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Arginine vasopressin and somatostatin receptors in rat astrocytes

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Arginine vasopressin and somatostatin receptors in rat astrocytes

By

Nasser Syed

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Toxicology

Program of Study Committee:
Walter H. Hsu, Major Professor
Richard J. Martin
Mary West Greenlee
Arthi Kanthasamy

Iowa State University

Ames, Iowa

2006

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DEDICATION

THIS THESIS IS DEDICATED TO ALLMIGHTY ALLLAH, MY GRAND PARENTS, MY PARENTS, MY WIFE AND MY DAUGHTER

TABLE OF CONTENTS

List of abbreviations

AC	Adenylyl cyclase
AVP	Arginine Vasopressin
cAMP	Cyclic adenosine monoposphate
$[Ca^{2+}]_i$	Intracellular calcium
DAG	Diacylglycerol
ER	Endoplasmic reticulum
GFAP	Glial Fibrillary acidic protein
G-protein	Guanine nucleotide-binding protein
GPCR	G-protein coupled receptor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HIT	Hamster insulin secreting tumor
IP_3	Inositol 1,4,5-triphosphate
mGluR	Metabotropic glutamate receptor
KRB	Krebs-Ringer bicarbonate buffer
PIP_2	Phosphatidylinositide 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PKC	Protein kinase C
SS	Somatostatin
SSTR	Somatostatin receptor

Abstract

We studied the effects of arginine vasopressin (AVP) and somatostatin (SS) on glutamate release and characterized the receptors that mediate the effects of these two peptides from rat astrocytes.

Aginine vasopressin (AVP) acts through specific G protein-coupled receptors and not only induces $[Ca^{2+}]_i$ increase in astrocytes, but also has been shown to regulate astrocytic cell volume changes. Here we report a novel finding that AVP induces glutamate release from astrocytes isolated from the cerebral cortex and hippocampus. We also investigated the type of AVP receptors involved in the AVP-induced increase in glutamate release from astrocytes isolated from the hippocampus and cortex of neonatal rats. We showed that the AVP (0.1 – 1000 nM)-induced increase in glutamate release and $[Ca^{2+}]_i$ is brought about by two distinct subtypes of AVP receptors (V_{1a} & V_{1b}). Our results suggested, that V_{1b} receptors are predominantly expressed in astrocytes isolated from the hippocampus and V_{1a} receptors are predominantly expressed in astrocytes isolated from the cortex of neonatal rats. In addition, the AVP-induced increase in glutamate did not contribute to an increase in $[Ca^{2+}]_i$, since blockade of metabotropic glutamate receptors did not alter the AVP-induced increase in $[Ca^{2+}]_i$. Also the administration of a phospholipase A_2 (PLA_2) inhibitor failed to alter AVP-induced $[Ca^{2+}]_i$ increase, suggesting the lack of involvement of PLA_2.

In the second part of the thesis, we investigated the effects of somatostatin (SS), a $G_{i/o}$-coupled receptor activating hormone on lowering of cAMP level, $[Ca^{2+}]_i$, and glutamate release from neonatal rat astrocyte cultures. Forskolin (10^{-7} to 10^{-5} M) increased glutamate release, cAMP levels and $[Ca^{2+}]_i$. in a concentration-dependent manner; forskolin-induced increase in $[Ca^{2+}]_i$ paralleled the increase in glutamate release, but the increase in cAMP

levels did not. SS alone did not have any effect on basal glutamate release, cAMP levels or $[Ca^{2+}]_i$, but inhibited the forskolin-induced glutamate release and increase in cAMP level in a concentration-dependent manner. Somatostatin also inhibited forskolin induced increase in $[Ca^{2+}]_i$. These effects were mimicked by the selective SSTR4 agonist L-803,087, but not by the selective agonists for SSTR1, SSTR2, SSTR3 or SSTR5. Although the inhibitory effect of SS and L-803,087 (10^{-9} to 10^{-6} M) on forskolin-induced increase in cAMP levels and glutamate release was concentration–dependent, the inhibition of glutamate release, however, was not apparent at $\geq 10^{-6}$ M of the agonists. This was probably due to the ability of SS and L-803,087 to increase $[Ca^{2+}]_i$ at $\geq 10^{-6}$ M. Pretreatment with U-73122, a phospholipase C inhibitor, blocked 10^{-6} M SS-induced increase in $[Ca^{2+}]_i$. Our findings suggest: In astrocytes, 1) SS inhibits forskolin-induced glutamate release by decreasing cAMP levels and at least partly by reducing $[Ca^{2+}]_i$. 2) SSTR4 mediates SS-induced decrease in cAMP levels, $[Ca^{2+}]_i$ and glutamate release. 3) SS at high concentrations ($\geq 10^{-6}$ M) may increase glutamate release by activating phospholipase C pathway. SS or SSTR4 agonists could be used to reduce glutamate release from astrocytes.

Chapter 1. General Introduction

Thesis Organization

This thesis is written in an alternative thesis format. It contains a general introduction including literature review and a list of references cited, two manuscripts and their conclusions along with acknowledgements.

Literature Review

Astrocytes:

Astrocytes (Fig. 1) are a type of glial cells of the central nervous system that were thought of as relatively uninteresting support cells of the central nervous system (Virchow, R., 1846). Since then studies have shown that astrocytes are complex and play a very important role in regulating and maintaining optimal conditions for the nervous system to function. Their role as regulators of brain homeostasis is just a part of their repertoire of many complex functions. To date, astrocytes are vital not only for the proper development of neurons, but also influence many aspects of neuronal functions (Nedergaard et al., 2003). Current studies also show that astrocytes may be involved in modulating synaptic transmission, CNS plasticity and brain homeostasis (Chen et al., 2000).

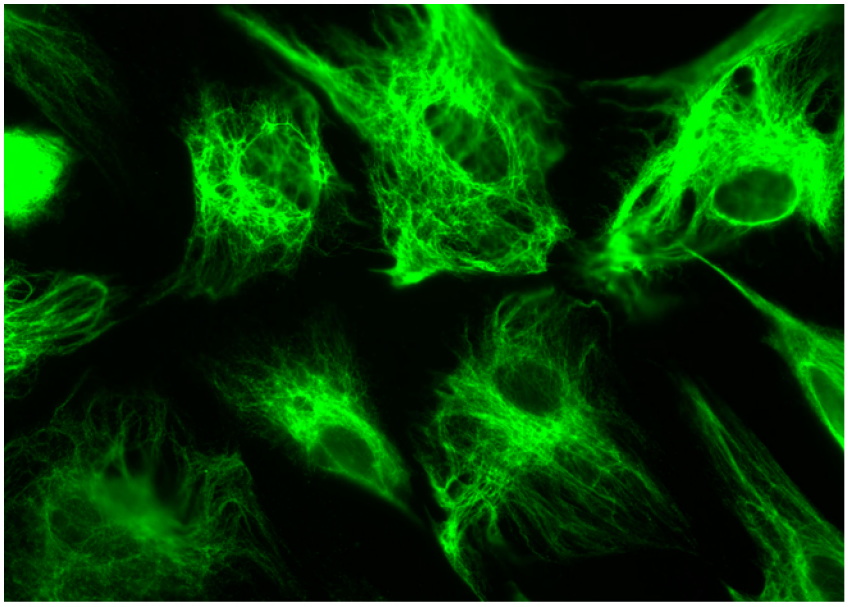


Figure 1. Image of Glial Fibrillary Acidic Protein (GFAP) expressing astrocytes. GFAP is an intermediate filament protein of 52kD found in glial cells such as astrocytes and ependymal cells and is used as a marker to identify astrocytes.

Arginine vasopressin:

Arginine vasopressin (AVP) is a nonapeptide (Table 1) with two cysteine residues in position 1 and 6 linked by a disulfide bridge (Barberis et al., 1998). The amino acids in positions 5 (asparagine), 7 (proline) and 9 (glycine) are conserved (Table 1). In all the mammals except swine the 8th amino acid is arginine, where as in swine and marsupials there is a lysine in the 8th position. AVP is synthesized by the magnocellular neurons of the hypothalamus in the para ventricular nucleus (PVN) and supraoptic nucleus (SON) (Swaab et al., 1975). AVP is transported (in its prohormone state with 168 amino acids) along the supraoptic–hypophyseal tract to the posterior pituitary (Fig. 2), from where it is released into the circulation. The preprohormone is processed to remove the signal peptide (residues -23 to -1) and is translocated as the prohormone through the rough endoplasmic reticulum to be incorporated into large membrane enclosed granules. The prohormone consists of three domains: VP (residues 1-9), VP-neurophysisn (residues 13-105) and VP glycopeptide (residues 107-145). In the secretory granule the prohormone is further processed to produce VP. Furthermore, significant levels of AVP have also been reported in cerebral cortex and hippocampus (Toide et al., 1995) pointing at other possible sources of AVP synthesis within the brain. AVP synthesis outside the hypothalamic-neurohypophyeal system has been reported in the heart (Hupf et al., 1999), pancreas (Méchaly et al., 1999) and pulmonary artery (Loesch et al., 1991). The magnocellular neurons of the SON are depolarized by hypertonic conditions (more AVP released) and hyperpolarized by hypotonic conditions (less AVP released).

The pressor effects of AVP were first observed in 1895 (Oliver et al., 1895) and was attributed to the pituitary extract from the posterior lobe of the pituitary gland. The

antidiuretic effects of the pituitary extract were first demonstrated in 1913 (von den Velden, 1913). AVP was isolated and synthesized in the 1950s and both the pressor and antidiuretic effects were attributed to the same hormone (Turner et al., 1951; Du Vigneaud et al., 1954).

AVP has been shown not only to regulate body fluid volume, osmolality and blood pressure, but also act as a neurotransmitter (Craig et al., 1997), regulate cell volume (Sarfaraz, 1999), plasma volume (Hayashi et al., 2006), potassium ion balance (Martha O'Donnell, 2005), chloride ion efflux (Good, 1990) and cognitive and behavioral functions and memory processes (Bielsky et al., 2003). In addition, AVP has also been reported to inhibit glutamate release from neurons of nucleus tractus solitarius (Bailey et al., 2006).

Hormone	Position of amino acid residues
Ancestral Molecule	Cys^{1}-Tyr^{2}-X^{3}-X^{4}-Asn^{5}-Cys^{6}-Pro^{7}-X^{8}-Gly^{9}-(NH_2)
Arginine vasopressin	Cys^{1}-Tyr^{2}-Phe^{3}-Gln^{4}-Asn^{5}-Cys^{6}-Pro^{7}-Arg^{8}-Gly^{9}-(NH_2)
Lysine Vasopressin	Cys^{1}-Tyr^{2}-Phe^{3}-Gln^{4}-Asn^{5}-Cys^{6}-Pro^{7}-Lys^{8}-Gly^{9}-(NH_2)

Table 1. Amino acid sequence of vasopressin (AVP) (modified from Hadley, 1992).

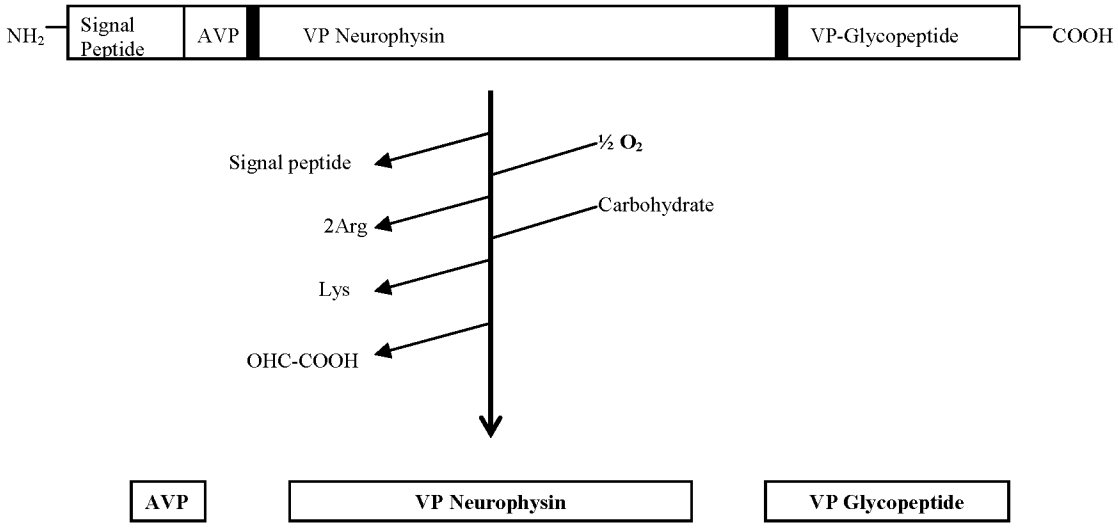


Figure 2. Processing of the 168-amino acid prepro-AVP to AVP, VP-Neurophysin and VP-Glycopeptide (modified from Pickering, 1995).

AVP release: Several factors influence AVP release and can be broadly classified as

Osmoregulation: Osmoreceptors in the hypothalamus regulate AVP release. Increases in plasma osmolality cause shrinkage of the osmoreceptor cells, which in turn alters the electric activity of the neurons and stimulates AVP release. The released AVP causes antidiuresis and makes urine more concentrated.

Volume regulation: Stretch receptors (baroreceptors) in the left atrium can sense the decrease in plasma volume and stimulate the release of AVP by reducing the tonic inhibitory pulses from the left atrium to the hypothalamus. The neural impulses reach the SON and PVN through the vagus nerve and stimulate AVP release into the circulation.

Baroreceptor regulation: Activation of carotid and aortic stretch receptors (baroreceptors) in response to hypotension also causes release of AVP. Hypotension-induced stimulus can raise the plasma concentration of AVP to 1000 times the normal levels and cause vasoconstriction.

Apart from the above mentioned stimuli for AVP secretion, cholinergic and beta-adrenergic stimulation of hypothalamus can increase AVP secretion while alpha 2-adrenergic

stimulation can inhibit AVP secretion. Emotional stress and pain may also increase AVP secretion (Alexander et al., 1988).

G-proteins:

The actions of AVP are mediated (Fig. 4) through specific receptors of the class G-protein coupled receptors (GPCRs). Guanine nucleotide binding proteins (G-proteins) are a family of proteins involved in second messenger signaling cascades and are found predominantly at the inner aspect of the plasma membrane. They form the link between the receptor and down stream effectors. G-proteins fall into two distinct classes – heterotrimeric G-proteins and monomeric G-proteins. The exchange of GDP for GTP activates the G-protein.

Heterotrimeric/large G-proteins are comprised of three different molecules (subunits) α, β and γ. The α-subunit has the binding site for GDP and GTP and hence acts like a regulator. GTP bound α-subunit is the active form, which dissociates from the βγ subunits and the GDP bound α-subunit is tightly associated with the βγ subunits. Based on sequence and functional similarities of the α-subunit, heterotrimeric G proteins can be classified into 4 families – G_s, $G_{i/o}$, G_q and $G_{12/13}$ (Simon et al., 1991). The G_s α subunit can stimulate adenylyl cyclase (Taussig et al., 1995). The $G_{i/o}$ family α subunits can inhibit adenylyl cyclase, while the α-subunit of the transducin, which also belongs to G_i family can stimulate cGMP-phosphodiesterase (Taussig et al., 1995). The α subunit of G_q family can stimulate phospholipase Cβ and G_{12} has been shown to activate several GTPases Ras, Rac, CDC42, and Rho (Radhika et al., 2001).

The monomeric G-proteins, usually referred to GTPases, are comprised of a single unit and are not directly coupled to the receptors. Their functions are similar to α subunit of

heterotrimeric G proteins. Normally they are bound to GDP and inactive and their activation is usually several steps downstream to the receptor activation. To date, about 150 eukaryotic small G proteins have been identified and divided into five families – Ras, Rho, Rab, Sar1/Arf and Ran (Takai et al., 2001). Ras and Rho families are involved in gene expression, Rho proteins regulate cytoskeleton reorganization, Rab and Sar1/Arf proteins influence vesicular transport. Ran has been shown to regulate nuclear transport and the cell cycle (Marcin et al., 2001).

AVP receptors structure, distribution and function:

Three types of receptors for AVP have been identified and are classified as V_{1a} (V_1), V_2 and V_{1b} (V_3) (Thibonnier et al., 1998). These receptors are GPCRs comprised of typical seven transmembrane α helices joined by intracellular and extracellular loops.

Gene for the V_{1a} (also called as V_1) maps to chromosome 12 (Thibonnier et al., 1996) and the V_{1a} receptor activates G-proteins of the $G_{q/11}$ family. V_{1a} receptors are widespread and are found in brain, testis, liver, cervical ganglion, liver, blood vessels and renal medulla (Phillips et al., 1990).

Gene for V_{1b} (also called as V_3) receptor maps to chromosome 1(Sugimoto et al., 1994; Rousseau et al., 1995). V_{1b} receptor acts through $G_{q/11}$ family of G-proteins and has been reported to be present in anterior pituitary (Thibonnier et al., 1998) and pancreas (Lee et al., 1995). V_{1b} receptor mRNA has also been reported in other peripheral tissues (kidney, thymus, heart, lung, uterus, breast) as well as in the anterior pituitary (Lolait et al., 1995).

Gene for V_2 receptor maps to chromosome 10. V_2 receptors, which act through G-proteins of the G_s class, have been reported to be present in the renal collecting tubules (Birnbaumer, 2000). The V_2 receptor differs from the V_1 (V_{1a} & V_{1b}) class of receptors in the

number of sites available for N-linked glycosylation; the V_1 (V_{1a} & V_{1b}) receptors have sites at both the amino-terminus and at the extracellular loop, whereas the V_2 receptor has a single site (Fig.3a & 3b) at the extracellular amino-terminus (Innamorati et al., 1996).

