cAMP regulates nitric oxide production and ouabain sensitive Na⁺, K⁺-ATPase activity in SH-SY5Y human neuroblastoma cells

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Summary We investigated the relation between cyclic AMP (cAMP) and nitric oxide (NO) production, as well as the effect of NO on Na⁺, K⁺-ATPase activity in the human neuroblastoma cell line SH-SY5Y. Two cAMP agonists, dibutyryl cAMP (DBC) and beraprost sodium (BPS), increased cAMP accumulation and NO production in a time and dose dependent manner at 50 mmol/l glucose. On the other hand, cellular sorbitol and myo-inositol contents and protein kinase C activity were not altered by DBC or BPS. A specific protein kinase A inhibitor, H-89, suppressed increases in nitrite/nitrate and cyclic GMP (cGMP) and protein kinase A activity stimulated by DBC or BPS. This finding suggests that cAMP stimulates NO production by activating protein kinase A via a pathway different from the sorbitol-myo-inositol-protein kinase C pathway. We observed that an NO donor, sodium nitroprusside, and an NO agonist, L-arginine, enhanced ouabain sensitive Na⁺,

K⁺-ATPase activity at 50 mmol/l glucose. We also found that a nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), inhibited Na⁺, K⁺-ATPase activity at 5 mmol/l glucose, and partially suppressed the enzyme activity stimulated by DBC or BPS. The results of this study suggest that cAMP regulates protein kinase A activity, NO production and ouabain sensitive Na⁺, K⁺-ATPase activity in a cascade fashion. The results also suggest that protein kinase A at least partially regulates Na⁺, K⁺-ATPase activity without mediation by NO in SH-SY5Y cells. We speculate that cAMP and NO are two important regulatory factors in the pathogenesis of diabetic neuropathy. [Diabetologia (1998) 41: 1451–1458]

Keywords Cyclic AMP, nitric oxide, cyclic GMP, nitrite, diabetic neuropathy, sodium potassium ATPase, protein kinase A, human neuroblastoma cells

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Abbreviations: cAMP, Cyclic AMP; cGMP, cyclic GMP; NO, nitric oxide; D-MEM, Dulbecco's modified eagle medium; D-PBS, Dulbecco's phosphate-buffered salines; BCS, bovine calf serum; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; BPS, beraprost sodium; DBC, dibutyryl cAMP; W-7, N-(6-aminoethyl)-5-chloro-1-naphthalenesulfate; L-NAME, N^G-nitro-L-arginine methyl ester; iNOS, inducible nitric oxide synthase (NOS isoform II); ncNOS, neural constitutive nitric oxide synthase (NOS isoform III); DTT, 1-threo-1,4-Dimer-capto-2, 3-butanediol.

Diabetic neuropathy has been observed in about 50 % of diabetic patients with long-standing disease [1]. Many factors have been shown to contribute to the pathogenesis of diabetic neuropathy, including activation of the polyol pathway, constriction of blood vessels and impairment of blood flow, and accelerated non-enzymatic glycosylation [2–7].

Using a rat model of diabetes, we showed in a previous study that cyclic AMP (cAMP) concentrations were reduced in the sciatic nerve of diabetics and that cAMP reduction was related to nerve conduction velocity and ouabain sensitive Na⁺, K⁺-ATPase activity [8–10]. These findings suggested that cAMP reduction is a cause of diabetic neuropathy. We referred to this idea as the “cAMP hypothesis” [8–10].

Another important factor in the pathogenesis of diabetic neuropathy could be nitric oxide (NO) deficiency. For example, experiments using streptozotocin-induced diabetic rats found that NO deficiency slowed nerve conduction velocity [11]. In addition, using the SH-SY5Y human neuroblastoma cell line as a model of peripheral nerve cells, we observed that a high-glucose media decreases NO production, alters myo-inositol metabolism, and decreases protein kinase C activity, suggesting that all of these are involved in the regulation of NO synthesis [12].

