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ATP-Stimulated c-*fos* and *zif268* mRNA Expression Is Inhibited by Chemical Hypoxia in a Rat Brain-Derived Type 2 Astrocyte Cell Line, RBA-2

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Abstract The stimulus-transcriptional coupling during ischemia/hypoxia was examined for ATP-stimulated expression of immediate early genes (IEGs; c-*fos*, *zif268*, c-*myc* and *nur77*) in a rat brain-derived type 2 astrocyte cell line, RBA-2. Incubation of cells with 1 mM of extracellular ATP stimulated time-dependent expression of c-*fos* and *zif268*. ATP induced the largest increases in *zif268* mRNA and a lesser one in c-*fos* mRNA. ATP also induced a slight increase in *nur77* mRNA but was ineffective in inducing c-*myc* expression in these cells. Brief exposure of cells to potassium cyanide to simulate chemical hypoxia induced 9-fold and 7-fold transient increases in c-*fos* and *zif268* expression, respectively, but did not affect c-*myc* or *nur77* expression. When cyanide and ATP were added together, the expression of c-*fos* and *zif268* expression was inhibited, and the effect was mimicked by simulating chemical hypoxia with sodium azide. To elucidate the mechanism involved, the effect of cyanide on ATP-stimulated increases in intracellular Ca^{2+} concentrations, $[Ca^{2+}]_i$, and phospholipase D (PLD) activities were measured. Cyanide induced an increase in $[Ca^{2\+}]_i$ and further enhanced the ATP-stimulated increases in $[Ca^{2+}]_i$ and PLD activities. Nevertheless, metabolic inhibitor, iodoacetate, blocked the ATP-induced c-*fos* and partially inhibited *zif268* expression, and deprivation of cells with glucose also inhibited the ATP-induced c-*fos* expression. Taken together, these results demonstrate that both extracellular ATP and chemical hypoxia induce c-*fos* and *zif268* expression in RBA-2 type 2 astrocytes. The chemical hypoxia inhibited ATP-stimulated c-*fos* and *zif268* expression is not due to alterations in Ca^{2+} and PLD signaling, and is at least partially related to metabolic disturbance in these cells. J. Cell. Biochem. 77:323–332, 2000.

Key words: ATP; c-*fos*; chemical hypoxia; *zif268*; IEG; type 2 astrocytes

ATP is recognized as a neurotransmitter with two large families of iontropic P2X and metabotropic P2Y purinergic receptors [Burnstock, 1997] that might mediate neuron-astrocyte interactions. In addition, astrocytes are known to play a neuroprotective role during anoxia/ischemia insult in the brain [Vibulsreth et al., 1987; Swanson and Choi, 1993; Maeda et al., 1994; Hori et al.,1996]. These studies were conducted using primary astrocyte cultures containing mostly type 1 astrocytes. Because of the low yield, information regarding the role of type 2 astrocyte during ischemia-hypoxia insult is very limited. Recently, we identified that a high concentration (1 mM) of extracellular ATP activated $P2X_7$ receptors, induced Ca^{2+} influx, and stimulated phospholipase D (PLD) in a type 2 astrocyte cell line, RBA-2 [Sun et al., 1999]. Because high concentrations of extracellular ATP could accumulate through (1) the release of cytosolic ATP via intrinsic plasma channels or pores in the absence of irreversible cytolysis, and (2) stimulation-induced release of cytosolic ATP upon sudden breakage of intact cells [Dubyak and El-Moatassim, 1993], activation of $P2X_7$ receptors may serve as a

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neuronal signal to stimulate type 2 astrocyte responses in ischemic/hypoxia brains.

The expression of immediate early genes (IEGs) such as c-*fos* has been studied extensively. It has become an important experimental model for the study of stimulus-transcription coupling because it is activated within minutes by many different stimuli in the nervous system [Morgan and Curran, 1991]. IEGs have been recognized as an intracellular signal after hypoxia [Nowak, 1993] and as one of the early events in genomic adaptations to hypoxia [Prabhakar et al., 1995]. An earlier report implied that induction of glial c-*fos* in areas in which there is neuronal loss may be important for its subsequent proliferation or for the production of growth factors [Gunn et al., 1990]. A rapid and transient induction of the c-*fos* gene was found in astrocytes during ischemia [Yu et al., 1995]. Prabhakar et al. [1995] reported hypoxia-induced cell-specific c-*fos* expression in PC12, hepatoblastoma (Heb3B), and fibroblasts, but not in neuroblastoma cells. A recent report demonstrated that the transcription factor, Egr-1, encoded by *zif268*, was required for the induction of a tissue factor gene during hypoxia [Yan et al., 1998]. Results from this heterogeneous system suggest that hypoxia-induced expression of various IEGs may involve multiple and/or overlapping mechanisms. In order to elucidate the transcription responses of RBA-2 astrocytes under hypoxic conditions, the present study examines the effects of cyanide-simulated chemical hypoxia on ATP-stimulated expression of four immediate early genes (IEGs): c-*fos*, *zif268* (NGFI-A/*nurr1*/*egr-1*/*Krox24*/TIS8), c-*myc*, and *nur77* (NGFI-B/N10/NAK1/TIS1).