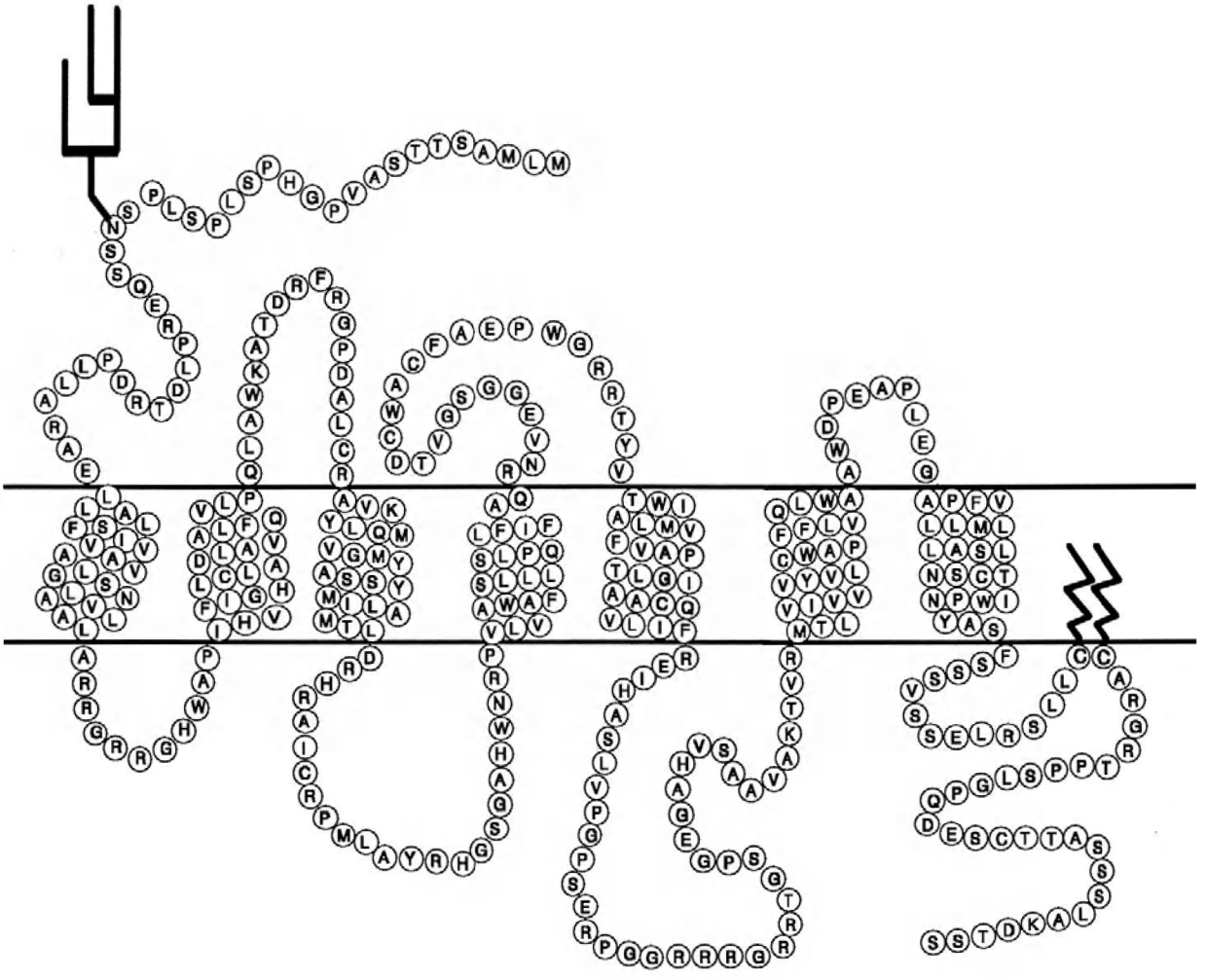


Figure 3a. Human V_2 receptor transmembrane topology showing the single site of N-linked glycosylation in the amino terminus (modified from Sadhegi et al. 1997)

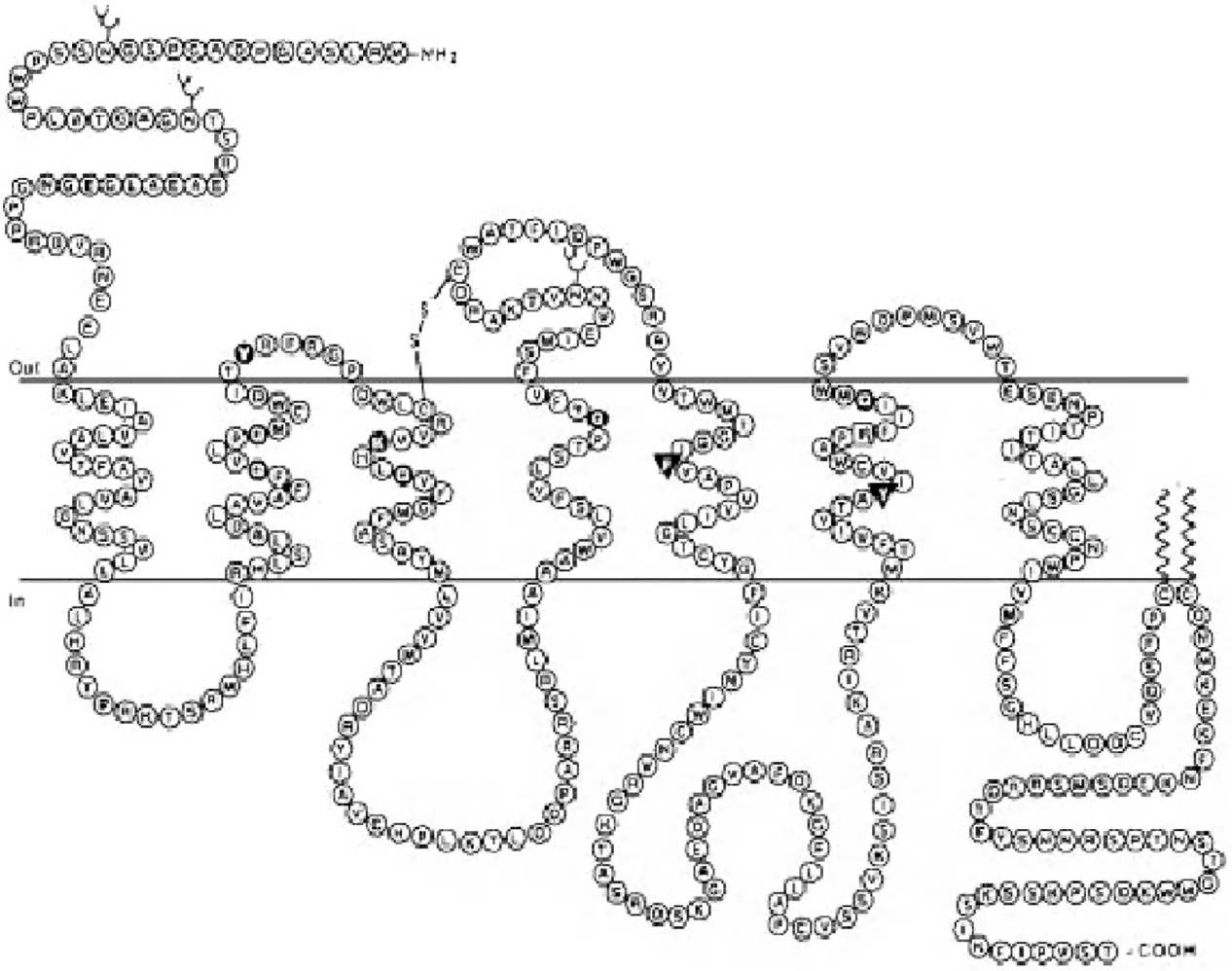


Figure 3b. Human V_{1a} receptor transmembrane topology showing sites of N-linked glycosylation in the amino terminus (modified from Barberis et al., 1998).

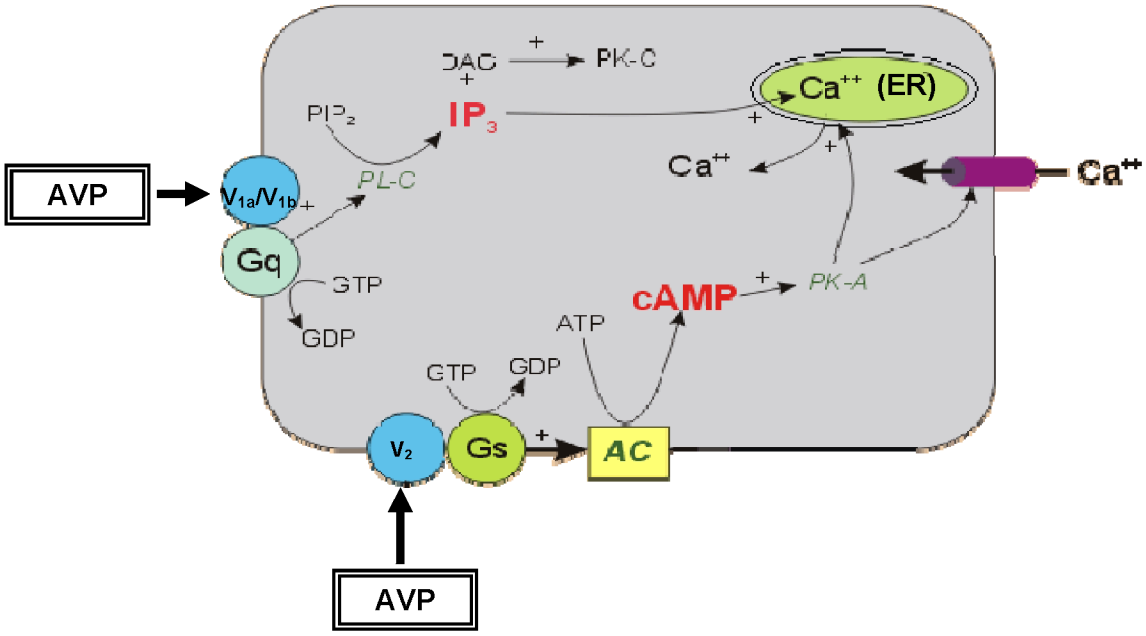


Figure 4. G_s and G_q signaling mechanism for AVP receptors.

ATP: Adenosine triphosphate, AC: Adenylyl cyclase, AVP: Arginine vasopressin, Ca^{++}: Calcium, cAMP: Cyclic adenosine monophosphate, DAG: Diacyl glycerol, ER: Endoplasmic reticulum, G_q: G_q coupled GPCR, G_s: G_s coupled GPCR, GTP: Guanosine triphosphate, GDP: Guanosine diphosphate, PKA: cAMP dependent protein kinase, PIP_2: Phosphatidyl bisphosphate, PLC: Phospholipase C.

G-protein mediated AVP action:

Binding of AVP with V_{1a}/V_{1b} receptors activates phospholipase C (PLC) (Barberis et al., 1998). Activation of PLC leads to hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP_2), which generates the second messengers inositol 1,4,5-tris-phosphate (IP_3) and diacylglycerol (DAG). IP_3 acts on its receptors on the endoplasmic reticulum leading to Ca^{2+} release from this organelle, while DAG activates protein kinase C (PKC) (Martin et al., 2004). The Ca^{2+} release activates Ca^{2+} release-activated Ca^{2+} current channels (ICRAC) on the plasma membrane (Mathes et al., 1998) leading to a sustained Ca^{2+} influx (Putney, 1990). Stimulation of V_{1a} receptors located in the vascular smooth muscle causes vasoconstriction,

whereas V_{1b} has been shown to regulate ACTH release by potentiating the effects of CRH (Aguilera et al., 2000), mediate insulin (Lee et al., 1995) and glucagon (Yibchok-anun et al., 1998) release from pancreatic islets.

Binding of AVP to V_2 receptors activates the adenylyl cyclase pathway, leading to increase in intracellular cAMP levels (Birnbaumer, 2000). The increased intracellular cAMP in the kidney (Orloff et al., 1967; Dousa et al., 1972) in turn triggers fusion of aquaporin-2-bearing vesicles with the apical plasma membrane of the collecting tubule, leading to increased water reabsorption (Harris et al., 1994).

AVP receptor antagonists:

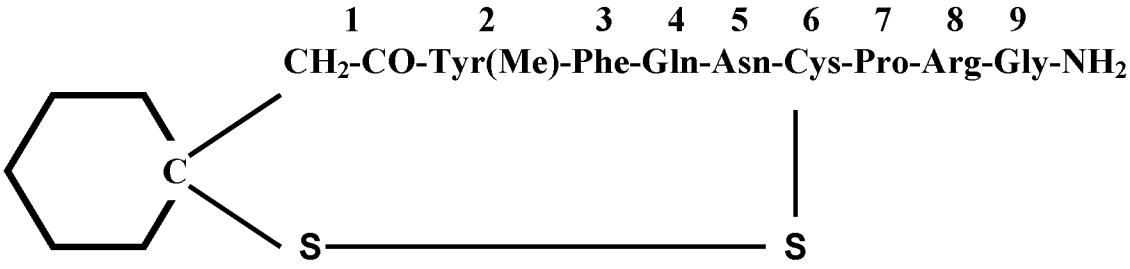
Several selective antagonists against V_{1a}, V_{1b} and V_2 have been synthesized ever since the structure of AVP had been deciphered (du Vigneaud et al., 1954). The antagonists (fig. 5) are available as both peptide and nonpeptide derivatives. The peptide antagonists are both cyclic and linear peptides. [1-(β-mercapto-β,β-cyclopentamethyleneproprionic acid),2-O-methyltyrosine]AVP also known as $d(CH_2)_5[Tyr(Me)^2]AVP$ (pA_2 = 8.62) (Manning et al., 1989) and $desGly^9d(CH_2)_5[Tyr(Et)^2]AVP$ (WK-3-6) (pA_2 = 8.17) (Jard et al., 1992) are potent V_{1a} receptor antagonists.

These antagonists are widely used in pharmacological studies but highly specific peptide antagonists for V_{1b} receptors are still not available currently. [1-deaminopenicillamine, 2-O-methyltyrosine]AVP ($dP[Tyr(Me)^2]AVP$) is the most potent V_{1b} antagonist (Manning et al., 1989) with a K_i of 9.7 nM. It also shows low affinity for V_2 receptors and also blocks V_{1a} receptors (pA_2 = 7.96) (Schlosser et al., 1994).

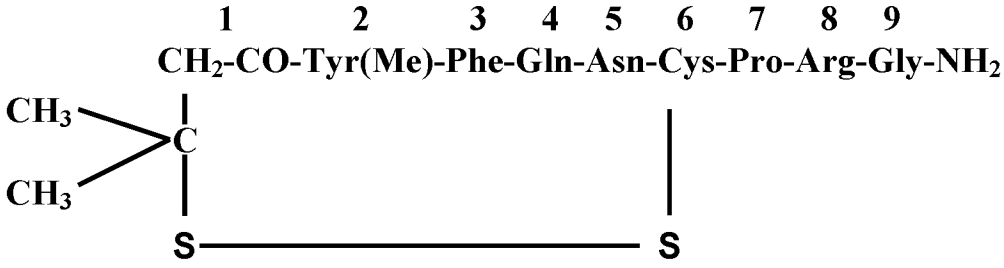
4-OHphenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL4-84) is a linear peptide antagonist that has a high affinity for V_{1b} receptor (K_i = 2.2 $\pm$ 0.1 nM) (Thibonnier et al., 1997). It also has potent anti V_{1a} (pA_2 = 8.74) activity (Jard et al., 1986).

$d(CH_2)_5$[D-Phe^2, Ile^4]-AVP (AO-2-44) is another potent peptide antagonist for $V_2/V_{1a}/V_{1b}$ receptors with pA_2 values of 7.83 and 8.16 for anti-V_{1a} and anti-V_2 respectively (Jard et al., 1986).

Several non-peptide antagonists (Fig. 6) have also been designed and are available for studies. ((2S, 4R)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxyphenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-N,N-dimethyl-2-pyrollidine carboxamide, isomer(-)) (SSR-149415) is a highly specific nonpeptide V_{1b} receptor antagonist (K_i = 1.3 to 5 nM) (Serradeil Le-Gal et al., 2002). (2S) 1-[(2R 3S)-(5-chloro-3-(2-chlorophenyl)-1-(3,4-dimethoxybenzene-sulfonyl)-3-hydroxy-2,3-dihydro-1H-indole-2-carbonyl]-pyrrolidine-2-carboxamide (SR-49059) (K_i = 1.6 $\pm$ 0.2 nM) is highly specific nonpeptide V_{1a} receptor antagonist (Serradeil Le-Gal et al., 1993). (1-[4-(N-tert-Butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]indoline-2-one, phosphate monohydrate; cis-isomer) (SR-121463B) is a selective nonpeptide antagonist for V_2 receptors (Viviane et al., 2003; Serradeil Le-Gal et al., 1996).

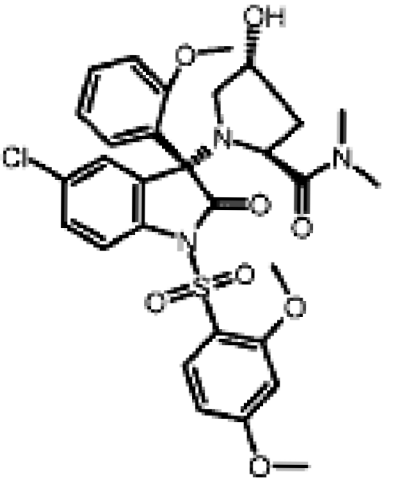


(A)

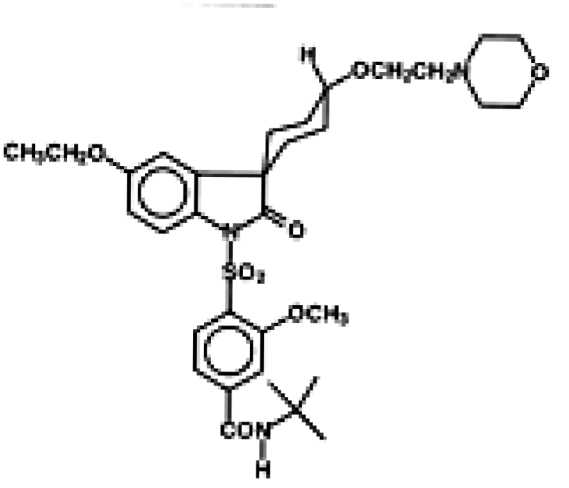


(B)

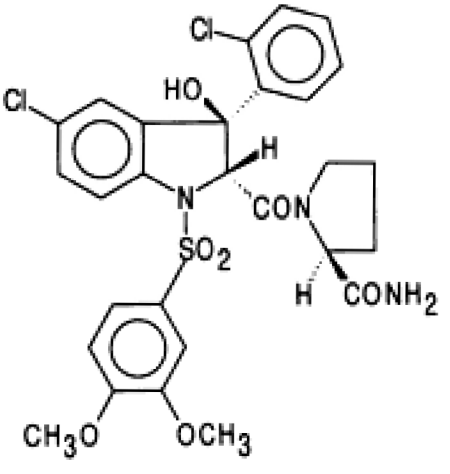
Figure 5. Structure of: AVP receptor antagonists A) d(CH2)5[Tyr(Me)2]AVP; B) dP[Tyr(Me)2]AVP (modified from Manning and Sawyer, 1989).



SSR-149415



SR-121463



SR-49059

Figure 6. Structure of nonpeptide AVP receptor antagonists (Modified from Serradeil-Le Gal et al. 2002, 1996, 1993).

Glutamate:

Glutamate was first proposed as a neurotransmitter by Hayashi (1954) and was shown to depolarize and excite central neurons (Curtis et al., 1959). Over the years glutamate has been shown to be a major excitatory neurotransmitter in brain (Lebon et al., 2001) and major pools of glutamate are concentrated in glutamatergic neurons (Shepherd, 1994) and astrocytes (Nicholls, 1989). The metabolism of glutamatergic neurons and astrocytes are coupled through a glutamate-glutamine cycle (Fig. 7), where in some of the glutamate released from nerve terminals, is transported into the surrounding glial cells. The glutamate is converted to glutamine which is then transported out of the glia and absorbed into neurons, where it is converted back to glutamate (Rothman et al., 2003). Apart from the glutamate taken up from the synaptic clefts, astrocytes utilize glucose to synthesize glutamate (Aureli et al., 1997). Interruption of this exchange leads to impairment of glutamatergic neurotransmission (Keyser et al., 1994). Astrocytes express the highest density of glutamate transporters (Huang et al., 2004) and hence take up a high quantity of synaptic glutamate (Magistretti et al., 1999) and regulate extracellular glutamate levels, thus protecting the neurons from glutamate toxicity. Furthermore, glutamate has also been shown to induce astrocytic swelling (Hansson et al., 1994).

Glutamate receptors:

Glutamate mediates its effects through specific receptors and these receptors fall under two major subgroups – ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). iGluRs directly gate ion channels, while mGluRs are coupled to other intracellular messenger systems and may also activate ion channels. The ionotropic

glutamate receptors are further classified into two groups based on whether they bind to the glutamate analogue N-Methyl-D-Aspartate (NMDA). The non-NMDA receptors are further divided into groups based on the selectivity by the agonists alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), Kainate and quisqualate. NMDA receptors, upon glutamate binding, open up nonselective cation channels, which are more permeable to Ca^{2+}. Non-NMDA receptors upon glutamate binding, open nonselective cation channels, which are more permeable to Na^{+} and K^{+} (Mayer et al., 1987). Metabotropic glutamate receptors are further classified (Table 2) into three groups (Group I, II & III) (Knopfel et al., 1995). Group I metabotropic glutamate receptors are coupled G_q class of GPCRs and its stimulation leads to increase in IP_3 and release of Ca^{2+} from endoplasmic reticulum. Group II and group III receptors are coupled to $G_{i/o}$ class of GPCRs which, when activated, decrease cAMP by inhibiting adenylyl cyclase (Pin et al., 1995). The Group II and Group III receptors are classified based on their agonist selectivity.

