Vasopressin Neuromodulation in the Hippocampus

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This study explored an effector mechanism associated with the arginine vasopressin (AVP) recognition site in the hippocampus, namely, potentiation of norepinephrine (NE)-induced cAMP accumulation in the surviving hippocampal slice. The biochemical mechanisms that underlie the AVP potentiation were investigated as follows: First, the actions of AVP upon NE-induced accumulation of cAMP in hippocampal slices from rat brain were specific to AVP and not shared by other closely related peptides, namely, oxytocin and AVP₄₋₉. Second, the AVP-induced neuromodulation involved beta-adrenergic receptors, with AVP having no effect on cAMP levels in the absence of NE. Third, the potentiation by AVP was biphasic, with lower AVP concentrations potentiating NE-induced cAMP accumulation, while higher concentrations did not potentiate. Fourth, an antagonist of V₁-type AVP receptors blocked AVP potentiation. Fifth, AVP potentiation was dependent upon extracellular calcium concentrations. Sixth, AVP potentiation was blocked by 50 μ M trifluoperazine, which is consistent with a calcium-calmodulin involvement but which might also implicate protein kinase C. These alternatives and the nature of the calcium involvement are discussed. AVP actions thus appear to involve interactions between several second-messenger systems and suggest a biochemical mechanism by which AVP exerts its centrally mediated behavioral effects.

The emerging knowledge of peptide actions in brain constitutes a major influence upon the growing understanding of neuronal response properties. The study of one such substance, vasopressin, has contributed insights into peptide effects on behavioral, biophysical, and biochemical aspects of neuronal function. Vasopressin is a 9 amino acid peptide which is biologically active in the periphery and in the CNS. Peripherally, vasopressin receptors and their associated effector mechanisms have been extensively characterized (for review, see Jard, 1983). In contrast, the exploration of CNS vasopressin receptors and effector mechanisms is still in an early stage of development (Brinton, 1987). Attention to the possible role of vasopressin as a neurally active peptide was initiated by the pioneering work of DeWied and his colleagues (DeWied, 1971, 1984; Kovacs et al., 1979a,

b). Two decades ago these investigators began to explore the behavioral effects of vasopressin and other peptides upon tasks thought to be indicators of mnemonic function (for review, see Strupp and Levitsky, 1985). Results of their studies have challenged us and others to explore the biochemical aspects of vasopressin action in brain.

Putative receptors for vasopressin have been detected by autoradiography in each of the developmental subdivisions of the brain (Biegon et al., 1984; Brinton et al., 1984; DeKloet et al., 1985). Of particular interest with respect to the present work are the arginine vasopressin (AVP) recognition sites within the hippocampal formation. In Ammon's horn, AVP receptors were observed in the pyramidal cell layer (Biegon et al., 1984; Brinton et al., 1984) and in the stratum oriens, radiatum, and lacunosum moleculare (Brinton et al., 1984). Within the dentate gyrus, AVP receptors were specifically localized to the molecular layer with no detectable recognition sites in the hilus (Biegon et al., 1984; Brinton et al., 1984). Receptor binding studies using synaptic membranes suggest that the recognition site for AVP in the hippocampus is a high-affinity binding site with a K_d of approximately 3 nM (Audigier and Barberis, 1985).

Electrophysiological evidence supports the postulate of functionally coupled AVP receptors in the hippocampus. Intracellular recordings from CA₁ pyramidal neurons showed that vasopressin induced a slow increase in spike discharge that was gradually reversible (Mizuno et al., 1984). Extracellular recordings showed a similar pattern of excitation with AVP inducing an increase in neuronal firing in the Ammon's horn of the hippocampus (Muhlenhaller et al., 1982). To date, the electrophysiological effects of AVP in the dentate gyrus are not known. However, recent data from patch-clamp analysis of sympathetic ganglion cells suggest the interesting possibility that AVP acts as a potent regulator of channel opening (Matsumoto et al., 1987).

Biochemical investigations of AVP action in hippocampus stem, in part, from the behavioral studies of Kovacs and co-workers (1979a, b). These investigators found that lesioning the dorsal noradrenergic bundle, which innervates the hippocampus (Moore and Bloom, 1979), abolished the behavioral effects of vasopressin (Kovacs et al., 1979a). This study provided the first evidence that AVP interacted with the monoamine norepinephrine (NE) to produce its behavioral effects. Exploring this interaction, Church (1983) and later we (Brinton and McEwen, 1985, 1986) and others (Newman, 1985) showed that AVP potentiated NE-induced accumulation of cyclic AMP in the hippocampus.

