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Differential Alteration of Catecholamine Release During Chemical Hypoxia Is Correlated With Cell Toxicity and Is Blocked by Protein Kinase C Inhibitors in PC12 Cells

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Abstract Release of neurotransmitters, including dopamine and glutamate, has been implicated in hypoxia/ischemia-induced alterations in neuronal function and in subsequent tissue damage. Although extensive studies have been done on the mechanism underlying the changes in glutamate release, few have examined the mechanism that is responsible for the changes in catecholamines. Rat pheochromocytoma-12 (PC12) cells synthesize, store, and release catecholamines including DA and NE. Therefore, we used HPLC and ED to evaluate extracellular DA and NE concentrations in a medium during chemical hypoxia in PC12 cells. Chemical hypoxia produced by KCN induced differential release of DA and NE. Under normal glucose conditions, KCN induced release of NE, but not DA. Under glucose-free conditions, KCN-induced release of DA was elevated transiently, whereas the release of NE increased progressively. Under parallel conditions, KCN biphasically elevated the level of cytosolic free calcium ($[Ca^{2+}]_i$) in glucose-free DMEM, peaking at 951 ± 18 nM at 1,107 ± 151 s, followed by a new plateau level at 249 ± 24 nM sustained from 4,243 ± 466 to 5,263 ± 440 s. Cell toxicity, as measured by LDH release, was increased significantly by KCN in glucose-free DMEM but was diminished in the presence of glucose, and was correlated with DA release by chemical hypoxia. The protein kinase C (PKC) inhibitor GÖ 6976 or staurosporine inhibited KCN-induced LDH release as well as the release of NE and DA. Taken together, selective activation of DA but not NE was correlated with the LDH release by chemical hypoxia, and was diminished with GÖ 6976 or staurosporine. These results suggest that selective activation of PKC isoforms is involved in the chemical hypoxia-induced DA release, which may lead to neuronal cell toxicity. J. Cell. Biochem. 79:191–201, 2000. © 2000 Wiley-Liss, Inc.

Key words: catecholamine; chemical hypoxia; cell toxicity; protein kinase C; PC12 cells

Calcium homeostasis and neurotransmitter release have been implicated in ischemia/hypoxia-induced alterations in neuronal function and in subsequent tissue damage [Siesjo, 1988]. Selective alteration of neurotransmitter release by low oxygen availability has been reported in neuronal cells and synaptosomes [Freeman et al., 1987; Gibson et al., 1989; Donnelly, 1993]. A reduction in oxygen tensions selectively altered the calcium-dependent efflux of these neurotransmitters, but did not change their calcium-independent release [Hirsch and Gibson, 1984; Freeman et al., 1987]. However, controversial results have been obtained: the release of acetylcholine was decreased [Freeman et al., 1987; Gibson and Peterson, 1981], or NE [Kumar et al., 1998], glutamate, and DA were increased, or NE and serotonin were not altered [Freeman et al., 1987]. It is known that the extracellular accumulation of glutamate during anoxia/ischemia is responsible for initiating neuronal injury.

Abbreviations used: cPKC, conventional protein kinase C; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ED, electrochemical detection; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glyco-bis (b-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; FBS, fetal bovine serum; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-(2-ethansulfonic acid); HPLC, high-performance liquid chromatography; KCN, potassium cyanide; LDH, lactate dehydrogenase; NE, norepinephrine; SOS, 1-octanesulfonate; THF, tetrahydrofuran.

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Little information is available on the release of catecholamines and whether the mechanism of their release resembles that of glutamate, which may itself influence the release of monoamines by activating presynaptic receptors [Santos et al., 1996].

Intracellular signals have been known to regulate neurotransmitter release [Meldolesi et al., 1988]. Hydrolysis of phosphoinositide produces Ins (1,4,5)P_3 and diacylglycerol; the former stimulates calcium release from the endoplasmic reticulum [Berridge, 1987], whereas the latter activates protein kinase C (PKC) via translocation from cytosol to particular fractions [Nishizuka, 1988], which results in the release of neurotransmitters [Robinson, 1991; Hens et al., 1995; Goodall et al., 1997]. Chemical hypoxia by KCN was used to inhibit oxygen utilization by cytochrome oxidase, and glucose was removed from the incubation media to inhibit glycolytic production of ATP [Gibson and Huang, 1992]. In our previous studies, during chemical hypoxia, the phosphoinositide cascade was initially activated, then declined after prolonged chemical hypoxia in synaptosomes [Huang and Gibson, 1989], whereas $[Ca^{2+}]_i$ increased in synaptosomes [Gibson et al., 1991; Huang et al., 1994] and in PC12 cells [Johnson et al., 1987b; Carrol et al., 1992; Huang et al., 1999]. The effects of histotoxic hypoxia on $[Ca^{2+}]_i$ paralleled those on glutamate and DA release [Ahnert-Hilger et al., 1987; Gibson et al., 1989]. Furthermore, protein kinase involvement has been suggested in the enhanced release of NE during hypoxia [Ahnert-Hilger et al., 1987; Kumar et al., 1998], but the types of protein kinases that are associated with hypoxia-induced catecholamine release remain unclear. Rat pheochromocytoma-12 (PC12) cells synthesize, store, and release catecholamines including DA and NE, providing a good model to study the mechanism of the release of these neurotransmitters. We previously observed that selective PKCγ isozyme was activated after KCN exposure in PC12 cells [Huang et al., 1999]; therefore, the involvement of PKC in hypoxia/ischemia-induced catecholamine release was examined in PC12 cells in the present study. Catecholamines including DA and NE were determined using HPLC/ED. Whether the DA and NE releases were associated with neuronal death during chemical hypoxia was also investigated.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from the following companies: DMEM and FBS from GIBCO (Grand Island, NY); horse serum from Hyclone (Logan, UT); GÖ6976 and staurosporine from Calbiochem (San Diego, CA); aprotinin, DA, EGTA, EDTA, SOS, leupeptin, HEPES, NE, phenyl-methylsulfonyl fluoride, and mercaptoethanol from Sigma (St. Louis, MO). HPLC grade acetonitrite (CH_3CN), THF, KCN, and NaOH were purchased from E. Merck (Darmstadt, Germany), and LDH standard and LDH kits from Boehringer Mannheim (Mannheim, Germany). Chloroacetic acid ($CH_2ClCOOH$) was purchased from BDH Laboratory Supplies (England). Unless otherwise stated, all reagents were of analytical quality.

