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THE ROLE OF ADENOSINE A_{2A} RECEPTORS ON NEUROMUSCULAR TRANSMISSION UPON AGEING

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Publications

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  (Chapter 4.1)

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Para ti, Beatriz.

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AC	Adenylate cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ADA	Adenosine deaminase
ADO	Adenosine
AK	Adenosine kinase
AKT	Threonine kinase
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine 5´-monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine 5´-triphosphate
A1R	Adenosine A1 receptor subtype
A2AR	Adenosine A2A receptor subtype
BDNF	Brain derived neurotrophic factor
BRET	Bioluminescense resonance energy transfer
CADO	2-chloroadenosine
cAMP	Cyclic adenosine monophosphate
CAT	Choline acetyl transferase
CGS21680	2-p-(2-carboxyethyl) phenethylamino]-5´-N-ethylcarboxamido adenosine hydrochloride
CREB	cAMP response element binding protein
CNS	Central nervous system
CPA	6-Cyclopentyladenosine
DAG	Diacylglycerol
DMSO	Dimethylsulphoxide

DPCPX	1,3-dipropyl-8-cyclopentylxanthine
EPC	Endplate currents
EPP	Endplate potential
ERK	Extracellular signal-regulated protein kinases
GMEPPs	Giant miniature endplate potentials
GPCR	G protein-coupled receptor
IMP	Inosine monophosphate
IP3	Inositol 1,4,5-triphosphate
ITU	5-Iodotubericidin
KCa	Ca2+-activated potassium channels
Ki	Equilibrium dissociation constant of the competitor
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MEPP	Miniature endplate potentials
MHC	Myosin heavy chain
NGF	Nerve growth factor
mAChR	Muscarinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor
NMJ	Neuromuscular junction
NMT	Neuromuscular transmission
NT	Neurotrophin
$p75^{NTR}$	$_{P}$75 neurotrophin receptor
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

QC	Quantal content
RRP	“Readily releasable” pool
SAH	S-adenosylhomocysteine
SSC	Spontaneous synaptic currents
TrkB	Tyrosine kinase B
U73122	1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione
VDCC	Voltage-dependent calcium channel
VGCC	Voltage-gated Ca^{2+} channels
ZM241385	4-(2-[7-amino-2-(2-furly)[1,4]triazolo[2,3-a][1,3,5]triazin5ylamino] ethyl) phenol

Abstract

Adenosine is a neuromodulator with important actions in the nervous system. The activation of adenosine A_{2A} receptors has been shown to modulate the action of other receptors. Considering that it was observed an interaction between adenosine A_{2A} receptors and TrkB receptors in hippocampus, I hypothesized that the activation of A_{2A} receptors could also facilitate BDNF actions on neuromuscular transmission. To answer that question I performed intracellular electrophysiological recordings in isolated preparations of the phrenic-nerve diaphragm from male infant rats. The data herein described clearly shows that there is a crosstalk between A_{2A} receptors and TrkB receptors at the neuromuscular junction, where A_{2A} receptors trigger the excitatory action of BDNF on neuromuscular transmission, through a mechanism that involves PLCγ. When I was investigating the interaction between adenosine A_{2A} receptors and TrkB receptors on neuromuscular transmission, I induced the increase of the endogenous adenosine level at the synaptic cleft, with an adenosine kinase inhibitor, 5`-iodotubericidin (ITU). It was observed that instead of inhibition, ITU greatly facilitated neuromuscular transmission. It was the first time that an excitatory effect induced by endogenous adenosine, at low frequency stimulation, was observed. Therefore I considered of interest to study the balance between adenosine A_1 and A_{2A} receptors at the neuromuscular junction upon ageing, since the activation of adenosine A_{2A} receptors has been shown to influence the actions of adenosine A_1 receptors and no one had looked at the excitatory actions of adenosine in aged rats at the neuromuscular junction. I performed intracellular electrophysiological experiments in isolated preparations of the phrenic-nerve diaphragm from four groups of male rats, with different ages: infant (3-4 weeks

old), young adult (12-16 weeks old), older adult (36-40 weeks old) and aged (70-80 weeks old). The data herein reported clearly showed that there is a predominance of excitatory adenosine effects at the motor nerve endings of infant rats. Moreover, ageing influenced adenosine A_1 and A_{2A} receptors, at the motor nerve terminals, in a different manner. The actions of A_{2A} receptors decreased with ageing, even disappearing in aged rats, whereas the actions of A_1 receptors remained unchanged upon ageing. Furthermore, the actions of adenosine A_{2A} receptors on neuromuscular transmission are dependent on adenosine A_1 receptors activation, since the excitatory effect of A_{2A} receptors was prevented by the adenosine A_1 receptor antagonist.

In conclusion, the data presented here demonstrate that adenosine A_{2A} receptors play a crucial role at the neuromuscular junction, since their effects on neuromuscular transmission predominate over adenosine A_1 receptors, in infant rats, and the excitatory effect of BDNF on neuromuscular transmission is dependent on their activation. The absence of adenosine A_{2A} receptors effects in aged rats might be one of the causes of the neuromuscular transmission impairment. The results herein described are relevant for a better understanding on the role of adenosine at the neuromuscular junction upon ageing, opening novel scientific questions such as investigate the role of adenosine A_{2A} receptors on neuromuscular transmission in the cenarios of neuromuscular disease as, for example, the amyotrophic lateral sclerosis disease.

Resumo

A adenosina é um importante neuromodulador do sistema nervoso. As suas acções estão dependentes da activação de receptores metabotrópicos associados às proteínas G. Os receptores A_{2A} e os receptores A_1 são considerados os receptores de alta afinidade para o ligando adenosina. A activação destes receptores desencadeia reacções intracelulares de natureza oposta. A activação dos receptores A_{2A} da adenosina, por estarem associados a uma proteína G excitatória causa o aumento da concentração de uma molecula intracellular que desempenha a função de segundo mensageiro, o monofosfato de adenosina (AMP) cíclico. Pelo contrário, a activação dos receptores A_1 da adenosina, que estão associados a uma proteína G inibitória, desencadeia a diminuição da referida molécula. Nos últimos anos tem sido demonstrado que os receptores A_{2A} da adenosina, além de exercerem efeitos directos na libertação de diferentes neurotransmissores, também modulam as acções de outros receptores, como por exemplo os receptores inibitórios A_1 da adenosina e os receptores do factor neurotrófico derivado do cérebro (BDNF, do inglês brain-derived neurotrophic factor), os receptores TrkB.

O objectivo do trabalho descrito nesta tese foi perceber o papel dos receptores A_{2A} na modulação da transmissão sináptica na junção neuromuscular de diafragma de rato ao longo da idade (desde os juvenis até aos animais próximo do termo de vida), bem como investigar a interacção destes receptores com os receptores TrkB do BDNF e com os receptores A_1 da adenosina. Utilizou-se uma abordagem funcional em que que se avaliou a transmissão neuromuscular evocada e espontanea, por métodos electrofisiológicos, em 4 grupos de animais com idades diferentes: juvenis (3-

4 semanas), jovens adultos (12-16 semanas), adultos de meia idade (36-40 semanas) e velhos (70-80 semanas).

Tendo em conta que no hipocampo de rato as acções do BDNF são dependentes da activação dos receptores A_{2A} da adenosina considerou-se importante avaliar se a interacção entre os receptores A_{2A} e as acções do BDNF, observada no hipocampo de rato, se observa também ao nível da junção neuromuscular. Os resultados obtidos e descritos nesta dissertação mostram que o efeito excitatório do BDNF na junção neuromuscular depende da activação dos receptores A_{2A} da adenosina. Mais, os resultados descritos nesta tese demonstram, pela primeira vez, que a interacção entre os receptores A_{2A} e TrkB, na junção neuromuscular, se verifica através da activação da via de transdução da cinase de proteína do tipo A pelos receptores A_{2A}, a qual permite a acção do BDNF, via receptores TrkB acoplados à lipase fosfatada do tipo C gamma, promovendo, desse modo, o aumento da transmissão neuromuscular.

Quando estávamos a avaliar se a activação dos receptores A_{2A} poderia facilitar o efeito do BDNF na transmissão neuromuscular, utilizámos uma ferramenta famacológica para promover o aumento dos níveis de adenosina endógena na fenda sináptica, isto é, adicionámos um inibidor da cinase de adenosina, iodotubericidina (ITU), ao banho de perfusão. A ITU impede a metabolização da adenosina intracelular em monofosfato de adenosina. O aumento da concentração de adenosina intracellular causa a sua libertação para a fenda sináptica através do transportador equilibrativo de nucleósidos. De modo inesperado a ITU, em vez de inibir, potenciou a transmissão neuromuscular de modo significativo. Este foi um resultado original, na medida em que nenhum autor, até agora, que seja do meu

conhecimento, tinha descrito um efeito excitatório da adenosina endogena via receptores A_{2A}, com um protocolo de estimulação de baixa frequência (0,5 Hz). Considerou-se, desse modo, importante investigar o equilibrio existente entre os receptores da adenosina A_1 e A_{2A}. Foi realizado um estudo farmacológico com o objectivo de investigar quais as condições que determinam a activação dos receptores A_1 ou dos receptores A_{2A} da adenosina. Os resultados que a seguir descrevemos documentam de modo claro que ambos os receptores de adenosina, A_1 e A_{2A}, presentes na junção neuromuscular de ratos jovens, podem ser activados por baixas concentrações de adenosina extracelular; no entanto a activação dos receptores A_{2A} predomina.

A maior parte das doenças neurodegenerativas tem incidência na velhice, no entanto existem poucos estudos em animais velhos ou muito velhos. Os receptores da adenosina têm assumido um papel cada vez mais relevante como alvo terapêutico para algumas doenças neurodegenerativas. Os poucos estudos que existem sobre o papel da adenosina na modulação da transmissão sináptica em animais envelhecidos foram realizados no sistema nervoso central. Por esse motivo considerámos importante investigar se o predomínio das acções excitatórias dos receptores A_{2A} sobre os receptores A_1, observado nos ratos juvenis, se mantem ao longo da idade. Este estudo permitiu obter um conjunto de dados que possibilitam caracterizar a fisiologia da transmissão neuromuscular ao longo da vida. Observámos que a transmissão sináptica, ao nível da junção neuromuscular de diafragma de rato, se mantém inalterada ao longo da vida, ficando comprometida apenas na velhice. Os animais muito velhos apresentam a amplitude e o conteúdo quântico dos potenciais evocados de placa motora, bem como a frequência

dos potenciais miniatura de placa motora significativamente diminuídos. Em relação ao efeito da idade nos receptores de adenosina, os resultados apresentados demonstram que a idade influencia os receptores de adenosina A_1 e A_{2A} de modo diferente. A amplitude dos efeitos excitatórios causados pela adenosina libertada endogenamente ou por um agonista inespecífico A_1/A_{2A} tendem a diminuir ao longo da vida, desaparecendo nos animais muito velhos, altura em que apenas a inibição medidada pela activação dos receptores A_1 é observada.

Em conclusão, os resultados obtidos neste trabalho demonstram que os receptores A_{2A} de adenosina têm um papel crucial na manutenção da transmissão neuromuscular, uma vez que as suas acções predominam sobre as dos receptores A_1 e as acções do BDNF dependem da sua activação. A ausência de efeitos excitatórios dos receptores A_{2A} nos animais muito velhos pode constituir uma das causas para o comprometimento da transmissão neuromuscular observada nessa idade. Os resultados que se descrevem na presente dissertação são relevantes para uma melhor compreensão do papel da adenosina na junção neuromuscular ao longo do envelhecimento, abrindo novas questões científicas, tais como investigar o papel dos receptores A_{2A} da adenosina na transmissão neuromuscular em situação de doença neuromuscular, por exemplo na esclerose lateral amiotrófica.

1. INTRODUCTION

1.1 THE NEUROMUSCULAR JUNCTION

The conection formed between a spinal motor neuron and a skeletal muscle cell has been studied extensively and most of the knowledge about chemical synapses derives from it: the neuromuscular junction.

1.1.1 Structural organisation of the neuromuscular junction

As illustrated in the Figure 1.1.1 the axon of the motor neuron innervates the muscle at a specialized region of the muscle membrane called the end-plate. At the region where the motor axon approaches the muscle fiber, the axon loses its myelin sheath and splits into several fine branches. The ends of the fine branches form multiple expansions or varicosities, called synaptic boutons, from which the motor neuron releases its transmitter. Each bouton is positioned over a junctional fold, a deep depression in the surface of the postsynaptic muscle fiber that contains the transmitter receptors. The transmitter released by the axon terminal is acetylcholine (ACh), and the receptor on the muscle membrane is the nicotinic type of ACh receptor.

The pre and postsynaptic membranes are separated by a synaptic cleft around 100nm wide. Within the cleft is a basement membrane composed of collagen and other matrix proteins. The enzyme acetylcholinesterase, which rapidly hydrolyses ACh, is anchored to the collagen fibrils of the basement membranes. In the muscle cell, in the region below the crest of the junctional fold and extending into the fold, the membrane is rich in voltage-gated Na^+ channels.

