Effects of dibutyl cAMP on stanniocalcin and stanniocalcin-related protein mRNA expression in neuroblastoma cells

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Abstract

Stanniocalcin is a polypeptide hormone that was first reported in fish as a regulator of mineral metabolism. Its recent identification in mammals has opened a new area of investigation in basic and clinical endocrinology. In the present study, regulation of the stanniocalcin (STC) and stanniocalcin related protein (STCrP) genes were investigated in mouse neuroblastoma cells (Neuro-2A) in relation to neuronal cell differentiation. Neuro-2A is an undifferentiated cell line that contains measurable levels of STCrP mRNA, but undetectable levels of STC mRNA. Treatment of the cells with either dbcAMP (1–4 mM) or 50 μ M euxanthone (PW1) resulted in extensive differentiation and neurite outgrowth. However, only neurites of dbcAMP-treated cells developed varicosities, a phenotypic marker of axon formation. Furthermore, following differentiation induced by dbcAMP, there was an upregulation of STC and downregulation of STCrP mRNA levels. In the first 24 and 48 h of treatments, there was a maximum twofold induction and 1.5-fold reduction in STC and STCrP mRNAs respectively. Following 96 h of treat-

ment, an additional 14-fold STC induction and 1.2-fold STCrP reduction were observed. The increase in STC mRNA levels was accompanied by a concomitant increase in axon-specific low molecular form microtubule-associated protein (MAP-2c) mRNA and varicosities on the neurites, suggesting a possible role for STC in axonogenesis. There was no induction of STC mRNA levels when PW1 was added into the culture media, whereas ionomycin (1–10 μ M) had no observable effects on cell differentiation or STC/STCrP mRNA. Immunocytochemical staining of dbcAMP-treated cells revealed abundant levels of immunoreactive STC, particularly in the varicosities, with only weak staining in control, untreated cells. Antisense oligodeoxynucleotides transfection studies indicated that the expression of STC was a cause of varicosity formation and a consequence of cell differentiation. Our findings lend further support to the notion that STC is involved in the process of neural differentiation.

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Introduction

Stanniocalcin (STC) is an endocrine hormone originally discovered in fish, and only recently identified in mammals. The primary function of STC in fish is the maintenance of serum calcium levels through the regulation of calcium and phosphate transport across the gills, gut and kidneys (Wagner 1994). Recent studies have shown that the mammalian hormone also plays a role in mineral metabolism, as human STC is capable of regulating renal and intestinal phosphate and/or Ca^{2+} transport in rodents and swine (Wagner *et al.* 1997, Madsen *et al.* 1998). However, unlike fish where STC is produced by one gland, the mammalian STC gene is widely expressed in tissues as diverse as

ovary, kidney, prostate, heart, adrenal and lung (Chang *et al.* 1995, Olsen *et al.* 1996, Varghese *et al.* 1998). Moreover, unlike fish STC, the mammalian hormone is not normally found in the blood, suggesting that it functions instead as an autocrine/paracrine mediator of cell function (Varghese *et al.* 1998, Niu *et al.* 2000). More recently, a cDNA encoding an STC-related protein (STCrP or STC2) with 58% homology to STC was cloned and sequenced (Chang & Reddel 1998, DiMattia *et al.* 1998, Ishibashi *et al.* 1998), implying that STC and STCrP comprise a gene family with origins in a common ancestral gene. There is also preliminary evidence indicating that they have opposing actions on renal phosphate transport (Wagner *et al.* 1997, Ishibashi *et al.* 1998), indicating that they may have complementary functions.

STCrP is highly expressed in brain tissue (Chang *et al.* 1995, Chang & Reddel 1998) whereas the presence of STC is still controversial (Chang *et al.* 1995, Moore *et al.* 1999). However, recent reports indicate that STC gene expression can be induced in a human neuronal cell line, Paju, following phorbol 12-myristate 13-acetate (PMA) treatment (Zhang *et al.* 1998). Furthermore, more recent experimental data suggest that STC can protect cerebral neurons against hypoxic/ischemic damage (Zhang *et al.* 2000). Nonetheless, the full spectrum of possible STC actions on neural tissue remains to be established. Since human STC was discovered in the process of identifying genes involved in cellular proliferation (Chang *et al.* 1995), it is possible that STC is involved in cell proliferation and differentiation. Therefore, in the present study the possible roles of STC and STCrP in neuronal cell differentiation were investigated in a mouse neuroblastoma cell line, Neuro-2A-BU1 (N2A-BU1). The study utilized dibutyryl cAMP, ionomycin, and euxanthone as possible inducers of cellular differentiation and neurite outgrowth, and correlated changes in cellular morphology with the levels of STC and STCrP mRNA and protein. The results suggest that STC has a role in neurite growth and differentiation.