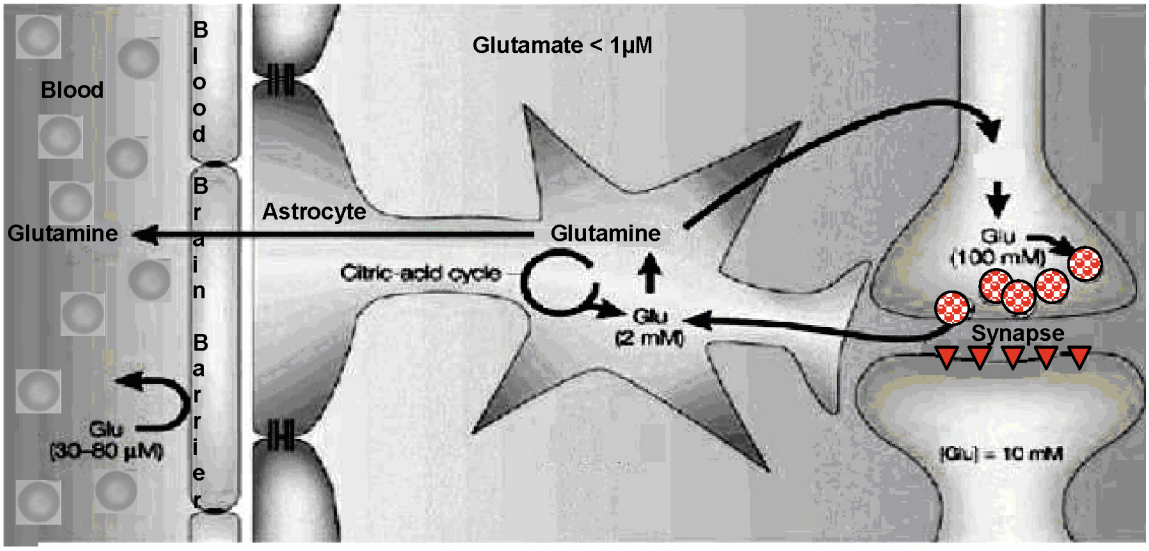


Figure 7. Astrocytes – Glutamate homeostasis (Modified from nature reviews).

Group	mGluR	Signal Pathway
I	**mGluR1** **mGluR5**	G_q
II	**mGluR2** **mGluR3**	$G_{i/o}$
III	**mGluR4** **mGluR6** **mGluR7** **mGluR8**	$G_{i/o}$

Table 2. Metabotropic glutamate receptors family and their functions.

Receptor subtypes	Species	Organ	Tissue/Cell type
GluR 2/3, Ka 2, NMDAR 1, mGluR 5, mGluR 2/3, mGluR 1	Rat/monkey	Heart	Atrium/septum conducting fibers, ganglia cells, nerve fibers, myocardiocytes, intercalated discs, blood vessels
GluR 2/3, Ka 2, NMDAR 1, mGluR 2/3	Rat/monkey	Ovary	Corpus luteum, primordial follicles, theca, granulosa cells, oocyte, blood vessels, nerve fibers
GluR 2/3, Ka 2, NMDAR 1, GluR 2/3	Rat/monkey	Uterus	Exocervix, myometrium, endometrial glands, epithelium of fallopian tubes, nerve fibers
GluR 2/3, Ka 2, NMDAR 1, mGluR 2/3	Rat	Kidney	Glomeruli, mesangium, podocytes, juxtaglomerular apparatus, tubules
GluR 2/3, Ka 2, NMDAR 1, mGluR 2/3	Rat	Testis	Germinal epithelium, interstitial cells
GluR 2/3, Ka 2, NMDAR 1, mGluR 2/3	Rat	Gastrointestinal	Enteroendocrine cells, parietal cells of the stomach, pancreatic islets, nerve fibers, ganglia cells, liver
GluR 2/3, Ka 2, NMDAR 1, mGluR 2/3	Rat	Others	Lungs, spleen, bone marrow (megakaryocytes), mast cells, inflammatory cells

Table 3. Distribution of glutamate receptors in peripheral tissue (Modified from Gill et al., 2001).

Somatostatin synthesis and release:

Since its unexpected discovery in 1972 (Brazeu et al., 1973) somatostatin (SS) has not only been reported to inhibit the release of growth hormone (Krulich et al., 1969), but also inhibit the secretion of endocrine pancreas (Skamene et al., 1984; Yao et al., 2001).

SS is synthesized as a preprohormone from the long arm of chromosome 3 (Patel et al., 1999) and the preproSS is further processed into a 14 amino-acid and a 28 amino-acid (SST-14 and SST-28) SS (Ganong, 2001). There is a considerable variation in the amount of SST-14 and SST-28 found in various tissues with SST-14 being exclusive form in enteric neurons and peripheral nerves in the retina and SST-28 being the dominant form in neural tissue, endocrine pancreas and the stomach (Patel et al., 1999).

The processed SS is released through an exocytotic pathway (Patel et al., 1999) from the neurons and δ cells of the pancreatic islets. SS release can be stimulated by growth hormone-releasing hormone, corticotropin-releasing hormone and neurotensin through G-protein coupled receptors (Patel et al., 1999). Gamma aminobutyrate (GABA) can inhibit SS release through both the ionotropic $GABA_A$ (hyperpolarization and inactivation of L-type VDCC) and $G_{i/o}$-coupled $GABA_B$ receptors. In the pancreas, SS release can be stimulated by glucagon, glucose, lipids and amino acids (Patel, 1999).

Somatostatin receptors:

Five receptor subtypes have been reported through which SS mediates its effects (Bruno et al., 1992, Yamada et al., 1992). All these receptors are GPCRs, sensitive to pertussis toxin (PTX) and are $G_{i/o}$ coupled (Patel, 1999) with a good degree of conservation among species (Reisine et al., 1995). The SSTR2 is subdivided into SSTR2A and SSTR2B due to the difference in the length of the cytoplasmic tail with the SSTR2A having a longer

cytoplasmic tail than SSTR2B. SSTR1-SSTR4 show a similar binding affinity towards both SST-14 and SST-28, but SSTR5 shows higher binding affinity to SST-28 (Patel et al., 1994; 1995; 1996). All 5 subtypes of SSTRs are expressed in pancreatic islets and stomach (Patel 1999; Weckbecker et al., 2003). SSTR1 and SSTR2 have been reported in the kidney and all subtypes have been reported in liver, lymphocytes, T cells, lungs and lymphoid tissue (Patel 1999; Weckbecker et al., 2003). SSTR1, 2, 3 and 4 have been reported in brain (Patel 1999; Bruns et al., 2003) with pituitary gland expressing SSTR1-3 and SSTR5 (Patel 1999; Weckbecker et al., 2003). Presence of multiple receptor subtypes in a given tissue and in different tissues suggests different physiological responses mediated by the different receptor subtypes.

Changes in SS binding sites have been associated with a number of pathological conditions. At the pituitary level, the number of SS binding sites has been negatively correlated in case of acromegaly and gliomas are rich in SS binding sites (Epelbaum et al., 1989). In neurodegenerative diseases such as Alzheimer's and Parkinson's, cortical SS concentrations are decreased and there is also a decrease in SS-binding sites in Alzheimer's disease (Kumar, 2005).

Actions of somatostatin:

The major actions of SS are antiproliferative (Patel et al., 1999), inhibition of hormone secretion - growth hormone from the pituitary and also inhibition of secretions from various organs (Bertherat et al., 2003). In the central and peripheral nervous system, SS acts as a neurotransmitter, inhibiting the release of other neurotransmitters and hormones such as glutamate (Dal Monte et al., 2003; Grilli et al., 2004)), dopamine, norepinephrine, thyrotropin releasing hormone and thyroid stimulating hormone (Gray et al., 1989; Patel,

1999). SS can also inhibit secretions of the gastrointestinal tract and pancreas, such as gastrin, vasoactive intestinal peptide, insulin, glucagon, gastric acid, pepsin and bile salt (Ganong, 2001; Patel, 1999).

SSTRs upon activation inhibit adenylyl cyclase activity (Patel et al., 1994) through α subunit of $G_{i/o}$, thus lowering the cAMP levels. However, at high agonist concentrations, human SSTR5 expressed in COS-7 (Akbar et al. 1994) or CHO-K1 cells (Carruthers et al. 1999) can also cause an activation of adenylate cyclase. SSTRs can also activate phospholipase A_2 (PLA_2) (Duerson et al., 1996) leading to production of arachidonic acid which has been implicated in inhibiting glutamate uptake by astrocytes (Marin et al., 1993). SS binding can directly activate diverse K^+ channels, such as large conductance Ca^{2+}-activated K^+ channels (White et al., 1991), inward- (Sims et al., 1991) and delayed rectifying K^+ channels (Wang et al., 1989; Raynor et al., 1991) and ATP-sensitive K^+ channels (de Weille et al., 1989). A few groups have even reported inhibition of inwardly rectifying K^+ channels (Karschin et al., 1994). In addition SS has also been shown to activate C (PLC)-PKC (Rosskopf et al., 2003), increase $[Ca^{2+}]_i$ (Cheng et al., 2002), modulate mitogen-activated protein (MAP) kinase signaling (Patel et al., 1999) and activate serine/threonine protein phosphatases such as calcineurin (Gromada et al., 2001). SS-induced inhibition of voltage dependent Ca^{2+} channels has been demonstrated in B lymphoblasts (Rosskopf et al., 2003). SS has been shown to down regulate the expression and release of endozepines from cultured astrocytes (Masmoudi et al., 2005).

SSTR peptide and non peptide agonists:

Peptide and nonpeptide agonists based on the structure of the different SSTRs and specific to each subtype SSTR are available. Their properties are summarized as shown in table 4. In the peptide agonists, Phe^7, Trp^8, Lys^9 and Thr^9 are important for the function and structural specificity with DTrp and Lys being the essential residues for the β turn (Patel, 1999). Most SSTR peptide agonists have higher affinities for more than one SSTR subtype and hence they are less selective than the nonpeptide agonists.

L-363,377 a cyclic hexapeptide SSTR2 antagonist forms the basis for modeling and synthesis of other SSTR subtype nonpeptide agonists (Schaeffer et al., 1998). Out of approximately 200,000 random compounds created from L-363,377 modeling L-264,930 is the most potent SSTR2 agonist. L-264,930 through further combinatorial chemistry has been used to develop L-797,591, L-779,976, L-796,778, L-803,087 and L-817,818 which are highly potent and specific SSTR1, SSTR2, SSTR3, SSTR4 and SSTR5 nonpeptide agonists, respectively (Schaeffer et al., 1998).

SSTR peptide and nonpeptide antagonists:

Peptide antagonists for SSTRs are based on the basic octapeptide template (Weckbecker et al., 2003) and have a Lys^4-$DTrp^5$, which forms the center of a β turn critical for the receptor recognition (Hocart et al., 1999). PRL-2868 and PRL-2903 are SSTR1 and SSTR2 octapeptide antagonists, respectively and have the β turn conformation with cyclic hexapeptide core having a disulfide bond between Cys^2 and Cys^7 (Hocart et al., 1999). SST3-ODN-8 is cyclic octapeptide antagonist for SSTR3, which has an N-methyl-amino-2-napthoyl aminoglycine instead of tryptophan in β turn and has a disulfide bond between Cys^1 and Cys^8 (Weckbecker et al., 2003). TT-232 a heptapeptide is an antagonist for SSTR4 and

has a cyclic pentapeptide core formed with a disulfide bond between Cys^2 and Cys^6 (Weckbecker et al., 2003). BIM-23056 is a linear octapeptide antagonist for SSTR5.

SRA-880 is a nonpeptide antagonist derived from octahydrobenzoquinoline (Hoyer et al., 2004) and is specific to SSTR1. BN-81674 is a nonpeptide antagonist for SSTR3 and is derived from tetrahydro-β-carboline (Poitout et al., 2001). Nonpeptide antagonists for SSTR2, SSTR4 and SSTR5 are still not available. Overall the nonpeptide antagonists are more selective and specific than the peptide antagonists (Table 4).

Compound	IC_{50} (nM) hSSTR1	hSSTR2	hSSTR3	hSSTR4	hSSTR5
Peptide agonists					
SMS 201-995	290	0.4-2.1	4.4-34.5	>1000	5.6-3.2
BIM23014	500	0.5-1.8	43-107	66	0.6-1.4
RC-160	>1000	5.4	31	45	0.7
DC32-87	7820	0.65	244	970	6.8
DC32-92	98	18	5.6	127	1.2
Nonpeptide agonists					
L-797,591	1.4	1875	2240	170	3600
L-779,976	2760	0.05	729	310	4260
L-796,778	1255	>10,000	24	8650	1200
L-803,087	199	4720	1280	0.7	3880
L-817,818	3.3	52	64	82	0.4
Peptide antagonists					
PRL-2868	37±9.5	176±88	382±181	>1000	971±411
PRL-2903	>1000	26±3.1	231±102	>1000	535±116
PRL-3195	1000	17.5±5.5	66±5.8	1000	5.98±0.91
SST3-ODN-8	>10,000	>10,000	6.7	>10,000	>10,000
TT-232	>10,000	>10,000	>10,000	900	>10,000
BIM23056	337	132	177	234	12.1
Nonpeptide antagonists					
SRA-880	9.8	36300	8710	15100	1445
BN-81674	>10000	>10000	0.92	>10000	>10000

Table 4. Agonists and antagonists for SSTRs (modified from Hocart et al., 1999; Patel 1999 and Weckbecker et al., 2003).

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Chapter 2. Arginine vasopressin increases glutamate release and intracellular Ca^{2+} concentration in hippocampal and cortical astrocytes through two distinct receptors

Nasser Syed, Catherine Martens, Walter H. Hsu.

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Abstract

Aginine vasopressin (AVP), released from the CNS, plays an important role in regulating several aspects of CNS functions including stress and cognition. AVP acts through specific G protein-coupled receptors and not only induces $[Ca^{2+}]_i$ increase in astrocytes, but also has been shown to regulate astrocytic cell volume changes. Here we report a novel finding that AVP induces glutamate release from astrocytes isolated from the cerebral cortex and hippocampus. We also investigated the type of AVP receptors involved in the AVP-induced increase in glutamate release from astrocytes isolated from the hippocampus and cortex of neonatal rats. We showed that the AVP (0.1 – 1000 nM)-induced increase in glutamate release and $[Ca^{2+}]_i$ is brought about by two distinct subtypes of AVP receptors (V_{1a} & V_{1b}). Our results suggested, that V_{1b} receptors are predominantly expressed in astrocytes isolated from the hippocampus and V_{1a} receptors are predominantly expressed in astrocytes isolated from the cerebral cortex of neonatal rats. In addition, the AVP-induced increase in glutamate did not contribute to an increase in $[Ca^{2+}]_i$, since blockade of metabotropic glutamate receptors did not alter the AVP-induced increase in $[Ca^{2+}]_i$. In addition, the administration of a phospholipase A_2 (PLA_2) inhibitor failed to alter AVP-induced $[Ca^{2+}]_i$ increase suggesting the lack of involvement of PLA_2.

Key Words: Hippocampus, cerebral cortex, V_{1a} receptors, V_{1b} receptors, V_2 receptors, glutamate release, phospholipase A_2.

Astrocytes express a repertoire of receptors and play a major role in brain homeostasis (Chen et al., 2000) by providing a link between vasculature and neurons. Astrocytes not only communicate with each other through Ca^{2+} waves, but also communicate with neurons through regulation of extracellular glutamate concentration (Schousboe et al., 2004). Although the exact mechanism of glutamate release from astrocytes is not yet clear, sufficient data exist to suggest a Ca^{2+}-dependent exocytotic process (Parpura et al., 2004). Several proteins and molecules required for exocytotic process have been shown to be present in astrocytes (Bezzi et al., 2004; Enrico et al, 2004; Montana et al., 2004). A highly effective regulatory mechanism must be in place to control the extracellular glutamate as increased extracellular concentrations can cause neurotoxicity and cell death (Schousboe et al., 2004). Thus, in glutamatergic neurotransmission astrocytic glutamate transporters are important in the termination of the neurotransmission process (Schousboe, 2003). The multifaceted role of astrocytes points in the direction that they are also involved in brain information processing, modulating synaptic transmission and CNS plasticity (De Luca et al., 1997; Klimkiewicz, 2001).

AVP, a neuropeptide synthesized in paraventricular, supraoptic and suprachiasmatic nuclei of the hypothalamus and the media amygdala, is not only involved in regulating the water balance (Dibas et al., 1998), but has also been shown to stimulate the Na^+-K^+-Cl^- cotransporter in the blood brain barrier (O'Donnell et al., 2005), and regulate cognitive functions, memory, aggression, stress and behavior (Bielsky et al., 2003). In addition, AVP-positive neurons have been reported in hippocampus (Tiberiis et al., 1983; Orlowska-Majdak

et al., 2003) and cerebral cortex (Delville et al., 1994) suggesting AVP synthesis outside the hypothalamus. To date, three types of G-protein coupled receptors (GPCRs) for AVP (V_{1a}, V_{1b} and V_2) have been identified (Thibonnier et al., 1998). The V_2 receptors are extensively/exclusively found in renal tubules, whereas the V_{1a} receptors are found in vascular smooth muscles, myometrium, urinary bladder, liver, platelets, spleen, testis, epithelial cells of the renal cortical collecting duct and many CNS structures including astrocytes in brain (Barberis et al., 1998; Jackson 2001). The V_{1b} receptors have been reported in adenohypophysis and the V_{1b} mRNA has been detected in kidneys, thymus, heart, lung, spleen, some regions of brain (Lolait et al., 1995) and in pancreas (Saito et al., 1995). However, little is known about the distribution of receptor subtypes in different regions of brain. The V_2 receptor activation stimulates G_s protein, adenylyl cyclase (Birnbaumer, 2000) and cAMP-dependent protein kinase (PKA). Activation of V_{1a} and V_{1b} receptors stimulates G_q proteins, phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA_2) (Briley et al., 1994). PLC activation results in production of IP_3 and diacylglycerol leading to an increase in $[Ca^{2+}]_i$, activation of protein Kinase C (PKC) and further downstream protein phosphorylation.

Our present study was to investigate whether AVP, which elicits a Ca^{2+} response in astrocytes, would also increase glutamate release and to characterize the specific receptors involved in the AVP-induced increase in glutamate release and $[Ca^{2+}]_i$. V_{1a} and V_{1b} receptors have been reported in astrocytes (Barberis et al., 1998) but whether these receptors are differentially expressed or homogenously distributed in astrocytes from different regions of the brain is still not yet clearly established. This identification is especially important since many treatments directed at AVP receptors have been proposed for various conditions

ranging from trauma-induced brain edema to disorders of memory, stress and cognitive functions. Taken together our results suggests that in neonatal rats, AVP-induced increase in glutamate release and $[Ca^{2+}]_i$ is mediated via V_{1b} receptors in astrocytes isolated from the hippocampus and by V_{1a} receptors in astrocytes isolated from the cerebral cortex. Furthermore, the AVP-induced increase in $[Ca^{2+}]_i$ was neither blocked by metabotropic glutamate receptor antagonist or the phospholipase A_2 inhibitor suggesting, glutamate release is secondary to increase in $[Ca^{2+}]_i$ and lack of PLA_2 involvement in this pathway respectively.

Materials and Methods

Chemicals: dP[Tyr(Me^2)]AVP, L-glutamic dehydrogenase (GDH), β-nicotinamide adenine dinucleotide (βNAD), Protease inhibitor cocktail, CelLytic-M lysis buffer (Sigma Chemicals, St. Louis, MO), fura-2 acetoxymethyl ester (fura-2AM) (TEF Labs, Austin, TX), , desGly^{9}d$(CH_2)_5$[Tyr(Et^2)]AVP (WK-3-6) (donated by Dr. M. Manning, University of Toledo, Toledo, OH), 7-(hydroxyimino)cyclopropan-[b]chromen-1a-carboxylic acid ethylester (CPCCOEt), N-(p-amylcinnamoyl)anthranilic acid (ACA) (Biomol, Plymouth Meeting, PA), (1-[4-(N-tert-Butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]indoline-2-one, phosphate monohydrate; cic-isomer) (SR-121463B) (donated by .Dr. C. Serradeil-Le Gal, Sanofi-Synthelabo Rech, Toulouse Cedex, France). Antisera vs. V_{1a} and V_{1b} receptors (Santa Cruz Biotech, Santa Cruz, CA).

