Effect of Potassium-induced Depolarization on Somatostatin Gene Expression in Cultured Fetal Rat Cerebrocortical Cells

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The stimulatory effect of potassium depolarization upon somatostatin (SS) mRNA levels in primary cultures of fetal cerebrocortical cells was analyzed. Depolarizing stimuli, such as 56 mM K⁺ exposure for 30 min, elicited an increase in immunoreactive somatostatin (IR-SS) release to the media and decreased SS mRNA levels. These were increased when exposure to depolarization stimuli was prolonged up to 3 or more hr. At this time, potassium (30 and 56 mM) acted as a secretagogue, stimulating SS secretion, but was also effective in stimulating SS mRNA levels, suggesting that SS secretion can be coupled to SS mRNA accumulation. These changes were inhibited by the Ca²⁺ channel antagonist verapamil. In contrast, Na⁺ channel blockade by TTX did not modify the 24 hr potassium-induced increase in SS mRNA, although it partially abolished potassium-induced SS secretion. Examination of the rate of disappearance of SS mRNA levels after inhibition of mRNA transcription by actinomycin-D revealed that K⁺ stimulation of cerebrocortical cells stabilized the SS mRNA. These results suggest that the induction of SS mRNA expression by K⁺ is dose dependent, and involves the modulation of ion channels. The time-course study confirmed that the K⁺-induced SS mRNA accumulation is time dependent, chronic activation of the Ca²⁺ channels being necessary to stimulate SS gene expression. K⁺ stimulation may also increase the level of SS mRNA in cerebrocortical cells by reducing its rate of degradation.

[Key words: somatostatin gene regulation, chronic depolarization, acute depolarization, potassium, potassium-induced somatostatin gene expression, brain somatostatin]

The neuropeptide somatostatin (SS) is widely distributed throughout the nervous system. In addition to its well-known role as a hypophysiotropic hormone, SS also appears to serve as a neurotransmitter or neuromodulator. SS release from neurons is, in turn, regulated by a number of classical neurotransmitters and neuropeptides (Arimura and Fishback, 1981; Reichlin, 1983).

Secretion of SS is increased by numerous agents that stimulate adenyl cyclase activity, including vasoactive intestinal peptide (Robbins and Landon, 1985; Tapia-Arancibia and Reichlin, 1985), glucagon (Shimatsu et al., 1982), and epinephrine (Chihara et al., 1979), suggesting that cAMP is a second messenger for SS release. cAMP also regulates SS biosynthesis in primary cultures of fetal rat hypothalamic cells and in clonal isolates of NIH-3T3 fibroblast cells transfected with the rat SS gene (Montminy et al., 1986a). Further studies have demonstrated a sequence within the SS gene that is necessary for its transcriptional regulation by cAMP (Montminy et al., 1986b), and a great deal is known about the molecular mechanisms by which cAMP regulates neural SS gene expression.

The regulation of neuronal gene expression by membrane depolarization is well documented. Recent studies have shown that membrane depolarization causes not only rapid alterations in protein phosphorylation (Nairn et al., 1985), but also activation of new programs of gene expression in neuronal-type cells (Greenberg et al., 1985, 1986; Morgan and Curran, 1986; Black et al., 1987; Saffen et al., 1988; Bartel et al., 1989). Moreover, an increase in mRNA has been measured with stimuli causing the activation of Ca²⁺ and Na⁺ channels and membrane depolarization (Loeffler et al., 1986; Morgan and Curran, 1986; Naranjo et al., 1986; Roach et al., 1987; Von Dreden et al., 1988). Genes regulated by depolarization include those that encode neuropeptides and neurotransmitter biosynthetic enzymes (Morris et al., 1988; Van Nguyen et al., 1990; Lu et al., 1991). Previous data from our laboratory (de los Frailes et al., 1990) demonstrated that when cultured cerebrocortical cells were exposed to high potassium concentrations for 24 hr, somatostatin release was not accompanied by intracellular depletion, suggesting an increment in its synthesis. Moreover, we also showed that chronic potassium treatment leads to posttranslational modifications of the peptide. Whether these changes in peptide synthesis reflect alterations in mRNA synthesis and/or processing, or whether they are also induced at the translational or posttranslational level is unknown.