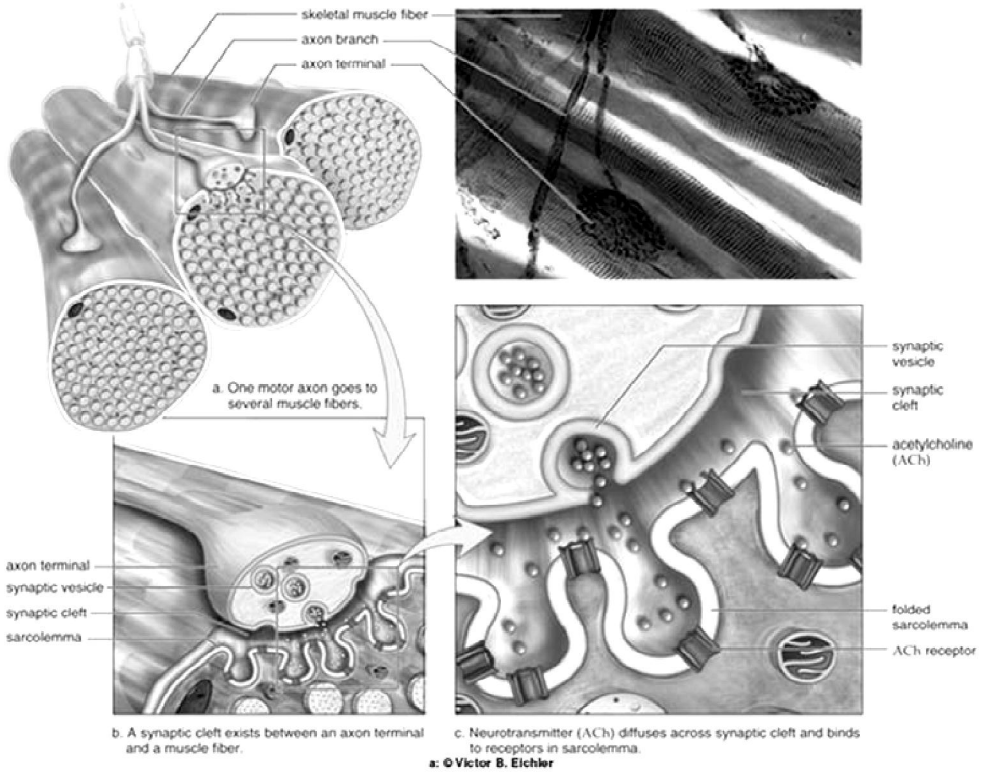


Figure 1.1.1 – Structure of the neuromuscular junction. (a) motor end plates at the neuromuscular junction. It is at the neuromuscular junction that the nerve fiber and the muscle fiber meet. (b, c) The motor end plate is the specialized portion of the sarcolemma of the muscle fiber. (Adapted from Fox, 2000).

Each presynaptic bouton contains all the machinery required to release neurotransmitter. This includes the synaptic vesicles, which contain the neurotransmitter ACh. Synaptic vesicle release at the neuromuscular junction is essential to sustain synaptic transmission. A subset of synaptic vesicles (defined as the “readily releasable” pool) is docked at active zones and available for release (Richards et al. 2003; Rizzoli and Betz 2004). However, only a fraction of these vesicles are released with each neuronal impulse. Synaptic vesicles located in close proximity to active zones do not contribute to neurotransmitter release until recruited to the “readily releasable” pool and thus constitute the “cycling” pool (Rosenmund and Stevens 1996; Zucker and Regehr 2002). Recycled vesicles can return to a “cycling” pool of vesicles, the “readily releasable” pool, or to a larger “reserve” pool located further away from the active zones (Richards et al. 2003; Sudhof 2004). Vesicles in the reserve pool are not released unless recruited to the cycling pool or “readily releasable” pool, usually to sustain long-term transmission.

1.1.2 Acetylcholine and its receptors

ACh is the neurotransmitter used by the motor neurons of the spinal cord and therefore is released at all vertebrate neuromuscular junctions. This neurotransmitter is synthesized in nerve terminals from acetyl coenzyme A (acetyl CoA, which is synthesized from glucose) and choline (derived from the diet), in a reaction catalysed by choline acetyl transferase (CAT). The postsynaptic action of ACh at the neuromuscular junction is not terminated by reuptake but by a powerful hydrolytic enzyme, acetylcholinesterase (AChE). This enzyme is concentrated in the synaptic cleft, ensuring a rapid decrease in ACh concentration after its release from the presynaptic terminal (Kandel et

al., 2000). The rapid hydrolysis of ACh limits the duration of the action of transmitter and also provides a local source of choline for re-utilization within the nerve terminal.

Two classes of cholinergic receptors have been identified: ionotropic receptors - nicotinic ACh receptors (nAChRs) and metabotropic receptors - muscarinic ACh receptors (mAChRs). In the nicotinic ACh-gated channel, at the nerve-muscle synapse, the pore in the membrane through which ions flow and the binding site for the chemical transmitter (ACh) that regulates the opening of the pore are all formed by a single macromolecule. This receptor consists of five polypeptide subunits that enclose the ion channel. All nACh receptors produce excitatory postsynaptic effects because they are nonselective cation channels, giving rise to currents with a reversal potential close to 0 mV (Kandel et al., 2000). Muscarinic ACh receptors (mAChRs) are metabotropic receptors that mediate many of the effects of ACh in nervous system and are formed from only a single subunit.

1.1.3 Neuromuscular transmission

The transmission of signals from nerve to muscle is an extremely reliable process. This is mainly because more transmitter is released from the motor nerve terminal by each nerve impulse than is required to excite the muscle fiber. The term safety factor for neuromuscular transmission is used to describe this excess. The release of transmitter is triggered by an increase in the concentration of free Ca^{2+} within the nerve terminal. This results from the opening of voltage-gated Ca^{2+} channels (VGCCs) by the depolarisation of the nerve membrane. In addition to Ca^{2+} channels, several forms of potassium channel are present in the nerve terminal, including voltage-gated and Ca^{2+}-

activated potassium channels (VGKC, KCa). There is some evidence that KCa channels are components of the active zones (Robitaille et al., 1993a,b). The potassium channels are likely to limit the duration of nerve terminal depolarisation and hence the extent of Ca^{2+} entry and transmitter release.

Each vesicle appears to contain 5000 – 10 000 molecules of ACh (Kuffler and Yoshikami, 1975). The ACh contained in a single vesicle is often referred to as a 'quantum' of transmitter, though its amount and effect on the postsynaptic membrane shows considerable statistical variation. One of the Katz´s seminal findings, in studies carried out with Paul Fatt (1952), was that spontaneous changes in the muscle cell membrane potential occur even in the absence of stimulation of the presynaptic motor neuron (Figure 1.1.2). Moreover Del Castillo and Stark (1954) reported that a reduction in calcium concentration have little effect on the spontaneous discharge. Spontaneous exocytosis of the content of individual vesicles occurs at most motor nerve terminals at a rate of several per minute to several per second. These give rise to small depolarisations (<1 mV) of the muscle fiber membrane, the miniature endplate potentials (MEPPs). Presynaptic action potential causes a synchronized release of many transmitter *quanta* producing an end plate potential (EPP) on the postjunctional cell. The magnitude of the end plate potential provides a convenient electrical assay of neurotransmitter secretion from a motor neuron terminal because it is made up of individual units (Katz, 1969).

The number of quanta released by a nerve impulse at a given neuromuscular junction is known as the 'quantal content' of the EPP. During continued stimulation of a motor nerve, the *quantal content* of an EPP is not maintained unless the frequency is less than 1 Hz. At higher frequencies there

is an initial fall, which is steepest during the first few impulses, after which EPP size is fairly well maintained at a level that is frequency dependent: the higher the frequency the lower the *quantal content* (Brooks and Thies, 1962).

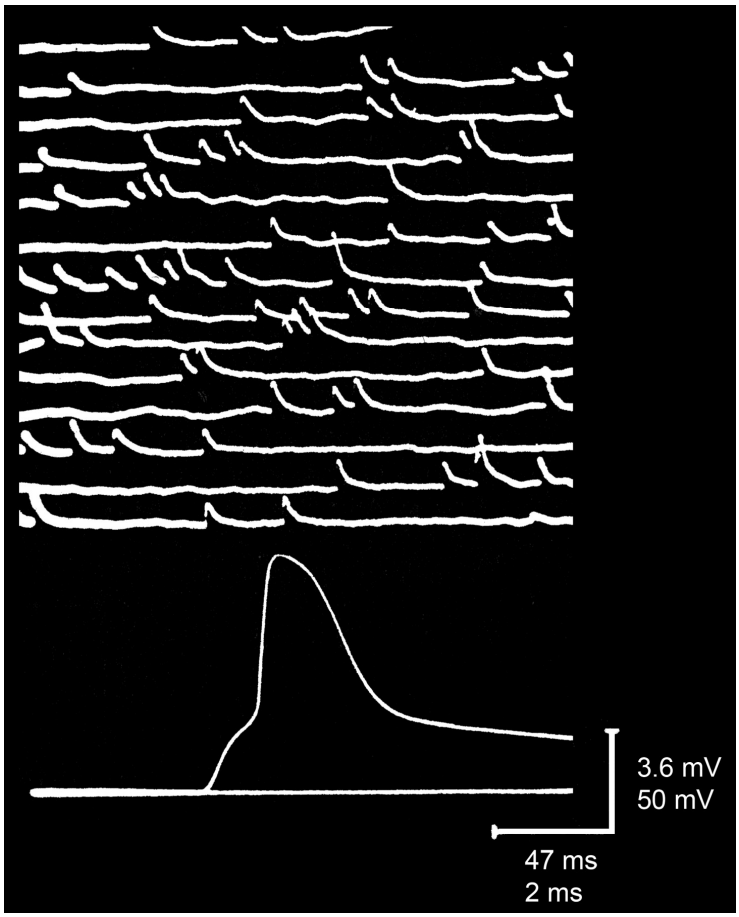


Figure 1.1.2 - Recording of spontaneous MEPPS (top) and of evoked EPP (bottom). The later is suprathreshold and elicits an action potential in the postsynaptic muscle fiber. Modified from Fatt & Katz (1952).

Large spontaneous EPPs have been observed under several conditions and were originally termed 'giants' (Liley, 1957). Later the term 'giant miniature endplate potential' (GMEPP) was adopted. The exact nature and origin of GMEPPs at the skeletal neuromuscular junction is unclear. It has been suggested that GMEPPs might result from a spontaneous multiquantal release of ACh insensitive to the influx of calcium (Gundersen, 1990). This might represent a form of constitutive exocytosis (Sellin et al., 1996) triggered by a Ca^{2+} release from intracellular stores or from an impaired processing of recycled synaptic vesicles (Rizzoli and Betz, 2002). GMEPPs have been observed in normal muscles (Liley, 1957; Menrath and Blackman, 1970; Jansen and Van Essen, 1976), and a high incidence can be observed during development (Bennett and Pettigrew, 1974; Blackshaw and Warner, 1976; Kullberg et al., 1977), in muscles innervated by regenerating (Miledi, 1960; Koenig and Pecot-Dechavassine, 1971; Bennet et al., 1973; Colmeus et al., 1982) or degenerating (Birks et al. 1960) nerve terminals, and in mice with motor endplate disease (Weinstein,1980).

1.1.4 Type of muscle fiber and neuromuscular transmission

The diaphragm muscle is a particularly active muscle of mixed fiber type composition. Based on myosin heavy chain (MHC) composition, diaphragm motor units differ in activation history and susceptibility to fatigue (Johnson and Siecke, 1993; Sieck and Fournier, 1989). The size of the “readily releasable” pool vary across diaphragm fiber types and RRP size is proportional to the number of active zones within a terminal, which in turn depends on terminal dimension. Presynaptic terminals at type I or IIa diaphragm fibers have smaller “readily releasable” pool size despite greater

average synaptic vesicle density at active zones (Mantilla et al., 2004), and lower quantal content compared with terminals innervating type IIx or IIb fibers (Rowley et al., 2007). The depolarisation caused by the flow of current into a muscle fiber is proportional to the 'input resistance' of the cell. This, in turn, is directly related to the resistance per unit area of surface membrane (closely related to permeability) and inversely related to the fiber diameter (Katz and Thesleff, 1957). Thus, if the number of ACh molecules per quantum is constant, then the mEPP amplitude should be greater for a small muscle fiber than for a large one. The lower the input resistance, the more AChR channels must be openned to depolarize enough to set up the muscle action potential. Thus small muscle fibers have larger endplate potentials. Muscle fiber diameter is increased by exercise or decreased by disuse. Such changes in diameter do not create a transmission problem because the number of quanta released from the motor nerve terminal can be altered to match the target cell (reviewed in Herrera and Werle, 1990).

1.1.5 Ageing and neuromuscular transmission

The neuromuscular junction has been reported to undergo significant detrimental changes during ageing that impair neuromuscular transmission (Figure 1.1.3). Early studies found that ageing was associated with morphological changes confined primarily to nerve endings with little or no degeneration or loss of primary axons, suggesting that ageing was associated with a functional denervation (Fujisawa, 1976; Gutmann and Hanzlikova, 1973). Subsequent studies revealed that age-related changes in neuromuscular junction morphology vary among different muscle types and could potentially be related to muscle activity levels (Prakash and Sieck,

1998). Ageing skeletal muscle is characterized by a progressive loss of muscle mass as well as a decrease in function (Narici and Maffulli, 2010). This age-acquired deficit known as sarcopenia, contributes profoundly to a decrease in the quality of life in elderly and predisposes them to an increased risk of morbidity, disability, and mortality (Janssen et al., 2004). Whether this loss of muscle mass is a cause or a consequence of nerve terminals degeneration remains to be clarified. Several authors reported morphological, molecular and functional age-related changes at the nerve cells. In aged nerves, wide incisures and infolded or outfolded myelin loops are frequent, resulting in an increased irregularity in the morphology of fibres along the internodes (Ceballos *et al*., 1999). Another study in rat diaphragm from older rats (28 months) found no change in endplate size, but there were an increased number of nerve terminals per endplate, decreased sprouting, and an increased number of synaptic vesicles (Smith and Rosenheimer, 1982). Balice-Gordon (1997) monitored age-related changes in the neuromuscular junction over time in C57BL6 mice (the most widely used mouse strain as "genetic background" for studies where genetically modified mice are used to study human disease). The author reported that following the developmental period of synapse elimination, neuromuscular junctions were stable over a long period of time. However, when the animals reached the age of 12–18 months, a small percentage of neuromuscular junctions started to display some loss of motor terminal branches and underlying ACh receptors. Furthermore, this investigator found that motor axons appear to compensate for the loss of synaptic sites by sprouting and adding new junctions. However, this compensatory effect diminished dramatically as animals reached the age of 18 months and older. The newly formed junctions were also more

vulnerable to degenerative changes and some of the junctions disappeared within weeks. By 24–36 months of age, the majority of neuromuscular junctions were dissociated from presynaptic terminals and postsynaptic endplates were severely fragmented.

Table 1.I – Age-related changes at the neuromuscular junction.