Materials and Methods

Cell culture

N2A-BU1, a subclone of Neuro-2A, was obtained by a limiting dilution method as described previously (Mak *et al.* 2000). The cells were grown in MEM supplemented with 10% FBS and antibiotics (50 U/ml penicillin, 50 µg/ml streptomycin and 10 µg/ml neomycin) at a density of 10^5 cm^{-2} in six-well plates (Nunc, Nalge Nunc, Denmark). The cells were incubated at 37 °C in a humidified 5% CO₂ incubator. After overnight incubation, N2A-BU1 cells were exposed for 24 h, 48 h and 96 h to one of the following treatments: (a) 1, 2 or 4 mM dibutyryl-cAMP (N⁶, 2'-o-dibutyryl-adenosine 3':5'-cyclic monophosphate: dbcAMP) (Roche Molecular Biochemicals, Indianapolis, IN, USA), (b) 1, 5 or 10 µM ionomycin (Calbiochem), (c) 50 µM euxanthone (PW1) or (d) 50 µM PW1 + 1 mM dbcAMP. Cells with a neurite length greater than the mean + 3 S.D. of neurite length measured in untreated control cells were determined to be differentiated cells (Mak *et al.* 2000). Six hundred cells were examined in 8–9 randomly chosen fields in each of 6 wells for each individual treatment.

RNA extraction and PCR product verification

Cells were dissolved in TRIzol reagent (GIBCO/BRL, Carlsbad, CA, USA). After 5-min incubation at room temperature (20 °C), chloroform was added for phase separation. The upper aqueous phase was collected and the

RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, air-dried and was finally redissolved in RNase-free water. A₂₆₀/A₂₈₀ ratios were between 1.6 and 1.8.

Stanniocalcin (STC), stanniocalcin-related protein (STCrP), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and axon-specific low molecular form microtubule-associated protein (MAP-2c) cDNAs were generated by PCR of total RNA derived from N2A-BU1 cells. The primers were designed on the basis of the published cDNA sequence of mouse STC: ATGTCCA AACTCAGCAGTGATTC-forward and AACTCA AAGTTGGTGTG-reverse (Wong *et al.* 1998); STCrP: AGAATCCAGGTCTCCAGGCG-forward and TGT GACATCCCCCTCTTTAATGT-reverse (Chang & Reddel 1998, Ishibashi *et al.* 1998); G3PDH: ATGGT GAAG GTCGTGTGAAC-forward and TTCAAGAGA GTAGGGAGGGC-reverse; MAP-2c: AACGGAGAG CTGACCTCAGCTGACA-forward and TCCGTC CTGAAGAGATGGAGCT-reverse (Beaman-Hall & Vallano 1993). The PCR was run for 35 cycles with a 56 °C or 60 °C (MAP-2c) annealing cycle (1 min), a 72 °C extension cycle (3 min), and a 95 °C denaturing cycle (50 s), plus final incubation at 72 °C for 10 min. The PCR products (~815 bp for STC, ~1000 bp for STCrP, ~1200 bp for G3PDH and ~404 bp for MAP-2c) were purified, subcloned into pUC 18 and subjected to dideoxy sequencing for verification.