To help define the mechanisms underlying depolarization-induced changes in SS gene expression, the role of Ca²⁺ and Na⁺ channels in SS mRNA induction by depolarization is examined in the present article. The possibility that K⁺ might act at posttranscriptional level by altering SS mRNA stability is also investigated. We provide evidence suggesting that chronic depolarization induces increments on SS mRNA levels. This effect involves the activation of Ca²⁺ channels, and is partly produced by reducing the rate of degradation of its mRNA.

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Materials and Methods

Reagents. Tetrodotoxin (TTX), verapamil (VPM) and actinomycin-D were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclic synthetic somatostatin (S-14) and Tyr¹-S-14 were obtained from Bachem, Inc. (La Jolla, CA). ¹²⁵I- and α -³²P-UTP were provided by New England Nuclear.

Buffers and media. Hank's Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), and Minimum Essential Medium (MEM) were obtained from M. A. Bioproducts (Walkersville, MD). MEM supplemented (MEM_s) consisted of MEM containing 10% fetal calf serum (FCS), 10% horse serum (HS) (M. A. Bioproducts, Walkersville, MD), glucose (6 g/l), insulin (80 mU/ml), streptomycin and penicillin (100 U/ml), and glutamine (200 mg/ml).

Cell culture. Preparation of primary long-term dispersed cell cultures of fetal rat cerebral cortex was done as previously described (de los Frailes et al., 1988). Timed pregnant Wistar rats were raised in our laboratory. On embryonic day 17, the embryos were removed from the pentobarbital-anesthetized mothers. The cerebral cortices were dissected under sterile conditions and the cells mechanically dispersed. The cells were suspended in MEM_s, plated in culture dishes at a density of 5×10^6 cells/35 mm plate, and kept in a humidified atmosphere of 5% CO₂, 95% air at 37°C. The medium was changed every 5–7 d.

Depolarization studies. After 7–9 d in culture, media were removed and 1.5 ml of fresh serum-free MEM_s, containing test substances (30 and 56 mM K⁺, 20 μ M VPM, 1 μ M TTX), was added for different time periods. At the end of incubation, cells and media were processed and used for immunoreactive somatostatin (IR-SS) and SS mRNA determinations.

mRNA stability studies. Actinomycin-D (5 μ g/ml), known to prevent RNA synthesis (Dani et al., 1984), was added to cells 24 hr after the addition of potassium (56 mM). Total RNA was extracted after 0, 1, 3, 6, 9, and 24 hr of incubation and analyzed by Northern blot.

Sample processing. At the termination of the experiments, the culture dishes were chilled on ice and 100 μ l of 1 N HCl was added to 1.5 ml of medium from the individual plates. Media were boiled for 5 min and spun down at 3000 rpm for 60 min, and the supernatants were kept frozen. The cells were scraped off into 1 ml 0.1 N HCl and extracted by sonication for 10 sec, followed by boiling (5 min) and centrifugation at 3000 rpm for 60 min. Supernatants of the media and cell extracts were kept at –20°C until assayed.

Radioimmunoassay. Immunoreactive somatostatin (IR-SS) was determined by a well-characterized radioimmunoassay (Patel and Reichlin, 1978), using an antiserum against S-14 raised in our laboratory by immunizing rabbits with S-14 bound to bovine thyroglobulin with glutaraldehyde. The initial dilution of the antiserum was 1:30,000 and the cross-reaction with S-28 was 20%. Assay sensitivity was 10 pg/ml. The intraassay and interassay variations were 8% and 15%, respectively.