Brief exposure of cells to cyanide has been used as a chemical hypoxia model system in cortical neuron cultures [Goldberg et al., 1987; Dubinsky and Rothman, 1991] and in PC12 cells [Carroll et al., 1992]. Because IEG expression is an important biochemical signal for both direct and indirect hypoxia insult, and extracellular ATP might play an important role in activating type 2 astrocytes during ischemia [Sun et al., 1999], we investigated the ATP- and cyanide-stimulated expression of c-*fos*, *zif268*, c-*myc*, and *nur77* in RBA-2 astrocytes. Our data showed that both ATP and cyanide induced c-*fos* and *zif268* expression. Nevertheless, cyanide inhibited ATP-stimulated c-*fos* and *zif268* expression. The possible biochemical mechanisms involved in the inhibition were then evaluated.

MATERIALS AND METHODS

Materials

ATP was purchased from Sigma Chemical Co. (St. Louis, MO) and phosphatidylethanol (PEt) from Biomol Research Laboratories (Plymouth Meeting, PA). Fetal bovine serum (FBS) and gentamicin were from Gibco-BRL (Gaithersburg, MD). Culture flasks and dishes were from Corning Laboratory Sciences (Corning, NY). Radioactively labeled [9,10-^{3}H]palmitic acid (spec act 51.0 Ci/mmol) was from NEN Research Products (Boston, MA). Enhanced chemiluminescence (ECL) System, Rediprime II, Hyperfilm-MP, and Nytran membranes were from Amersham Life Science (Buckinghamshire, UK). HPTLC plates (Kieselgel 60, 10 $\times$ 10 cm) and organic solvents were from Merck KGaA (Darmstadt, Germany). Medical x-ray film was from Fuji Photo Film Co., (Tokyo, Japan).

Cell Culture of RBA-2 Astrocyte Cell Line

RBA-2 type 2 astrocytes have been established and characterized as small size (15–20 μ), having stellate morphology, and expressing glial fibrillary acidic protein (GFAP), A2B5 and glutamine synthetase antigens [Sun et al., 1999]. These cells were continuously maintained in F10 medium (pH 6.2, adjusted with bicarbonate) supplemented with 10% heat-inactivated FBS and gentamycin (50 μg/ml).

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from RBA-2 cells by a single-step acid guanidium thiocyanate-phenol-chloroform extraction method [Chomczynski and Sacchi, 1987]. For Northern blot analysis, RNA samples (5–20 μg/lane) were applied to 1.2% agarose gel in the presence of 2.2 M formaldehyde. After electrophoresis, gels were transblotted onto Nytran membranes and then prehybridized at 60°C in a solution containing 1% sodium dodecyl sulfate (SDS), 1 M NaCl, 10% dextran sulfate, and 100 μg/ml of sheared salmon sperm DNA. Complementary DNA (cDNA) probes of c-*fos* [Curran et al., 1987], *nur77* [Hazel et al., 1988], c-*myc* [Land et al., 1986], *zif268* [Lbrandt, 1987], and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [Fort et al., 1985] were labeled with [^{32}P]-dCTP, using the random priming labeling system (Rediprime II). Radioactive probes (1×10^6 cpm/ml) were added directly to the prehybridization solution. After hybridization, incu-

bation for 2 h at 60°C, membranes were washed twice in 2× SSC at room temperature for 15 min each, followed by two 15-min washes at 60°C in 2× saline-sodium citrate (SSC)/1% SDS, and two 15-min washes at 60°C in 0.1× SSC. Each membrane was then exposed to Hyperfilm-MP.

Quantitative Analysis of IEG Expression

The radioactive bands of each IEG and GAPDH on the blots were quantified by a phosphorimager (Molecular Dynamics, CA). Expression of IEGs was standardized with the respective GAPDH. The results are presented as ratios of IEG/GAPDH in fold of controls. The experiment was performed three times with three separate cultures. The difference between two means was calculated by Student's *t*-test and considered statistically significant when $P \leq 0.05$.

Measurement of $[Ca^{2+}]_i$

Increases in $[Ca^{2+}]_i$ were determined by using the fluorescent Ca^{2+} indicator fura-2 methods described by Grynkiewicz et al. [1985] and Sun et al. [1999]. RBA-2 astrocytes were resuspended in culture medium at a density of 1×10^7 cells/ml and incubated with fura-2-AM (5 μM) for 30 min at 37°C. The cell suspension was then rinsed twice with serum free F10 culture medium to remove the excess fura-2-AM and resuspended in culture medium at a density of 4×10^6/ml. It was then incubated for 20 min at room temperature to hydrolyze the entrapped ester completely. The cell suspension (0.5 ml) was then washed, resuspended in 2.5 ml loading buffer, and then transferred to a 3-cm^3 cuvette positioned in the thermostat-regulated (37°C) sample chamber of a dual-excitation beam spectrofluorometer (SPEX, model CM1T111). The loading buffer consisted of 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 5 mM glucose, and 10 mM Hepes at pH 7.4.

