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Activation of protein kinase C attenuates the cyclic GMP responses to C-type natriuretic peptide in cultured mouse astrocytes

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C-type natriuretic peptide (CNP), a recently discovered natriuretic peptide, has a potent stimulatory effect on cyclic GMP (cGMP) formation in cultured mouse astrocytes. Pretreatment of astrocytes with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), attenuated CNP-induced cGMP responses in a dose-dependent manner, with a half-maximal inhibitory concentration of 6 nM, whereas the inactive phorbol ester analog, 4α-phorbol 12,13-didecanoate, was without effect. In the presence of staurosporine, a PKC inhibitor, the inhibitory effect of PMA on CNP-stimulated cGMP production was reversed. These results suggest that PKC is an inhibitory modulator of CNP-stimulated cGMP responses in astrocytes and that CNP may interact with neuropeptides which stimulate PKC.

Protein kinase C; C-type natriuretic peptide; Cyclic GMP; Astrocyte

1. INTRODUCTION

C-type natriuretic peptide (CNP), a third natriuretic peptide, was recently identified in porcine brain [1]. It is present in 2 major endogenous forms, designated CNP-53 and CNP-22, containing 53 and 22 amino acids, respectively, the former carrying the bioactive CNP-22 sequence at its C-terminus [2,3]. CNP has a highly conserved amino acid sequence across different species, human CNP-22 being identical to rat and porcine CNP-22 [4,5]. CNP-22 also shows sequence homology to atrial (ANP) and brain (BNP) natriuretic peptides and exerts similar, though less potent, pharmacological effects in regard to natriuresis, diuresis and hypotensive activity [1].

CNP is present in high concentrations in the central nervous system (CNS) but not peripheral tissues [3,6,7]. In the rat, CNP mRNA is expressed only in the brain [4]. These findings suggest that CNP may function mainly as a neuropeptide. The CNS actions of CNP remain to be defined, although Samson et al. [8] have shown recently that, in contrast to ANP, the intracerebroventricular injection of CNP-22 can stimulate a drinking response in the rat.

Our group and others have recently demonstrated that CNP, like ANP and BNP, stimulates cyclic GMP (cGMP) production in cultured mouse astrocytes and rat glioma cells. Furthermore, CNP is several-fold more potent than ANP and BNP in stimulating cGMP formation in these cells [9,10]. The protein kinase C (PKC) family of enzymes are particularly abundant in the CNS and are important for such cellular processes as ion conductance, cell proliferation and cell differentiation [11–13]. Distinct isoforms of PKC have lately been identified in different types of glial cells and may mediate various functions within these cells [14]. We have therefore investigated the possible interaction between PKC and CNP-dependent cGMP responses in primary cultures of mouse astrocytes.

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2. MATERIALS AND METHODS

C-type natriuretic peptide (CNP_{1-22}) was purchased from Peninsula (USA). Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), 4α-phorbol 12,13-didecanoate (αPDD), 3-isobutyl-1-methylxanthine (IBMX), HEPES, and bovine serum albumin (BSA) were purchased from Sigma (USA). Staurosporine was from Calbiochem (USA). The cGMP radioimmunoassay kit was from Amersham International (UK).

Primary cultures of mouse astrocytes were prepared in 35-mm-diameter culture dishes using the method described by Hertz et al. [15] with some modifications [16,17]. Regular monitoring with indirect immunofluorescence using anti-glial fibrillary acidic protein (GFAP) antiserum showed that approximately 95% of the cells were GFAP positive.

