UNIVERSIDADE DA BEIRA INTERIOR
Ciências

The role of cGMP on adenosine A_1 receptor-mediated inhibition of synaptic transmission at the hippocampus

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Abstract

The adenosine A1 receptor is highly expressed in hippocampus where it inhibits synaptic transmission and has neuroprotective activity. Similar actions are obtained by increasing the concentration of the second messenger cGMP and recently it was found that activation of adenosine A1 receptor increased cGMP levels in the nervous system, but the role of cGMP on adenosine A1 receptor mediated inhibition of synaptic transmission remains to be established. This work addressed if the adenosine A1 receptor inhibitory effect on neurotransmission is dependent on the cGMP pathway. To answer this question investigated in what extent increasing the levels of cGMP (with a phosphodiesterases inhibitor and a cGMP analog), or blocking the cGMP pathway (using nitric oxide synthase, protein kinase G and soluble guanylyl cyclase inhibitors) modify the inhibitory effect of an A1 receptor agonist on synaptic transmission. The hippocampal slice was used as an experimental model and neurotransmission evaluated through extracelular electrophysiological recording technique, specifically by measuring the slope of field excitatory postsynaptic potentials (fEPSPs) evoked by electrical stimulation.

N^6-cyclopentyladenosine (CPA, 15nM), a selective adenosine A1 receptor agonist, reversibly decreased the fEPSPs by 48% ± 2.1% (n=5; P<0.05). Incubation of the slices with a phosphodiesterase inhibitor Bay 60-7550 (100 nM), in order to prevent cGMP degradation, did not modify the CPA (15 nM) inhibitory effect on fEPSPs (50%±2.8%, n=5; P>0.05 when compared with CPA alone). The presence of a membrane-permeable analog of cGMP, 8-pCPTP-cGMP (10 μM), also did not significantly affect (P>0.05) the CPA (15nM) inhibitory effect on fEPSPs (59.0%±4.5% in the absence and 50%±8.6% in the presence of 8-pCTP-cGMP, n=4). On the other hand, inhibition of nitric oxide synthase (NOS) by L-NAME (200 μM) decreased (P<0.05) the CPA (15nM) inhibitory effect on fEPSPs (54%±5.3% in the absence and 23%±5.7% in the presence of L-NAME, n=5, female rats). In male rats, the presence of a Protein Kinase G (PKG) inhibitor (KT5823, 1nM) decreased the inhibitory effect of CPA (15nM) on fEPSPs by 45.0%±8.9% (n=4, P<0.05 compared with zero); similar results were obtained in females. Finally the effect of an inhibitor of soluble guanylyl cyclase (sGC), ODQ (10 μM), on the CPA (15 nM) inhibitory action on fEPSPs was investigated. In males ODQ decreased (P<0.05) the CPA inhibitory effect (50%±4.3% in the absence and 39%±6.0% in the presence of ODQ, n=4), but only when adenosine deaminase (1 U/ml) was present; similar results were found in females.

In conclusion, the results strongly suggest that inhibitory action of adenosine A_1 receptors on glutamatergic neurotransmission at the hippocampus is, at least partially, mediated by activation of the NOS/sGC/cGMP/PKG pathway.

Keywords

Adenosine A1 receptor, cGMP, hippocampus, synaptic transmission, soluble guanylyl cyclase, protein kinase G, phosphodiesterase

Resumo alargado

A adenosina é libertada para o meio extracelular em situação de actividade neuronal prolongada ou após insulto neurotóxico. A adenosina, via activação do receptor A1, inibe a transmissão sináptica e previne o dano neuronal causado por isquémia, excitotocicidade ou episódios epiléticos.

Os receptores A1 diminuem a neurotransmissão quer pré-sinapticamente, por inibibição da libertação de glutamato, quer pós-sinapticamente, por activação dos canais de potássio que provocam hiperpolarização dos neurónios pós-sinápticos. Os receptores A1 produzem este efeito inibitório através da modulação de vários efectores e mensageiros intracelulares, tais como o cAMP, canais de cálcio e potássio e fosfatos de inositol.

O cGMP é produzido pelas ciclases do guanililo que são uma família de enzimas que convertem o GTP em cGMP. Existem dois tipos de ciclases do guanililo: a forma solúvel (sGC) activada pelo oxido nítrico e a forma particulada, a qual constitui um receptor membranar para ligandos extracelulares como o péptido natriurético. O cGMP é degradado por acção de fosfodiesterases que catalisam a hidrólise do cGMP e também do cAMP (algumas formas), desempenhando um importante papel na regulação da via dos nucleótidos cíclicos e na comunicação celular.

O cGMP através da activação da cinase G de proteínas (PKG) diminui a transmissão sináptica reduzindo a actividade neuronal e exerce uma acção neuroprotectora face a insultos neurotóxicos.

Uma via importante que modula os níveis de cGMP no cérebro em resposta à atividade sináptica é a via oxido nítrico/sGC. O oxido nitrico age como mensageiro retrogrado, uma vez que além de activar a ciclase do guanililo solúvel em neurónio pós-sinápticos pode difundir para o meio extracelular e pré-sinapticamente estimular a sGC produzindo cGMP diminuindo assim a libertação de glutamato.

Estudos anteriores mostraram que doadores de oxido nítrico inibiram a transmissão sináptica em fatias de hippocampo e essa inibição foi bloqueada por antagonistas do receptor A1 (Boulton et al., 1994; Broome et al., 1994). Em contraste, a inibição da ciclase do guanililo solúvel não afectou o efeito inibitório do doador de oxido nitrico na transmisão sináptica (Arrigoni et al., 2006). Por outro lado, o óxido nítrico mostrou aumentar o efeito inibitório da 2-chloroadenosine (CADO) na transmissão sináptica e esse aumento foi bloqueado por inibidores da sGC (Fragata et al., 2006). No entanto, o papel do cGMP na mediação do efeito inibitório do receptor A1 na transmissão sináptica permanece por esclarecer. Observámos recentemente que a activação do receptor A1 aumenta a formação de cGMP no cérebro. Com o presente projecto pretende-se investigar se o efeito inibitório do receptor A1 na transmissão sináptica glutamatérgica serão mediados, pelo menos em parte, pela via do cGMP. Para responder à questão anterior investigou-se em que medida fármacos que aumentam os níveis de cGMP (e.g. inibidor de fosfodiesterases e análogo do cGMP) ou que bloqueiem a via do cGMP (e.g. inibidores da sintase do óxido nítrico, da PKG e da sGC) modificarão o efeito de um agonista selectivo do receptor A1 na transmissão sináptica. A fatia de hipocampo de rato foi utilizada como modelo experimental. A neurotransmissão foi avaliada por electrofisiologia através do registo e medição do declive dos potenciais pós-sinápticos excitatórios de campo (fEPSPs) evocados por estimulação eléctrica.

A N6-ciclopentiladenosina (CPA, 15 nM), um agonista selectivo do receptor A1 de adenosina diminuiu reversivelmente os fEPSPs em 48% ± 2,1% (n = 5, P<0,05). A incubação das fatias com um inibidor de fosfodiesterases, o Bay 60-7550 (100 nM), a fim de evitar a degradação do cGMP, não alterou o efeito inibitório da CPA (15 nM) nos fEPSPs (50% ± 2,8%, n = 5, P>0,05 quando comparado com a CPA sozinha). A presença de um análogo do cGMP permeável à membrana, o 8-pCPTP-cGMP (10 μM), também não afectou significativamente (P>0,05) o efeito inibitório da CPA (15 nM) nos fEPSPs (59% ± 4,5%, na ausência e 50% ± 8,6% na presença de 8-PCTP-cGMP, n = 4). Por outro lado, a inibição da sintase do óxido nítrico (NOS) pelo L-NAME (200 μM) diminuiu (P<0,05) o efeito inibitório da CPA (15 nM) nos fEPSPs (54% ± 5,3 % na ausência e 23% ± 5,7% na presença de L-NAME, n = 5, ratos fêmeas). Em ratos machos, a presença de um inibidor (KT5823, 1 nM) da PKG diminuiu o efeito inibitório do CPA (15 nM) nos fEPSPs em 45.0%±8.9% (n=4, P<0,05 comparado com zero); resultados semelhantes também foram obtidos em fêmeas. Finalmente, o efeito de um inibidor da ciclase do guanililo solúvel, o ODQ (10 μM), na acção inibitória da CPA (15 nM) nos fEPSPs foi investigado. Em machos o ODQ diminuiu (P<0,05) o efeito inibitório da CPA (50% ± 4,3%, na ausência e 39% ± 6,0% na presença de ODQ, n=4), mas apenas quando a desaminase da adenosina (1 U/ml) estava presente; resultados semelhantes foram obtidos em fêmeas.

Em conclusão, os resultados sugerem que a acção inibitória dos receptores A1 da adenosina na neurotransmissão glutamatérgica ao nível do hipocampo é, pelo menos em parte, mediada pela activação da via NOS/sGC/cGMP/PKG.

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8-pCPT-cGMP: 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate
ADA: Adenosine deaminase
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP: Adenosine 5´-triphosphate
CA: Cornus ammonis
cGMP: Cyclic guanosine monophosphate
CNG: cyclic nucleotide-gated
CPA: N6-cyclopentyladenosine
EAATS: excitatory amino acid transporter
eNOS: Endothelial nitric oxide synthase
fEPSP: Field excitatory post-synaptic potentials
iNOS: Inducible nitric oxide synthase
IP3: Inositol trisphosphate
L-NAME: N^G-nitro-L-arginine methylester (L-NAME)
MAPK: Mitogen-activated protein kinase
NMDA: N-methyl D-aspartate
ODQ: 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one
PDE: Phosphodiesterase
pGC: particulate guanylyl cyclase
PIP2: Phosphatidylinositol 4,5-bisphosphate
PKA: cAMP-dependent protein kinase
PKC: Protein kinase C
PKG: Protein kinase G
PLC: Phospholipase C
NO: Nitric oxide
NOS: Nitric oxide synthase
nNOS: Neuronal nitric oxide synthase
sGC: Soluble guanylyl cyclase
VGLUT: vesicular glutamate transporter

Chapter 1

Introduction

1.1 Hipocampus

The hippocampus is a complex and fascinating region of the brain that has enormous clinical significance. It is an important part of the limbic system, which plays vital role in the behavioral, emotional and memory processes (Smith et al, 2006). The hippocampal formation consists of two C-shaped interlocking cell layers: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon's horn or cornus ammonis (CA) and subiculum (see Fig. 1). Both granular and pyramidal cells use glutamate as neurotransmitter. Ammon´s horn is subdivided into four zones: CA1, CA2, CA3 and CA4 (Hayman et al, 1998). The CA1 and CA2 fields contain small pyramidal cells, the CA3 field forms a curve and loose band of pyramidal neurons and the CA4 field, also referred to as a hilar region, is formed by loosely structured pyramidal cells, which are surrounded by dentate gyrus (Sendrowski, 2013). The dentate gyrus subdivides into the molecular or dendritic layer, stratum granulosum (granule cell bodies) and the hilar region or stratum polimorphum, characterized by widely scattered polymorph neurons, it is separated from CA1-CA3 by the hippocampal sulcus (Duvernoy et al., 2013).

Hippocampal circuitry has been studied for decades because of its essential role in memory function. It's trisynaptic circuit consists of dentate gyrus granule cells, CA3 pyramidal neurons, and CA1 pyramidal cells, and is integrated by the terminal axons of the enthorrinal cortex-proceeding perforant pathway, which establish synaptic contacts with the dendritic spines located in dendritic segment of the dentate gyrus granule cells. The axons from dentate gyrus granule cells (mossy fibers) stimulate the CA3 pyramidal cells and the Schaffer collaterals of CA3 axons excite the CA1 pyramidal cells. These pyramidal cells also receive GABAergic synaptic inputs from interneurons (10% of neuronal population of hippocampus), and glutamatergic inputs from cortical pyramidal cells and subcortical sources (Scoville et al., 2000; González-Burgos et al., 2009; Klausberger, 2009; Mcbain et al, 1999).

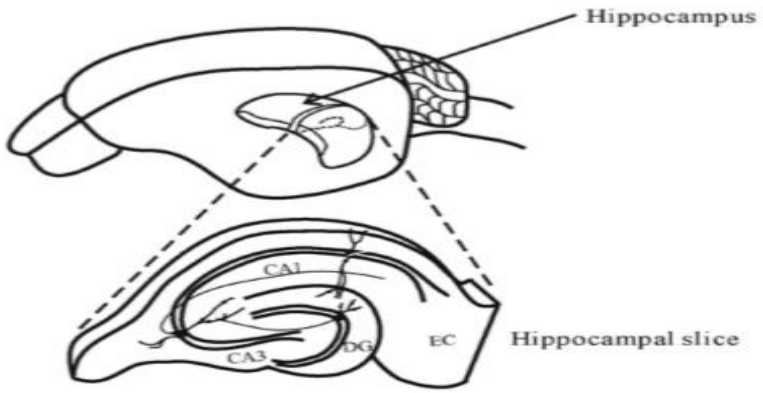

Fig.1 Localization of the hippocampus in the brain and the general layout of the trisynaptic loop. Orientation of the hippocampus in the rat brain allows for visualization of a hippocampal slice as it would appear in situ. Each slice includes a portion of the entorhinal cortex (EC), the dentate gyrus (DG), field CA3 (CA3) and field CA1 (CA1). Thin lines represent axonal projections (adapted from Wojtowicz, 2012).

1.2 The role of glutamate in central nervous system

Glutamate is the major excitatory neurotransmitter in central nervous system (CNS). Its involved in numerous cellular functions, including regulation of cellular, motor and synaptic activity, plasticity, cell death and survival, learning and memory (Brynes et al., 2009).

The metabolism of glutamate in the brain involves both neurons and astrocytes. Glutamate is packaged into presynaptic vesicles through vesicular glutamate transporter (VGLUT) proteins, these vesicles fuse with plasma membrane through interactions with Snare proteins. After membrane depolarizes and influx of calcium occurs glutamate is then released into the synaptic cleft where it binds glutamate receptors. It is reuptaked through excitatory amino acid transporter (EAATs) expressed predominantly in astrocytes, where it is converted to glutamine by glutamine synthetase and exported again to neurons (Niciu et al., 2011) (see Fig. 2).

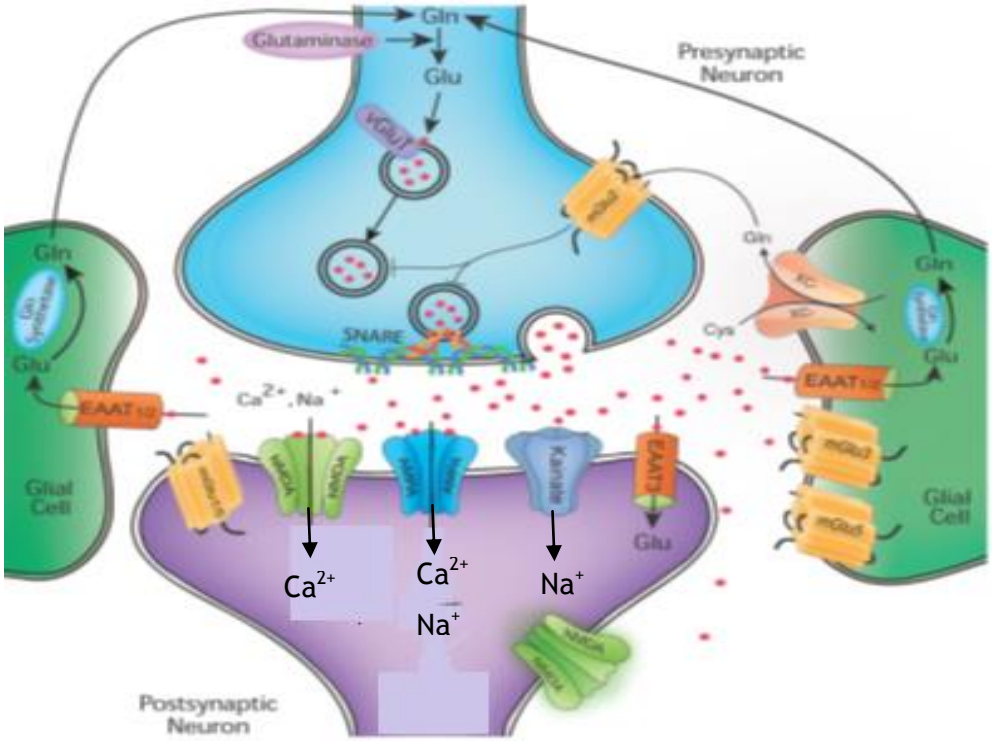

Fig.2 Glutamatergic neurotransmission: due to the risk of excitotoxic damage as consequence of excessive glutamatergic stimulation, precise physiological control of glutamate must be maintained in the mammalian CNS. Glutamine (Gln) is converted to glutamate (Glu) by glutaminase. Glu is packaged into presynaptic vesicles trough vesicular Glu transporter (VGLUT) proteins and synaptically released in a voltage-dependent manner through vesicular interactions with SNARE proteins. Synaptically-released Glu is reuptaked from the extracellular space by excitatory amino acid transporters (EAATs) expressed predominantly on astroglia. In astrocytes, Glu is converted to Gln by Gln synthetase and exported extracellularly to be taken up again by neurons. Additionally, system x-C is a cystine/glutamate antiporter expressed on glia that also contributes to Glu recycling. Glu receptors are present on presynaptic and postsynaptic neurons as well as on glial cells. These include both ionotropic receptors (NMDA, AMPA, KA) and metabotropic receptors (mGluRs) (Adapted from Niciu et al., 2011).

In normal conditions, neurons are exposed to small and transient impulses of glutamate, regulated by efficient uptake/removal mechanisms. However, they may be subjected to excessive glutamatergic stimulation due to overstimulation of pós-sinaptic glutamate receptors, a phenomenon called excitotoxicity, considered the major mechanism of cell death in CNS (Kostandy, 2012, Wang et al., 2010).