Cell Cultures

Rat pheochromocytoma cells (PC12) were grown in NUNC T75 flasks (Naperville, IL) in DMEM supplemented with 5% horse serum and 10% FBS, and were maintained in a 10% CO_2 humidified incubator at 37°C as previously described [Huang et al., 1998, 1999]. The cells were subcultured and grown on 12-well plates, 3.5-cm Petri dishes for 2 days before the experiments.

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

$[Ca^{2+}]_i$ was determined as described previously [Huang et al., 1998]. Briefly, cells on polylysine-coated glass coverslips were loaded with 2 μM fura-2AM at 37°C for 30 min, then they were rinsed with HEPES buffer. The cells on the coverslips were then transferred to a microchamber on the stage of an inverted Olympus microscope IX-70 (Hatagaya Shibuya-Ku, Tokyo, Japan) and viewed under bright light and UV illumination via a 40× oil immersion fluorescence objective. $[Ca^{2+}]_i$ was monitored for 1 min to obtain the basal level. Then, KCN (0.5 mM) was added and the $[Ca^{2+}]_i$ was monitored by alternating excitation wavelengths of between 340 and 380 nm with emission at 510 nm using a Delta Scan System (Photon Technology International; Princeton, NJ) and was calculated using the method of Grynikewicz et al. [1985].

Measurement of LDH

Cells were grown on 24-well plates for 2 days, washed with DMEM-HEPES, and treated with KCN (0.5 mM) for the various indicated times. After incubation, the cultured media were collected and centrifuged at 10,000*g* for 5 min. Fifty microliters of the supernatants were transferred to microtiter plates and the activity of LDH released into the medium was measured at 492 nm at a reference wavelength of 630 nm using a spectrophotometric plate reader (MRX, Dynatech, Chantilly, VA) as previously described [Huang et al., 1998].

Catecholamine (DA and NE) Measurements

Catecholamine measurements have been described previously [Cheng et al., 1996, 2000]. Dialysis probes were perfused with Ringer's solution at 1 μl/min using a CMA/100 microinfusion pump. After 2 h of basal level collection, KCN (final concentration 0.5 mM) was added and the dialysates were either subjected to an on-line HPLC system or collected (in 5 μl of 0.1M HCl containing 10^{-7} M ascorbic acid to preserve biogenic amines) in a CMA/140 fraction collector then analyzed for extracellular DA and NE every 30 min. The HPLC/ED comprised a Microtech Ultra Plus pump and pump controller (Micro-tech Scientific, Sunnyvale, CA), a CMA 260 degasser, two BAS-4C electrochemical detectors (ED), a Beckman I/O 406 dual-channel interface (AI-406), and Beckman System Gold Data Analysis Software (Beckman Instrument Inc., Taiwan Branch), and a microprobe reversed-phase column filled with Inertial ODS-2 (GSK-C18, 5-μm ODS, 1.0 × 150 mm I.D., GL Sciences Inc., Tokyo, Japan). Potentials for the anodic and cathodic glassy carbon working electrodes were held independently by two amperometric detectors at +0.75 V and +0.05 V, with respect to an Ag/AgCl reference electrode. The chromatograms of the redox reactions were simultaneously recorded on the AI-406, and analyzed via the Beckman System Gold Data Analysis Software. To increase the detection sensitivity of the HPLC system, a very thin spacer (16 μm) was used instead of a conventional one (51 μm) to create a submicroliter thin-layer electrochemical cell, and very slow flow rates were used to minimize pulse fluctuations.

RESULTS

Catecholamine Release With Hypoxia and /or a Lack of Glucose

Because DA and NE are the major catecholamines in PC-12 cells, temporal responses in the release of DA and NE by chemical hypoxia (i.e., 0.5 mM KCN) were examined in glucose-present or glucose-free media. KCN induced differential temporal patterns in the release of DA and NE. As shown in Figure 1, under the normal glucose condition, KCN increased NE release gradually over time to a level of 10 ng/ml after treatment for 5 h, whereas DA release was small but detectable, ranging between 0.3 and 0.5 ng/ml. Thus, under the normal glucose condition, chemical hypoxia induced release of NE but not DA. On the other hand, the combination of a lack of glucose and diminished oxygen availability increased DA release transiently to a peak of 5 ng/ml after 30 min of KCN treatment, but the release of NE increased progressively to a level of 15 ng/ ml after treatment for 5 h (Fig. 2). Control media did not affect the level of NE or DA either under a glucose-present (data not shown) or a glucose-free condition (Fig. 2).