Each experiment was performed in duplicate or triplicate at 22°C and repeated three times. The concentrations given for the various reagents are final concentrations, the total volume of incubation being 1 ml. To study the influence of phorbol esters on CNP-dependent cGMP generation, monolayer astrocytes in 35-mm-diameter culture dishes were washed twice with buffer containing 5 g/l BSA and 10 mM HEPES in serum-free minimal essential medium (pH 7.3), then incubated with varying concentrations of phorbol esters in buffer supplemented with 1 mM of the phosphodiesterase inhibitor IBMX at 22°C for 1 h. Following preincubation, 10 nM CNP was added to mouse astrocytes for 10 min, the time taken for CNP to achieve maximum

stimulation of cGMP [10]. The incubation was terminated by aspirating the solution and rinsing the cells with 2 ml of ice-cold phosphate-buffered saline. Intracellular cGMP was released with 1 ml 6% (w/v) trichloroacetic acid (TCA), which was subsequently removed by extraction with water-saturated diethyl ether. Following acetylation [18], cGMP in the sample was measured by radioimmunoassay [17].

To examine possible antagonism by staurosporine, a PKC inhibitor, on CNP-induced cGMP production, astrocytes were incubated with 100 nM staurosporine in buffer supplemented with 1 mM IBMX at 22°C for 15 min. Incubation was continued for another hour in the presence or absence of 10 nM PMA, followed by the addition of 10 nM PMA, followed by the addition of 10 nM CNP for 10 min and subsequent determination of cGMP levels.

Statistical analysis was performed with 2-way ANOVA and Dunnett's *t*-test for multiple comparisons. Statistical significance was taken as $P < 0.05$.

3. RESULTS

Preliminary experiments showed that pre-incubation of astrocytes with 1 μM PMA for times spanning 15 min to 7 h progressively suppressed the CNP-induced increment of cGMP up to 2 h. The longer periods of incubation did not produce any further change in the incremental cGMP response (data not shown). Subsequent dose-response experiments with phorbol esters were therefore conducted using a 1-h preincubation time.

In agreement with our previous results [10], 10 nM CNP stimulated cGMP production vigorously from a basal value of 1.1 ± 0.1 pmol/mg protein to 80.7 ± 8.2 pmol/mg protein (mean ± S.E.M., $n = 6$) (Fig. 1). Pretreatment with PMA for 1 h had no effect on basal cGMP production, whereas PMA suppressed CNP-stimulated cGMP production in a dose-dependent manner, with a half-maximal inhibitory concentration (IC_{50}) of 6 nM and maximal inhibition of 22.3 ± 4.6 pmol cGMP/mg protein at 1 μM of PMA (Fig. 1). Likewise, PDBu inhibited the CNP-induced cGMP response in a concentration-dependent manner. PDBu, at 10 nM, significantly suppressed the cGMP increment from 74.6 ± 6.6 pmol/mg protein to 39.9 ± 2.7 pmol/mg protein ($P < 0.01$), whereas αPDD, which does not stimulate PKC, had no significant suppressive effect up to a concentration of 1 μM (62.4 ± 5.8 pmol/mg protein) (Fig. 2).