The present experiments were undertaken to explore the mechanism by which AVP modulates NE-induced accumulation of cAMP. The data show that in the hippocampal slice, vasopressin modulates NE-induced accumulation of cAMP in

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a dose-dependent and peptide-specific manner. The data further suggest that the neuromodulatory effect of AVP in the hippocampus involves a calcium-dependent process that is inhibited by a V_1 -type vasopressin receptor antagonist and by a calmodulin and protein kinase C antagonist, trifluoperazine. These findings further our understanding of vasopressin action in the CNS and may also serve to provide insights into the biochemical basis of the behavioral effects attributed to vasopressin. Preliminary forms of some of the results presented in this paper have been presented previously (Brinton and McEwen, 1985, 1986).

Materials and Methods

Experiments to investigate the effect of AVP upon NE-induced accumulation of cAMP were performed in hippocampal slices from male Sprague-Dawley rats (150–175 g; Charles River, Kingston, New York). Hippocampal tissue was cut using a McIlwain tissue chopper into 325 micron slices then turned 90° and sliced again. (In our experiments there was no difference between results obtained in singly or doubly cut slices.) Slices were then incubated in constantly oxygenated (95% O_2 /5% CO_2) modified Na bicarbonate Krebs Ringer for 35 min in a 37°C shaking water bath. The medium was then exchanged with one containing 30 μ M Rolipram (a phosphodiesterase inhibitor that does not inhibit adenosine-stimulated adenylate cyclase; gift of N. Sprzgala, Schering Pharmaceuticals AG, FRG) and the incubation continued for another 20 min. Another phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (Sigma Chemical Company, St. Louis), was also tested, and while lower basal levels were observed, there was no difference in the magnitude of the potentiating effect of AVP. Following the incubation in the presence of Rolipram, samples for basal levels of cAMP accumulation were taken. At this point incubation in the presence of the phosphodiesterase inhibitor and the test substances was initiated and the incubation continued for an additional 12 min at 37°C in constantly oxygenated Krebs Ringer. Following both the 20 min preincubation period in the presence of the phosphodiesterase inhibitor and in the incubation in the presence of test substances, 500 μ l samples containing approximately 500 μ g of wet weight tissue were aliquoted into 1.7 ml conical capped microfuge tubes resting in a bath of ice water. Basal cAMP accumulation was measured in single samples from each test vial, and basal cAMP accumulation was averaged across all conditions within an individual experiment. Each experimental condition was measured in triplicate within each experiment. Aliquoted slices quickly settled to the bottom of the tube and the Krebs Ringer was vacuumed off followed by the immediate addition of 250 μ l of ice-cold 7% trichloroacetic acid (TCA) to terminate the accumulation of cAMP. Samples were then sonicated and centrifuged at $15,000 \times g$ for 15 min. The supernatant was removed and 250 μ l of 0.2 N NaOH added to the pellet for protein determination by the method of Bradford (1976). TCA supernatants were processed as described below.

All experimental conditions contained 0.01% ascorbic acid and 2 μ M bacitracin (Sigma). For experiments performed in the absence of added calcium, the osmolarity was adjusted by increasing $MgCl_2$ to 2.6 mM. Otherwise, the modified Krebs Ringer (pH 7.4) consisted of the following (in mM): 124 NaCl, 5 KCl, 26 $NaHCO_3$, 1.3 $MgCl_2$, 1.4 KH_2PO_4 , 0.8 $CaCl_2$, 10 dextrose. NE [(α)-arterenol; Sigma] at a concentration of 10 μ M was used in all experiments. Oxytocin and AVP_{4-9} were obtained from Peninsula. AVP and the V_1 receptor antagonist [1-(beta-mercapto-beta, beta'-cyclopentamethylene propionic acid), 2-CO-(methyl) tyrosine] arg_8 -vasopressin [$d(CH_2)_5$ Try(Me)AVP] were a generous gift of Dr. Maurice Manning (Medical College of Ohio, Toledo, OH). These peptides, as well as the calmodulin antagonist trifluoperazine (Sigma), were diluted into Krebs Ringer immediately prior to use. BAY K 8644 (gift of Dr. A. Scriabine, Miles Institute, New Haven, CT) and forskolin (Sigma) were diluted into 95% ethanol, and 10 μ l of concentrated stock solution was added to 10 ml of Krebs Ringer for use in the experiments. Experiments utilizing BAY K 8644 were performed in greatly subdued lighting. Phorbol 12, 13-dibutyrate (PDBu; LC Services Corp., Woburn, MA) was dissolved in 100% dimethyl sulfoxide (DMSO) and diluted to a final DMSO concentration of 0.01% for experimental use. Control experiments in which 10 μ l of 95% ethanol was diluted into 10 ml Krebs Ringer or Krebs solution containing 0.01% DMSO showed no effect upon NE-induced accumulation of cAMP.

