Failure of Ca2+-activated, CREB-dependent transcription in astrocytes

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Abstract

Astrocytes participate in signaling via Ca^{2+} transients that spread from cell to cell across a multicellular syncytium. The effect, if any, of these Ca^{2+} waves on the transcription of CREB-dependent genes is not known. We report here that, unlike neurons, increasing intracellular Ca^{2+} in cultured mouse cortical astrocytes failed to activate CREB-dependent transcription, even though CREB was phosphorylated at serine 133. In contrast, both CREB phosphorylation and CREB-dependent transcription were robustly stimulated by increasing cAMP. The failure of Ca^{2+}-activated transcription in astrocytes was correlated with the absence of CaMKIV, a Ca^{2+}-dependent protein kinase required for Ca^{2+}-stimulated gene transcription in neurons. The inability of Ca^{2+} to signal via CaMKIV may insulate CREB-dependent gene transcription in astrocytes from activation by Ca^{2+} waves.

Keywords

Ca^{2+}/calmodulin-dependent protein kinase; Ca^{2+} imaging; Ca^{2+} waves; cyclic AMP; okadaic acid; protein phosphatase

INTRODUCTION

Although Ca^{2+} ions comprise a universal signaling mechanism in the nervous system, they play different roles in neurons and astrocytes. In neurons, action potentials and excitatory synaptic activity lead to brief Ca^{2+} transients, due to entry of Ca^{2+} through voltage-gated Ca^{2+} channels and Ca^{2+}-permeant neurotransmitter receptors, as well as Ca^{2+} release from intracellular endoplasmic reticulum stores. As mediators of neurotransmitter release and dendritic integration, these neuronal Ca^{2+} signals are generally transient and localized due to rapid intracellular buffering, extrusion across the plasma membrane, and uptake into intracellular stores. Nevertheless, increased duration and/or intensity of excitatory stimulation can raise intracellular $[Ca^{2+}]$ to levels high enough to induce transcription of activity-dependent genes, a process required for learning and memory.

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In contrast to neurons, astrocytes also utilize Ca^{2+} for intercellular signaling. Stimulation of cultured astrocytes leads to the generation of Ca^{2+} transients that are substantially longer than those of neurons and that can propagate from cell to cell across a multicellular syncytium (Cornell-Bell et al., 1990; Charles et al., 1991; Scemes and Giaume, 2006). These propagating waves are mediated by gap junctions (Finkbeiner, 1992; Scemes and Giaume, 2006) and by release of ATP, which raises intracellular $[Ca^{2+}]$ in adjacent astrocytes by activating purinergic P2 receptors (James and Butt, 2001). Thus, these Ca^{2+} waves provide a mechanism for intercellular signaling across astrocyte networks that may operate in parallel with faster communication among neurons mediated by action potentials and synaptic transmission.

These astrocytic Ca^{2+} waves have the potential to induce sustained activation of Ca^{2+}-dependent genes, which, in neurons, would occur only transiently in response to excitatory input. In this report we investigated the extent to which Ca^{2+} can activate gene expression in astrocytes. Surprisingly, we found that in astrocytes, transcriptional activation regulated by the transcription factor, Ca^{2+}/cAMP-regulatory element binding protein (CREB), failed to respond to increased intracellular $[Ca^{2+}]$, even though it was strongly activated by cAMP. Our results indicate that astrocytes lack the intracellular Ca^{2+} signaling cascades required to induce CREB-dependent gene transcription in neurons.

MATERIALS AND METHODS

Cell Culture and Transfection

Astrocytes were isolated from embryonic day 16 or postnatal day 1 mouse cerebral cortex (C57Bl/6J, Jackson Laboratories, Bar Harbor, ME). Cells were mechanically dissociated by trituration, plated onto poly-L-lysine-coated (Sigma-Aldrich Co., St. Louis, MO) T75 flasks (2 brains/flask) and were maintained in DMEM/F12 medium (1/1) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and penicillin G (100 U/ml) and streptomycin (100 μg/ml) (both Invitrogen, Carlsbad, CA) in 5% CO_2 at 37°C. Starting on day 3 after plating, medium was replaced every two days. After 10–13 days, cells were trypsinized and replated in same medium without penicillin/streptomycin at 5 x 10^5/well in 24-well plates for luciferase assays and 2.0 x 10^6/well in 35-mm dishes for RNA and protein analyses.