Cell culture

Cell cultures were established according to a previously described procedure (Jeftinija et al. 1996). Briefly, primary culture of astrocytes was established from cells isolated from the hippocampus and cerebral cortex of 1 – 4 day old Sprague-Dewley rats. Following

trypsinization, the cells were plated in culture flasks containing α–MEM supplemented with 10% FBS and maintained at 37^0C until they reached 90% confluency. The confluent cultures were further processed to obtain enriched astrocyte type I cultures by preshaking at 260 RPM in an orbital shaker for 2 h to remove microglia and dividing astrocytes. After preshaking the media was changed and the cultures were incubated for 1 h to equilibrate before they were shaken overnight at 260 RPM. Following the shaking the supernatant was discarded and the adhering astrocytes were trypsinized and resuspended in new flasks with FBS supplemented α-MEM. Cells from passage 2 to 6 were used for all the experiments. For glutamate measurement, cells were plated on 2-mm coverslips and used after they reached confluency by placing the cover slip in an open perifusion chamber. For calcium imaging experiments, the astrocytes were plated in a central 15-mm diameter well in a 35-mm dish and used after 24 h of culture.

Immunocytochemistry

The cells were confirmed as astrocytes by their morphology and positive staining for glial fibrilary acidic protein (GFAP) marker using immunocytochemistry (Hsu et al., 1981). Immunocytochemistry was performed by using antibodies against GFAP.

Glutamate measurement

Glutamate levels were detected using an enzymatic assay as previously described (Nicholls et al., 1986; Nicholls et al., 1987; Ayoub et al., 1998; Innocenti et al., 2000). L-glutamic dehydrogenase (GDH) converts β-nicotinamide adenine dinucleotide (βNAD) to NADH in the presence of glutamate and NADH fluoresces when excited with UV light. By providing GDH and βNAD in the medium in which astrocytes are bathed, the glutamate released into the medium can be detected as an increase in NADH fluorescence signal.

The enzymatic assay solution in which the cells were bathed was composed of: NaCl, 137 mM; KCl, 5 mM; $MgCl_2$, 2 mM; $CaCl_2$, 2 mM; HEPES, 10 mM; glucose, 10 mM. The assay solution was supplemented with 50 U/ml GDH and 1 mM NAD^+ for glutamate detection.

All experiments were performed on the stage of an epifluorescence (Nikon) microscope equipped with fluorescence microscopy. NADH fluorescence measurement and the fluorescence changes were expressed as dF/F_0 (%) (F_0 = fluorescence level before cells are stimulated, dF = Change in fluorescence). The light from a xenon arc lamp was delivered to the enzymatic assay solution in which the cells were bathed at 360 nm wavelength through 60X oil immersion objective and the fluorescent emission collected through a dichroic mirror (Chroma) was detected using a quantex CCD camera. The changes in fluorescence were recorded for 300 seconds.

Intracellular calcium imaging

Krebs-Ringer bicarbonate buffer (KRB) containing 136 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, 0.1% BSA, pH 7.35 was used for all the $[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ measurements were performed using fura-2 dye. Briefly, cells were loaded with 2 μM fura-2 AM for 30 min at room temperature after which the cells were washed with KRB. The fura-2 dye loaded cells were monitored for $[Ca^{2+}]_i$ by using Nikon microscope connected to Quantex CCD camera and Metafluor software.

Immunocytochemistry

The astrocytes were identified and confirmed by their morphology and immunoreactivity to specific astrocytic marker glial fibrillary acidic protein (GFAP).

Western blot analysis

The presence of specific AVP receptor subtypes was confirmed by their immunoreactivity to AVP receptor subtype specific antibodies after electrophoreisis and subsequent western blot. Briefly, 2 million astrocytes each from hippocampus and cerebral cortex cultures were lysed in a lysis buffer (Sigma CelLytic M) having protease inhibitors (Sigma protease inhibitor cocktail). 15 µg of protein from each lysate sample was separated by 10% SDS-PAGE. Subsequently, western blot analysis was performed by transferring the proteins to a nitrocellulose membrane and the membranes were probed with specific antibodies (1:700) for the presence of the receptor proteins. For the negative control only secondary antibodies were used to probe the nitrocellulose membrane.

Data Analysis

Unless mentioned all data are expressed as mean $\pm$ S.E.M. with n = 3. EC_{50} and IC_{50} values were calculated using graphpad prism software. One way ANOVA analysis was used to compare individual means using jmp(SAS) with the significance level set at $p < 0.05$.

Results

AVP (0.1 nM – 1000 nM) induced a concentration-dependent increase in glutamate release (Fig.1) in the astrocytes from both the hippocampus and cerebral cortex with EC_{50} of 0.76 $\pm$ 0.08 nM and 0.86 $\pm$ 0.16 nM respectively. A similar AVP (0.1 nM – 1000 nM)-induced increase in $[Ca^{2+}]_i$ was observed in astrocytes from the hippocampus and cortex (Fig.2) with the maximal increase at 1µM. The EC_{50} for AVP-induced increase in $[Ca^{2+}]_i$ in astrocytes from hippocampus was 1.46 $\pm$ 0.28 nM and in astrocytes from cortex was 1.31$\pm$ 0.22 nM. For further investigations 1 nM AVP was used based on the EC_{50} values of AVP in its increase in $[Ca^{2+}]_i$ and glutamate release.

To characterize the AVP receptor subtypes, specific antagonists were used to determine the inhibition of 1 nM AVP-induced increase in glutamate release and $[Ca^{2+}]_i$. Figs. 3 and 4 show the effects of 1 nM dP[Tyr(Me^2)]AVP, a V_{1b} receptor antagonist (Lee et al, 1995), and 1 nM WK-3-6, a V_{1a} receptor antagonist (Lee et al, 1995), on 1 nM AVP-induced glutamate release from the hippocampal and cortical astrocytes. The V_{1b} antagonist blocked the AVP-induced glutamate release from hippocampal astrocytes (Fig. 3) but had no effect in cortical astrocytes. In contrast, the V_{1a} antagonist blocked the AVP-induced glutamate release from the cortical astrocytes (Fig. 4) and had no effect on glutamate release from the hippocampal astrocytes. Fig.5 and Fig.6 show the effects of V_{1b} and V_{1a} antagonists on 1 nM AVP-induced increase in $[Ca^{2+}]_i$. The V_{1b} receptor antagonist abolished the effects of 1 nM AVP in a concentration-dependent manner in the hippocampal astrocytes with IC_{50} of 0.88 $\pm$ 0.15 nM, whereas 1 nM V_{1a} receptor antagonist had no effect (Fig.5). The V_{1a} receptor antagonist abolished the effects of 1 nM AVP in a concentration-dependent manner in the cortical astrocytes with IC_{50} of 0.98 $\pm$ 0.13 nM, whereas 1 nM of the V_{1b} receptor antagonist had no effect (Fig.6). SR-121463B, a non peptide V_2 receptor antagonist did not alter the 1 nM AVP-induced increase in $[Ca^{2+}]_i$ in both hippocampal and cortical astrocytes (data not shown).

Western blot data showed a strong presence of the V_{1b} (Fig. 7a) receptor protein and a weak presence of V_{1a} (Fig. 7b) receptor protein in hippocampal astrocytes, whereas in cortical astrocytes only V_{1a} receptor protein was detected (Fig. 8a) and there was no detection of the V_{1b} receptor protein (Fig. 8b).

AVP has been reported to stimulate phospholipase A_2 in several cell types and cell lines (Chen et al., 1999) and PLA_2 enzymes have been shown to be involved in AVP-induced

signal transduction (Spatz et al., 1994). It has been postulated that accumulation of arachidonic acid (AA) in the astrocytes can inhibit glutamate reuptake (Marin et al., 1991), thus leading to accumulation of glutamate outside the cell, which in turn can activate metabotropic glutamate receptors on the membrane causing an increase in $[Ca^{2+}]_i$ (Marin et al., 1993). In addition, glutamate increases $[Ca^{2+}]_i$ in cultured astrocytes through metabotropic glutamate receptor mGluR5 (Nakahara et al., 1997). Since AVP increased glutamate release and $[Ca^{2+}]_i$ in the astrocytes, we investigated whether the increase in $[Ca^{2+}]_i$ was at least partially due to the accumulation of glutamate in the media brought about by PLA_2 activation and accumulation of AA. The metabotropic glutamate receptor antagonist CPCCOEt (Annoura et al., 1996; Hermans et al., 1998), at 100 μM abolished the 1 μM L-glutamate-induced increase in $[Ca^{2+}]_i$, but had no effect on 1 nM AVP-induced increase in $[Ca^{2+}]_i$ in astrocytes from both the hippocampus and the cerebral cortex (Fig. 9). Pretreatment of the astrocytes with 1 μM ACA, a PLA_2 inhibitor, failed to inhibit the 1 nM AVP-induced increase in $[Ca^{2+}]_i$ (Fig. 10).

Discussion

Since the increase in $[Ca^{2+}]_i$ can trigger glutamate release from astrocytes (Jeremic et al., 2001; Parpura et al.; 2004, Hua et al., 2004), we determined whether AVP, which elicits $[Ca^{2+}]_i$ elevations in these cells, would evoke glutamate release. Indeed, AVP (0.1 - 1000 nM) increased the glutamate release in a concentration-dependent manner. The 1 nM AVP-induced increase in glutamate release and $[Ca^{2+}]_i$ was blocked by dP[Tyr(Me^2)]AVP, a V_{1b} receptor antagonist (Lee et al., 1995), in the hippocampal astrocytes and by WK-3-6, a V_{1a} receptor antagonist (Lee et al., 1995), in the cortical astrocytes. The latter finding is in agreement with several studies on cortical astrocytes (Hatton et al., 1992) and in

disagreement with the report of Chen et al. (2000) who claimed V_{1b} receptors mediate AVP-induced increase in $[Ca^{2+}]_i$ in cortical astrocytes. One explanation for this difference is the concentrations of the agonist AVP and the antagonists dP[Tyr(Me^2)]AVP used in these studies. In the work by Chen et al. 1 μM AVP was used along with 2.5 μM V_{1b} antagonist, which are rather high concentrations as opposed to 1 nM AVP and 1 nM V_{1b} antagonist used in the present study. Since the V_{1b} antagonist dP[Tyr(Me^2)]AVP also has low affinity for V_{1a} receptors in rat liver (pK_i of 8.36) (Arsenijevic et al., 1994), the concentration used by Chen et al. may have antagonized V_{1a} receptors as well. Nevertheless, our Western blot findings indicate that only V_{1a} receptors are expressed in the astrocytes isolated from cerebral cortex, whereas V_{1b} receptors are expressed predominantly in the astrocytes isolated from hippocampus. Although others show the presence of V_{1a} (Ostrowski et al., 1992) and V_{1b} mRNA transcripts in different regions of the brain (Christopher et al., 1998; Hurbin et al., 1998), sufficient data in terms of actual receptor protein expression is lacking. To the best of our knowledge, the Western blot data from the present study are the first ones to demonstrate the AVP receptor protein expression in astrocytes.

Since glutamate has been reported to increase $[Ca^{2+}]_i$ in astrocytes through metabotropic glutamate receptors (Nakahara et al., 1997), we investigated whether the AVP-induced increase in $[Ca^{2+}]_i$ is due to the glutamate released by the astrocytes. The metabotropic glutamate receptor antagonist CPCCOEt failed to inhibit the AVP-induced increase in $[Ca^{2+}]_i$ pointing in the direction that the glutamate release is secondary to the increase in $[Ca^{2+}]_i$. Since PLA_2 activation has been implicated in glutamate release (Marin et al., 1993), we also investigated the role of PLA_2 in AVP-induced increase in $[Ca^{2+}]_i$. The PLA_2 inhibitor, ACA did not alter the AVP-induced increase in $[Ca^{2+}]_i$. Therefore, it is

unlikely that PLA_2 or glutamate release plays a noticeable role in AVP-induced increase in $[Ca^{2+}]_i$ in astrocytes.

AVP has been functionally characterized as a regulator of cell volume and has been implicated in trauma-induced brain edema (O'Donnell et al., 2005). It has been shown to modulate regulated cell volume changes (Latzkovits et al., 1993; Sarfaraz et al., 1999; Hertz et al., 2000) in astrocytes and affect activation and translocation of aquaporins (Niermann et al., 2001). Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II can attenuate water permeability in astrocytes (Gunnarson et al., 2005) and glutamate can induce astrocytic swelling (Han et al., 2004; Hansson et al., 1994). Therefore, the resulting changes in astrocyte cell volume may be due to the combined effects of increased $[Ca^{2+}]_i$ and glutamate release. Excess glutamate can lead to neuronal cell hyperexcitability and cell death, therefore astrocytes must have elaborate mechanisms working simultaneously to control the levels of extracellular glutamate as indicated by a report that astrocytes express the highest density of glutamate transporters (Huang et al., 2004). However, what physiological relevance does such AVP-induced release of glutamate through Ca^{2+}-dependent mechanism has apart from neuronal excitation and synaptic plasticity within the nervous system warrants further investigation. Nevertheless, some of the toxic effects of AVP during brain trauma may be due to increased glutamate release.

Our study demonstrated that AVP-induced increase in glutamate release and $[Ca^{2+}]_i$ from astrocytes through V_{1b} receptors in the hippocampal astrocytes and V_{1a} receptors in the cortical astrocytes. To the best of our knowledge this is the first report showing AVP-induced glutamate release and the presence of different receptor subtypes for AVP in astrocytes from different regions of brain. Neither the metabotropic glutamate receptor antagonist nor the

PLA_2 inhibitor blocked the AVP-induced increase in $[Ca^{2+}]_i$ suggesting that the glutamate release is secondary to the increase in $[Ca^{2+}]_i$. However, it is not yet clear as to what differences may exist in V_{1a}- and V_{1b}-based signaling mechanisms beyond the accompanying Ca^{2+} changes and glutamate release, since both receptors are mediated through $G_{q/11}$ and PLC-β. The presence of different receptor subtypes for AVP in astrocytes from different regions of the brain suggests functional specializations of astrocytes from various regions of the brain and goes beyond the commonly accepted role of AVP as just a regulator of osmotic balance. Use of AVP receptor antagonists for treating brain edema and several cognitive and memory disorders have been proposed and V_1 receptor antagonists have been shown to significantly decrease brain edema and play a neuroprotective role in brain trauma-induced edema (Kagawa et al., 1996). Since conditions like cerebral ischemia can increase water and AVP content even in the hippocampus (Liu et al., 1996), the use of more specific receptor antagonists aimed at both V_{1a} and V_{1b} may show different, if not better, results. The understanding of the differential expression of the AVP receptor subtypes in different regions of the rat brain can be a novel approach to the use and therapeutic values of the AVP receptor specific agonists and antagonists. Treatments targeted at the AVP receptors needs to take into consideration this differential expression pattern, the effect of AVP on glutamate release and design methods of delivering the receptor specific treatments targeted to specific areas of brain. With the availability of nonpeptide antagonists for AVP receptors this approach might become possible.

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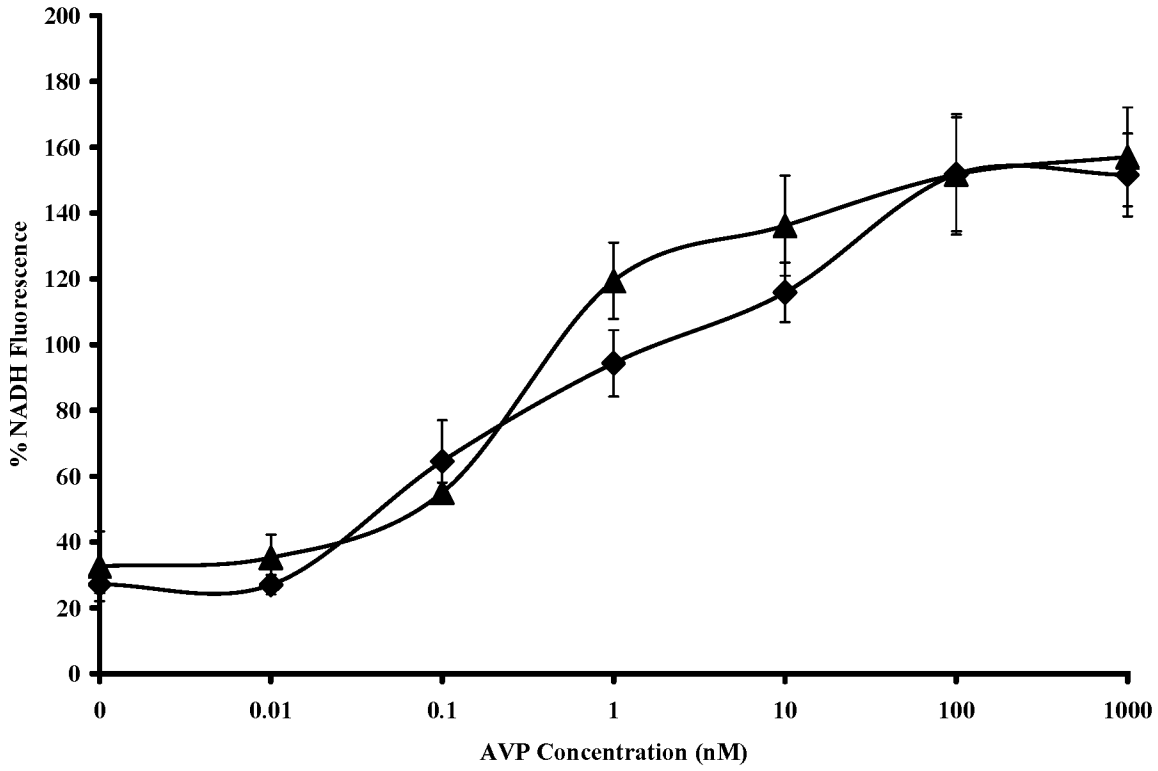


Fig. 1. Effect of AVP on glutamate release from astrocytes. Glutamate release was measured indirectly as fluorescence of NADH in hippocampal (◆) and cortical (▲) astrocytes. Values are the mean ± S.E. (n = cell preparations from 3 different rats).

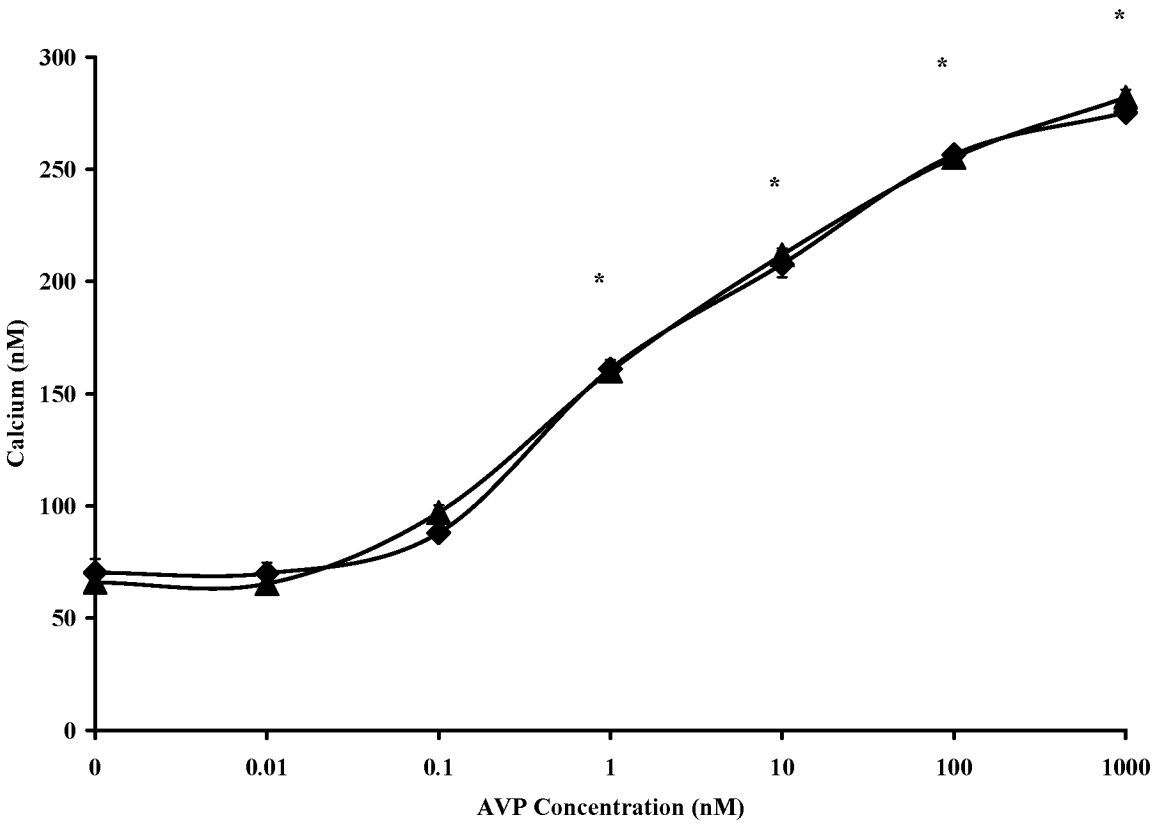


Fig. 2. AVP-induced increase in $[Ca^{2+}]_i$ in hippocampal (◆) and cortical (▲) astrocytes. Values are the mean ± S.E. (n = cell preparations from 3 different rats).