Cyclic AMP accumulation was determined using a protein kinase

Figure 1. AVP neuromodulation of NE-induced accumulation of cAMP in slices from rat hippocampus. AVP (250 nM) alone has no effect upon cAMP accumulation while significantly potentiating that induced by NE. Data were analyzed by Student's *t* test, * = $p < 0.05$ vs. NE alone, $n =$ at least 6 for each condition.

competitive protein binding assay (modified from Gilman, 1970). Experimental samples and cAMP standards dissolved in 7% TCA were extracted 3 times in 6 volumes of diethyl ether. Extracted samples (50 μ l) and standards (50 μ l) were incubated in an ice-water bath in the presence of 150 μ l [5,8- 3H]-adenosine 3',5' cyclic phosphate (Amersham) containing approximately 40,000 cpm/150 μ l and 100 μ l protein kinase (7.5 μ l/tube; Sigma) for 90 min. The binding reaction was terminated by the addition of 100 μ l of activated charcoal/BSA solution; the samples were then vortexed and centrifuged at $15,000 \times g$ for 10 min. Aliquots of supernatant (250 μ l) were counted for radioactivity in a scintillation counter. Cyclic AMP content was determined by linear-regression analysis based on a log/logit transformation. Data were analyzed either by a Student's *t* test or by 1-way analysis of variance followed by a Newman-Keuls test for multiple comparisons.

Results

Dose-response for vasopressin potentiation of NE-induced accumulation of cAMP

In the hippocampal slice preparation, *in vitro* addition of vasopressin did not increase cAMP accumulation above basal levels, whereas addition of 10 μ M NE resulted in a 3-fold increase over basal levels (Fig. 1). The simultaneous presence of 250 nM AVP and NE resulted in approximately a 5-fold increase in cAMP accumulation over baseline and a 40% increase in cAMP above that which accumulated in response to NE alone (Fig. 1). Thus, while 250 nM AVP had no effect alone upon cAMP accumulation, AVP significantly [$F(5,44) = 5.1$, $p < 0.001$] potentiated the effect of NE upon cAMP accumulation. The modulatory effect of AVP shows an inverted U-shape relationship within a concentration range of 10–1000 nM. Lower concentrations (50–250 nM) of AVP potentiated NE-induced accumulation of cAMP, while the highest concentration (1000 nM) did not potentiate (Table 1).

Peptide specificity

Because the metabolite peptides of vasopressin, AVP_{4-8} and AVP_{4-9} (Burbach and Leboville, 1983), have been shown to be behaviorally active (Burbach et al., 1983; DeWied et al., 1984), we tested whether the metabolite, AVP_{4-9} , also modulated NE-induced accumulation of cAMP. Additionally, we tested whether oxytocin, a peptide closely related in amino acid composition to vasopressin that has been shown to have behavioral effects

Table 1. Effect of AVP concentration on NE-induced accumulation of cAMP in the hippocampus

Condition	cAMP/mg protein ^a (pmol)	Percent of NE control
A. Basal	79 ± 9 (46)	33
B. NE, 10 μ M	236 ± 16 (16)	100
C. NE + 10 nM AVP	222 ± 24 (8)	94
D. NE + 50 nM AVP	267 ± 11 (3)	113
E. NE + 100 nM AVP	342 ± 39 (6) ^b	144
F. NE + 250 nM AVP	350 ± 27 (12) ^b	148
G. NE + 1000 nM AVP	260 ± 37 (5)	110

NE concentration = 10 μ M in all cases. Mean values $\pm$ SEMs are given (*n*). The data were analyzed by 1-way ANOVA followed by a Newman-Keuls test.

^a *n* given in parentheses.

^b *p* < 0.05 vs. conditions B and C.

opposite to those of AVP (Kovacs et al., 1979b), would influence NE-induced accumulation of cAMP. As seen in Table 2, neither 10 or 100 nM AVP₄₋₉ nor 10–1000 nM oxytocin significantly affected NE-induced accumulation of cAMP.