Extraction of RNA from primary cultures. Total RNA was extracted by a modification of the method described by Favaloro et al. (1980). Cells were washed with Ca-free Hank's solution and resuspended in 10 mM Tris (pH 7) 0.15 M NaCl/2 mM MgCl₂ containing 10 U of RNasin (Promega Biotech, Madison, WI). The cell suspension was treated with 0.5% NP-40 for 5 min at 0°C and the nuclear pellet and cytoplasm were separated by centrifugation (14,000 rpm). The supernatant containing cytoplasmic RNA was added to an equal volume of 10 mM Tris (pH 7.6) 0.15 M NaCl/5 mM EDTA and extracted with phenol:chloroform. RNA in the aqueous phase was precipitated by ethanol. The RNA pellet was dissolved in 2.3 M ammonium acetate and precipitated by ethanol. The quality of the RNA was monitored by the ratio of UV absorbance at 260 and 280 nm. A_{260}/A_{280} ratios were consistently 1.95–2.0, indicating that the samples were essentially free of contaminating protein.

Antisense RNA probe labeling. Levels of somatostatin mRNA were quantitated by Northern blot hybridization using an antisense somatostatin RNA probe. The antisense probe was constructed by inserting a fragment of the rat preprosomatostatin cDNA (Goodman et al., 1983) into the expression vector pSP 65 (Promega Biotech). The vector containing the somatostatin cDNA insert was linearized with SalI and a ³²P-labeled antisense RNA probe produced with an SP6 polymerase. Labeled antisense somatostatin RNA probes were prepared according to the method recommended by the manufacturer (Boehringer Mannheim, Indianapolis, IN) using ³²P-UTP (800 Ci/mmol) as radionucleotide. The size of the radiolabeled riboprobe was confirmed by gel electrophoresis [1% agarose, formaldehyde/MOPS (20 mM 3-[N-morpholino]propane sulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH

7.0)] followed by autoradiography. Transcription efficiency was assessed by trichloroacetic acid (TCA) precipitation onto Whatman GF/C paper followed by liquid scintillation counting. Efficiency ranged from 50% to 75%.

Northern blot analysis. Ten micrograms of total RNA from each sample were desiccated in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY) and reconstituted in 10 μ l of denaturing buffer composed of 50% deionized formamide, 16% formaldehyde, and 1× MOPS. RNA samples were then heated at 65°C for 15 min, quick-cooled, and loaded onto a 1% agarose-formaldehyde gel. Running buffer was 1× MOPS and 15% formaldehyde. After size separation, RNA samples were transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH) at room temperature for 24 hr in 1× TAE (10 mM Tris/5 mM sodium acetate/0.5 mM EDTA).

Membranes were exposed to short-wave UV light (0.12 J) in a UV cross-linker (Hoefer Scientific Instruments, San Francisco, CA) and sequentially hybridized to a ³²P-labeled somatostatin antisense RNA probe. Blots were prehybridized for 2 hr at 65°C in a solution containing 5× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate), 50 mM PO₄ pH 6.5, 10× Denhardt's (1× Denhardt's = 0.02% Ficoll-400/0.02% BSA/0.02% polyvinylpyrrolidone-40), 50% deionized formamide, and 500 μ g/ml denatured salmon sperm DNA. Hybridization was performed for 24 hr at 65°C in the same buffer, after adding 4 × 10⁶ cpm/ml labeled probe.

After hybridization, the blots were washed twice for 30 min in 2× SSC, 0.1% SDS, 65°C, and twice for 30 min in 0.2× SSC, 0.1% SDS. Autoradiograms were developed after exposure to x-ray film for 10 hr at –80°C using two intensifying screens.

To ensure that equal amounts of RNA were loaded and the transfer was quantitative, RNAs were stained with methylene blue after transfer from agarose/formaldehyde gels to nylon membrane (Herrin and Schmidt, 1988).

Statistical analysis of the data was done using the Student's *t* test.

Results

Effect of acute depolarization by potassium

The potential role of membrane depolarization was assessed by incubating fetal cerebrocortical cells kept in culture for 8 d with high potassium concentrations (56 mM) for 30 min. In some cases, agents blocking the sodium (TTX) or calcium (VPM) ion channels were simultaneously added. Cell exposure to 56 mM potassium in the presence of 2.2 mM calcium resulted in a significant stimulation of IR-SS release (Fig. 1). This effect was completely prevented by the addition of VPM and partially abolished by TTX (Fig. 1). Northern blot hybridization assays were performed to determine whether the increase caused by 56 mM potassium was associated with an increase in SS mRNA accumulation. Potassium decreased SS mRNA accumulation (Fig. 2). Block of calcium and sodium entry with VPM and TTX reversed potassium-induced SS mRNA decrease, suggesting that voltage-dependent Ca²⁺ and Na⁺ channels are involved in the potassium-induced decrease in SS mRNA accumulation.