Glutamate acts on four different postsynaptic receptors: N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), kainate and metabotropic receptors. AMPA and NMDA receptors are the main types of glutamate receptor mediating fast glutamatergic neurotransmission. NMDA receptors are heteromeric, ligand-gated ion channels which have critical roles in synaptic transmission, plasticity and excitotoxicity in the CNS. These receptors have high permeability to Ca^{2+}, Na^{+} and K^{+} and are blocked by extracelular Mg^{2+}, under resting membrane potentials, with unusually slow 'activation/deactivation' kinetics (Candy et al.,2001).

AMPA receptors are glutamate-gated ion channels permeable to sodium and potassium but, usually, not calcium and when glutamate binds to these receptors causes depolarization of the membrane potential due to influx of sodium ions and consequently removes the magnesium plug that blocks the NMDA receptor (Fleming et al., 2010).

Kainate receptors contribute to excitatory synaptic transmission at certain synapses through regulation of glutamate release (Kostandy, 2012).

Metabotropic receptors are G-protein coupled receptors that are grouped into three classes (Groups I-III) based on pharmacological properties, signal transduction mechanisms and sequence similarities. They modulate synaptic neurotransmission through the binding of glutamate at its large extracellular domain, however, its signalling response to glutamate may be modulated by compounds that bind to allosteric sites located in the less highly conserved transmembrane regions (Con et al., 2009, Gu et al., 2008).

1.3 Role of adenosine in central nervous system as a neuromodulator

The first evidence that extracellular adenosine is able to modify physiological processes appeared when Drury and Szent-Gyorgyi (1929) reported that adenosine injected in mammals causes decreased arterial blood pressure and dilatation of the coronary arteries. The first observation on the relevance of adenosine in the nervous system were made in the seventies, when it was reported that adenosine decreases the release of acetylcholine from motor nerve terminals and affects cAMP accumulation in nerve cells (Gingsborg et al., 1972; Sattin et al., 1970). Since then, many studies extend these observations to every transmitter of peripheral and central nervous system, considering that adenosine is involved in a very sophisticated interplay between its own receptors and the receptors of several other synaptic mediators (Sebastião and Ribeiro, 1996; Dunwiddie and Masino, 2001).

Adenosine is a ribonucleoside released from most cells, including neurons and glia where it is formed as a by-product of purine nucleotides metabolism and other key metabolic cell processes. Adenosine is a neuromodulator because it isn't stored and released from vesicles, it is generated by the highly regulated intracellular metabolism of AMP and transported out of the cell through bi-directional facilitated diffusion transporters (Wei et al, 2011).

The various actions of adenosine are mediated through specific receptors in the brain, involved in normal and pathological processes, including regulation of sleep, synaptic plasticity and neuroprotective actions; it is also capable of affecting neuronal excitability through inhibition of release of various neurotransmitters, including glutamate, γ-aminobutyric acid (GABA), acetylcholine, and dopamine (see Lopes et al., 2011; Sebastião et al., 1996).

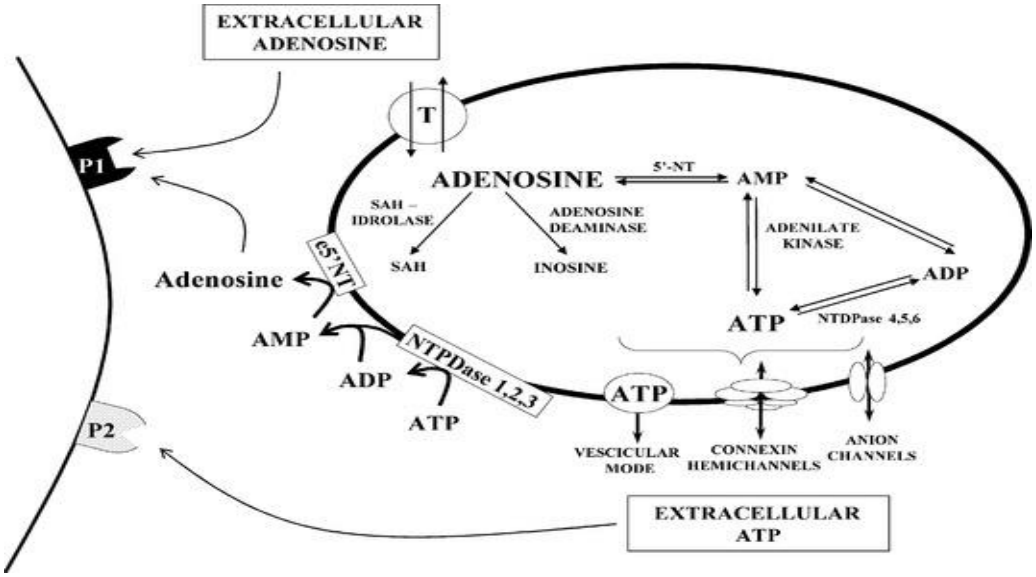

Fig. 3 Schematic drawing of intracellular and extracellular adenosine formation. In the extracellular space, adenosine and ATP act on own purinergic receptor subtypes: P1 and P2 receptors, respectively. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; e5′-NT, ecto-5′-nucleotidase; 5′-NT, 5′-nucleotidase; NTDPase, ecto-nucleoside triphosphate diphosphohydrolases; P1, adenosine receptor; P2, ATP receptor; SAH, S-adenosylhomo-cysteine; T, bidirectional nucleoside transporte (adapted from Pedata et al,2007).

1.4 Adenosine metabolism

Understanding the metabolic pathways which are involved in formation and removal of adenosine and the multiple mechanisms by which adenosine can be released into the extracellular space is very important and has been extensively studied (Latini and Pedata, 2001) (see Figure 3).

Intracellular adenosine is formed from degradation of AMP through an action of 5-endonucleotidase or by hydrolysis of S-adenosil-homocysteine (SAM). Extracellular adenosine originate from intracellular adenosine, which can pass the plasma membrane via an equilibrative nucleoside transporter (ENT) or by hydrolysis of released adenine nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and to a lesser extent cyclic adenosine monophosphate (cAMP) through the action of ecto-nucleotidases (see Figure 3). It is catabolised by the action of enzymes such as adenosine kinase and adenosine deaminase or inactivation of the reversible reaction catalyzed by S-adenosyl homocysteine (SAH) hydrolase (Dunwiddie et al., 1997, Zimmerman, 1996). In the brain, the major route of adenosine catabolism in under normal conditions is phosphorylation to AMP by adenosine kinase (Paul et al, 2011) (see Fig. 4).

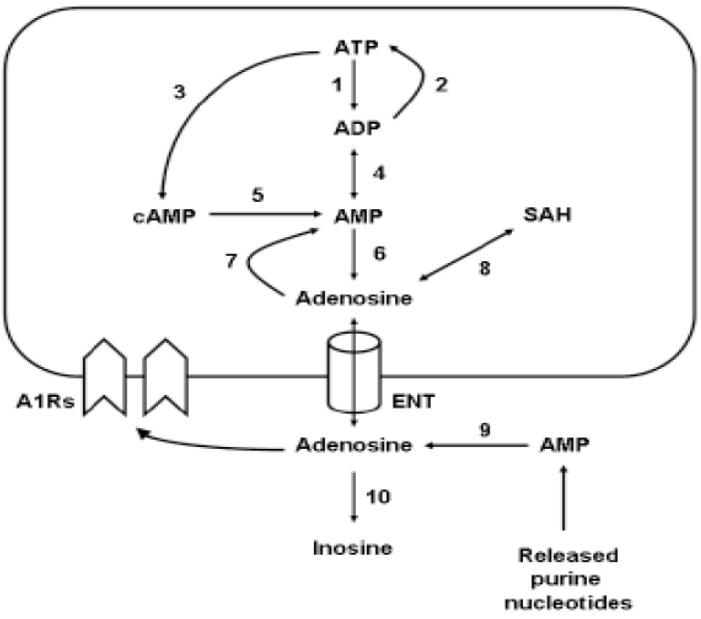

Fig. 4 Metabolic scheme showing the pathways of adenosine formation and removal. Enzymes involved are: 1. ATPases, 2. oxidative phosporylation (and creatine kinase), 3. adenylyl cyclase, 4. adenylate kinase, 5. phosphodiesterase, 6. 5'-endonucleotidase, 7. adenosine kinase, 8. SAH hydrolase, 9. ecto-5'-nucleotidase (CD73), 10. adenosine deaminase (adapted from Paul et al., 2011).

1.5 Adenosine receptors

The effects of adenosine on neuronal activity are mediated by specific receptors located in the plasma membrane. Four types of adenosine receptors are presently known: A_1, A_{2A}, A_{2B} and A_3, which belong to the G-protein coupled receptor (GPCR) family and all have been cloned and characterized from several mammalian species including humans (for review see Fredholm et al., 2001).

The adenosine receptors have been shown to couple to different G proteins and to different transducing systems in different cell types (Cunha, 2005). A1 and A3 receptors are usually coupled to 'inhibitory' G-proteins (G_i and G_o), negatively coupled to adenylyl cyclase, whereas the A2A and A2B receptors are couple to 'stimulatory' G-proteins (G_s), positively coupled to adenylyl cyclase. In the striatum, A2A receptors are mainly coupled to G_{olf}, a G-protein abundant in this brain area that activates adenylyl cyclase (Fredholm et al., 2001). A1 receptor is highly expressed in cortex, cerebellum, hippocampus, and dorsal horn of spinal cord, A2A is highly expressed in the striato-pallidal GABAergic neurones. Little is known about A2B and A3 receptors due to their low level of expression and density in brain tissues (Dixon et al., 1996; Ribeiro et al., 2003).

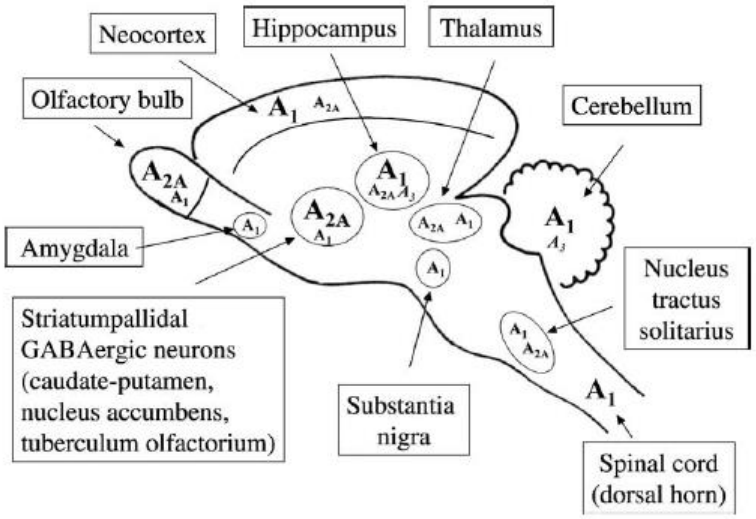

Fig. 5 Distribution of high affinity adenosine receptors (A1, A2A and human A3) in the main regions of the central nervous system where adenosine has been proposed to interfere with brain functions and disease. High levels of expression are indicated by bigger alphabets (Ribeiro et al., 2003).

1.5.1 Adenosine A_1 Receptor

A1 receptors cause inhibition of synaptic transmission in the hippocampus through activation of presynaptic and postsynaptic receptors. Presynaptic receptors reduce calcium influx through inhibition of N-type and Q/P-type voltage operated Ca^{2+} channels and decrease the release of several neurotransmitters such as glutamate, acetylcholine, dopamine, noradrenaline, and serotonin. Postsynaptic receptors activate potassium conductance causing hyperpolarization of the plasma membrane (see Ribeiro et al., 2003; Cunha et al., 2005).

A1 receptors can operate on different transducing systems, such as adenylyl cyclase and phospholipase C (PLC) payhway. Adenylate cyclase is responsible for the production of cAMP, which activates PKA, while phospholipase C catalyzes the degradation of phosphatidylinositol bisphosphate (PIP2) in diacylglycerol (DAG) and inositol triphosphate (IP3) (see Fig. 6). DAG is an activator of PKC, whereas IP3 induces calcium release into the cytosol, through a complex mechanism involving the endoplasmic reticulum (Biber et al., 1997; Berridge, 1993).

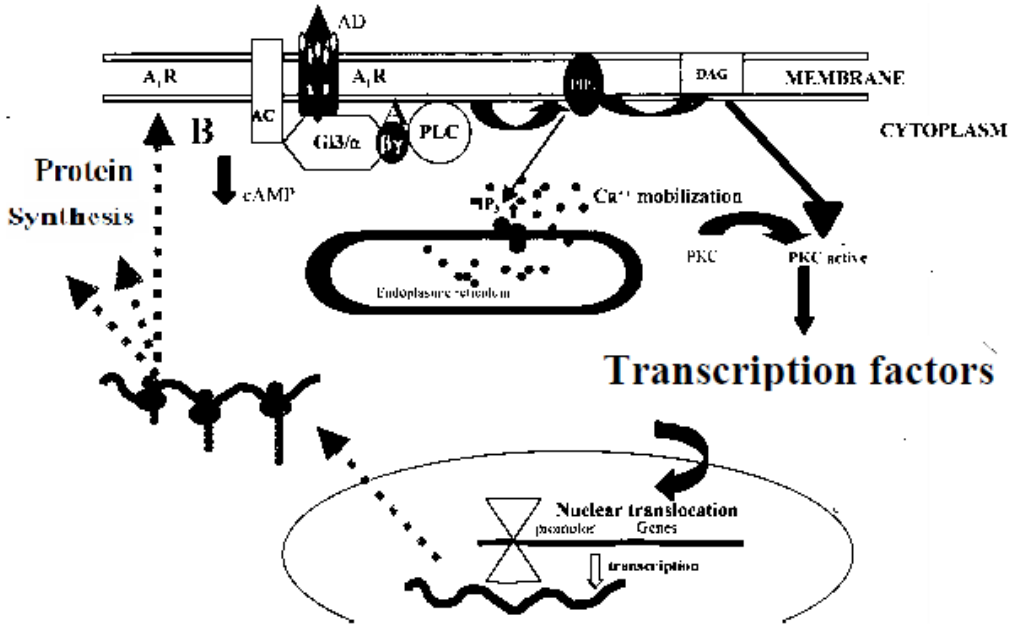

Fig.6 Model of possible adenosine effects mediated through the A1 adenosine receptor. The A1 adenosine receptor (A1R) is coupled to Gi3 protein that can either activate the pathway B, by inhibiting adenylyl cyclase (AC), or increase in adenosine levels might activate another pathway A by activating PLC, resulting in the activation of PKC and the mobilization of internal Ca^{2+} by IP3. PKC can phosphorylate transcription factors which facilitate transcription of genes in the nucleus and protein expression (adapted from Basheer et al., 2001).

1.5.1.1 The role of A1 receptor in neuroprotection

Adenosine is an endogenous neuroprotective agent in the CNS, since it prevents the damage of neuronal tissue caused by ischemia, excitotoxicity or epileptic seizures (de Mendonça et al.,2000). Glucose is the main energy resource of the brain, therefore neurons are the most vulnerable cells to ischemic injury when compared to other cell and the hippocampus is the most susceptible area in the brain. Ischemia occurs through the reduction or interruption of oxygen and nutrients supply to neuronal tissue. When this happens the production of ATP is halted and there is no energy left to maintain the membrane potential, which results in depolarization of the membrane and consequent excessive glutamate release which leads to excitotoxicity. Excitotoxicity contributes to neuronal damage and death by calcium deregulation. The calcium ion plays a fundamental part in excitotoxicity because the large influx of calcium through NMDA receptors and calcium ion channels activates caspases and proteases involved in apoptose and induces formation of reactive oxygen species causing oxidative stress which results in DNA and lipid membrane damage (Gupta et al.,2011, Kitagawa,2012).

The neuroprotective actions of adenosine against excitotoxicity are mediated via A1 receptor which inhibits synaptic transmition by: presynaptically inhibiting glutamate release from glutamatergic nerve terminals and by decreasing the influx of Ca2+ and postsynaptically by inhibiting NMDA receptors and by increasing the efflux of K+, which leads to hyperpolarization of postsynaptic neurons (see Fig. 7). These actions limit calcium entry and decrease metabolic demand, which help to preserve ATP stores essential for pumping Ca^{2+} out of the cell (de Mendonça et al., 1995, Dunwiddie e Masino, 2001).

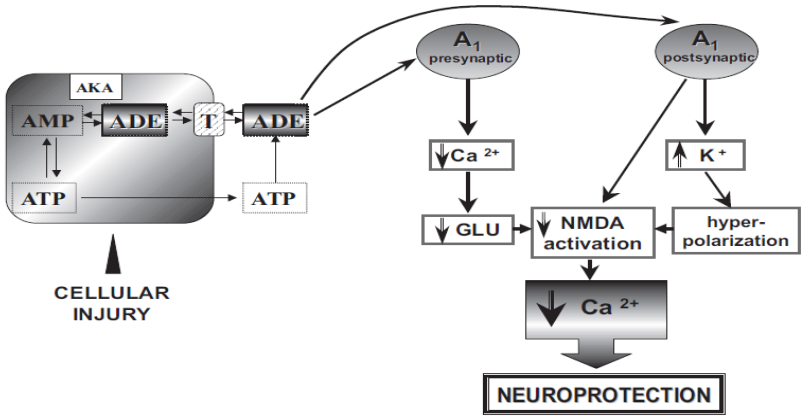

Fig.7 Schematic representation of possible mechanisms responsible for the neuroprotective action of adenosine A1 receptor agonists. ADE – adenosine; A1 – adenosine A1 receptor; AKA- adenosine kinase; Ca^{2+} – calcium ion; GLU – glutamate; K^+ – potassium ion; NMDA- N-methyl-D-aspartate receptor; T – bidirectional nucleoside transporter; ↓ – decrease; ↑ – increase. Following cellular injury, extracellular level of adenosine is markedly increased *via* an enhanced release of intracellular ADE and/or ATP, which is rapidly degraded to ADE by ectonucleotidases. Once in the extracellular space, adenosine acting through presynaptic A1 receptors may attenuate the influx of Ca^{2+} through voltage-dependent calcium channels, and thus decrease the release of glutamate. On the other hand, adenosine acting through postsynaptic A1 receptors may activate K^+ channels, which leads to hyperpolarization of postsynaptic neurons and directly inhibits NMDA receptor activation. All these effects limit the opening of voltage-dependent Ca^{2+} channels and the neuronal Ca^{2+} influx, which contributes to neuroprotection (Wardas, 2002).