Beta-adrenergic receptor antagonist and forskolin activity

Propranolol (10 μ M), a beta-adrenergic receptor antagonist, effectively blocked [*F* (2,15) = 28.9, *p* < 0.0001] both the NE-induced accumulation of cAMP and the potentiating effect of AVP (Table 3). This finding supports the hypothesis that vasopressin acts to modulate beta-adrenergic receptor-coupled adenylate cyclase. This hypothesis was further supported by experiments carried out using the adenylate cyclase activator forskolin (Seamon and Daly, 1986). At 10 μ M, forskolin induced a greater than 2-fold increase in cAMP accumulation. In the presence of the same concentration of forskolin, vasopressin neither potentiated nor inhibited cAMP accumulation (Table 3). This result provides evidence that vasopressin is not modulating adenylate cyclase directly but is more likely acting to modulate a receptor-coupled event.

Vasopressin V₁ receptor antagonist activity

Behavioral studies had shown that the memory-enhancing effect of AVP was abolished with the V₁ receptor antagonist d(CH₂)₅Tyr(Me)AVP (LeMoal et al., 1981; DeWied et al., 1984). This antagonist is specific for the V₁ vasopressin receptor in liver and blood vessels (Kruszynski et al., 1980). Because of the antagonism of the behavioral effect, we tested whether d(CH₂)₅Tyr(Me)AVP would antagonize the AVP-induced neuromodulation in the hippocampus. As shown in Figure 2, this antagonist significantly [*F* (2,6) = 9.2, *p* < 0.01] blocked the AVP potentiation of NE-induced accumulation of cAMP. Vasopressin antagonists specific for the V₂ type of vasopressin receptor did not antagonize the AVP potentiation (Brinton and McEwen, 1986).

Calcium pathway experiments

Because the peripheral V₁ receptor is linked to calcium mobilization (Kirk et al., 1981) and because the V₁ receptor antagonist blocked the potentiating effect of AVP, we tested the calcium dependency of the AVP-induced neuromodulation. When extracellular calcium was absent, the potentiating effect of AVP was lost (Fig. 3). In contrast, replacement of extracellular calcium restored the AVP-induced potentiation (Fig. 3). Further

Table 2. Effect of vasopressin metabolite peptide, AVP 4–9, and oxytocin (OXY) upon NE-induced accumulation of cAMP in the hippocampus

Condition	cAMP/mg protein (pmol)	Percent of NE
Basal	62 ± 6	22
NE, 10 μ M	280 ± 20	100
NE + AVP, 100 nM	455 ± 45 ^a	163
NE + AVP ₄₋₉ , 10 nM	290 ± 15	104
NE + AVP ₄₋₉ , 100 nM	262 ± 25	94
NE + OXY, 10 nM	239 ± 16	84
NE + OXY, 100 nM	217 ± 12	77
NE + OXY, 1000 nM	224 ± 47	80

NE concentration was 10 μ M in all cases. Values are means $\pm$ SEMs from at least 3 separate determinations. Data were analyzed by 1-way ANOVA followed by Newman-Keuls test.

^a *p* < 0.05 vs. NE alone.

experiments showed that a higher concentration (2.5 mM) of calcium no longer enhanced the AVP-induced potentiation (Fig. 3). The accumulation of cAMP during basal conditions and in response to NE was not significantly affected by the presence or absence of extracellular calcium. Thus, it appears that it is the AVP neuromodulatory influence that is selectively sensitive to concentrations of extracellular calcium.

The dependency upon extracellular calcium suggested that calcium access could be a factor in the mechanism by which AVP modulated NE-induced accumulation of cAMP. We therefore tested whether the calcium channel activator BAY K 8644 (Schramm et al., 1983) would influence the neuromodulatory effect of AVP. In the absence of extracellular calcium (Fig. 4) or at a low concentration (0.4 mM Ca²⁺; data not shown), neither AVP nor BAY K 8644 had any potentiating effect upon NE-induced accumulation of cAMP. In the presence of 0.8 mM calcium, the potentiating effect of AVP was restored, while BAY K 8644 alone had no effect. However, BAY K 8644 + AVP in combination significantly [*F* (2,12) 12.2, *p* < 0.001] potentiated the effect of NE (Fig. 4). While the potentiation observed in the presence of AVP + BAY K 8644 was not significantly greater than that seen in the presence of AVP alone, the potentiation observed with AVP and BAY K 8644 nevertheless represented a total increase of 83% over the cAMP produced by NE alone and a 23% increase over that produced in the presence of NE + AVP (Fig. 4).

The biphasic nature of both the AVP dose dependency and the calcium sensitivity of the AVP-induced neuromodulation was reminiscent of calcium/calmodulin regulation of adenylate cyclase (Brostrom et al., 1975, 1978; Cheung, 1980; Treisman et al., 1983). We hypothesized that if AVP-induced neuromodulation involved a calmodulin-dependent process, then trifluoperazine (TFP), a calmodulin antagonist (Levin and Weiss, 1977), should block the potentiating effect of AVP. Data presented in Table 4 show that TFP, while having no effect upon cAMP accumulation in response to NE alone, significantly blocked the potentiating effect of AVP [*F* (3,16) = 10.0, *p* < 0.001].