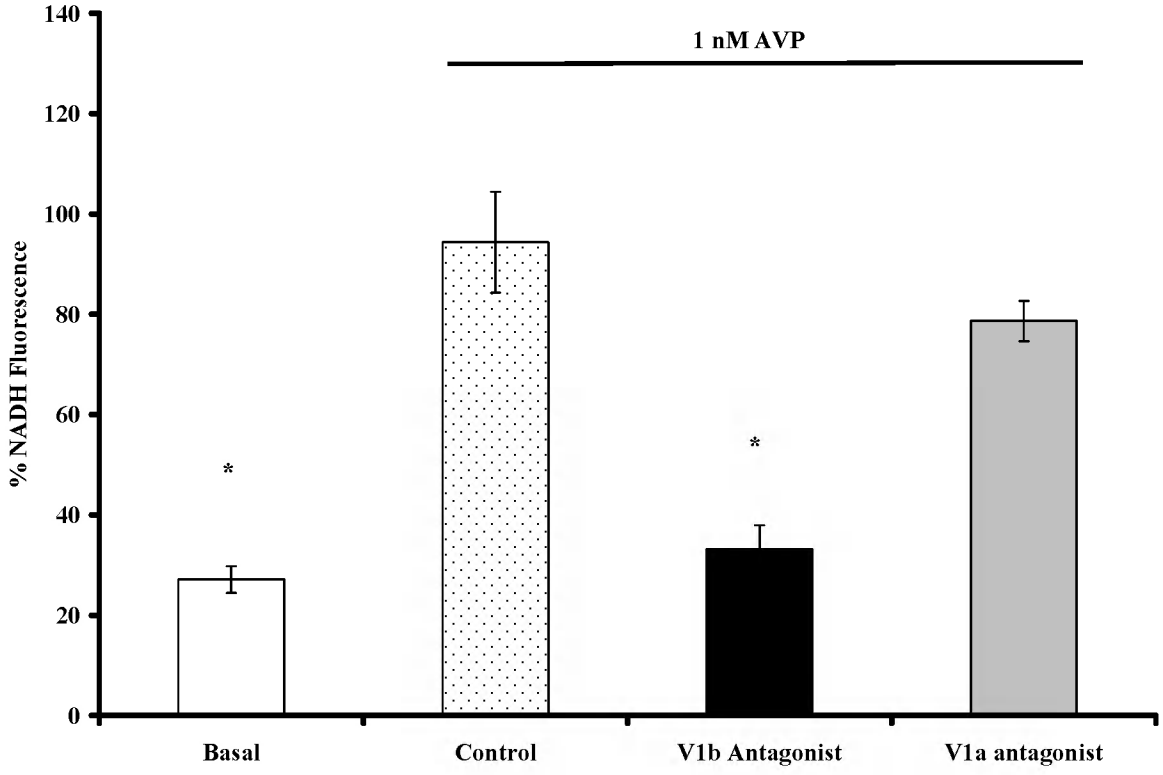


Fig. 3. Effect of 1 nM dP[Tyr(Me^2)]AVP (■), a V_{1b} receptor antagonist, and 1 nM WK-3-6 (■), a V_{1a} receptor antagonist, on AVP-induced increase in glutamate release from hippocampal astrocytes. *, $p < 0.05$ compared with the basal group. Values are the mean ± S.E. (n = 3 n = cell preparations from 3 different rats).

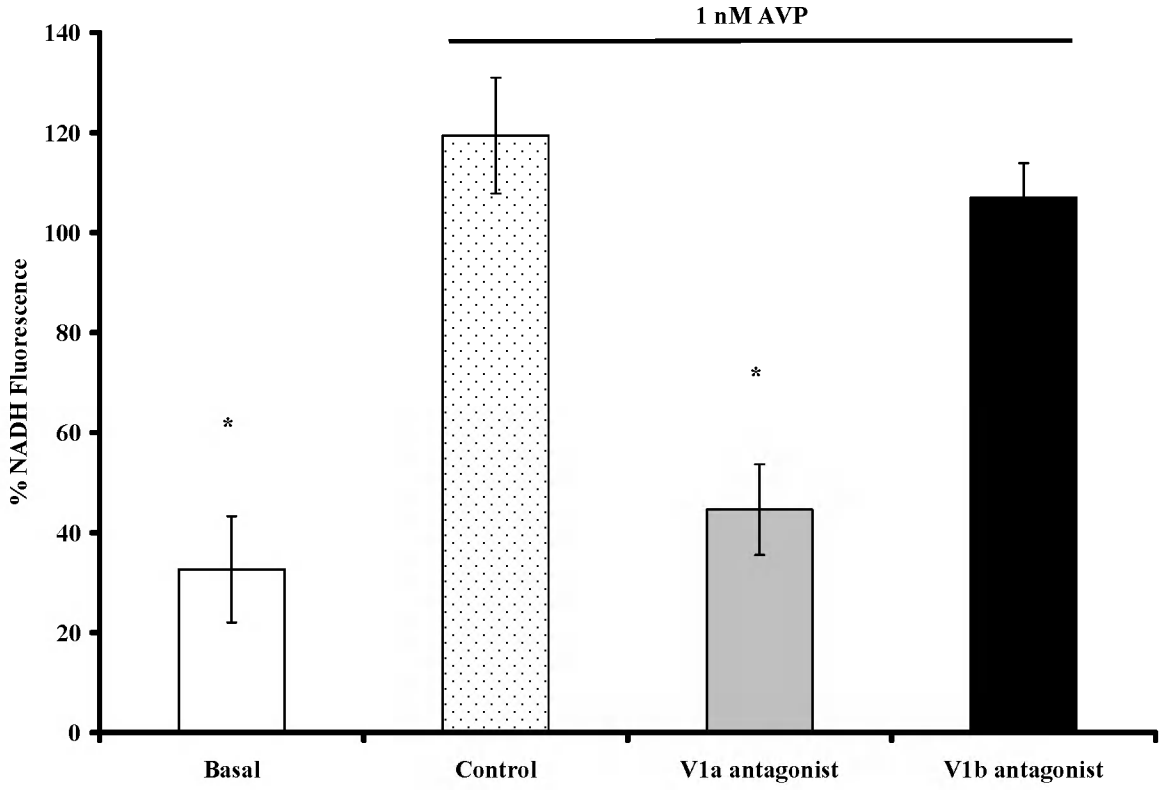


Fig. 4. Effect of 1 nM WK-3-6 (▒), a V_{1a} receptor antagonist, and 1 nM dP[Tyr(Me^2)]AVP (■), a V_{1b} receptor antagonist, on AVP-induced increase in glutamate release from cortical astrocytes. *, $p < 0.05$ compared with the basal group. Values are the mean ± S.E. (n = cell preparations from 3 different rats).

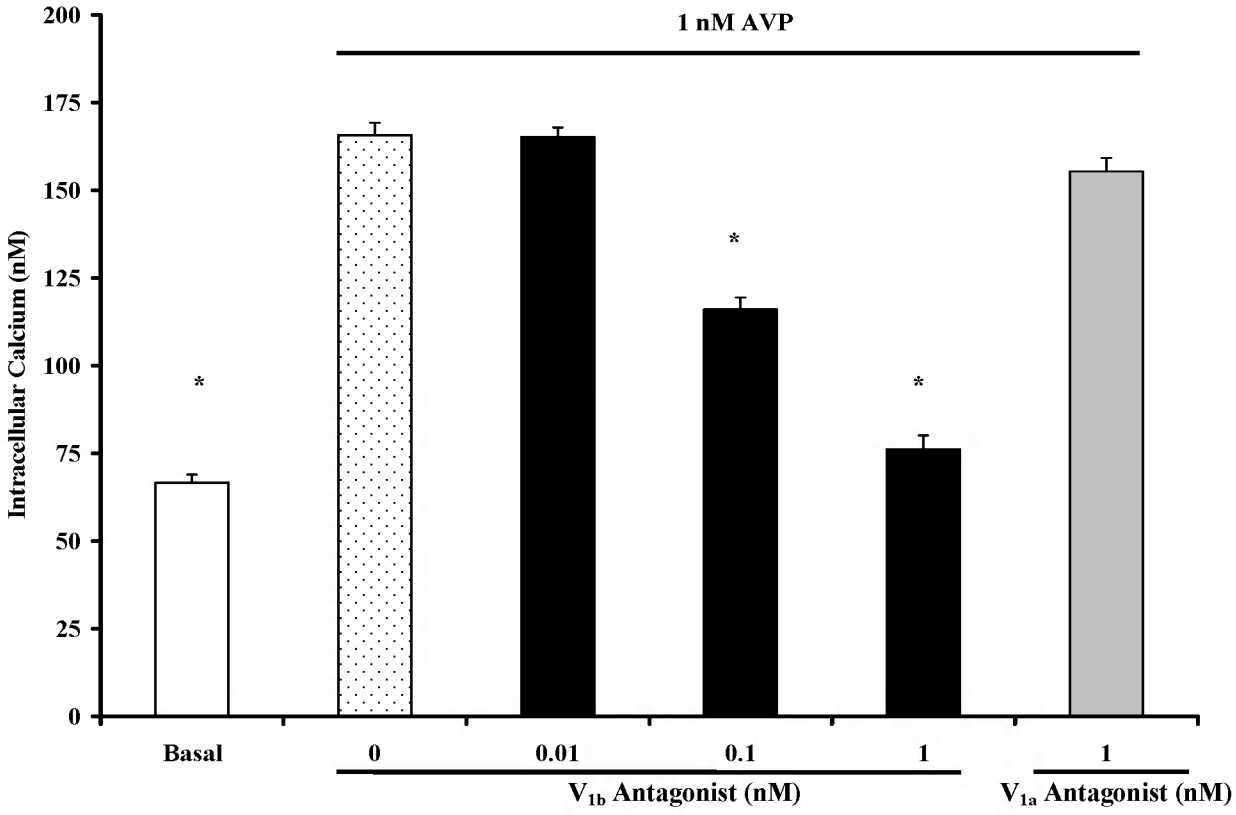


Fig. 5. Effect of dP[Tyr(Me^2)]AVP (■), a V_{1b} receptor antagonist, and WK-3-6 (■), a V_{1a} receptor antagonist, on AVP-induced increase in $[Ca^{2+}]_i$ in hippocampal astrocytes. *, $p < 0.05$ compared with the basal group. Values are the mean ± S.E. (n = cell preparations from 3 different rats).

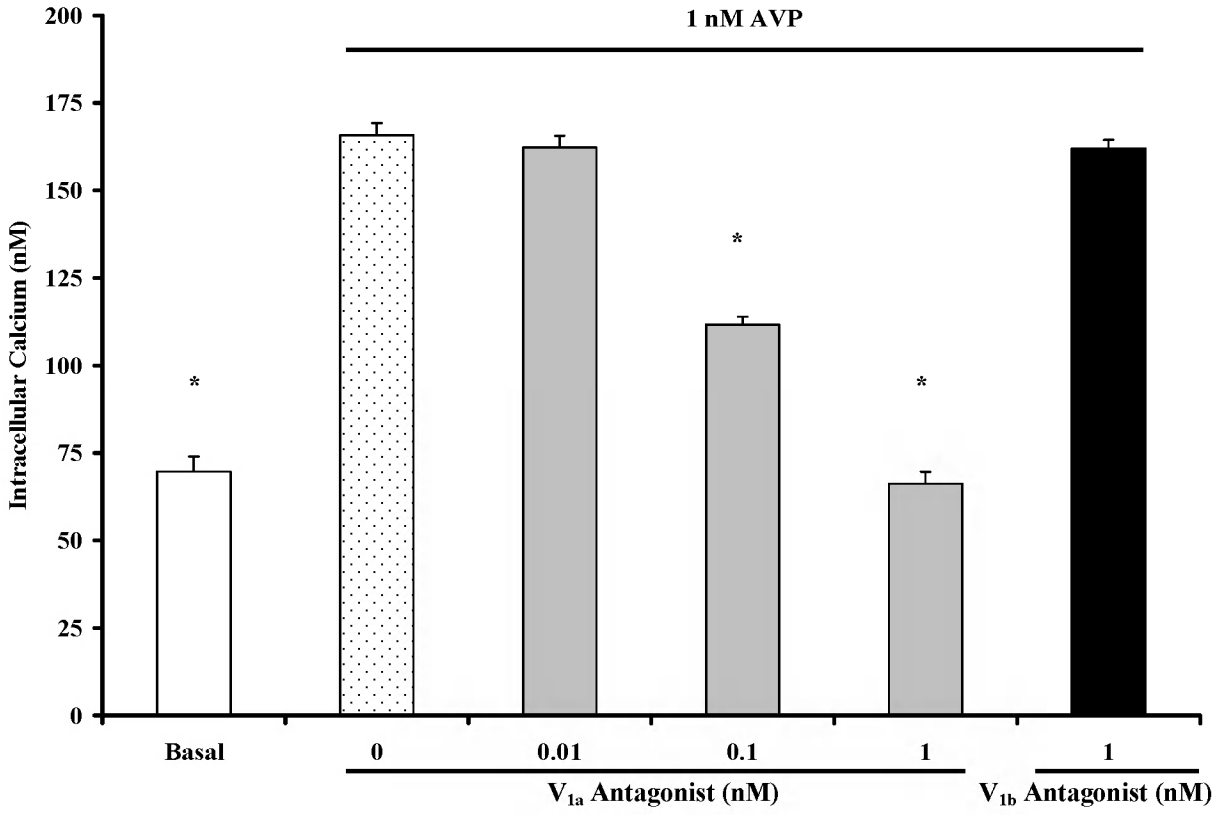
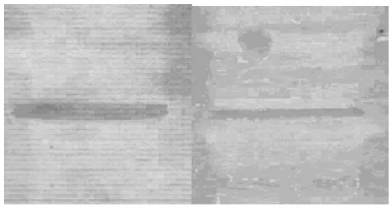
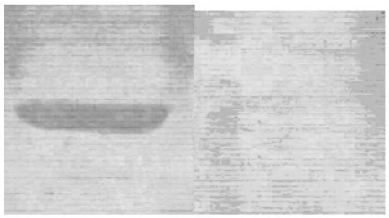


Fig. 6. Effect of WK-3-6 (■), a V_{1a} receptor antagonist abolished and dP[Tyr(Me^2)]AVP (■), a V_{1b} receptor antagonist on AVP- induced increase in $[Ca^{2+}]_i$ in cortical astrocytes. *, $p < 0.05$ compared with basal. Values are the mean ± S.E. (n = 3 independent cell preparations).



(a) **(b)**

Fig. 7. Western blots of hippocampal astrocytes showed strong staining for anti-V_{1b} (a) and a weak staining for anti-V_{1a} (b). Atleast 3 independent cell preparations were used.



(a) **(b)**

Fig. 8. Western blots of cortical astrocytes showed strong staining for anti-V_{1a} (a) and a weak staining for anti-V_{1b} (b). 3 independent cell preparations were used.

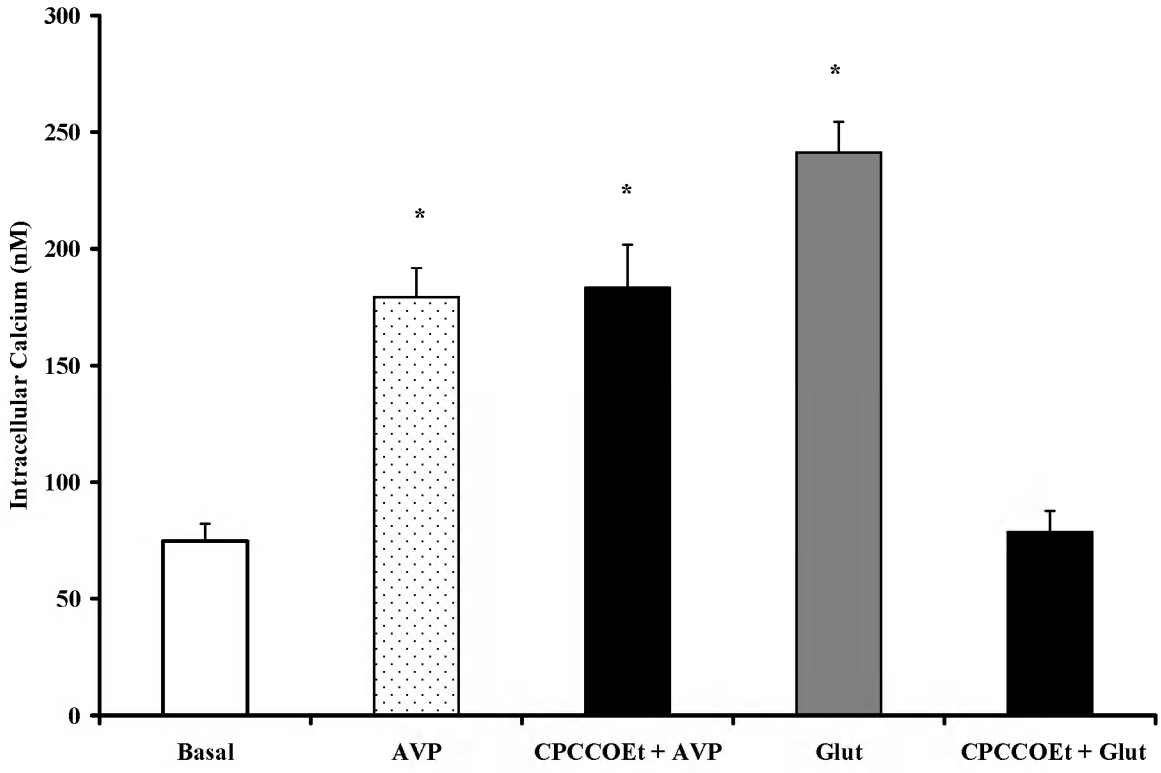


Fig. 9. Effect of CPCCOEt (■) a metabotropic glutamate receptor antagonist on AVP-induced increase in $[Ca^{2+}]_i$ and L-Glutamate-induced increase in $[Ca^{2+}]_i$ in both hippocampal and cortical astrocytes. *, $p < 0.05$ compared with basal. Values are the mean $\pm$ S.E. ($n = 6$ independent cell preparations). Astrocytes from cerebral cortex and hippocampus were used.