Effect of chronic depolarization by potassium

Figure 3 shows the effect of chronic potassium depolarization on IR-SS in the presence of drugs that modulate Na⁺ and Ca²⁺ channels. Exposure to 56 mM K⁺ for 24 hr resulted in a significant stimulation of IR-SS release. This effect was calcium dependent, since it was partially abolished by 20 μ M VPM. On the contrary, TTX (1 μ M) addition did not prevent potassium stimulatory effect, possibly because Na⁺ entry is not an absolute requirement for chronic K⁺-induced SS secretion. Intracellular IR-SS content was increased by K⁺ treatment; thus, total IR-SS content was increased, suggesting an increment in its synthesis. Neither VPM nor TTX alone modified basal SS release. As opposed to acute depolarization, chronic exposure to elevated concentrations of potassium elicited a very large increase

Figure 1. Effect of acute potassium depolarization on IR-SS release (upper panel) and cell content (lower panel) in cultured cerebrocortical cells. Cells were obtained from 17-d-old fetuses and maintained in culture for 7–10 d. Cultures were exposed to 56 mM K⁺ alone or combined with 20 μ M verapamil (VPM) or 1 μ M tetrodotoxin (TTX). Control (C) received MEM alone. The values represent the mean $\pm$ SEM of IR-SS ($n = 4$). ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

in SS mRNA accumulation (Fig. 4). In addition, mRNA stimulation was blocked by VPM (Fig. 4), indicating that the potassium effect was, indeed, mediated by voltage-dependent calcium channels. TTX showed no modulating effect (Fig. 4), reflecting that the voltage-dependent Na⁺ channels are not involved in the chronic potassium-induced SS mRNA accumulation. VPM and TTX did not affect the basal level of SS gene expression. This indicates that, when at rest, extracellular Ca²⁺ and Na⁺ and/or their voltage-dependent channels by themselves are probably not involved in regulating basal SS mRNA.

Dose response experiments

To confirm the action of chronic exposure to K⁺ on SS mRNA levels, cells were incubated with two different K⁺ concentrations for 24 hr. The Northern blot on Figure 5 shows that even at concentrations of 30 mM K⁺, SS mRNA levels were increased, and that this effect was higher in the presence of 56 mM K⁺. This study reveals a clear dose-dependent response of the SS mRNA levels to K⁺ treatment.

Time course of potassium effect

Incubation of the cells with potassium (56 mM) for up to 24 hr resulted in an increase in media IR-SS content (Fig. 6), at 30 min and 24 hr, whereas cell IR-SS content remained below that

Figure 2. SS mRNA accumulation in primary cultures of cerebrocortical cells: Northern blot analysis of total RNA extracted from cultures incubated for 30 min with 56 mM K⁺ alone or combined with VPM (20 μ M), and/or TTX (1 μ M). Control received MEM alone. Each lane contained 10 μ g total cellular RNA. Blots were hybridized to a ³²P-labeled SS antisense RNA probe.

Figure 3. Effect of chronic potassium depolarization on IR-SS release (upper panel) and cell content (lower panel) in cultured cerebrocortical cells. Cells obtained from 17-d-old fetuses and maintained in culture for 7–10 d were incubated for 24 hr with 56 mM K⁺ and/or VPM (20 μ M), and/or TTX (1 μ M). C (control). The values represent the mean $\pm$ SEM ($n = 4$). ***, $p < 0.001$; **, $p < 0.01$.