Luciferase Assays

Astrocytes were transiently transfected 1 day after replating using a Lipofectamine 2000 diluted 1:33 in Opti-MEM (Invitrogen). 4x*CRE*- (Stratagene, La Jolla, CA) and *TRKB P2*- (Kingsbury et al., 2003) luciferase reporters were introduced at 250 ng/well; cells were cotransfected with 35 ng/well TK *Renilla* plasmid (Promega, Madison, WI). One day after transfection, cultures were stimulated for 5 h, washed with PBS and harvested in 110 μl *Renilla* luciferase lysis buffer (Promega). Five μl of extract were assayed for reporter (luciferase assay reagent, Promega) and *Renilla* (*Renilla* luciferase assay system; Promega) activity. Promoter-driven luciferase activity was normalized to *Renilla* activity to correct for variability among wells.

Western Blot

Cells were harvested directly into 2X Laemmli sample buffer (Sigma-Aldrich), heated to 100°C for 5 min, fractionated by SDS-PAGE on 4–12% Bis-Tris gels run in MOPS buffer; gels were transferred to a PVDF membrane (Invitrogen). Western blotting was conducted using antibodies to CREB (Cell Signaling; 1:1000), phospho-CREB (specific for phospho-Ser133; Cell Signaling; 1:1000), CaMKIV (Cell Signaling; 1:1000), and actin (Sigma-Aldrich; 1:2000). Secondary antibody conjugated to horseradish peroxidase (Jackson

ImmunoResearch Laboratories, Inc., West Grove, PA) was used at 1:5000. Blots were incubated with antibodies overnight at 4°C in blocking buffer (TBS-t, 5% bovine serum albumin and 5% dehydrated milk), developed with ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, New Jersey) and analyzed on an Image Station 2000R (Eastman Kodak, Rochester, NY).

Quantitative, Real-time PCR (Q-PCR)

Following stimulation, astrocytes were harvested using Qia-shredder (Qiagen, Germantown, MD), and RNA was isolated using RNeasy RNA extraction kit (Qiagen). 0.5–1 μg was used for reverse transcription with Superscript II (Invitrogen) for 50 min at 42°C. RNA processed in the absence of Superscript II served as control. Q-PCR was conducted using a LightCycler 480 Q-PCR thermal cycler (Roche, Basel, Switzerland) SYBR Green I Mastermix (Roche), and a final primer concentration of 500 nM. The Q-PCR protocol included an initial hold step for 5 min at 95°C, and cycles of 10 sec at 95°C, 15 sec at 58°C, and 1 min at 72°C, repeated for 45 cycles. Final samples were analyzed by gel electrophoresis to confirm that the correct products were amplified. Forward and reverse primer sequences were: for CaMKIV, GATGCAGGTGTAAAAGAGG and CATCCTGCTGTGGAACCC and for β-actin, ATCGTGGGCCGCCCTAGGCA and TGGCCTTAGGGTTCGAGAGG (obtained from Invitrogen or IDT, Coralville, IA).

Ca^{2+} Imaging

Intracellular Ca^{2+} levels were determined using fura-2 imaging as described by Bambrick et al. (1997).

Reagents

ATP, KCl, ionomycin and okadaic acid were purchased from Sigma-Aldrich; forskolin, cyclopiazonic acid and BAPTA-AM from Calbiochem (San Diego, CA); 4-Br A23187 from Alexis Biochemicals (San Diego, CA); and [Phe(2),Orn(8)]-oxytocin from Bachem Bioscience, Inc. (King of Prussia, PA).

RESULTS

To compare the regulation of CREB-dependent genes in astrocytes and neurons, cultures were transfected with a luciferase reporter construct driven by four tandem *CRE* sequences (4x*CRE*). In neurons, both depolarization by addition of 50 mM KCl and increasing cAMP by addition of forskolin stimulated 4x*CRE* reporter activity (Fig. 1A). Depolarization-induced gene transcription in neurons was blocked by Ca^{2+} chelators and L-type Ca^{2+}-channels antagonists, confirming that the effect is mediated by Ca^{2+} (Kingsbury et al., 2003). In contrast, ATP (10 μM) and depolarization with elevated KCl, which raised [Ca^{2+}] in astrocytes by 3–6-fold (Fig. 1C,D), failed to stimulate 4x*CRE* in astrocytes (Fig. 1B). Higher concentrations of ATP did not significantly stimulate 4x*CRE* expression (+10 μM ATP: 1.5 ± 0.4, p>0.05; +30 μM ATP: 1.1 ± 0.1, p>0.05; +100 μM ATP: 1.6 ± 0.3, p>0.05; +10 μM forskolin: 17.8 ± 3.1, p<0.001; n=3). Moreover, additional stimuli reported to raise intracellular [Ca^{2+}], including the ionophores, ionomycin and 8-Br-A23187, the endocrine hormone, oxytocin, and cyclopiazonic acid, an inhibitor of uptake of Ca^{2+} into internal stores, all failed to stimulate 4x*CRE* expression (Fig. 1B). It should be noted that 10 μM 8-Br-A23187 is used in the calibration of Ca^{2+} imaging experiments in astrocytes (Parpura and Haydon, 2000) in order to set the intracellular Ca^{2+} concentration at a very high level. Although Ca^{2+} failed to induce 4x*CRE* expression in astrocytes, forskolin stimulated 4x*CRE* expression by 40-fold (Fig. 1B). We also examined the effects of forskolin and ATP on 4x*CRE* expression in astrocyte cultures prepared from postnatal (P1) mouse cortex. As with embryonic cortex (Fig. 1B), the results demonstrated that ATP did not induce CREB-

