH3T3 or Neuro2a cells.

cells by delivering the same intensity stimuli in 50 or 12 trains at high frequency, *arg3.1/arc* but not *c-fos* was consistently induced in the ipsilateral dentate gyrus 1 hr after stimulation (Fig. 1*H-L,Q,R*). The absence of *c-fos* mRNA induction with weaker LTP-producing stimulation is not a consequence of the short half life of *c-fos* mRNA, because it was also observed at an earlier time

Figure 4. S1 nuclease mapping of the transcription start site. *Right lane* (*P*), Undigested S1-*arg3.1/arc* probe. *Middle lane* (*S1*), S1 nuclease-digested S1-*arg3.1/arc* oligonucleotide after hybridization with total RNA prepared from 60 min serum-stimulated NIH3T3 cells. The sequence of M13 on the *left* served as a length marker.

point (Worley et al., 1993). This is contrasted by the robust induction of *arg3.1/arc*. When stimulus intensities were increased (50-train LTP protocol), one of five animals showed induction of *c-fos* transcripts, indicating that *c-fos* can be selectively induced in the stimulated dentate gyrus and that the stronger stimulus parameters used here are presumably close to the threshold of *c-fos* activation (Fig. 1*H*). These results demonstrate that *arg3.1/arc* and *c-fos* possess distinct thresholds to synaptic activation and that the induction of *arg3.1/arc* is most highly associated with LTP. Previous studies have demonstrated different thresholds for the activation of immediate early genes and that *c-fos* is unresponsive to weak LTP-producing stimulation but can be induced when stimulation intensity is increased and more persistent forms of LTP are generated (Abraham et al., 1993; Worley et al., 1993).

Membrane depolarization increases *arg3.1/arc* mRNA expression

Membrane depolarization of PC12 cells induces *c-fos* transcription by calcium influx through voltage-sensitive calcium channels (VSCCs) (Greenberg et al., 1986; Morgan and Curran, 1986). We exposed PC12 cells to potassium chloride and monitored the expression of *arg3.1/arc* and *c-fos* mRNA at various times after membrane depolarization (Fig. 2). We will refer to this treatment also as calcium-mediated induction. As was reported earlier (Bartel et al., 1989), the greatest increase in *c-fos* mRNA levels was