Morphological	
Loss of primary axons and nerve terminals degeneration.	Gutmann and Hanzlikova, 1973 Fujsawa, 1976
Loss of synaptic vesicles per nerve terminal.	Banker et al. 1983
Loss of motor nerve terminal branches	Balice-Gordon, 1997
Preferential loss of unmyelinated axons than myelinated fibres	Ceballos et al., 1999

Molecular	
Reduction of mitochondrial content at the nerve terminal	Fahim and Robbins, 1982
Reduction of ACh receptors	Banker et al., 1983
Increased activity of Choline-o-acetyltransferase	Washio et al., 1987
Less ACh per terminal	Smith and Weiler, 1987
Decline in the expression of the major myelin proteins.	Ceballos et al., 1999 Rangaraju et al., 2009

Functional	
Decreased axonal transport	Gutman and Hanzlihova, 1973
Reduction of the safety factor	Smith, 1979
Impairment of evoked transmission	Kelly and Robins, 1983
Impairment of MEPPs frequency	Banker et al., 1983

Molecular changes were also reported. Old rats have less ACh per terminal than mature rats (Smith and Weiler, 1987). However, incorporation of choline into ACh is faster in the diaphragms of old rats, and choline-o-acetyltransferase activity is elevated (Smith and Weiler, 1987; Washio et al., 1987). The amount of ACh released by stimulation is not altered by age, but the amount of ACh released from resting preparations is substantially faster in the old rats. It seems that old rats have less ACh per terminal because they are leaking it away faster (Smith, 1990). It has been postulated that the age-related changes in the neuromuscular junction are also associated with alterations in axonal transport (Gutmann and Hanzlikova, 1973). Jacob and Robbins (1990) reported that in old mice, the motor neurons loose the ability to expand the field of innervation in response to partial denervation, what might be related to a decline in axonal transport during ageing, since it can affect parameters such as availability of trophic factors as well as movement of organelles such as mitochondria that are critical for neuronal survival.

Molecular changes influence neuromuscular transmission. Age-related changes in the spontaneous release occur most rapidly in the first 6 weeks of life (Kelly, 1978) as a consequence of growth of muscle fibers and nerve terminals (Banker et al., 1983), whereas changes in evoked transmitter release begin in mid-life (Kelly and Robbins, 1983). The effect of age on neuromuscular transmission depends on the muscle type, being the diaphragm the most preserved muscle (Banker et al., 1983).

1.2 ADENOSINE

Several modulatory substances are present at a given synapse. Among the diversity of neuromodulators at the nervous system; adenosine and/or adenosine triphosphate (ATP) have important roles as fine-tuners. As shown

in Figure 1.2.1 one can see the details of the different aspects related to release, metabolism, degradation and adenosine receptors involved in neuromuscular transmission at the neuromuscular junction. Purines and purine nucleotides are essential constituents of all living cells (Arch and Newsholme, 1978; van de Berghe et al., 1992). ATP is used as an energy source of nearly all celular activity, whereas adenosine is a component of nucleic acids. Both purines are released from neurons and other cells and produce widespread effects on multiple organ systems by binding to purine receptors on the cell surface. The first observations on the relevance of adenosine in the nervous system were made in the seventies, when it was reported that adenosine decreased the release of ACh from motor nerve terminals (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975) and affected cAMP accumulation in nerve cells (Sattin and Rall, 1970). Several studies have been conducted since then that confirm and further detail the action of adenosine as neuromodulator of almost every neurotransmitter in the nervous system (see Ribeiro and Sebastião, 2010 for review).

1.2.1 Pathways: synthesis and degradation of adenosine

Formation and metabolism of intracellular adenosine

Adenosine is at a crossroad between several key metabolic pathways in cellular metabolism, it is a product of ATP degradation and is also a substrate for its synthesis (see Fredholm, 1997). Inside the cells, adenosine is formed by the action of endo-5´-nucleotidase over AMP (Phillis and Newsholme, 1979) and also by hydrolysis of S-adenosilhomocysteine (SAM) (Nagata et al., 1984), reaction catalised by SAM hydrolase. Judging by the kinetic constants of some of the major enzymes involved, the intracellular

concentration of adenosine at equillibrium is probably around 100 nM (Meghji, 1992) This level is controled by the rapid phosphorylation of adenosine by adenosine kinase and also by its conversion to inosine by adenosine deaminase (Wu and Phillis, 1984; Nagy et al., 1990). Adenosine deaminase is mainly cytosolic but it also occurs as a surface ecto-enzyme (Franco et al., 1997).

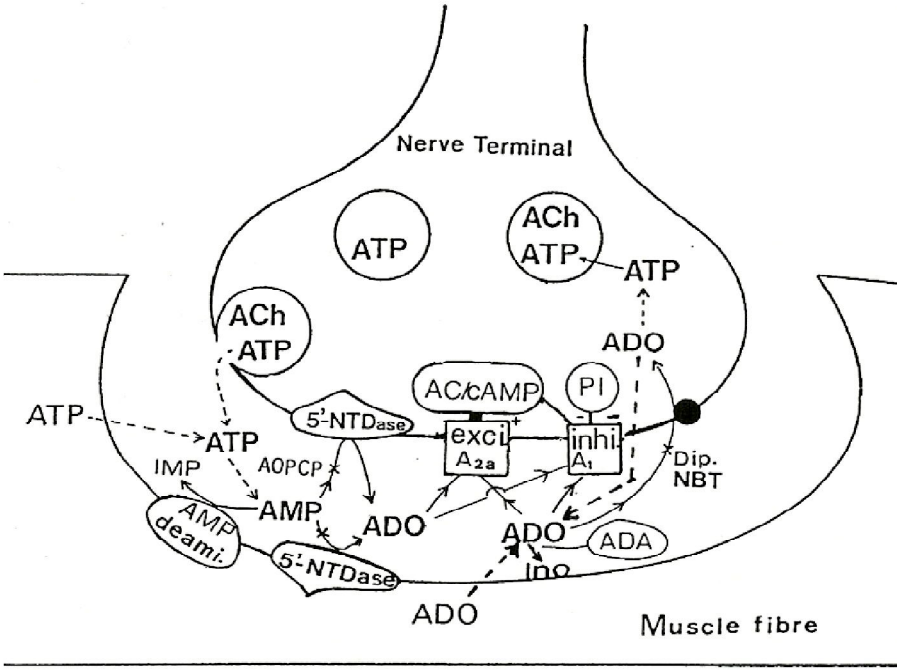


Figure 1.2.1 – Schematic representation of a cholinergic nerve terminal innervating a skeletal muscle fiber. ATP is released into the synaptic cleft and it is hydrolised to adenosine (ADO), which is the active substance, and prefferentialy activates excitatory adenosine A2a receptors positively coupled to adenylate cyclase (AC). Adenosine is inactivated through uptake or deamination. (Adapted from Ribeiro et al., 1996).

Origin of extracellular adenosine

Adenosine is neither stored nor released as a classic neurotransmitter since it does not accumulate in synaptic vesicles. Instead it is released from the cytoplasm into the extracellular space through a nucleoside transporter (see e.g. Ribeiro et al., 2003). In the case of the neuromuscular junction, it is generally accepted that ATP is stored together with ACh in cholinergic synaptic vesicles (Dowdall et al., 1974). ATP can be co-released with ACh from motor nerve terminals (Silinsky, 1975) or released independently of ACh secretion (Rabasseda et al., 1987; Sebastião and Ribeiro, 1988). ATP and ACh are released from rat phrenic-nerve diaphragm muscle preparations stimulated at physiological frequencies; ATP is rapidly hydrolysed to ADP (Silinsky, 1975). Because large amounts of ATP are found in cholinergic synaptic vesicles (Dunant and Muller, 1986; Whittaker, 1984), they are thought to be the source of adenosine. When ATPase is inhibited in preparations of *Torpedo* synaptossomes, the ACh/ATP released is almost identical to that in the synaptic vesicles (Yu and Van der Kloot, 1990), which is an important evidence for vesicular release. When ATP is released at the neuromuscular junction, it is degraded into ADP, AMP, IMP, adenosine, inosine and hypoxantine (Cunha and Sebastião, 1991) through the ecto-nucleotidase pathway (Cunha et al., 1996) (Figure 1.2.2).

The concentration of extracellular endogenous adenosine is around 120-200 nM (Dunwiddie and Diao, 1994). The amount of adenosine released, upon electrical stimulation of innervated skeletal muscle preparations, in the presence of a supramaximal concentration of tubocurarine is approximately half the amount released in the absence of tubocurarine, suggesting that half

of the adenosine being released comes from the nerve and the other half comes from contracting muscle fibers (Cunha and Sebastião, 1993).

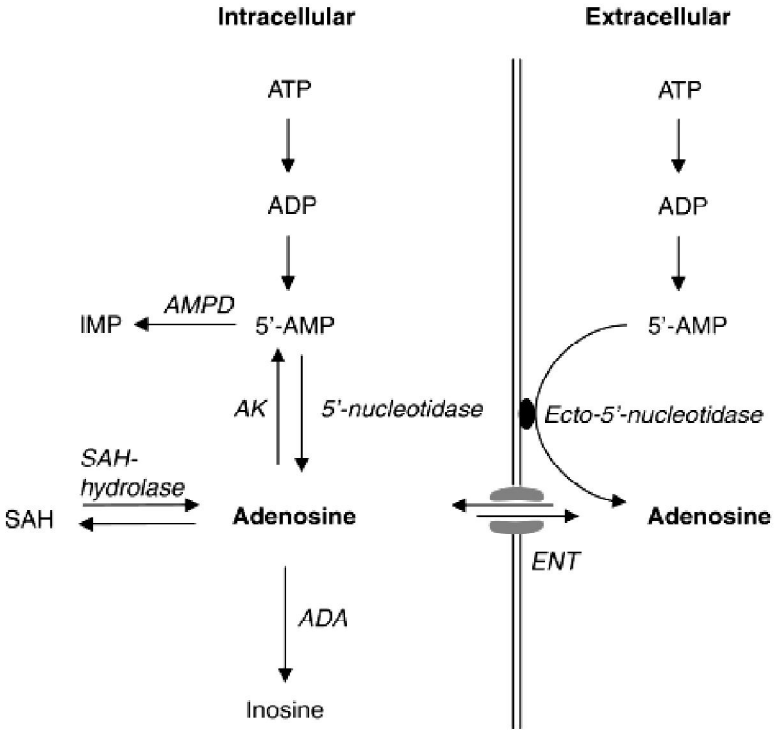


Figure 1.2.2 – **Pathways involved in the syntesis and degradation of adenosine.** Adenosine can be formed both intra and extracellularly, and cross the plasma membrane through equilibrative (ENT) or concentrative transporters. Degradation of extracellular adenosine occurs either through deamination to inosine or phosphorylation to 5´-adenosine monophosphate (5´-AMP). Abreviations: ATP – adenosine triphosphate; ADP – adenosine monophosphate; AMPD – AMP deaminase; IMP, inosine monophosphate; AK – adenosine kinase; ADA – adenosine deaminase; SAH – S-adenosylhomocysteine. (Adapted from Meijer et al., 2008).

Inactivation of extracellular adenosine

Inactivation of extracellular adenosine at the rat diaphragm, in contrast with what occurs in other preparations from the peripheral and central nervous systems might occur through both uptake and deamination (Sebastião and Ribeiro, 1988). The nucleoside transporters constitute an extended family (Agbanyo et al, 1990) with different kinetic and pharmacological properties (Schubert et al., 1984). In the central nervous system (CNS), the existing classes of transporters all share the ability of transporting adenosine more efficiently (Bender et al., 1980) Thampy and Barnes, 1983; Geiger et al., 1988) although they also transport other nucleosides. Adenosine transportation is bi-directional, results from facilitated diffusion process (Plagemann and Wohlhueter, 1980) and is kinetically independent of the intracellular metabolism of adenosine (Gu et al., 1993) (Figure 1.2.2).

1.2.2 Adenosine Receptors

Once in the extracellular space, adenosine modifies cell functioning by operating G-protein coupled receptors (GPCR). All of these receptors possess seven transmembrane domains and are linked to a variety of transducing mechanisms. Four major classes of adenosine receptors have been pharmacologically defined and cloned. These are the adenosine A_1 and A_3 receptors (see Linden, 1994), which are coupled to G_i protein, and the A_{2A} and A_{2B} receptors (Daly et al., 1983), which coupled to G_s protein (Figure 1.2.3).

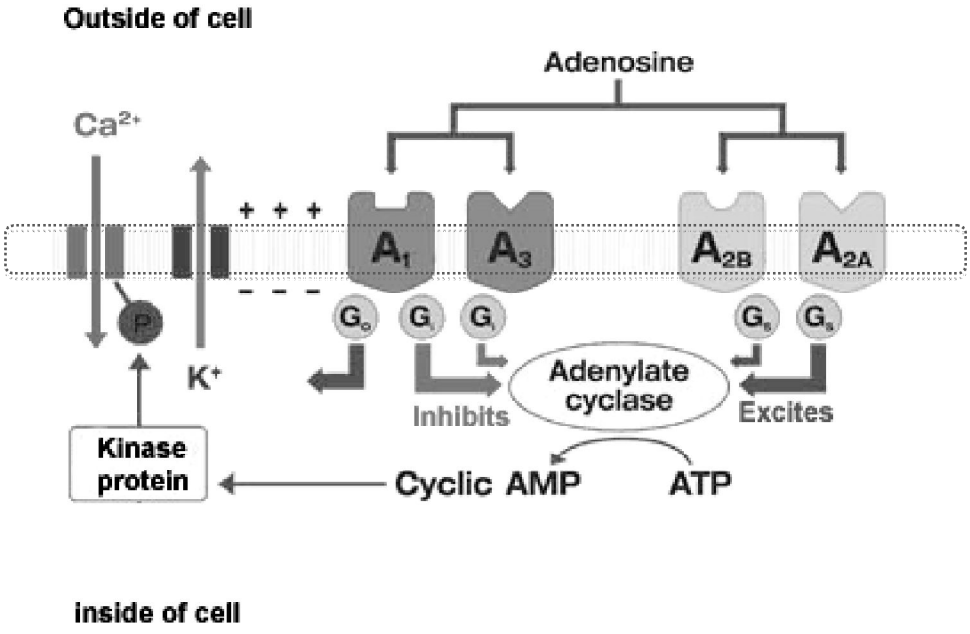


Figure 1.2.3 – Adenosine receptors. Adenosine can activate A_1, A_{2A}, A_{2B} and A_3 receptors. All adenosine receptors are G-protein coupled receptors, where A_1 and A_3 are mainly linked to the activation of Gi and A_{2A} and A_{2B} are mainly coupled to Gs proteins. Probable coupling to Gq proteins has also been reported for A_3 and A_{2B} receptors (Adapted fom Linden et al., 1999).