Effects of Chemical Hypoxia on $[Ca^{2+}]_i$

Previous studies showed that in the presence of glucose, KCN at 0.5 mM did not increase $[Ca^{2+}]_i$, whereas in the absence of glucose, KCN gradually increased the $[Ca^{2+}]_i$ up to 20 min in PC12 cells [Huang et al., 1999]. In the present study, we further monitored the complete profile of Ca^{2+} response to 0.5 mM KCN in the presence or absence of glucose for up to 2 h. KCN gradually increased $[Ca^{2+}]_i$, peaking at 95 ± 18 nM at 1,107 ± 151 s, followed by a new plateau level at 249 ± 24 nM sustained from 4,243 ± 466 to 5,263 ± 440 s (Fig. 3). Conversely, the initial peak but not the new plateau level of Ca^{2+} by KCN was diminished in the presence of glucose. As shown in Figure 3, although KCN in the glucose-present condition did not increase the initial peak of Ca^{2+}, similar to the glucose-free condition it still induced a prolonged duration of Ca^{2+} at 181 ± 134 nM, sustained from 60 to 110 min.

Effects of Chemical Hypoxia on LDH

To assess whether the increase in DA or NE during chemical hypoxia was secondary to cell injury, we monitored the LDH activity of PC12

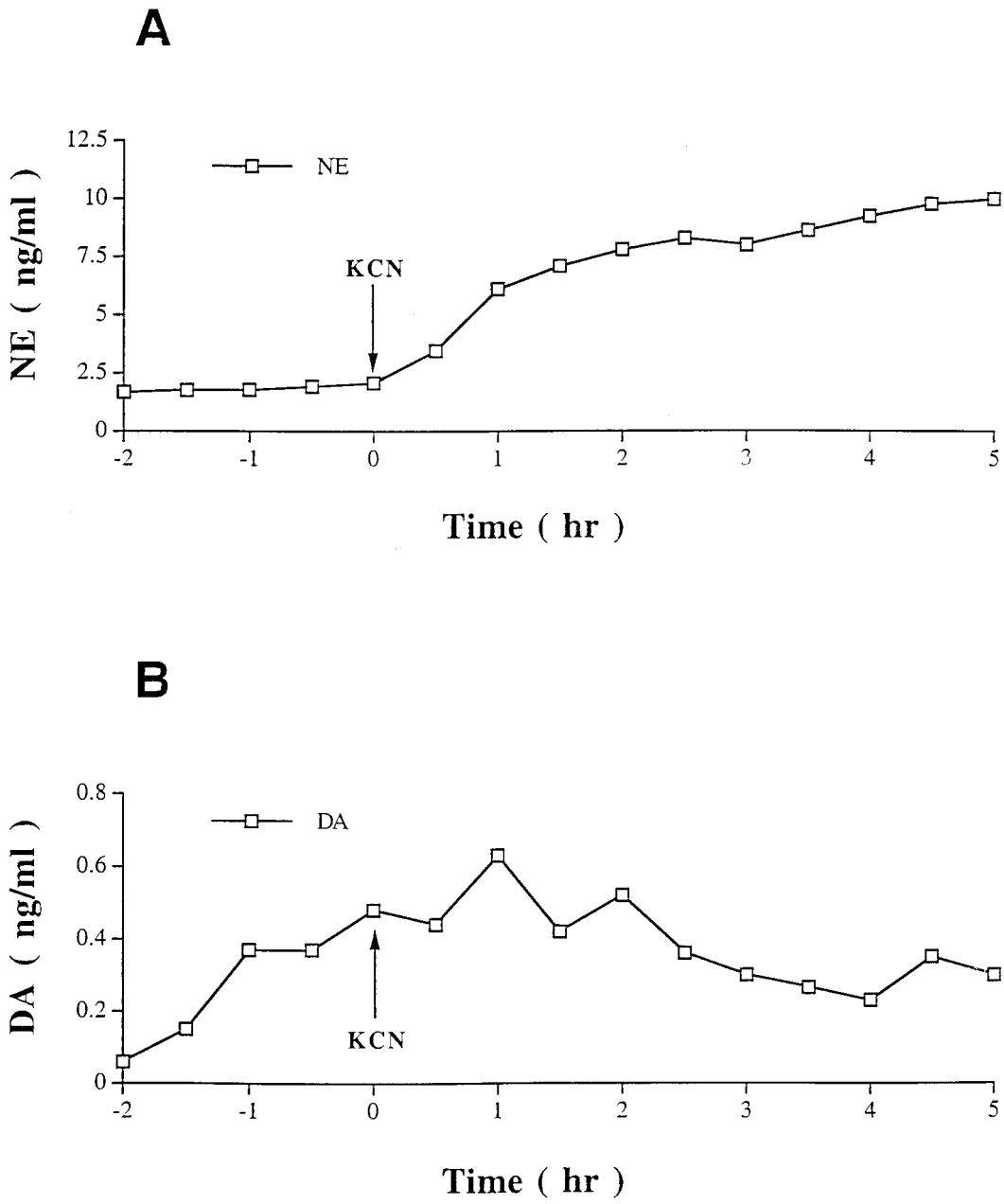

Fig. 1. NE and DA release with chemical hypoxia in the presence of glucose. The cells were treated with normal DMEM. After an equilibration period of 2 h to establish a basal level, potassium cyanide (KCN; final concentration 0.5 mM) or control medium (data not shown) was added, and the dialysates were subjected to either an on-line HPLC system or collected (in 5 μl of 0.1M HCl containing 10^{-7} M ascorbic acid to preserve biogenic amides) in a CMA/140 fraction collector and then were analyzed for extracellular NE (**A**) and DA (**B**) every 30 min. This experiment was replicated with similar results.