dependent gene transcription in P1 astrocytes, while forskolin induced a robust stimulation (+10 µM ATP: 0.91 ± 0.1, $p > 0.05$; +50 µM forskolin: 20.3 ± 8.0, $p < 0.05$; n=3). Thus, CREB-dependent gene transcription is not stimulated by increased intracellular $[Ca^{2+}]$ in astrocytes, even though cAMP can induce robust stimulation.

The presence of *CRE*s in gene promoters confers cAMP- and Ca^{2+}-responsiveness to many genes in multiple cell types (Mayr and Montminy, 2001; Zhang et al., 2005). The observation that *4xCRE* is not activated by Ca^{2+} in astrocytes suggests that the promoters of endogenous genes requiring CREB activation for Ca^{2+}-responsiveness will not be activated by Ca^{2+} in astrocytes. To test this, we measured luciferase reporter activity driven by the downstream promoter (P2) of the gene encoding the brain-derived neurotrophic factor (BDNF) receptor, *TRKB* (Barettino et al., 1999). P2 contains two *CRE*s and, as previously reported, is activated by both cAMP and Ca^{2+} in neurons (Kingsbury et al., 2003). Neither ATP (10 µM) nor KCl (50mM) activated the *TRKB* P2-luciferase reporter in astrocytes, while forskolin treatment induced a 4–5-fold stimulation (Figure 2). The ability of cAMP, but not Ca^{2+}, to activate *TRKB* P2-luciferase matches the stimulus pattern of 4x*CRE*-luciferase, consistent with the notion that the inability of Ca^{2+} to activate *CRE*-luciferase reflects a difference in Ca^{2+} signaling mechanism in astrocytes rather than a unique property of 4x*CRE*.

Since phosphorylation of serine 133 is required for CREB-dependent gene transcription (Mayr and Montminy, 2001), we determined if ATP was capable of inducing CREB phosphorylation in astrocytes. CREB phosphorylation was monitored by Western blot analysis using an antibody that binds CREB only when it is phosphorylated at serine 133. Both ATP and forskolin stimulated CREB phosphorylation, however, whereas forskolin treatment resulted in phospho-CREB levels that remained significantly above control at least 2 hr, ATP-induced phospho-CREB levels had returned to baseline within 1 hr (Figure 3A,B). Preincubation with the membrane permeant Ca^{2+} chelator BAPTA-AM (150 µM) for 30 min inhibited the ATP-induced increase in phospho-CREB (Figure 3C). Taken together, these results demonstrate that, although ATP can induce Ca^{2+}-dependent CREB phosphorylation, the time-course is transient compared to that induced by forskolin.

Transient CREB phosphorylation may not be sufficient to induce CREB-dependent gene transcription (Bito et al., 1996). To test the hypothesis that the failure of ATP to activate 4x*CRE* is due to acid (OA), an inhibitor of protein phosphatases PP1 and PP2A, which account for the majority of cellular protein phosphatase activity (Cohen et al., 1990; Shenolikar, 1994; Bito et al., 1996). OA prolonged ATP-induced CREB serine 133 phosphorylation, with phospho-CREB levels still elevated at 2 hrs as they were following forskolin treatment (Figure 4A). Nevertheless, ATP still failed to stimulate 4x *CRE* activity in the presence of OA (Figure 4B). Forskolin increased 4x*CRE* activity over 30-fold (Figure 4B), confirming that the failure of the combined OA and ATP treatment to activate 4x*CRE* expression was not due to nonspecific inhibitory effects of OA. Thus insufficient duration of CREB phosphorylation, alone, cannot account for the failure of ATP to activate 4x*CRE*.