There have been many reports concerning the interactions between cAMP and NO [13, 14]. For example, cAMP has been found to stimulate inducible nitric oxide synthase (iNOS) mRNA expression in some cell lines [13, 14]. The effect of cAMP on neuronal constitutive nitric oxide synthase (ncNOS)-catalysed NO production, however, is not known. Since we reported previously that SH-SY5Y cells express ncNOS [12], in this study we investigated the relation between cAMP and NO in SH-SY5Y cells, as well as the effect of NO on Na^+ , K^+ -ATPase activity.

Materials and methods

Cell culture. SH-SY5Y cells (passage 73–85, kindly provided by Dr. D.A. Greene of the University of Michigan, Ann Arbor, Mich., USA) were grown in Delbecco's modified eagle medium (D-MEM) (Gibco BRL Grand Island, N.Y., USA) containing 10% bovine calf serum (BCS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Gibco BRL), and 5 mmol/l or 50 mmol/l glucose, in humidified 10% CO_2 . The media was changed every 3 days. Cells were plated in a 6-cm dish, and 1 day later exposed to 5 mmol/l or 50 mmol/l glucose for 5–6 days. The cells were then exposed to beraprost sodium (BPS) (Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan), dibutyryl cAMP (DBC) (Daiichi Co., Tokyo, Japan), N-[2-(p-bromocinamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) (Seikagaku Corp. Tokyo, Japan), 12-O-tetradecanoylphorbol-13-acetate (TPA), N-(6-aminoethyl)-5-chloro-1-naphthalenesulfate (W-7), sodium nitroprusside, L-arginine or N^G -nitro-L-arginine methyl ester (L-NAME) (all from Sigma Chemical Co., St. Louis, Mo., USA) for the durations indicated in the text and figures.

Assays

Nitrite/nitrate. Nitrite/nitrate contents were measured using commercially-available kits (Cayman Chemical Co., Ann Arbor, Mich., USA) [15, 16]. Briefly, 80 µl aliquots of phenol-red free D-MEM from the culture dishes, 10 µl of nitrate reductase and 10 µl of enzyme co-factor were added to ELISA plates and incubated at room temperature for 1 h. The reaction was terminated by the addition of 50 µl each of Griess reagent 1 and 2. After 10 min, the difference in absorbance at 650 nm and 540 nm was measured with a microplate reader (Toyo Soda, Tokyo, Japan) and compared with a standard curve generated by 0–20 µmol/l NaNO_3 .

Cyclic GMP (cGMP) and cAMP. Cells and media were each homogenized with ethanol (final 50%) and then centrifuged at $900 \times g$ for 5 min at 4°C. Supernatants were evaporated under a N_2 stream and the residues were resuspended in an appropriate volume of imidazole buffer. RIA assays for cAMP and cGMP were done with commercially available kits (Yamasa Co., Chiba, Japan) [8–10, 17] and the results were normalized against cell protein concentration.

Since the cross reactivities of DBC in the cAMP RIA system and cAMP in the cGMP RIA system were 0.02% and 0.05%, respectively, the influences of DBC on cAMP and cAMP on cGMP determinations were negligible.

Ouabain sensitive Na^+ , K^+ -ATPase activity. Sodium-potassium activated ATPase activity was measured in freshly prepared homogenates of SH-SY5Y cells. Cells were rinsed with Dulbecco's phosphate-buffered salines (D-PBS) and scraped into buffer containing 50 mmol/l imidazole (pH 7.4), 100 mmol/l KCl, 4 mmol/l MgCl_2 , and 1 mmol/l EDTA, and sonicated with a Sonifier 250 (Branson, Conn., USA). The crude homogenate was then centrifuged at $200 \times g$ for 5 min at 4°C and the supernatant was assayed for ATPase as described previously [18, 19].