PLD Assay

PLD activity was measured by analyzing the accumulation of phosphatidylethanol (PEt) in the presence of 300 mM ethanol as described previously [Sun et al., 1999]. In brief, RBA-2 astrocytes (1×10^6 cells/dish) were subcultured into 60-mm dishes and cultured for 2 days. Cells were then labeled with 1 μCi/ml of [9,10(n)-^{3}H]palmitic acid (spec act 51.0 Ci/mmol) in F10 media, at pH 6.2, and supplemented with 2.5% FBS for 18 h. After the labeled medium was removed, the cells were rinsed with wash buffer and cultured in 2 ml loading buffer supplemented with 300 mM ethanol in the presence of the agonist at 37° C for the lengths of time indicated. The reactions were stopped by aspiration, followed by addition of 1.3 ml ice-cold methanol. Cells were scraped from the culture dish and transferred to a borosilicate glass test tube (13 × 100 mm); the dish was scraped again after the addition of 1 ml water. Lipids were extracted by the addition of 2.7 ml chloroform, after which the sample was mixed by vortexing and centrifuging at 400*g* for 5 min to permit phase separation. The lower organic phases were transferred to new test tubes in which they were evaporated to dryness. The lipids were then redissolved in 200 μl chloroform and applied to 1% potassium oxalate pre-impregnated 10 × 10 cm Kieselgel 60 HPTLC plates (Merck, Darmstadt, Germany). Additional unlabeled PEt was also applied to each sample, and the plates were separated by a one-dimensional solvent system using the upper layer of a mixture of ethyl acetate: iso-octane: acetic acid: H_2O (65:10:10:50, by vol). After development, the plates were dried, the lipid bands were visualized by exposure to iodine vapor, and the PEt and phospholipid (PL) bands were scraped into scintillation vials for counting by scintillation spectrometry. The radioactivity of PEt was standardized as dpm PEt/100,000 dpm in PL [El-Moatassim and Dubyak, 1993].

RESULTS

ATP Stimulated c-*fos*, *zif268*, and *nur77*, But Not c-*myc* Expression, in RBA-2 Astrocytes

The cells were subcultured in 100-mm dishes for 2–3 days, treated with 1 mM ATP for 0–60 min and the expression of c-*fos*, *zif268*, c-*myc* and *nur77* were analyzed by Northern blots. As shown in Figure 1A, ATP rapidly stimulated time-dependent c-*fos*, *zif268* and *nur77* expression. An initial induction was observed at 15 min and peak levels at 45 min. Although all three IEGs were induced by ATP, there are striking differences in the levels of induction among these three IEGs. The peak levels of c-*fos*, *zif268*, and *nur77* mRNA were 27.6-, 74.4-, and 3.7-fold, as compared with the controls, respectively (Fig. 1B). ATP-induced *zif268* expression was far greater than that of

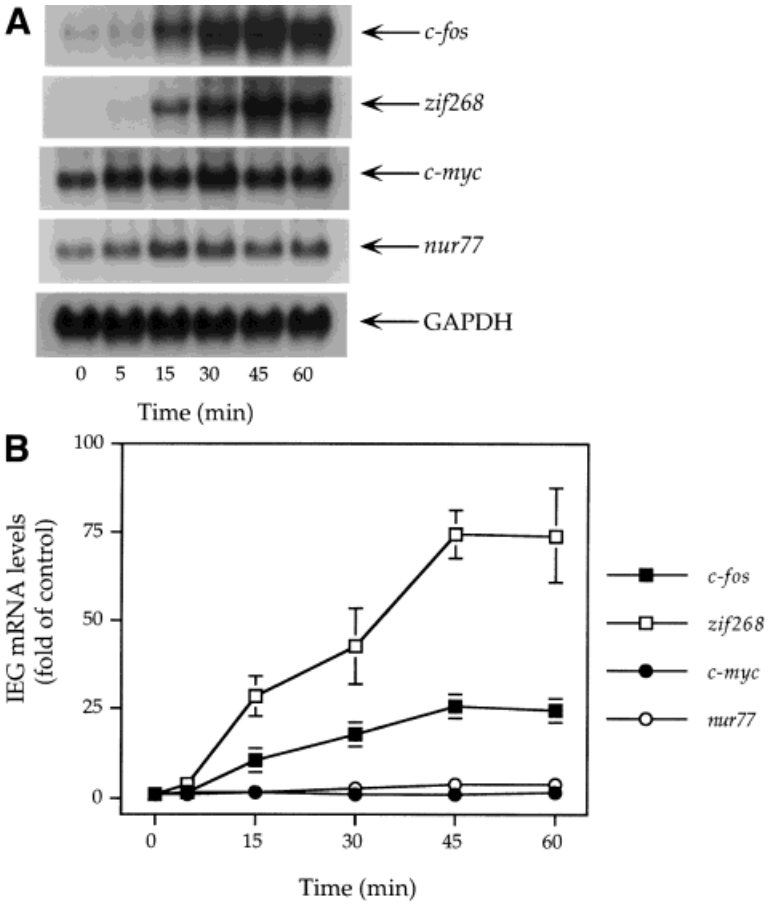

Fig. 1. Analysis of extracellular ATP-induced immediate early genes (IEGs: c-*fos*, *zif268*, c-*myc*, and *nur77*) expression. RBA-2 astrocytes were subcultured on 10-cm dishes for 2–3 days and treated with 1 mM extracellular ATP for 0–60 min. Total RNA extraction and Northern blot analyzsis for c-*fos*, *zif268*, c-*myc*, *nur77*, and GAPDH are described under Materials and Methods. After Northern blot analysis, the blot was stripped and hybridized again with a probe for GAPDH. (**A**) Autoradiographs for ATP-induced c-*fos*, *zif268*, c-*myc*, *nur77*, and GAPDH expression, and (**B**) quantitative analysis of c-*fos*, *zif268*, c-*myc*, and *nur77* were determined by phosphorimage-densitometry analysis and ratio of IEG/GAPDH calculated. Data represent fold of control of mean ± SD from three separate cultures.

c-*fos* and *nur77* expression in these cells. Basal levels of c-*myc* mRNA were high, and ATP failed to elicit an increase in c-*myc* mRNA level in these cells (Fig. 1B).