1.5.1.2 Potential therapeutic effect of the A1 receptor

Adenosine A1 receptor may be a potential target for the treatment of neurodegenerative diseases. During epileptic seizures, adenosine accumulates in the extracellular space and suppresses epileptic seizure activity exerting an anticonvulsant effect through A1 adenosine receptors by modulating ionic currents postsynaptically and reducing excitatory neurotransmitter release (During et al., 1992; Ribeiro et al., 2003).

Alzheimer's disease is the most common form of dementia and one of the most serious health problems. It is characterized by synaptic loss and neuronal cell death as well as the presence of extracellular amyloid plaques, composed of the amyloid-β protein and intracellular neurofibrillary tangles. Studies with post-mortem brain with Alzheimer's disease show a decrease of density of A1 receptors between 40-60% in hippocampus (Kalaria et al,1990).

Spinal cord injury (SCI) produces mechanical and physical damage which may lead to inflammation and neuronal cell death. Adenosine receptors have been shown to have a major role in regulating the inflammatory responses, since, the adenosine A1 receptor has been involved in mediating neuroprotective effects against SCI including reduction of hyperalgesia which involve an attenuation of hypersensitivity to pain, usually caused by damage to nociceptors (pain receptors) and/or peripheral nerves (Horiuchi et al.,2010). Adenosine receptor antagonists, such as caffeine, have a broad potential as therapeutic tools since they have shown to reduce the physical, cellular and molecular damages caused by stroke (cerebral infarction) and neurodegenerative diseases, such as Alzheimer disease (Ulas et al, 1993) probably by blocking A2A receptors. In case of A1 receptor agonists, they could be effective for the treatment of anxiety, since A1 receptor agonists have anxiolytic activity in rodent models of anxiety, whereas A1 receptor antagonists have anxiogenic properties (Florio et al., 1998). The A1 receptor may also be useful against insomnia, since reports described that administration of a highly selective A1 receptor agonist resulted in an increased propensity to sleep, while an A1 receptor antagonist increased waking and decreased sleep (Bennington et al.,1995; Schwierin et al., 1996).

1.6 Cyclic guanosine monophosphate.

Cyclic guanosine monophosphate (cGMP) is a cyclic nucleotide which is formed from guanosine triphosphate (GTP) by the action of guanylyl cyclases. It was discovered in the early 1960s as the result of a search for analogs of the second messenger cyclic AMP (cAMP). However, cGMP remained rather obscure because initially no clear physiological function of cGMP was found due to the lack of selective agonists for guanylyl cyclases. The interest in cGMP signalling changed with the discovery in the 1970s that NO-donating vasodilators relax smooth muscle by activating a soluble form of guanylyl cyclase and the subsequent discovery of endogenous activators of the cGMP-signalling pathway, such as natriuretic peptides and nitric oxide (NO), led to the recent appreciation of the importance of cGMP in the pathophysiological regulation of numerous cellular processes (Arnold et al., 1977; Schmidt et al., 1993). cGMP is an important second messenger involved in many physiological functions, such as vision process, vasodilatation, muscle relaxation, inhibition of platelet aggregation and anti-inflammatory and anti-apoptotic effects (Russwurm et al., 2013) (see Fig. 8). cGMP is also a second messenger in the nervous system present in cortex, caudate-putamen, cerebellum and hippocampus where it plays an important role in regulation of neurotransmission and synaptic plasticity in mechanism of long term potentiation (LTP) and long term depression (LTD), suggesting its participation in learning and memory process (Boulton et al., 1995; Monfort et al., 2002).

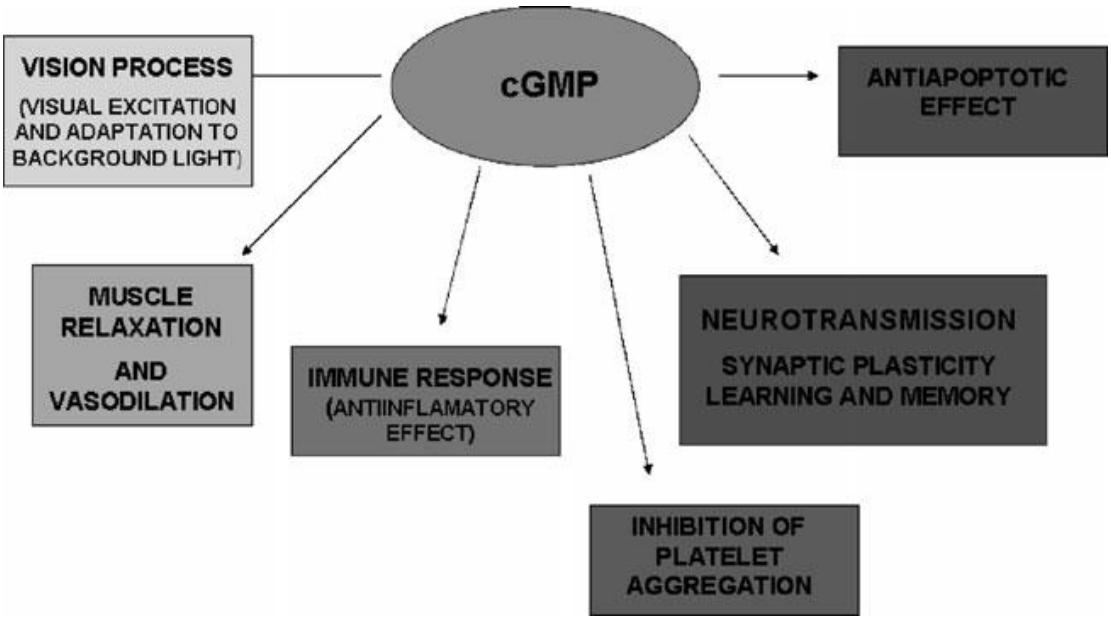

Fig. 8 cGMP functions in living organisms (adapted from Domek-Łopacińska et al., 2010)

1.6.1 cGMP metabolism

cGMP is produced by the action of guanylyl cyclases (GC), which are a family of enzymes that catalyse the conversion of GTP to cGMP. There are two types of GC, the soluble form (sGC) activated by nitric oxide (NO) and the particular form (pGC) that is a receptor for extracellular ligands, such as natriuretic peptides (Lucas et al., 2000). sGC is a heterodimeric enzyme containing one α and one ß subunits and each subunit has two isoforms (α1, α2, ß1, ß2). It contains a heme group, which can bind NO and induce a conformational change in the enzyme increasing its activity. Modulation of sGC by NO plays an important role in the modulation of cerebral processes including the release of some neurotransmitters in certain brain areas, intercellular communication, long-term potentiation and some forms of learning and memory (Boulton et al., 1995). pGCs are homodimers or homotetramers of single membrane-spanning subunits that contain a N-terminal extracellular ligand binding domain and intracellular regulatory and catalytic domains, which mediate important cardiovascular, renal and anti-inflammatory actions (Lucas et al., 2000). cGMP is eliminated by phosphodiesterases and transported out of the cell by ATP-dependent cGMP transporters. Cyclic nucleotides phosphodiesterases (PDEs) are enzymes that catalyze the hydrolysis of cAMP and cGMP and they are important in regulation of cyclic nucleotide signalling and cellular communication. PDEs are divided into three categories based on their substrate specificity: PDE 4, 7, and 8 hydrolyze cAMP; PDE 5, 6, and 9 hydrolyze cGMP; and PDE 1, 2, 3, 10, and 11 are dual substrate enzymes hydrolyzing both cAMP and cGMP. In brain, cGMP is degraded mainly by phosphodiesterases 2, 5 and 9 distributed in the frontal cortex, hippocampus, amygdala, olfactory bulb, striatum, hypothalamus, and cerebellum (Kleppisch, 2009) (see Fig. 9).

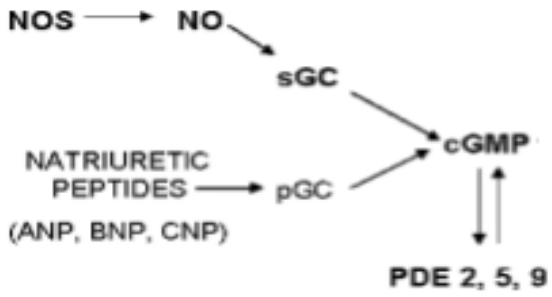

Fig.9 NO/cGMP signaling pathway. Cyclic GMP (cGMP) can be synthesized via soluble guanylyl cyclase (sGC) activated by nitric oxide (NO), which is liberated by its synthase (nitric oxide synthase–NOS). The second possibility is cGMP synthesis via particular guanylyl cyclase (pGC) activated by natriuretic peptides (atrial–ANP, brain–BNP, and C type–CNP) (adated from Domek-Łopacińska et al., 2010).

1.6.2 NO-cGMP signalling

An important pathway which modulates the levels of cGMP in brain in response to synaptic activity is the NO-cGMP pathway. NO is a free radical and a small-molecule gas that is capable of diffusing across membranes where it can act as a second messenger in signalling cascades. NO acts as the intracellular ligand for the sGC (Garthwaite, 2007). The group of enzymes responsible for NO production was first described in 1989 to be the Ca^{2+}/calmodulin (CaM)-dependent NO synthases (NOS), and was subsequently characterised throughout the 1990's. The isolation and cloning of NOS from rat cerebellum was rapidly conjoined with immunohistochemical evidence demonstrating NOS not only to be localized in endothelial cells but also to be highly expressed in the brain (Bredt and Snyder, 1990; Bredt et al., 1990, 1991; Vincent and Kimura, 1992).

NOS has three different isoforms, neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). nNOS can be predominately found in the neurons, heart and skeletal muscle and its activity is regulated by Ca^{2+} and calmodulin. iNOS is highly expressed in cells of the immune system such as macrophages and it is induced by lipopolysacharide, glucocorticoids and cytokines. eNOS is mostly found in endothelial cells and it is activated by Ca^{2+} or phosphorylation through Akt and AMPK, induced by insulin or estrogen (Murad, 2006; Forstermann et al., 2012). NO is an important regulator of cGMP levels acting as a retrograde messenger. NO activates sGC in post-synaptic neurons or diffuses to the extracellular medium were it can act pre-synaptically stimulating sGC to produce cGMP, which decreases the release of glutamate (Feil and Kleppisch, 2008; but see Russwurm et al., 2013).

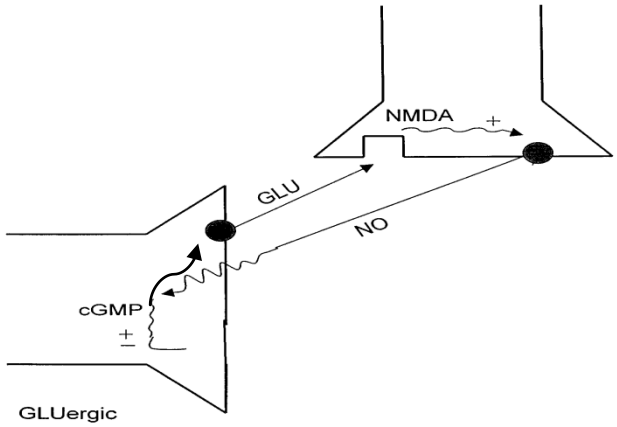

Fig. 10 The scheme of NO/cGMP role in glutamatergic signaling pathway (adated from Prast and Philipu,2001)

The main effector of cGMP is protein kinase G (PKG). Kinases are enzymes that transfer phosphate groups from a donor, usually a nucleoside triphosphate (e.g., ATP), to specific substrates. This phosphorylation results in a functional change of the substrate protein. The most important families are tyrosine kinases and serine/threonine kinases. PKG belong to the serine/threonine kinases family (Smolenski et al.,1998). Therefore, PKG phosphorylates serines and threonines on many cellular proteins resulting in changes in activity or function, subcellular localization, or regulatory features. PKG is activated when two molecules of cGMP bind to the regulatory subunits of this

protein, releasing the autoinhibitory domain of the regulatory subunit from the catalytic subunit. The proteins that are modified by PKG commonly regulate calcium homeostasis, calcium sensitivity of cellular proteins, gene expression, feedback of the NO-signaling pathway, and other processes. PKG participate in the negative feedback of cGMP signalling by phosphorylating and activating PDEs, which decrease the levels of cGMP (Francis et al, 2010).

Cyclic nucleotide-gated (CNG) channels are heteromeric proteins which have weak sensitivity to voltage, and open upon direct binding of either cGMP or cAMP. In the absence of extracellular Ca^{2+}, these channels are permeable to Na^{+} and K^{+} (Kaupp and Seifert, 2002). The ability of cGMP to activate these cation-selective channels was first documented in retinal rod photoreceptors (Fesenko et al., 1985). CNG channels are present in photoreceptors of the retina, neurons of the olfactory epithelium and non-sensory cells and their basic function consists in providing a cGMP-dependent entry pathway for Ca^{2+} (Biel et al, 1996). In the hippocampus, in situ hybridisation studies and patch clamp-recordings from cultured neurons demonstrated the presence of both olfactory and rod-type CNG channels in this brain region, localized in neuronal somata and dendrites of pyramidal neurons, as well as in granule cells of the dentate gyrus (Leinders-Zufall et al., 1995; Kingston et al., 1996). Olfactory-type CNG channels activation support LTP in the Schaffer collateral/CA1 synapse recorded from hippocampal slices (Parent et al., 1998).

Finally, the activity of some PDE isoforms can be modulated negatively or positively by cGMP, either directly by cGMP binding to a regulatory domain of the PDE, or indirectly via cGMP-dependent phosphorylation of the PDE (Bender and Beavo, 2006) (see Fig.10).

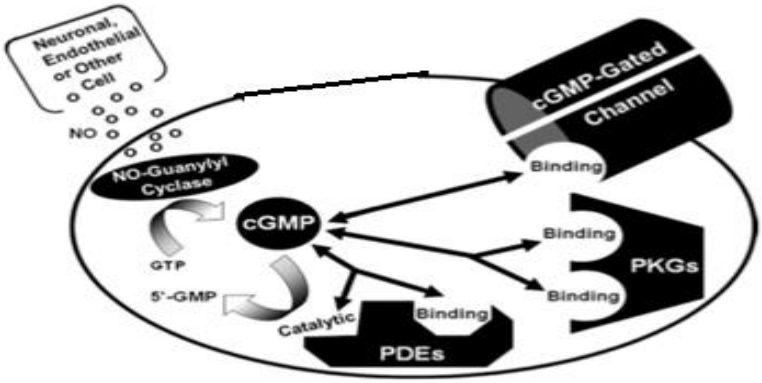

Fig.11 Cellular targets of cGMP. Multiple intracellular proteins can interact with cGMP, including the cGMP-gated cation channel, PKG and allosteric sites and catalytic sites on certain PDEs. The level of cellular cGMP is determined largely by the balance between its synthesis by guanylyl cyclase and breakdown by PDEs. The different shapes of the pockets on the respective proteins indicate that the catalytic sites of PDEs (shown as half-diamonds), the allosteric sites of PDEs (shown as half-octagons) and the allosteric sites on PKG and cation channels (shown as half-circles) are structurally and evolutionarily unrelated (adapted from Francis et al., 2010).

1.6.3 cGMP as a therapeutic target for neurodegenerative diseases

Alzheimer's disease (AD) is the most common form of dementia. In AD patients, memory loss is accompanied by the formation of beta-amyloid plaques and the appearance of tau in a pathological form (hyperphosphorylated). During the aging process NOS activity is increased, which leads to increased NO production. At the same time, the activity of cGMP hydrolyzing PDEs is increased, cGMP levels are decreased, and the LTP process is less efficient in the senescent compared with the adult brain, resulting in worse cognitive performance of aged animals (Domek-Łopacińska et al, 2010).

A promissing selective PDE5 inhibitor exerts potent anti-AD effects when administered at advanced stages of the disease in different mouse models of AD, resulting in the reversal of cognitive impairment. However, the mechanism of action remains unclear (Puzzo et al., 2009).

Liver disease may lead to altered cerebral function resulting in hepatic encephalopathy (HE), a complex neuropsychiatric syndrome leading a wide array of neurological and psychiatric alterations including cognitive and motor impairment which can progress to coma and death. Hyperammonemia is a main factor contributing to the cerebral alterations in HE, once it affects glutamatergic neurotransmission (Cauli et al., 2009; Monfort et al., 2009; Montoliu et al.,2010).

Chronic hyperammonemia in vivo also impairs LTP in hippocampus by altering activation of PKG and, subsequently, of phosphodiesterase 5 (Monfort et al., 2005). Hippocampal LTP in hyperammonemia and HE may contribute to the reduction in the ability to learn some types of tasks, including spatial learning (Monfort et al., 2007). Treatment of rats with chronic hyperammonemia with inhibitors of phosphodiesterase 5 reduces the degradation of cGMP, restoring its levels in the brain and restoring learning abilities, which are also restored by administration of cGMP itself (Erceg et al., 2005).

1.7 Could cGMP play a role in mediating the A_1 receptor inhibitory effect on neurotransmission?

The adenosine A1 receptor and cGMP are involved in similar regulatory processes in the nervous system, since both module neurotransmission, synaptic plasticity and have neuroprotective activity (Santschi et al., 2006). A1 receptor reduces synaptic transmission by inhibiting the presynaptic release of neurotransmitters and by reducing the excitability of postsynaptic neurons (Ribeiro et al, 2003). The A1 receptor-mediated post-synaptic effect involves membrane hyperpolarization due to activation of K^+ and Cl^- channels. On the other, the presynaptic effect involves inhibition of N- and P/Q-type Ca^{2+} channels (Wu and Saggau, 1994), but this mechanism seems to only partially explain the presynaptic effect of the A1 receptor, suggesting that other mechanisms might mediate the inhibitory effect of adenosine A1 receptors on presynaptic neurotransmitter release. cGMP was found to decrease synaptic transmission by inhibiting the pre-synaptic release of glutamate and modulating the response of postsynaptic receptors (Prast and Philipus, 2001). Furthermore, we recently observed that activation of A1 receptor increased cGMP levels in the hippocampus. However, it is not clarified if part of the inhibitory effects of A1 receptor on neurotransmission are mediated by cGMP in the hippocampus. To answer this question we tested if increasing the levels of cGMP, or blocking components of the cGMP pathway, will modify the inhibitory effect of A1 receptors in synaptic transmission at the hippocampus.

