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Vasopressinergic Regulation of the Hypothalamic Pituitary Adrenal Axis and Stress Adaptation

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Vasopressin (VP) stimulates pituitary ACTH secretion through interaction with receptors of the V1b subtype (V1bR, V3R), located in the plasma membrane of the pituitary corticotroph, mainly by potentiating the stimulatory effects of corticotropin releasing hormone (CRH). Chronic stress paradigms associated with corticotroph hyperresponsiveness lead to preferential expression of hypothalamic VP over CRH and upregulation of pituitary V1bR, suggesting an important role for VP during adaptation of the hypothalamic–pituitary–adrenal (HPA) axis to stress. Vasopressinergic regulation of ACTH secretion depends on the number of V1bRs as well as coupling of the receptor to phospholipase C (PLC) in the pituitary. Regulation of V1bR gene transcription may involve a number of regulatory elements in the promoter region, of which a GAGA box was shown to be essential. Although V1bR gene transcription is necessary to maintain V1bR mRNA levels, the lack of correlation between VP binding and V1bR mRNA suggests that regulation of mRNA translation is a major regulatory step of the number of V1bRs. V1bR translation appears to be under tonic inhibition by upstream minicistrons and positive regulation through protein kinase C (PKC) activation of an internal ribosome entry site (IRES) in the 5′ untranslated region (5′UTR) of the mRNA. The data provide mechanisms by which regulation of hypothalamic VP and pituitary V1bR content contribute to controlling HPA axis activity during chronic stress.

Keywords: Corticotroph; Hypothalamic paraventricular nucleus; Vasopressin transcription; V1b receptor; V1b receptor mRNA

INTRODUCTION

In addition to its recognized role in water conservation, the nanapeptide vasopressin (VP) plays a role in stress adaptation acting as a neurotransmitter in the brain (Engelmann *et al.*, 1996) as well as a regulator of pituitary corticotroph function (McCann and Brobeck, 1954; Antoni, 1993; Aguilera, 1994). VP transported from neurons of the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei to the pituitary neural lobe and secreted into the peripheral circulation under osmotic stimulation, is responsible for the antidiuretic effects of the peptide. Magnocellular VP can stimulate ACTH secretion under acute conditions (Holmes *et al.*, 1986; Irvine *et al.*, 1989; Aguilera, 1994) but prolonged activation of magnocellular VP neurons inhibits rather than stimulates the hypothalamic–pituitary–adrenal (HPA) axis (Dohanics *et al.*, 1990; Chowdry *et al.*, 1991; Aguilera, 1994). On the other hand, VP responsible for ACTH secretion is produced in parvocellular neurons of the PVN and secreted into pituitary portal capillaries from axon terminals in the external zone of the median eminence (Antoni, 1993). VP gene expression in parvocellular neurons is responsive to stress and changes in plasma glucocorticoid levels but not to osmotic changes (Aguilera *et al.*, 2000).

While acute stress causes rapid and transient increases in plasma ACTH and corticosterone, chronic stress can lead to habituation of the responses depending on the nature of the stimulus (Dallman *et al.*, 1987; Aguilera, 1994). However, ACTH responses to a novel heterotypic stress are usually enhanced, irrespective of the responses to the persistent homotypic stimulus. There is evidence indicating that VP could contribute to this phenomenon. For example, during chronic stress there is an increase in expression in parvocellular PVN neurons and presumably an increase in the release of VP into the hypophysial

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circulation, as well as an increase in pituitary VP receptors. Also, VP markedly potentiates the stimulatory effects of corticotropin releasing hormone (CRH) on ACTH secretion (Gilles *et al.*, 1982; DeGoeij *et al.*, 1991; Aguilera, 1994).

The actions of VP are mediated by plasma membrane receptors of the guanyl nucleotide binding protein (G-protein) coupled receptor family (Jard *et al.*, 1987; Peter *et al.*, 1995). Molecular cloning and pharmacological tools have identified three major receptor subtypes encoded by different genes; kidney V2 receptors, which are coupled to the G-protein, Gs, and adenylate cyclase; V1a receptors, present in smooth muscle and liver, coupled to phospholipase C (PLC); and the pituitary V1b receptors (V1bR or V3), also linked to PLC; (Lolait *et al.*, 1992; Morel *et al.*, 1992; Seibold *et al.*, 1992; Sumimoto *et al.*, 1994; Lolait *et al.*, 1995). The V1b receptor is responsible for the effects of VP in the pituitary corticotroph and regulation of V1bR expression in the pituitary appears to play an important role in corticotroph responsiveness as suggested by the good correlation between receptor content in the pituitary and ACTH responses (Aguilera, 1994). The isolation of the V1bR cDNA (Sumimoto *et al.*, 1994; Lolait *et al.*, 1995) and the more recent identification and characterization of genomic clones and 5′flanking region have facilitated the understanding of the molecular mechanisms of regulation of the receptor (De Keyser *et al.*, 1994; Ventura *et al.*, 1999; Rabadan-Diehl *et al.*, 2000; Rene *et al.*, 2000). This review discusses current knowledge of the regulation of VP expression in parvocellular neurons of the PVN, as well as the role of pituitary V1bR regulation in controlling the responsiveness of the pituitary corticotroph during stress and other alterations of the HPA axis.