Adenosine controls the release of neurotransmitters by activating inhibitory A_1 and facilitatory A_{2A} adenosine receptors, which may coexist in the same nerve terminal (Correia de Sá et al, 2001). A_1 receptors are coupled to pertussis toxin sensitive G-proteins (Gi/Go) that lead to an inhibition of the activity of the enzyme adenylate cyclase (through subunits α and $\beta\gamma$) (see Cunha, 2001). The inhibitory effects of adenosine are attenuated by activators

of protein kinase C or by Li^+, which is known to affect phosphoinositide metabolism. However the effects are not attenuated by activators of protein kinase A (Sebastião and Ribeiro, 1990). Depression of ACh release via presynaptic A_1 receptors is by inhibition of N-type Ca^{2+} channels (Schwartz et al., 2003), but is not the basis of tetanic fade at the rat neuromuscular junctions (Malinowsky et al., 1997). Tetanic depression is overcome by tonic adenosine A_{2A} receptor facilitation of Ca^{2+} influx through L-type channels at the rat motor nerve terminals (Oliveira et al., 2004). Those receptors have been demonstrated in motor nerve terminals by functional studies (Correia de Sá et al., 2001). Baxter and collegues (2005) using immunofluorescence techniques revealed A_{2A} receptors immunoreactivity at neuromuscular junctions in a variety of skeletal muscles including the rat diaphragm and demonstrated that those receptors are localised only at the neuromuscular junction nerve terminal.

It appears that instead of the classical view that endogenous adenosine activates A_1 receptors regardless of whether it is released as adenosine or formed from adenine nucleotides; adenosine formed from adenine nucleotides acts preferentially on A_{2A} receptors and adenosine released as such acts preferentially on A_1 receptors (Cunha et al., 1996). This might result from a different localization of A_1 and A_{2A} receptors in relation to adenosine release sites and to the localization of the enzyme, ecto-5-nucleotidase, which hydrolyses the released nucleotides into adenosine (Reddy et al., 1995).

Adenosine Heteromers

During decades, in the field of adenosine neuromodulation, A_{2A} receptors were considered as isolated entities. However, the ability of these receptors to

form heteromers with other GPCRs, such as the dopamine and glutamate receptors, and heteromers constituted by different adenosine receptor subtypes (Ferré et al., 2007) is now established at the anatomic, biochemical and functional levels. Co-immunoprecipitation and bioluminescence resonance energy transfer (BRET) techniques have shown the existence of A_1−A_{2A} receptor heteromers in co-transfected HEK cells, as well as the existence of an intermolecular crosstalk, and radioligand-binding techniques have allowed the identification of an intramembrane receptor–receptor interaction in the A_1−A_{2A} receptor heteromer (Ciruela et al. 2006). The presence of A_1-A_{2A} heteromers at the neuromuscular junction and their functional implications were not studied yet, although presynaptic interactions between A_1 and A_{2A} receptors were observed at motor nerve terminals (Correia de Sá et al. 1996).

1.2.3 Adenosine and ageing

There are no many studies on adenosine in aged ainmals at the neuromuscular junction, although several studies performed in the central nervous system showed that the neuromodulatory action of adenosine changes with age. Ageing decreases the ability of adenosine A_1 receptors to inhibit neuronal activity (Sebastião et al., 2000). This may be a function of an age-related decrease in the density of A_1 receptors in the brain, which has been shown in both mice (Pagonopoulou and Angelatou, 1992) and humans (Meyer et al., 2007). Low A_1 receptor density and function, however, can be compensated for by higher levels of extracellular adenosine, which keep tonic inhibition high in aged animals (Bauman et al., 1992). In contrast to A_1 receptors, A_{2A} receptors has a different pattern of change with age wich

depends on the brain area, as indicated: there is a significant increase in the density of A_{2A} receptors in the cortex (Cunha et al., 1995) and hippocampus (Diogenes et al. 2007) of aged rats, which correlates with their enhanced ability to facilitate glutamatergic synaptic transmission (Rebola et al., 2003) and ACh release (Lopes et al., 1999b) in the hippocampus. In the striatum there is a tendency for a decrease in A_{2A} receptor density in aged rats (Cunha et al., 1995), and within the striatum, age may influence the A_{2A} receptors in glutamatergic, dopaminergic or GABAergic nerve terminals in different ways (Corsi et al., 1999, 2000). There are age-related shifts in the A_1 receptor inhibitory/A_{2A} receptor excitatory balance, and this shift may be different in different areas of the brain. Due to the influence of A_{2A} receptors on other receptors (Sebastião and Ribeiro, 2000), the change in the A_{2A} receptor influence upon ageing may markedly affect the action of other modulators. However, at the neuromuscular junction, one of the few preparations where it is experimentally possible to separate the neuromodulatory and homeostatic roles of adenosine, the tonic A_1 receptor inhibition of ACh release is not modified in aged rats (Pereira et al., 2000). The role of adenosine excitatory receptors upon ageing remains to be clarified.

1.3 BDNF AND MOTOR NEURONS

It was some decades ago that Levi-Montalcini and Hamburger (1953) discovered the first growth factor, NGF (nerve growth factor), a protein required for the survival of certain sympathetic and sensory neurons. The discovery represents an important milestone in neurobiology and since then on, several other trophic factors were identified. In 1982, Barde and

collaborators isolated a neurotrophin from a pig brain, designated as brain-derived neurotrophic factor (BDNF).

1.3.1 BDNF as a trophic factor and as a neuromodulator

BDNF trophic actions

Interactions among presynaptic motor neuron, postsynaptic muscle, and Schwann cells play critical role in growth, maintenance, and survival of synapses. The exchange of trophic factors has been implicated in pre- and postsynaptic development as well as preserving neuronal and synaptic plasticity at the neuromuscular junction. Neurotrophins are known to participate in activity-induced modification of synaptic transmission (Schinder et al., 2000; Schinder and Poo, 2000). The expression levels of BDNF are low at birth but increase dramatically during the first weeks of postnatal development. Consistent with the classic neurotrophic hypothesis, the muscle targets produce BDNF, which promote the survival of innervating motor neurons that express the corresponding receptor tyrosin kinase B (TrkB) (Funakoshi et al., 1993). Chronic treatment of the nerve-muscle culture with neurotrophins promoted the maturation of the neuromuscular junction. Thus, the spontaneous and evoked synaptic currents recorded at the neuromuscular synapses exhibited more mature properties (Wang et al., 1995).

BDNF fast actions

Similar to what was observed in the central nervous system, neurotrophins not only regulate neuronal survival, but also modulate the synaptic activity of neuromuscular junction, via both pre and postsynaptic mechanisms (Levine et

al., 1998; Li et al., 1998). In *Xenopus laevis* nerve-muscle co-culture, acute exposure to BDNF rapidly increases the frequency of spontaneous synaptic currents (SSCs) and the amplitude of impulse-evoked end-plate currents (EPCs) (Lohof et al., 1993). Several lines of evidence (mainly from studies performed in the central nervous system) point to a presynaptic site of action of BDNF (McAllister, 2002), namely by inducing neurotransmitter release. BDNF (1) enhances ACh release from hippocampal synaptosomes (Knipper et al., 1994); (2) induces neurotransmitter release in cultured hippocampal neurones (Li et al., 1998); (3) increases the number of docked vesicles at the active zones (Tyler and Pozzo-Miller, 2001) and (4) enhances the expression levels of several synaptic vesicles proteins (Takei et al., 1997). At the neuromuscular junction, the action of this neurotrophin seems to be facilitated by presynaptic depolarization (Boulanger and Poo, (1999a). Moreover, it was demonstrated that synaptic potentiation induced by BDNF is regulated by cyclic AMP (cAMP) (Boulanger and Poo, 1999b). More recent studies reported that adenosine A_{2A} receptor activation facilitates BDNF-induced potentiation of synaptic transmission in the rat hippocampal slices (Diógenes et al., 2004).

1.3.2 BDNF receptors: TrkB and p75

All neurotrophins have a common basic structure, along with variable domains that determine the specificity of biological actions resulting from activation of the two types of receptors: high affinity receptor tyrosine kinases (Trk) and the low affinity panneurotrophin receptor ($p75^{NTR}$) (Chao, 1994; Kaplan and Miller, 2000).

The expression of a particular Trk receptor subtype confers on that neuron the capacity to respond to the corresponding neurotrophin. BDNF excertes its

action through activation of TrkB receptors. Researches have also turned their attention to the status of the p75 receptor. It binds all neurotrophins with the same affinity but different kinetics. Its intracellular domain has no catalytic activity but interacts with a great variety of adaptor molecules mediating such diverse actions as apoptosis, rearrangement of the cytoskeleton or arrest of the cell cycle. When Trk and $p75^{NTR}$ receptors are activated in concert, they enhance the potency and specificity of the binding to the neurotrophin (Hempstead, 2002). At the neuromuscular junction TrkB mRNA is expressed in motoneurons (Ernfors et al., 1992). The TrkB receptor for BDNF is found at diaphragm muscle neuromuscular junctions (in both presynaptic and postsynaptic sites) (Escandon et al., 1994), providing a potential physiologic basis for the effect of BDNF on neuromuscular transmission at diaphragm muscle fibers (Mantilla et al., 2004).

Segal and Greenberg (1996) reported that the phosphorylated TrkB serve as protein-interaction sites for adapter protein SHC, phospholipase C-γ (PLC-γ), and phosphotidylinositol-3 kinase (PI-3K), the intracellular molecules that lead to the activation of these major signalling pathways (Figure 1.3.1): (1) tyrosine phosphorylation of SHC triggers SHC/Grb2/SOS interaction, Ras activation and a series of phosphorylation reactions that include Raf, MAPK/ERK kinase (MEK), and mitogen-activated protein kinase (MAPK); when activated, MAPK is translocated to the nucleus and influences gene expression (Messaoudi et al., 2002); (2) the activation of PLC- γ induces the cleavage of phosphotidylinositol-4,5-biphosphate to generate inositoltriphosphate (IP3) and diacylglycerol (DAG), which in turn induces the release of Ca^{2+} from internal stores and activates protein kinase C (PKC), respectively (Johnson, 1999). Franke and collaborators (1995) also reported

that (3) phosphotidylinositol-2 (PI-3K) activation promote neuronal survival through activation of serine and threonine kinase AK-T.

TrkB can be expressed as truncated receptors, which lack intracellular catalytic domains. The function of these truncated forms of the Trk receptors is currently not understood, but they are highly abundant in both the central and peripheral nervous systems.

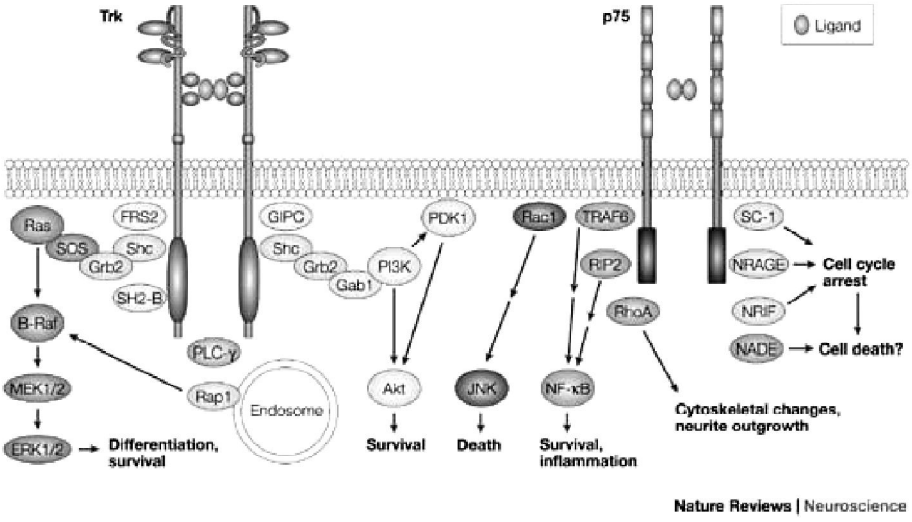


Figure 1.3.1 – Neurotrophin receptors and signalling cascades involved in neurotrophin signalling. The intracellular domain of the activated Trk receptors serves as docking site for many proteins, leading to the activation of MAPK (mitogen-activated protein kinase), Akt (threonine kinase) and PLCgamma (phospholipase C-gamma) pathways. P75 receptors activated pathways include activation of JNK, NF-KB and Rho kinases (From Chao, 2003).

1.3.3 BDNF and ageing at the neuromuscular junction

The exact role or the identity of neurotrophic and/or myotrophic factors that promotes survival and maintenance of presynaptic and postsynaptic apparatus at the neuromuscular junction in the context of ageing has not been fully determined. However, some studies indicate that a variety of trophic factors play a modulatory role in neuromuscular system to a different extent during ageing. Furthermore, BDNF have been shown to be important for maintenance of ACh receptors clustering in the neuromuscular junction since if neurotrophin signaling is interrupted or neurotrophin expression is suppressed, ACh receptors are dispersed and neuromuscular junction is fragmented and affected muscles show increased fatigue (Gonzalez et al., 1999), morphological changes that are described in aged animals.

1.4 Interaction between Adenosine and BDNF at the neuromuscular junction

Presynaptic depolarization (Boulanger and Poo 1999a) - which is known to increase extracellular adenosine levels, as well as the enhancement of intracellular cAMP (Boulanger and Poo 1999b), the most frequent A_{2A} receptor transducing pathway, triggers synaptic actions of BDNF. On the other hand, A_{2A} receptors are known to transactivate TrkB receptors in the absence of the neurotrophin (Lee and Chao, 2001). This transactivation requires long-term incubation with G-protein coupled receptor agonists and receptor internalization (Rajagopal et al., 2004). Indeed, it has recently been recognized that adenosine A_{2A} receptor activation is a crucial prerequisite for the functioning of neurotrophic receptors at synapses. This has been shown for the facilitatory actions of BDNF on synaptic transmission (Diógenes et al.,

2004; Tebano et al., 2008) and on LTP (Fontinha et al., 2008) at the CA1 area of the hippocampus.