Chapter 2

Methods

2.1 Extracellular electrophysiology.

Extracellular electrophysiology was used to record field excitatory postsynaptic potentials (fEPSPs) in response to an evoked electric stimulus in Schaffer collaterals with the objective of analysing synaptic transmission in hippocampal slices.

The fEPSP can be subdivided into three components: the stimulus artefact, the presynaptic volley and the postsynaptic potential (Fig. 12). The stimulus artefact appears when an electric stimulus reaches Schaffer collaterals. When this signal propagates to CA1 dendritic region it originates the presynaptic volley. Glutamate released from Schaffer collaterals terminals causes postsynaptic depolarization of CA1 pyramidal neurons generating the postsynaptic potential (Anderson and Collingridge, 2001; Mathis et al., 2011).

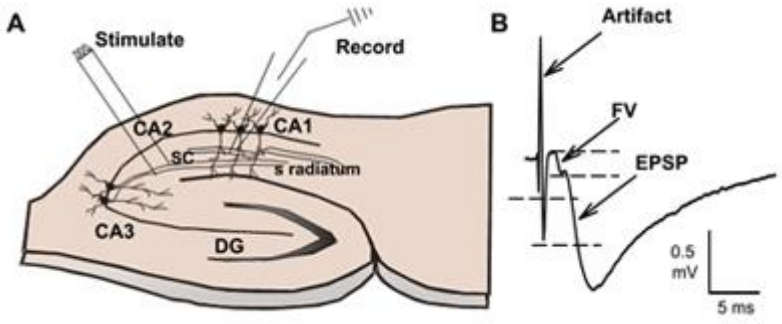

Fig.12 Hippocampal slice illustration and extracellular waveforms. A: Cartoon of a transverse hippocampal section used in electrophysiology experiments. CA = cornus ammonis. DG = dentate gyrus. SC = Schaffer collaterals. S radiatum = stratum radiatum. B, Electrical stimulation of the SC (a CA3 axon tract) elicits a stimulus artefact, followed almost immediately by a presynaptic population spike, or fiber volley (FV). The amplitude of FV is directly proportional to the number of SC fibers activated. The slope of the negative-going phase of the field excitatory postsynaptic potential (EPSP) corresponds directly to the activation of depolarizing synaptic currents in CA1 pyramidal neurons in response to glutamate release from SC terminals (Mathis et al., 2011).

2.1.1 Hippocampal Slices Preparation

Young adult male and female Wistar rats (8-10 weeks old) were used throughout the study. The animals were handled according to European Community guidelines and Portuguese law concerning animal care. The rats were anaesthetized with Isoflurane before decapitation, the brain was removed and placed into a Petri dish containing ice-cold Krebs-Henseleit buffer (pH 7.4), gassed with a O_2 (95%) and CO_2 (5%) mixture, and the hippocampus were dissected free.

After dissection the hippocampus was placed in a McIlwain tissue chopper, and cut perpendicularly to its long axis (400 µm). Slices were then carefully placed in different holding chambers with Krebs-Henseleit buffer at room temperature (22-25ºC) and left to stabilize for at least one hour or until they were required.

From the holding chamber the slices were transferred to a recording chamber (1ml capacity) and continuously perfused at 3 ml/min with Krebs-Henseleit maintained at 32ºC by a TC-202A temperature controller. Drugs to be tested were applied to the perfusion buffer.

The Krebs-Henseleit buffer contained (mM): NaCL 118, $NaHCO_3$ 25, KCl 4.7, glucose 11.6, KH_2PO_4 1.2, $MgSO_4$ 1.2 and $CaCl_2$ 1.3.

2.1.2 Recording field excitatory post-synaptic potentials (fEPSP)

Field excitatory postsynaptic potentials (fEPSPs) were recorded through an extracellular pulled glass capillary microelectrode (2-8 MΩ resistance, filled with a 4 M NaCl solution) placed in stratum radiatum of Cornus Ammonis 1 (CA1). Stimulation consisted in rectangular 0.1 milliseconds pulses, delivered once every 15 seconds through a bipolar concentric wire electrode positioned in the Schaffer collaterals-commissural fibers, in the stratum radiatum near the CA3-CA1 border (see Fig. 12). The intensity of stimulus (80-200 µA intensity) was adjusted to obtain a large fEPSP with a minimum population spike contamination. Extracellular recordings were obtained with an Axoclamp 2B amplifier and digitized. Individual fEPSPs were monitored, and averages of eight consecutive responses were recorded and analyzed by the software LTP 230d.

2.1.3 Data analysis and statistics

Data was analysed by GraphPad Prism and expressed as mean ± standard error of the mean (SEM) from n independent experiments. To allow comparisons between different experiments slope and amplitude values were normalized, taking as 100% the averaged five values obtained immediately before applying the test drug.

Statistically significant differences between the mean values obtained in control and test conditions were evaluated with the paired Student´s t-test and considered significant for values of $P<0,05$.

2.1.4 Drugs

Al drugs used were: N6-cyclopentyladenosine (CPA; Tocris), Bay 60-7550 (Cayman Chemical), KT5823 (Santa Cruz Biotecnology), 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP; Sigma), 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ; Sigma), N^G-nitro-L-arginine methylester (L-NAME; Sigma), Adenosine deaminase (ADA; Roche). All other reagents used were from analytical grade.

Chapter 3

Results

3.1. Effect of the selective A1 receptor agonist (CPA) on fEPSP

To assess the action of adenosine A1 receptor activation on synaptic transmission, we started by testing the effect of an adenosine A1 receptor selective agonist, CPA, on the evoked fEPSP at the rat hippocampal slice. Two consecutive applications of CPA (15 nM) separated by 90 minutes were performed. The effect of CPA (15 nM) produced by each application was calculated 40 min after CPA addition, when the CPA effect stabilized. The CPA effect was washed out within 40 min. There was no significant difference between the fEPSP inhibition produced by the two consecutive applications of CPA; when the hippocampus slice was exposed to the first application of CPA (15 nM) the fEPSP decreased 40% ± 2% and in the second application there was an inhibition of 41% ± 2% in the fEPSP (n=3, paired Student's t-test; P>0.05; see Fig. 13).

In the whole set of experiments the effects on fEPSPs produced by the 1st application of CPA (15 nM) was used as internal control, when testing the effect of a drug on the effect of a second application of CPA.

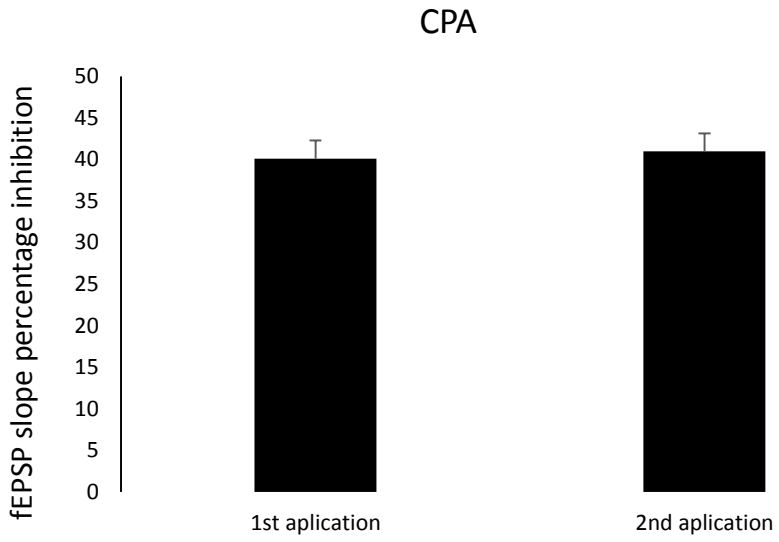

Fig.13 Effect of CPA (15 nM) on the slope of fEPSPs in hippocampal slices of male rats. The graph sumarizes the percentage of inhibitory effect of two consecutive aplication of CPA separated by 90 minutes. No significant differences were observed between the two applications of CPA. The bars represented the average ± SEM of three independent experiments.

3.2. The effect of CPA was not modified in presence of an inhibitor of phosphodiesterases nor in the presence of a cGMP analog

We continued by testing if preventing intracellular cGMP degradation, and consequently increasing its accumulation, would modify the CPA inhibitory effect on fEPSP. For that purpose we used Bay 60-7550, mostly a selective inhibitor of PDE2, the main enzyme responsible for cGMP degradation at the hippocampus (Bartus et al., 2013).

In these experiments, the application of Bay 60-7550 (100 nM) during 30 minutes increased the fEPSPs by 25% ± 3.5% (n=5; P<0.05 when compared with zero). CPA (15 nM) in the presence of Bay 60-7550 (100 nM) decreased the fEPSP slope by 50% ± 2.8% after 40 minutes; a similar effect was observed in the same slice with CPA (15 nM) alone (n=5, paired t-test; P>0.05; Fig.14 and Fig. 15).

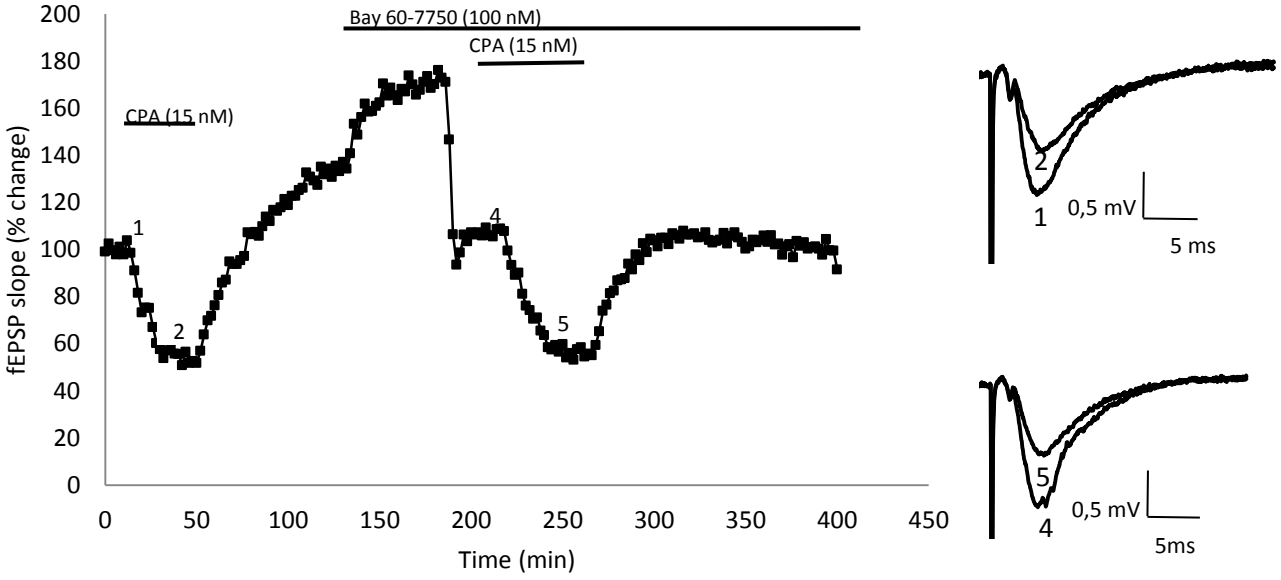

Fig.14 Effect of CPA (15 nM) alone and in presence of the phosphodiesterase inhibitor Bay 60-7750 (100nM) on the slope of fEPSPs recorded in the CA1 area of an hippocampal slice of a male rat. A time course of a representative experiment is shown in the left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) reversibly decreased the fEPSP slope by 45 %, while in the presence of Bay 60-7750 (100 nM) CPA (15 nM) caused a decrease of 48 % in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

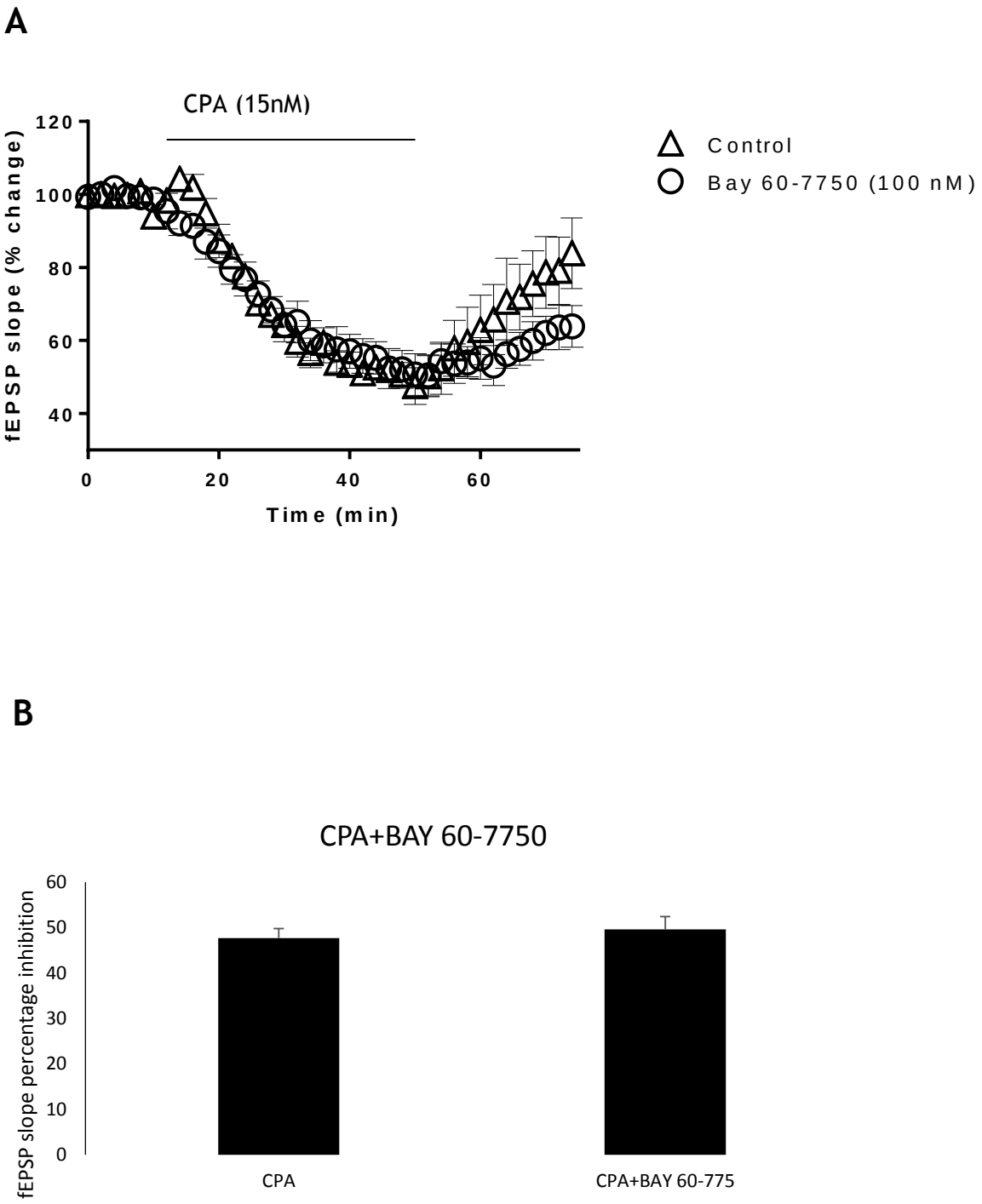

Fig.15 Inhibitory effect of CPA (15 nM) alone and in the presence of Bay 60-7750 (100 nM) on the slope of fEPSPs in hippocampal slice of male rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of five experiments; the time-distance between points corresponds to two minutes B: Comparison between average of percentage inhibition produced by CPA in the absence (left) or in the presence (right) of Bay 60-7750; 100% in A represents average fEPSP slope recorded for 10 min before applying CPA under each testing condition. The bars represent the average ± SEM of five independent experiments.

Next we tested the effect of 8-pCPTP-cGMP, a membrane-permeable analog of cGMP to mimic the effects of cGMP in synaptic transmission. Application of 8-pCPT-cGMP (10 μM) during 30 minutes was variable in the whole set of experiments, since it either increased, decreased, or unaffected the fEPSP slope. CPA (15 nM) alone depressed the fEPSP by 59% ± 4.5% after 40 minutes (n=4); in the presence of 8-pCPT-cGMP (10 μM) the effect of CPA (15 nM) was slightly lower, 50% ± 8.6% (n=4) decrease of the fEPSP slope, although not significantly different from the effect of CPA alone (P>0.05, paired Student´s t-test; Fig. 16).