DIFFERENTIAL REGULATION OF HYPOTHALAMIC CRH AND VP DURING STRESS

Acute stress leads to rapid release of CRH and VP into the pituitary portal circulation from parvocellular neurons of the PVN (Berkenbosh *et al.*, 1989; Plotsky, 1991; Kovacs and Sawchenko, 1996). The use of intronic *in situ* hybridization techniques to detect heteronuclear (hn) RNA has revealed concomitant activation of CRH and VP gene transcription but with different temporal patterns. While the increases in CRH gene expression are transient and correlate with the stimulation of ACTH secretion, stimulation of VP gene expression is delayed and more prolonged (Aguilera *et al.*, 2000). In general, the changes in plasma ACTH and corticosterone concentrations parallel changes in CRH gene transcription. For example, desensitization of ACTH responses to repeated restraint is associated with progressive blunting of the CRH hnRNA response in parvocellular PVN neurons, proportional to the frequency of exposure to the stressor, while preserved plasma ACTH concentrations observed after repeated intraperitoneal hypertonic saline injection or a heterotypical stress are accompanied by preserved CRH transcription responses. In contrast to the varying CRH responses, VP hnRNA and mRNA responses to a repeated homotypic stress are preserved or increased, even when there is desensitization of CRH gene responses (Aguilera *et al.*, 2000) (Fig. 1A). The mechanism of the differential transcription responses of the CRH and VP genes remains to be elucidated but it could involve differential activation of signaling pathways by various stressors, as well as the presence of distinct interactions of the promoter of the two genes with glucocorticoid receptors and other transcription factors.

During chronic stress, the content of immunoreactive CRH and VP in nerve endings of parvocellular PVN neurons in the external zone of the median eminence increases in parallel with changes in transcriptional activity. In chronic somatosensory stress paradigms, CRH stores remain unchanged but there is a progressive increase in VP stores and in the number of CRH nerve endings containing VP (DeGoeij *et al.*, 1991). On the other hand, osmotic stimulation has no effect on immunoreactive parvocellular terminals in the median eminence (Fig. 1B).

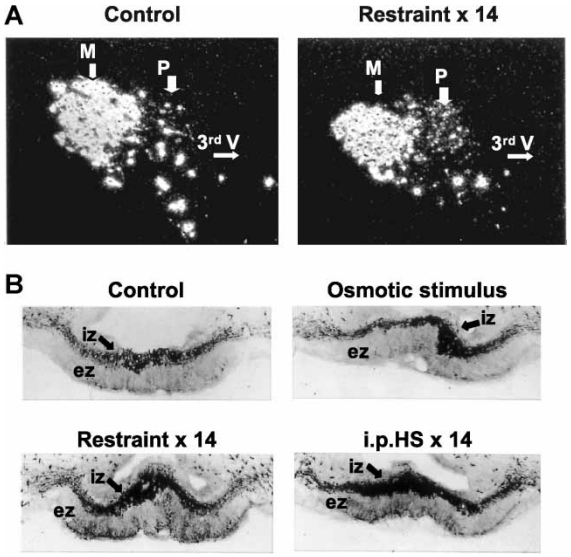

FIGURE 1 Repeated stress increases VP expression in parvocellular neurons. (A) Dark field microphotograph from VP mRNA *in situ* hybridization illustrating changes in VP expression in parvocellular neurons of the PVN in control rats and in rats subjected to 1 h restraint stress daily for 14 days. Parvocellular (P) and magnocellular (M) neurons stained for VPmRNA are indicated by the arrows. Third ventricle (3rd V). (B) Immunohistochemical staining for VP in the median eminence. Computerized semi-quantification of the immunostaining using the public domain program NIH Image (developed at the US National Institutes of Health, and available via the Internet at URL: http//rsb.Info.nih.gov/nih-image) showed changes in immunoactive VP in parvocellular axon terminals of the external zone of the median eminence (ez) following repeated restraint for 1 h for 14 days (restraint × 14) and intraperitoneal hypertonic saline injection for 14 days (i.p. HS × 14). No changes were observed following the predominant osmotic stimulus of water deprivation. Internal zone (iz) of the median eminence containing magnocellular axons.