To determine inorganic phosphate concentrations, 750 µl of a reducing agent solution containing 3.5% ferrous ammonium sulphate, 1.0% thiourea, and 1.0% H_2SO_4 , and 150 µl of an ammonium molybdate solution containing 4.4% ammonium molybdate and 9% of H_2SO_4 were added to 750 µl of the solution to be assayed. After a 10 min incubation at room temperature, the absorbance at 750 nm was measured with a spectrophotometer (Hitachi, Tokyo, Japan).

Sorbitol. After aspirating the media, the cells were rinsed with D-PBS, scraped into 1 ml of pure water, and sonicated. An equal volume of 6% perchloric acid and a 1/10 volume of 2.5 mmol/l K_2CO_3 were added to the homogenate, and the mixture was centrifuged at $900 \times g$ at 4°C for 5 min. Sorbitol concentrations were determined by a fluorometric enzyme assay as described previously [20, 21].

Myo-inositol. A myo-inositol assay was done according to the spectrofluorometric enzymatic procedure reported previously [22], with slight modifications. Briefly, SH-SY5Y cells were washed with D-PBS, scraped into pure water and sonicated. The homogenate was deproteinized with 0.15 mol/l ZnSO_4 and $\text{Ba}(\text{OH})_2$, then centrifuged at $1,000 \times g$. The supernatant was mixed with glycine-NaOH buffer (final 60 mmol/l, pH 9.5) containing 0.5 mmol/l NAD (Sigma) and absorbance was measured with a spectrophotometer (Hitachi, Tokyo, Japan) both before and 1 h after adding 0.5 U of myo-inositol dehydrogenase (Sigma) (excitation λ : 340 nm, emission λ : 460 nm.)

Protein Kinase A. Cells were collected and lysed with a sonicator in ice-cold buffer A (5 mmol/l Tris-HCl (pH 7.2), 0.25 mol/l sucrose, 5 mmol/l EGTA, 1 mmol/l PMSF, 0.1 mmol/l 1-threo-1,4-Dimercapto-2,3-butanediol (DTT), 0.1% Triton X-100, and 10 µg/ml leupeptin/aprotinin; all from Sigma). The lysate was centrifuged at $14,000 \times g$ for 15 min at 4°C, and the supernatant was saved for enzyme activity. The phosphotransferase activity of Protein Kinase A was measured using a cAMP-dependent protein kinase assay kit (Upstate Biotechnology, N.Y., USA) according to the manufacturer's instructions. Briefly, 500 pmol of a specific protein kinase A substrate, kemptide (LRRASLG), was diluted in either 20 µl of assay dilution buffer B (20 mmol/l 3-morpholinopropanesulphonic acid (MOPS) (pH 7.2), 25 mmol/l β -glycerophosphate, 5 mmol/l EGTA, 1 mmol/l sodium vanadate, and 1 mmol/l

DTT) or 20 μ l of assay dilution buffer B containing 20 μ mol/l of a protein kinase C inhibitor peptide (protein kinase C pseudosubstrate, RFARKGALRQKNV) and 20 μ mol/l of a calmodulin-dependent protein kinase inhibitor (calmidazolium chloride, R24571). The reaction mixtures were supplemented with 50 μ g of sonicated cellular extract (5.0 μ g protein/ μ l) and 10 μ l of kinase reagents (75 mmol/l MgCl_2 , 500 mmol/l ATP, and 10 μ Ci [γ - ^{32}P] ATP at 3000 Ci/mmol). Kinase reactions were conducted for 10 min at 30°C, and then 25 μ l aliquots were spotted onto Whatman P81 phosphocellulose paper (Whatman, Clifton, N.J., USA) and dried at 25°C. The P81 phosphocellulose paper was washed five times with 0.75% H_3PO_4 and once with acetone. The amount of radioactivity trapped on the P81 phosphocellulose paper was measured using a liquid scintillation counter.