Chemical Hypoxia Enhanced c-*fos* and *zif268* Expression, But Not *nur77* or c-*myc* Expression, in RBA-2 Astrocytes

Before this study, the effect of cyanide on the viability of RBA-2 astrocytes was checked by MTT assays [Mosmann, 1983; Carmichael et al., 1987]. Our results showed that 60-min treatment of cells with 0–1.5 mM cyanide did not lead to significant cell death. Nevertheless, 0.5 mM cyanide decreased intracellular ATP concentration by 50% in these cells (data not shown). Therefore, expression of c-*fos*, *zif268*, c-*myc* and *nur77* was investigated in RBA-2 cells treated with 0.5 mM cyanide for 0–60 min (chemical hypoxia) and times after removal of treatment of cyanide for 60 min (postchemical hypoxia). As shown in Figure 2A, cyanide rapidly induced c-*fos* and *zif268* mRNA expression; significant increases were observed at 15 min with peak levels reached at 30 min. At 1 h after removal of cyanide (postchemical hypoxia), IEG levels returned to the control levels (Fig. 2A). Peak levels of cyanide-induced c-*fos* and *zif268* expression were 8.7- and 6.8-fold, compared with the individual controls, respectively. At 60 min of chemical hypoxia, the mRNA levels of c-*fos* and *zif268* remained at 3.4- and 2.8-fold compared to the individual controls, respectively (Fig. 2B). In addition, cyanide did not cause rapid induction of c-*myc* or *nur77*, but 2.2- and 1.7-fold increases were observed, respectively, at 60 min in these cells.

ATP-Stimulated c-*fos* and *zif268* Expression Was Inhibited in Chemical Hypoxia-Treated RBA-2 Astrocytes

Because both ATP and cyanide induced c-*fos* and *zif268* expression, we then investigated whether chemical hypoxia affected ATP-stimulated IEG expression in these cells. As shown in Figure 3A and 3B, 15-min treatment of cells with 0.5 mM cyanide induced 3.2- and 4.1-fold increases in c-*fos* and *zif268* expres-

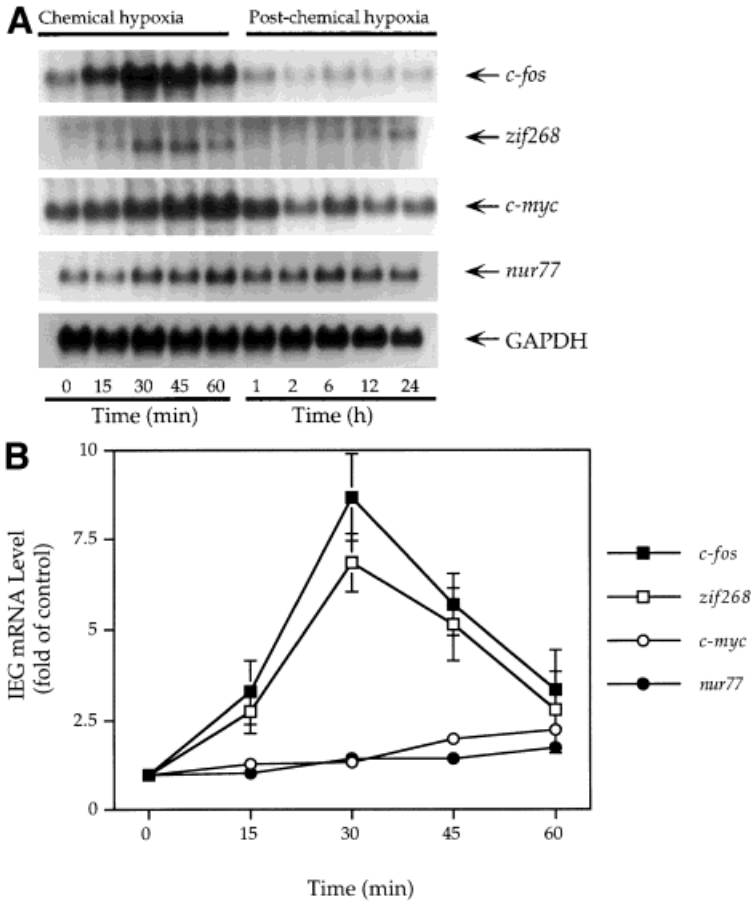

Fig. 2. Analysis of chemical hypoxia-induced immediate early genes (IEGs: c-*fos*, *zif268*, c-*myc*, and *nur77*) expression. RBA-2 astrocytes were subcultured on 10-cm dishes for 2–3 days and treated with 0.5 mM potassium cyanide to induce chemical hypoxia for 0–60 min. Total RNA extraction and Northern blot analysis for c-*fos*, *zif268*, c-*myc*, *nur77*, and GAPDH were performed as described under Materials and Methods. In the case of postchemical hypoxia, cells were treated with 0.5 mM cyanide for 1 h, medium replaced with fresh culture medium and further cultured for 1–24 h, RNA extraction and Northern blot analysis were performed. **A:** Autoradiographs for chemical hypoxia-induced and postchemical hypoxia c-*fos*, *zif268*, c-*myc*, *nur77* and GAPDH mRNA expression. **B:** Quantitative analysis of c-*fos*, *zif268*, c-*myc*, and *nur77* was determined by phosphorimage-densitometry analysis and ratio of IEG/GAPDH calculated. Data represent fold of control of mean ± SD from three separate cultures.

sion, respectively. Treating the cells with ATP for 15 min induced 8.5- and 9.7-fold increases in c-*fos* and *zif268* expression, respectively. However, when treating cells with cyanide plus ATP for 15 min, the inductions of c-*fos* and *zif268* were 2.8- and 4.7-fold of controls, respectively (Fig. 3B). Thus, ATP-stimulated c-*fos* and *zif268* expression was inhibited in the presence of cyanide.