cells. Toxicity of KCN treatment to the cells was assessed by LDH release into the culture medium. Because the LDH release by KCN was not evident in the presence of glucose [Huang et al., 1999], the subsequent experiments were performed in glucose-free medium. The LDH release by KCN was not evident at 30 min, but increased significantly by 60 min in glucose-free DMEM (Fig. 4), and it occurred later than DA release during chemical hypoxia (30 min). In the presence of glucose, NE but not DA nor LDH was released by KCN treatment;

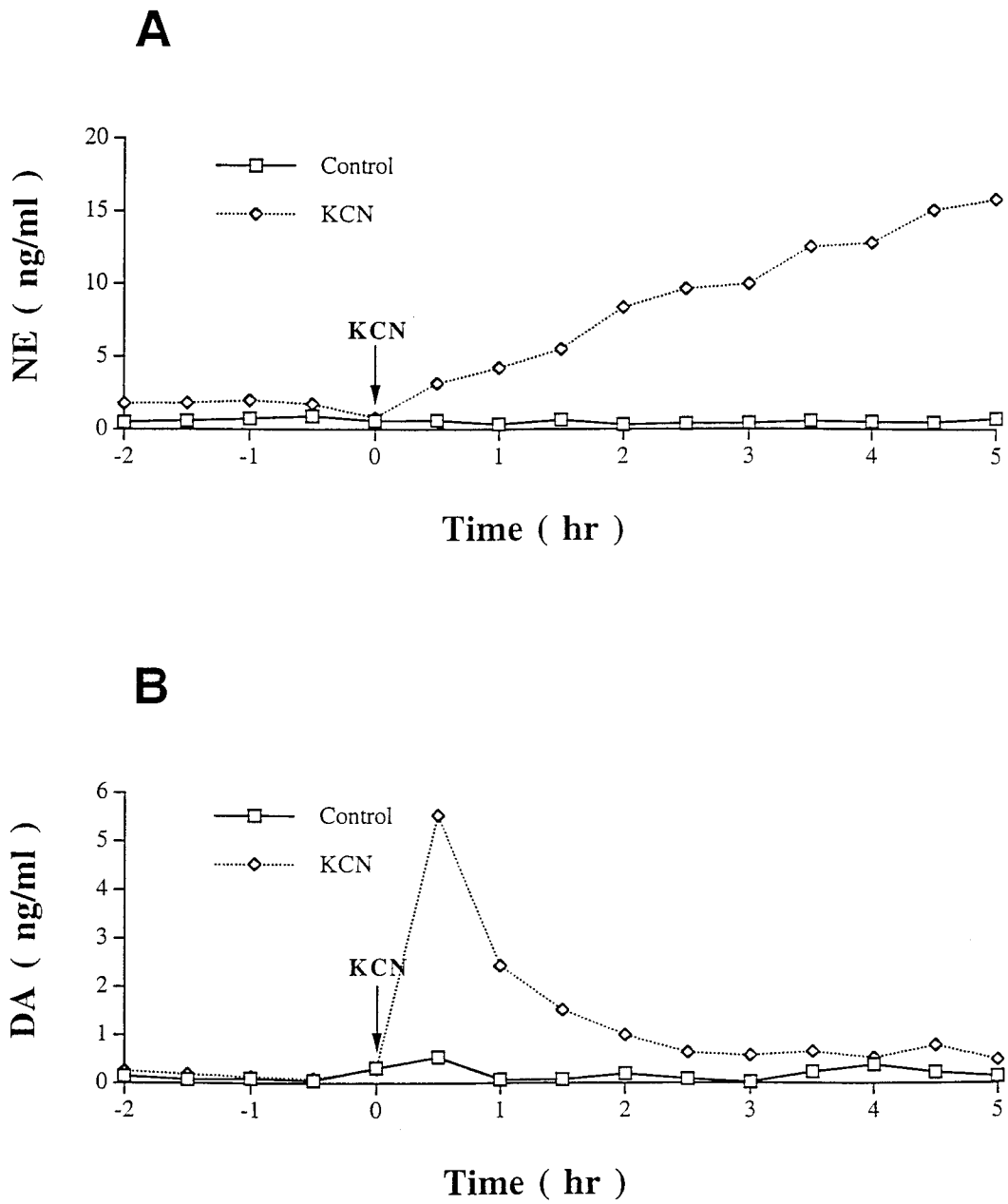

Fig. 2. NE and DA release with chemical hypoxia in the absence of glucose. The cells were treated with glucose-free DMEM. After an equilibration period of 2 h to establish a basal level, potassium cyanide (KCN; final concentration 0.5 mM) or a control medium was added and the dialysates were subjected to either an on-line HPLC system or collected (in 5 μl of 0.1M HCl containing 10^{-7} M ascorbic acid to preserve biogenic amides) in a CMA/140 fraction collector and then were analyzed for extracellular NE (**A**) and DA (**B**) every 30 min. This experiment was replicated with similar results.

thus, the release of LDH was not parallel with that of NE by KCN.