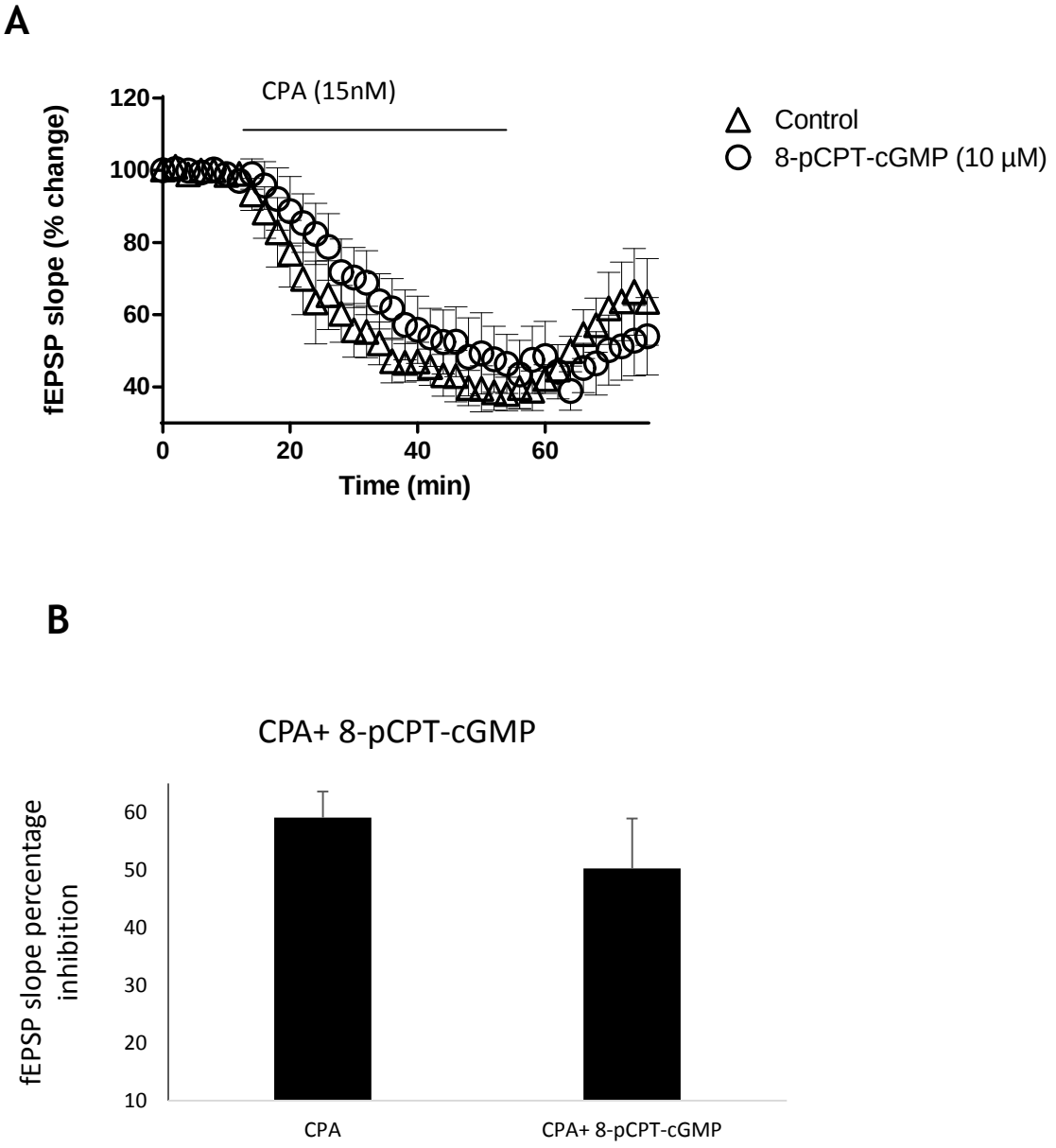

Fig.16 Inhibitory effect of CPA alone (15 nM) and in the presence of 8-pCPT-cGMP (10 μM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of male rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of four experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition produced by CPA in the absence (left) or in the presence (right) of 8-pCPT-cGMP; 100% in A represents average fEPSP slope recorded for 10 min before applying CPA under each testing condition. The bars represented the average ± SEM of four independent experiments.

3.3 A1 receptor activity is dampened by an NO synthase antagonist

To determine if the synthesis of NO, an agonist for soluble guanylate cyclase, interferes with A1 receptor activity, we tested the effect of CPA in the presence of L-NAME, an NO synthase antagonist, in young male and female rats.

In female rats, L-NAME (200 μM) strongly dampened the inhibitory effect of CPA (15 nM), as ilustred in Fig. 17 and 18. CPA (15 nM) alone depressed the fEPSP slope by 54% ± 5.3%, whereas CPA (15 nM) in the presence of L-NAME (200 μM) decreased the fEPSP slope by 23% ± 5.7% (n=5; $P<0.05$, paired Student´s t-test, compared with CPA alone). Application of L-NAME (200 μM) during 30 minutes tended to increase the fEPSP slope (49% ± 25%, n=4), however this tendency was not statistically significant ($P>0.05$, Student's t-test, when compared with zero) since in two experiences L-NAME failed to modify synaptic transmission.

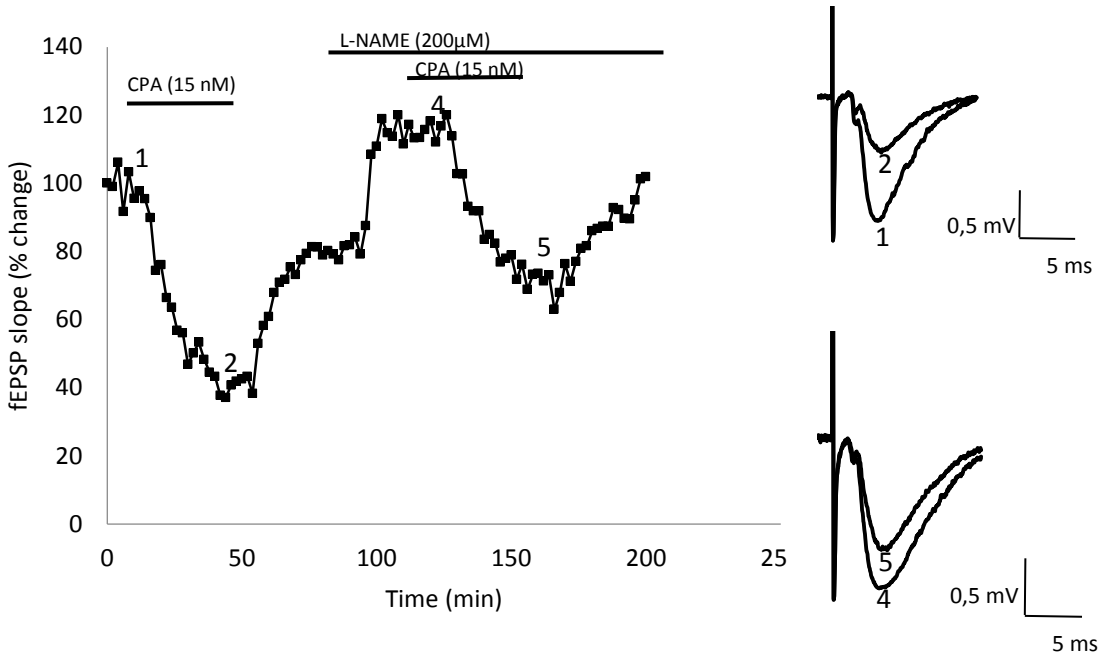

Fig.17 Effect of CPA (15 nM) alone and in presence of inhibitor of NOS, L-NAME (200 μM), on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of a female rat. A timecourse of a representative experiment is shown in left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) reversibly decreased the fEPSP slope by 61%, while in the presence of L-NAME (200 μM) CPA (15 nM) caused a decrease of 22% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

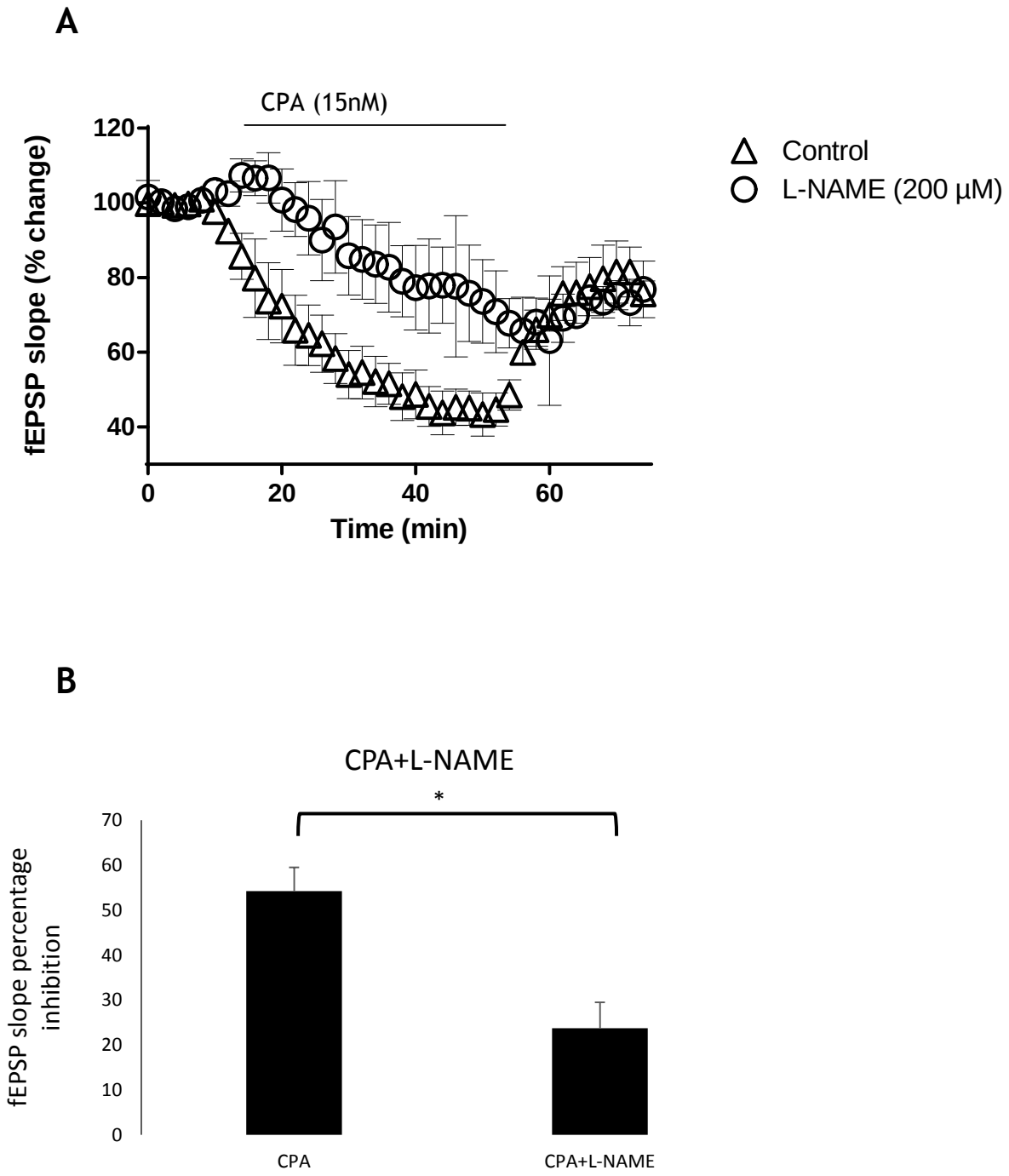

Fig.18 Inhibitory effect of CPA alone (15 nM) and in the presence of L-NAME (200 µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of female rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean ± SEM of five experiments; the time-distance between points corresponds to two minutes B: Comparison between average of percentage inhibition produced by CPA in the absence (left) or in the presence (right) of L-NAME; 100% in A represents average fEPSP slope recorded for 10 min before applying CPA under each testing condition. The bars represented the average ± SEM of five independent experiments. * P<0.008 (Paired Student´s t-test).

In experiments performed with male rats the results obtained are similar to those obtained with females, as illustrated in Fig. 19.

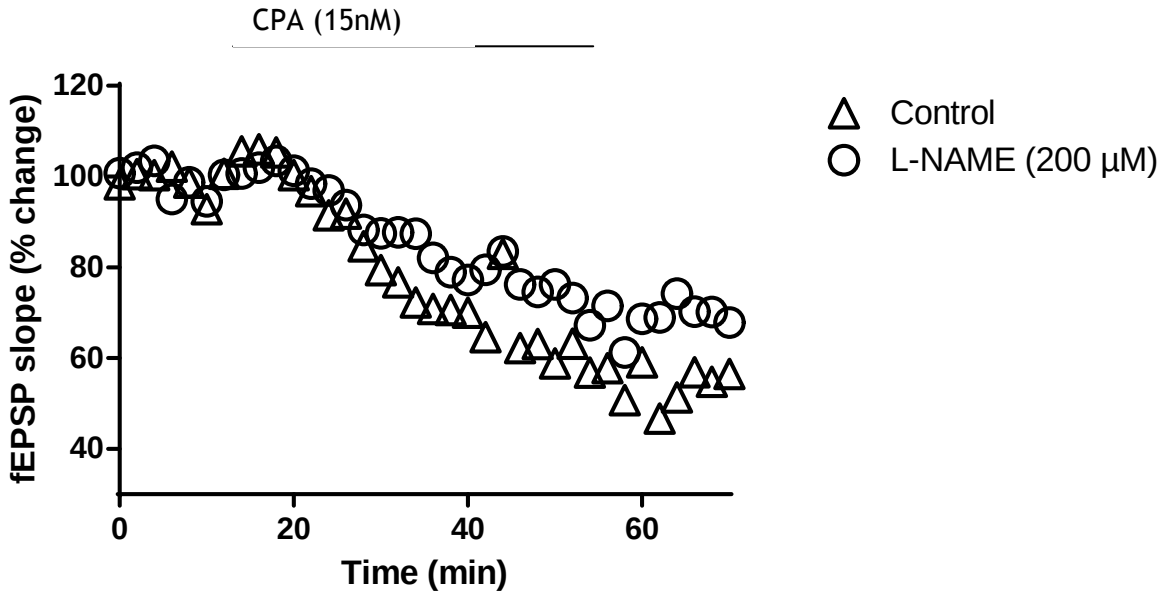

Fig. 19 Inhibitory effect of CPA alone (15 nM) and in the presence of L-NAME (200 µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of male rats. Superimposition of the time courses; each point in the ordinates corresponds to the mean of two experiments; the time-distance between points corresponds to two minutes. fEPSP slopes recorded for 10 min before application of CPA were normalized to 100% to allow comparison between the effects of CPA in the two experimental conditions

3.4. CPA effect on fEPSP slope is depressed by Protein Kinase G (PKG) inhibitor

To elucidate if the effect of A1 receptors in synaptic transmission depended on PKG activity, the CPA effect was evaluated in the absence and in the presence of a selective PKG inhibitor (KT5823) in young male and female rats (Fig. 20 and Fig. 21).

In male rats, CPA (15nM) alone depressed the fEPSP slope by 44% ± 10.6% while CPA in the presence of KT5823 (1nM) decreased the fEPSP slope by 22% ± 3% after 40 minutes (n=4), which corresponds to a 45 ± 8.9 attenuation ($P<0.05$, compared with zero, Student´s t-test) of the CPA effect caused by KT5823. Application

of KT5823 during 30 minutes tended to increase the fEPSP slope (23% ± 14.2% increase, n=4), although this increase was not statistically significant ($P>0.05$, Student's t-test, when compared with zero).

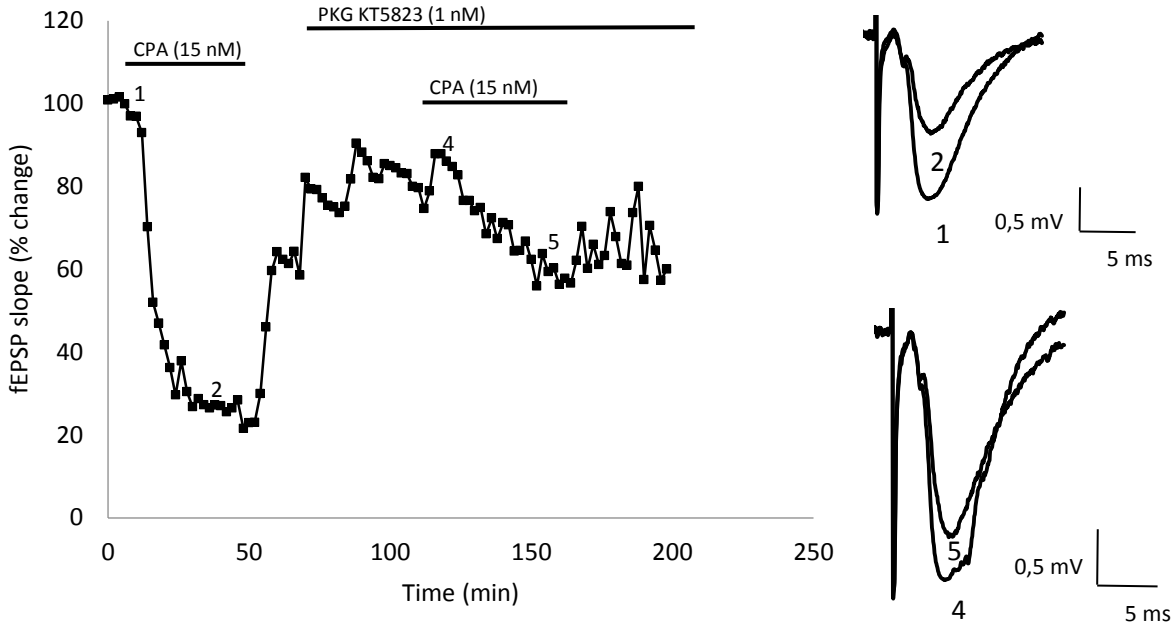

Fig.20 Effect of CPA (15 nM) alone and in presence of inhibitor of PKG, KT5823 (1 nM), on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of a male rat. A timecourse of a representative experiment is shown in left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) reversibly decreased the fEPSP slope by 74% , while in the presence of KT5823 (10 μM) CPA (15 nM) caused a decrease of 27% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