-1737	CCTGCAG	CTCCCGGCTC	CCAGGATTCC	TCCAGTAGGA	AAACTCTAGT	TAGTCCCTAT
-1680	TCTAGCAATT	TCTCTGCTTG	TTTCCCCTCC	CTGCAGTGAT	GCCCCTCTGC	CTGCCTTCTG
				TCF	SRE	
-1620	TGTCCGCCTA	CTCACCTCTA	ATTAGAAATG	CAGGAGAC	CATTTTAGG	GAGTTTTTTT
-1560	TCCCCAGAAA	GACATTCTCT	TCAGGGATGG	AAGCCCTGGG	TGTGTGTGGG	GGGGGGGGGG
-1500	AGGGGGGACA	GGTTGGCTCT	AAGAGAGGCA	GAGCTCCCCA	AAGCTTCTTG	AAAACAGAGG
-1440	AGGGGGGCTCT	GAGGGATATG	AGGTTAGGAG	AGTTCTGTAG	GTGTGTGAGC	AAAAGTGGGA
-1380	TGGGAGAAGC	TGAGATGTGA	GTACACCTGG	ATTACAAGTG	GGGGCCACTG	GTGTGTGTGT
-1320	GGAAGGTCAT	GAGTGGGGGG	TATGAGTCCA	CAGAGAGGGC	CGTGAGTGGG	GGGCATGAGT
-1260	CTACATGGAA	GGGTGTGAGT	GGGCTAAGTG	CTGGTTGGTA	TGAGTGTTC	GTGTGTATGT
-1200	ATGAGTGTGA	GAGAGGGGGA	GCTGGCAGAG	AGGAAAGGGG	GTCTTTCGAG	GCACCATGTG
-1140	TCTGTGGGCC	CCTGTAGGCT	TCTGCTGTGG	GCAGTGATAT	GTCCCAAGT	AACATCAGGA
-1080	AATGGGAGAC	CTAGACCTGT	GTTCTGGCT	TGGTGTAAAT	CAAGAAGGAT	GCCCTCCCCC
-1020	ACTGCACCAC	AGCGCTGTTG	GCTGTTGCCA	GGGAATCGGA	AGGGGCCCTC	CTCTAGAGGG
		ETS	SRE	E-Box		
-960	AATCTCA	GCT TCCGAGCCC	CATTCTTAT	ATGGCATCTT	GCTTTCCTCT	GCAGGCTCCT
-900	TCCCCTCCTT	CCTCCTTCTC	CCTCCCTCCT	TCCTCCGGAC	TAACCTCTTG	GAAGGCTTGA
-840	GAAGCTTCTG	GTCCAGGTGT	GGCTGGGTCC	TAGGGACTTC	AGGGTTGAGC	AATGTTGCCT
-780	GCACATCTTG	ATATCCCTAT	GACCTACCCA	AGTGTCTGTA	CAGGTTCAAG	TGATACATCT
					AP-1	
-720	GTTCAGGGC	ACTGCGGCC	TGTTACAGCA	CTGACACACA	GACAGGGCC	CTCAGTGTCC
-660	ACAAACCTAT	GAAGGGGTCC	AGAAAAAGCC	TTGCCTGAGT	GGAGTTCTCG	AACAATATTG
-600	GACCAGGCTA	GGATTGAGAC	TTATGACTCT	TGGGGCAAGG	TTGGCAGGA	AGTGGTGTG
					ETS	
-540	TGTGTGTGTG	TGTGTGTGTG	TGTGTGTGTG	TGTGTGTGTG	TGTGTCCCGC	TAACCAAAGG
-480	CAGCTGAGGA	TAGAAGGTT	TGGAGTGAGA	GATTTCCTACC	AGGCACCTCT	GAAGTTGCAT
-420	ACTGTGTGGT	CAGGATGGGC	AAGAGTCAGG	ATGGGCGCAT	CAAAGATTCA	GAGGGGTAAAC
-360	ATCCAGCTGC	TCCCTAAGAA	AAGAGATCAT	GAGGCCCCAG	GGCAACACAT	AGGCAATAAC
-300	CTGCCCTTAGC	CTCATCTCTG	CTGCCAGGA	CTAGGTAGAG	GAGAGTGTCT	CCGGTCCCCA
				AP-1	SP-1	
-240	AAATCCCTCC	CGGTGGGAGG	CGCGCACAGA	GCACATTAGT	CACTCGGGC	TGTGAAGGGG
					SP-1	
-180	CGGGTCTCTG	AGGGCACCCA	CGGGAGGGGA	GCGAGTAGGC	GCGGAAGGCG	GGCCTGCGG
		SP-1			CAAT-Box	
-120	CAGGAGAGGG	CGCGGGCGG	CTCTGGCGCG	GAGCCTGGGC	GCCGCCAATG	GAGCCAGGG
		SP-1		TATA-Box		
-60	CTCCACGAGC	TGCGGCCAC	GGGCCCGCG	CAGCATAAAT	AGCGCTGGT	GGCGGTTTCG

1	GTGCAGAGCT	CAAGCGAGTT	CTCCCGCAGC	CGCAGTCTCT	GGGCTCTCT	AGCTTCAGCG
61	GCGACGAGCC	TGCCACACTC	GCTAAGCTCC	TCCGGCACCG	CACACCTGCC	ACTGCCGCTG
121	CAGCCGCCGG	CTCTGCTCC	TCCGGCTTC	TGCTCAGAG	GAGTTCTTAG	CCTGTTCCGA
181	GCCGACGAC	CGACGACCAG	ATGGAGCTGG	ACCATATGAC	CACCGGCGGC	CTCCACGCCT
			M E L D	H M T	T G G	L H A Y