HYPOTHALAMIC VP EXPRESSION AND GLUCOCORTICOID FEEDBACK

Circulating glucocorticoids exert a negative feedback effect on the expression of CRH and VP in parvocellular PVN neurons (Dallman *et al.*, 1987), but there is evidence of preferential inhibition of VP compared with CRH expression (Ma *et al.*, 1997; Ma and Aguilera, 1999; Kovacs *et al.*, 2000). Glucocorticoids are likely to act at multiple sites, inhibiting VP secretion by direct actions on the parvocellular neuron, and indirectly by modifying the activity of neural pathways to the parvocellular neuron such as GABAergic and glutamatergic afferents (Herman and Cullinan, 1997; Kiss, 1998). Recent studies show that glucocorticoids induce the release of endogenous cannabinoids in the PVN, which can mediate rapid indirect, non-genomic glucocorticoid feedback in the parvocellular neuron (Di *et al.*, 2003). On the other hand, the VP gene promoter contains an active glucocorticoid response element (GRE; Burke *et al.*, 1997) capable of mediating transcriptional inhibition by glucocorticoids. Studies in hypothalamic organotypic cultures containing parvocellular neurons revealed that glucocorticoids inhibit forskolin-stimulated VP hnRNA expression in the presence of tetrodotoxin, which inhibits synaptic transmission (Kuwahara *et al.*, 2003). This indicates that glucocorticoids can directly inhibit VP gene transcription in the parvocellular PVN neuron. While indirect actions of glucocorticoids are likely to mediate most rapid feedback effects of glucocorticoids, direct inhibitory effects on CRH and VP synthesis may account for long term feedback inhibition.

Experiments using adrenalectomized rats and glucocorticoid administration have shown that the parvocellular neuron becomes more sensitive to stress in the absence of glucocorticoids (Makino *et al.*, 1995; Ma and Aguilera, 1999; Kovacs *et al.*, 2000). This suggests that in normal conditions, basal levels of glucocorticoids limit the responsiveness of the parvocellular PVN neuron to stress, serving as a protective mechanism to prevent inappropriate activation of the HPA axis in response to frequent minor stimuli. While glucocorticoid withdrawal sensitizes responses of both CRH and VP genes to stress, exogenous glucocorticoid administration in adrenalectomized rats has differential effects with a preferential inhibition of the VP gene over the CRH gene. Corticosterone markedly decreases the elevated basal VP hnRNA levels in parvocellular PVN neurons observed 48 h or 6 days after adrenalectomy (Ma *et al.*, 1997; Ma and Aguilera, 1999). In 6-day adrenalectomized rats, injection of high doses of corticosterone had no significant effect on CRH transcription, while completely abolishing VP responses (Ma and Aguilera, 1999). Similarly, Kovacs *et al.* (2000) showed that adrenalectomy potentiates CRH and VP transcription responses to ether exposure and has no effect on the kinetics of CRH responses while advancing VP responses. High circulating corticosterone concentration prevented VP gene transcriptional responses to ether but only partially inhibited the ability of the stronger stressor, restraint, to increase VP hnRNA (Ma and Aguilera, 1999). The preservation of VP gene transcription in the presence of elevated glucocorticoid levels is likely to contribute to the high levels of VP expression characteristic of chronic stress paradigms. The molecular mechanisms involved in blunting glucocorticoid inhibition by stress probably involve induction of second messengers by stress-activated neurotransmitters, leading to the production of intermediate early genes or transcription factors capable to interact with glucocorticoid receptors and to modify their activity at the responsive element in the gene promoter (Diamond *et al.*, 1990; Morgan and Curran, 1991; Webster and Cidlowski, 1999). In addition to blunting the inhibitory effect of glucocorticoids on VP gene transcription, additional actions of stress-dependent neural and signaling pathways involving mRNA stability or translation may contribute to the marked increase in VP expression during stress.

VASOPRESSINERGIC REGULATION OF THE PITUITARY CORTICOTROPH

In humans and rodents, VP alone is a weak stimulus of ACTH secretion, but through its interaction with CRH it contributes to maintaining corticotroph responsiveness in spite of elevated glucocorticoid levels during chronic stress (Gilles *et al.*, 1982; Antoni, 1993; Aguilera, 1994). The mechanism of the synergism between CRH and VP includes protein kinase C-(PKC) mediated potentiation of CRH-stimulated cAMP production, as well as post-cAMP dependent mechanisms (Bilezikjian *et al.*, 1987; Carvallo and Aguilera, 1989). In addition, VP could decrease the sensitivity of glucocorticoid feedback by inducing transcription factors with capacity to interact with the glucocorticoid receptor (Diamond *et al.*, 1990; Morgan and Curran, 1991; Webster and Cidlowski, 1999). In contrast to the facilitation of ACTH secretion by VP, the peptide inhibits basal and CRH-stimulated POMC gene transcription *in vitro* (Levin and Roberts, 1991) and *in vivo* (Ochedalski and Aguilera, unpublished). This effect of VP is probably mediated by PKC but its physiological significance for pituitary corticotroph responsiveness is unknown.