PCR primer dropping

Semiquantitative RT-PCR was conducted as described previously using the housekeeping gene, G3PDH, as an internal standard (Wong *et al.* 1994). RT-PCR was performed using Superscript One-Step RT-PCR system (GIBCO/BRL) and was calibrated using STC/STCrP and G3PDH primer pairs. The number of cycles was varied to determine the optimal number that would allow detection of the amplified products while keeping amplification for these genes in the log phase. The following amplification cycles were used to compare levels of gene expression: G3PDH 22 cycles and STCrP 30 cycles. Because lower levels of STC were expressed in the differentiating cell, different cycles were used for the PCR: G3PDH 17 cycles and STC 30 cycles. For the assay, total RNA was diluted to 0.4 µg/µl in RNase-free water, mixed with 0.5 µg pd(T)_{12–18}, 25 µl of 2 × reaction mix, 1 µl RT/Taq mix and 22.6 µl of RNase-free water to a final volume of 50 µl in a reaction tube. The reaction was incubated at 45 °C for 30 min, followed by 95 °C for 2 min to inactivate the reverse transcriptase and to completely denature the template. Gene specific primer sets (STC/G3PDH or STCrP/G3PDH) were added into the reaction according to its corresponding pre-calibrated cycle number. Reactions were run for the optimized cycles with a 56 °C annealing cycle (1 min), 72 °C

extension cycle (3 min), and a 95 °C denaturing cycle (1 min). Control amplifications were carried out either without RT or without RNA. All glass- and plastic-ware was treated with diethyl pyrocarbonate and autoclaved.

Northern blot analysis

Control and stimulated N2A-BU1 cells were harvested and total RNA was isolated as outlined above. Thirty micrograms RNA per lane were resolved on 1% agarose/formaldehyde gels, and subjected to Northern blot analysis as previously described by using random-primed, ³²P-labelled mouse STC, STCrP and G3PDH cDNA probes under conditions of high stringency (Varghese *et al.* 1998). After exposure to X-ray film, the STC, STCrP and G3PDH bands were quantified by scanning densitometry.

RT-PCR

MAP-2c mRNA expression was determined using the Superscript One-Step RT-PCR system (GIBCO/BRL). Total RNA from control and various treatments was reverse transcribed and amplified with a MAP-2c primer set using a 60 °C annealing temperature for 35 cycles to produce PCR products 404 bp in size.

Radioimmunoassay (RIA) and immunocytochemistry (ICC)

For RIA, conditioned media from control, dbcAMP-, PW1- or dbcAMP+PW1-treated cells were analysed in triplicate using a double antibody human STC (hSTC) RIA that has been validated for measurements of STC in rodents (Niu *et al.* 2000). For ICC, N2A-BU1 cells were grown on 6-well plates with or without addition of 4 mM dbcAMP for 96 h. The cells were then fixed with 3% freshly made paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. Cells were then permeabilized by treatment with PBS containing 0.05% Nonidet P-40 for 10 min. The staining procedure involved pretreatment of the cells with 10% normal goat serum in PBS to reduce non-specific staining, followed by a 1-h incubation with hSTC antiserum (1:1000) and a 1-h incubation with goat anti-rabbit IgG coupled to FITC (1:200). The wells were washed three times for 15 min in PBS after each antiserum application. Control procedures included the application of preimmune rabbit serum or antiserum preabsorbed with hSTC in lieu of antiserum alone (Varghese *et al.* 1998). The immunostained wells were examined in a laser confocal microscope using an argon light source (Bio-Rad, MRC-600).

S-oligodeoxynucleotide transfection

The antisense (5'-GTTTGGAGCATTCT-3') and sense (5'-AGAATGCTCCAAAAC-3') phosphorothioate oligodeoxynucleotides (S-ODN) specific for the initiation

codon region of mouse STC cDNA were synthesized (Gibco/BRL). For the preparation of transfection, N2A-BU1 cells with about 80% confluence grown on 6-well plates were washed with fresh FCS-free MEM, followed by addition of 950 µl FCS-free MEM. The cells were transfected with the antisense or sense S-ODN (total volume 50 µl) by using FuGENE 6 transfection reagent according to the instructions of the manufacturer (Roche Molecular Biochemicals). A FuGENE 6 reagent only control and a normal cell control (untreated) were also included. After incubation for 8 h at 37 °C, 1 ml 20% FCS in MEM was added into each well to make a final FCS concentration of 10%. After a further 1-h incubation, the cells were exposed for 24 h to 4 mM dbcAMP. The cells were examined in 8–9 randomly chosen fields in each of 6 wells. STC and MAP-2c mRNA expression were determined as described previously.

Statistical analysis

All data are represented as means ± S.E. Statistical significance is tested by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. Groups were considered significantly different if *P* < 0.05.