Although TFP is a potent calmodulin antagonist, it can also antagonize protein kinase C activity (Schatzman et al., 1981; Wrenn et al., 1981; Nishizuka, 1984). We therefore undertook experiments using the phorbol ester PDBu to test the effect of protein kinase C activation upon NE-induced cAMP accumu-

Figure 2. V_1 -receptor antagonist peptide $d(CH_2)_5Tyr(Me)AVP$ antagonizes the potentiating effect of AVP upon NE-induced accumulation of cAMP in the hippocampus. NE concentration, 10 μM ; AVP concentration, 250 nM; $d(CH_2)_5Tyr(Me)AVP$ concentration, 100 nM. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls test, ** = $p < 0.01$ vs. NE alone; * = $p < 0.05$ vs. NE + AVP.

lation. In the presence of 100 nM PDBu and without NE, cAMP accumulation increased approximately 2-fold over basal levels [cAMP in pmol/mg protein $\pm$ SEM (n); basal = 39 ± 5 (3); PDBu = 88 ± 17 (3)]. At 1 μM PDBu, cAMP accumulation increased 3-fold over basal levels (Fig. 5). Finally, cAMP accumulation induced by the simultaneous presence of both PDBu and NE was essentially equal to the summation of cAMP accumulation in response to NE alone plus PDBu alone (Fig. 5). Thus, PDBu does not mimic the effects of AVP, in that it activates the cAMP response both in the absence of and in the presence of NE.

Discussion

The present series of experiments demonstrates that vasopressin exerts a modulatory effect upon beta-adrenergic receptor-induced accumulation of cAMP in hippocampal slices from rat brain. In addition, the potentiation by AVP was biphasic and specific to vasopressin, as neither oxytocin nor the metabolite peptide AVP_{4-9} mimicked the effect of AVP in the 10–1000 nM concentration range. The AVP potentiation was blocked by an

Table 3. Effect of propranolol on AVP-induced neuromodulation and effect of AVP upon forskolin stimulated adenylate cyclase in the hippocampus

Condition	cAMP/mg protein above basal (pmol)
A. NE	127 ± 17 (5)
B. NE + AVP	187 ± 15^a (5)
C. NE + AVP + propranolol	30 ± 10^b (5)
D. Forskolin	157 ± 11 (3)
E. Forskolin + AVP	168 ± 21 (3)

NE concentration, 10 μM ; AVP concentration, 250 nM; propranolol concentration, 10 μM ; forskolin, 10 μM . Values are means $\pm$ SEM (n). Basal cAMP levels were 79 ± 6 (5) for propranolol experiments and 76 ± 16 (3) for forskolin experiments. Statistical analysis was by 1-way ANOVA, followed by a Newman-Keuls test.

^a $p < 0.05$ vs. NE alone.

^b $p < 0.05$ vs. NE + AVP.

Figure 3. Calcium dependency of AVP neuromodulation of NE-induced accumulation of cAMP in the hippocampus. In the absence of extracellular calcium, NE-induced accumulation of cAMP is unaffected while the potentiating effect of AVP is lost. In the presence of 0.8 mM calcium, the neuromodulatory action of AVP is restored. In contrast, in the presence of 2.5 mM calcium, AVP modulation is inhibitory upon NE-induced accumulation of cAMP. NE concentration, 10 μM ; AVP concentration, 250 nM. Data were analyzed using a paired Student's t test, * = $p < 0.05$.

antagonist of V_1 receptors, which implicates the phosphoinositol pathway. A calcium-related mechanism was suggested by a dependency of the AVP potentiation upon extracellular calcium. Moreover, the potentiation induced by AVP was blocked by the calmodulin and protein kinase C antagonist TFP. The main findings described in the present report in the hippocampus, that of AVP potentiation and the calcium dependency of AVP-induced neuromodulation, are consistent with those recently reported for AVP modulation of NE-induced cAMP accumulation in isolated rat superior cervical ganglion (Petit et al., 1988). Related data and major issues raised by these findings are discussed below.