Influence of PKC in Hypoxia-Induced Release of Catecholamine and LDH

Our previous studies have shown that the PKCγ isoform is selectively activated during chemical hypoxia [Huang et al., 1999]; therefore, we assessed the role of PKC in hypoxia-induced catecholamine release and cell toxicity. The potent broad-spectrum PKC inhibitor staurosporine or the more selective conventional cPKC isozyme inhibitor GÖ6976 [for Ca^{2+}-dependent PKC (α,β,γ)] was used to ex-

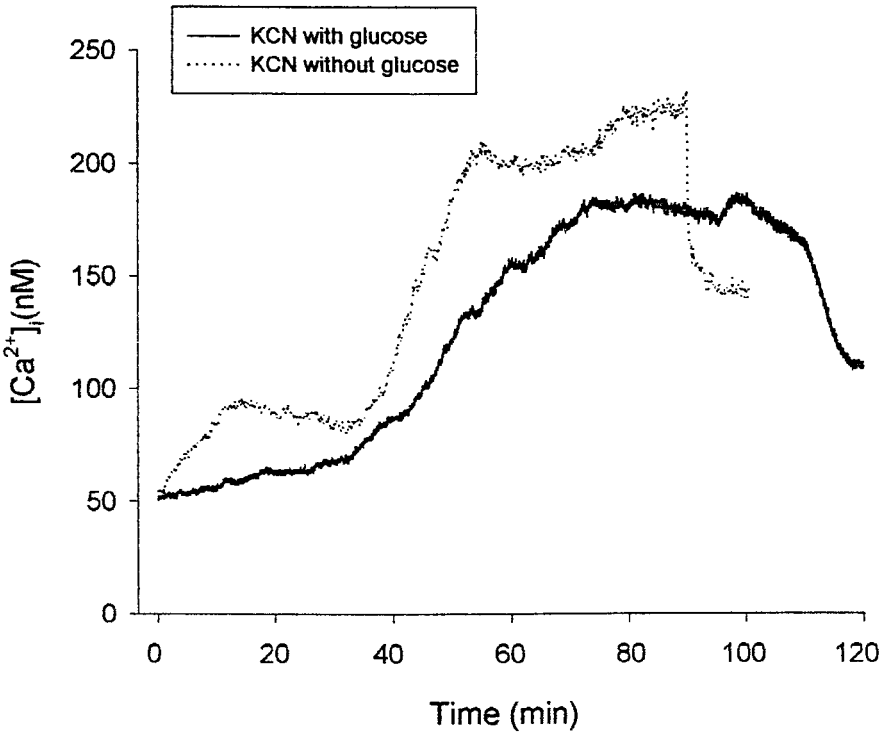

Fig. 3. Effect of KCN on $[Ca^{2+}]_i$. On the day of the experiment, the medium was removed and the resting levels of $[Ca^{2+}]_i$ were recorded for 60 s, then the cells in the absence of glucose were treated with KCN (0.5 mM), and $[Ca^{2+}]_i$ levels were recorded for 2 h. The tracings are averages from four samples (glucose-free) or two samples (glucose-present), and separate experiments produced similar results.

amine the relevance of PKCs to catecholamine release and cell toxicity during chemical hypoxia. GÖ6976 or staurosporine alone at the concentrations that we used did not affect the LDH release, whereas they dose-dependently attenuated KCN-induced LDH release. As shown in Figure 5, in glucose-free DMEM, GÖ6976 at concentrations as low as 1 nM partially inhibited and at 10 nM completely inhibited KCN-induced LDH release, and staurosporine at a higher concentration (1 μM) inhibited KCN's toxicity. However, at such concentrations, GÖ6976 or staurosporine did not increase LDH release in the medium significantly (Fig. 5).

Because KCN induced the release of DA and LDH, and this only occurred in the absence of glucose, this condition was adapted for the subsequent experiment. The release of DA and NE during chemical hypoxia was examined in the presence or absence of GÖ6976 or staurosporine at the concentrations that were effective in reducing LDH (Fig. 5). The results are shown in Figure 6, with both GÖ6976 at 10 nM and staurosporine at 100 nM effectively inhibiting the release of DA and NE by KCN, and parallel with the diminishing release of LDH (Fig. 5). This demonstrates that PKC is involved in the DA and NE release during chemical hypoxia. Taking the results together, in the absence of glucose, the time of DA release by KCN (i.e., 30 min) occurred before that of LDH (i.e., 60 min), and staurosporine or GÖ6976 inhibited DA release by KCN dose dependently, suggesting that the release of DA may lead to the neuronal cell injury.

DISCUSSION

The present study demonstrated the existence of differential temporal patterns in the release of DA and NE during chemical hypoxia in PC12 cells. In the presence of glucose, KCN induced the release of NE but not DA. However, in the absence of glucose, KCN induced the release of DA transiently, while NE increased progressively, and the release of LDH increased time dependently. The correlation of the release of LDH with that of DA by chemical hypoxia under glucose-free conditions was mediated by the activation of cPKC.