Chemical Hypoxia Enhanced ATP-Stimulated Increases in Intracellular Ca^{2+} Concentration and Phospholipase D (PLD) Activities

To elucidate whether chemical hypoxia-inhibited ATP-induced c-*fos* and *zif268* expression was due to alterations in ATP-stimulated signal transduction pathways, we then measured the effect of cyanide on ATP-stimulated Ca^{2+} signaling and PLD activities in these cells. As shown in Figure 4A, both ATP and cyanide stimulated rapid and sustained increases in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$). The ATP-stimulated increase in $[Ca^{2+}]_i$ was more rapid and larger than that of cyanide, and cyanide further enhanced the ATP-stimulated Ca^{2+} signaling. Quantitative analysis of the net increases in $[Ca^{2+}]_i$ revealed that the cyanide- and ATP-induced net increases $[Ca^{2+}]_i$ were 115.5 ± 17.5 and 46.2 ± 8.5 nM, respectively. The ATP-plus-cyanide-induced net increases in $[Ca^{2+}]_i$ was 159.5 ± 14.7 nM (n = 3). The effect was additive, suggesting that ATP- and cyanide-stimulated increases in $[Ca^{2+}]_i$ are mediated through separate mechanisms in RBA-2 astrocytes.

In order to elucidate whether $P2X_7$ receptor-mediated signaling pathways might be altered by cyanide, PLD activities were measured in these cells. As shown in Figure 4C, ATP stimulated PLD activity, and cyanide further enhanced the ATP-stimulated PLD activity. Therefore, cyanide-inhibited ATP-stimulated c-*fos* and *zif268* expression could not be due to an alteration in the $P2X_7$ receptor-mediated signaling system. The results also suggest that the $P2X_7$ receptors remain intact in the cyanide-treated RBA-2 cells.

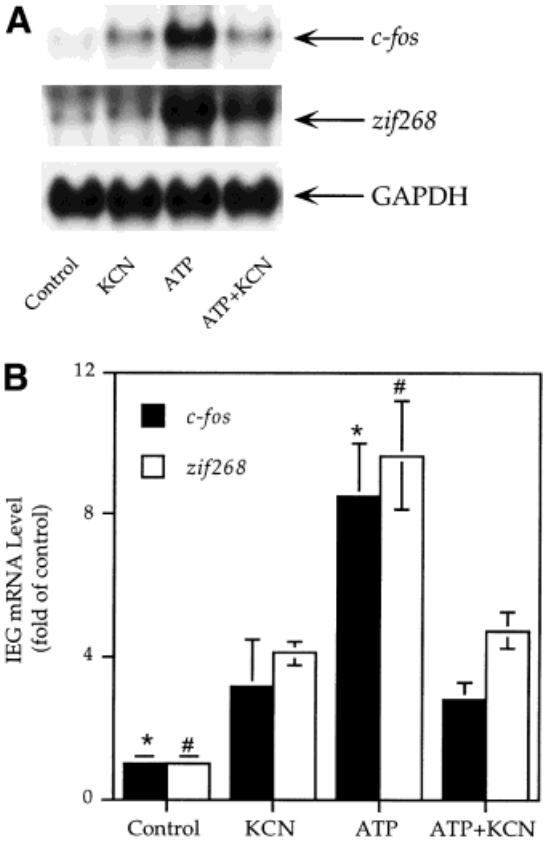

Fig. 3. Effect of chemical hypoxia on ATP-induced c-*fos* and *zif268* expression. RBA-2 astrocytes were subcultured on 10-cm dishes for 2–3 days and treated with 0.5 mM cyanide, ATP, or ATP plus cyanide for 15 min. Total RNA extraction and Northern blot analysis for c-*fos*, *zif268*, and GAPDH were as described under Materials and Methods. **A:** Autoradiographs for the effect of chemical hypoxia and ATP-induced c-*fos* and *zif268* mRNA expression. **B:** Quantitative analysis of c-*fos* and *zif268* determined by phosphorimage-densitometry analysis and ratio of IEG/GAPDH calculated. Data represent fold of control of mean ± SD from three separate cultures. *, #Significantly different means, as compared with cyanide-induced (KCN) c-*fos* or *zif268* expression by Student's *t*-test with $P \leq 0.05$, respectively.

Sodium Azide and Iodoacetate Decreased ATP-Stimulated c-*fos* and *zif268* mRNA Expression

To mimic the effect of cyanide, cells were incubated in the presence of sodium azide to inhibit mitochondrial respiration [Rose et al., 1998]. As shown in Figure 5, ATP-stimulated c-*fos* and *zif268* expression was 13- and 17-fold that of the controls, respectively. Induction of c-*fos* and *zif268* mRNA by ATP plus cyanide (ATP+KCN) and by ATP plus sodium azide (ATP+NaAzide) were 1.2- and 5.6-fold, and 1.8- and 5.1-fold, respectively, compared to that of the controls. Thus, cyanide and sodium azide caused a similar magnitude of inhibition on ATP-stimulated c-*fos* and *zif268* expression.

To elucidate the mechanism further, cells were incubated with a metabolic inhibitor, iodoacetate. As shown in Figure 5B, iodoacetate inhibited ATP-induced c-*fos* and *zif268* mRNA expression by 67% and 43%, respectively. In addition, when cells were preincubated in glucose-free medium for 6 h, ATP-induced c-*fos* and *zif268* mRNA expression were 6.1- and 16.9-fold compared with the controls. Deprivation of glucose inhibited 53% of ATP-induced c-*fos* expression but did not affect *zif268* expression in these cells. Taken together, cyanide-inhibited ATP-stimulated c-*fos* and *zif268* expression was, at least in part, due to metabolic disturbance and cell ATP depletion.