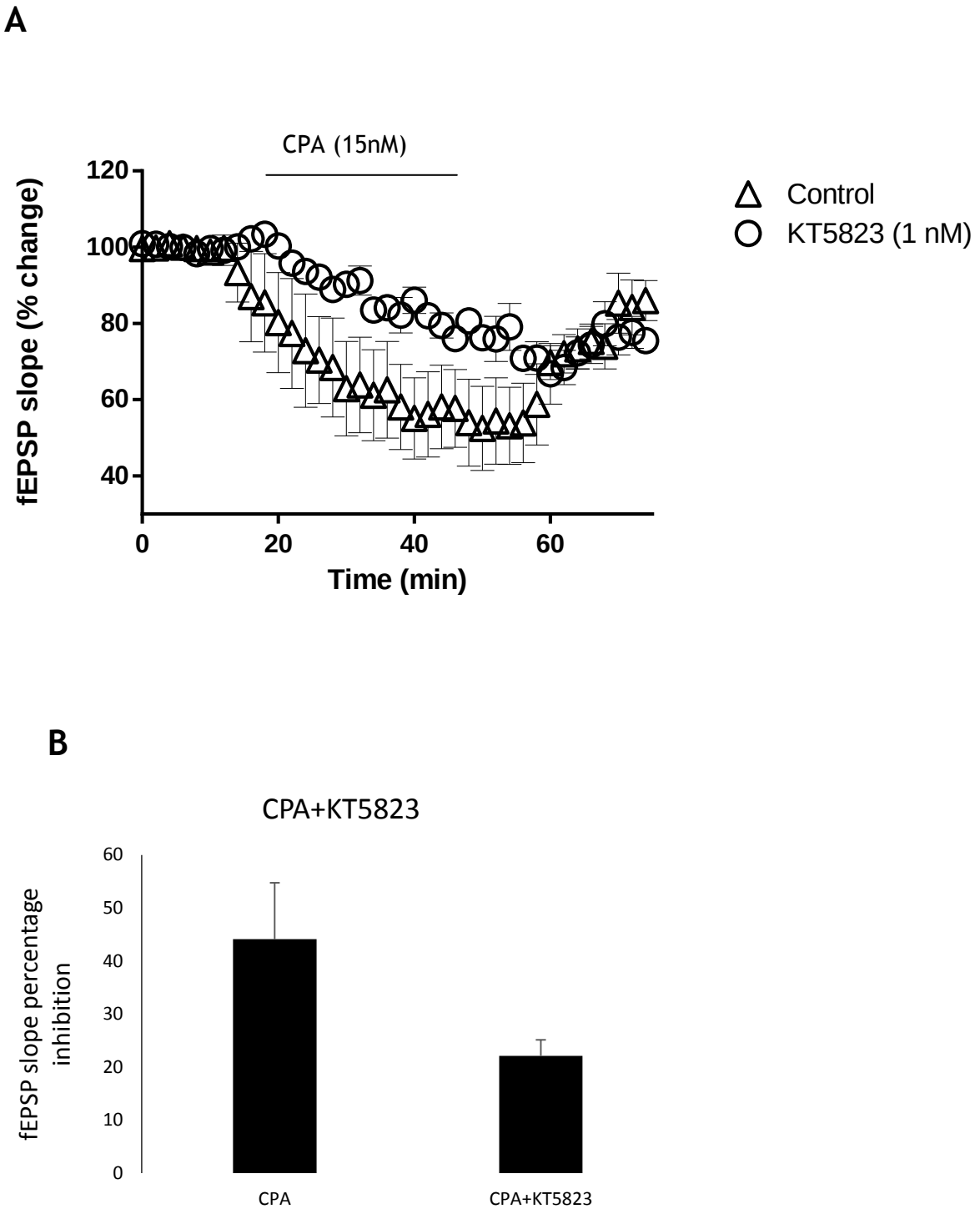

Fig.21 Inhibitory effect of CPA alone (15 nM) and in the presence of KT5823 (1 nM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of male rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of four experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition caused by CPA in the absence (left) or in the presence (right) of KT5823; 100% in A represents average fEPSP slope recorded for 10 min before applying CPA under each testing condition. The bars represent the average ± SEM of four independent experiments.

With female rats the results were similar to those obtained with male rats. CPA (15nM) alone depressed the fEPSP slope by 50% ± 5% whereas CPA (15nM) in the presence of KT5823 (1nM) decreased the fEPSP slope by 33% ± 2.6% (n=4; P<0.05, paired Student's t-test, when compared with CPA alone). Application of KT5823 (1nM) during 30 minutes increased the fEPSP slope by 25%±5.2% (P<0.05, when compared with zero, Student's t-test).

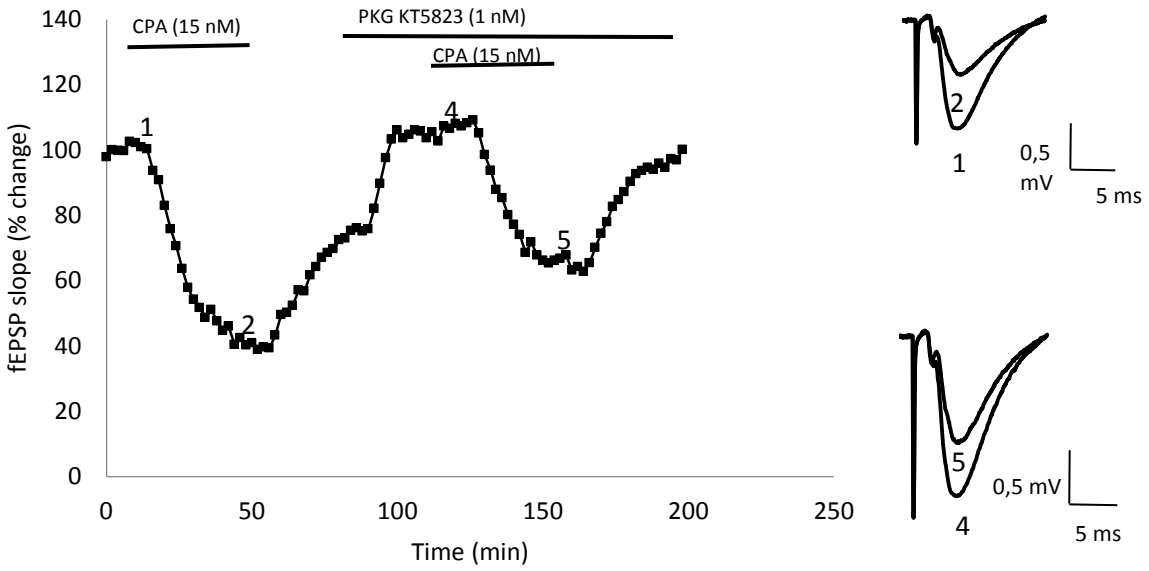

Fig.22 Effect of CPA (15 nM) alone and in presence of inhibitor of PKG KT5823 (1 nM) on the slope of fEPSPs recorded the CA1 area of a hippocampal slice of a female rat. A timecourse of a representative experiment is shown in left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) reversibly decreased the fEPSP slope by 60%, while in the presence of KT5823 (10 μM) CPA (15 nM) caused a decrease of 24% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 minutes before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

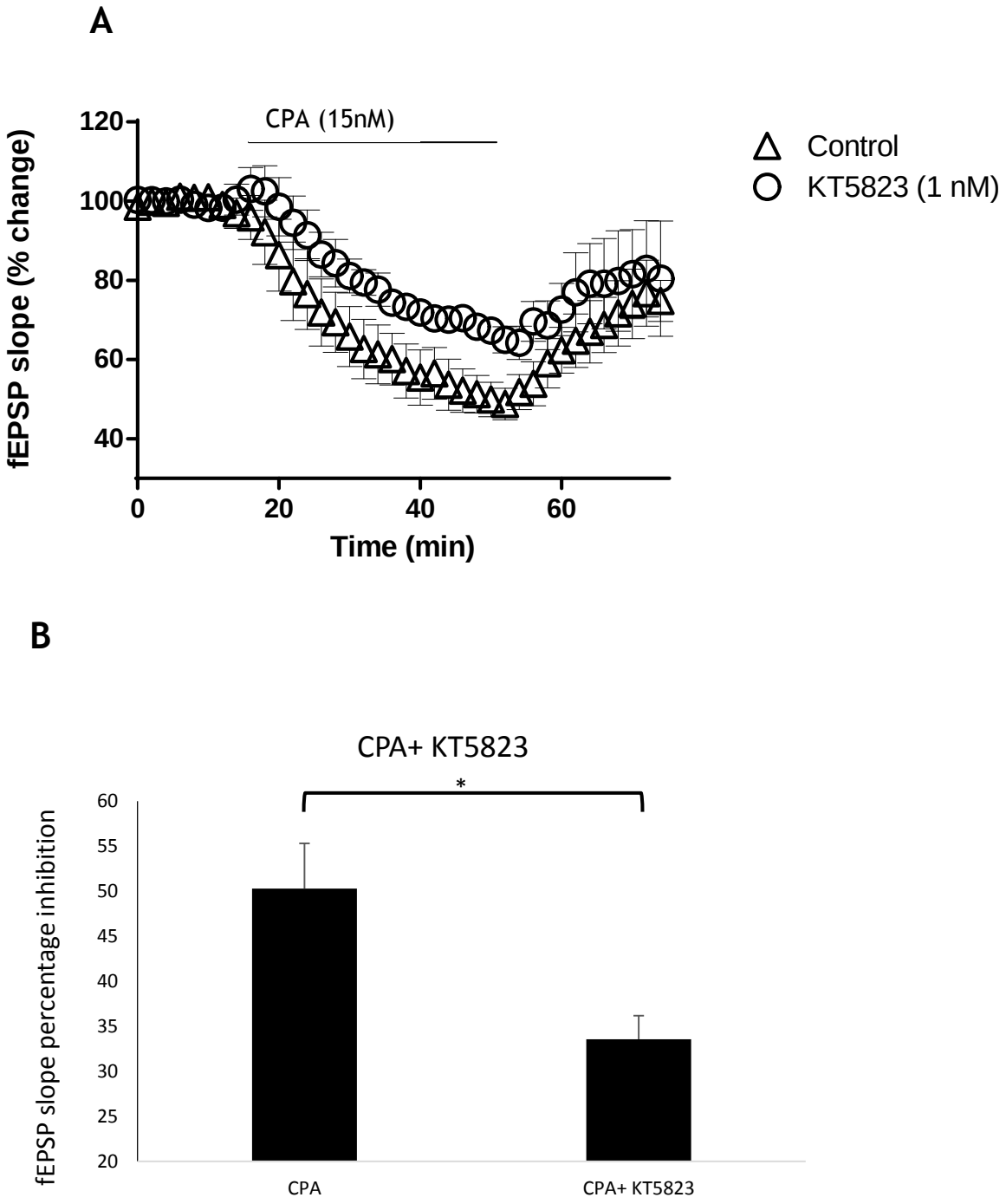

Fig.23 Inhibitory effect of CPA alone (15 nM) and in the presence of KT5823 (1 nM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of female rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of five experiments; the time-distance between points corresponds to two minutes B:Comparison between average of percentage inhibition caused by CPA in the absence (left) or in the presence (right) of KT5823; 100% in A represents average fEPSP slope in the recorded for 10 minutes before applying CPA under each testing condition. The bars represented the average ± SEM of five independent experiments. * P<0.038 (Paired Student´s t-test).

3.5 Inhibition of soluble guanylyl cyclase decreased the effect of CPA on fEPSP slope and required the presence of adenosine deaminase

Since it was previously observed, in section 3.3., that the effect of A1 receptors in synaptic transmission was strongly attenuated by inhibiting NOS-mediated NO production, it was then investigated if the effect of A1 receptors could be mediated through NO-activated sGC in young adult male and female rats. For that purpose the effect of CPA in the presence of ODQ, a soluble guanylyl cyclase irreversible inhibitor, was studied.

In male rats, CPA (15 nM) alone depressed the fEPSP slope by 35% ± 6.4% (n=3) after 40 minutes, whereas CPA (15nM) in the presence of ODQ (10 μM) yield a similar effect, decreasing the fEPSP slope by 32%±3.2% (n=3; P>0.05, paired Student's t-test, compared with CPA alone) (see Fig. 24 and Fig. 25). Aplication of ODQ (10 μM) during 30 minutes increased the fEPSP by 17%±2.8% (n=3, P<0.05, when compared with zero, Student's t-test).

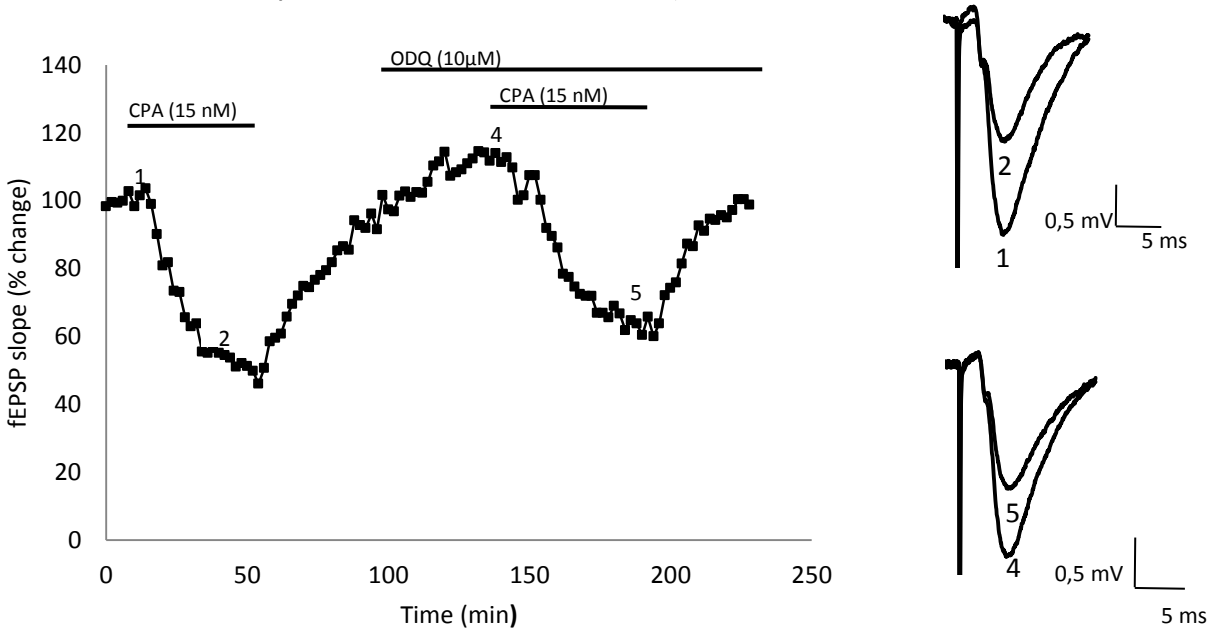

Fig.24 Effect of CPA (15 nM) alone and in presence of the inhibitor of soluble guanylyl cyclase ODQ (10μM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of a male rat. A timecourse of a representative experiment is shown in left panel and representive traces of averaged fEPSPs recorded at the times indicated are shown in the right panels . Application of CPA (15 nM) reversibly decreased the fEPSP slope by 47%, while in the presence of ODQ (10 μM) CPA (15 nM) caused a decreased of 34% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. . The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

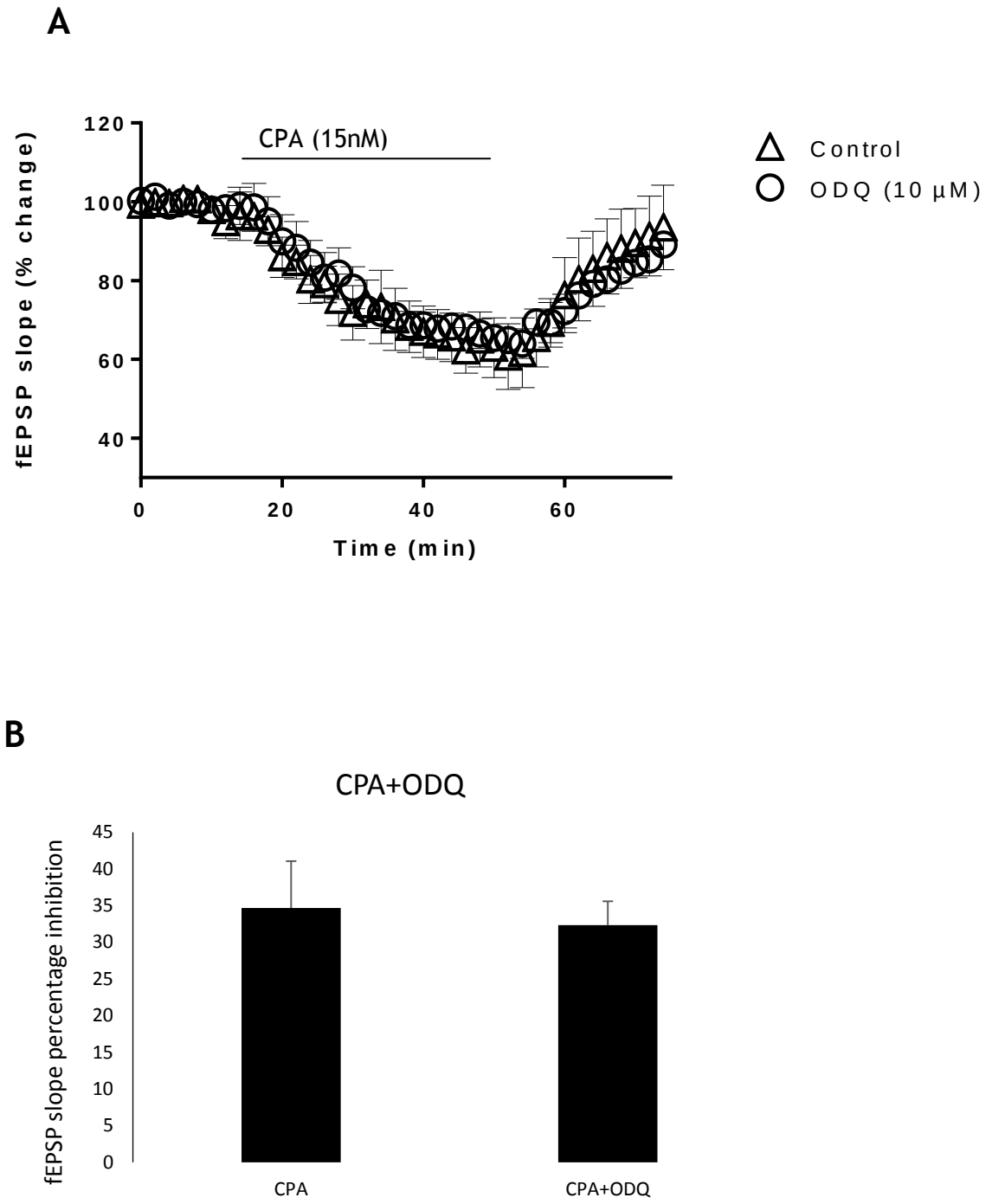

Fig.25 Inhibitory effect of CPA alone (15 nM) and in the presence of ODQ (10 µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of male rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of three experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition caused by CPA in the absence (left) or in the presence (right) of ODQ; 100% in A represents average fEPSP slope recorded for 10 minutes before applying CPA under each testing condition. The bars represented the average ± SEM of three independent experiments.