2. OBJECTIVES

The present work was focused on the investigation of the role of adenosine A_{2A} receptors on neuromuscular transmission upon ageing. This question was addressed considering the interaction of adenosine A_{2A} receptors with other receptors, namely TrkB receptors and A_1 receptors. Thus the specific objectives of the work were:

1. To investigate whether activation of adenosine A_{2A} receptors can modulate BDNF action on neuromuscular transmission, at young ages.
2. To understand the influence of ageing on neuromuscular transmission.
3. To study the balance between the effects of adenosine A_{2A} and A_1 receptors on neuromuscular transmission upon ageing.

3. METHODS

3.1 Why did we use the neuromuscular junction model?

Experiments were performed at the innervated rat diaphragm neuromuscular junction. This preparation has some advantages since (1) there is only one type of transmitter, which chemical nature is well known; (2) it is easy to obtain intracellular recordings from the postsynaptic region; (3) in the absence of postsynaptic modifications, the response recorded from one cell is a function of the output of the transmitter released by only one nerve terminal and (4) it is possible to distinguish between pre- and postsynaptic effects (for a review see Ribeiro, 1991). It is possible to perform this study on this experimental model because of the existence of ATP in ACh vesicles and high expression of adenosine A_{2A} receptors (Baxter et al., 2005) as well as TrKB receptors (Escandon et al., 1994) in the presynaptic component.

3.2 Animals

The experiments were performed on isolated preparations of the phrenic-nerve diaphragm from male Wistar rats obtained from Harlan Interfauna Iberia, SL (Barcelona). Four groups of rats were used: infant (3-4 weeks old), young adult (12-16 weeks old), older adult (36-40 weeks old) and aged (70-80 weeks old). The animals, handled according to European Community guidelines and Portuguese Law on animal care, were killed by decapitation under halothane anaesthesia.

3.3 The rat phrenic-nerve diaphragm preparation

The experiments were performed on left phrenic-nerve-hemidiaphragm preparations. After opening the torax, the left phrenic-nerve was exposed and the left hemi-diaphragm, with the phrenic-nerve attached carefully dissected and removed with few ribs attached. The preparation was then placed on a large (approx. 50 ml) chamber filled with krebs solution for further dissection of a diaphramn strip (about 5 mm wide) with the phrenic-nerve attached. This procedure was done under a dissecting microscope; the muscle was carefully cut parallel to the orientation of muscle fibers, so that the number of individual muscle fibers that were cut was minimized. The highest density of phrenic-nerve endings was at the middle portion of the muscle strip. To facilitate dissection, the strip was slightly stretched by pulling surgical threads applied on the extremities of the preparation.

The strip was mounted in a Perspex chamber of 3 ml capacity and was stretched so that its final length corresponded to about two times its length in the resting position. The normal bathing solution (pH 7.4) contained (mM) NaCl 117, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.2. It was continuously gassed with 95% O2 and 5% CO2 (pH 7.4) and kept at room temperature (22-25ºC).

Muscle twitches were prevented by increasing the bath concentration of Mg^{2+} to 19mM or by appling Tb (1.5 μM).

3.4 Electrophysiological Intracelular Recordings

Except otherwise indicated, the solutions were at room temperature (22-23ºC). In some experiments the perfusion solutions were warmed up to the physiological temperature (36-37ºC). The strips (approx. 10 mm wide) were

mounted in a Perspex chamber of 3 ml capacity through which the solutions flowed continuously at a rate of 3 ml min^{-1} via a roller pump, the bath level being kept constant by suction. The solutions were changed by transferring the inlet tube of the pump from one flask to another.

The endplate potentials were recorded in the conventional way (Fatt and Katz, 1951) with intracellular electrodes filled with KCl (3 M) and of 10-20 MΩ resistance inserted in the motor endplate site (Figure 3.4.1). The reference electrode was a Ag-AgCl pellet. The nerve was sucked by a stimulator electrode coupled to a syringe and stimulated supramaximally with rectangular pulses of 20 µs duration applied every 2 s.

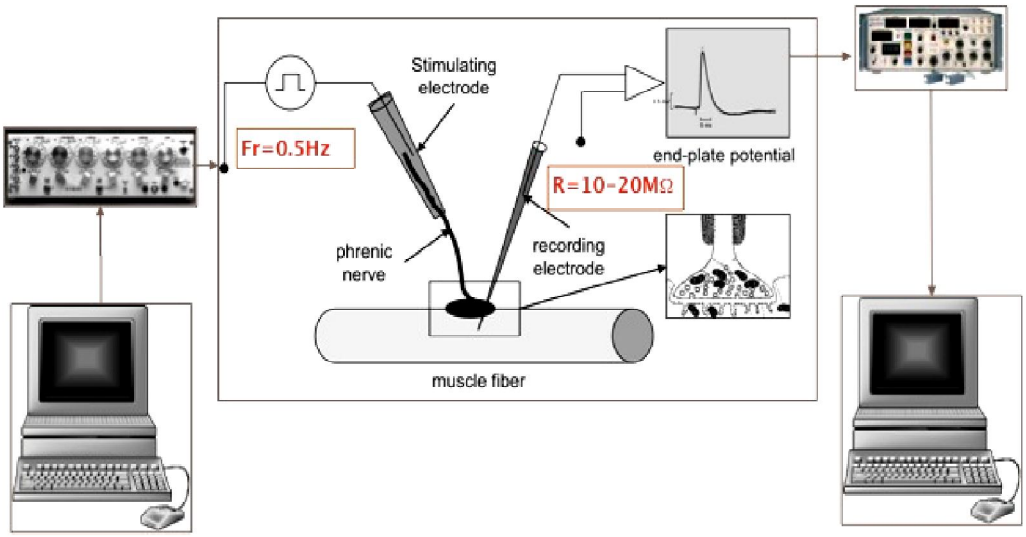


Figure 3.4.1 - Scheme of the Electrophysiological Setup and intracellular recordings (Fr – frequency of electric stimulation, R – recording electrode resistance).

3.4.1 Chemical approaches to prevent muscle contraction

High concentration of Magnesium

Magnesium is a physiologically occurring cation which blocks neuromuscular transmission in concentrations which do not prevent either nerve conduction or the muscle response to direct stimulation. This block is counteracted by increasing the calcium concentration (see Engbaek, 1952). High concentrations of magnesium decrease the amount of transmitter released at the motor nerve terminals, diminishing the depolarizing action of ACh at the end-plate and depressing the excitability of the muscle fiber membrane (Castillo and Engbaek, 1954).

Tubocurarine

The underlying event that triggers the action potential can be revealed by taking advantage of curare, an arrow poison used by some South American Indians. It is well stablished that tubocurarine reduces the amplitude of end-plate potentials by binding to ACh receptors on the postsynaptic membrane (Jenkinson, 1960; Adams, 1975; Colquhoun et al., 1979). Curare blocks the endplate potential because it is a competitive inhibitor of ACh, the transmitter released at the presynaptic terminal. Curare does not block the voltage-dependent Na^+ conductance or the voltage-dependent K^+ conductance that underlies the muscle action potential. Curare affects the stimulus which normally leads to the initiation of the muscle action potential. Thus the muscle cell doesn´t dispolarise enough to activate the voltage gated ion channels that are involved in the action potential generation. An animal that is poisoned with curare will asphyxiate because the process of neuromuscular transmission at respiratory muscles is blocked.

3.4.2 End-Plate Potentials

EPPs and MEPPs were recorded in the conventional way (Fatt and Katz, 1951), with intracellular electrodes filled with KCl (3M) and 10-20MΩ resistance, inserted in the motor end-plate. The reference electrode was an Ag-AgCl pellet. The nerve was stimulated supramaximally (rectangular pulses of 20 μs duration applied once every 2s) through a suction electrode. Individual EPPs, as well as the resting membrane potential of the muscle fibre, were continuously monitored. Muscle fibers with a resting membrane potential less negative than -60mV, were rejected. Throughout the experiment, EPPs and the resting membrane potential were continuously monitored and digitally stored on a personal computer with the Clampex® programme (pCLAMP 10 Axon Instruments, Foster City, CA). The results were later analysed off-line and each 60 consecutive EPPs were averaged. The n used in statistical analyses was number of experiments, which corresponds to a single neuromuscular junction per preparation/animal. The neuromuscular junctions from which data that were considered for further analysis was selected by the following parameters: the stability of the resting membrane potential, which could not spontaneously vary by more than 5% while assaying a drug effect, the initial value of the membrane potential, which ranged between -60mV – 80mV, and the initial amplitude of EPPs, which ranged between 2mV – 4mV. MEPPs were sampled and stored before the application of the drugs, at 16 and 30 min and in some experiments 46 min, after starting drug perfusion. Later, the samples were analysed off-line. MEPPs were detected by an event detection protocol. The threshold for detection of MEPPs was set at the level of 0.25 mV of amplitude and 2 ms of duration to prevent the contamination of the signal with electric noise. Mean MEPP frequency was measured by

counting the number of MEPPs acquired in gap free mode for 100 sec periods, at the following times: -10 min (before adding the drug), 0 min (immediately before addition) and 30 min after starting drug perfusion. The values were only considered for posterior analysis whenever MEPP frequency at -10 min and 0 min did not differ by more than 5%. The mean amplitude of MEPPs was calculated considering the average of the mean amplitude of 100 consecutive MEPPs. In the calculation of the mean amplitude of the MEPPs, the occasional “giants” (above 1mV) were neglected, since it has been shown that they do not contribute to the components of evoked release (Menrath and Blackman, 1970). A change in MEPP frequency that was not accompanied by a change in MEPP amplitude was interpreted as a change in spontaneous transmitter release (Katz, 1969). Quantal contents were estimated as the ratio of the average evoked response to the average amplitude of the MEPPs recorded during the same period.

3.5 Drugs

Table – Drugs used in the experimental work

Abbreviation	Designation	Function	Supplier	Stock solution
ADA	Adenosine deaminase (EC 3.5.4.4)	**Adenosine deamination promoting enzyme**	Roche Diagnostics Corporation (Germany)	
ADO	Adenosine	**Endogenous ligand**	Sigma-RBI (St. Louis, USA)	
AICAR	5-aminoimidazole-4-carboxamide-1-beta-d-ribofranoside	**AMPK activator**		5 mM stock solutions in DMSO*#
BDNF	Brain-derived neurotrophic factor	**Neurotrophin**	Regeneron Pharmaceuticals (Tarrytown, NY)	Supplied in 1mg/ml solution in 150 mM NaCl, 10mM Na2PO4 buffer, 0.004% Tween20*
2-CADO	2-chloroadenosine	**Stable adenosine analog**	Sigma-RBI (St. Louis, USA)	
CGS21680	2-p-(2-carboxyethyl) phenethylamino]-5´-N-ethylcarboxamido adenosine hydrochloride	**Adenosine A_{2A} receptor agonist**	Tocris (Bristol, UK)	5 mM stock solutions in DMSO*#
CPA	6-Cyclopentyladenosine	**Adenosine A_1 receptor agonist**	Sigma-RBI (St. Louis, USA)	5 mM stock solutions in DMSO*#
DPCPX	1,3-dipropyl-8-cyclopentylxanthine	**Adenosine A_1 receptor antagonist**	Tocris (Bristol, UK)	5 mM stock solutions in DMSO*#

Tb	d-tubocurarine chloride	**Nicotinic receptor blocker**	Sigma-RBI (St. Louis, USA)	5 mM stock solutions in water
H-89	N-(2-[-p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride	**Protein kinase inhibitor**	Sigma-RBI (St. Louis, USA)	5 mM stock solutions in DMSO*#
ITU	5´-iodotubericidin	**Adenosine kinase inhibitor**	Sigma-RBI (St. Louis, USA)	5 mM stock solutions in DMSO*
K-252a	9-hydroxy-9-methoxycarbony-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-cycloocta	**Inhibitor of tyrosine protein kinase inhibitor**	Calbiochem (La Jolla, USA)	1 mM stock solutions in DMSO*
U73122	1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione	**PLCγ inhibitor**	Tocris (Bristol, UK)	5 mM stock solutions in DMSO*
ZM241385	4-(2-[7-amino-2-(2-furly)[1,4]triazolo[2,3-a][1,3,5]triazin 5ylamino] ethyl) phenol	**Adenosine A_{2A} receptor antagonist**	Tocris (Bristol, UK)	5 mM stock solutions in DMSO*

* Aliquots of these stock solutions were kept frozen at -20ºC until used. The stock solution of ITU was kept in the dark to prevent photodecomposition. #The maximum concentration of DMSO applied to the preparations (0.02% v/v) was devoid of effect on EPPs.

3.6 Statistics

The data are expressed as mean ± SEM from *n* experiments. To allow comparisons between different experiments, the EPP amplitude, as well as the MEPP frequency or amplitude and the quantum content, were normalised, with 100% determined as the averaged values obtained during 10 min immediately before applying the test drug. The significance of the differences between means was evaluated by Student´s t test, when only two means were compared, or by one-way ANOVA followed by the Tukeys test or by the Bonferroni (as indicated) test whenever multiple comparisons were made. Values of $P<0.05$ were considered to represent statistically significant differences.

4. RESULTS

4.1 TRIGGERING OF BDNF FACILITATORY ACTION ON NEUROMUSCULAR TRANSMISSION BY ADENOSINE A_{2A} RECEPTORS.

4.1.1 Rationale

BDNF facilitates neuromuscular transmission at developing neuromuscular junctions (Boulanger and Poo, 1999a) , an action regulated by cyclic AMP (Boulanger and Poo, 1999b). In the rat hippocampus the effect of this neurotrophin is enhanced when adenosine A_{2A} receptors are activated (Diógenes et al., 2004). Since, adenosine A_{2A} receptors are present in the rat motor nerve terminals (Baxter et al., 2005), facilitate neuromuscular transmission (Correia de Sá et al., 1991) and are coupled to cyclic AMP (Correia de Sá et al., 1994), it is of interest to investigate if A_{2A} receptors could influence the action of BDNF on neuromuscular transmission at the rat neuromuscular junction. The mechanisms involved in the potential interplay between adenosine A_{2A} receptors and BDNF were also investigated. Since the actions of neurotrophins are expected to be more relevant at young ages, 3-week-old animals were used.

4.1.2 Pre-depolarization induced by high K^+ facilitates BDNF excitatory action on neuromuscular transmission through A_{2A} receptors.

