



Neuropeptide Y Activates Protein Kinase C in Hamster Suprachiasmatic Nuclei Brain Slices

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Abstract

The mammalian circadian clock in the suprachiasmatic nuclei (SCN) can be phase-shifted by neuropeptide Y applied in the subjective day. Previous studies suggested that neuropeptide Y might act through a protein kinase C (PKC)-dependent mechanism. We directly measured PKC activity in suprachiasmatic nuclei brain slices following application of neuropeptide Y. PKC activity increased 5 min after neuropeptide Y application, with a return to baseline levels 15 min after application. An initial small decrease in PKC activity 1 min after neuropeptide Y application was also observed after control applications of artificial cerebrospinal fluid. Our results support the hypothesis that phase shifts induced by neuropeptide Y involve activation of PKC.

Keywords: circadian, protein kinase C assay, neuropeptide Y, rhythm, suprachiasmatic.

Introduction

The mammalian circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Klein et al., 1991). Protein kinase C (PKC) was first identified in the rat SCN by autoradiographic localization of phorbol ester binding (Nadakavukaren et al., 1990). Immunocytochemical studies have shown that specific PKC isoenzymes are present in the SCN of a variety of animal species including the hamster (Biello et al., 1997; Van der Zee & Bult, 1995). In addition, mRNAs of various PKC isoenzymes are rhythmically expressed in constant darkness (Cagampang et al., 1998). However, the functional role of PKC in mammalian clock function is not understood.

The hypothalamic brain slice preparation provides a means of measuring direct circadian clock output by detection of cellular firing rate for up to three to four 24 h cycles (Klein et al., 1991). Application of neurochemicals, such as neuropeptide Y or melatonin, to the SCN can alter the phase of the circadian rhythm. Using PKC inhibitors and activators, studies have suggested that neuropeptide Y and melatonin phase shift the circadian clock by a PKC-dependent signal transduction pathway (Biello et al., 1997; McArthur et al., 1997). While one study indicated that melatonin increases PKC activity in a biochemical assay using suprachiasmatic tissue, a similar direct kinase activity assay has not been conducted following neuropeptide Y treatment. A recent study indicates that PKC activators may not mimic effects of neuropeptide Y at all circadian phases (Schak & Harrington, 1999), increasing the need for a direct assay to test the hypothesis that neuropeptide Y should increase PKC activity in the hamster SCN.

Material and Method

Animals and tissue preparation

Male golden hamsters (LVG, Charles River Laboratories, Kingston, NY) were housed in a light:dark cycle 14h:10h. Zeitgeber time was defined with ZT12 as the time of lights-off. Hamsters were overdosed with halothane anesthesia and decapitated at ZT3–5. A punch containing the SCN was removed from each of two coronal hypothalamic slices (500 μ m) from each hamster and placed in a gas-fluid interface slice chamber supplied with warm, humidified gas (95% O₂:5% CO₂), continuously bathed in artificial cerebrospinal fluid (ACSF; 125.2 mM NaCl, 3.8 mM KCl, 1.2 mM KH₂PO₄, 24.8 mM NaHCO₃, 10 mM D-glucose, 1 mM MgSO₄, 2.5 mM CaCl₂, pH 7.4, 34.5°C, 1 mL/min). All chemicals were supplied by Sigma unless otherwise stated.

Drugs and treatments

On the first day *in vitro*, a 200 nL microdrop of neuropeptide Y (NPY; 200 ng; 47 pmol) or ACSF was administered to the SCN region using a 1 μ L Hamilton syringe at ZT6. Drug application followed at least 1 h after dissection. Tissue was collected various times after treatment.

Tissue fractionation

The two SCN punches from one animal were placed in a microcentrifuge tube with 350 μ L ice-cold homogenization buffer (50 mM pH 7.4 HEPES, 4 mM EGTA, 2 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 5 μ M leupeptin, 1.5 mM DTT, 100 μ L phosphatase inhibitor cocktail 1 (Sigma P2850) at 100-fold dilution). Samples were sonicated while kept on ice (four 15 sec duration sonications, with 35 sec rest periods in between; microprobes at maximum output, VibraCell, Sonics and Materials Inc., Danbury, CT). We then removed a 200 μ L sample for the whole tissue assay. The

remaining 150 μ L was transferred to a centrifuge tube with 100 μ L homogenization buffer and centrifuged ($100,000 \times g$ for 30 min at 4°C). The supernatant was saved as the cytosol fraction. The pellet was resuspended in 350 μ L ice-cold homogenization buffer with 1% Triton X-100, stirred for 15 min at 20°C, and centrifuged ($20,000 \times g$ for 5 min at 4°C), with the supernate saved as the membrane fraction.

Protein kinase C activity assay

Activity was based on the incorporation of [γ - 32 P]ATP (Amersham) into a selective protein kinase C substrate ('Selectide', neurogranin 28–43; Calbiochem) at 30°C. Phosphorylation was conducted in an incubation volume of 50 μ L for 10 min, containing 20 mM HEPES (pH 7.4), 4 mM EGTA, 2 mM EDTA, 10 mM MgCl₂, 3.15 mM CaCl₂ and 10 μ M PKC Selectide Substrate, 100 μ M [γ - 32 P]ATP (0.5 μ Ci/50 μ L sample, specific activity 4×10^2 cpm/pmol), and with or without (2 μ M) PKC inhibitor, PKC(19–36) (Life Technologies). Samples were placed onto phosphocellulose disks, and rinsed with 0.85% phosphoric acid. The protein content of each sample was determined using the Lowry assay (Lowry et al., 1951), and results were expressed as pmol/min/ μ g protein. Results are shown as the percentage of the control (untreated) sample. All time points were within the linear portion of the assay.