Results

Morphological differentiation of neuroblastoma cells

Untreated N2A-BU1 cells grew as densely packed, rounded and flattened cells (Fig. 1A). After treatment with 1–4 mM dbcAMP, all the cells elaborated neurites which were long, less branching and bipolar (Fig. 1B–D). The cell bodies were generally narrower and neurites were substantially decorated with varicose processes, raising the possibility that these neurites may be axon-like. Treatment with PW1 produced cells with flattened cell bodies, long and multipolar neurites but no varicosities (Fig. 1E). Co-treatment of PW1 treated cells with 1 mM dbcAMP produced varicosities as well (Fig. 1F). These morphological changes reached a maximum by 96 h of treatment. The effects of dbcAMP on varicosity formation were dose- and time-dependent. Treatment of N2A-BU1 cells with 1, 5 or 10 µM ionomycin had no observable effect (results not shown).

Gene expression of STC and STCrP mRNAs

Using the PCR primer dropping method (Fig. 2), STC and STCrP mRNAs were detected and quantified. The levels of STC and STCrP transcripts were measured in control and treated cells 24, 48 and 96 h post-treatment (Fig. 3). The control cells expressed measurable levels of STCrP mRNA (in arbitrary units: 1.2–1.35) whereas STC

Figure 1 A-F. Phase contrast comparison of neuroblastoma (N2A-BU1) cell response to different stimulations. N2A-BU1 cells were grown in 10% FBS/MEM and exposed for 48 h to (B) 1 mM, (C) 2 mM, (D) 4 mM dbcAMP, (E) 50 μ M PW1 and (F) 50 μ M PW1 + 1 mM dbcAMP. Control cells were untreated (A). The cells were photographed without prior fixation. Varicosities are indicated by arrows.

mRNA was undetectable. Following differentiation induced by dbcAMP, there was a large increase in STC mRNA but a reduction in the level of STCrP. In the first 24 and 48 h of treatment, there was a doubling in STC and a 1.5-fold reduction in STCrP mRNA levels by cells exposed to 1–4 mM dbcAMP ($P < 0.05$). However, the most pronounced concentration-dependent effects on the

cells were observed between 48 and 96 h when STC mRNA levels increased nearly 14-fold ($P < 0.001$) and STCrP decreased nearly 1.2 fold ($P < 0.005$) in response to 4 mM dbcAMP. No significant changes in STC and STCrP mRNAs were detected in PW1-induced cells although cell differentiation in the absence of varicosity formation had obviously taken place. However, when

Figure 2 Representative results of PCR primer dropping. PCR products of (A) G3PDH and STC (size of ~1200 bp and ~815 bp) and (B) G3PDH and STCrP (size of ~1200 bp and ~1000 bp) are shown. The number above each lane indicates the total number of PCR cycles completed for each set of primers.

1 mM dbcAMP was added together with PW1 into the culture media, markedly greater increases in STC mRNA levels were observed than with 1 mM dbcAMP alone (~34-fold between 24 and 96 h ($P<0.001$) (Fig. 4) as compared with only 16-fold in the presence of 1 mM dbcAMP). Similar trends of induction and reduction of STC and STCrP transcripts were confirmed by Northern blot analysis (Fig. 5). There was no induction of STC mRNA levels when ionomycin was added into the media. Variations in the levels of STCrP mRNA in response to the PW1 and ionomycin treatments were small and not statistically significant.

MAP-2c mRNA expression

For the detection of MAP-2c mRNA, the MAP-2c-F/MAP-2c-R primer set amplifies a ~404 bp fragment. The band was detected in the cells treated with dbcAMP alone or PW1 plus dbcAMP, but was barely detectable in the control and PW1-treated cells (Fig. 6).

RIA and ICC

When the conditioned media were assayed for STC content, none was detected even in cells which showed an upregulation of STC gene expression. Hence ICC was conducted to determine if there had been an upregulation of intracellular STC protein levels. Following 96-h of 4 mM dbcAMP treatment, high levels of STC protein were detected in cell bodies and varicosities, with low levels detected in neurite compartments (Fig. 7). There was little or no staining in the control cells. The use of

nonimmune serum or antiserum preabsorbed with hSTC abolished staining in all cells (results not shown).