Another possible explanation for the failure of ATP to stimulate 4x*CRE* expression is that Ca^{2+} may activate a parallel pathway that inhibits CREB-dependent gene transcription. For example, it has been reported that Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylates CREB at serine 142 in addition to serine 133 in response to Ca^{2+} resulting in inhibition of CREB-dependent gene transcription (Sun et al., 1994). In cultured hippocampal neurons, Ca^{2+} entering via extrasynaptic NMDA receptors has been reported to antagonize activation of CREB-dependent genes induced by Ca^{2+} entering through synaptic NMDA receptors (Hardingham et al., 2002). To test the possible role of Ca^{2+} in initiating a CREB inhibitory-pathway, astrocytes were treated with ATP and the membrane-permeant cAMP

analog, 8-Br-cAMP, alone or in combination (Fig. 5). If Ca^{2+} activates a CREB-inhibitory pathway, ATP should antagonize stimulation of CREB-dependent gene transcription by cAMP. Like forskolin, 30 and 100 μM 8-Br-cAMP treatment resulted in 4x*CRE* activation. Importantly, activation of 4x*CRE* by ATP + 8-Br-cAMP is not significantly different from that by 8-Br-cAMP alone. Thus, the failure of ATP to induce CREB-dependent transcription in astrocytes is likely due to the absence of a necessary transcription-activating signal and not to the stimulation of a Ca^{2+}-dependent CREB-inhibitory pathway.

Phosphorylation of CREB at serine 133 is generally necessary but not sufficient for activation of *CRE*-containing genes. CREB phosphorylation leads to the recruitment of a co-activator such as CREB binding protein (CBP) to the transcription initiation complex. In neurons, Ca^{2+}-induced activation of CREB-dependent gene transcription requires concurrent activation of CBP by CaMKIV (Chaw-la et al., 1998; Impey et al., 2002). We postulated that failure to activate CBP by CaMKIV-mediated phosphorylation could explain the inability of Ca^{2+} to stimulate CREB-dependent gene transcription in astrocytes. As shown in Figure 6, CaMKIV expression was virtually undetectable in astrocytes. Using Q-PCR, CaMKIV mRNA was easily detected in neurons, but was expressed only marginally above genomic DNA expression in astrocytes (Fig. 6A), determined by omitting reverse transcriptase from the reaction generating cDNA. Consistent with the absence of CaMKIV mRNA in astrocytes, we were unable to detect significant CaMKIV protein expression by Western blot analysis (Fig. 6B). As a positive control, analysis of cortical neurons revealed a strong a double band at the expected molecular weights of 61 and 63 kDa, corresponding to the CaMKIVα and CaMKIVβ isoforms (Ohmstede et al., 1989). Given its vital role in the Ca^{2+}-dependent activation of CREB in neurons, the lack of CaMKIV expression in astrocytes provides a plausible explanation for the failure of Ca^{2+} to activate CREB-dependent gene transcription in astrocytes.

DISCUSSION

Ca^{2+} fails to activate CREB-dependent gene transcription in astrocytes

Our results reveal that, in cultured cortical astrocytes, elevated $[Ca^{2+}]$ does not activate either a simple, synthetic CREB-dependent promoter (4x*CRE*) or a complex promoter of an endogenous gene (*TRKB* P2), despite the fact that both are robustly stimulated by cAMP. The use of multiple stimuli to raise intracellular $[Ca^{2+}]$, validated by Ca^{2+} imaging (Fig. 1), and the demonstration that ATP stimulated CREB phosphorylation in a Ca^{2+}-dependent manner (Fig. 3C), confirmed that $[Ca^{2+}]$ was both elevated and biologically active under conditions where the CREB-dependent reporters were not activated. Thus, Ca^{2+} fails to stimulate both synthetic (Fig. 1) and endogenous (Fig. 2) CREB-dependent promoters, leading to the conclusion that the results obtained are due to a Ca^{2+} signaling deficit in astrocytes. Notably, in neurons from the same embryonic mouse cortices, Ca^{2+} stimulated both 4x*CRE* (Fig. 1A) and *TRKB* P2 (Kingsbury et al., 2003) expression. Even under conditions where cAMP and Ca^{2+} induced comparable increases in CREB phosphorylation in astrocytes (Fig. 4A), only cAMP activated CREB-dependent gene transcription (Fig. 4B). Consequently, the failure of Ca^{2+} to stimulate CREB-dependent gene transcription is most likely due to the lack of Ca^{2+}-dependent coactivator activation, a signaling step accomplished by CaMKIV in neurons (Impey et al., 2002).

Absence of Ca^{2+} signaling pathways in astrocytes

Although this is the first report of the failure of Ca^{2+} to regulate CREB-dependent genes in astrocytes, there is evidence in the literature suggesting that astrocytic Ca^{2+} signaling pathways may be defective. Watterson et al. (2001) reported that CaMKIV, was undetectable on Western blots from rat hippocampal astrocytes. Recently, Cahoy et al.