Results

Application of ACSF induced a small decrease in PKC activity as seen in Figure 1. A similar decrease was observed following application of NPY, but was followed by a dramatic increase in activity 5 min following NPY application (see Fig. 1). Activity returned to baseline levels by 15 min after application. Results from cytosol and membrane fractions did not indicate any consistent effect of NPY on relative levels of PKC activity in these fractions. The PKC inhibitor decreased activity to background levels in all cases.

Discussion

Results from this study indicate that neuropeptide Y can increase the activity of PKC when applied in the subjective day to the SCN. These results support findings from a previous study indicating that phase-shift responses to neuropeptide Y are mediated by PKC, since these responses can be blocked by co-application of specific inhibitors of PKC activation (Biello et al., 1997). Our results are similar to those from a study of the effects of melatonin on SCN tissue (McArthur et al., 1997), although melatonin increased PKC activity by 30 sec after application, while the effect of NPY was slower to appear. The two neuromodulators could be working through the same pathway, although it is also quite possible that they act through different PKC isoenzymes.

The PKCs are a subgroup of the serine/threonine-specific protein kinase family. The PKC isoenzymes can be classified into three groups: (1) the conventional PKCs,

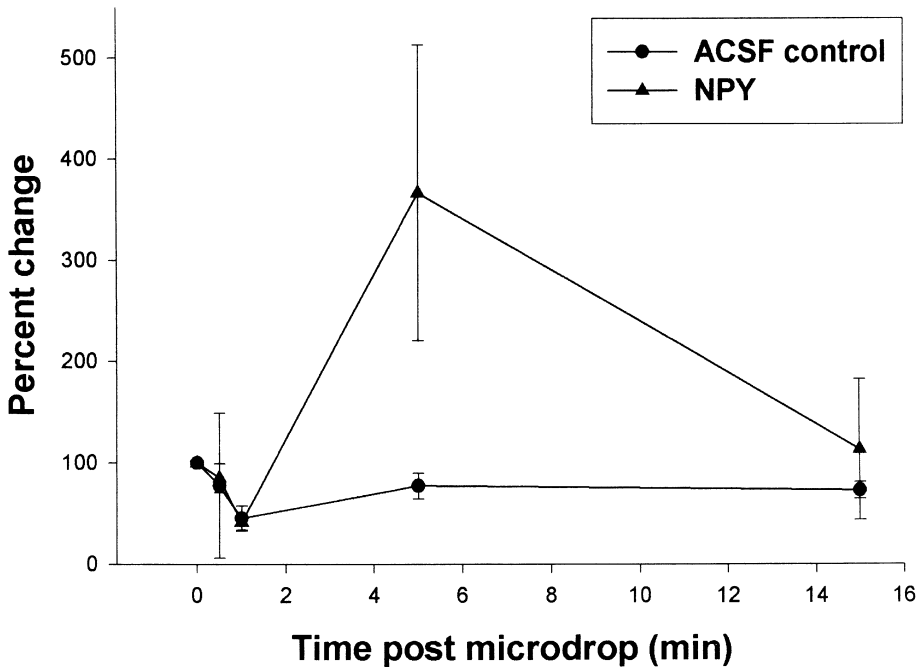


Figure 1. Percentage change in PKC activity measured relative to the control value for each run for samples treated with either NPY (triangles) or ACSF (circles). Samples were collected either 30 s, 1 min, 5 min or 15 min after treatment ($n = 2-3$ at each point; error bars represent SEM).

α , β I, β II, and γ , which require negatively charged phospholipids, diacylglycerol or phorbol ester, and calcium for optimal activation; (2) the novel PKCs, δ , ϵ , θ , η /L (mouse/human), and μ , which require negatively charged phospholipids, diacylglycerol or phorbol ester, but no calcium for optimal activation; and (3) the atypical PKCs λ /I (mouse/human), and ζ , which do not require calcium, diacylglycerol, or phorbol ester, but only negatively charged phospholipids for activation (Tanaka & Nishizuka, 1994). The calcium-dependent conventional isoforms are found in the SCN of several species, with PKC α being generally the most abundant as indicated by immunocytochemical staining (Bult & Smale, 1999; Van der Zee & Bult, 1995). Levels of immunocytochemically detected PKC α decreased during the light phase in vole SCN (Jansen et al., 1998). Levels of mRNA for the calcium-dependent PKC isoforms α , β I, β II, and γ vary during the circadian cycle in the rat SCN, with PKC α showing peak expression in the early subjective night, and PKC β I, β II, and γ showing peaks both in the early subjective day and the early subjective night (Cagampang et al., 1998). The one calcium-independent PKC isoform examined, PKC ϵ , did not show circadian variation in mRNA levels in the rat SCN (Cagampang et al., 1998). The atypical PKCs λ /I (mouse/human), and ζ , which do not show responsiveness to

phorbol esters, have not, to our knowledge, been examined in SCN tissue, but appear to play a role in insulin-secreting cells (Selbie et al., 1993; Yedovitzky et al., 1997) and in chick sympathetic neurons (Boehm et al., 1996).

PKC may have a complex role in SCN function, modulating many aspects of neuronal function. For example, PKC can enhance the effect of metabotropic glutamate receptor activation, inhibiting glutamate-induced calcium responses in SCN neurons (Haak, 1999), while PACAP-mediated increases in calcium in SCN neurons were partially mediated by PKC (Kopp et al., 1999). It is likely that PKC can play an important role in setting circadian clock phase, although the exact functional significance of this role is not yet understood.

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