Figure 4. Effect of AVP and BAY K 8644 upon NE-induced accumulation of cAMP in 0.0 and 0.8 mM calcium. All conditions occurred in the presence of 10 μM NE to stimulate adenylate cyclase activity. In the presence of 0.0 mM Ca^{2+} , 2.6 mM $MgCl_2$, neither 250 nM AVP nor 5 μM BAY K 8644 singly or in combination affected cAMP accumulation induced by NE. In the presence of 0.8 mM Ca^{2+} , AVP potentiation was restored. BAY K 8644 was again ineffective when present singly but significantly enhanced the potentiating effect of AVP when both were present in the incubation medium. Data were analyzed by 1-way ANOVA followed by a Newman-Keuls test, * = $p < 0.05$ vs. NE alone, $n = 5$ in each condition.

Figure 5. Effect of phorbol 12, 13-dibutyrate, PDBu, upon cAMP accumulation in the hippocampus. PDBu alone induces a 2-fold increase over basal accumulation of cAMP. When NE and PDBu were present in combination, NE/PDBu, cAMP accumulation was additive to the sum of NE alone plus PDBu alone minus basal (since basal cAMP accumulation has contributed twice under the arithmetic addition condition and only once to the condition where NE and PDBu were both present during the incubation). NE concentration, 10 μ M; PDBu concentration, 1 μ M.

Dose-response and peptide specificity

The biphasic character of vasopressin action is not unique to its neuromodulatory effect. An inverted-U shape dose relationship has also been observed in the ability of AVP to induce neurite outgrowth in embryonic neurons growing in culture (Brinton and Gruener, 1987). Lower concentrations of AVP enhanced neurite outgrowth, while higher concentrations markedly inhibited neuritic extension in culture.

The potentiating effect of AVP on NE-induced accumulation of cAMP was specific to vasopressin and not mimicked by oxytocin or by the metabolite peptide AVP₄₋₉. These results do not preclude oxytocin or AVP₄₋₉ from having AVP-like effects at higher concentration. The inability of the metabolite peptide AVP₄₋₉, which has been shown to be behaviorally more potent than AVP (Burbach et al., 1983; DeWied et al., 1984), to modulate NE-induced cAMP accumulation in a concentration range at which AVP itself is effective implies that modulation of cAMP accumulation may not be the basis for these effects which are common to both peptides. There is evidence that AVP₄₋₉ binding sites and putative AVP receptors are found in different places; i.e., putative AVP receptors are found in pyramidal cell layers and dentate gyrus of the hippocampus (Biegon et al., 1984; Brinton et al., 1984), where specific binding for the metabolite peptide is absent. Instead, recognition sites for AVP₄₋₉ are detected only in the hilus of the dentate gyrus (Brinton et al., 1986). Thus, the similarity in the behavioral effects of AVP and its metabolite AVP₄₋₉ may not be due to a convergence upon a common biochemical process but rather to a convergence upon the same anatomical substrate. Such a case could be envisaged where AVP acted directly to influence dentate granule cell activity, whereas AVP₄₋₉ acted indirectly upon dentate granule cells via a feedforward circuit between the polymorph cells of the hilar region and the granule cells of the dentate gyrus (Bayer, 1985). Such a postulate is consistent with the different distribution of recognition sites for AVP and its metabolite peptide, AVP₄₋₉.

Table 4. Effect of trifluoperazine (TFP) upon AVP-induced neuromodulation in the hippocampus

Condition	cAMP/mg protein (pmol)	Percent of NE
A. Basal	56 $\pm$ 7 (10)	27
B. NE	205 $\pm$ 19 (5)	100
C. NE + AVP	291 $\pm$ 6 (5) ^a	142
D. NE + TFP	220 $\pm$ 12 (5)	107
E. NE + AVP + TFP	229 $\pm$ 11 (5) ^b	112

NE concentration, 10 μ M; AVP concentration, 250 nM; TFP concentration, 50 μ M. Values represent means $\pm$ SEMs (*n*). Statistical analysis was by 1-way ANOVA followed by a Newman-Keuls test.

^a *p* < 0.01 vs. NE alone.

^b *p* < 0.01 vs. NE + AVP.

AVP modulation of beta-adrenergic-stimulated adenylate cyclase

Propranolol significantly blocked the cAMP accumulation which occurred in response to both NE and NE + AVP, suggesting the involvement of beta-adrenergic receptors. This hypothesis is supported by the findings of Petit et al. (1988), who found that in the superior cervical ganglion AVP significantly potentiated the cAMP accumulation in response to the specific beta-agonist isoproterenol. These investigators also found that phenolamine, an alpha-adrenergic antagonist, had no effect upon cAMP accumulation induced by NE nor upon the AVP potentiation of NE-induced cAMP accumulation. Taken together, these data support the hypothesis that AVP modulates the cAMP accumulation induced by beta-receptor activation.