(2008) published a transcriptome database tabulating the genes expressed by neurons, astrocytes and other brain cells. Consistent with the results reported here, Ca^{2+} signaling pathways were highly enriched in neurons compared to astrocytes, and CaMKIV transcripts in particular were strongly expressed in neurons but nearly undetectable in astrocytes.

Ca^{2+}-dependent genes can be regulated in astrocytes by other signaling pathways

Even though the CREB-dependent promoters utilized in this study were insensitive to Ca^{2+} in astrocytes, those promoters were still activated by forskolin (Fig. 1) and 8-Br-cAMP (Fig. 5). Thus, the cAMP-dependent signaling mechanisms (e.g., PKA) that activate CREB and necessary coactivators (c.f. Brindle et al., 1995) are intact in astrocytes. Recently, Canellada et al. (2008) reported that Ca^{2+} stimulates transcription of the genes encoding cyclooxygenase-2 and Rcan1–4 in astrocytes. Interestingly, this effect was mediated by the Ca^{2+}-dependent, calcineurin/NFAT signaling pathway. In addition, the immediate early gene, *c-fos*, can be activated in astrocytes by Ca^{2+}-dependent signaling mechanisms due to the presence of a serum response element and the repressor element, *DRE,* in its promoter (Edling et al., 2006). Thus, genes such as *c-fos*, which have complex promoters, may be activated by Ca^{2+}-dependent mechanisms that are independent of *CRE*. Moreover, CREB-dependent genes may also be activated by the mitogen-activated protein kinase and phosphoinositide-3 kinase signaling pathways, independently of Ca^{2+} (Schinelli et al., 2001).

CREB-dependent gene transcription and Ca^{2+} waves in astrocytes

The failure of Ca^{2+} to regulate CREB-dependent genes in astrocytes may reflect the unique mechanism of Ca^{2+} signaling that arises as intracellular transients, which can propagate from cell to cell across a multicellular astrocytic syncytium. If intracellular Ca^{2+} signaling were intact, as it is in neurons, these Ca^{2+} waves would be expected to provide constitutive drive on CREB-dependent genes, overexpression of which could be detrimental to normal cell function. There are hundreds, and possibly thousands, of CREB-dependent genes (Zhang et al., 2005) that potentially would be stimulated as a result of these Ca^{2+} transients, representing such diverse cellular functions as intracellular signaling, transcriptional regulation, energy metabolism and cellular differentiation (Su et al., 2000; Mayr and Montminy, 2001; Lonze and Ginty, 2002, Impey et al., 2004; Takanaga et al., 2004). Based on the analysis of 4x*CRE* and *TRKB* P2 promoter activation reported here, we suggest that many of these genes may effectively be insulated from the astrocytic Ca^{2+} waves, because the absence of CaMKIV expression prevents Ca^{2+} from activating CREB-dependent gene transcription. Nevertheless, these genes need not always be silenced in astrocytes; they can still be activated by cAMP.