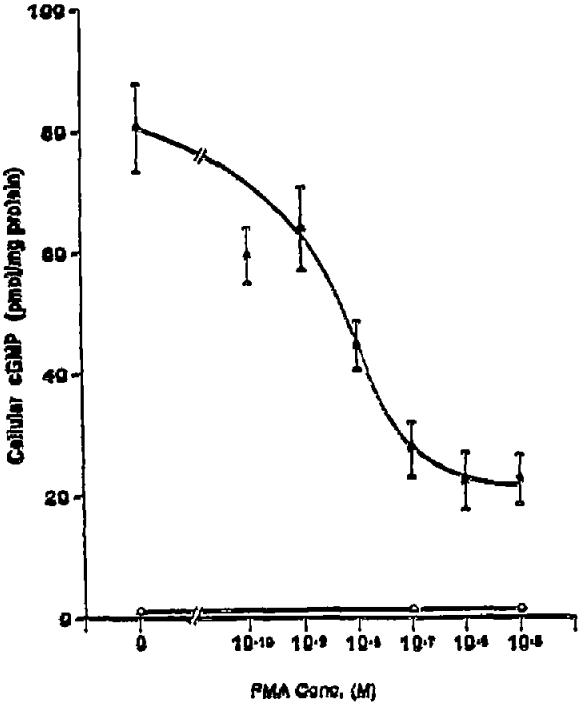

Fig. 1. Dose-dependent inhibition of CNP-stimulated cyclic GMP production by PMA in primary cultures of mouse astrocytes. Cells were treated with various concentrations of PMA for 1 h, then exposed to 10 nM CNP for 10 min. Following incubation, cGMP was released with 6% TCA and quantified as described in section 2. Data are mean ± S.E.M. values of 6 data points derived from duplicate determinations in three separate experiments. (○) without CNP, (●) with 10 nM CNP.

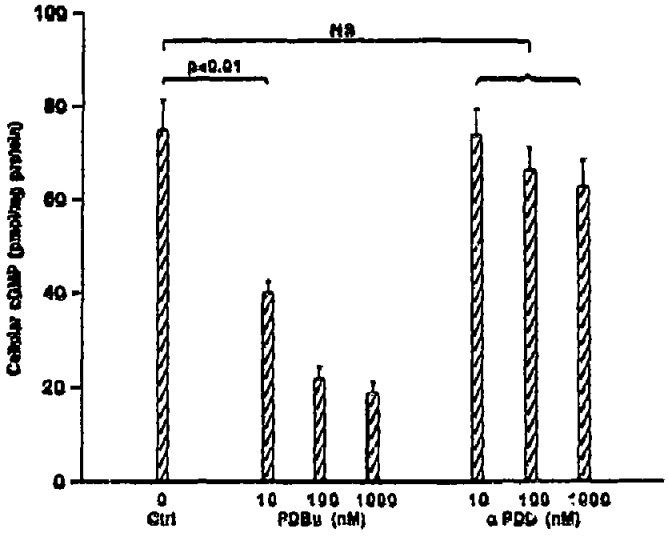

Fig. 2. Effect of phorbol ester analogues on CNP-stimulated cGMP generation in primary cultures of mouse astrocytes. Experimental conditions were identical to those in Fig. 1 and cells were treated with three concentrations of PDBu and αPDD prior to exposure to 10 nM CNP. Control (Ctrl) samples were incubated with 10 nM CNP in the absence of phorbol esters. Results are mean ± S.E.M. of dupliente determinations from three separate experiments. NS = not statistically significant.

In separate experiments incorporating staurosporine, 10 nM PMA reduced CNP-dependent cGMP formation from 62.3 ± 3.9 to 32.9 ± 1.3 pmol/mg protein ($P < 0.01$). In the absence of PMA, 100 nM staurosporine did not affect CNP-induced cGMP production. However, it blocked completely the PMA-mediated suppression of the CNP-stimulated cGMP response (Fig. 3).

4. DISCUSSION

Our results indicate that CNP-induced cGMP production in astrocytes can be suppressed by the phorbol esters PMA and PDBu, both of which are activators of