An additional mechanism by which VP could influence ACTH secretion is regulation of CRH receptors in the pituitary. *In vitro* experiments have shown that VP increases the number of cells responsive to CRH *in vitro* (Jia *et al.*, 1992), possibly by increasing the number of cells expressing CRH receptors. Although VP is known to potentiate homologous desensitization of the CRH receptor (Aguilera, 1994), low levels of VP seem to be necessary for the upregulation of CRHR-1 expression observed during stress, possibly by enhancing CRH receptor synthesis.

REGULATION OF V1b RECEPTORS DURING STRESS

In most stress paradigms there is a good correlation between changes in pituitary V1b receptor levels and pituitary ACTH responsiveness (Aguilera, 1994). For example, reduced ACTH responses during chronic osmotic stimulation are associated with pituitary VP receptor downregulation, whereas somatosensory stressors leading to ACTH hyperresponsiveness to a novel stress (repeated immobilization or repeated i.p. hypertonic saline injections, an osmotic stimulus with a predominant painful component) are associated with VP receptor upregulation (Aguilera, 1994). In all conditions studied, changes in VP receptors reflect changes in the number of binding sites, with no significant alteration in affinity. This positive correlation between VP receptor content and pituitary ACTH responsiveness suggests that VP receptor regulation plays a role in controlling the capacity of corticotrophs to respond to a novel stimulation during chronic alteration of the HPA axis (Fig. 2A). On the other hand, V1bR mRNA levels do not always correlate with the changes in VP binding (Fig. 2B). In conditions such as chronic osmotic stimulation (water deprivation for 48 h or 2% NaCl in drinking water for 6 days), or after adrenalectomy or glucocorticoid administration, VP binding downregulation is observed in spite of normal or increased V1bR mRNA expression. This suggests that VP receptor content is controlled at the translational and post-translational levels rather than by the levels of V1bR mRNA (Rabadan-Diehl *et al.*, 1995; 1997).

The factors responsible for V1bR regulation probably involve the effects of hypothalamic CRH and VP released into the pituitary portal circulation during stress and the increase in adrenal glucocorticoid secretion. Administration of glucocorticoid reduces the number of pituitary VP receptors but it increases V1b-R mRNA levels. While it is possible that glucocorticoids decrease VP binding by inhibiting V1bR mRNA translation, other factors secreted during stress such as CRH and VP could overcome glucocorticoid inhibition resulting in VP receptor upregulation. In addition to receptor synthesis, the number of active VP receptors could be influenced by ligand binding, leading to receptor desensitization and internalization as occurs with other G-protein coupled receptors (Catt *et al.*, 1979; Ferguson and Caron, 1998). The participation of such mechanisms in the regulation of the V1b receptor remains to be elucidated but they are likely to be involved in the rapid downregulation of VP binding that occurs following acute stress (Rabadan-Diehl *et al.*, 1995).

Receptor activity depends on the coupling of the receptor to signaling transduction systems. It is well recognized that the stimulatory effect of VP on ACTH secretion is relatively refractory to glucocorticoid feedback. *In vivo* and *in vitro* experiments have shown that glucocorticoids increase the coupling efficiency of the V1b receptor to PLC, resulting in enhanced VP-stimulated inositol phosphate formation in spite of downregulation of VP binding. This effect of glucocorticoids is in part mediated by increase in the content of the coupling protein Gq in the pituitary (Rabadan-Diehl and Aguilera, 1998) (Fig. 3). Such an increase in receptor coupling provides an additional mechanism by which VP can contribute to maintaining corticotroph responsiveness in spite of high circulating levels of glucocorticoid during stress.

MOLECULAR MECHANISMS REGULATING V1bR EXPRESSION

The molecular mechanisms regulating the expression of the V1bR has been facilitated by the cloning and characterization of the receptor gene. The gene has three