BDNF (20-100 ng/ml, n=4), when applied alone at the rat diaphragm neuromuscular junction, did not influence the amplitude of EPPs within 60 min of its application (Fig. 4.1.1). To evaluate if a BDNF action could be triggered by depolarization, the depolarizing conditions used were inspired by those that

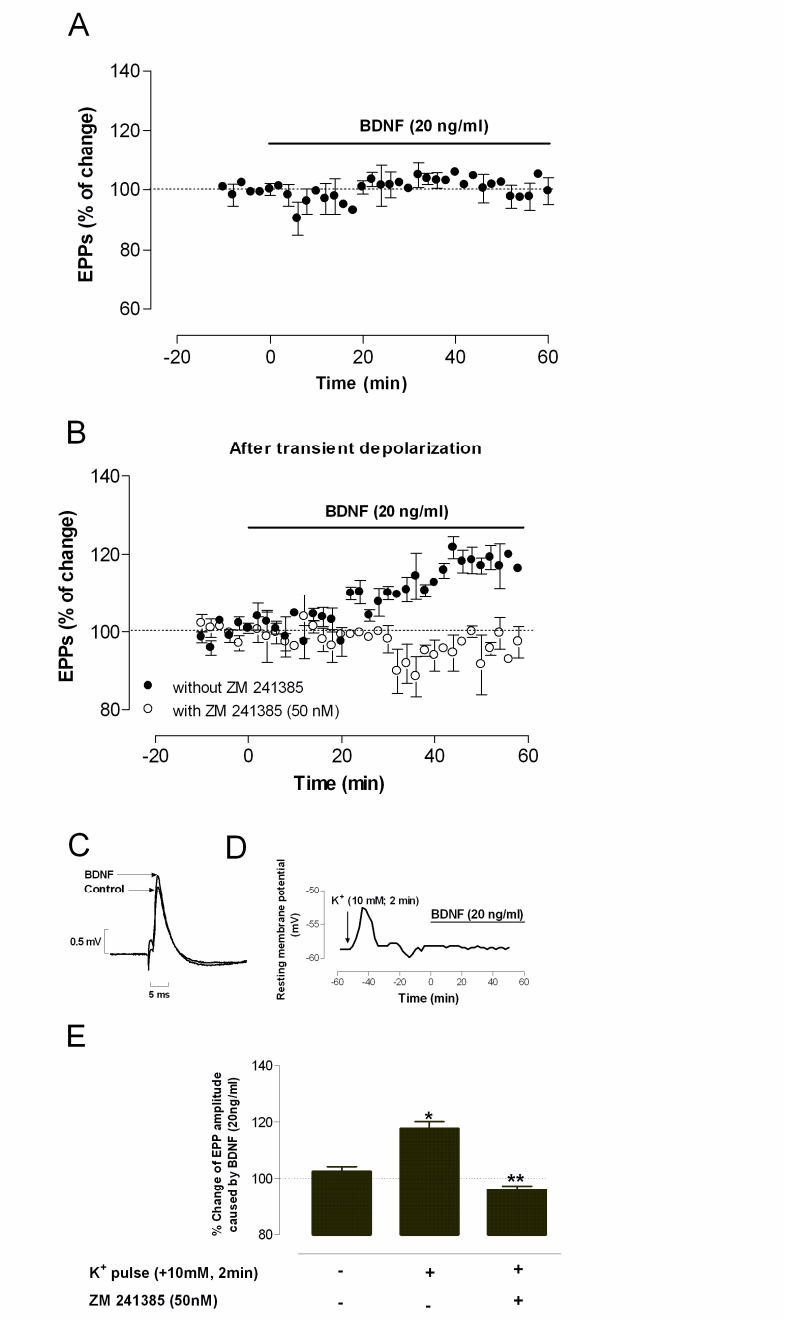
proved to trigger an excitatory action of BDNF on hippocampal synaptic transmission (Diógenes et al., 2004); so, the bathing solution was changed for 2 min by a solution where 10 mM KCl had been added, therefore the potassium concentration in the bath was transiently shifted to 15 mM. As illustrated in Fig. 4.1.1D, the membrane potential was transiently depolarized during the increase of KCl in the bath and returned to its resting level within 20 min after returning to the normal KCl concentration (5 mM) in the bath. The EPP amplitude values changed during the period of membrane depolarization but a stable value could be again recorded within 30 min after returning to the normal bathing solution. BDNF (20 ng/ml) was added 46 min after the K^+ pulse, therefore after the resting membrane potential and the amplitude of the EPPs became stable again. When applied to innervated diaphragm preparations under these experimental conditions, the neurotrophin caused an increase in EPP amplitude (18.0 ± 2.6% increase, *n*=3, $p<0.05$; Fig. 4.1.1B, E), while being virtually devoid of effect on the resting membrane potential of the muscle fiber (Fig. 4.1.1D). As also illustrated in Fig. 4.1.1, the enhancement caused by BDNF on EPP amplitude in preparations that had been pre-depolarized by the K^+ pulse was fully prevented (n=3) when A_{2A} adenosine receptors were blocked with ZM 241385, at a concentration (50 nM) that selectively blocks A_{2A} receptors in the rat (Poucher et al., 1995) and that by itself was virtually devoid of effect on EPPs (n=3).

4.1.3. The excitatory effect of BDNF on neuromuscular transmission requires PLCγ, through a mechanism that involves A_{2A} receptor and PKA activation.

To further evaluate the interplay between adenosine A_{2A} receptors and the action of BDNF at the neuromuscular junction, we investigated if the agonist, CGS 21680 at a concentration (10nM) selective for A_{2A} receptors (Jarvis et al., 1995) could trigger a BDNF action even in preparations that had not been pre-depolarized by K^+. CGS 21680 was added to the preparation at least 40 min before BDNF application and caused an increase in EPPs amplitude of 23.0 ± 4.4% (*n*=7; Fig. 4.1.2A). CGS 21680 (10nM) was not causing maximum enhancement of neuromuscular transmission since in one experiment it was observed that excitatory effect of CGS 21680 (50nM) on EPP amplitude was about twice of that observed in the same endplate with CGS 21680 (10nM). As illustrated in Fig. 4.1.2, when BDNF (20 ng/ml) was applied after the full effect of CGS 21680 (10 nM) had been observed, it induced a further enhancement (18.0 ± 1.6%, *n*=5, $p<0.05$) of the EPP amplitude. Both the effect of CGS 21680 (10 nM) as well as the effect of BDNF in the presence of CGS 21680 were prevented by the A_{2A} receptor antagonist, ZM 241385 (50 nM, n=3) (Fig. 4.1.2). The enhancement caused by BDNF on EPP amplitude most probably involves Trk phosphorylation since it was abolished (% change caused by BDNF: -6.0 ± 5.7%, *n*=3) in the presence of the inhibitor of Trk phosphorylation, K252a (200 nM) (Berg et al., 1992), which was added to the perfusion solution approximately 25 min after CGS 21680 (10 nM) and 15 min before BDNF (20 ng/ml). BDNF preferentially activates TrkB receptors (Lewin and Barde, 1996), which are present at the neuromuscular junction (Gonzalez et al., 1999). So, the presently observed effect of BDNF is likely due to TrkB

receptor activation. K252a (200 nM) did not influence ($p>0.05$) the excitatory effect of CGS 21680 (10 nM) on EPP amplitude either when applied before (n=3) or after (n=3) the A_{2A} receptor agonist.

Figure 4.1.1. Depolarization triggers an excitatory action of BDNF on transmission at the rat innervated diaphragm, an action requiring co-activation of adenosine A_{2A} receptors. In (A) and (B) are shown the time course of the changes in EPP amplitude induced by BDNF (20 ng/ml) when applied to preparations that had not been pre-depolarized (A) or to preparations that had been depolarized with 10 mM KCl for 2 min 46 min before BDNF application (B) in the absence (•) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM). Panel (C) shows superimposed traces of averaged e.p.ps recorded from one experiment immediately before (control) and 60 min after addition of BDNF (20 ng/ml). In panel(D) are shown the time course of changes in the resting membrane potential recorded from the same experiment as in (C); the application of the K^+ pulse is indicated by the arrow and that of BDNF by the horizontal bar. Panel (E) shows the averaged increase in EPP amplitude induced by BDNF (20 ng/ml) under the experimental conditions indicated below each column. The change of e.p.ps amplitude caused by BDNF in predepolarized preparations (middle column) was significantly different (*$P<0.05$; ANOVA one way analysis of variance followed by Bonferroni multiple comparisons test) from the effect of BDNF applied alone (left column) as well as from the effect of BDNF when applied in predepolarized preparations superfused with ZM 241385 (right column, **$P<0.05$). 100% in A, B and E represents the averaged amplitude of EPPS recorded for 10 min immediately before starting BDNF perfusion, which was 1.4 ± 0.1mV, n=4 (A, and E left colum), 1.4 ± 0.2mV, n=3 (B •, and E middle colum) and 1.5 ± 0.2mV, n=3 (B o, and E right column). Resting membrane potential in all experiments at time zero (i.e. before BDNF) ranged from -52 to -65 mV.


A
EPPs (% of change)
BDNF (20 ng/ml)
Time (min)
B
After transient depolarization
EPPs (% of change)
BDNF (20 ng/ml)
without ZM 241385
with ZM 241385 (50 nM)
Time (min)
C
BDNF
Control
0.5 mV
5 ms
D
Resting membrane potential (mV)
K+ (10 mM; 2 min)
BDNF (20 ng/ml)
Time (min)
E
% Change of EPP amplitude caused by BDNF (20ng/ml)
K+ pulse (+10mM, 2min)
ZM 241385 (50nM)

Adenosine A_{2A} receptors in most preparations, including the rat neuromuscular junction (Correia de Sa and Ribeiro, 1994) are coupled to adenylate cyclase. We therefore evaluated how a PKA inhibitor, H-89 Chijiwa et al., 1990), would influence the actions of CGS 21680 and BDNF on neuromuscular transmission. H-89 (1μM) was added 30 min before CGS 21680 (10 nM) and prevented ($P<0.05$) the excitatory effect of the A_{2A} receptor agonist on EPP amplitude (% change caused by CGS 21680 in the perfusion of H-89: 3.0 ± 3.2%, n=3). As illustrated in Fig. 4.1.2E, the enhancement of EPP amplitude caused by BDNF in the presence of CGS 21680 (10 nM) was also prevented ($P<0.05$, n=3) by H-89 (1 μM); indeed, in the presence of H-89 + CGS 21680, BDNF even slightly decreased (% change: - 8 ±0.4%, n=3, $p<0.05$) rather than increased EPP amplitude.

The phosphorylated TrkB receptor serve as protein-interaction sites for adapter protein SCH, phosphotidylinositol-3 kinase (PI-3K) and phospholipase C-γ (PLC- γ) (Segal and Greenberg, 1996). BDNF slow actions, i.e. related to its influence on gene expression and leading to neuronal survival, are probably mediated through SHC (Messaoudi et al., 2002) and the PI-3K (Franke et al., 1995). In contrast, BDNF fast actions are usually related to the recruitment of PLC- γ, which induces the cleavage of phosphotidylinositol-4,5-biphosphate to generate inositoltriphosphate (IP3) and diacylglycerol (DAG), therefore inducing the release of Ca^{2+} from internal stores and promoting protein kinase C (PKC) activation (Johnson, 1999). In the present work the PLCγ inhibitor, U73122 (5 μM) Bleasdale et al., 1990), added 20 min before CGS 21680 (10nM), did not influence ($P>0.05$) the excitatory action of the A_{2A} receptor agonist on EPP amplitude (% change caused by CGS 21680 in the presence of U73122: 26.0 ± 9.2%, n=3), but abolished ($P<0.05$, n=3) the

enhancement of EPP amplitude caused by BDNF (10 ng/ml) in the presence of CGS 21680 (10 nM) (Fig. 4.1.2E).

4.1.4. Discussion

The present results clearly show that pre-depolarizing conditions and A_{2A} receptor activation trigger an excitatory action of BDNF on neuromuscular transmission at the rat innervated diaphragm. The facilitation of the excitatory effect of BDNF caused by depolarization can result from the following mechanisms: (1) recruitment of TrkB receptors to the plasma membrane since in cultures of spinal motor neurons it was reported that within minutes of depolarization, there is a rapid recruitment of TrkB receptors to the plasma membrane by translocation from intracellular stores (Meyer-Franke et al., 1998); (2) increase in cyclic AMP levels, since it was demonstrated that the catalytic unit of adenylate cyclase is sensitive to the membrane potential, being active under depolarization condition (Reddy et al., 1995); it is also known that cyclic AMP can induce expression of mRNA for TrkB receptors and neurotrophin in primary astroglial cultures (Condorelli et al., 1995); (3) increase in extracellular adenosine levels (Latini and Pedata, 2001), which in turn can activate A_{2A} membrane receptors and induce an increase in cyclic AMP levels. Depolarization triggers adenosine release (Pazzagli et al., 1993) and we found that the action of BDNF after a pre-depolarizing pulse was prevented when adenosine A_{2A} receptors are blocked.