Protein Kinase C. Cells were collected and lysed with a sonicator in ice-cold buffer A. The lysate was centrifuged at $1000 \times g$ for 10 min at 4°C, and the supernatant was centrifuged again at $100,000 \times g$ for 1 h at 4°C. The pellet and supernatant fractions were saved as the membrane and cytosolic fractions, respectively. Phospho-transferase activity of protein kinase C was measured using a protein kinase C assay kit (Upstate Biotechnology, N. Y., USA) according to the manufacturer's instructions. Briefly, 500 pmol of a specific protein kinase C substrate, kemptide (QKRPSQRSKYL), was diluted in either 20 μ l of assay dilution buffer C (buffer B without EGTA and with 1 mmol/l CaCl_2) or 20 μ l of assay dilution buffer C containing 2 μ mol/l protein kinase A inhibitor peptide (PKI) and a calmodulin-dependent protein kinase inhibitor (calmidazolium chloride, R24571), and 10 μ l of dilution buffer C containing a protein kinase C lipid activator (0.5 mg/ml phosphatidylserine and 0.05 mg/ml diglyceride). The rest of the procedures for the measurement of protein kinase C activity were identical to those of protein kinase A.

Protein contents. Cellular protein content was determined by the BCA method, using commercially-available kits (Sigma) [23].

Statistical analysis. Results are shown as the means $\pm$ SEM. Statistical analyses were performed on replicate ($n = 6$ or 8) samples determined in one experiment in all Figures and in Table 1. The difference among groups was evaluated for significance by one way analysis of variance (ANOVA), followed by post hoc multiple comparison test (Scheffe F -test) using Statview software (Abacus Concepts, Inc. Calif., USA).

Results

Effect of DBC and BPS on NO production. To determine the effect of cAMP accumulation on NO production, we used the cAMP agonist DBC and BPS [24]. In SH-SY5Y cells placed in 5 or 50 mmol/l glucose, both agents increased NO production (nitrite/nitrate and cGMP concentrations) at 30 min, 6 h, 12 h and 48 h after treatment (data not shown). Nitrite/nitrate concentrations of cells placed in 50 mmol/l glucose were lower than those of cells placed in 5 mmol/l glucose (Fig. 1)($p < 0.05$). We also observed lower cGMP concentrations in SH-SY5Y cells placed in 50 mmol/l glucose than in those placed in 5 mmol/l glucose ($p < 0.05$). Both DBC and BPS

Fig. 1a,b. Effect of dibutyryl cyclic AMP (DBC) or beraprost sodium (BPS) on nitric oxide (NO) production in SH-SY5Y cells 30 min after treatment. **a** Nitrite/nitrate concentration. **b** Cycliv GMP (cGMP) concentrations. Cells were cultured in 5 or 50 mmol/l glucose for 6 days, and then incubated with 10 μ mol/l DBC or 100 μ mol/l BPS. $\square$, 5 mmol/l glucose; $\blacksquare$, 50 mmol/l glucose. $n = 8$ for each. *: significant difference ($p < 0.05$)

induced higher cGMP concentrations than control in cells placed in 50 mmol/l glucose at 30 min after treatment (Fig. 1) ($p < 0.05$).

Both DBC and BPS induced higher intracellular nitrite/nitrate and cGMP concentrations at higher concentrations of the agonist (data not shown) and the former induced higher levels than the latter.

We observed no changes in cell morphology or growth rate following incubation with DBC or BPS for 48 h (data not shown). The effects of DBC or BPS on differentiated SH-SY5Y cells treated with 500 μ mol/l TPA for 3 days [25, 26] were almost identical to those on undifferentiated SH-SY5Y cells (data not shown).

Effect of DBC or BPS on cAMP accumulation and protein kinase A activity. Although SH-SY5Y cells placed in 50 mmol/l glucose had lower cAMP accumulations than those placed in 5 mmol/l glucose ($p < 0.05$), both DBC (1 μ mol/l) and BPS (10 μ mol/l) increased cAMP accumulation at 30 min after treatment ($p < 0.01$) (Fig. 2). Results similar to those observed at 30 min were seen in cells incubated for 6, 12, and 48 h (data not shown).