Figure 4. Stimulation of SS mRNA accumulation in primary cultures of cerebrocortical cells: autoradiogram of a Northern blot containing cerebrocortical cell cytoplasmic RNA hybridized to a ^{32}P -labeled SS antisense RNA probe. Cells were exposed to 56 mM KCl (K^+), and/or VPM (20 μM), and/or TTX (1 μM) for 24 hr. C, control (each lane contained 10 μg total cellular RNA). *Top*, Influence of calcium channel blocker verapamil (VPM). *Bottom*, Influence of sodium channel blocker tetrodotoxin (TTX).

of control at all times tested, except at 24 hr. When SS mRNA was measured for the same time period, it was decreased at the earliest time tested (30 min), and a progressive increase was observed after 3 hr (Fig. 7).

Effect of potassium on somatostatin mRNA stability

In order to study whether potassium may influence SS mRNA half-life, we carried out actinomycin-D decay curves in control cells and in K^+ -treated cells. Cells were removed 0, 1, 3, 6, 9, and 24 hr after actinomycin-D addition. Total RNA was extracted and analyzed by Northern blot. As is shown in Figure 8, potassium stimulation of cerebrocortical cells clearly reduced the rate of disappearance of SS mRNA.

Discussion

The present work provides evidence that chronic membrane depolarization, elicited by elevated extracellular K^+ concentrations, increased the level of SS mRNA. In agreement with previous data, we have observed that acute (30 min) stimulation by depolarizing concentrations of potassium leads to an increase in SS peptide release (Mudge et al., 1977; Patel et al., 1977; Wakabayashi et al., 1977; Berelowitz et al., 1978; Iversen et al.,

Figure 5. Dose-response effects of K^+ on SS mRNA levels: Northern blot analysis of total RNA extracted from control (C), and cerebrocortical cells treated with 30 and 56 mM KCl for 24 hr. Each lane contained 10 μg total cellular RNA. Blots were hybridized to a ^{32}P -labeled SS antisense RNA probe.

1978; Maeda and Frohman, 1980; Bonanno et al., 1991), along with a decrease in SS mRNA. From our results, it is evident that with short potassium treatment there is some dissociation between the accumulation of SS peptide and SS mRNA. In fact,

Figure 6. Time course of the effect of high potassium concentrations on IR-SS release (*upper panel*) and cell content (*lower panel*) in cerebrocortical cultures. Cells were incubated with 56 mM K^+ or MEM alone (control) for up to 24 hr. The values represent the mean $\pm$ SEM of IR-SS ($n = 4$). C, control. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

Figure 7. Time dependence of SS mRNA accumulation in primary cultures of cerebrocortical cells treated with 56 mM KCl. Cells were incubated with 56 mM K⁺ (K⁺) or MEM alone (Control) for up to 24 hr. This autoradiogram of a Northern blot depicts the amount of hybridizable SS mRNA. Ten micrograms of total cellular RNA from each sample were electrophoresed, blotted, and hybridized with a ³²P-labeled SS antisense RNA probe.

we observed that a 30 min exposure to K⁺ leads to a high increment in the concentration of medium SS, along with a decrease of the intracellular content. When the amount of SS in both compartments is summed, it is higher in the K⁺-treated plates than in the control plates, suggesting that an increase in the translation activity has taken place. To explain the controversy between reduced SS mRNA and higher IR-SS levels, several mechanisms could be suggested; for example, short-term K⁺ exposure could decrease SS mRNA stability; SS transcription induced by K⁺ could be delayed with respect to SS translation. Although there is not evidence in the literature supporting these hypothesis, these or other mechanisms could account for the dissociation between the activation of SS translation and transcription that occurs at 30 min. Moreover, previous data from our laboratory (de los Frailes et al., 1990) demonstrated that potassium treatment modified the forms of SS released, causing an increase in high-molecular-weight forms, indicating modification in the processing of SS. Based on these data, we could hypothesize that the effects observed on SS peptide, after acute exposure, could reflect posttranslational mod-

ification of the peptide and/or variations in the translational activity. In the present study, the K⁺-induced increase in SS release was almost completely blocked by VPM and TTX, indicating that the Ca²⁺ and Na⁺ entry through voltage-dependent Ca²⁺ and Na⁺ channels is a requirement for these responses. Therefore, these results suggest that in cultured cerebrocortical cells, Ca²⁺ and Na⁺ entry through voltage-dependent Ca²⁺ and Na⁺ channels is an adequate and sufficient event to produce an increase in acute somatostatin release.