Acknowledgments

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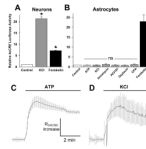
Fig. 1. Ca^{2+} activated 4x*CRE*-luciferase reporter in neurons but not astrocytes
(**A**) 4x*CRE* was activated in neurons by both depolarization (50 mM KCl) and forskolin (50 μM). Error bars show mean ± sem. *, significantly different from control ($p<0.05$). (**B**) Multiple stimuli that raise [Ca^{2+}]$_i$ failed to activate 4x*CRE*-luciferase in astrocytes. None of the treatments, which included ATP (10 μM; $n = 7$ experiments), KCl (50 mM; $n = 7$), ionomycin (2.5 μM; $n = 3$), A23187 (10 μM; $n = 3$), [Phe(2), Orn(8)]-oxytocin (10 nM; $n = 6$), and cyclopiazonic acid (10 μM; $n = 3$), significantly stimulated 4x*CRE* activity. In contrast, forskolin stimulated 4x*CRE* activity by 20-fold. Error bars show mean ± sem. ns, not significantly different from control ($p>0.05$) (**C,D**) ATP (10 μM, panel C) and KCl (50 mM, panel D) each caused a rapid rise in [Ca^{2+}]$_i$ that was maintained for the duration of the treatment. Vertical lines show ± sem for 30 cells (each experiment was repeated 3 times with similar results). The vertical calibration bar indicates a 1.5- or 3-fold increase in R340/380 in **C** and **D**, respectively.
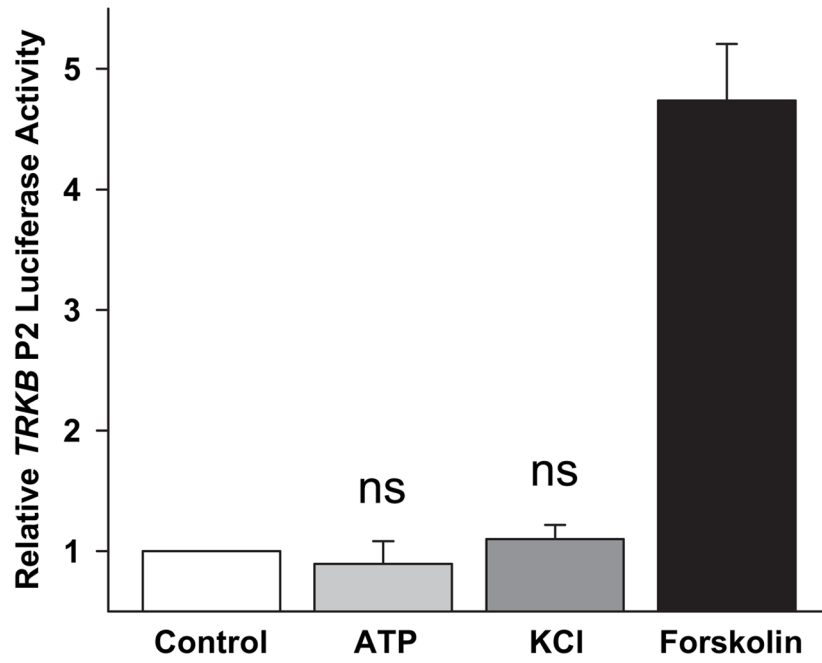
Fig. 2. *TRKB* P2 was not activated by ATP in astrocytes
ATP and KCl did not significantly activate *TRKB* P2. Error bars show mean ± sem ($n = 4$ experiments). ns, not significantly different from control ($p > 0.05$).
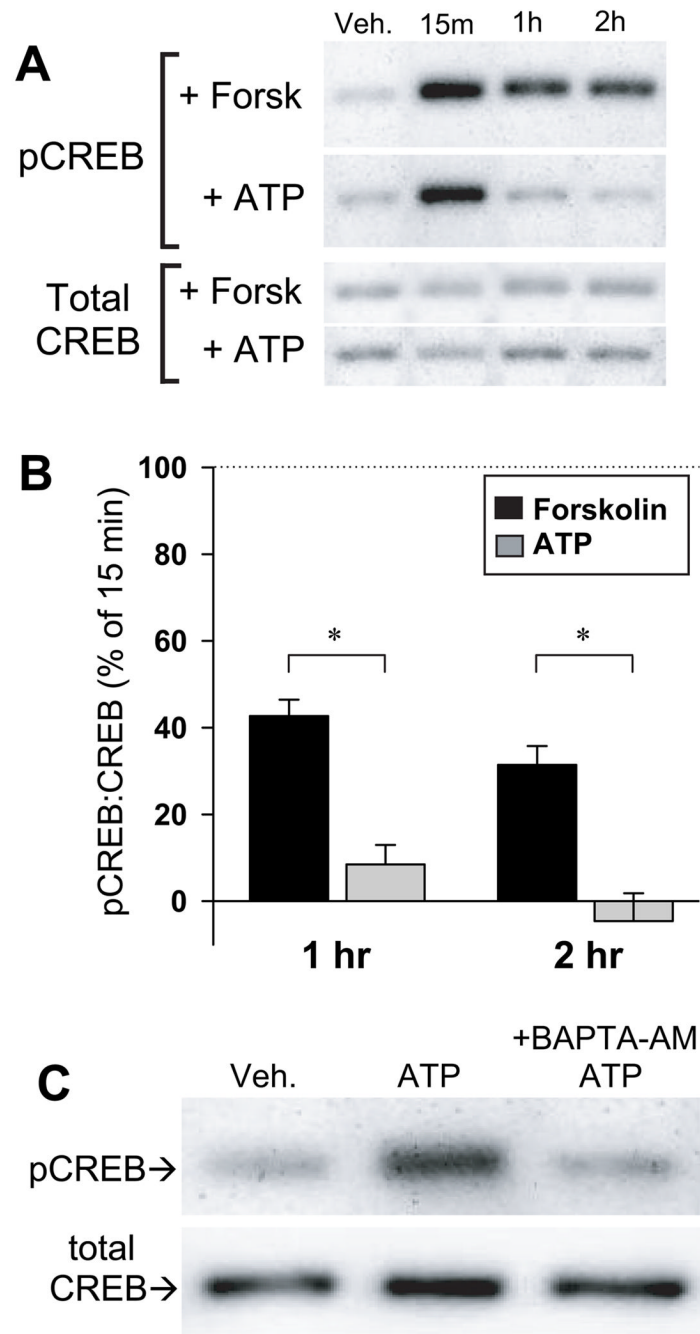
Fig. 3. ATP-induced CREB phosphorylation was transient compared to that induced by forskolin