The PKA activity in SH-SY5Y cells placed in 50 mmol/l glucose was lower ($p < 0.05$) than that of cells placed in 5 mmol/l glucose (498 ± 28 vs 1036 ± 56 pmol phosphate incorporated/mg protein, respectively) (Fig. 2). Both 1 μ mol/l DBC and 10 μ mol/l BPS induced higher protein kinase A activities than control cells, when placed in 50 mmol/l glucose (Fig. 2). Higher concentrations of DBC or BPS tended to induce higher activities than lower concentrations.

Effect of DBC or BPS on sorbitol contents, myo-inositol contents and protein kinase C activity. Sorbitol

Fig. 2a,b. Effect of DBC or BPS on cyclic AMP (cAMP) accumulation **a** and protein kinase A (PKA) activity **b** in SH-SY5Y cells. Cells were cultured at 5 or 50 mmol/l glucose for 6 days and incubated with the indicated concentration of BPS or DBC for 30 min. **a** □, 5 mmol/l glucose; ■, 50 mmol/l glucose. $n = 8$ for each. *: significant difference ($p < 0.05$) **b** □, 5 mmol/l glucose; ■, 50 mmol/l glucose; ▨, 50 mmol/l glucose with DBC; ▩, 50 mmol/l glucose with BPS. The precise incubation conditions are described below the Figure 2b. $n = 6$ for each. *: significant difference ($p < 0.05$)

concentrations were higher in cells placed in 50 mmol/l glucose than in cells placed in 5 mmol/l glucose ($p < 0.05$) and neither DBC nor BPS had any effect on sorbitol concentrations (Table 1). On the other hand, myo-inositol concentrations in cells placed in 50 mmol/l glucose were lower than those in cells placed in 5 mmol/l glucose ($p < 0.05$). Again, neither DBC nor BPS had any effect on cellular myo-inositol concentrations (Table 1).

The total protein kinase C activities of cells placed in 50 mmol/l glucose were lower than those of cells placed in 5 mmol/l glucose ($p < 0.05$) (Table 1). Neither DBC nor BPS had any stimulatory effect on these total activities. In addition, neither DBC nor

BPS affected the cytosolic (without stimulators: 4.35 ± 0.21 with DBC: 4.64 ± 0.31 , with BPS: 4.96 ± 0.42 nmol phosphate incorporated/mg protein, respectively) and the membrane-associated protein kinase C activities of cell placed in 50 mmol/l glucose (without stimulators: 8.20 ± 0.57 , with DBC: 7.88 ± 0.39 , with BPS: 8.69 ± 0.49 nmol phosphate incorporated/mg protein, respectively).

Effect of H-89 on NO production and protein kinase A activity. A protein kinase A inhibitor, H-89 [27], inhibited both NO production (Fig. 3) and protein kinase A activity (Fig. 3) in cells placed in 5 or 50 mmol/l glucose in a dose dependent manner. H-89 also inhibited NO production (Fig. 3) and protein kinase A activity (Fig. 3) stimulated by DBC in cells placed in 50 mmol/l glucose.

Effects of H-89 and W-7 on NO production. To determine whether the effect of DBC and BPS on NO production is due to increased cAMP accumulation, we measured cellular cGMP in the presence of H-89. In the absence of cAMP agonists, 5 μmol/l H-89 did not decrease cGMP contents of cells placed in 5 or 50 mmol/l glucose (data not shown). H-89 inhibited the increases in cGMP and cellular nitrite/nitrate con-

Table 1. Effects of DBC or BPS on sorbitol and myo-inositol contents and protein kinase C (PKC) activity