Figure 5. Sequence of the arg3.1/arc promoter region. The transcription start site is marked with asterisks. Promoter element consensus sequences are boxed. For a detailed description of the regulatory elements, refer to Results. A satellite sequence is underlined. The first codons of the reading frame are translated.

observed at 30 min and 1 hr after depolarization. Although present at higher baseline levels, arg3.1/arc mRNA levels were similarly increased, but in contrast to *c-fos*, they remained elevated for at least 4 hr. Ten hours after stimulation, arg3.1/arc mRNA levels were slightly below control levels. Inhibition of protein synthesis did not abolish the induction of *c-fos* or arg3.1/arc mRNA, as is typical for IEGs. However, in contrast to *c-fos*, arg3.1/arc showed no superinduction in the presence of protein synthesis inhibition. This observation resembles arg3.1/arc expression in the hippocampus *in vivo*, where seizures in the presence of cycloheximide increase arg3.1/arc mRNA without apparent superinduction (Link et al., 1995; Wallace et al., 1998). The lack of superinduction might be the result of a higher stability of arg3.1/arc transcripts, which may also explain the longer time of elevated arg3.1/arc levels compared with *c-fos*.

Arg3.1/arc expression is activated by cAMP in PC12 cells but not in NIH3T3 or Neuro2a cells

Pharmacological and genetic experiments have demonstrated that cAMP plays a critical role in L-LTP (Frey et al., 1993; Abel et al., 1997). With an interest in arg3.1/arc involvement in this phenomenon, we determined whether arg3.1/arc expression is responsive to activation by cAMP. PC12 pheochromocytoma cells, NIH3T3 fibroblasts, and Neuro2a neuroblastoma cells were stimulated with forskolin, a strong activator of adenylyl cyclase (Fig. 3). We will refer to this treatment also as cAMP-mediated induction. In PC12 cells, arg3.1/arc transcript levels were elevated rapidly by

cAMP (Fig. 3A) and increased fourfold ($n = 2$) and fivefold ($n = 6$) at 30 min and 1 hour after stimulation, respectively. In contrast to the effects of membrane depolarization, arg3.1/arc transcripts were downregulated below baseline levels by 4 hr after forskolin stimulation. Such a return of arg3.1/arc transcripts to below baseline levels has been observed previously 4 hr after seizure activity in the intact animal and might reflect an active transcriptional shutoff mechanism (Link et al., 1995). As reported elsewhere (Krujer et al., 1985), *c-fos* transcript levels were equally increased with a similar time course of induction (Fig. 3A). Forskolin-stimulated increases in arg3.1/arc and *c-fos* mRNA levels were dependent on transcriptional activation of these genes, because the induction of both genes was completely blocked by actinomycin D, a potent inhibitor of transcription (Fig. 3B). In strong contrast, no cAMP-mediated induction of arg3.1/arc was seen in NIH3T3 cells, although *c-fos* transcript levels were clearly elevated within 30 min after stimulation (Fisch et al., 1989) (Fig. 3C). Similarly, arg3.1/arc was unresponsive to cAMP stimulation in Neuro2a cells, although increased *c-fos* transcript levels could be observed 1 and 4 hr after stimulation (Fig. 3D). These findings indicate that induction of arg3.1/arc transcription by cAMP is dependent on a signaling pathway that is present in PC12 cells but absent in NIH3T3 or Neuro2a cells. In contrast, *c-fos* transcripts were readily increased in these cells, indicating that cAMP-mediated induction of *c-fos* is not solely dependent on the pathway present in PC12 cells.