DISCUSSION

Activation of P2Y receptor has been shown to associate with induction of c-*fos*, c-*jun*, *junB*, and TIS11 in astrocytes [Priller et al., 1998], suggesting that extracellular ATP activated a mechanism or mechanisms to stimulate transcriptional responses of IEGs in astrocytes. The present study demonstrates that 1 mM extracellular ATP selectively induced a maximal of 28- and 74-fold increases in c-*fos* and *zif268* mRNA expression, respectively. Because RBA-2 astrocyte may only possess $P2X_7$ receptors [Sun et al., 1999], ATP-induced c-*fos* and *zif268* mRNA expression is likely to be mediated through activation of $P2X_7$ receptors. In addition, short-term exposure of cells to 0.5 mM cyanide to simulate chemical hypoxia also stimulated 9- and 7-fold increases in c-*fos* and *zif268* expression, respectively. Thus cyanide also activate a mechanism or mechanisms to induce the expression of these two IEGs.

It is well documented that Ca^{2+} is an important regulator for c-*fos* transcription [Sheng et al., 1988; Morgan and Curran, 1992]. ATP has been shown to activate $P2X_7$ receptors and stimulate Ca^{2+} influx in RBA-2 astrocytes [Sun et al., 1999]; therefore, the ATP-stimulated c-*fos* and *zif268* expression is probably mediated through increases in $[Ca^{2+}]_i$ or through a Ca^{2+}-dependent pathway. However, peak levels of ATP-induced *zif268* expression (74-fold) far exceed those of c-*fos* expression (28-fold). ATP only slightly induced *nur77* expression (4-fold), whereas it did not affect c-*myc* expression in these cells. The cause of this discrepancy is not known at this moment, and is prob-

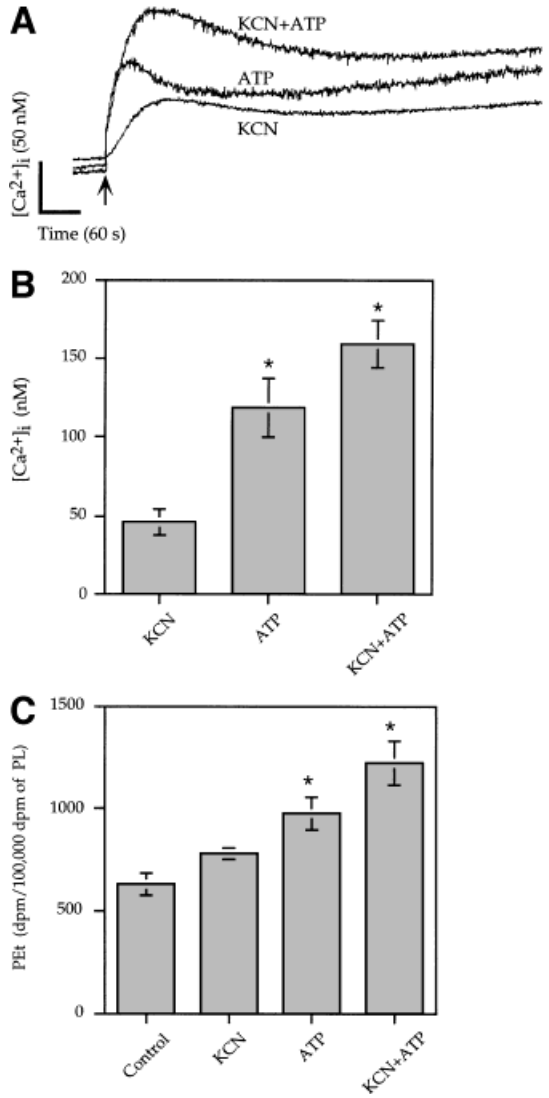

Fig. 4. Effect of chemical hypoxia on ATP-stimulated Ca^{2+} influx and phospholipase D (PLD) activities. **A:** RBA-2 astrocytes were preloaded with fura-2, rinsed, resuspended in loading buffers, and then placed inside a thermostat-regulated sample chamber. The addition of ATP (1 mM) or cyanide (0.5 mM) is indicated by an arrow; increases in $[Ca^{2+}]_i$ are shown by recording changes in fluorescences of fura-2 and fura-2-Ca^{2+} as described under Materials and Methods. The experiments were performed at least three times and results were reproducible. **B:** Quantitative analysis of ATP- and cyanide-stimulated Ca^{2+} signaling. Data represent mean ± SD of net increases in $[Ca^{2+}]_i$ from three determinations. *Significant difference of means as calculated by Student's *t*-test with $P \leq 0.05$, as compared with the controls. **C:** RBA-2 astrocytes were subcultured in 60-mm dishes (1×10^6 cells/dish), labeled with ^{3}H-palmitate for 18 h, washed, and incubated in loading buffer containing 300 mM ethanol in the presence or absence of 1 mM ATP or 0.5 mM cyanide, and incubated for 15 min at 37°C. PLD activities were assayed by measuring the accumulation of PEt and standarized with 1×10^5 dpm in phospholipid (PL) as described under Materials and Methods. Data represent the mean ± SD of dpm PEt/100,000 dpm in PL from three determinations. *Significant difference of means as calculated by nonpaired Student's *t*-test with $P \leq 0.05$, as compared with the controls.

ably due to mechanisms intrinsic to each IEG in RBA-2 astrocytes.