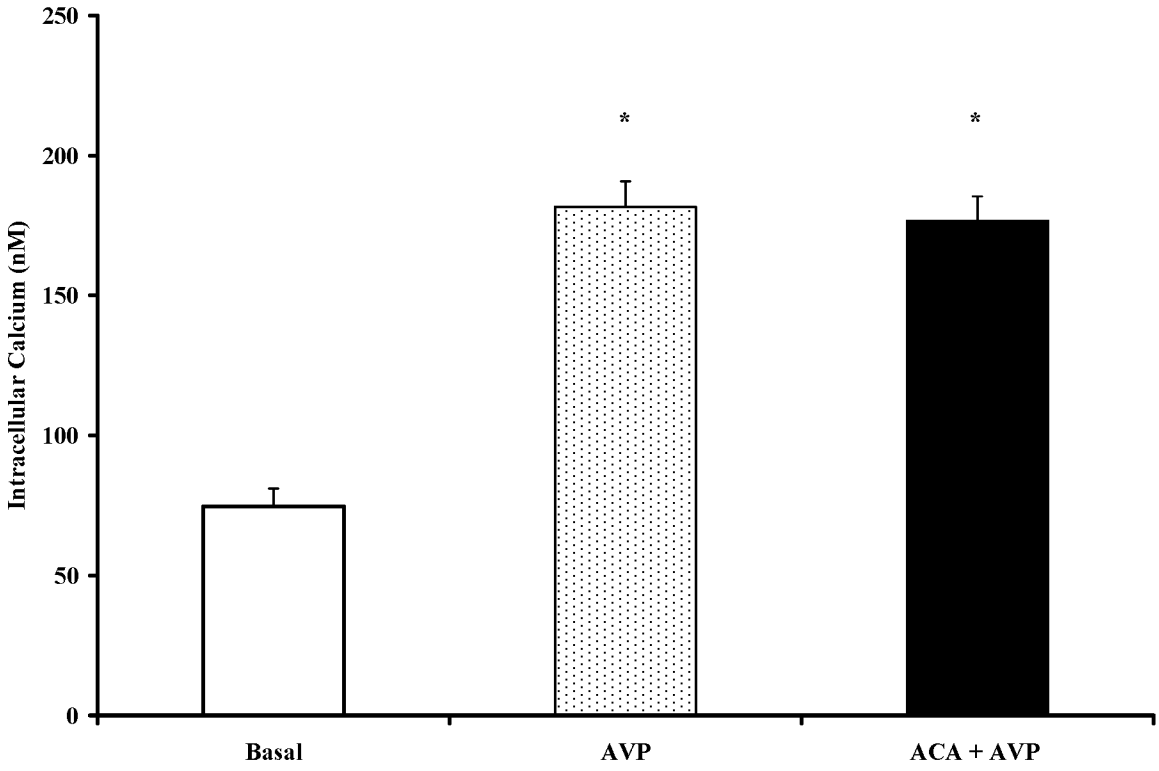


Fig. 10. Effect of 100 μM ACA (■) a PLA_2 inhibitor on AVP-induced increase in $[Ca^{2+}]_i$ in both hippocampal and cortical astrocytes. *, $p < 0.05$ compared with basal. Values are the mean ± S.E. (n = 6 independent cell preparations). Astrocytes from cerebral cortex and hippocampus were used.

Chapter 3. Somatostatin inhibits forskolin-induced increase in cyclic AMP levels, intracellular Ca^{2+} concentrations and glutamate release via SSTR4 in rat astrocytes

Nasser Syed, Catherine Martens, Walter H. Hsu.

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Abstract

Increase in cAMP levels has been shown to facilitate glutamate release from nerve endings. Here we investigated the effects of somatostatin (SS), a $G_{i/o}$-coupled receptor activating hormone on lowering of cAMP level, $[Ca^{2+}]_i$, and glutamate release from neonatal rat astrocyte cultures. Forskolin (0.1 to 10 μM) increased glutamate release, cAMP levels and $[Ca^{2+}]_i$. in a concentration-dependent manner; forskolin-induced increase in $[Ca^{2+}]_i$ paralleled the increase in glutamate release, but the increase in cAMP levels did not. SS alone did not have any effect on basal glutamate release, cAMP levels or $[Ca^{2+}]_i$, but inhibited the forskolin-induced glutamate release and increase in cAMP level in a concentration-dependent manner. SS also inhibited forskolin induced increase in $[Ca^{2+}]_i$. These effects were mimicked by the selective SSTR4 agonist L-803,087, but not by the selective agonists for SSTR1, SSTR2, SSTR3 or SSTR5. Although the inhibitory effect of SS and L-803,087 (1 nM to 1 μM) on forskolin-induced increase in cAMP levels and glutamate release was concentration–dependent, the inhibition of glutamate release, however, was not apparent at ≥1 μM of the agonists SS and L-803,087. This was probably due to the ability of SS and L-803,087 to increase $[Ca^{2+}]_i$ at ≥1 μM. Pretreatment with U-73122, a phospholipase C inhibitor, blocked 1 μM SS-induced increase in $[Ca^{2+}]_i$. Our findings suggest: in astrocytes, 1) SS inhibits forskolin-induced glutamate release by decreasing cAMP levels and at least partly by

reducing $[Ca^{2+}]_i$. 2) SSTR4 mediates SS-induced decrease in cAMP levels, $[Ca^{2+}]_i$ and glutamate release. 3) SS at ≥1 μM may increase glutamate release by activating phospholipase C pathway. SS and SSTR4 agonists could be used to reduce glutamate release from astrocytes.

Key words: Somatostatin, Astrocytes, cAMP, Glutamate release, βNAD.

Somatostatin (SS), a peptide hormone synthesized in neurons, δ cells of the pancreatic islets and other cells (Patel et al., 1999). It is present as a 14 amino-acid and a 28 amino-acid active forms (Ganong, 2001). The 14 amino-acid form is exclusive in enteric neurons, the retina and the 28 amino-acid form is the dominant one in neural tissue, endocrine pancreas and the stomach (Patel et al., 1999). Since its discovery as an inhibitor of growth hormone release, SS has been shown to be important for many functions in the brain, pituitary, pancreas, gastrointestinal tract, adrenals, thyroid, kidney and immune system (Ferone et al., 2004). The actions of SS include inhibition of endocrine and exocrine secretions, modulation of neurotransmission, motor and cognitive functions, inhibition of intestinal motility, absorption of nutrients and ions, vascular contractility, and proliferation of cells (Guillermet-Guibert et al., 2005).

Somatostatin (SS) acts through specific G-protein coupled receptors (GPCRs) and to date, there are 5 subtypes of SS receptors whose distribution vary in different tissues (Reisine et al., 1993). Presence of genes for SSTR1, SSTR2 and SSTR4 have been reported in cultured rat astrocytes (Feindt et al., 1995) and genes for all 5 subtypes of SSTRs with low levels of SSTR5 has also been reported in the various parts of rat brain (Feindt et al., 1995). SSTR2 and SSTR4 are expressed predominantly in cortical neurons along with low level expression of SSTR1, SSTR3 and SSTR5 (Kumar, 2005). A recent report on endozepine

synthesis and release has also demonstrated the presence of SSTR1, SSTR2 and SSTR4 in astrocytes (Masmoudi et al., 2005). All the SSTRs are coupled to $G_{i/o}$ class of GPCRs, which upon activation, inhibit adenylyl cyclase activity, thus lowering cAMP levels. Upon binding of the agonist SS, the activated receptors exert mainly inhibitory and antiproliferative effects (Patel et al., 1999). SS is a known neurotransmitter/neuromodulator (Grouselle et al., 1998) and acts on both neurons and astrocytes (Krisch et al., 1994). SS inhibits glutamate release from neurons by activating SSTR2 (Grilli et al., 2004). In addition, SS has been shown to be important for cognitive functions (Dournaud et al., 1996).

cAMP is a major intracellular signaling molecule that has been shown to increase glutamate release from astrocytes (Shiga et al., 2006) and neurons (Herrero et al., 1996; Dohovics et al., 2003). Adenylyl cyclase activation through G_s-coupled receptors; subsequent increase in cAMP levels and activation of cAMP-dependent protein kinase (PKA) is an important regulator for the glutamate release in neurons (Dohovics et al., 2003; Wang et al., 2003, Grilli et al., 2004). Since SS inhibits adenylyl cyclase activity and has been shown to inhibit glutamate release from neurons (Dal Monte et al., 2003; Grilli et al., 2004), we investigated whether SS would inhibit glutamate release from astrocytes. If so, what would be the underlying mechanism of action? In addition, we characterized the subtype of SS receptors involved in SS-induced reduction of glutamate release and cAMP levels in cultured rat astrocytes. Our results suggest that SS decreases the forskolin-induced glutamate release, cAMP levels and $[Ca^{2+}]_i$ in astrocytes and this effect is mediated by SSTR4.

Materials and Methods

Chemicals: Somatostatin, L-glutamic dehydrogenase (GDH), β-nicotinamide adenine dinucleotide (βNAD), cAMP (Sigma chemical), fura-2 acetoxymethyl ester (fura-2AM)

(TEF Labs, Austin, TX), U-73122, U-73343 (Biomol), L-797,591, L-779,976, L-796,778, L-803,087, L-817,818 (donated by Merck), TT-232 (donated by Dr. Keri Gyorgi, Hungary).

Cell culture

Cell cultures were established according to a previously described procedure (Jeftinija et al., 1996). Briefly primary culture of astrocytes was established from cells isolated from the cerebral cortex of 1 – 4 day old Sprague-Dewley rats. Following trypsinization, the cells were plated in culture flasks containing α–MEM supplemented with 10% FBS and maintained at 37^0C until they reached 90% confluency. The confluent cultures were further processed to obtain enriched astrocyte type I cultures by preshaking at 260 RPM in an orbital shaker for 2 h to remove microglia and dividing astrocytes. After the preshaking, the media was changed and the cultures were shaken overnight at 260 RPM. Following the shaking the supernatant was discarded and the adhering astrocytes were trypsinized and resuspended in new flasks with FBS supplemented α-MEM. The cells were confirmed as astrocytes by their morphology and positive staining for glial fibrilary acidic protein (GFAP) marker using immunocytochemistry (Hsu et al., 1981). Cells from passage 2 to 6 were used for all the experiments. For glutamate measurement, cells were plated on 2-mm coverslips and used after they reached confluency by placing the cover slip in an open perifusion chamber. For cAMP measurements, the astrocytes were plated in 12 well plates and were used when the cells reached 90% confluency.

cAMP assay

Krebs-Ringer bicarbonate buffer (KRB) containing 136 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES, 10 mM glucose, 0.1% BSA, pH 7.35 was used for all the cAMP measurements. After the astrocytes reached 90%, confluency media was

removed and cells were washed twice with KRB and were incubated with various treatments (forskolin, somatostatin, somatostatin receptor specific agonists and antagonists) for 15 min after which the cells were washed twice with KRB. The cells were detached from the wells using 0.01 N HCl and transferred to glass tubes. This was followed by heat inactivation of the phosphodiesterases by immersing the tubes in 70^0 C water bath for 10 min. Subsequently, the extracts were centrifuged at 2000 x *g* for 5 minutes at 4^0 C, the supernatants were transferred to fresh tubes and neutralized with equal amounts of 0.01 N NaOH. cAMP concentrations were determined using radioimmunoassay as described previously (Richards et al., 1979).

Glutamate measurement

Glutamate levels were detected using an enzymatic assay as previously described (Nicholls et al., 1986; Nicholls et al., 1987; Ayoub et al., 1998; Innocenti et al., 2000). L-glutamic dehydrogenase (GDH) β-nicotinamide adenine dinucleotide (βNAD) to NADH in the presence of glutamate and NADH fluoresces when excited with UV light. By providing GDH and βNAD in the medium in which astrocytes are bathed, the glutamate released into the medium can be detected as an increase in NADH fluorescence signal.

The enzymatic assay solution in which the cells were bathed was composed of NaCl, 137 mM; KCl, 5 mM; $MgCl_2$, 2 mM; $CaCl_2$, 2 mM; HEPES, 10 mM; glucose, 10 mM. The assay solution was supplemented with 50 U/ml GDH and 1 mM NAD^+ for glutamate detection.

All experiments were performed on the stage of an epifluorescence (Nikon) microscope. NADH fluorescence measurement and the fluorescence changes were expressed as dF/F_0 (%) (F_0 = fluorescence level before cells are stimulated, dF = Change in

fluorescence). The light from a xenon arc lamp was delivered to the enzymatic assay solution in which the cells were bathed at 360 nm wavelength through 60X oil immersion objective and the fluorescent emission collected through a dichroic mirror (Chroma) was detected using a quantex CCD camera. The changes in the fluorescence were recorded for 300 seconds.

Intracellular Ca^{2+} imaging

KRB was used for all the $[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ measurements were performed using fura-2 dye. Briefly cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2AM) for 30 min at room temperature; subsequently the cells were washed with KRB and left for 15 to 20 min for de-esterification. The fura-2 dye loaded cells were monitored for $[Ca^{2+}]_i$ by using Nikon diaphot microscope connected to Quantex CCD camera and Metafluor software.

Immunocytochemistry

The astrocytes were identified and confirmed by their morphology and immunoreactivity to specific astrocytic marker glial fibrillary acidic protein (GFAP) (Hsu et al., 1981).

Data Analysis

Unless mentioned, all data are expressed as mean $\pm$ S.E. with n = 3. EC_{50} and IC_{50} values were calculated using graphpad prism software. One way ANOVA was used to compare individual means using jmp(SAS) with the significance level set at $p < 0.05$. Basal group represents the basal level when no agonists or antagonists are used.

Results

Forskolin-induced increase in glutamate release, cAMP levels and $[Ca^{2+}]_i$ in astrocytes

Forskolin (0.1 μM – 10 μM) induced a concentration-dependent increase in glutamate release from astrocytes (Fig. 1) with maximal increase at 1 μM. The EC_{50} for forskolin-induced increase in glutamate release was 95 ± 18 nM. A similar forskolin (0.1 μM – 10 μM)-induced increase in cAMP levels (Fig. 2) was observed with maximal increase at 10 μM. The EC_{50} for forskolin-induced increase in cAMP levels was 135 ± 13 nM. On an average, the 1 μM forskolin induced a 4 fold increase in cAMP, whereas 10 μM forskolin induced a 10 – 12 fold increase in cAMP over the basal. For all further experiments we used 1 μM forskolin to study the inhibitory effect of SS and SSTR agonists. Forskolin (1 nM – 10 μM) also caused a concentration-dependent increase in $[Ca^{2+}]_i$ with maximal increase at 1 μM (Fig. 3). The EC_{50} for forskolin-induced increase in $[Ca^{2+}]_i$ was 168 ± 11 nM. The forskolin-induced $[Ca^{2+}]_i$ increase paralleled that of glutamate release.

Effect of SS on forskolin-induced glutamate release

SS (100 nM) alone did not have any effect on glutamate release from the astrocytes, but SS (10 nM & 100 nM) decreased the forskolin (1 μM)-induced glutamate release in a concentration-dependent manner with maximal decrease at 100 nM (Fig. 4). However, at 1 μM, SS did not inhibit the glutamate release. The IC_{50} for SS-induced decrease in glutamate release was 6 ± 3 nM.

Effect of SS on forskolin-induced increase in cAMP levels

SS alone even at 1 μM did not have any effect on basal cAMP levels in astrocytes (data not shown), but SS (0.1 μM & 1 μM) inhibited the forskolin (1 μM)-induced increase

in intracellular cAMP levels in a concentration-dependent manner with maximal inhibition at 1 μM (Fig. 5). The IC_{50} for SS-induced inhibition of cAMP levels was 112 ± 7 nM.

Effect of SS on forskolin-induced increase in $[Ca^{2+}]_i$.

Since forskolin increased $[Ca^{2+}]_i$ in astrocytes, we determined if SS would inhibit the forskolin-induced increase in $[Ca^{2+}]_i$. SS (0.1 μM) alone did not have any effect on $[Ca^{2+}]_i$, but reduced the forskolin (1 μM)-induced increase in $[Ca^{2+}]_i$ (Fig. 6).

Effect of SSTR agonists on forskolin-induced increase in cAMP levels

Initially, all 5 SSTR agonists were tested at 0.1 μM to determine which of them would inhibit the forskolin (1 μM)-induced increase in cAMP levels. SSTR1 agonist L-797,591, SSTR2 agonist L-779,976, SSTR3 agonist L-796,778, and SSTR5 agonist L-817,818 failed to inhibit the forskolin-induced increase in cAMP levels (Fig. 7). In contrast, SSTR4 agonist L-803,087 (0.1 μM) inhibited the forskolin-induced increase in cAMP levels.

Concentration-dependency of L-803,087 in inhibiting forskolin-induced increase in cAMP levels

L-803,087 (1 nM – 1 μM) inhibited the forskolin-induced increase in cAMP levels in a concentration-dependent manner with 1 μM causing the maximal inhibition (Fig. 8). The IC_{50} for L-803,087-induced inhibition of cAMP levels was 5 ± 3 nM.

Effect of L-803,087 on forskolin-induced glutamate release

L-803,087 (10 nM & 100 nM) decreased the forskolin-induced glutamate release with maximal decrease at 100 nM; but at ≥1 μM L-803,087 failed to inhibit the forskolin-induced glutamate release (Fig. 9). The IC_{50} for L-803,087-induced inhibition of glutamate release was 7 ± 2 nM.

Effect of SSTR4 antagonist TT-232 on SS- and L-803,087-induced decrease in cAMP levels

Pretreatment with TT-232 (10 μM) blocked the SS- and L-803,087-induced inhibition of cAMP levels (Fig. 10). TT-232 (10 μM), a specific SSTR4 antagonist (Weckbecker et al., 2003), alone at 10 μM did not have any effect on cAMP levels.

Effect of SS and L-803,087 on $[Ca^{2+}]_i$ in astrocytes

Since SS and L-803,087 at $\geq$ 1 μM failed to inhibit the forskolin-induced glutamate release, this suggested these agonists may exert a stimulatory effect in addition to the inhibitory effect. Thus, we investigated if SS and L-803,087 would elicit an increase in $[Ca^{2+}]_i$. SS and L-803,087 (1 nM – 100 nM) had no effect on $[Ca^{2+}]_i$ in astrocytes; however, at $\geq$1 μM, both SS and L-803,087 increased the $[Ca^{2+}]_i$ (Figs. 11a & 11b). The SS-induced increase in $[Ca^{2+}]_i$ was blocked by 1 μM U-73122, a phospholipase C (PLC) inhibitor, while its structural analog U-73343, which does not inhibit PLC, had no effect on SS-induced increase in $[Ca^{2+}]_i$ (Fig. 12). These results suggest that SS-induced increase in $[Ca^{2+}]_i$ is through activation of PLC. Neither U-73122 alone nor U-73343 alone caused any significant changes in $[Ca^{2+}]_i$ (data not shown).

Discussion

The results in the present study provide for the first time evidence that SS inhibits forskolin-induced glutamate release from astrocytes. SS has been traditionally shown to be inhibitory in function; in the CNS it has been shown to inhibit glutamate release from the neurons of the cerebral cortex (Grilli et al., 2004) and retina (Dal Monte et al., 2003). The effects of SS on astrocytes beyond lowering of cAMP levels are still not well-understood, but it has been shown to inhibit endozepine release from astrocytes (Masmoudi et al., 2005).

Here we showed that SS not only reduced the forskolin-induced increase in cAMP levels and $[Ca^{2+}]_i$, but it also reduced the forskolin-induced glutamate release.

Cyclic AMP, a major signaling molecule that has been shown to induce glutamate release from astrocytes (Shiga et al., 2006), activates L-type voltage-dependent Ca^{2+} channels in astrocytes (Barres et al., 1989) and enhances capacitative Ca^{2+} entry (Wu et al., 1999; Grimaldi et al., 1999). SS has been shown to inhibit Ca^{2+} influx though L-type channels in rat neurons (Johnson et al., 2001) and human neuroendocrine tumor cells of the gut (Glassmeier et al., 1998). Since SS is a known inhibitor of adenylyl cyclase, the reduction in glutamate release by SS and L-803,087, a SSTR4 agonist, in astrocytes may be through a similar adenylyl cyclase-PKA dependent pathway as in the SS-induced inhibition of glutamate release from cortical neurons (Grilli et al., 2003). Interestingly, the glutamate release was inhibited only at agonist concentrations of $\leq$100 nM; at $\geq$1 μM, SS and L-803,087 did not inhibit the forskolin-induced glutamate release. Since the increase in $[Ca^{2+}]_i$ can trigger glutamate release from astrocytes (Jeftinija et al., 2001; Parpura et al., 2004; Takano et al., 2005; Montana et al., 2004), the failure to inhibit glutamate release by SS and L-803,087 may be due to their ability to increase $[Ca^{2+}]_i$ at $\geq$1 μM. SS has been shown to activate PLC and increase $[Ca^{2+}]_i$ in COS-7 cells (Akbar et al., 1994), smooth muscle cells (Murthy et al., 1996) and NG108-15 cells (Rhie et al., 2003). We further showed that U-73122, a PLC inhibitor, blocked SS-induced increase in $[Ca^{2+}]_i$, suggesting this increase is mediated by IP_3-mediated Ca^{2+} release from the endoplasmic reticulum.