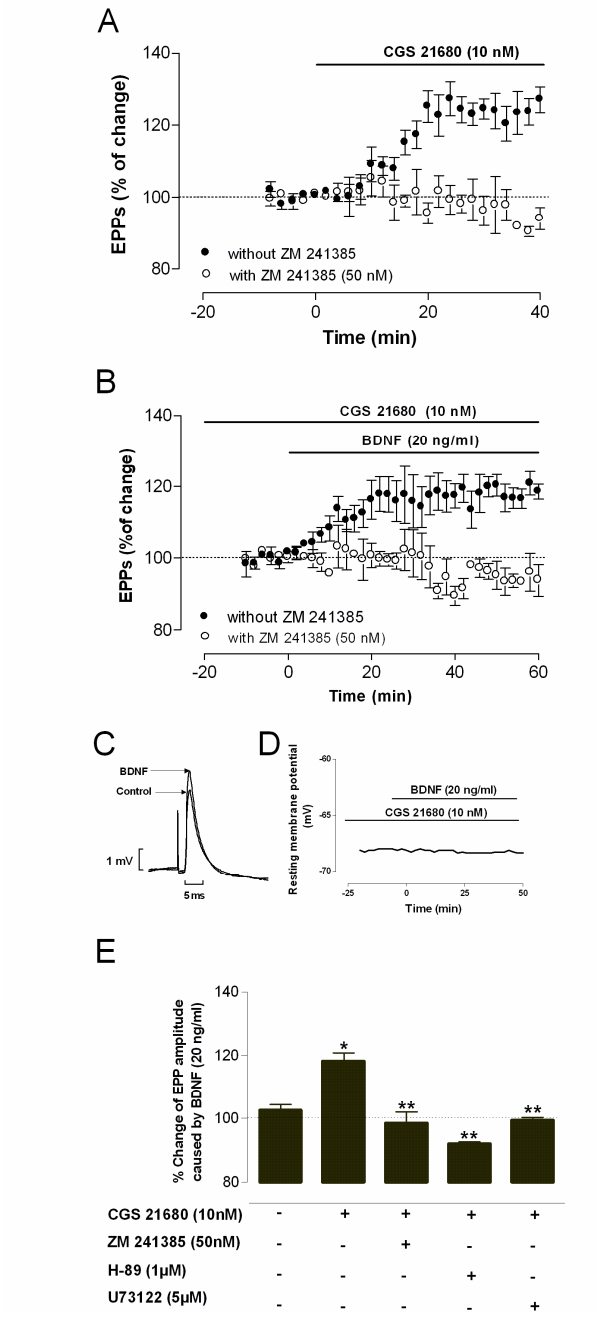

A
CGS 21680 (10 nM)
EPPs (% of change)
without ZM 241385
with ZM 241385 (50 nM)
Time (min)
B
CGS 21680 (10 nM)
BDNF (20 ng/ml)
EPPs (%of change)
without ZM 241385
with ZM 241385 (50 nM)
Time (min)
C
BDNF
Control
1 mV
5 ms
D
BDNF (20 ng/ml)
CGS 21680 (10 nM)
Resting membrane potential (mV)
Time (min)
E
% Change of EPP amplitude caused by BDNF (20 ng/ml)
CGS 21680 (10nM)
ZM 241385 (50nM)
H-89 (1μM)
U73122 (5μM)

Figure 4.1.2. The excitatory effect of BDNF on EPPs amplitude at the rat diaphragm neuromuscular junction requires PLCγ, through a mechanism that involves A_{2A} receptor and PKA activation. In (A) and (B) are shown the time course of the changes in EPP amplitude induced by CGS 21680 (10nM) and BDNF (20 ng/ml), respectively, when applied to preparations in the absence (•) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM). Panel (C) shows superimposed traces of averaged EPPs recorded from one experiment immediately before (control) and 60 min after addition of BDNF (20 ng/ml). In panel (D) are shown the time course of changes in the resting membrane potential recorded from the same experiment as in (C). Panel (E) shows the averaged increase in EPP amplitude induced by BDNF (20 ng/ml) under the experimental conditions indicated below each column. The change of EPPs amplitude caused by BDNF in the presence of CGS 21680 (10nM) (second column) was significantly different (*$p<0.05$; ANOVA one way analysis of variance followed by Bonferroni multiple comparisons test) from the effect of BDNF applied alone (first column), an effect prevented (**$P<0.05$) by: the selective adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM) (third column); the PKAs inhibitor, H-89 (1μM) (fourth column) or the PLCγ blocker, U73122 (5μM) (last column). 100% in (A) represents the averaged EPP amplitude recorded for 10 min immediately before addition of CGS 21680 (10nM) and in (B) to the averaged EPP amplitude recorded for 10 min immediately before addition of BDNF (20ng/ml) and was: 2.2 ± 0.4 mV, n=7 (A), 2.9 ± 0.5 mV; *n*=5 (B •, and E second column), 2.3 ± 0.5 mV, n=3 (B o, and E third column), 1.4 ± 0.1 mV, n=4 (E first column), 3.6 ± 0.2 mV, n=3 (E fourth column) and 1.7 ± 0.5 mV, n=3 (E fifth column). Resting membrane potential in all experiments at time zero (i.e. before BDNF) ranged from -58 to -71 mV.

This suggests that adenosine is a link between depolarization and BDNF actions at the neuromuscular junction. Corroborating this hypothesis is our finding that in the absence of pre-depolarizing conditions, A_{2A} receptor activation with a selective agonist can also trigger the action of BDNF on neuromuscular transmission. TrkB receptors in cultured hippocampal neurons can be activated by A_{2A} receptor agonists, even in the presence of BDNF (Lee and Chao, 2001). This transactivation of TrkB receptors is rather slow being

evident only after 2 h of incubation with CGS 21680. The triggering of the excitatory action of BDNF by CGS 21680 observed in the present work occurs faster (within 45 min after CGS 21680 application). Furthermore, the excitatory action of CGS 21680 by itself on EPPs was not influenced by K252a, indicating that CGS 21680 does not facilitate neuromuscular transmission by inducing transactivation of TrkB receptors. A common feature between our results and those by Lee and Chao (2001) is that in both cases there is a cross talk between adenosine A_{2A} receptors and TrkB receptors.

Extracellular adenosine mediates two independent actions on neuromuscular transmission: a fast and easily washable inhibitory action, mediated by A_1 receptors (Ginsborg and Hirst, 1972; Ribeiro and Sebastião, 1987; Sebastião et al., 1990) and a slower A_{2A} receptor mediated enhancement of EPPs, which as pointed out previously Correia de Sa et al., 1991) is hardly reversible. The reason for this slower and sustained adenosine A_{2A} receptor mediated action might be the transducing system operated by the receptor, which involves cyclic AMP formation and PKA activation Correia de Sa and Ribeiro, 1994) with subsequent protein phosphorylation, whereas in the case of adenosine A_1 receptors, in mammalian nerve terminals, it involves a fast inhibition of calcium entry (Ribeiro et al., 1979; Silinsky, 2004). It is therefore conceivable that the consequences of adenosine A_{2A} receptor activation extend far beyond receptor activation and adenosine removal from the synaptic cleft, whereas the consequences of A_1 receptor activation vanish shortly after dissociation of adenosine from the receptor.

Since BDNF actions are facilitated by cyclic AMP (Boulanger and Poo, 1999; Diógenes et al., 2004) and we observed that PKA inhibition prevents both the action of the A_{2A} receptor agonist as well as the effect of BDNF on

neuromuscular transmission, one can suggest that after depolarization there is an increase of the extracellular adenosine levels, which activates A_{2A} receptors coupled to adenylate cyclase leading to increase in cyclic AMP formation, followed by PKA activation, which finally facilitates the BDNF action through TrkB receptors. Whether PKA operates by inducing recruitment of TrkB receptors to the plasma membrane (Meyer-Franke et al., 1998) or by promoting the ability of TrkB receptors to recruit PLCγ, and consequently to mobilize calcium leading to enhancement of neuromuscular transmission, requires further investigation. The presently observed action of BDNF on neuromuscular transmission most probably involves PLCγ since it was prevented by a PLCγ inhibitor. Interestingly, the PLCγ inhibitor did not influence the excitatory action of the A_{2A} receptor agonist, CGS 21680 on neuromuscular transmission, indicating that PLCγ is not required for the ability of A_{2A} receptors to enhance neuromuscular transmission but is required for the facilitatory action of BDNF.

In summary, the results described in this chapter clearly indicate that there is a cross-talk between adenosine A_{2A} receptors and TrkB receptors at the neuromuscular junction, where A_{2A} receptors trigger the excitatory action of BDNF on neuromuscular transmission. This cross talk involves the cyclic AMP/PKA transducing mechanism, required for the action of A_{2A} receptors and PLCγ, required for the action of BDNF.

4.2 PREDOMINANCE OF ADENOSINE EXCITATORY OVER INHIBITORY EFFECTS ON TRANSMISSION AT THE NEUROMUSCULAR JUNCTION OF INFANT RATS

4.2.1 Rationale

It is well established that adenosine decreases the amplitude and the quantal content (q.c.) of EPPs at the neuromuscular junctions of adult rats (Ginsborg and Hirst, 1972) and frogs (Ribeiro and Walker, 1975). It is also known that in the same motor nerve ending, both inhibitory adenosine A_1 receptors and excitatory adenosine A_{2A} receptors coexist, modulating the evoked release of ACh (Correia-de-Sá et al., 1991). The way in which adenosine is able to achieve a balance in the control of neurotransmission depends on the extracellular concentration of the nucleoside, which subsequently depends on extracellular adenosine generation and inactivation (via cellular uptake and/or extracellular deamination) (Sebastião and Ribeiro, 1988). At the rat neuromuscular junction extracellular adenosine can be originated by transport-mediated release (Cunha and Sebastião, 1993), in parallel with its formation from released ATP (Smith, 1991).

Adenosine kinase (AK) is an intracellular enzyme that catabolizes adenosine to AMP and, therefore, its inhibition leads to enhancement of adenosine release into the extracelllar space (see Arch and Newsholme, 1978). While exploring the action of endogenous adenosine on neuromuscular transmission by using the adenosine kinase inhibitor (AKI), 5`-iodotubericidin (ITU), we observed, as reported in the present work, that instead of inhibition, ITU greatly facilitated neuromuscular transmission. In addition to this effect of ITU, it seemed, therefore, of interest to re-evaluate the excitatory effect of

adenosine at the rat neuromuscular junction, by using the endogenous ligand, adenosine and the stable adenosine analogue, 2-chloroadenosine (CADO). CADO has about the same affinity as the endogenous ligand for adenosine receptors (see e.g. Ribeiro and Sebastião, 1985) with the advantage of not being metabolized, allowing to evaluate its action at defined concentrations.

4.2.2 Adenosine kinase inhibition facilitates neuromuscular transmission through adenosine A_{2A} receptors activation.

Fig.1 illustrates the effect of the AKI, 5´-iodotubericidin (ITU, 10µM) on the amplitude of EPPs. As shown, 40 min after its application, ITU enhanced the amplitude of EPPs by 25 ± 6.0% (n=4, Fig. 4.2.1B) while being virtually devoid of effect on the resting membrane potential of the muscle fiber (Fig. 4.2.1A). This effect was sustained during the period of application of ITU and EPPs amplitude did not return to the baseline within 30 min after reperfusion with the normal bathing solution (Fig. 4.2.1A). At lower concentration (1 µM) ITU caused a smaller (15 ± 1.8%, n=3) increase in the amplitude of EPPs.

To evaluate if this excitatory effect could be attributed to activation of A_{2A} receptors by released adenosine, ITU (10µM) was perfused in the presence of the A_{2A} receptor selective antagonist, ZM241385 (Poucher et al., 1995). In these conditions, the excitatory effect of ITU was transformed into an inhibitory effect (-28 ± 2.4%, n=3; Fig. 4.2.1C), which can be related with A_1 receptor activation by released adenosine, since it was prevented by the adenosine A_1 receptor selective antagonist, DPCPX (10nM). Surprisingly, in the presence of DPCPX, ITU (10µM) failed to enhance neuromuscular transmission (1 ± 1.8%, n=6; Fig. 4.2.1D) indicating a possible interplay

between high affinity A_1 receptors and A_{2A} receptors. To further evaluate the interaction between these adenosine receptors, we investigated if the A_1 receptors antagonist, DPCPX (10nM), could prevent the effect of the A_{2A} receptors selective agonist, CGS21680 (Jarvis et al., 1989). As illustrated in the Fig. 4.2.2, this agonist enhanced EPP amplitude (23 ± 4.4%, n=7), an effect completely abolished (0 ± 3.2%, n=6) by the A_1 receptor antagonist, DPCPX. As expected the A_{2A} receptor antagonist, ZM241385 (50nM), also abolished the facilitatory action of CGS21680 (10nM) on EPP amplitude.

To further confirm that the action of ITU on neuromuscular transmission are due to changes in the extracellular levels of adenosine, experiments were performed in the presence of both adenosine A_1 receptor and A_{2A} receptor antagonists, DPCPX (10nM) and ZM241385 (50nM), which were added together to the preparations before addition of ITU (10 μM). Under these conditions, and as expected, ITU did not modify the amplitude of EPPs (Fig. 4.2.1E).

The above-described effects of ITU upon neuromuscular transmission suggest that by enhancing extracellular adenosine it is possible to induce a long lasting facilitation of neuromuscular transmission due to A_{2A} receptor activation. This long lasting facilitatory effect is not reverted by inactivation of extracellular adenosine with adenosine deaminase (ADA, 2U/ml), an enzyme that converts adenosine into inosine (see Arch and Newsholme, 1978); thus, in four experiments where ADA (2U/ml) was perfused after the full excitatory effect of ITU (10μM), the amplitude of EPPs did not decrease towards pre-ITU levels. However, when ADA (2U/ml) was applied before ITU (10μM), a time lag of about 10 min occurred before any facilitation of neuromuscular transmission started to be observed. After this time lag, the amplitude of EPPs

started to increase probably because the rate of adenosine production started to be faster than the rate of adenosine deamination by the concentration of ADA used (2U/ml). Higher concentrations of ADA were not tried to avoid the risk of non-enzymatically related actions (Cunha et al., 1996).

When applied alone, ADA (2U/ml), caused only a slight decrease in the amplitude of EPPs (-6.8 ± 1.8%, n=5; $P<0.05$). The selective A_{2A} receptor antagonist, ZM241385 (50nM) or the selective A_1 receptor antagonist, DPCPX (10nM), did not modify the amplitude of EPPs (3 ± 6.7%, n=5; 0 ± 5.5%, n=7, respectively).