Because cyanide stimulated increases in $[Ca^{2+}]_i$ and induced a similar magnitude of c-*fos* and *zif268* expression, the effect of cyanide on c-*fos* and *zif268* expression was probably mediated through the same mechanism, likely, an increase in $[Ca^{2+}]_i$. It has been shown that cyanide stimulated Ca^{2+} entry through voltage-sensitive Ca^{2+} channels in nerve growth factor-differentiated PC 12 cells [Gibson et al., 1997] and ATP-stimulated Ca^{2+} influx through $P2X_7$ receptors in RBA-2 astrocytes [Sun et al., 1999]. Thus, the synergistic effect of ATP and cyanide on increases in $[Ca^{2+}]_i$ is mediated through the two separate mechanisms. This result also agrees with an earlier finding in hippocampal neurons that cyanide enhanced glutamate-stimulated increases in $[Ca^{2+}]_i$ [Dubinsky and Rothman, 1991]. Nevertheless, their results showed that cyanide-induced increases in $[Ca^{2+}]_i$ exceeded those induced by glutamate. By contrast, the ATP-induced increases in $[Ca^{2+}]_i$ exceeded those induced by cyanide in RBA-2 astrocytes. Because cyanide might affect Ca^{2+} channels [Gibson et al., 1997], the result suggests a differential distribution of these channels between neurons and RBA-2 astrocyte. Together, these results imply a differential Ca^{2+} response subsequent to neurotransmitter and chemical hypoxia stimulation between neurons and astrocytes.

The discrepancy between the magnitudes of c-*fos* and *zif268* induced by ATP may be due to a differential sensitivity to changes in $[Ca^{2+}]_i$ in RBA-2 astrocytes. Werlen et al. [1993] have reported that transcription initiation of c-*fos* and *zif268* were extremely sensitive to $[Ca^{2+}]_i$ in HL-60 myeloid leukemia cells. In addition, Enslen and Soderling [1994] demonstrated a distinct calcineurin-mediated regulation between *zif268* and *nur77* in PC12 cells. However, ATP can not induce IEG expression using a serum-free buffer system in RBA-2 astrocytes (data not shown). This finding correlates with earlier findings that elevation of intracellular Ca^{2+} alone cannot induce expression of c-*fos* [Mehment et al., 1990] or *zif268* [Jamieson et al., 1989]. Therefore, discrepancies between the magnitudes of ATP-stimulated c-*fos* and *zif268* are probably due to differential requirements for other co-factors. In the present study, cyanide did not affect *nur77* expression in RBA-2 astrocytes. Conversely, *nur77* induc-

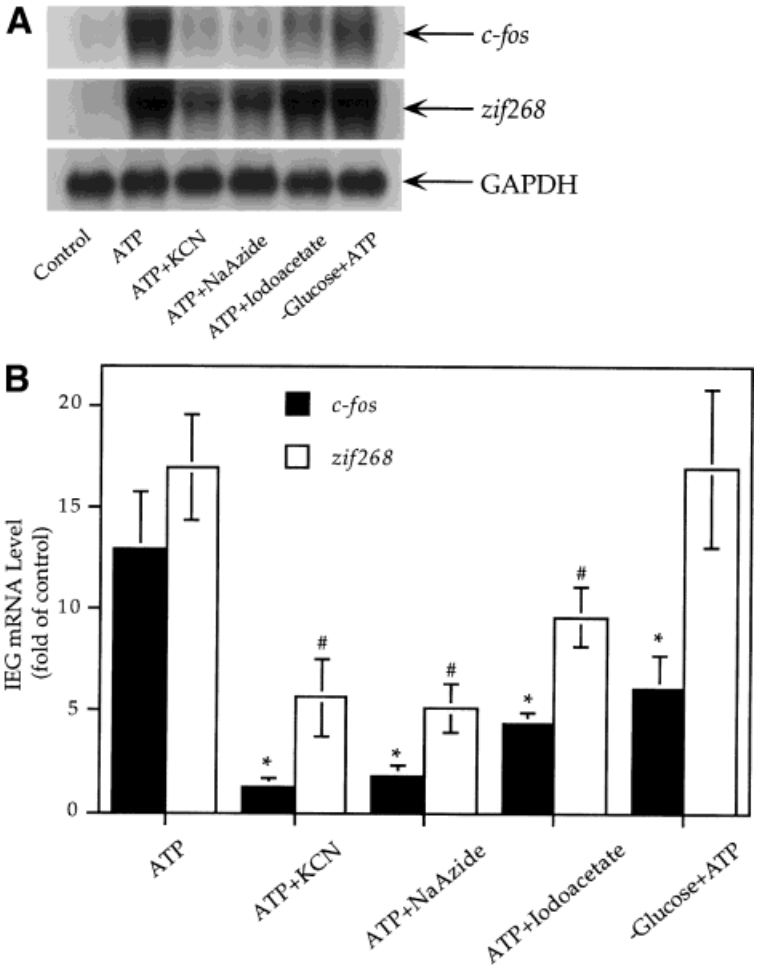

Fig. 5. Effects of metabolic inhibitors on ATP-induced c-*fos* and *zif268* expression. RBA-2 astrocytes were subcultured on 10-cm dishes for 2–3 days and treated with ATP (1 mM) in the presence or absence of 0.5 mM cyanide or 4 mM sodium azide (NaAzide) or 0.5 mM iodoacetate or cells deprived of glucose for 6 h as indicated for 15 min. In the case of iodoacetate, cells were pretreated with iodoacetate for 15 min before the addition of ATP. Total RNA extraction and Northern blot analysis for c-*fos*, *zif268*, and GAPDH were as described under Materials and Methods. **A:** Autoradiographs of c-*fos* and *zif268* mRNA expression. **B:** Quantitative analysis of c-*fos* and *zif268* expression was determined by phosphorimage-densitometry analysis and ratio of IEG/GAPDH calculated. Data represent the fold of control of mean ± SD from three separate cultures. $^{*,\#}$Significantly different means as compared with ATP-induced c-*fos* or *zif268* expression calculated by nonpaired Student's *t*-test with $P \leq 0.05$, respectively.