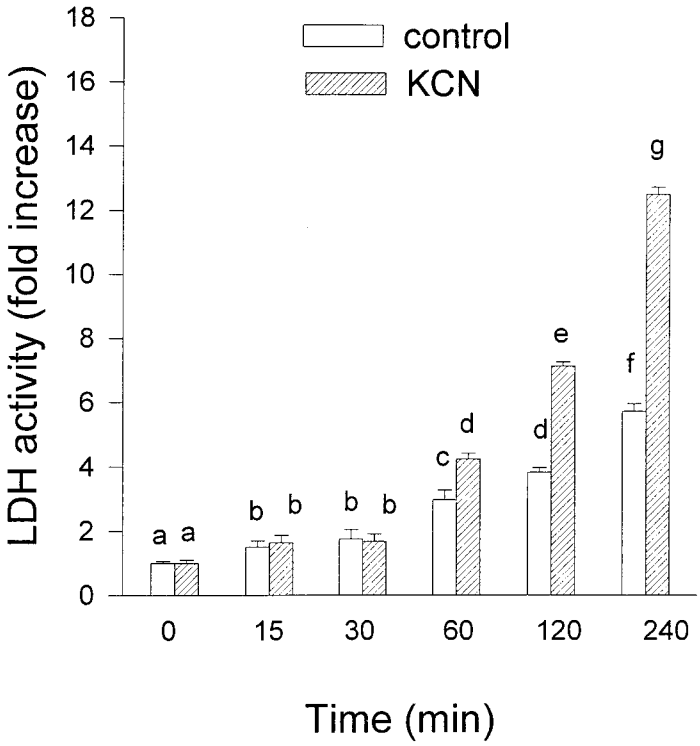

Fig. 4. Effect of KCN on the activity of LDH in PC12 cells. The cells were treated with KCN (0.5 mM) in the absence of glucose for 0, 15, 30, 60, 120, and 240 min, and the release of LDH from the cell culture medium was determined. Data are the mean ± SEM from two to four separate experiments each performed in four dishes (n = 8–16). Values in each group with different letters (a, b, c, d, e, f, g) varied significantly ($P < 0.05$) by analysis of variance followed by the Student–Newman–Keul's test.

DA was released transiently and then diminished during chemical hypoxia in the presence of glucose, whereas NE was released progressively during chemical hypoxia and decreased 50% in the presence of glucose. However, the mechanism for the differential release of DA and NE was not clear. It may have been because of their different sensitivities to the ATP level, since previous studies have shown that ATP concentrations in a glucose-free medium are reduced severely, i.e., 80% or 90% by 0.5 mM KCN in PC12 cells [Carrol et al., 1992; Huang et al., 1993]. Therefore, DA release may be more sensitive than NE to the glucose or ATP level during chemical hypoxia. It has been reported that release of noradrenaline, dopamine, 5-hydroxytryptamine, and glutamate correlates well with ATP depletion in anoxic and ischemic conditions [Santos et al., 1996]. Alternatively, $[Ca^{2+}]_i$ increased progressively with the duration of chemical hypoxia in the absence of glucose (Fig. 3). The difference in the release of DA and NE may have been because of their different sensitivities to Ca^{2+}. In the absence of glucose, the initial peak of Ca^{2+} was correlated with the transient increase in DA release by KCN (Figs. 2 and 3), whereas in the presence of glucose, the initial increases of Ca^{2+} as well as DA (Figs. 1 and 3) by KCN were diminished. Furthermore, NE levels can be regulated by reuptake [Uchida et al., 1998]. Lack of reuptake of DA in PC12 cells [Greene and Rein, 1977] may explain the transient increase of this neurotransmitter by KCN. Additionally, NE and DA are stored in separate vesicles, and they may be regulated by independent mechanisms [Kumar et al., 1998].

Changes in the activation of cPKC with hypoxia may lead to hypoxia-induced release of catecholamines, as supported by two lines of evidence. Staurosporine has been shown to inhibit many different protein kinases [Herbert et al., 1990] and induces apoptosis [Kruman et al., 1998]. Cells pretreated with staurosporine (100 nM) or a cPKC inhibitor GÖ6976 (10 nM) completely inhibited KCN-induced release of DA and NE (Fig. 6), suggesting that cPKC was involved in this process, in agreement with the results of Kumar et al. [1998]. In addition, our previous studies showed that selective translocation of specific protein kinase C isoforms occurs during chemical hypoxia, with only Ca^{2+}-dependent PKC-γ and not the other PKC isozymes -α, -δ, or -ϵ increasing significantly in the membrane and nuclear fractions after KCN treatment of PC12 cells [Huang et al., 1999].