The results observed with female rats were similar to those obtained with male rats (Fig. 26 and Fig. 27). In female rats CPA (15nM) alone depressed the fEPSP slope by 39% ± 5.6% whereas CPA (15nM) in the presence of ODQ (10 µM) decreased the fEPSP slope by 35% ± 4.9% (n=4, P>0.05, paired Student's t-test, compared with CPA alone). Application of ODQ during 30 minutes increased the fEPSP slope by 19% ± 9.7% (n=4; P<0,05, when compared with zero, Student's t-test).

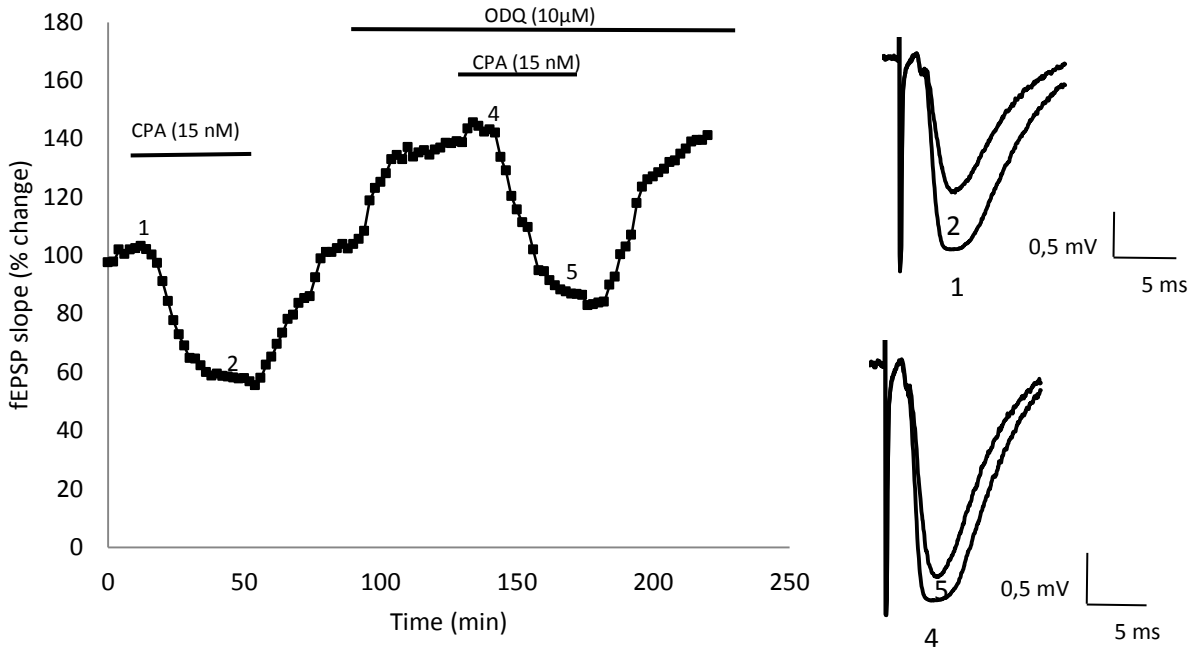

Fig.26 Effect of CPA (15 nM) alone and in presence of the inhibitor of soluble guanylyl cyclase ODQ (10µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of a female rat. A timecourse of a representative experiment is shown in left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) reversibly decreased the fEPSP slope by 41%, while in the presence of ODQ (10 µM) CPA (15 nM) caused a decreased of 38% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

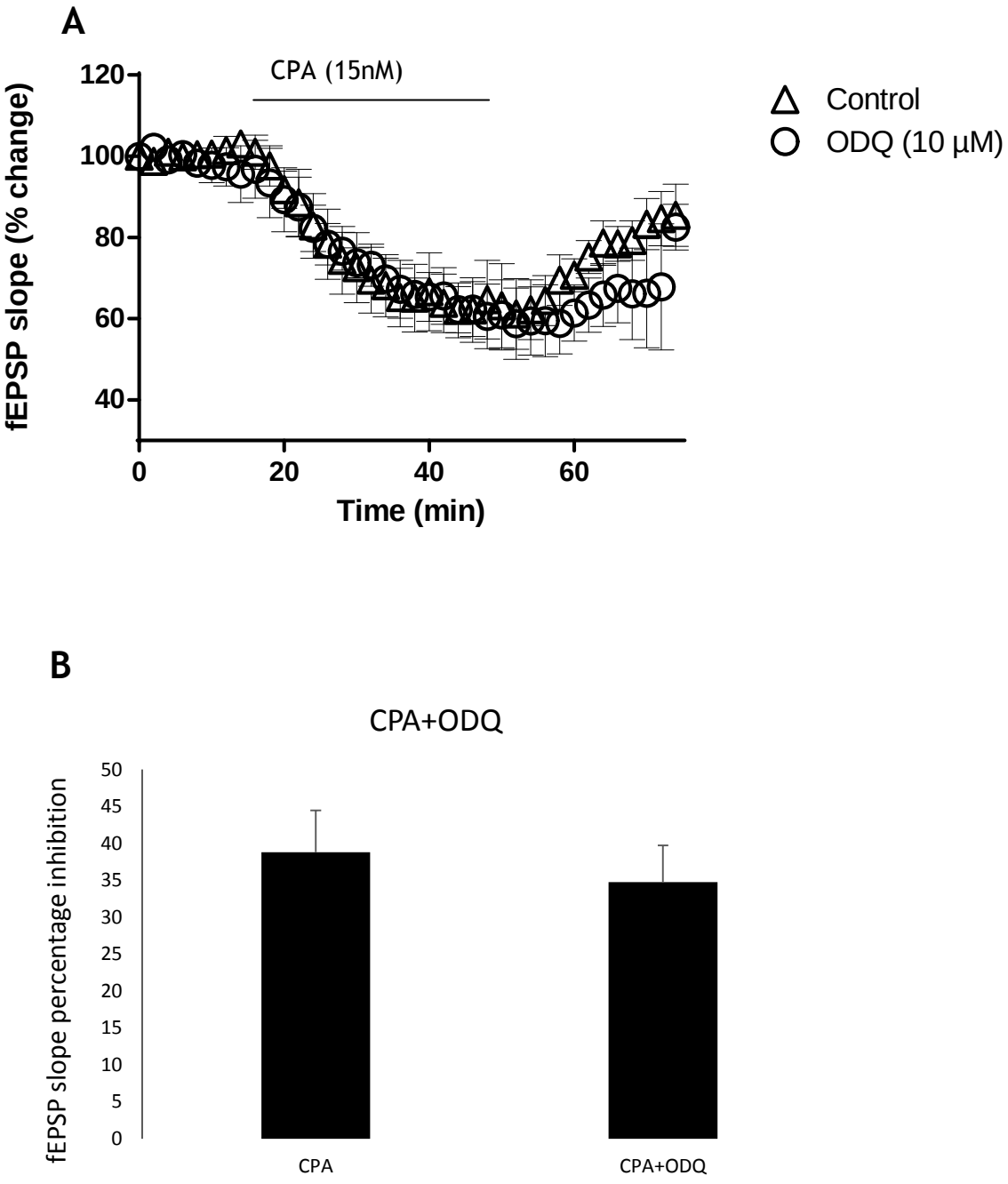

Fig.27 Inhibitory effect of CPA alone (15 nM) and in the presence of ODQ (10 µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of female rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of four experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition caused by CPA in the absence (left) or in the presence (right) of ODQ; 100% in A represents average fEPSP slope in the recorded for 10 minutes before applying CPA under each testing condition. The bars represented the average ± SEM of four independent experiments.

Considering that the presence of ODQ (10 μM), both in males and female rats, didn't modify the effect of CPA (15nM) on neurotransmission, we then decided to perform the same experiments but in the presence of Adenosine deaminase (ADA) in order to remove the interference of endogenous adenosine on adenosine receptors activity, which could be preventing the observation of ODQ effect on the A1 receptor action.

In male rats, in the presence of ADA (1U/ml) CPA (15nM) decreased the fEPSP slope by 50% ± 4.3% (n=4) after 40 minutes, however CPA in presence of ODQ (10 μM) and ADA caused a smaller effect, depressing the fEPSP slope by 39% ± 6% (n=4; $P<0.05$, compared with CPA in the presence of ADA, paired Student's t-test) (Fig. 28 and Fig. 29). Aplication of ODQ during 30 minutes in presence of ADA increased the fEPSP slope by 23% ± 4.4% (n=4, $P<0.05$, when compared with zero, Student's t-test).

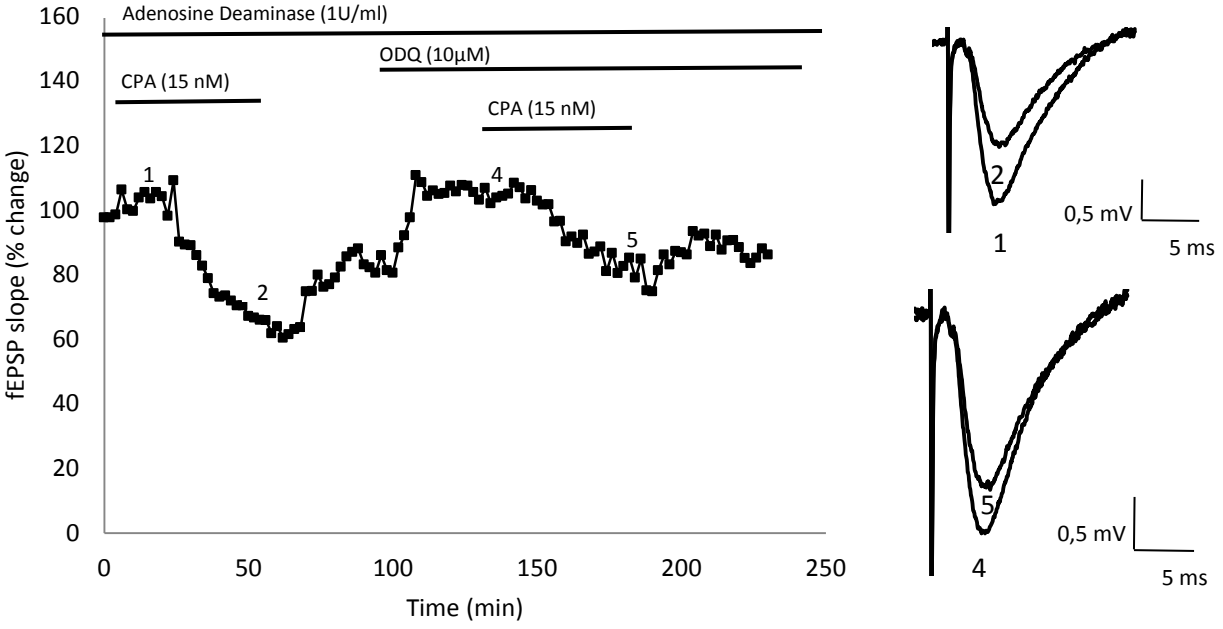

Fig.28 Effect of CPA (15 nM)) in the presence of ADA (1U/ml) and in the absence or in the presence of the inhibitor of soluble guanylyl cyclase ODQ (10μM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of a male rat. A timecourse of a representative experiment is shown in left panel and representatives traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) in the presence of ADA reversibly decreased the fEPSP slope by 35%, while in the presence of ODQ (10 μM) and ADA (1 U/ml) CPA (15 nM) caused a decrease of 21% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

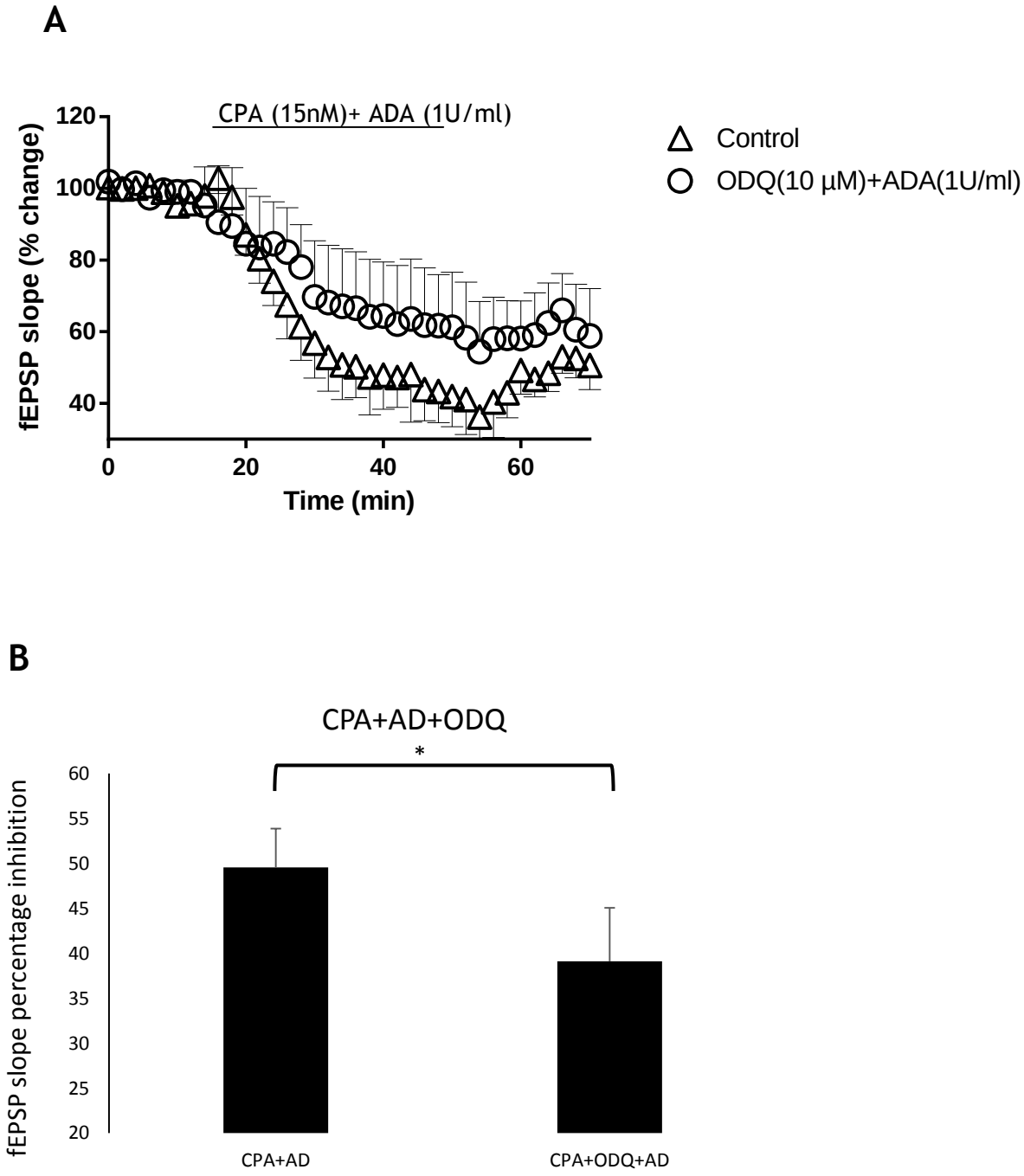

Fig.29 Inhibitory effect of CPA (15 nM)) in the presence of ADA (1U/ml) and in the absence or in the presence of the inhibitor of soluble guanylyl cyclase ODQ (10µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of male rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of five experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition caused by CPA in the absence (left) or in the presence (right) of ODQ;100% in A represents average fEPSP slope in the recorded for 10 min before applying CPA under each testing condition. The bars represented the average ± SEM of four independent experiments. * P<0.018 (Paired Student´s t-test).

The results observed with female rats were similar to those obtained with male rats (Fig. 30 and Fig. 31). In female rats, CPA (15nM) in presence of ADA (1U/ml) decreased the fEPSP slope by 52% ± 4.9%, however CPA in presence of ODQ and ADA (1U/ml) depressed the fEPSP slope by 38% ± 5.5% after 40 minutes (n=4; P<0,05, compared with CPA in the presence of ADA, paired Student's t-test). Application of ODQ (10 μM) during 30 minutes in presence of ADA (1U/ml) showed a tendency to increase, by 29% ±12.7%, the fEPSP slope, although this effect was not statistically significant (n=4; P>0.05, when compared with zero, Student's t-test).