	<i>n</i>	sorbitol (nmol/mg protein)	myo-inositol (nmol/mg protein)	total PKC activity (nmol phosphate incorporated/ mg protein)
5 mmol/l glucose	6	1.08 ± 0.17^a	44.28 ± 2.35^a	15.63 ± 0.65^a
50 mmol/l glucose	6	3.27 ± 0.24	21.40 ± 0.78	11.91 ± 0.47
50 mmol/l glucose + DBC 10 μmol/l	6	3.88 ± 0.39^{NS}	19.28 ± 1.26^{NS}	12.01 ± 0.51^{NS}
50 mmol/l glucose + BPS 100 μmol/l	6	3.68 ± 0.29^{NS}	24.50 ± 1.69^{NS}	13.05 ± 0.49^{NS}

Values are expressed as the means $\pm$ S.E.M.
 n : number of samples. ^a $p < 0.05$ vs 50 mmol/l glucose

NS: not significant vs 50 mmol/l glucose
 Significance was determined by one-way analysis of variance

Fig. 3a,b. Inhibitory effect of H-89 on cGMP concentrations **a** and protein kinase A (PKA) activity **b**. Cells were grown at 5 or 50 mmol/l glucose, and then incubated for 6 h with the indicated concentration (0.1 ~ 10 $\mu\text{mol/l}$) of H-89. ○, 5 mmol/l glucose; ●, 50 mmol/l glucose; ▲, 50 mmol/l glucose with 10 $\mu\text{mol/l}$ DBC. $n = 6$ for each. *: $p < 0.05$ vs 5 mmol/l glucose without H-89. #: $p < 0.05$ vs 50 mmol/l glucose with DBC without H-89. +: $p < 0.05$ vs 50 mmol/l glucose without H-89

Fig. 4. Effect of W-7 on cGMP content. Cells were grown in 5 or 50 mmol/l glucose and then incubated for 6 h with 1 $\mu\text{mol/l}$ DBC or 10 $\mu\text{mol/l}$ BPS in the presence or absence of 30 $\mu\text{mol/l}$ W-7. □, 5 mmol/l glucose; ■, 50 mmol/l glucose. $n = 8$ for each. *: significant difference ($p < 0.05$)

centrations (data not shown) induced by DBC or BPS at both concentrations of glucose. We also used a calmodulin antagonist, W-7 [28], to investigate whether DBC or BPS had any effect on Ca^{2+} /CaM-mediated activation of ncNOS. In the absence of cAMP agonists, W-7 (30 $\mu\text{mol/l}$) did not alter basal cGMP concentrations of cells placed in 5 or 50 mmol/l glucose (Fig. 4) but reduced cellular cGMP ($p < 0.05$, in all cases) (Fig. 4) and nitrite/nitrate concentrations (data not shown) enhanced by DBC or BPS.

Effect of NO or cAMP on Na^+ , K^+ -ATPase activity. In preliminary experiments, Na^+ , K^+ -ATPase activity in

Fig. 5. Effect of NO on Na^+ , K^+ -ATPase activity. Cells were grown in 5 or 50 mmol/l glucose, and then incubated with 2 mmol/l L-arginine, 1 $\mu\text{mol/l}$ sodium nitroprusside (SNP) or 10 $\mu\text{mol/l}$ L-NAME for 2 h. □, 5 mmol/l glucose; ■, 50 mmol/l glucose. $n = 8$ for each. *: significant difference ($p < 0.05$)

Fig. 6. Effect of L-NAME on Na^+ , K^+ -ATPase activity in cells treated with DBC or BPS. Cells were grown in 5 or 50 mmol/l glucose, and then incubated for 6 h with 10 $\mu\text{mol/l}$ DBC or 100 $\mu\text{mol/l}$ BPS in the presence or absence of 10 $\mu\text{mol/l}$ L-NAME. □, 5 mmol/l glucose; ■, 50 mmol/l glucose. $n = 8$ for each. *: significant difference ($p < 0.05$)