Possible synaptic localization of AVP neuromodulation within the hippocampus

Within the hippocampus, AVP neuromodulation of NE-induced cAMP accumulation may occur most prominently in the dentate gyrus. This postulate is based on the observation that binding sites for the V₁ antagonist which blocked the AVP potentiation are localized to the dentate gyrus and do not occur in other regions of the hippocampus (van Leeuwen et al., 1986; R. E. Brinton, H. I. Yamamura, and B. S. McEwen, unpublished observation). The localization of antagonist binding sites, together with the presence of AVP binding sites in Ammon's horn and the dentate gyrus, implies heterogeneity of putative AVP receptors in the hippocampal formation.

Is the effect of AVP on NE-induced accumulation of cAMP a pre- or postsynaptic event? The initial studies of Kovacs et al. (1979a) demonstrated for the first time that the behavioral actions of AVP depended on the presence of NE by showing that lesions of the dorsal noradrenergic bundle abolished the behavioral effects of AVP. These studies did not, however, establish a pre- or postsynaptic site of action. Subsequent work, including the present studies, has supported a postsynaptic mechanism. First and foremost, AVP does not by itself increase cAMP accumulation. A presynaptic regulation of NE release would be evidenced by an increase in cAMP accumulation in response to AVP alone. The lack of effect of AVP alone upon cAMP accumulation has been reported in hippocampal slices (this study; Church, 1983; Newman, 1985), as well as in the superior cervical ganglion (Petit et al., 1988). Moreover, direct measurements indicate that AVP does not alter uptake or release in hippocampal slices (Hagan and Balfour, 1983). Supportive

evidence from autoradiography indicates that ^3H -AVP binding sites are not lost or diminished following 6-hydroxydopamine lesions of the dorsal noradrenergic bundle which markedly reduce NE levels in the hippocampus (R. E. Brinton, Th. A. Voorhuis, and E. R. DeKloet, unpublished observations).

Involvement of V_1 -type receptors in AVP potentiation

Experiments with a peptide antagonist of the V_1 receptor, $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ (Kruszynski et al., 1980), which has also been found to block the behavioral effects of AVP (LeMoal et al., 1981; DeWied et al., 1984) that are dependent on NE (Kovacs et al., 1979a), indicate that a V_1 -type of receptor is involved in the AVP effects on NE-induced cAMP accumulation. Because V_1 receptors involve activation of phosphoinositol (PI) metabolism in the periphery (Bone et al., 1984; Hanley et al., 1984; Kiraly et al., 1986) and in the hippocampus (Stephens and Logan, 1986), it is likely that potentiating effects of AVP on NE-induced cAMP accumulation may involve all or part of this pathway. A similar inference has been made for the ability of AVP in the anterior pituitary to activate PI metabolism through V_1 -like receptors (Raymond et al., 1985) and to potentiate the cAMP response to corticotrophin-releasing factor (Giguere and Labrie, 1982).

Possible mechanisms of calcium dependency of AVP-induced neuromodulation

The AVP modulation of NE-induced cAMP accumulation is dependent on Ca^{2+} ions in the external medium, not only in surviving hippocampal slices (this study) but also in the isolated surviving rat superior cervical ganglion (Petit et al., 1988). The basis for this calcium dependency may be due to an interaction with a pool of calcium/calmodulin-dependent adenylate cyclase (Brostrom et al., 1975). This hypothesis is supported by the inhibition of AVP-induced potentiation by the calmodulin antagonist TFP. While the data supports this hypothesis, it is not proved by the TFP effect since TFP has been found to antagonize protein kinase C (Schatzman et al., 1981; Wrenn et al., 1981). However, the present experiments revealed an important distinction between the effects of AVP and those of protein kinase C stimulation by the phorbol ester PDBu, namely, that AVP alone did not induce cAMP accumulation, whereas PDBu did. This result might be explained by the observations of Nichols et al. (1986), who found that PDBu stimulates the release of NE and other neurotransmitters. In light of these findings and the present data, the additivity of PDBu and NE upon cAMP accumulation suggests that PDBu action may be due to the release of neurotransmitters other than NE which stimulate cAMP formation. Alternatively, PDBu might have an effect via protein kinase C activation upon adenylate cyclase activity, as has been demonstrated in some other systems (Cronin and Canonico, 1985; Nabika et al., 1985; Sugden et al., 1985; Abou-Samra et al., 1987). Regardless of which possibility may apply, the additivity of PDBu and NE upon cAMP accumulation in hippocampal slices suggests an activation of 2 separate compartments of adenylate cyclase, whereas the synergy of AVP with NE suggests a convergence upon the same compartment. In the superior cervical ganglion, where AVP also potentiates NE-induced cAMP accumulation in a calcium-dependent fashion, the addition of a different protein kinase C activator, TPA, had no effect upon NE-induced cAMP accumulation (Petit et al., 1988). Therefore, the weight of the evidence based upon the fact that activation of protein kinase C by PDBu did not mimic the effect of AVP,

and that TFP inhibited AVP-induced potentiation, favors the involvement of a calcium/calmodulin-dependent mechanism.