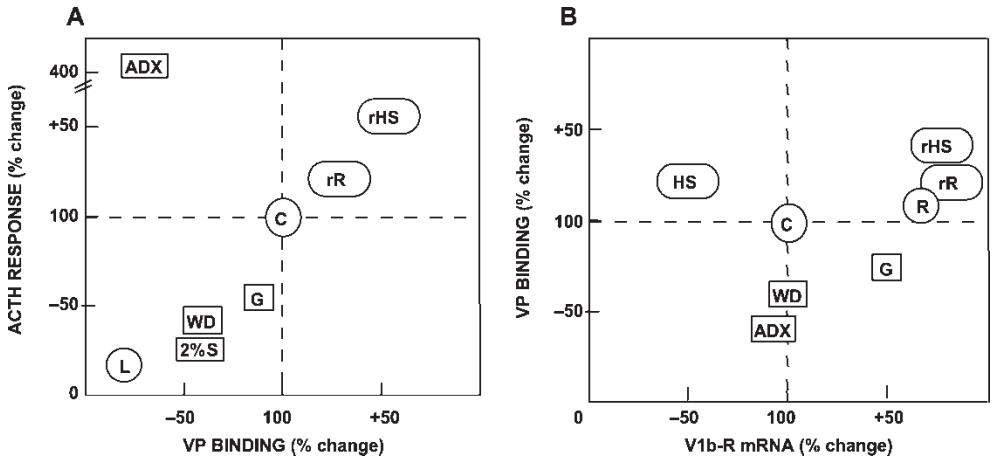

FIGURE 2 (A) Correlation between ACTH responsiveness to a novel stress and changes in VP binding in the pituitaries from rats subjected to experimental manipulations of the HPA axis. With the exception of adrenalectomy (ADX) there is a direct correlation between VP receptor content and ACTH responsiveness. (B) Lack of correlation between the changes in VP binding and changes in V1bR mRNA in the pituitary of rats subjected to chronic manipulations of the HPA axis. Abbreviations: C, controls; ADX, 6 days adrenalectomy; L, lactation; R, single restraint for 1 h; rHS, repeated ip hypertonic saline injection for 14 days; rR, repeated restraint, 1 h per day for 14 days; WD, water deprivation for 60 h; G, glucocorticoid administration for 6 days; 2% S, 2% NaCl to drink for 6 days.

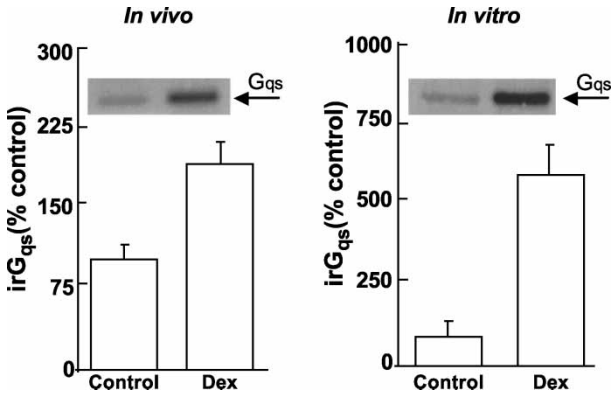

FIGURE 3 Glucocorticoids increase Gqs expression in the pituitary. *In vivo*, rats were subjected to dexamethasone injection (50 μg/day) for 7 days, or, *in vitro*, primary pituitary cells cultures were incubated with 10 nM dexamethasone for 24 h. Levels of Gqs were measured by Western blot.

exons and two introns (Fig. 4). A first short intron (161 bp), is located in the 5′untranslated region (5′UTR) from +124 to +285 bp and a second 8.6 kb is present at the end of the 6th transmembrane domain. Two major putative transcription initiation points (tsp) have been identified, and the proximal tsp was assigned as +1, and the distal −31. No proximal TATA box was detected but several activator protein-1 (AP1) and AP2 sites, a cluster of Sp1 sites upstream of the AP2 sites, and a glucocorticoid responsive element (GRE) have been identified by computer analysis. The region proximal to the tsp contains two unusual elements in G protein coupled receptor promoters, including large stretches of CA repeats (CACA box) and CT repeats (inverted GAGA box) (Rabadan-Diehl *et al.*, 2000).

Transfection of a V1bR promoter-luciferase construct containing 92 bp of exon 2, intron 1, exon 1 and 2161 bp of 5′flanking region, induce transcriptional activation of luciferase gene irrespective of the ability of the cells to express endogenous V1bRs (Rabadan-Diehl *et al.*, 2000; Volpi *et al.*, 2002). Deletion of the first intron abolished promoter activity, suggesting that the intron is essential for basal receptor transcription (Rabadan-Diehl *et al.*, 2000; Volpi *et al.*, 2002). Deletion of sequences upstream of −294 increases promoter activity without changes in transfection efficiency, strongly suggesting that the region between −294 and −2161 bp of the V1bR promoter contains repressor elements (Nomura *et al.*, 2001; Volpi *et al.*, 2002).

TRANSCRIPTIONAL REGULATION OF THE V1bR

The rapid increases in V1bR mRNA expression in the anterior pituitary observed shortly after acute stress strongly suggest that stress activates V1bR gene transcription. Analysis of V1bR promoter activity has revealed that the GAGA box is important for activation of V1bR gene transcription. The dramatic reduction of promoter activity following deletion of 213 bp containing the GAGA box (Volpi *et al.*, 2002), suggests that the GAGA repeats are required for initiation of transcription, similar to observations in the type-1 angiotensin receptor and serine protease inhibitor genes (Simar-Blanchet *et al.*, 1998; Wyse *et al.*, 2000).