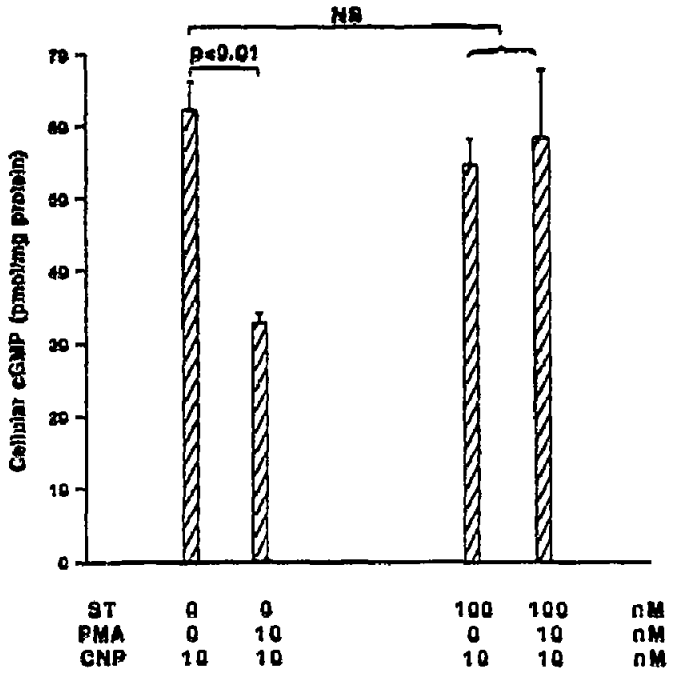

Fig. 3. Effect of CNP, CNP plus PMA, CNP plus staurosporine (ST), or CNP plus staurosporine and PMA on cGMP formation in cultured mouse astrocytes. Cells were treated with either 100 nM staurosporine or buffer alone for 15 min, then incubated with 10 nM PMA or buffer alone for 1 h before exposure to 10 nM CNP. Results are mean ± S.E.M. of three experiments performed in duplicate. NS = not statistically significant.

PKC [19,20]. This is analogous to the inhibitory action of PMA on ANP-dependent cGMP production in rat adrenocortical carcinoma cells [21]. The role of PKC activation in mediating this effect is supported by 2 other observations. First, αPPD, an inactive phorbol ester analog that does not stimulate PKC [19], did not suppress CNP-induced cGMP response. Secondly, staurosporine, a potent PKC inhibitor [22], blocked the inhibitory action of PMA. It is known that CNP acts upon an isoform of receptor guanylate cyclase (GC-B), which consists of 4 domains [23,24]. Attenuation of the CNP-induced cGMP response by PKC could be mediated by phosphorylation of receptor guanylate cyclase, leading to a decrease in CNP binding and/or suppression of the activity of the kinase-like or catalytic domain.

Astrocyte cGMP production, in response to 10 nM CNP, is attenuated, but not fully blocked, even with high doses of PMA and PDBu, suggesting that other regulatory mechanisms must also be involved. In accord with this, PMA attenuates only moderately ANP-dependent cGMP responses in cultured vascular smooth muscle cells whereas the PKC-activating hormones vasopressin and angiotensin II [25,26], markedly inhibit cGMP accumulation in these cells within 1 min of incubation, an effect attributed to their ability to increase cytoplasmic free calcium by stimulating inositol phospholipid turnover [27]. Endothelin has also been shown to inhibit ANP-augmented cGMP responses in glial cells within 5 min, possibly by altering the activity of kinases other than PKC [28]. However, a contributory effect of PKC activation with more prolonged incubation with endothelin, a known PKC stimulator [29], cannot be excluded. Recently, Haneda et al. [30] have reported that PKC activation is necessary for the sustained suppressive action of angiotensin II on ANP-induced cGMP production in glomerular mesangial cells. Since these hormones also exist as neuropeptides [31,32], our findings, taken together with the observations of others, raise the possibility that a number of neuropeptides may interact with CNP (in at least some instances through modulation of PKC) in the CNS. Work is currently in progress to investigate this issue.