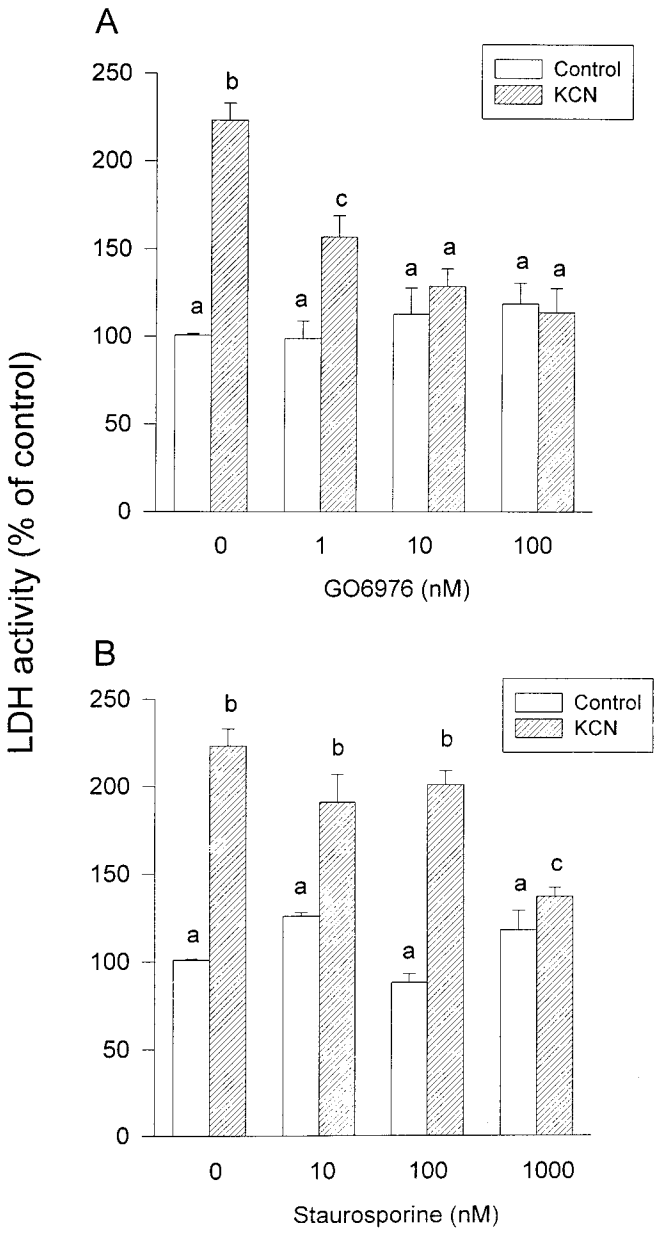

Fig. 5. (**A**) Effects of the PKC inhibitors GÖ6976 and staurosporine (**B**) on the KCN-induced release of LDH in PC12 cells. On the day of the experiment, the culture medium was removed and the cells were treated with glucose-free DMEM with PKC inhibitor GÖ6976 (1–100 nM) or staurosporine (10–1,000 nM) plus KCN (0.5 mM) for 120 min. The release of LDH from the cells into the cultured media was determined. Data are the mean ± SEM from three separate experiments each performed in four dishes (n = 12). Values in each group with different letters (a, b, c) varied significantly ($P < 0.05$) by analysis of variance followed by the Student–Newman–Keul's test.

KCN gradually increased $[Ca^{2+}]_i$ from a basal level of 51 nM to a peak level at 95 nM by 20 min (n = 4), followed by a decline in level within 40 min, then an increase to a new plateau level that was sustained from 60 to 90 min (Fig. 4). Therefore, we proposed that hypoxic stress in these cells activates signal transduction pathways, possibly involving calcium influx from an extracellular source, which results in the induction of PKCγ [Huang et al., 1999]. These results were consistent with those of the others in that cyanide induced PKC translocation [Rathinavelu et al., 1994; Yamaoka et al., 1993], and transient ischemia induces PKCδ activation [Miettinen et al., 1996]. Cyanide-induced catecholamine release is Ca^{2+} mediated and is involved in neurotoxicity [Kanthasamy et al., 1991]. In addition, other Ca^{2+}-dependent kinases or calmodulin-dependent kinases have been reported to enhance Ca^{2+}-dependent noradrenaline uptake in PC12 cells [Uchida et al., 1998], which may also contribute to the increase in extracellular catecholamine concentration during chemical hypoxia. Acute hypoxia caused a dose-dependent stimulation of DA release that was inhibited by ni-