The identity of the binding proteins interacting with GAGA repeats in mammalian genes is not yet known. However, transfection of an expression vector for a GAGA binding protein identified in Drosophila (Benyajati *et al.*, 1997) increases V1bR mRNA in H32 or MCF-7 cells, which express endogenous receptor (Volpi *et al.*, 2002)

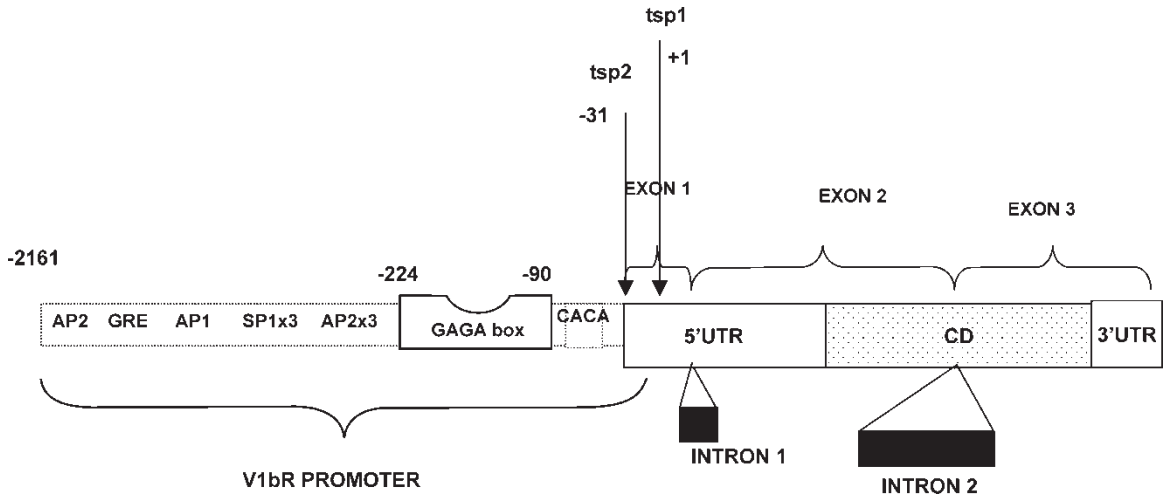

FIGURE 4 Structure of the rat V1bR gene extrapolated from the genomic clone (Rabadan-Diehl *et al.*, 2000) and reported cDNA sequences (Lolait *et al.*, 1995). The three exons are represented by the box and the shaded area shows the coding regions. Two major transcription start points identified by 5′ rapid amplification of cDNA end (5′RACE) and RNA protection assay are indicated by arrows (tsp1 and tsp2). The putative regulatory elements in the V1b receptor promoter determined by computer analysis are depicted upstream of tsp 2. As shown, the promoter contains no TATA box, but AP1 and AP2 sites, a cluster of SP1 sites, a GRE, a GAGA box and a CACA box. Exon 1 and intron 1 and the GAGA box are required for basal promoter activity in transfected cells.