Figure 6. Response of the arg3.1/arc promoter to forskolin. Arg3.1/arc promoter deletions extending from position -1737 to +250, -834 to +250, and -177 to +250 were fused to a luciferase reporter. The reporter constructs were used to stably transfect PC12 cells. Cells were stimulated with forskolin for 1 hr. Inducibility of the arg3.1/arc promoter deletion constructs and that of the endogenous arg3.1/arc promoter was determined by Northern blot analyses. *A*, Autoradiograph of a Northern blot hybridized with a probe specific for luciferase to measure the inducibility of the transfected arg3.1/arc promoter deletions. Hybridization with a probe specific for GAPDH was used as a loading control. Numbers on the left indicate the 5' deletion endpoints of the arg3.1/arc promoter constructs. Plus signs in the corresponding line indicate the presence of the respective constructs. The first three lanes from the left contained RNA from forskolin-stimulated cells; the three lanes on the right contained RNA from mock-stimulated cells. *B*, Autoradiograph of a Northern blot hybridized with a probe specific for arg3.1/arc to measure the inducibility of the endogenous arg3.1/arc promoter and a probe specific for GAPDH. Labels are as in *A*. *C*, Quantification of Northern blots ($n = 3$). Hybridization signals were normalized against mock-stimulated control and GAPDH signals. Error bars represent the SEM; $p < 0.05$ compared with control (two-tailed Student's *t* test). Note that transient transfection experiments indicated that the observed weak residual inducibility represents a function of the basic vector itself and not of the inserted arg3.1/arc sequences (data not shown).

The arg3.1/arc promoter does not contain a CRE consensus sequence

A ubiquitously found cAMP-dependent signaling mechanism is the cAMP/PKA/CREB pathway (Montminy, 1997). For example, in NIH3T3 cells the transcriptional activation of CRE-containing genes such as *c-fos* is mediated by the PKA-induced phosphorylation of CREB (Fisch et al., 1989; Gonzalez and Montminy, 1989). These observations indicated to us that the arg3.1/arc promoter might not contain a functional CRE site and that other signaling pathways are responsible for its induction by cAMP in PC12 cells. To test this assumption we isolated from a mouse 129/Sv (ev) genomic library a 21 kb fragment containing the arg3.1/arc gene, including 10 kb of the 5' flanking sequence, the entire transcribed region, and 6.5 kb downstream from the poly(A)⁺ signal. We determined the transcription start site using nuclease S1 analysis (Kuhl et al., 1987) (Fig. 4) and sequenced 1737 bp of the promoter as well as the entire transcribed region (GenBank accession number AF177701). Figure 5 shows the sequence of the promoter. The first 200 bp upstream from the transcription initiation site are of high GC content (75%), as is typical of a promoter region. At position -28 there is a TATA-box and at position -77 a CAAT-box. Four SP-1 sites were detected, at positions -48, -107, -135, and -184. These elements are presumably responsible for the constitutive activity of the promoter (Dierks et al., 1983). At positions -936 and -1581 we found serum response element (SRE) consensus sequences, which might account for the serum responsiveness of this gene (Link et al., 1995). Similar to *c-fos*, the core SRE sequence of arg3.1/arc is flanked by sequences that support a stable formation of a ternary complex (Treisman et al., 1992). At positions -205 and -671 we detected AP-1 consensus sequences that are also contained within the *c-fos* promoter. Despite these similarities to *c-fos*, 1737 bp of the arg3.1/arc promoter do not contain the CRE consensus sequence TGACGTCA. To test whether this arg3.1/arc promoter segment is inducible by cAMP, we fused it with a luciferase reporter gene and generated stable transfected PC12 cells. In addition, we tested two smaller fragments deleting one or both of the SRE. The SRE can be activated by MAPK in hippocampal cultures by Elk-1 (Xia et al., 1996), and during LTP stimulation Elk-1 is phosphorylated in a MAPK-dependent manner (Davis et al., 2000). The arg3.1/arc promoter contains two SRE consensus sequences. However, our deletion analysis of arg3.1/arc promoter constructs in PC12 cells shows that these SRE sites are without influence on the transcriptional activation of the gene by cAMP. Figure 6 shows that in contrast to the endogenous arg3.1/arc gene all three deletion constructs were only modestly inducible by forskolin. Moreover, transient transfection experiments indicated that the observed weak residual inducibility represents a function of the basic vector itself and not of the inserted arg3.1/arc sequences (data not shown). These experiments indicate that 1737 bp of the arg3.1/arc promoter, containing SRE and AP1 sites, are not sufficient to mediate induction by forskolin. Certain aspects of chromatin structure that are not fully conserved in the transfection assay might be necessary for the controlled expression of arg3.1/arc, or alternatively, additional elements positioned outside of the tested promoter region may be required for induction. Such elements could be CRE sites that are located more distantly.