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REFERENCES

[1] Sudoh, T., Minamino, N., Kangawa, K. and Matsuo, H. (1990) Biochem. Biophys. Res. Commun. 168, 863–870.
[2] Minamino, N., Kangawa, K. and Matsuo, H. (1990) Biochem. Biophys. Res. Commun. 170, 973–979.
[3] Minamino, N., Makino, Y., Tateyama, H., Kangawa, K. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 179, 535–542.
[4] Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1990) FEBS Lett. 276, 209–213.
[5] Tawaragi, Y., Fuchimura, K., Tanaka, S., Minamino, N., Kangawa, K. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 175, 645–651.
[6] Ueda, S., Minamino, N., Aburaya, M., Kangawa, K., Matsukura, S. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. 175, 759–767.
[7] Komatsu, Y., Makao, K., Suga, S., Ogawa, Y., Mukoyama, M., Ari, H., Shirakami, G., Hosoda, K., Nakagawa, O., Hama, N., Kishimoto, I. and Imura, H. (1991) Endocrinology 129, 1104–1106.
[8] Samson, W.K., Skala, K.D. and Huang, F.-L.S. (1991) Brain Res. 568, 285–288.
[9] Eguchi, S., Hirata, Y., Imai, T., Kanno, K., Ohta, K., Emori, T. and Marumo, F. (1992) Eur. J. Pharmacol. 225, 79–82.
[10] Yeung, V.T.F., Ho, S.K.S., Cockram, C.S., Lee, C.M. and Nicholls, M.G. (1992) J. Neurochem, in press.
[11] Shearman, M.S., Sekiguchi, K. and Nishizuka, Y. (1989) Pharmacol. Rev. 41, 211–237.
[12] Kikkawa, U., Kishimoto, A. and Nishizuka, Y. (1989) Annu. Rev. Biochem. 58, 31–44.
[13] Nishizuka, Y. (1989) Cancer 63, 1892–1903.
[14] Masliah, E., Yoshida, K., Shimohama, S., Cage, F.H. and Saitoh, T. (1991) Brain Res. 549, 106–111.
[15] Hertz, L., Schousboe, A., Boechler, N., Mukerji, S. and Fedoroff, S. (1978) Neurochem. Res. 3, 1–14.
[16] Kum, W., Cockram, C.S., Zhu, S.Q., Teoh, R., Vallance-Owen, J. and Young, J.D. (1987) J. Neurochem. 49, 1293–1297.
[17] Yeung, V.T.F., Lai, C.K., Cockram, C.S., Young, J.D. and Nicholls, M.G. (1991) J. Neurochem. 56, 1684–1689.
[18] Brooker, G., Harper, J.F., Terasaki, W.L. and Maylan, R.D. (1979) Adv. Cyclic Nucleotide Res. 10, 1–33.

[19] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[20] Stassen, F.L., Schmidt, D.B., Papadopoulos, M. and Sarau, H.M. (1989) J. Biol. Chem. 264, 4916–4923.
[21] Jaiswal, R.K., Jaiswal, N. and Sharma, R.K. (1988) FEBS Lett. 227, 47–50.
[22] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[23] Schulz, S., Singh, S., Bellet, R.A., Singh, G., Tubb, D.J., Chin, H. and Garbers, D.L. (1989) Cell 58, 1156–1162.
[24] Koller, K.J., Lowe, D.G., Bennett, G.L., Minamino, N., Kangawa, K., Matsuo, H. and Goeddel, D.V. (1991) Science 252, 120–123.
[25] Erusalimsky, J.D. and Rozengurt, E. (1989) J. Cell. Physiol. 141, 253–261.
[26] Chardonnens, D., Lang, U., Capponi, A.M. and Vallotton, M.B. (1989) J. Cardiovasc. Pharmacol. 14, S39–S44.
[27] Smith, J.B. and Lincoln, T.M. (1987) Am. J. Physiol. 253, C147–C150.
[28] Levin, E.R., Frank, H.J.L. and Pedram, A. (1992) J. Neurochem. 58, 659–666.
[29] Simonson, M.S. and Dunn, M.J. (1990) FASEB J. 4, 2989–3000.
[30] Haneda, M., Kikkawa, R., Maeda, S., Togawa, M., Koa, D., Horide, N., Kajiwara, N. and Shigeta, Y. (1991) Kidney Int. 40, 188–194.
[31] Phillips, M.I. (1983) Federation Proc. 42, 2667–2672.
[32] MacCumber, M.W., Ross, C.A. and Snyder, S.H. (1990) Proc. Natl. Acad. Sci. USA 87, 2359–2363.