(**A**) Western blots showing time-course of CREB phosphorylation in the presence of forskolin (50 μM) and ATP (10 μM). Blots were probed with an antibody against CREB phosphorylated at serine 133 (top), stripped, and reprobed with an antibody against total CREB (bottom). (**B**) Analysis of four experiments. For each experiment, the magnitude of the Phospho-CREB:total CREB ratio in the presence of ATP or forskolin minus the unstimulated value was set to 100%. CREB phosphorylation at 1 hr and 2 hr is expressed as a percentage of this 15 min value. Although both forskolin and ATP rapidly raise phospho-CREB within 15 min (A), forskolin induced CREB phosphorylation remained significantly

elevated up to 2 hr, whereas ATP-induced phospho-CREB levels returned to baseline levels by 1 hr. *, $p < 0.0001$ for forskolin vs. ATP at 1 hr ($n = 5$ experiments) and at 2 hrs ($n = 4$ experiments). (**C**) Western blot showing Ca^{2+}-dependence of ATP-induced CREB phosphorylation in astrocytes. Cells were pretreated for 30 min with BAPTA-AM (150 μM) and then treated with vehicle (DMSO), forskolin (50 μM) or ATP (10 μM) for 15 min as indicated.
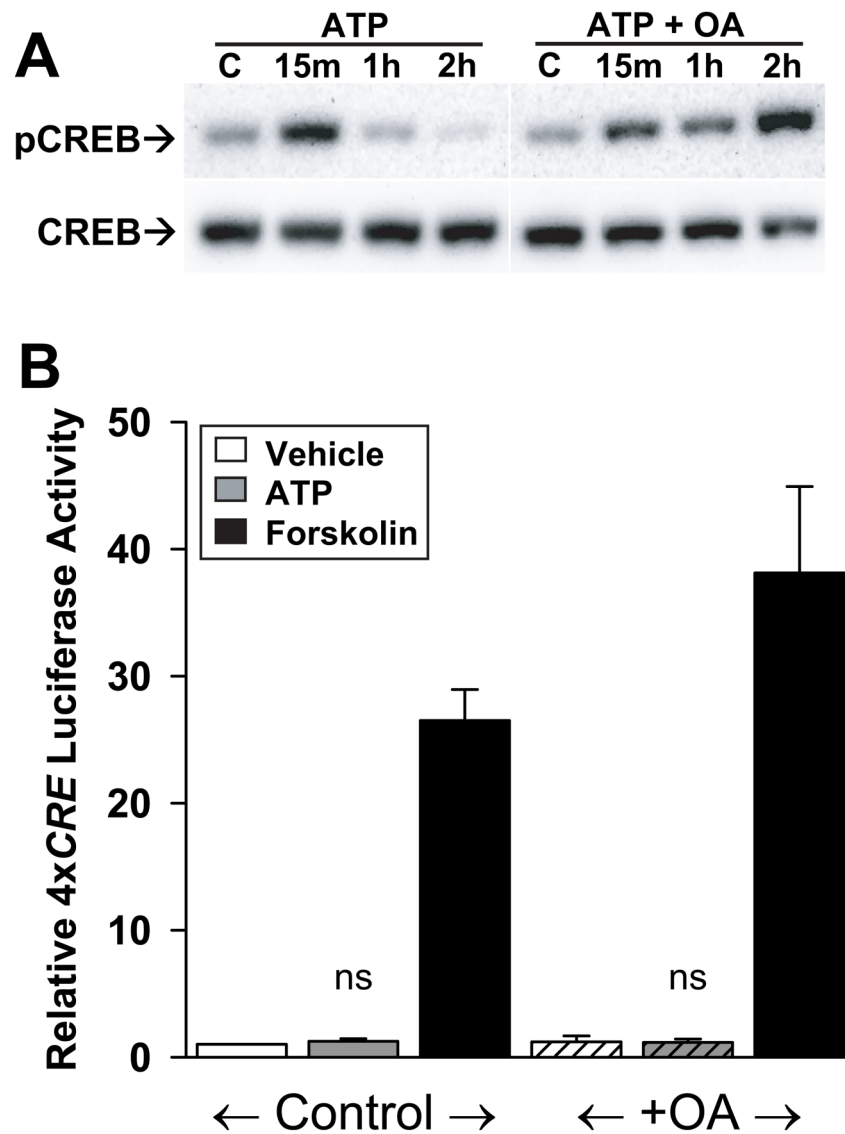
Fig. 4. Extending duration of CREB phosphorylation did not enable ATP-induced transcription