SH-SY5Y was at a maximum in cells placed in 5 mmol/l glucose, and was reduced in a dose dependent manner from 5 to 50 mmol/l of glucose (data not shown). The activity of Na^+ , K^+ -ATPase was lower in SH-SY5Y cells placed in 50 mmol/l glucose than in cells placed in 5 mmol/l glucose ($p < 0.05$) (Fig. 5). Both sodium nitroprusside (1 $\mu\text{mol/l}$) and L-arginine (2 mmol/l) enhanced (but not completely) Na^+ , K^+ -ATPase activity in cells placed in 50 mmol/l glucose ($p < 0.05$, in both cases) but did not show significant effects in cells placed in 5 mmol/l glucose (Fig. 5). On the other hand, L-NAME [29], reduced (but not completely) Na^+ , K^+ -ATPase activity in cells placed

in 5 mmol/l glucose ($p < 0.05$), although showing no significant effect on cells placed in 50 mmol/l glucose (Fig. 5).

Both DBC (10 μ mol/l) and BPS (100 μ mol/l) increased Na^+ , K^+ -ATPase activity in cells placed in 50 mmol/l glucose and L-NAME inhibited (but not completely) this stimulated activity ($p < 0.05$) (Fig. 6).

Discussion

In another series of experiments, we found that culture glucose concentrations lowered cAMP accumulations in a dose dependent manner from 5 to 50 mmol/l glucose in SH-SY5Y cells. This effect was D-glucose specific, and L-glucose or mannitol did not affect cAMP accumulation (Shindo et al. paper in preparation). Based on these results, we did experiments using concentrations of 5 and 50 mmol/l glucose in media as models of normal and diabetic milieu, respectively.

It was reported previously that protein kinase A stimulates myo-inositol transport [30]. In our study, although accumulations of cAMP in SH-SY5Y cells was accompanied by an increase in NO production and protein kinase A activity, cAMP accumulation did not affect sorbitol or myo-inositol concentrations, or protein kinase C activity. Thus, we think that SH-SY5Y cells have a system in which cAMP stimulates NO production via a pathway different from the myo-inositol-PI synthesis-protein kinase C pathway as mentioned previously [12].

We think that the effects caused by DBC or BPS treatment are not due to further differentiation of SH-SY5Y cells [31, 32] for two reasons. Firstly, we did not observe any changes in morphology or growth rate following agonist treatment. Secondly, these agents had almost identical effects on NO production in SH-SY5Y cells pre-exposed to TPA to those observed in cells that were not treated with TPA.

Since we reported previously that SH-SY5Y cells express ncNOS mRNA but not iNOS mRNA [12], we think that cAMP increases NO production through activation of ncNOS. We speculate, based on the observed inhibitory effect of H-89, that protein kinase A is involved in the activation of ncNOS. We are not certain, however, whether ncNOS is activated as a result of direct phosphorylation by protein kinase A, or whether other intermediates are involved. In addition, the observation that W-7 inhibited NO production stimulated by DBC or BPS suggests that both cAMP, and possibly protein kinase A as well, also regulate ncNOS activity by affecting Ca^{2+} influx or binding of calmodulin to ncNOS to both.

It has been reported that protein kinase A regulates Na^+ , K^+ -ATPase activity in many cell lines and