Effects of STC antisense S-ODN on the differentiation of N2A-BU1 cells

The effects of antisense or sense S-ODN to the differentiation of N2A-BU1 cells are shown in Fig. 8. Dibutyryl-cAMP stimulated the FuGENE 6 reagent control, the normal cell control (6.1 ± 1.2 varicosities per cell, $n=575$) and sense S-ODN (6.0 ± 0.8 varicosities per cell, $n=517$) transfected cells to develop neurites with substantially decorated varicose processes. However, antisense S-ODN transfected cells elaborated long and bipolar neurites but contained far fewer varicosities (0.3 ± 0.05 varicosities per cell, $n=536$) ($P<0.05$). The levels of STC mRNA were found to be significantly reduced (by $70 \pm 6.3\%$, $P<0.05$) after antisense S-ODN transfection (Fig. 9A). All transfected and non-transfected cells treated with dbcAMP expressed MAP-2c mRNA (Fig. 9B).

Discussion

The present study has examined the roles of STC and STCrP in neuronal cell function with particular reference to the process of differentiation in mouse neuroblastoma cells (N2A-BU1), a subclone of Neuro-2A. Neuroblastoma cell lines show morphological, biochemical and electrophysiological characteristics of neurons and are widely utilized models for studying the neurite outgrowth

Effects of dbcAMP on STC mRNA Levels in Neuro-2A Cells.

(B)

Effects of dbcAMP on STCrP mRNA Levels in Neuro-2A Cells.

phase of neuronal differentiation. It has previously been reported that treatment with dbcAMP, ionomycin, H7 and PW1 produce differing degrees of cell differentiation and neurite outgrowth in N2A cells (Tsuda *et al.* 1989, Breen & Anderton 1990, Wu *et al.* 1998, Mak *et al.* 2000). In the present study, three currently used neuritogenic agents were applied to the cultures, to determine their relative abilities to induce cell differentiation, and in particular the nature of neurite outgrowth in relation to STC and STCrP gene expression. Being able to correlate STC and STCrP gene expression with a specific type of neurite outgrowth (i.e. axonogenesis or dendritogenesis) could prove valuable in revealing the cellular functions of these hormones. Further, as both concentration and time of exposure to stimulatory agents has proven to be crucial in determining expression of the neurite phenotype (Wu *et al.* 1998), the time- and concentration-related effects of these agents were also investigated.

Our results showed that both dbcAMP and PW1 induced extensive differentiation of the cells, whereas ionomycin had no observable effects. Our results with ionomycin differed from those in a previous study on the same cell line (Wu *et al.* 1998). This is not surprising as variable responsiveness to ionomycin has been reported in a number of different neuroblastoma cell studies (Shea *et al.* 1985, Fischer *et al.* 1986) and subclone variability may, in part, account for this. Cell differentiation was induced by both dbcAMP and PW1 treatment; however distinctive morphological differences in the nature of neurite outgrowth were observed in response to each agent. It is well known that neurite extension is a morphological expression of neuronal differentiation and produces structural and functional polarity related to outgrowth of axonal and dendritic processes (Wu *et al.* 1998). In the present study, cells treated with dbcAMP produced neurites with distinctive varicose structures, which were rarely found in PW1-treated cells. These varicosities are frequently observed along the axons of neurons (Wouterlood & Groenewegen 1985) and are reported to be associated with the process of axon formation. The lengthy, bipolar appearance of neurites in the present study also supported this viewpoint. Furthermore, our analysis of MAP-2c transcript levels in dbcAMP-treated cells revealed marked increases as compared with control, PW1- and ionomycin-treated cultures. Since low molecular form MAP-2c is primarily found in axons and plays a role in maintaining plasticity of the neuronal

cytoskeleton (Riederer & Matus 1985, Garner & Matus 1988), it further supports the notion that neurites in cells treated with dbcAMP were axonal, and not dendritic in nature. Similar results have also been reported in other studies where the application of dbcAMP to Neuro-2A cells produced neurites with axonal characteristics (Shea *et al.* 1985, Fischer *et al.* 1986). On the other hand, the morphological characteristics of the neurite outgrowth induced by PW1 (multipolar nature) were more indicative of a dendritic character (Mak *et al.* 2000) and there was no MAP-2c upregulation in PW1-treated cells.