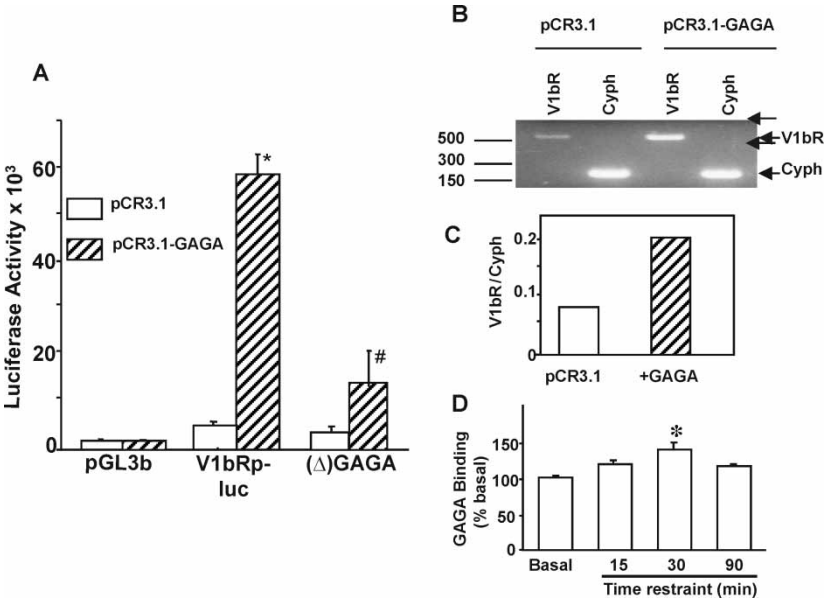

FIGURE 5 GAGA repeats in the V1bR promoter play a role in the transcriptional regulation of the receptor. (A) Transfection of an expression vector for Drosophila GAGA binding protein (pCR3.1-GAGA) increases V1bR promoter activity in the hypothalamic cell line, H32. Cells were co-transfected with empty luciferase vector pGL3 basic (pGL3b) or a luciferase construct driven by 830 bp of the proximal V1bR promoter (V1bRp-luc), or the construct lacking the GAGA box (ΔGAGA). Promoter activity is expressed as percentage of pGL3-basic luciferase activity. $*P < 0.01$ vs. pCR3.1; $\#P < 0.01$ vs. V1bRp-luc co-transfection with GAGA; $n = 3$. (B) Effect of GAGA binding protein on endogeneous V1bR mRNA expression in H32 cells. Cells were transfected with the empty expression vector (pCR3.1) or containing Drosophila GAGA factor. V1bR (V1bR) and cyclophillin (Cyph) mRNAs were measured by semiquantitative RT-PCR. Samples without the reverse transcription step were used as controls to exclude genomic contamination (not shown). (C) Quantification of PCR bands using a KODAK ID Image Analysis system. $*P < 0.05$ vs. cells transfected with the empty vector; $n = 3$. (D) Restraint stress, a condition associated with V1bR mRNA upregulation, increases GAGA binding activity of pituitary nuclear extracts.

(Fig. 5B and C). Drosophila GAGA factor also stimulates V1bR promoter activity when co-transfected with a V1bR promoter-luciferase construct in the same cell lines (Volpi *et al.*, 2002). This increase in V1bR promoter activity by Drosophila GAGA factor is mediated by the GAGA box in the promoter, since the effect was abolished by deletion of the main stretch of GAGA repeats (Fig. 5A). Gel shift assays have shown that pituitary nuclear extracts contain an endogenous protein complex that is able to bind the V1bR GAGA box. The native nuclear protein complex shows an apparent molecular mass of $\sim 127 \pm 12$ kDa after size exclusion chromatography. Treatment of nuclear extracts with deoxycholate abolished GAGA binding activity from all fractions. However, activity was reconstituted by addition of NP-40 to fractions in the 70 kDa range, suggesting that the active complex corresponds to a homo- or heterodimer of proteins of about ~ 70 kDa (Volpi *et al.*, 2002).

Experimental conditions such as restraint stress leading to increases in V1bR expression are associated with parallel changes in GAGA binding activity in nuclear extracts from pituitary (Volpi *et al.*, 2002) (Fig. 5D). The effect of stress appears to be specific for the pituitary since in the same experiments no differences between control and stressed rats were found in the electrophoresis mobility shift assay analysis of liver nuclear proteins. The increase in GAGA binding activity during stress is transient and occurs early (30 min), suggesting the involvement of phosphorylation in the activation of GAGA binding proteins. Recent studies show that transactivation of the EGF receptor by VP mediates rapid increases in GAGA binding activity in nuclear extracts of H32 cells, supporting the proposal that activation of the GAGA binding complex occurs through phosphorylation (Volpi *et al.*, unpublished). The above data suggest that the GAGA box in the V1bR promoter is important for activation of transcription and contributes to the V1bR upregulation during stress.

POST-TRANSCRIPTIONAL REGULATION OF V1bR EXPRESSION

In several experimental conditions, such as chronic osmotic stimulation, adrenalectomy or glucocorticoid administration, V1bR mRNA levels do not correlate with the content of VP receptors in the pituitary (Aguilera, 1994; Rabadan-Diehl *et al.*, 1995). A similar lack of correlation between VP binding and receptor mRNA has been observed for the V1a and V2 VP receptors (Terada *et al.*, 1993; Young *et al.*, 1993). This suggests that receptor synthesis does not depend simply on the presence of mRNA but that mechanisms exist to control translation of V1bR mRNA into receptor protein. Post-translational events such as receptor internalization and recycling to the membrane may also play a role.

Recent studies have focused on the translational regulation of the V1bR, and especially on the role of the 5′UTR (Nomura *et al.*, 2001; Rabadan-Diehl and Aguilera, 2002; Rabadan-Diehl *et al.*, 2003). The V1bR

5′UTR is long, structurally complex, and displays several minicistrons or small open reading frames located upstream (uORF) of the V1bR main ORF. Upstream ORFs have been described in yeast and several eukaryotic mRNAs and are recognized to reduce the ability of ribosomes to linearly scan the V1b 5′UTR mRNA according to conventional scanning models and therefore to slow translation (Kozak, 1991).