The other limb of the PI pathway involves generation of the metabolite, IP_3 (Berridge and Irvine, 1984). If V_1 -receptor activation of PI metabolism is involved in AVP potentiation in the hippocampal slice, then how might this mechanism operate in relation to the dependency on external calcium? One possibility is that the primary actions of PI metabolites such as IP_3 , which cause mobilization of internal Ca^{2+} stores, is dependent upon or regulated by external Ca^{2+} levels. Thus, a reduction in external Ca^{2+} could reduce the amount of mobilized Ca^{2+} available to internal compartments, where, for example, adenylate cyclase activation occurs. This possibility is supported by the data of Kirk et al. (1981) and LeBoff et al. (1986), who found that mobilization of internal stores of Ca^{2+} was influenced by Ca^{2+} levels in the external medium. However, in these systems AVP-induced PI turnover does not decline to zero in the absence of external Ca^{2+} . This is in contrast to AVP-induced neuromodulation in the hippocampus (this report) and in the superior cervical ganglion (Petit et al., 1988) and is also in contrast to AVP activation of glycogen phosphorylase in liver (Blackmore et al., 1978). In each of these latter instances, the absence of Ca^{2+} in the external medium results in a loss of AVP-induced effects. Thus, there appear to be events dependent on V_1 -receptor activation in both the periphery and the CNS which are distinguished by their relative versus absolute requirement upon external calcium.

The dependency on calcium ions in the hippocampal slice was biphasic, in that concentrations up to 1 mM facilitated the AVP potentiation effect, whereas a concentration of 2.5 mM no longer facilitated the potentiation. These effects were observed in the presence of a phosphodiesterase inhibitor and might therefore represent a direct effect of calcium to inhibit adenylate cyclase allosterically, as has been described (Hanski et al., 1977). In order for calcium to have this effect in the presence of AVP, whereas it did not inhibit cAMP accumulation with NE alone, it is necessary to postulate an effect of AVP that increases access of internal pools to external calcium concentrations.

These findings collectively raise questions regarding possible additional factors that determine Ca^{2+} flux between the external environment and internal pools. In the present study, experiments in which Ca^{2+} flux was manipulated by the calcium channel activator BAY K 8644 showed that this compound alone had no effect upon NE-induced cAMP accumulation. However, a trend to enhance the AVP potentiation was observed in the presence of BAY K 8644. It should be noted that BAY K 8644 activates Ca^{2+} flux only when the cell is depolarized (Nowicky et al., 1985). Petit et al. (1988) observed a similar trend to potentiate NE-induced cAMP accumulation in the superior cervical ganglion using the calcium ionophore A23187. Thus, we conclude, albeit cautiously, that the weight of evidence favors an AVP-stimulated calcium entry mechanism, potentially involving AVP activation of calcium channels. More work on this topic is clearly required and might best be carried out in cultured cells in which survival is not as great a problem as in the hippocampal slice. Such a postulate is supported by recent studies in sympathetic ganglion cultures showing that AVP increases channel opening (Matsumoto et al., 1987).

In summary, in the hippocampal slice, vasopressin potentiates the accumulation of cAMP in response to NE in a manner that is dependent on calcium in the external medium and that is blocked by the calmodulin antagonist TFP under conditions

in which protein kinase C involvement is a less likely alternative. The weight of evidence supports a postsynaptic V_1 receptor, which is probably involved in activating phosphoinositol metabolism. At this stage, in order to account for the observed dependence on external Ca^{2+} , it can only be speculated that V_1 -receptor activation somehow leads to enhanced Ca^{2+} access and entry, as well as stimulation of PI turnover. Modulating cAMP accumulation via a calcium dependent step provides a pathway whereby it is possible to influence both cAMP-dependent and calcium/calmodulin-dependent protein kinases (see Browning et al., 1985; Cohen, 1985; McGuinness et al., 1985). The capacity to potentially influence both classes of protein kinases, and thus their associated substrates, could impact significantly upon the functional correlates of the hippocampus, including those involved with mnemonic processes.

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