The present study also demonstrated that undifferentiated N2A-BU1 cells contained measurable levels of STCrP mRNA but not STC mRNA. However, following differentiation there was selective upregulation and down-regulation of the STC and STCrP genes respectively, but only in response to dbcAMP. This response was both time- and dose-dependent. Furthermore, as differentiation induced by dbcAMP was also correlated with the appearance of varicosities and upregulation of MAP-2c, this further suggests that STC is somehow associated with axonal growth. The fold inductions of STC mRNA between 48 and 96 h of treatments were striking, suggesting that time-of-exposure is crucial for the expression of the neurite phenotype and the necessary genes (e.g. STC). Previous studies (Wu *et al.* 1998) on the N2a-W28 subclone showed that these cells required six days of exposure to dbcAMP in order to achieve neurite maturation, which highlights the importance of time-of-treatment on neuronal cell differentiation. In addition, we found the STC mRNA induction resulting from dbcAMP-induced varicosities-associated outgrowth was enhanced by concomitant PW1 treatment throughout the induction period, although PW1 on its own had no effect. The apparent synergism between dbcAMP and PW1 is likely to be mechanistically relevant as these two treatments have in common the ability to stimulate neurite outgrowth. The underlying mechanisms are not known, but PW1 treatment obviously sensitized the STC gene to induction by dbcAMP. A second important finding was that there was no induction of STC gene expression by ionomycin, indicating that increased cytosolic Ca^{2+} levels are not involved in upregulating STC gene activity by these cells (Zhang *et al.* 1998). Extracellular calcium-invoked upregulation of STC mRNA and/or protein synthesis have been demonstrated in SUSM-1 (Chang *et al.* 1995) and Paju cells (Zhang *et al.* 2000) respectively,

Figure 3 Messenger RNA levels of STC and STCrP in N2A-BU1 cells. Cells were incubated for 24, 48 and 96 h in 10% FBS/MEM containing 1, 2 and 4 mM dbcAMP. Four hundred nanograms total RNA of each sample were reverse-transcribed and amplified for 30 and 17 cycles (with STC and G3PDH) or 30 and 22 cycles (with STCrP and G3PDH). PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide. (A) Upper panel: inverse image of the gel; lower panel: the left y-axis represents the densitometric analysis of STC relative to G3PDH levels while the right y-axis denotes the fold inductions of STC mRNA with the zero fold increase set at the 24 h, 1 mM dbcAMP level of signal intensity. (B) Densitometric analysis of STCrP mRNA relative to G3PDH levels. Data (means $\pm$ S.E.M.) are from six separate experiments. Values with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's Multiple Range test ($P < 0.05$).

Effects of PW1 or PW1 + dbcAMP on STC mRNA and STCrP mRNA Levels in Neuro-2A Cells

Figure 4 Effect of PW1 and PW1+dbcAMP on STC and STCrP mRNA levels in N2A-BU1. Cells were incubated for 24, 48 and 96 h in 10% FBS/MEM containing 50 μ M PW1 (P) or 50 μ M PW1+1 mM dbcAMP (P+C). Four hundred nanograms total RNA of each sample were reverse-transcribed and amplified for 30 and 17 cycles for STC and G3PDH or for 30 and 22 cycles for STCrP and G3PDH respectively. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide. Upper panel: inverse image of the gel; lower panel: the left y-axis represents the densitometric analysis of STC and STCrP relative to G3PDH levels while the right y-axis denotes the fold inductions of STC mRNA with the zero fold increase set at the 24 h, P+C level of signal intensity. Data (means $\pm$ S.E.M.) are from six separate experiments. Note how the STC transcript is selectively upregulated in dbcAMP-treated cells. Values with the same letter are not significantly different according to the results of one-way ANOVA followed by Duncan's Multiple Range test ($P < 0.05$).

although the calcium levels required (5.4 mM) were considerably above the physiological range. In fish, both STC secretion and mRNA levels are regulated by changes in extracellular calcium within the physiological range (0.7–1.9 mM); however the overall importance of changes

Figure 5 Northern analysis of STC and STCrP expression in N2A-BU1 cells following dbcAMP, PW1 and dbcAMP+PW1 treatments for 96 h. Total RNA (30 μ g/lane) was fractionated in 3% formaldehyde–1% agarose denaturing gel, transferred, and hybridized with a mouse STC or STCrP cDNA probe. G3PDH hybridization is shown to normalize the STC or STCrP signal in each lane. Note the upregulation of STC and downregulation of STCrP mRNAs observed following dbcAMP treatments.