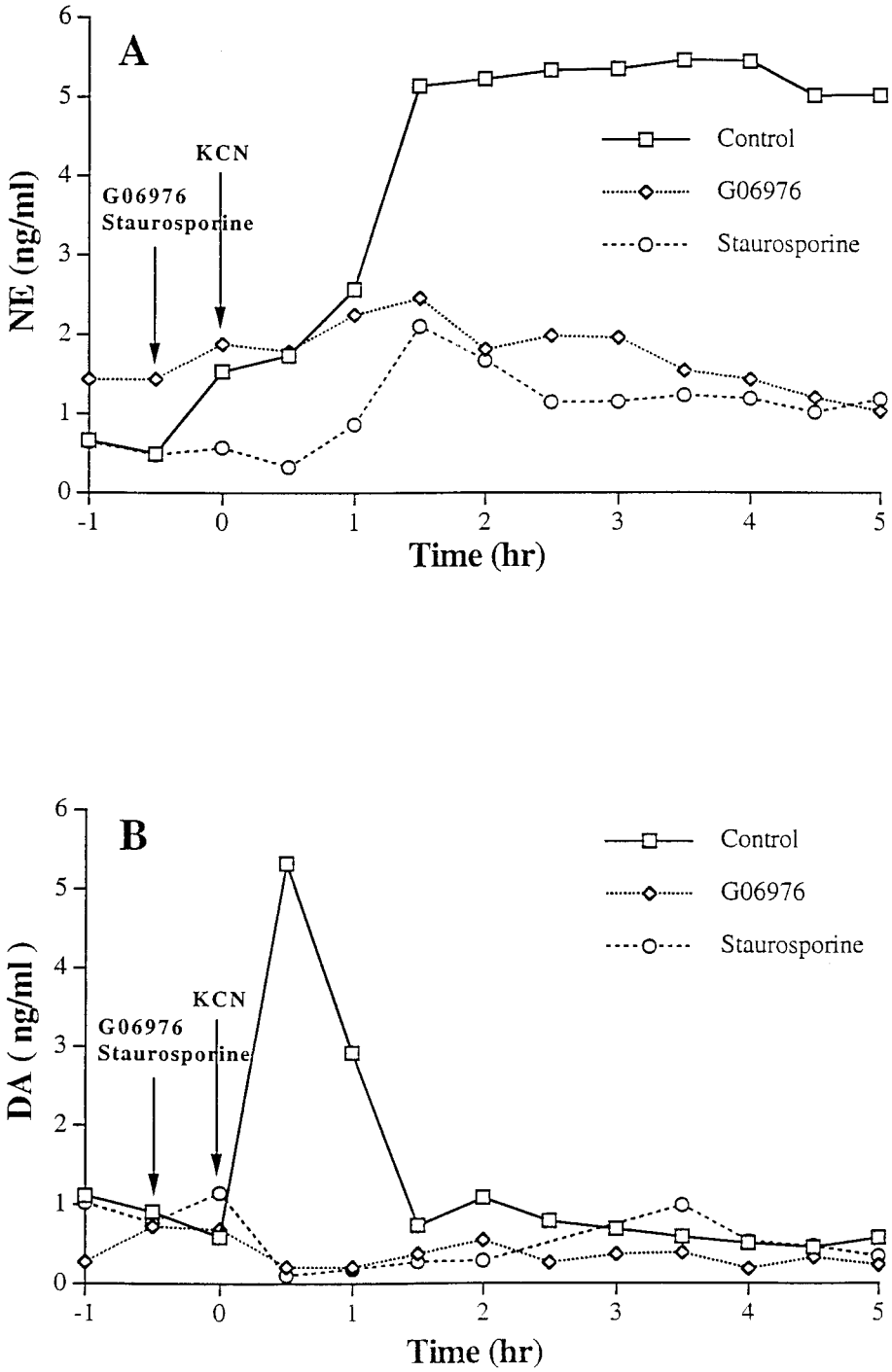

Fig. 6. Effects of the PKC inhibitor GÖ6976 and staurosporine on the KCN-induced DA and NE release in the glucose-free medium. The cells were incubated with glucose free DME medium and equilibrated for 2 h to establish a basal level. Staurosporine (1 μM) or GÖ6976 (10 nM) was added and incubated for 15 min, then KCN (final concentration 0.5 mM) was added and dialysates were subjected to either an on-line HPLC system or collected (in 5 μl of 0.1 M HCl containing 10^{-7} M ascorbic acid to preserve biogenic amides) in a CMA/140 fraction collector and then were analyzed for extracellular NE (**A**) and DA (**B**) every 30 min. This experiment was replicated with similar results.

fedipine [Jackson and Nurse, 1997]. In addition, a Ca^{2+} channel blocker attenuated NE and DA release during hypoxia in PC12 cells [Kumar et al., 1998], which suggests that L-type voltage-gated Ca^{2+} channels contribute, in part, to hypoxia-induced catecholamine release. However, whether chemical hypoxia acts through the same mechanism as that in nonchemical hypoxia requires further investigation.

To test for the correlation between the KCN-induced cell injury and DA release in the PC12 cells, the toxicity of KCN treatment to the cells was assessed by the release of LDH into the culture medium. Under a parallel condition, LDH release into the medium in response to KCN was evident with glucose deprivation (Fig. 4), but was abolished in the presence of glucose [Huang et al., 1999], a result that was in agreement with DA release (Figs. 1 and 2). In addition, PKC inhibitors attenuated LDH as well as DA release by KCN, suggesting that PKC was involved in hypoxia-induced toxicity [Maduh et al., 1995; Pavlakovic et al., 1995]. The results support the hypothesis that neuronal damage produced by chemical hypoxia is associated with excessive release of DA.

In glucose-free medium, KCN not only elevated DA and NE releases but also increased $[Ca^{2+}]_i$, in agreement with the results of LDH release. However, NE release was not parallel with LDH release caused by chemical hypoxia, because NE release by KCN could also be activated in the presence of glucose, whereas LDH release was not evident. Depletion of ATP and elevation of $[Ca^{2+}]_i$ during chemical hypoxia, along with glucose deprivation, may activate catecholamine release and trigger chemical hypoxia-induced cell death. These differences may have important implications for pharmacological intervention in neurotransmitter release.

The molecular mechanism by which a catecholamine induces neuronal toxicity remains unclear. Metabolites of NE and DA, including autooxidized dopamine, generate reactive oxygen species, i.e., hydrogen peroxide, superoxide, and the hydroxyl radical, which are potentially cytotoxic [Cadet and Brannock, 1998]. Moreover, the autooxidation of catecholamines may contribute to the peroxidation of neuronal membranes [Maduh et al., 1988; Johnson et al., 1987a], which has been proposed to be involved in the pathogenesis of many human diseases [Lai and Yu, 1997]. Dopamine can induce apoptosis in PC12 cells by inducing DNA fragmentation and activating c-jun N-terminal kinase/stress-activated protein kinase [Kang et al., 1998].

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