tissues [33, 34]. Furthermore, NO has been found to stimulate Na^+ , K^+ -ATPase activity directly in the vascular wall [35, 36] and cGMP-dependent protein kinase, activated by NO and cGMP, has been reported to modulate Na^+ , K^+ -ATPase activity in renal tubular endothelium [37]. Concerning the relation between cAMP and Na^+ , K^+ -ATPase activity and diabetic neuropathy, we showed for the first time that Na^+ , K^+ -ATPase activity is regulated by cAMP and that NO regulates Na^+ , K^+ -ATPase activity. We have proposed the "cAMP hypothesis" as a possible explanation for diabetic neuropathy [8–10]. Based on the results of our study, we postulate that cAMP regulates protein kinase A, NO production and Na^+ , K^+ -ATPase activity in a cascade fashion, at least in SH-SY5Y cells. We also postulate that the regulation of Na^+ , K^+ -ATPase activity by cAMP is at least partially mediated by protein kinase A directly. On the other hand, we reported previously that protein kinase C regulates NO production [12]. So, we also speculate that Na^+ , K^+ -ATPase activity is regulated by non-cAMP-mediated NO. Therefore, we believe that cAMP and NO are two important regulatory factors for diabetic neuropathy.

A possible discrepancy in our experiments is the observation that Na^+ , K^+ -ATPase activity was not further stimulated in cells placed in 5 mmol/l glucose by agonists which increase cAMP and was not inhibited in cells placed in 50 mmol/l glucose by L-NAME. We feel, however, that the above observation itself indicates that at least in SH-SY5Y cells, Na^+ , K^+ -ATPase activity is close to maximum at 5 mmol/l glucose and close to minimum at 50 mmol/l glucose. This was supported by our preliminary experiments, in which we observed that glucose inhibited Na^+ , K^+ -ATPase activity in a dose dependent manner from 5 to 50 mmol/l of glucose. Since the present experiment is the first report of ouabain sensitive Na^+ , K^+ -ATPase activity in SH-SY5Y cells, we can not compare our results with other data.

In our experiments using cultured cells, a factor equivalent to blood flow in vivo is neglected. Thus, the hypotheses we draw from our experiments considers only metabolic factors as causes of diabetic neuropathy. In an animal study, it was reported that a NO deficiency slowed nerve conduction velocity [11]. The presence of ncNOS in large nerve fibre which dominate nerve conduction velocity has, however, not been confirmed. As a result the researcher suggested that NO deficiency had an indirect effect on nerve function, probably via reducing to nerve blood flow. It has also been reported that blood flow to the nerve is decreased by a combination of impaired NOS activity and increased endothelin [38] and that NO donor increases blood flow to the nerve and improves nerve function [39]. Furthermore, the existence of ncNOS has not been reported in peripheral motor neurons. Therefore, some investigators

think that the main effect of NO in diabetic neuropathy could be due to increased blood flow to the nerve.

Recent studies have shown, however, that NO possibly contributes to the modulation of certain autonomic functions by acting as a non-adrenergic non-cholinergic transmitter in the peripheral autonomic nerve system [40, 41]. Moreover, other studies have suggested that NO is involved in many pathologic conditions, such as sensory disturbance, hyperalgesia and the recovery process after axonal injury [42, 43]. Therefore, our theory could be relevant to peripheral sensory neuropathy or autonomic neuropathy or both.

In the treatment of diabetic neuropathy, agents which increase intracellular cAMP, such as cilostazol [44, 45], prostaglandin E_1 analogues [46, 47] and prostacyclin analogues [48, 49], have been reported to be effective in the treatment of clinical diabetic neuropathy. These agents possibly improve nerve function both by the metabolic effects of cAMP and NO, and by increasing blood flow to the nerve. Cyclooxygenase inhibitors, such as flurbiprofen, which are expected to reduce intracellular cAMP have been reported to impair both blood flow to the nerve and nerve function [50, 51]. We believe that these agents affect nerve function by altering cAMP or NO concentrations in nerve tissue. On the other hand, some vasodilators improve nerve function without affecting cAMP or NO concentrations [52, 53]. Thus, a deficiency in cAMP or NO is perhaps not always the main cause of all types of diabetic neuropathy. Therefore, the aetiology of diabetic neuropathy could be more heterogeneous than we think. Nevertheless, the results of many clinical findings and animal studies [8–11], together with those of this study, strongly suggest the importance of cAMP and NO deficiencies in the pathogenesis of diabetic neuropathy.

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