SSTR1, 2 and 4 have been found in cultured astrocytes (Feindt et al., 1995; Masmoudi et al., 2004). However, in the present study, SSTR4 seemed to be the only one that was consistently expressed, since SSTR4 agonist and antagonist were the only pharmacologic

agents that influenced cAMP levels and glutamate release in astrocytes. One reason for a strong expression of SSTR4 in our astrocytes cultures could be due to the difference in tissue collection and culture conditions. The cerebral cortex was collected in the present study, but cerebral hemispheres were collected by Masmoudi et al. (2005). We used α-MEM media as opposed to DMEM/Ham's F12 used by Masmoudi et al. The study by Feindt et al. (1995) used RT-PCR to probe only for the presence of gene transcripts for different SSTR subtypes, however no assays were done to look into actual receptor expression. In addition, SS has been shown to inhibit glutamate release from cerebrocortical neurons (Grilli et al., 2004) and mouse retinal cells (Dal Monte et al., 2003) through SSTR2. In the present study, we showed that SS decreased cAMP levels and glutamate release from cultured neonatal rat astrocytes through SSTR4. An important outcome of these studies is the potential targets for drugs to alleviate the effects of excessive glutamate transmission and glutamate induced neurotoxicity and more specific therapeutic interventions.

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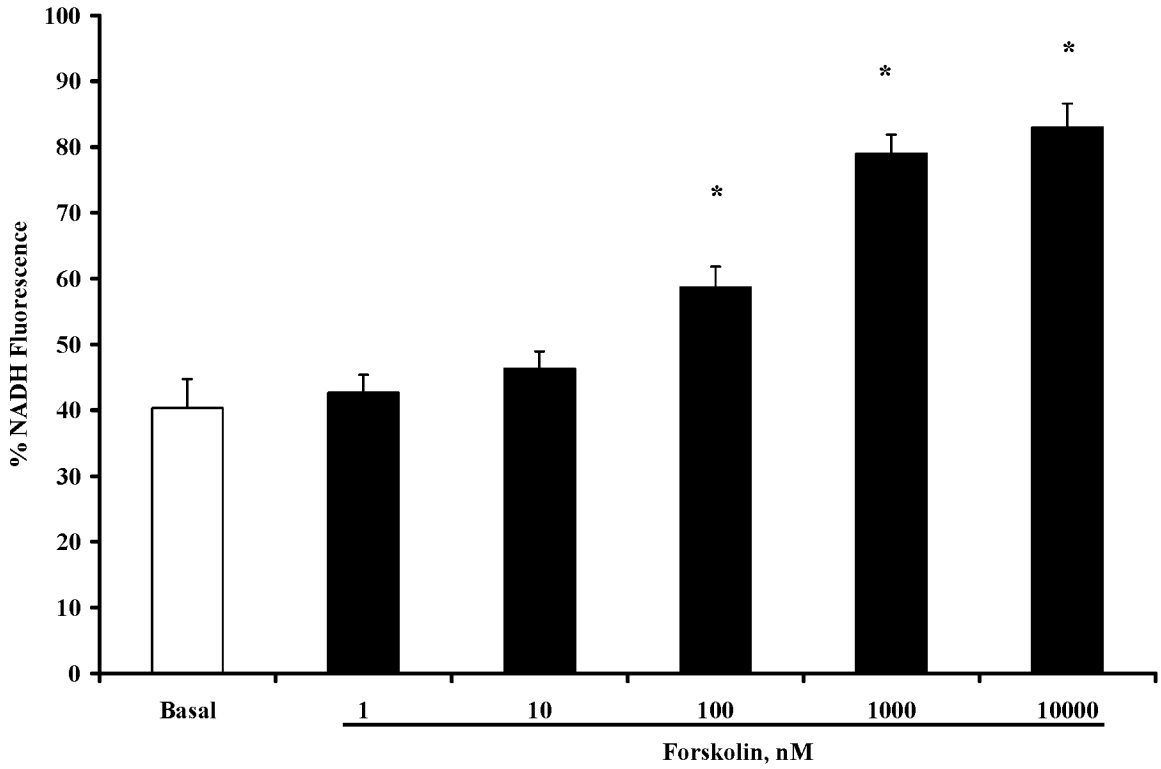

Fig. 1. Effect of forskolin on glutamate release from astrocytes. *, $p < 0.05$ compared with the basal group. Values are the mean $\pm$ S.E. ($n = 4$ independent cell preparations).

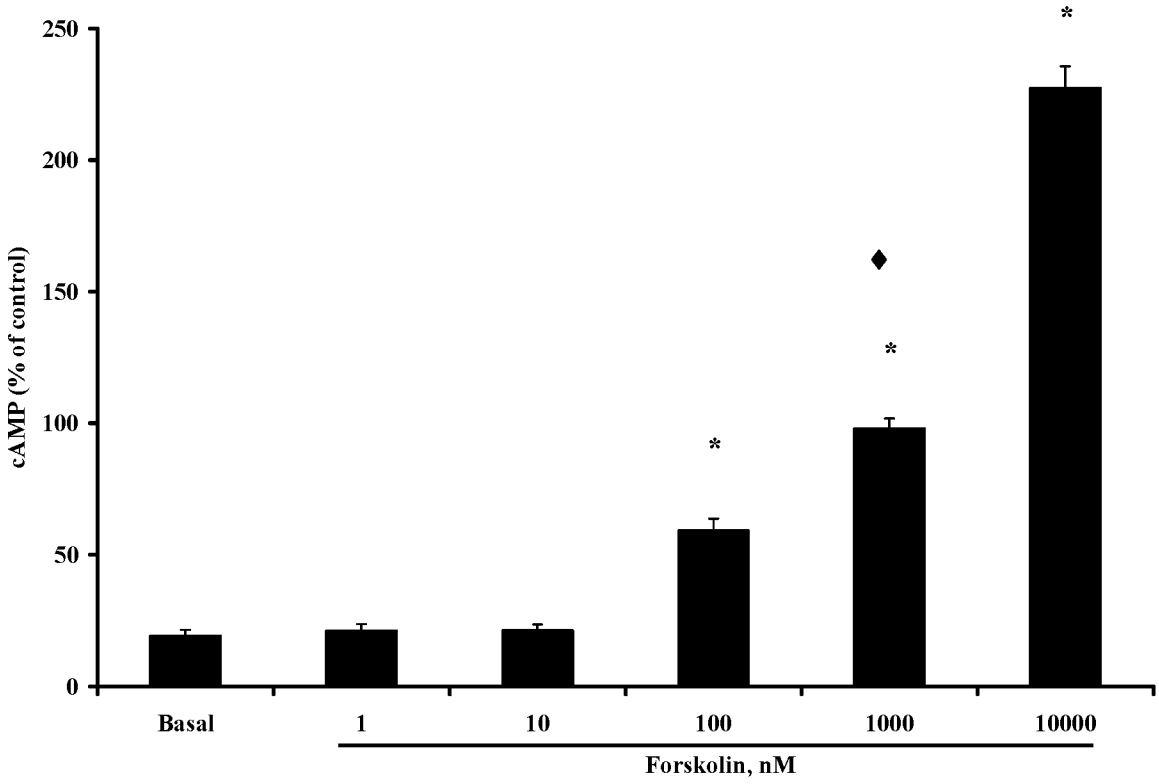

Fig. 2. Effect of forskolin on cAMP levels in astrocytes. Each value is expressed as a percentage of control (1 μM forskolin (◆)). *, $p < 0.05$ compared with the basal group. Values are the mean ± S.E. (n = 3 independent cell preparations).

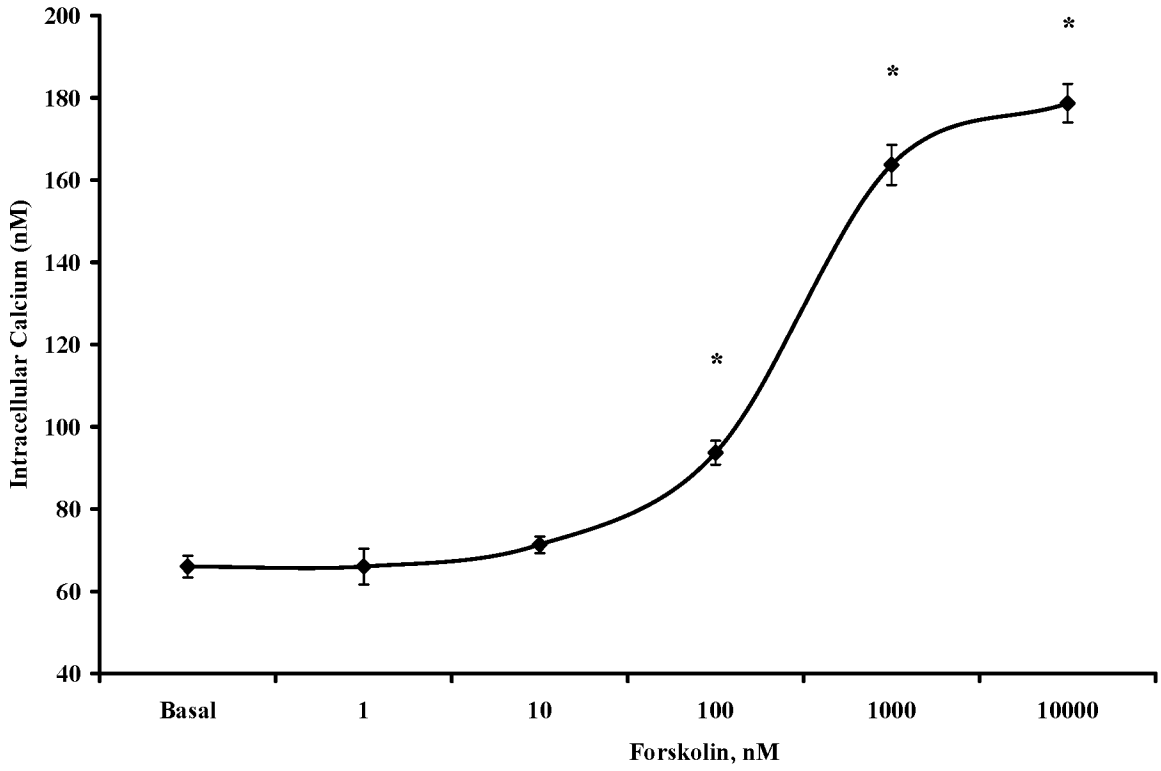

Fig. 3. Effect of forskolin on $[Ca^{2+}]_i$. Forskolin induced a concentration-dependent increase in $[Ca^{2+}]_i$ with maximal increase at 1 μM. Values are the mean ± S.E. (n = 3 independent cell preparations with ≥ 15 cells per experiment).

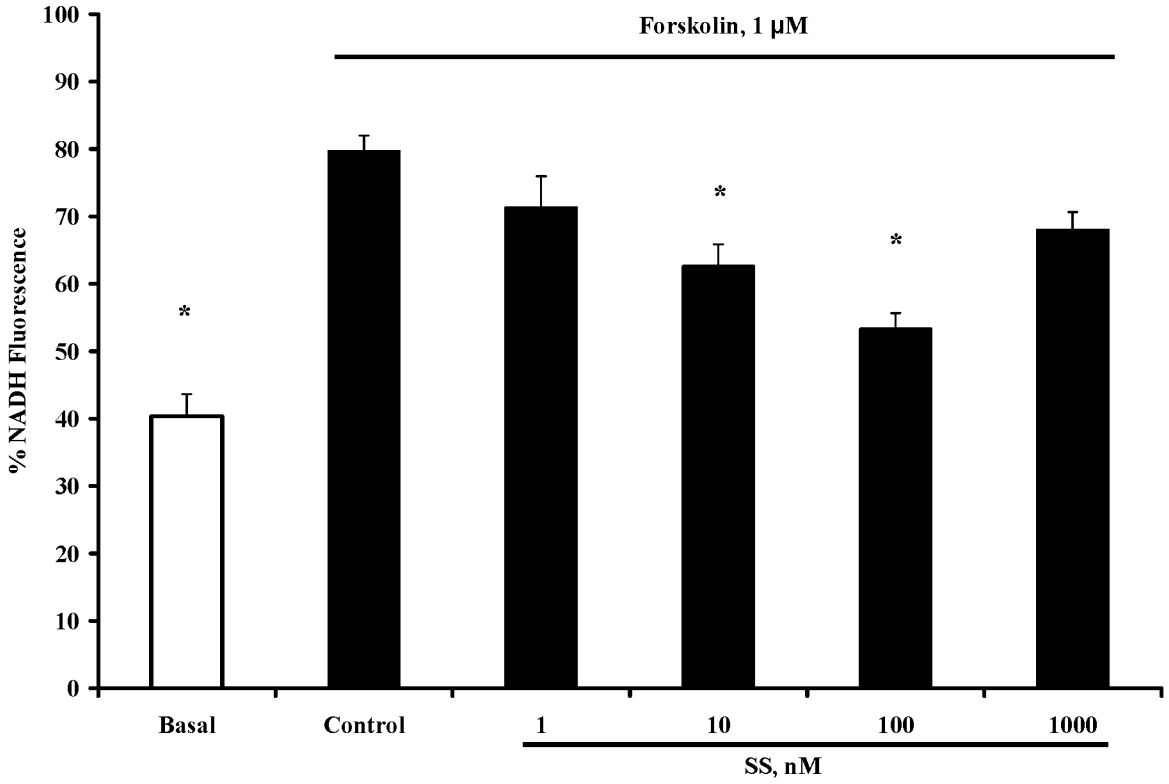

Fig. 4. Effect of SS on forskolin-induced increase in glutamate release. *, $p < 0.05$ compared with control (1 µM Forskolin). Values are the mean ± S.E. (n = 3 independent cell preparations).

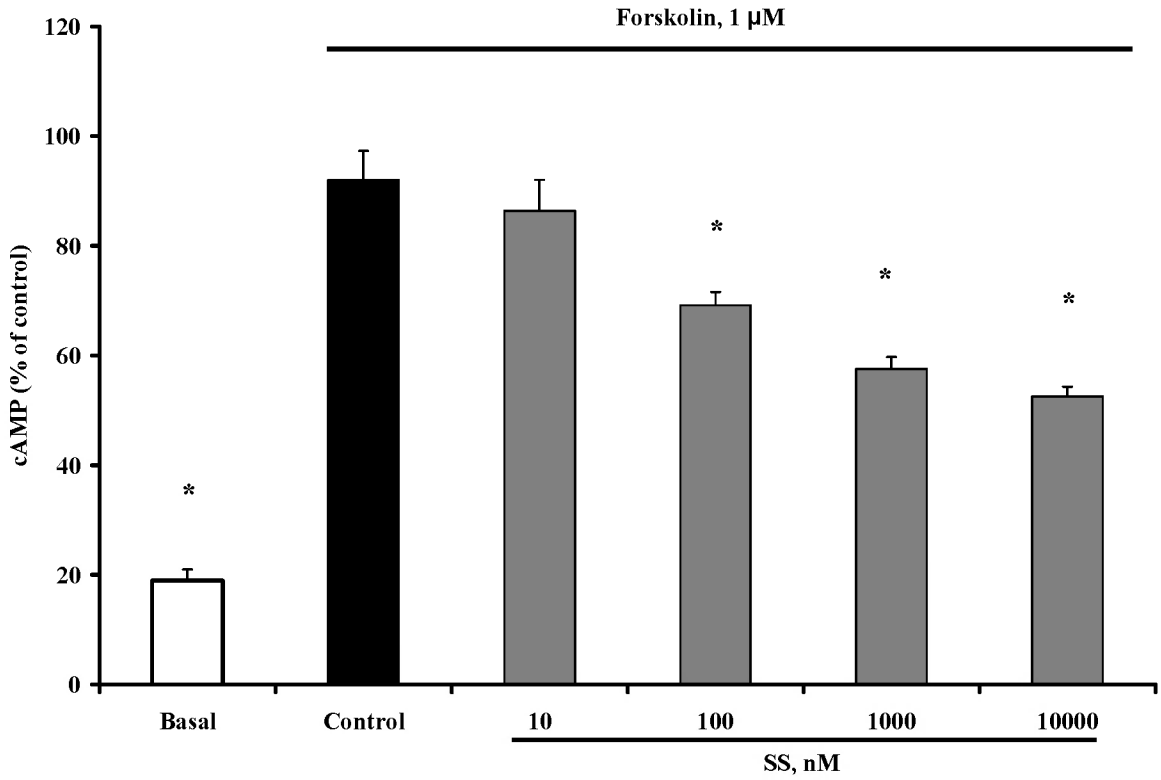

Fig. 5. Effect of SS on forskolin-induced increase in cAMP levels, expressed as percentage of control (1 μM forskolin). *, $p < 0.05$ compared with control. Values are the mean $\pm$ S.E. (n = 3 independent cell preparations).

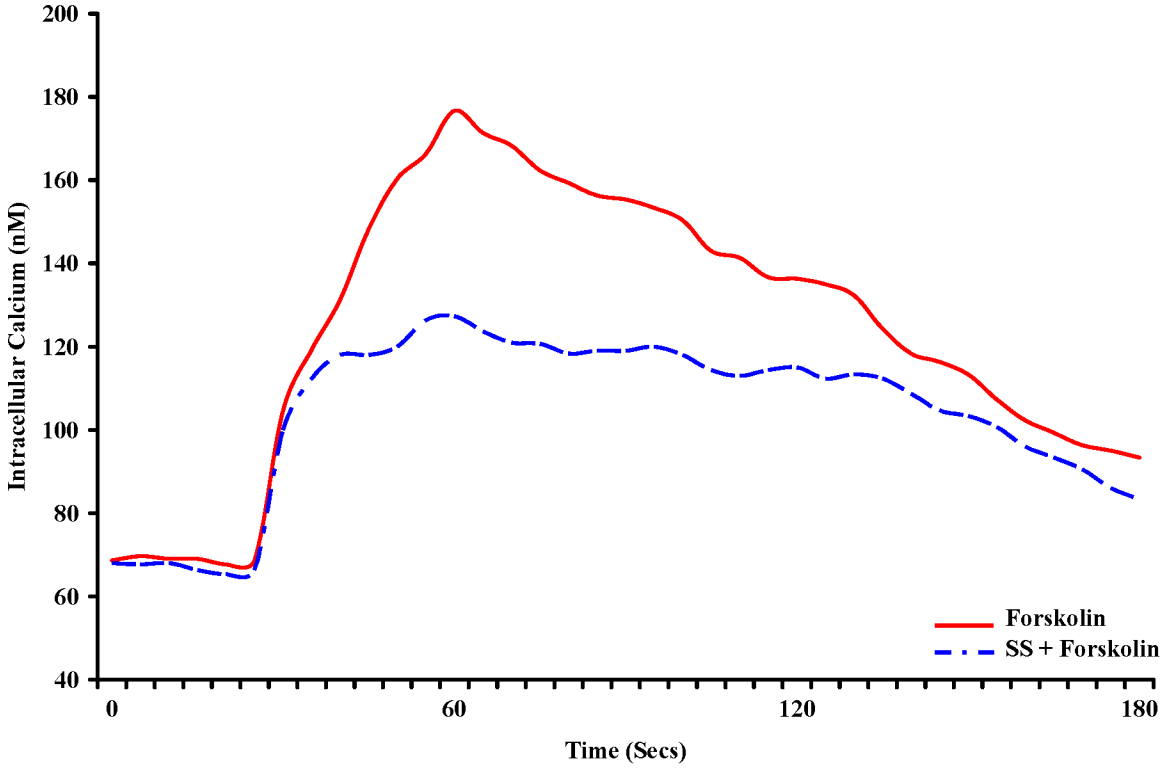

Fig. 6. Effect of SS on forskolin-induced increase in $[Ca^{2+}]_i$. SS (100 nM) decreased but did not abolish the 1 μM forskolin-induced increase in $[Ca^{2+}]_i$. The graph shows a representative trace of $[Ca^{2+}]_i$ from 3 experiments with ≥ 15 cells per experiment.