Figure 6 Analysis of MAP-2c mRNAs in N2A-BU1 cells by RT-PCR. Cells were incubated for 96 h in 10% FBS/MEM containing 1, 2, 4 mM dbcAMP, 50 μ M PW1 and 50 μ M PW1+1 mM dbcAMP. Eight hundred nanograms total RNA of each sample were reverse-transcribed and amplified for 35 cycles. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide. The inverse image of the gel is presented. Note how the MAP-2c transcript is selectively upregulated in dbcAMP- and PW1+dbcAMP-treated cells.

in intracellular calcium has not been investigated (Wagner & Jaworski 1994). Similarly, the importance of intracellular calcium levels to STC gene activity in mammalian nervous tissue also needs to be clarified.

We failed to detect the presence of any STC immunoreactivity in the conditioned media of dbcAMP-treated cells in spite of the marked upregulation in mRNA levels. Possible explanations for this are that STC may not be secreted by N2A cells or is immediately taken up by the cells following its release. It is also possible that STC is rapidly degraded and rendered non-immunoreactive upon release from N2A cells, as observed *in vivo* in the case of human STC when administered intravenously to rats in a bolus fashion (Niu *et al.* 2000). Whichever proves to be

Figure 7 Immunocytochemical localization of STC in (A) control and (B-C) 4 mM dbcAMP-treated N2A-BU1 cells. High levels of STC immunoreactivity are apparent in the cell bodies and varicosities of dbcAMP-treated cells only (white arrows).

the case, the upregulation of STC gene expression following dbcAMP treatment clearly resulted in enhanced STC production by the cells. Following 96 h of dbcAMP treatment (4 mM), high levels of STC protein were detected in the cells, particularly in the varicosities, with only low levels detected, by comparison, in the neurite

Figure 8 Phase-contrast comparison of the effects of STC sense or antisense oligodeoxynucleotides (S-ODN) transfections on varicosity formation in differentiating N2A-BU1 cells. N2A-BU1 cells were grown in 10% FBS/MEM and exposed for 24 h to 4 mM dbcAMP. Wild-type (A), STC sense S-ODN transfected (B) and STC antisense S-ODN transfected cells (C). Varicosities are indicated by arrows.

compartment. This might be indicative of orthograde transport of STC protein to the varicosities.

The concomitant elevation in STC mRNA levels and appearance of varicosities in N2A-BU1 cells is not sufficient evidence by itself to conclude that the upregulation of STC is a cause or consequence of cell differentiation. This is an important question that needs addressing and was demonstrated in the transfection studies. Our results

Figure 9 Analysis of (A) STC and (B) MAP-2c mRNAs in N2A-BU1 cells by RT-PCR. Wild-type, STC sense S-ODN transfected and STC antisense S-ODN transfected cells were incubated for 24 h in 10% FBS/MEM containing 4 mM dbcAMP. PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide. The inverse images of the gels are presented. Note a reduction of STC transcript in STC antisense S-ODN transfected cells.

demonstrated that STC antisense S-ODN specifically inhibited the formation of varicosities in dbcAMP-stimulated N2A-BU1 cells but had no effect on MAP-2c mRNA expression and cell differentiation. The observation implied that STC was possibly a consequence of cell differentiation, but a cause of varicosity formation. Nevertheless, our results provide the first hint of the possible complementary actions of STC and STCrP in

neuronal cells, supporting the hypothesis that they have opposite regulatory effects in the renal system (Ishibashi *et al.* 1998, Honda *et al.* 1999). The existence of STC in both lower (bony fish) and higher (mammals) vertebrates has opened up a new area of investigation in basic and comparative endocrinology, and will hopefully lead to a more complete understanding of its basic biology and possible clinical significance. Our findings lend further support to the notion that STC is involved in the process of neural differentiation and, in particular, of varicose formation. The implications for normal neuronal cell functions and pathophysiology could be widespread.

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