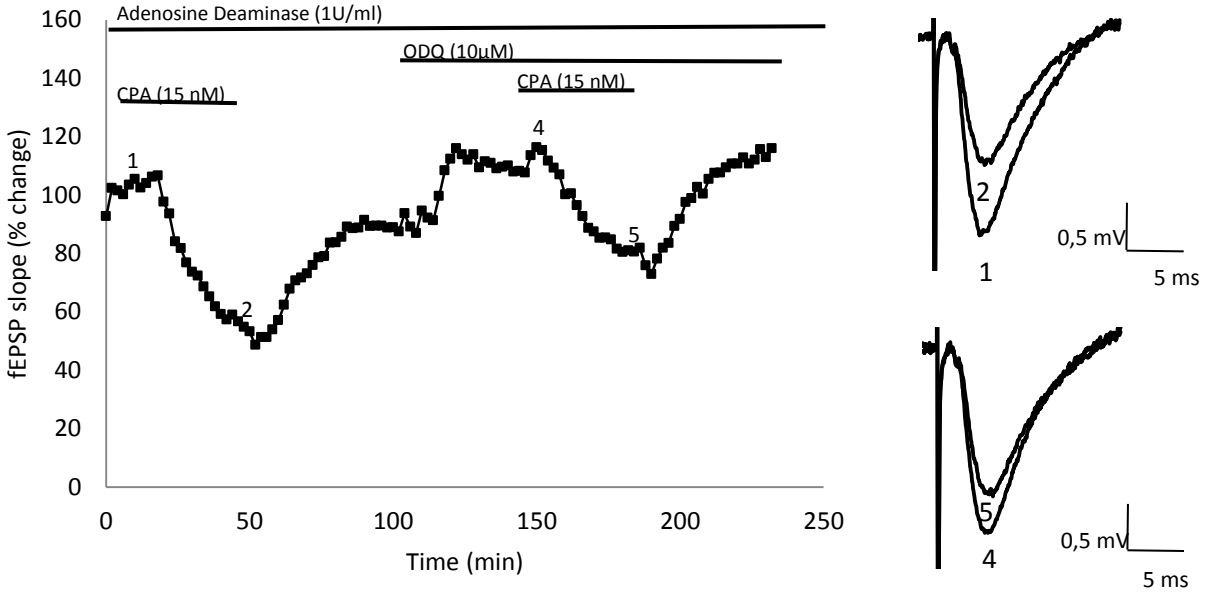

Fig.30 Effect of CPA (15 nM) in the presence of ADA (1U/ml) and in the absence or in the presence of the inhibitor of soluble guanylyl cyclase ODQ (10μM) on the slope of fEPSPs recorded the CA1 area of a hippocampal slice of a female rat. A timecourse of a representative experiment is shown in left panel and representative traces of averaged fEPSPs recorded at the times indicated are shown in the right panels. Application of CPA (15 nM) in the presence of ADA reversibly decreased the fEPSP slope by 43%, while in the presence of ODQ (10 μM) and ADA (1 U/ml) CPA (15 nM) caused a decrease of 25% in the fEPSP. Each point, in the ordinates, corresponds to the mean of eight consecutive fEPSPs; the time-distance between points corresponds to two minutes. The amplitudes of fEPSPs recorded for 10 min before each application of CPA were normalized to 100% to allow comparison between the effects of CPA under the two experimental conditions.

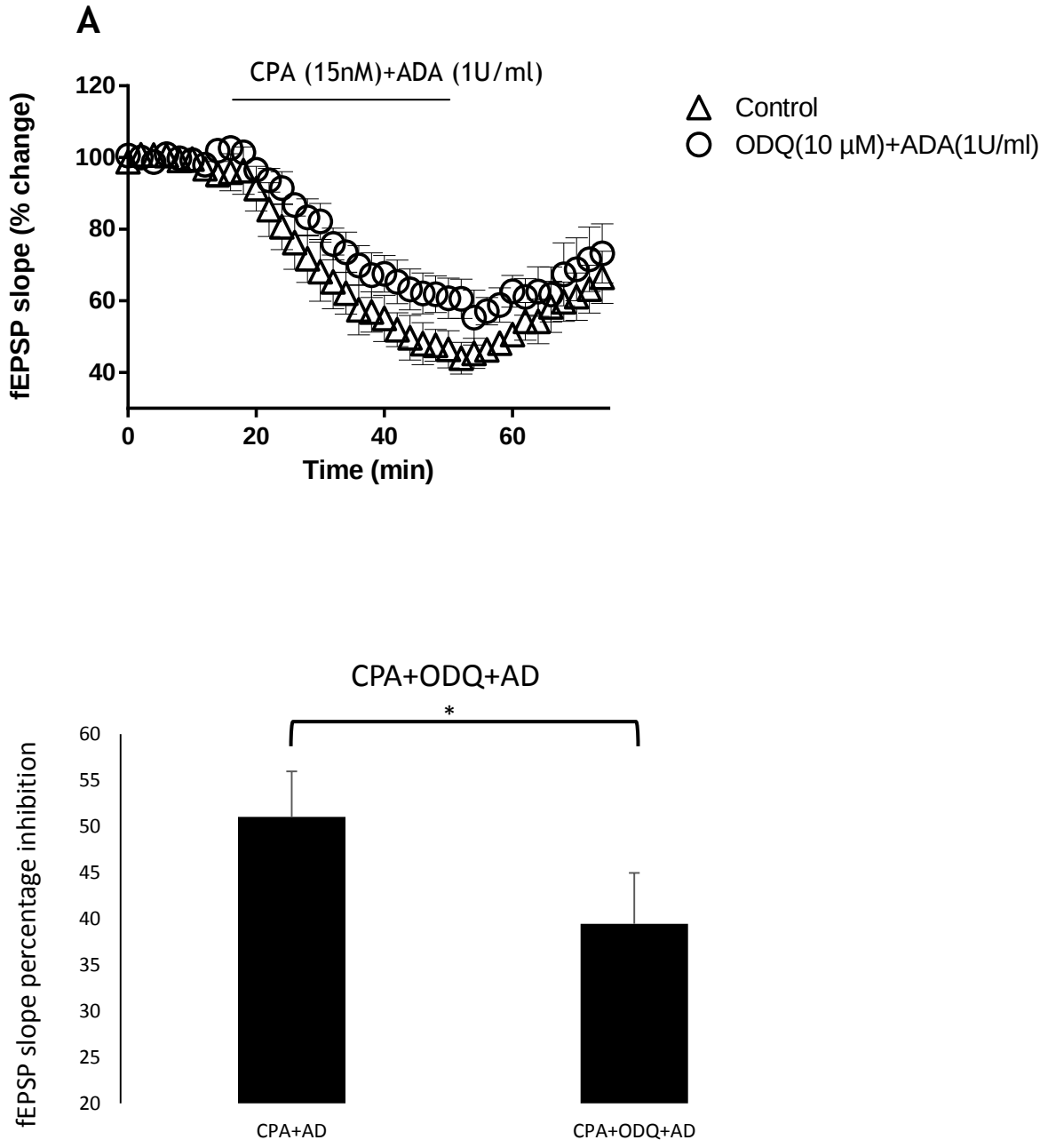

Fig.31 Inhibitory effect of CPA (15 nM) in the presence of ADA (1U/ml) and in the absence or in the presence of the inhibitor of soluble guanylyl cyclase ODQ (10µM) on the slope of fEPSPs recorded in the CA1 area of a hippocampal slice of female rats. A: Superimposition of the time courses; each point in the ordinates corresponds to the mean of four experiments; the time-distance between points corresponds to two minutes. B: Comparison between average of percentage inhibition produced by CPA in the absence (left) or in the presence (right) of ODQ; 100% in A represents average fEPSP slope in the recorded for 10 min before applying CPA under each testing condition. The bars represented the average ± SEM of four independent experiments. * $P<0.026$ (Paired Student´s t-test).

Chapter 4

Discussion

4.1 Increasing cGMP levels didn't modify A1 receptor inhibitory activity

4.1.1 Phosphodiesterase 2 (PDE2)

One important hallmark of the hippocampus is the very high activity of the cyclic nucleotide degrading enzyme phosphodiesterase (PDE), which contributes to the difficulty to measure detectable GMP levels. Several isoforms of PDE have been demonstrated to be localized in the rat hippocampus by means of *in situ* hybridisation and immunohistochemical approaches. These include PDE 1, 2, 3 and 4, as well as PDE 9 and, to a weaker extent, PDE 5 (Ludvig et al., 1991; Repaske et al., 1993; Furuyama et al., 1994; Reinhardt and Bondy, 1996). However, the principal PDE iso-enzyme responsible for cGMP hydrolysis in the hippocampus appears to be PDE2 (Repaske *et al.*, 1993; Suvarna and O'Donnell, 2002). Despite it being able to degrade both cAMP and cGMP, evidence coming from studies on cortical neurons and hippocampal slices indicates that cGMP levels are primarily elevated upon PDE2 inhibition (Martins et al, 1982; Suvarna and O'Donnell, 2002; Boess et al., 2004; ,Bender and Beavo, 2006). PDE2 has been proposed to regulate cGMP levels that arise in response to NMDA receptor activation in rat neurons from cortex and hippocampus (Suvarna and O'Donnell, 2002). Furthermore, PDE2 has been implicated in synaptic plasticity, learning and memory. Accordingly, inhibition of PDE2 has been reported to enhance hippocampal long-term potentiation (LTP) (Boess et al., 2004). In cultured neurons, BAY 60-7550, a selective PDE2 inhibitor, increases cGMP levels in the presence of NMDA or guanylyl cyclase activators but also increases cAMP levels (Masood et al., 2009). In hippocampus slices Bay 60-7750 also increased cAMP (Boess et al.,2004). If the adenosine A1 receptor inhibitory effect on neurotransmission is mediated by an increase on cGMP concentration, then inhibiting cGMP degradation with Bay 60-7750 should potentiate the A1 receptor-mediated effect. However, according with the results obtained in the present work, inhibition of PDE2, did not modify the effect of CPA on EPSP slope, which could be consequence of the effect of Bay 60-7550 on cGMP levels being probably counterbalanced by increases of cAMP levels, since these cyclic nucleotides have opposite effects on synaptic transmission. In fact, in the present study, Bay 60-7750 by itself increased the EPSPs slope at the hippocampal slices, suggesting that its effect on increasing cAMP levels (stimulating neurotransmission) surpassed its effect on increasing cGMP levels.

4.1.2 cGMP analog

cGMP is a critical second messenger that regulates multiple targets including protein kinase G (PKG), phosphodiesterases and cyclic nucleotide-gated ion channels (Francis et al., 2010). Stimulating nitric oxide synthase, activating soluble guanylyl cyclase or elevating concentrations of intracellular cGMP depressed excitatory synaptic transmission in CA1 hippocampal neurons (Lei et al., 2000). Similarly, 8-Br-cGMP, a membrane-permeable cGMP analogue, depressed the amplitude of mEPSCs, evoked field potentials and EPSCs in CA1 neurons (Lei at al., 2000). In hippocampus slices, 8-pCPT-cGMP, another membrane-permeable cGMP analogue, also depressed the fEPSPs (Arrigoni et al., 2006). cGMP induces long-term changes in efficacy at glutamatergic synapses through activation of protein kinase G (PKG) (Serulle et al., 2007), so it is possible that the inhibition of the fEPSP by a cGMP analog is due to postsynaptic depression of the glutamatergic receptors.

We tried to investigate if using a membrane-permeable cGMP-analogue, 8-pCPT-cGMP, to mimic the A1 receptor action, could occlude the A1 receptor-mediated inhibitory effect on synaptic transmission. However, in the present study we obtained different responses of 8-pCPT-cGMP since in some experiments it increased and in others depressed or had no effect on synaptic transmission. This could be consequence of the fact that cGMP not only depress but can also stimulate synaptic transmission (Russwurm et al., 2013), depending probably on the predominant cGMP targets present, which might vary from cell to cell. In the present work, 8-pCPT-cGMP did not modify the effect of A1 receptors possibly because cGMP might act in many different targets simultaneously.

4.2 Influence of blocking components of the NOS/sGC/PKG pathway on the adenosine A1 receptor activity

4.2.1 Nitric oxide synthase (NOS)

Nitric oxide is a retrograder messenger produced by nitric oxide synthases. It induces cGMP-mediated signal transduction cascade by activating soluble guanylyl cyclase (Masters et al., 1996; Murad et al., 1993). The function of NO in the CNS has been studied most extensively in synaptic plasticity, including long-term depression (LTD) in the cerebellum and striatum and long-term potentiation (LTP) in the hippocampus and cerebral cortex (Garthwaite and Boulton, 1995; Hawkins et al., 1998). In cerebellar and striatal LTD, NO generated presynaptically or in interneurons acts postsynaptically, whereas in hippocampal and cortical LTP, NO is synthesized postsynaptically and acts on pre synaptic terminals (Ko and Kelly, 1999).

Nitric oxide plays a main role in LTP, as indicated by experiments showing that LTP is eliminated or blocked significantly by inhibitors of nitric oxide synthase (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991). Furthermore, inhibitors of sGC or PKG also block the induction of LTP (Zhuo et al., 1994; Blitzer et al., 1995; Boulton et al., 1995). On the other hand, presynaptic injection of cGMP produces activity-dependent long-term potentiation in cultured hippocampal neurons (Arancio et al.,1995).

NO/cGMP signaling cascade has previously been proposed to play a role in synaptic transmission. Chetkovich et al. (1993) reported that application of a high-frequency stimulation induces a transient increase in cGMP in area CA1 of the hippocampus. In hippocampus slices, NO donors depress fEPSPs via the NO-cGMP pathway (Boulton et al, 1994). In addition, several studies have shown that nitric oxide stimulates adenosine release. In the ventral striatum inhibition of nitric oxide synthase (NOS) activity decreases extracellular adenosine concentrations, suggesting that endogenous NO modulates adenosine release (Fischer et al. 1995). Inhibition of field EPSPs by NO may, at least in part, be due to the release of adenosine that subsequently acts to depress neurotransmission through A1 receptors (Fallahi et al. 1996). Accordingly, nitric oxide inhibited synaptic transmission in hippocampal slices and this inhibition was blocked by an adenosine A1 receptor antagonist (Boulton et al,1994; Broome et al,1994). However, this NO effect seems not to be mediated by sGC activation, since inhibition of soluble guanylyl cyclase did not affect the inhibitory effect of nitric oxide at the synaptic transmission (Arrigoni et al., 2006). On the other hand, nitric oxide was shown to increase the inhibitory effect of 2-chloroadenosine (CADO) in synaptic transmission and this increase was blocked by inhibitors of sGC (Fragata et al., 2006). However the possibility that NO formation might mediate the adenosine A1 inhibition of synaptic transmission has not been investigated. The results obtained in the present study demonstrated that when NO production was halted by L-NAME, an inhibitor of NOS, the effect of A1 receptor in synaptic transmission was strongly attenuated in hippocampus slices, suggesting that NO and cGMP contribute to A1 receptor-mediated inhibition of synaptic transmission, since NO stimulates the cGMP pathway.

4.2.2 Protein kinase G (PKG)

Studies in cortical slices and cortical neurons suggests that the major actions of cGMP at synapses are mediated by PKG (Barnstable et al., 2004). Inhibitors of PKG prevented the induction of LTP in hippocampal CA1 region (Zhuo et al., 1994, Boulton et al.,1995), whereas activators of PKG lowered the threshold for the induction of LTP (Arancio et al., 1995) Moreover, previous studies showed that PKG inhibition prevents LTD (Reyes-Harde et al., 1999). On the other hand, according with Doerner and Alger (1988) cGMP depressed hippocampal $Ca2^{+}$ currents through a PKG-independent mechanism. In other tissues such as heart and smooth muscle, cGMP inhibits Ca2+ currents by stimulating PKG (Clapp and Gurney, 1991). The present work provided evidence that a PKG inhibitor increased fEPSP slope in hippocampus slices, suggesting that inhibition of components of cGMP pathway increased synaptic transmission. The present work also showed that blockade of PKG activation depressed the effect of A1 receptor in synaptic transmission, demonstrating that part of the inhibitory effect of A1 receptors are mediated by cGMP signalling in the hippocampus. Accordingly, inhibitors of NOS, sGC and PKG decreased A1 receptor mediated nociceptive effects (Lima et al, 2010).

The inhibitory effect of adenosine A1 on neurotransmitter release receptor has been shown to involve inhibition of N- and P/Q-type Ca^{2+} channels (Wu and Saggau, 1994), but this mechanism seems to only partially explain the presynaptic effect of the A1 receptor. The results obtained in the present study suggest that stimulation of cGMP and PKG activation, might constitute another parallel mechanism mediating the presynaptic inhibitory effect of adenosine A1 receptors on synaptic transmission. Alternatively, since PKG can inhibit N-type Ca^{2+} channels (D'Ascenzo et al., 2002), cGMP/PKG might probably mediate, at least in part, the inhibitory action of A1 receptors on N-type channels.

4.3.3 Soluble guanylyl cyclase (sGC)

Nitric oxide activates soluble guanylyl cyclase (sGC), increasing the formation of cGMP. According to Arrigoni et al (2006), ODQ, an inhibitor of soluble guanylate cyclase, failed to modify the DEA/NO-induced adenosine-mediated inhibition of the fEPSP. In contrast, other studies point in other directions, since nitric oxide-mediated adenosine release was blocked by ODQ (Saransaari and Oja, 2004), while nitric oxide-dependent potentiation of the inhibitory effect of the adenosine analog CADO on synaptic transmission is also blocked by ODQ. (Fragata et al,2006). Now we tested if blocking sGC would interfere with the A1 receptor-mediated activity on synaptic transmission. Since our initial experiments with ODQ failed to modify the activity of A1 receptors, we proceeded by performing the experiments in the presence of ADA. Endogenous adenosine can exert an inhibitory tonic modulation of CA1 neurons that is regulated by adenosine uptake and deamination. Furthermore, endogenous adenosine accumulation during conditions of increased metabolic demand provides a negative feedback that reduces electrophysiological activity (Maitre et al., 1974; Haas et al., 1984). ADA is an enzyme involved in purine metabolism which converts adenosine in inosin, removing endogenous adenosine (Ruiz et al,2000). Our reasoning was correct, since the presence of ADA unmasked the dampening effect of ODQ on A1 receptor-mediated inhibition of synaptic transmission indicating that endogenous adenosine needs to be removed by ADA to allow the influence of ODQ on A1 receptor activity to express itself.

Conclusion

Although increasing cGMP levels failed to modify A1 receptor activity, blocking components of cGMP pathway dampened A1 receptor inhibitory activity on synaptic transmission, revealing that part of the effect of A1 receptors depends on activation of the cGMP-pathway, suggesting cGMP as another second messenger in the sophisticated A1 receptor-mediated neurmodulatory activity at the hippocampus.

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