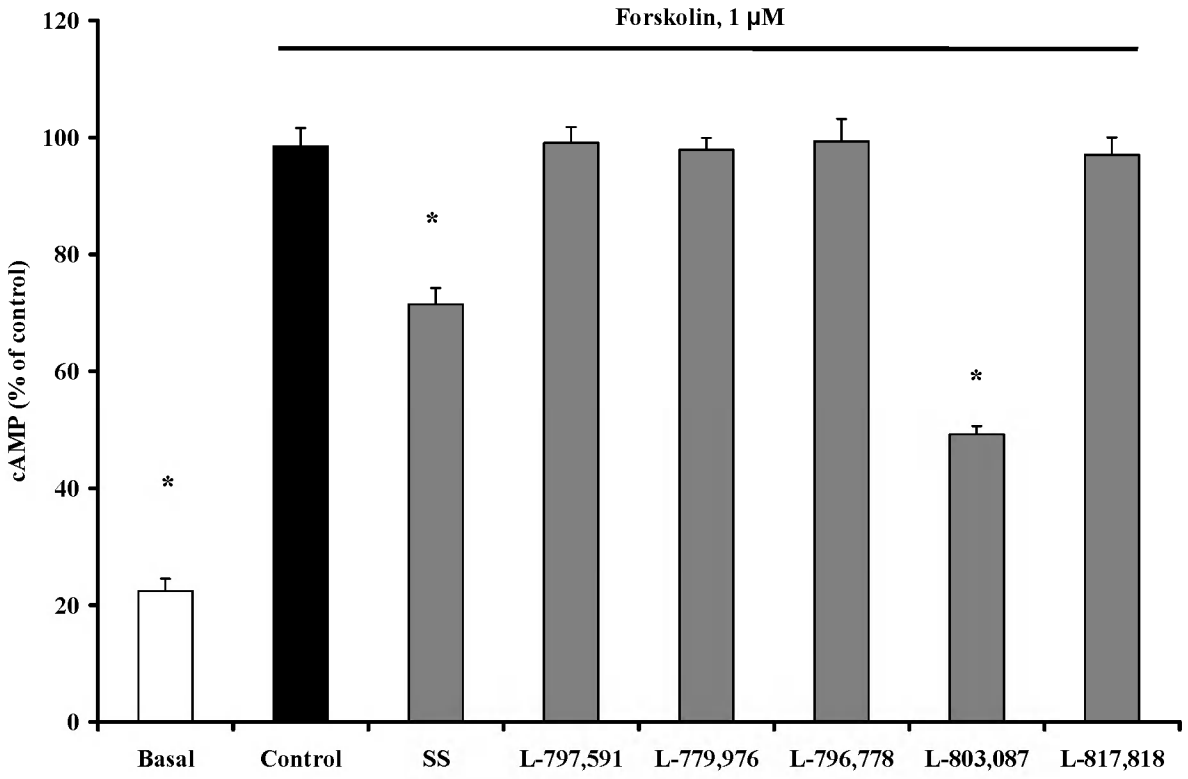

Fig. 7. Effect of SSTR agonists on forskolin-induced increase in cAMP levels expressed as percentage of control (1 μM forskolin). All 5 SSTR agonists were tested at 100 nM concentration. *, $p < 0.05$ compared with control. Values are the mean $\pm$ S.E. ($n = 3$ independent cell preparations).

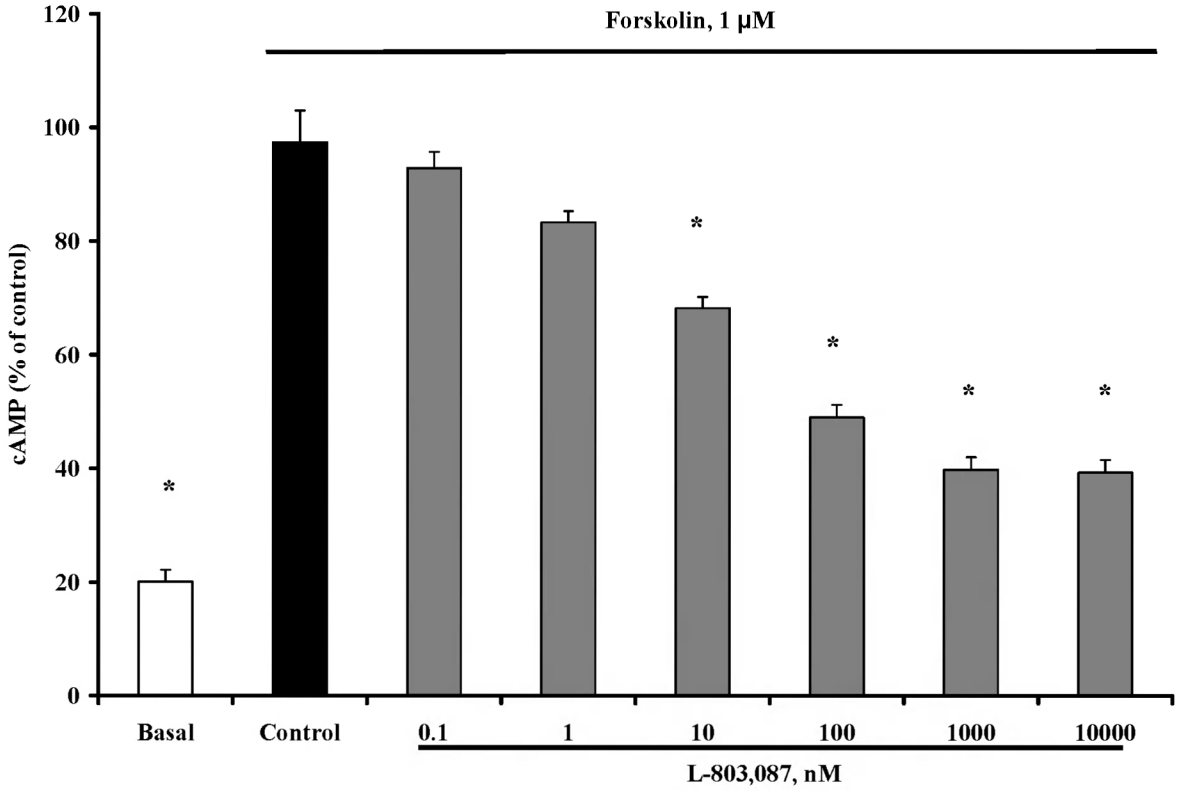

Fig. 8. Effect of SSTR4 agonist, L-803,087 (100 pM – 10 µM) on 1 µM forskolin-induced increase in cAMP levels expressed as percentage of control (1 µM forskolin). *, $p < 0.05$ compared with control. Values are the mean ± S.E. (n = 3 independent cell preparations).

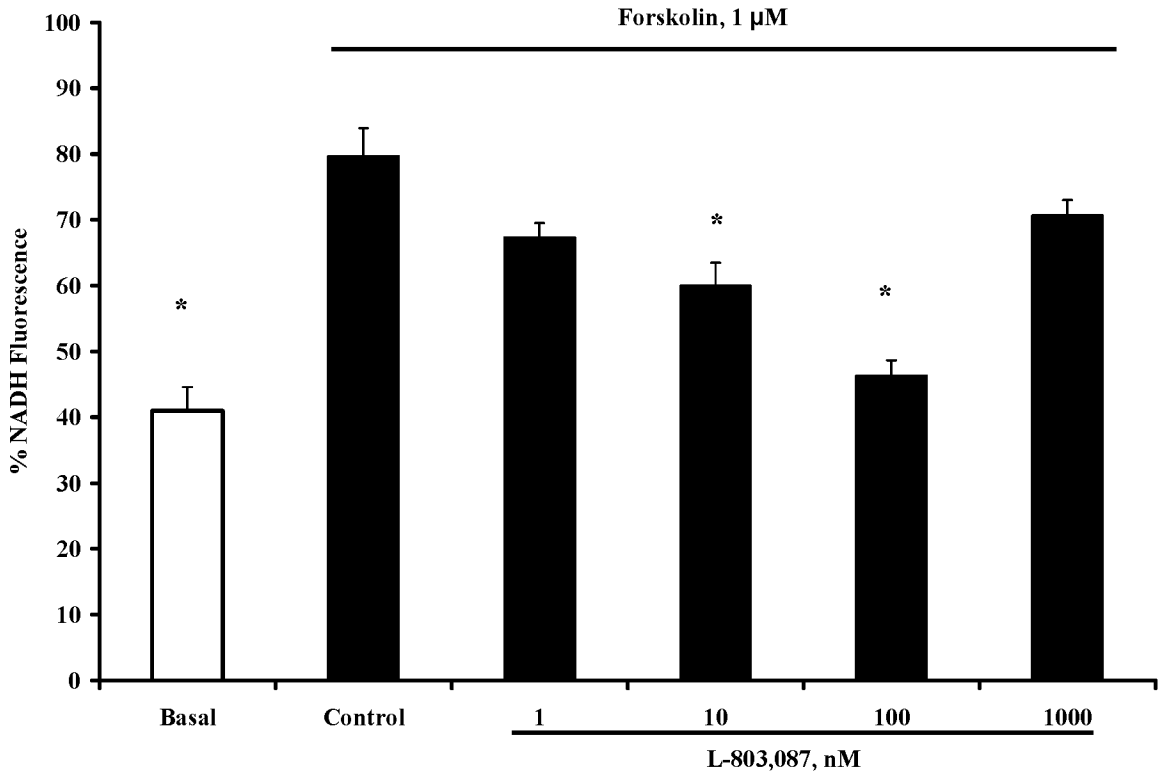

Fig. 9. Effect of SSTR4 agonist, L-803,087 (10 nM – 1 μM) on forskolin-induced increase in glutamate release. *, $p < 0.05$ compared with control (1 μM Forskolin). Values are the mean ± S.E. (n = 3 independent cell preparations).

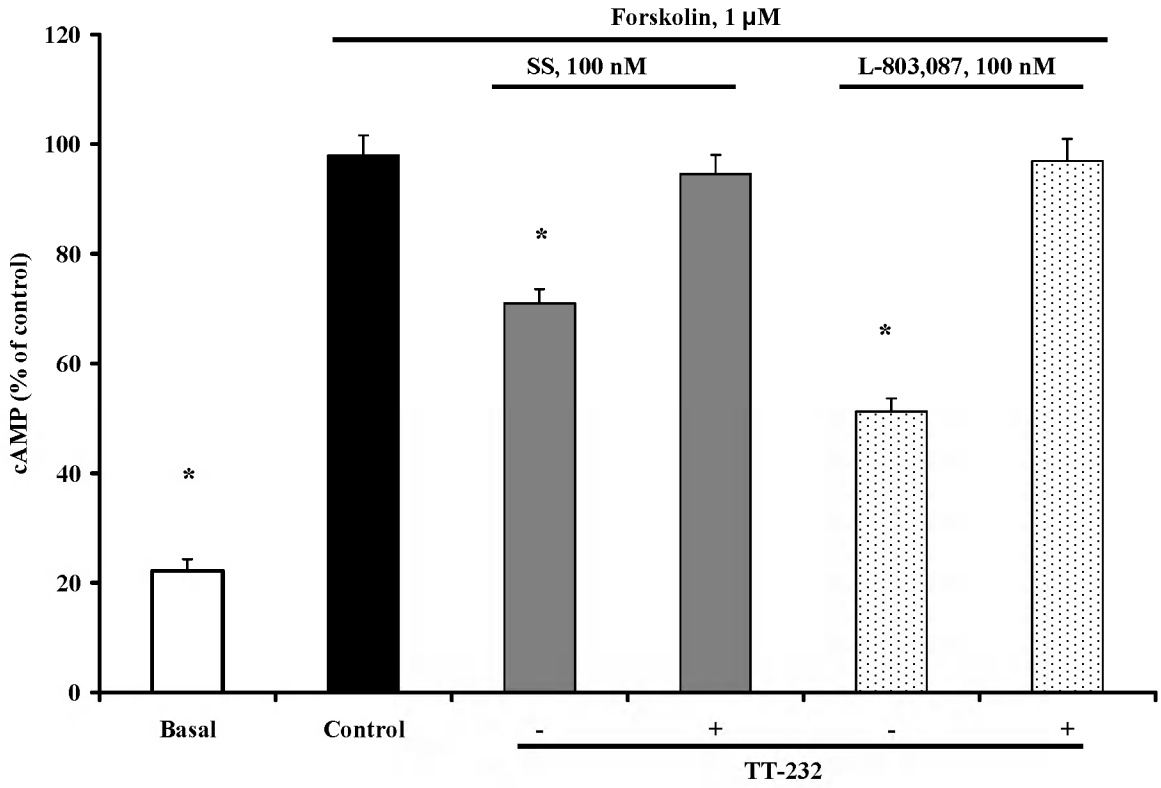

Fig. 10. Pretreatment with TT-232 (10 μM) antagonized the SS (100 nM)- and SSTR4 agonist L-803,087 (100 nM)-induced decrease in cAMP levels. cAMP levels expressed as percentage of control (1 μM forskolin). *, $p < 0.05$ compared with control. Values are the mean $\pm$ S.E. (n = 3 independent cell preparations).

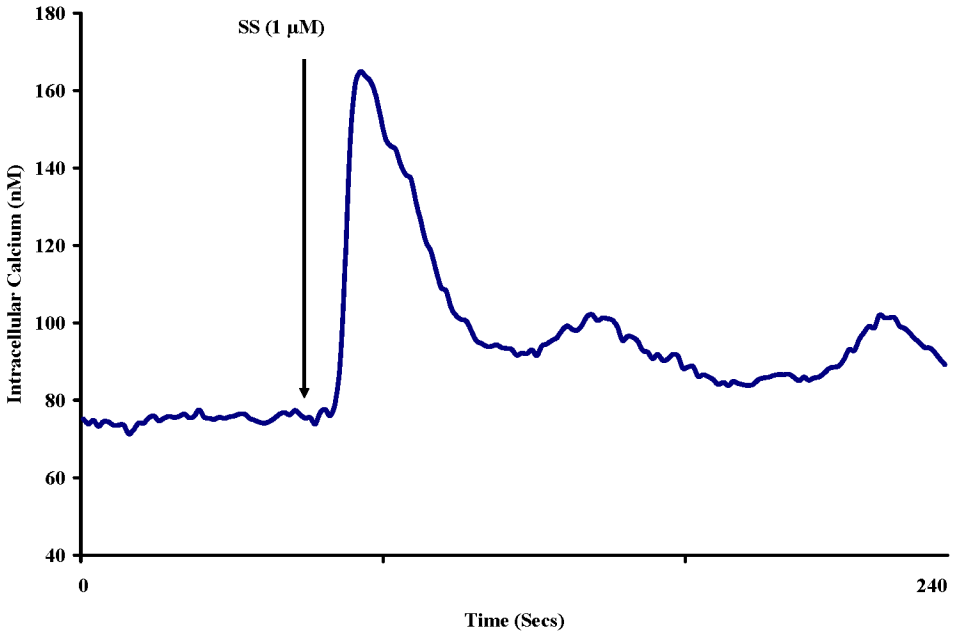

Fig. 11a. SS (1 µM)-induced increase in $[Ca^{2+}]_i$. The graphs are representative traces from four experiments with ≥ 15 cells per experiment

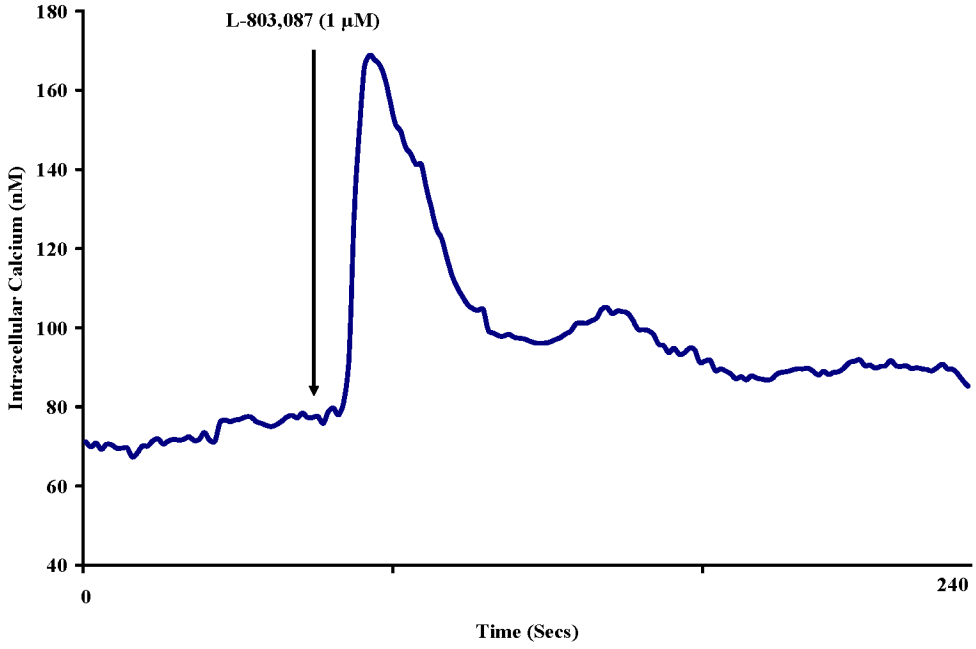

Fig. 11b. L-803,087 (1 µM)-induced increase in $[Ca^{2+}]_i$. The graphs are representative traces from four experiments with ≥ 15 cells per experiment.

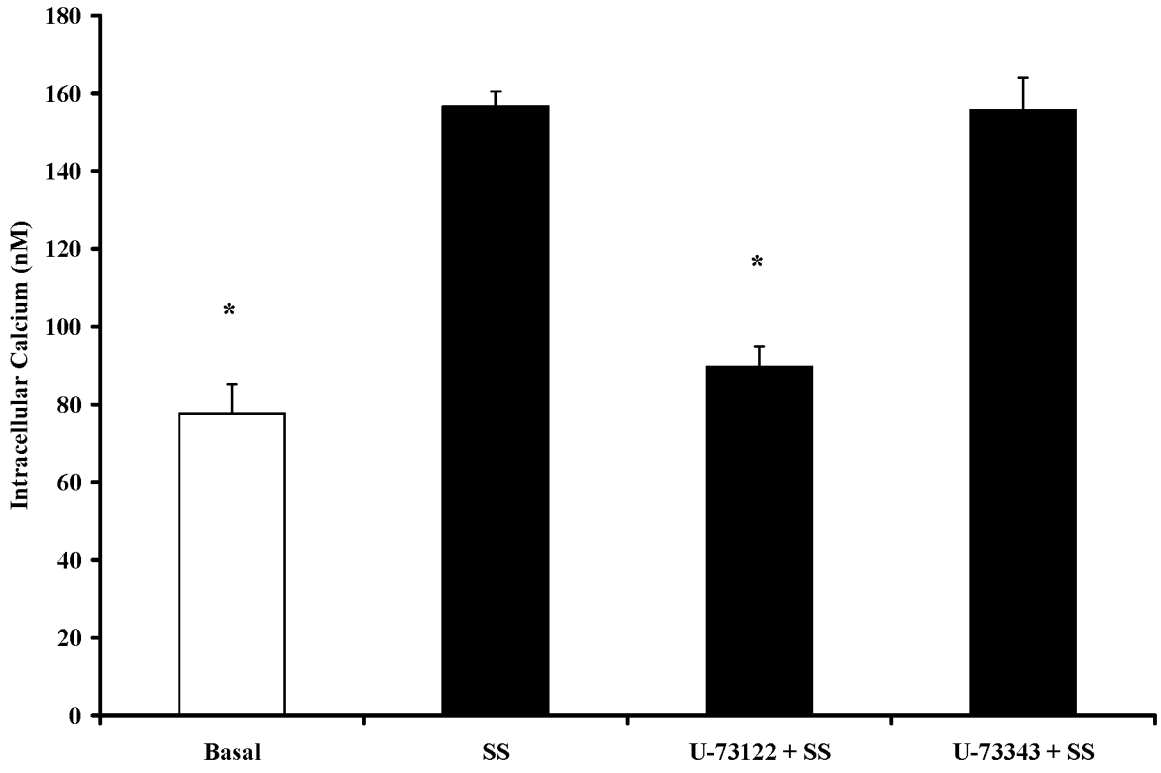

Fig. 12. Effect of U-73122 and U-73343 (1 μM) on SS (1 μM)-induced increase in $[Ca^{2+}]_i$. *, $p < 0.05$ compared with SS. Values are the mean ± S.E. (n = 3 independent cell preparations with ≥ 15 cells per experiment).

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