Figure 7. Induced levels of arg3.1/arc and *c-fos* mRNA can be blocked by calcium channel and protein kinase A inhibitors in PC12 cells. PC12 cells were pretreated for 30 min with the indicated inhibitors and subsequently stimulated by the addition of either KCl or forskolin. *A, D*, Autoradiographs of Northern blots hybridized to probes specific for arg3.1/arc, *c-fos*, and GAPDH. *B, E*, Quantification of hybridization signals after forskolin stimulation. *C, F*, Quantification of hybridization signals after KCl depolarization. *A–C*, Effect of the calcium channel blocker nifedipine (10 μ M) on induced arg3.1/arc and *c-fos* mRNA levels ($n = 2$). *D–F*, Effect of the protein kinase A blocker H-89 (20 μ M) on induced arg3.1/arc and *c-fos* mRNA levels ($n = 3$). Error bars represent SEM; * $p < 0.05$ (two-tailed Student's *t* test); NS, not significant, $p > 0.05$. The induction of arg3.1/arc mRNA by forskolin and KCl was completely blocked by the PKA inhibitor.

Induced expression of arg3.1/arc is dependent on the activation of PKA

To delineate the signaling pathways that are required for the activation of arg3.1/arc transcription, we made use of various pharmacological inhibitors. Nifedipine, a blocker of VSCCs (Toll, 1982), effectively blocked the induction elicited by membrane depolarization of *c-fos* (Morgan and Curran, 1986) and arg3.1/arc but had no significant effect on the cAMP-induced expression of either gene (Fig. 7*A–C*). These results demonstrate that calcium influx through VSCCs is necessary for membrane depolarization-induced expression of arg3.1/arc mRNA. We next asked whether the calcium- and cAMP-induced expression of arg3.1/arc mRNA in PC12 cells is dependent on PKA. H89 is a potent and selective inhibitor of PKA but does not inhibit CaM kinase, protein kinase C, casein kinase II, or cGMP-dependent protein kinase at the concentrations used in the present experiments (Chijiwa et al., 1990). In PC12 cells both KCl- and forskolin-induced expression of arg3.1/arc mRNA was completely abolished in the presence of the PKA inhibitor H-89. Similarly, induced expression of *c-fos* was also greatly diminished (Ginty et al., 1991) (Fig. 7*D–F*). The small remaining increase in *c-fos* mRNA levels in the presence of the PKA blocker might be accounted for by cAMP-mediated activation of VSCCs (Sculptoreanu et al., 1993), leading to calcium influx and stimulation of calcium-calmodulin-dependent kinases, which phosphorylate CREB (Sheng et al., 1991; Deisseroth et al., 1996). Alternatively, it is conceivable that the inhibitor might have an effect on protein synthesis and thereby stabilize *c-fos* mRNA. In any case, these data indicate that calcium- and cAMP-induced expression of arg3.1/arc and *c-fos* mRNA is dependent on the activation of PKA.