(**A**) Western blot analysis revealed that OA pretreatment prolonged ATP-induced CREB phosphorylation. Blots were analyzed as described in Fig. 3. This experiment was conducted four times with similar results. (**B**) Even after pretreatment with OA, ATP did not significantly affect 4x*CRE* activity. Error bars show mean ± sem (*n* = 6 experiments). ns, not significantly different from control (*p*>0.05).
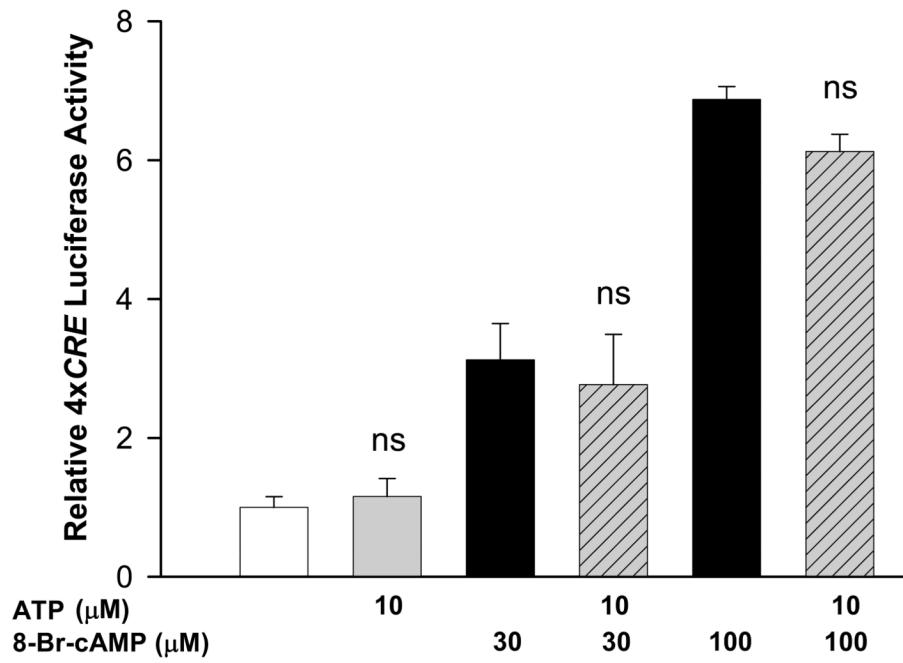
Fig. 5. ATP did not inhibit activation of 4x*CRE* by cAMP
Error bars show mean ± sem for 4 replicate determinations. ns, not significantly different
from the 4x*CRE* activity in the absence of ATP (p>0.05).
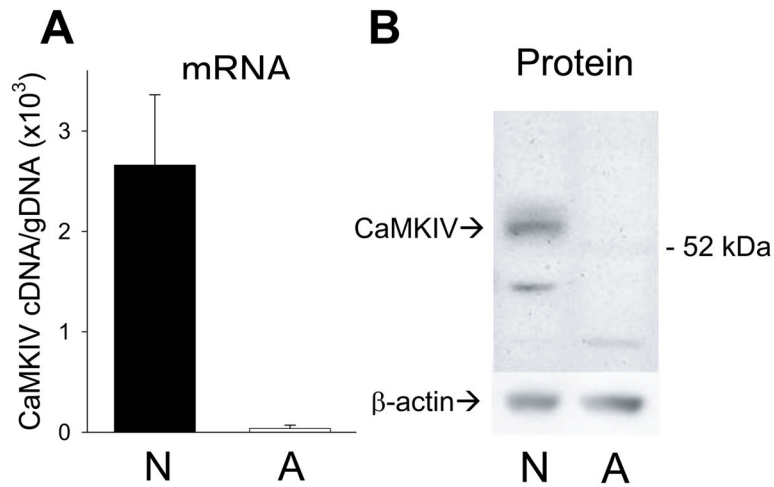
Fig. 6. Astrocytes did not express CaMKIV
CaMKIV mRNA and protein expression was analyzed in neurons (N) and astrocytes (A).
(**A**) CaMKIV RNA expression determined by Q-PCR, relative to the signal obtained when
reverse transcriptase was omitted (gDNA). Error bars show mean ± sem, $n = 4$ experiments.
(**B**) Western blot analysis of CaMKIV expression, which was detected in neurons (*left lane*)
but not in astrocytes (*right lane*). Neuronal bands at 61 and 63 kDa corresponded to the α-
and β-isoforms of CaMKIV. Blots were stripped and reprobed for β-actin. CaMKIV protein
expression in astrocytes, normalized to β-actin expression, was 3.2 ± 2.4% of that in neurons
($n = 3$ experiments).