tion was found in cerebral cortex in acute carbon monoxide-intoxicated hypoxic mice [Tang et al., 1997]. However, ipsilateral induction of *nur77* mRNA and bilateral inductions of *zif268* were found in the focal cerebral ischemia-reperfused rat brain [Lin et al., 1996]. Thus, the induction of IEGs may be cellular specific in the brain.

Because IEGs encode transcription factors, the observed ATP- and cyanide-stimulated IEG expression may model a response of astrocytes by which transcription factors are involved in the induction of delayed genes. A rapid co-induction of c-*fos* and *zif268* has been shown in ischemic-hypoxic immature rat brain, and is associated with a delayed astrocyte response as shown by increasing GFAP expression [Gubits et al., 1993]. Hypoxia-stimulated c-*fos* is associated with induction of cytoskeleton protein genes, i.e., GFAP, vimentin, and β-actin expression in astrocytes [Yu et al., 1995]. A recent report indicated that the hypoxia-induced *zif268* expression correlated with an increase in tissue factor expression and pulmonary fibrin deposition in lung [Yan et al., 1998]. Our recent studies demonstrated that ATP stimulated transforming growth factor-β1 (TGF-β1) expression in RBA-2 astrocytes (data not shown). TGF-β1 expression has been shown to be mediated through Zif268 [Liu et al., 1996]. Therefore, the mark induction of *zif268* may implicate a functional significance in cytokine production of type 2 astrocytes. Taken together, ATP- and chemical hypoxia-induced c-*fos* and *zif268* expression may be pathophysiologically important in stimulating growth or proliferation of RBA-2 astrocytes after hypoxic insult.

In the present study, cyanide and ATP have a synergistic effect on Ca^{2+} and PLD signaling, whereas ATP-stimulated c-*fos* and *zif268* expression was inhibited by cyanide. Although the effect of cyanide on other ATP-induced signaling pathway is not known at this moment,

inhibitions being mimicked by sodium azide and by iodoacetate suggest that the effect may be associated with metabolic disturbance and cell ATP depletion in RBA-2 astrocytes. Early studies showed that 30-min treatment of PC12 cells with cyanide reduced intracellular ATP concentrations ($[ATP]_i$) by 92% [Carroll et al., 1992]. Iodoacetate inhibited $[ATP]_i$ by 40% in cortical neuron cultures [Uto et al., 1995]. Sodium azide combined with acidosis caused a near-complete cell ATP depletion in astrocytes [Swanson et al., 1997]. Furthermore, iodoacetate plus cyanide produced a marked depletion of cell ATP [Lash et al., 1996]. Cyanide decreased $[ATP]_i$ by 50% in RBA-2 astrocytes (data not shown). Thus, the cyanide-, iodoacetate-, and sodium azide-inhibited ATP-stimulated c-*fos* and *zif268* expression may be related to metabolic disturbance and cell ATP depletion in RBA-2 astrocytes. However, the differential effects between cyanide and iodoacetate suggesting that other mechanisms may be involved. Additional effects by iodoacetate and cyanide have been reported. Uto et al. [1995] demonstrated that the delayed neuronal death induced by iodoacetate and cyanide was probably mediated by free-radicals. Furthermore, iodoacetate, but not cyanide, produced marked increase in plasma membrane permeability to Na^+, K^+, Pi and small molecules such as lucifer yellow [Kempson et al., 1991], and the release of preincorporated [^{3}H]arachidonic acid [Webster et al., 1994]. In addition, cyanide induced a differential activation/translocation of protein kinase C isozymes [Huang et al., 1999]. In the present study, deprivation of cells with glucose for 6 h inhibited ATP-stimulated c-*fos* expression by 50%, whereas it did not affect *zif268* expression. Thus, induction of *zif268* may be less energy dependent and regulated by other mechanisms. In addition, astrocytes possess glycogen stores [Hamprecht and Dringen, 1995; Swanson and Choi, 1993] and removal of glucose may stimulate an increase in anaerobic glycolysis [Peuchen et al., 1996]. Therefore, the treatment may result in incomplete ATP depletion that lead to partial inhibition of c-*fos* expression. Taken together, cell ATP depletion may mediate, in part if not all, the cyanide inhibited ATP-induced c-*fos* and *zif268* expression in RBA-2 astrocytes.

In conclusion, the results showed that extracellular ATP induced large increases in c-*fos* and *zif268* mRNA expression in RBA-2 type 2 astrocytes. Chemical hypoxia simulated by cyanide also induced c-*fos* and *zif268* mRNA expression. By contrast, cyanide treatment blocked ATP-stimulated c-*fos* and *zif268* expression; the blockage may be partially related to ATP depletion, and is independent of extracellular ATP- and cyanide-stimulated increases in $[Ca^{2+}]_i$ or PLD signaling pathway. In addition, cyanide may also stimulate a separate mechanism to inhibit the ATP-induced *zif268* expression.

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