cAMP-induced expression of arg3.1/arc is dependent on the activation of MAPK

After cAMP stimulation PKA can activate MAPK in PC12 cells (Vossler et al., 1997). In this instance, PKA phosphorylates the small G-protein Rap1 and increases GTP loading (Altschuler and Lapetina, 1993). This in turn leads to the activation of B-Raf, which can phosphorylate the MAPK/ERK kinase (MEK) and finally results in the activation of MAPK. Interestingly, this pathway is absent in NIH3T3 cells, which do not express B-Raf at significant levels (Vossler et al., 1997). With an interest in the possibility that cAMP/PKA-mediated induction of arg3.1/arc is dependent on this signaling mechanism, the effect of the selective MEK inhibitor PD098059 (Alessi et al., 1995) was examined. In the presence of the MEK inhibitor, cAMP-mediated induction of arg3.1/arc in PC12 cells was greatly reduced, whereas the induction of *c-fos* was largely retained (Fig. 8*A,B*). Similar to PC12 cells (Figs. 7*D,E*, 8*A,B*), in primary cultures of hippocampal neurons, expression of both arg3.1/arc and *c-fos* mRNA was strongly induced by forskolin (Fig. 8*C,D*). The PKA blocker H-89 dramatically reduced cAMP-induced arg3.1/arc mRNA levels to below constitutive levels. Induced transcription of *c-fos* was also greatly reduced. In neurons pretreated with the MEK inhibitor PD098059, activation of arg3.1/arc transcription by forskolin was blocked. In contrast, the activation of *c-fos* by cAMP was only slightly attenuated by the MEK inhibitor (Fig. 8*C,D*).

DISCUSSION

Activity-dependent alterations in synaptic efficacy are thought to underlie learning and memory, epileptogenesis, drug addiction, and several neurological diseases (Nestler and Aghajanian, 1997;

Figure 8. Forskolin-induced arg3.1/arc mRNA levels are blocked by MAPK/ERK kinase inhibitor in PC12 cells and primary cultures of hippocampal neurons. PC12 cells and primary cultures of hippocampal neurons were pretreated with the MAPK/ERK kinase blocker PD098059 (50 μ M) and subsequently stimulated by forskolin ($n = 3$). Hippocampal neurons were also pretreated with the PKA blocker H-89 (compare with Fig. 7D,E). *A*, *C*, Autoradiographs of Northern blots loaded with RNA from PC12 cells (*A*) and RNA from hippocampal neurons (*C*). Hybridization was with probes specific for arg3.1/arc, c-fos, and GAPDH. *B*, *D*, Quantification of arg3.1/arc and c-fos hybridization signals from analyses of PC12 cells and hippocampal neurons, respectively. Error bars represent SEM; * $p < 0.05$ (two-tailed Student's t test); NS, not significant, $p > 0.05$. Although the induction of arg3.1/arc mRNA by forskolin was blocked in PC12 cells and hippocampal neurons by the MAPK/ERK kinase inhibitor, induction of c-fos mRNA was affected to a much lesser extent.

Milner et al., 1998). To stabilize changes in synaptic strength, neurons activate a program of gene expression that results in alterations of their molecular composition and structure. Among activity-dependent genes, arg3.1/arc is thus far unique, because its mRNA has the potential to be locally translated at stimulated synapses and consequently might play a key role in synapse-specific modifications (Kuhl and Skehel, 1998). Importantly, arg3.1/arc is the first and only gene with expression that has been directly linked to information processing (Guzowski et al., 1999). Moreover, arg3.1/arc is reliably induced with LTP-producing stimulation. The strict association of induced arg3.1/arc expression in cells that have established LTP further indicates that arg3.1/arc plays a role in the maintenance or consolidation phase of this process. In this study we analyzed the signaling pathways that contribute to the induction of arg3.1/arc. Of particular interest to us was the examination of signaling pathways that had been shown to be important for the establishment of long-lasting synaptic plasticity.