We have previously demonstrated, using media and cell levels of IR-SS as indices of SS biosynthesis (de los Frailes et al., 1989), that chronic exposure of cultured cerebrocortical cells to high potassium concentrations increased SS biosynthesis. The present data demonstrate that chronic K⁺-induced depolarization also induces an increment in SS mRNA accumulation. As shown in the time-course study, the effect of K⁺ is time dependent, and requires between 3 and 6 hr to show up. This time dependence could explain the discrepancy with the data published by Montminy (1986a), which showed that treatment of the diencephalic cells with 30 mM KCl for 4 hr diminished SS mRNA accumulation.

We have found that depolarization-induced mRNA is almost inhibited by the calcium channel blocker VPM, which is consistent with calcium being the effector of the transcriptional response to depolarization. These data are in agreement with previous reports describing that the calcium channel antagonist D₆₀₀, VPM, and Co²⁺ (Kley et al., 1986) or low calcium media (Waschek et al., 1987; Morris et al., 1988; Sheng et al., 1990; Lu et al., 1991) inhibit veratridine- or KCl-induced activation of proenkephalin gene expression in bovine adrenal chromaffin cells. They further support the hypothesis that one of the biological roles of voltage-sensitive calcium channels located in neuronal cell bodies may be the transduction of depolarizing signals to the nucleus to regulate gene expression. Alterations in intracellular calcium levels activate several transduction pathways, resulting in distinct patterns of gene expression. Many responses to a Ca²⁺-mediated agonist involve interaction with calmodulin and subsequent activation of calcium-dependent protein kinases (Dash et al., 1991; Sheng et al., 1991). In addition, in many cell types, the Ca²⁺ pathway of agonist action interacts synergistically with the diacylglycerol pathway (Nishizuka, 1984). This study opens up the possibility that one of these intracellular pathways may be involved in potassium-induced SS gene expression. None of the Ca²⁺ and Na⁺ channel-blocking drugs had a significant effect on the basal level of SS mRNA and IR-SS. This indicates that, in the resting state, extracellular Ca²⁺ and Na⁺ and/or the Ca²⁺ and Na⁺ channels

Figure 8. Actinomycin-D decay curves: Northern blot analysis of total RNA extracted from control and K⁺-treated cells (for 1, 3, 6, 9, and 24 hr) after addition of actinomycin-D (5 μg/ml) at time 24 hr. Each lane contained 10 μg total cellular RNA. Blots were hybridized to a ³²P-labeled SS antisense RNA probe.

themselves are probably not involved in regulating basal mRNA levels and secretion.

It was observed that VPM does not completely inhibit the increase of SS mRNA induced by depolarization. This could be due to the action of potassium stimulation on the steady-state levels of SS mRNA, reducing the rate of degradation of SS mRNA. The molecular mechanisms accounting for the K⁺-mediated regulation of mRNA stability are unknown. However, it could be possible that K⁺ induces the synthesis of a new protein through transcriptional activation of its gene. This protein then directly or indirectly interacts with SS mRNA, increasing its stability. There is evidence that other hormones, such as prolactin (PRL) (Guyette et al., 1979; Eisenstein and Rosend, 1988), estrogen (Brock and Shapiro, 1983), glucocorticoids (Vannice et al., 1984), and thyroid hormone (Simonet and Ness, 1989) may induce some of their actions by stabilizing certain mRNA species.

As our cultures are based on a mixed cell system, we have to consider that all the effects evoked by K⁺-induced depolarization can be attributed not only to a direct action on somatostatinergic neurons but also to the release by other cell types of a secondary effector acting on these SS neurons.

In summary, the data presented here demonstrate that in fetal neuronal cells chronic K⁺-induced depolarization activates SS gene expression through a calcium-dependent pathway and, at least in part, by increasing the stability of the SS mRNA.

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