Mutations that inactivate the upstream ORFs in the V1bR mRNA have been shown to increase *in vitro* translation of a 46 kDa band corresponding to the V1bR (Rabadan-Diehl and Aguilera, 2002), or luciferase activity using reporter gene constructs (Nomura *et al.*, 2001). The more proximal uORF encodes a putative peptide of 38 aa that can be translated *in vitro* and in cells. The production of *in vitro* translated V1bR protein decreased when a construct containing a mutation of the uORF was co-translated with a construct encoding the 38 aa peptide suggesting that the peptide exerts tonic inhibition of V1bR translation (Rabadan-Diehl and Aguilera, 2002). There is evidence that upstream peptides encoded by uORFs of the mRNAs of the CRH type 1 receptor, β_2-adrenergic and type 1 angiotensin II receptors may also mediate inhibition of translation of the receptor proteins (Parola and Kobilka, 1994; Mori *et al.*, 1996; Xu *et al.*, 2001).

Complex 5′UTRs can not only inhibit translation but can facilitate translation in situations in which cap-dependent translation and mRNA scanning are inefficient. In viruses and in some low abundance eukaryotic proteins, such as G-protein coupled receptors, translation is mediated by ribosome binding to an internal ribosome entry site (IRES) in a cap-independent manner (Sonnenberg, 1996). The complex structure of the V1bR 5′UTR suggested the involvement of an IRES in activating receptor translation. Experiments using bicistronic vector technology showed that insertion of 500 bp of the V1bR 5′UTR between the coding regions of two cistrons, renilla and firefly luciferase, increased luciferase activity of the second cistron when transfected into MCF-7 cells, or in an *in vitro* translation system. The stimulation of translation by the V1bR 5′UTR was preserved after inhibition of cap-dependent translation suggesting that it contains an IRES, capable to stimulate cap-independent translation. The phorbol ester PMA, a PKC activator, increases IRES-mediated translation, suggesting that V1bR upregulation observed during stress may involve IRES activation by PKC-coupled regulators, such as VP itself, the secretion of which increases during stress (Rabadan-Diehl *et al.*, 2003).

In summary, while regulation of V1bR levels in the anterior pituitary involves transcriptional activation, mRNA levels do not determine receptor protein synthesis. The existence of mechanisms capable of inhibiting and activating mRNA translation at the level of the 5′UTR appears to be critical for regulation of V1bR expression (Fig. 6). Minicistrons located upstream of the V1bR open reading frame able to repress translational activity of the main V1bR ORF may account for the low V1bR content in basal conditions. On the other hand, activation of translation through an IRES could mediate the rapid V1bR upregulation observed during stress.

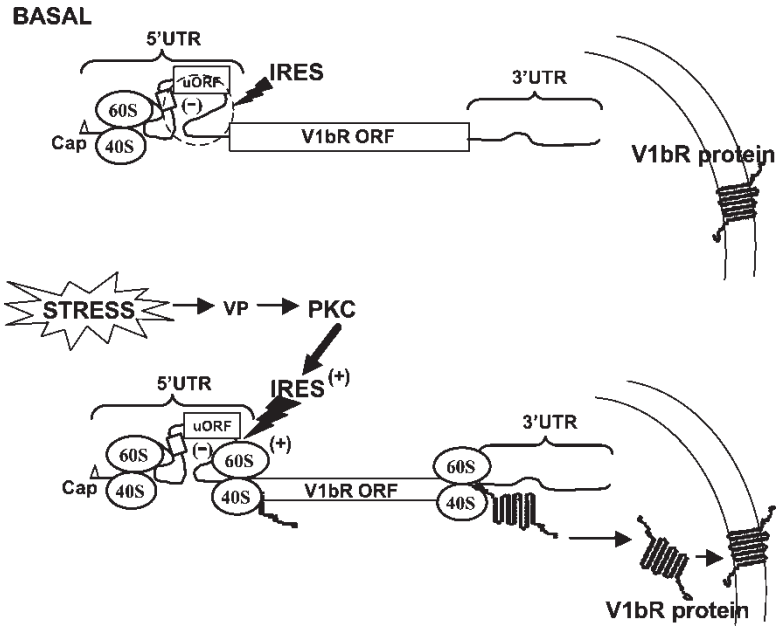

FIGURE 6 Diagram depicting mechanisms for negative and positive regulation of translation by the 5′UTR of the V1bR mRNA. (A) In basal conditions the complexity of the 5′ untranslated region (5′UTR) and the presence of several upstream open reading frames (uORF) could slow-down cap-mediated translation and scanning of the mRNA by the translational apparatus, and account for low levels of translation of the main V1bR open reading frame (V1bR ORF). (B) Activation of neural pathways during stress can initiate translation through protein kinase C (PKC) mediated activation of an internal ribosome entry site (IRES) present in the 5′UTR of the V1bR mRNA. This may mediate positive regulation of V1bR translation during stress.

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