Here we demonstrate that transcription of arg3.1/arc mRNA in neurons can be induced by calcium and cAMP. This inducibility is strictly dependent on the activation of PKA and the MAPK/ERK kinase signaling pathways, which have been demonstrated to play specific roles in learning and memory and synaptic plas-

ticity (Frey et al., 1993; Abel et al., 1997; English and Sweatt, 1997; Martin et al., 1997; Berman et al., 1998; Blum et al., 1999; Coogan et al., 1999; Rosenblum et al., 2000). Moreover, our experiments suggest indirectly that induction of arg3.1/arc may not require the transcription factor CREB. This assumption is based on three independent observations, but alternative explanations may exist, as indicated below.

First, CRE sites are typically located within the first few hundred nucleotides of cAMP-responsive genes (Sassone-Corsi, 1995; Montminy, 1997). We did not detect a CRE site within 1737 bp of the arg3.1/arc promoter or the entire transcribed region of 3.5 kb. At position +111 the arg3.1/arc promoter contains a single CGTCA sequence; however, such an incomplete CRE site is not active (Fink et al., 1988) and does not bind to CREB by itself (Nichols et al., 1992). However, we note that 1737 bp of the arg3.1/arc promoter proved unresponsive to forskolin stimulation in transfection experiments; therefore, these data do not exclude the possibility that unidentified CRE sites lie outside of this region. Second, in PC12 cells and hippocampal neurons, both arg3.1/arc and c-fos transcripts are strongly induced by cAMP. In strong contrast, although CREB is activated by cAMP stimulation in NIH3T3 and Neuro2a cells and the transcription of the CRE-containing c-fos gene is consequently induced (Fisch et al., 1989; Gonzalez and Montminy, 1989), we observed no induction of arg3.1/arc in these two cell lines. Third, in cAMP-stimulated PC12 cells and hippocampal neurons that had been pretreated with a MAPK/ERK kinase inhibitor, the induction of arg3.1/arc was effectively blocked. Similar to NIH3T3 cells in which B-Raf is not expressed at levels sufficient to activate MAPK/ERK kinase (Vossler et al., 1997), cAMP can mediate induction of c-fos transcription in the MAPK/ERK kinase inhibitor-treated neurons, presumably through the PKA/CREB or calcium-calmodulin kinase/CREB pathway (Deisseroth et al., 1996). These data argue, at face value, against an involvement of CREB in the induction of arg3.1/arc. However, an alternative explanation may exist if we assume that the expression of arg3.1/arc is under both positive and negative control. In such a scenario cAMP-induced expression of arg3.1/arc would be under the control of a repressor and a positive activator, which may be CREB. Release of the repressor would depend strictly on the MAPK pathway, and in cell lines that carry a defect in this pathway, arg3.1/arc would be unresponsive to stimulation, although CREB was activated.

Phosphorylation of CREB directed by cAMP- and calcium-influenced signaling pathways has been shown to be important in the neuronal signaling processes leading to the formation and retention of long-term memory in invertebrates and vertebrates (Dash et al., 1990; Bourchuladze et al., 1994; Bartsch et al., 1995; Yin and Tully, 1996; Guzowski and McGaugh, 1997). Although CREB plays a crucial role in memory in *Aplysia* and *Drosophila*, recent studies on mice with hypomorphic CREB alleles suggest that more complex pathways may exist in mammals (Gass et al., 1998). Moreover, the transcription factor CREB is activated by diverse extracellular stimuli through multiple signaling cascades and in addition to synaptic plasticity seems to play a role in developmental and adaptive responses (Datta et al., 1999; Finkbeiner, 2000; Walton and Dragunow, 2000). Although the programs of gene expression in these distinct biological functions of CREB might overlap and specificity might be brought about by cooperation with other transcriptional factors, our studies suggest that in addition parallel pathways may exist. To our knowledge arg3.1/arc is the first activity-dependent gene with stimulus-dependent transcriptional activation that relies solely on the

MAPK/ERK pathway. It will be interesting to determine whether other plasticity-associated genes are regulated in the same manner.

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