ITU has been reported to inhibit AMP-activated protein kinase (AMPK) (Musi et al., 2001). To evaluate if inhibition of this enzyme could mimic the effect of ITU on neuromuscular transmission, we tested the AMPK activator, 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (AICAR). However, this compound even at a lower concentration (300 μM) than that usually used to activate AMPK (1 mM, see Giri et al., 2004), caused a marked hyperpolarization (by about 10 mV), and therefore any changes in EPP amplitude were masked by this postsynaptic effect. When used at 1mM, AICAR caused a similar (~10 mV) membrane hyperpolarization. Since this marked hyperpolarization was not mimicked by any adenosine receptor agonist, neither by ITU, no further protocols were designed with AICAR. Membrane hyperpolarization caused by submilimolar concentrations of AICAR is probably due to changes in membrane K^+ conductance (Klein et al., 2009)

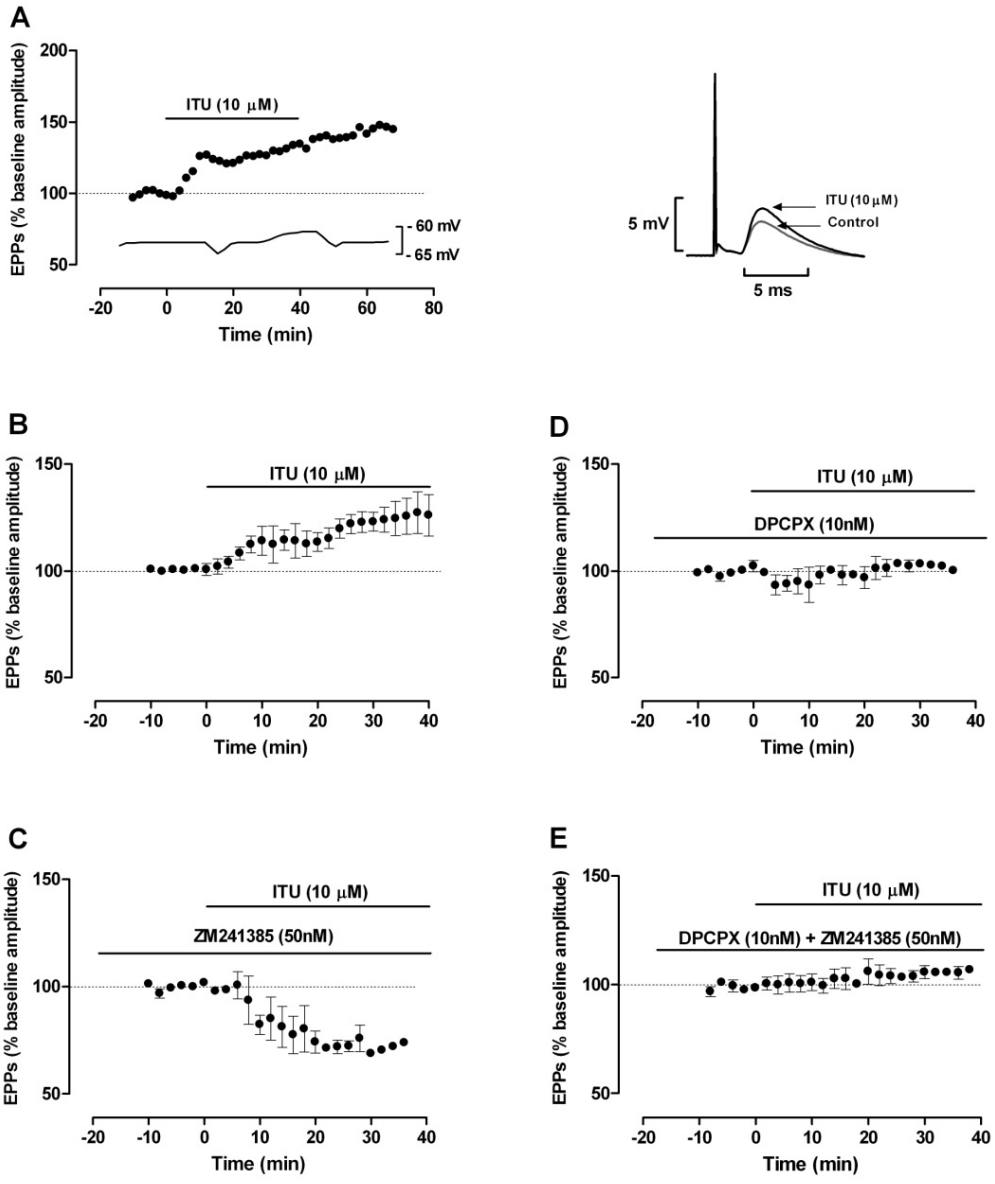

A
EPPs (% baseline amplitude)
200
150
100
50
ITU (10 μM)
- 60 mV
- 65 mV
-20
0
20
40
60
80
Time (min)
5 mV
ITU (10 μM)
Control
5 ms
B
EPPs (% baseline amplitude)
150
100
50
ITU (10 μM)
-20
-10
0
10
20
30
40
Time (min)
D
EPPs (% baseline amplitude)
150
100
50
ITU (10 μM)
DPCPX (10nM)
-20
-10
0
10
20
30
40
Time (min)
C
EPPs (% baseline amplitude)
150
100
50
ITU (10 μM)
ZM241385 (50nM)
-20
-10
0
10
20
30
40
Time (min)
E
EPPs (% baseline amplitude)
150
100
50
ITU (10 μM)
DPCPX (10nM) + ZM241385 (50nM)
-20
-10
0
10
20
30
40
Time (min)

Figure 4.2.1. Adenosine kinase inhibition influence the amplitude of evoked end-plate potentials (EPPs) recorded from a muscle fiber of the rat diaphragm. In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by the adenosine kinase inhibitor iodotubericidine, ITU (10μM), in one experiment. In the lower panel in (A), are shown superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ITU (10μM). In (B) is shown the averaged change in the amplitude of EPPs caused by ITU (10μM) in 4 experiments. In (C), (D) and (E) are shown the averaged changes in the amplitude of EPPs caused by ITU (10μM), when it was applied (C) in the presence of the selective antagonist of adenosine A2A receptors, ZM 241385 (50nM), (D) in the presence of the selective antagonist of adenosine A1 receptors, DPCPX (10nM) and (E) in the presence of the co-administrated selective antagonist of adenosine A1 receptors, DPCPX (10nM) with the selective antagonist of adenosine A2A receptors, ZM 241385 (50nM). In the ordinates, 100% represents the averaged amplitude of EPPs recorded for 10 min immediately before starting ITU perfusion, which was 3.2mV, n=1 (A); 3.9 ± 0.7mV, n=4 (B); 3.7 ± 1.2mV, n=3 (C); 3.2 ± 1.4mV, n=4 (D), 1.3 ± 2mV. Each point represents the averaged amplitude of 60 successive EPPs. The horizontal bars indicate the period of drugs perfusion. Resting membrane potential in all experiments at time zero (i.e. before ITU addition) ranged from -60 to -72mV. Solutions contained a submaximal concentration (1.5 μM) of tubocurarine, which decreased EPP amplitude below threshold of action potential generation therefore preventing muscle twitches in response to nerve stimulation.

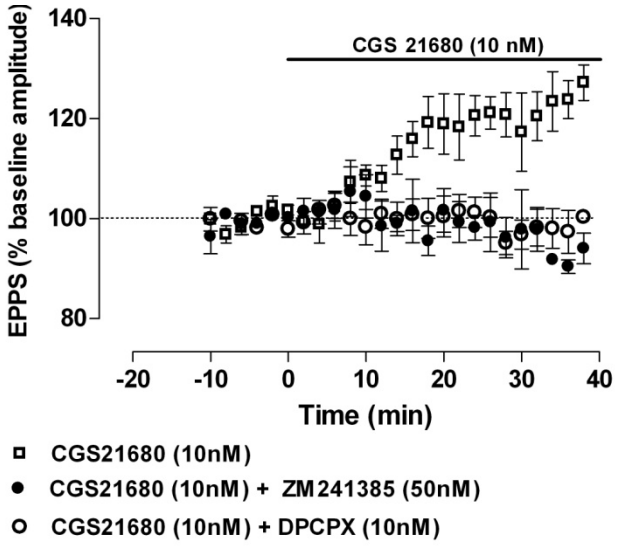


Figure 4.2.2. The selective A2A receptor agonist enhances the amplitude of end plate potentials (EPPs), an action blocked by either A1 or A2A receptor selective antagonists. The time course of the changes in EPP amplitude induced by the A2A receptor agonist, CGS 21680 (10nM), when applied to preparations in the absence (£), in the presence (o) of the selective adenosine A1 receptor antagonist, DPCPX (10nM), or in the presence (•) of the selective adenosine A2A receptor antagonist, ZM241385 (50nM) is shown. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CGS 21680 (10nM): 2.2 ± 0.4 mV, n=7 (£); 2.5 ± 0.6 mV, n=4 (○); 1.6 ± 0.5 mV, n=6 (•). Resting membrane potential in all experiments at time zero (i.e. before CGS 21680 addition) ranged from -60 to -72 mV. Perfusing solutions contained tubocurarine (1.5µM).

4.2.3 At submicromolar concentrations, adenosine enhances both evoked and spontaneous neuromuscular transmission.

As seen in Fig. 4.2.3, adenosine (300nM) enhanced neuromuscular transmission by 38 ± 6.8% (n=5), an effect that was not diminished in the next 30 minutes after stopping adenosine perfusion (Fig. 4.2.3). This excitatory effect of a submicromolar concentration of adenosine was consistently observed (Fig. 4.2.3B) and was antagonized by the selective A_{2A} receptor antagonist, ZM241385 (50nM, Fig. 4.2.3C). When concentrations of adenosine above 10µM were applied to the preparations, the amplitude of EPPs clearly decreased, in accordance with what has been previously described (see Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). To evaluate if adenosine A_{2A} receptors were fully activated in the presence of adenosine concentrations that induced a decrease in the amplitude of EPPs, we applied the selective A_{2A} receptor agonist, CGS21680, to the bath solution after the full inhibitory effect of adenosine (25µM) was observed. In these conditions CGS 21680 induced an increase in the amplitude of EPPs (Fig. 4.2.3D), indicating that adenosine (25µM) might not be able to completely activate A_{2A} receptors or that CGS 21680 is more efficient than adenosine itself to attenuate A_1 receptor functioning. Since CGS 21680 has higher selectivity and affinity for A_{2A} receptors than adenosine itself, it is plausible that it more efficiently attenuates A_1 receptor mediated inhibition (Cunha et al., 1994) than the endogenous agonist, adenosine, which activates both A_1 and A_{2A} receptors.

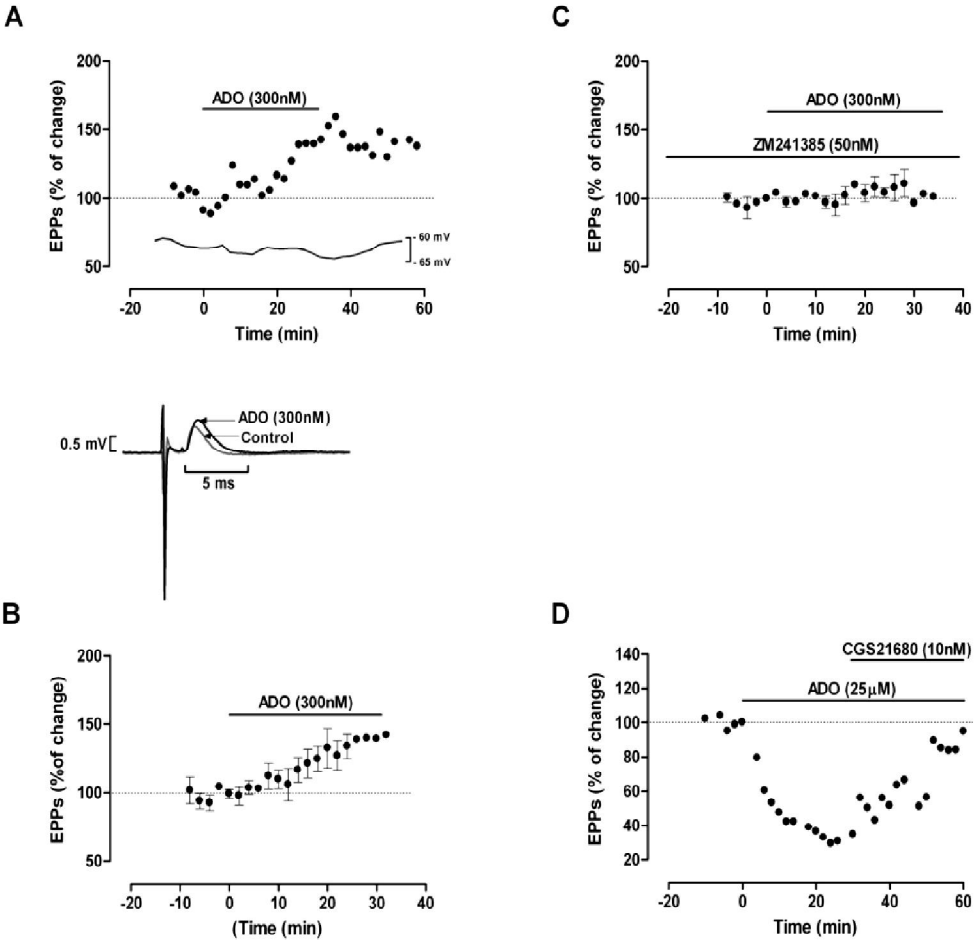

A
EPPs (% of change)
ADO (300nM)
60 mV
65 mV
Time (min)
0.5 mV
ADO (300nM)
Control
5 ms
B
EPPs (%of change)
ADO (300nM)
(Time (min)
C
EPPs (% of change)
ADO (300nM)
ZM241385 (50nM)
Time (min)
D
EPPs (% of change)
CGS21680 (10nM)
ADO (25μM)
Time (min)

Figure 4.2.3. At submicromolar concentrations, adenosine enhances the amplitude of evoked end-plate potentials (EPPs). From (A) to (C) are shown the time course of the changes in EPP amplitude induced by ADO (300nM) when applied to preparations in the absence (A, B) or in the presence (C) of the selective adenosine A_{2A} receptor antagonist, ZM 241385 (50nM). In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by ADO (300nM) in one experiment. In the lower panel, in (A), are shown superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ADO (300nM). In (D) is shown the effect of the selective agonist of adenosine A_{2A} receptors, CGS 21680, when applied in the presence of a concentration of ADO (25µM), which markedly inhibited the amplitude of EPPs. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of the drugs indicated above the bars: 0.8mV, n=1 (A); 1.7 ± 0.3 mV, n=5 (B); 3.4 ± 0.8 mV, n=3 (C); 3.0mV, n=1 (D). Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -63 to -72 mV. Perfusing solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

Figure 4.2.4 summarizes the results from experiments where MEPPs and EPPs were recorded simultaneously with Mg^{2+} 19mM paralyzed preparations, to evaluate changes in the q.c. of EPPs. Adenosine (300nM) increased the frequency of MEPPs (n=3) by 40 ± 2.5%, without changing its average amplitude. The q.c. of EPPs in the presence of adenosine (300nM) was increased by 55 ± 9.7% (n=3). This facilitation caused by submicromolar concentrations of adenosine contrasts with adenosine´s inhibitory action at micromolar concentrations that inhibited both the MEPP frequencies and q.c. of EPPs (see Ginsborg and Hirst, 1972 and Ribeiro and Walker, 1975).

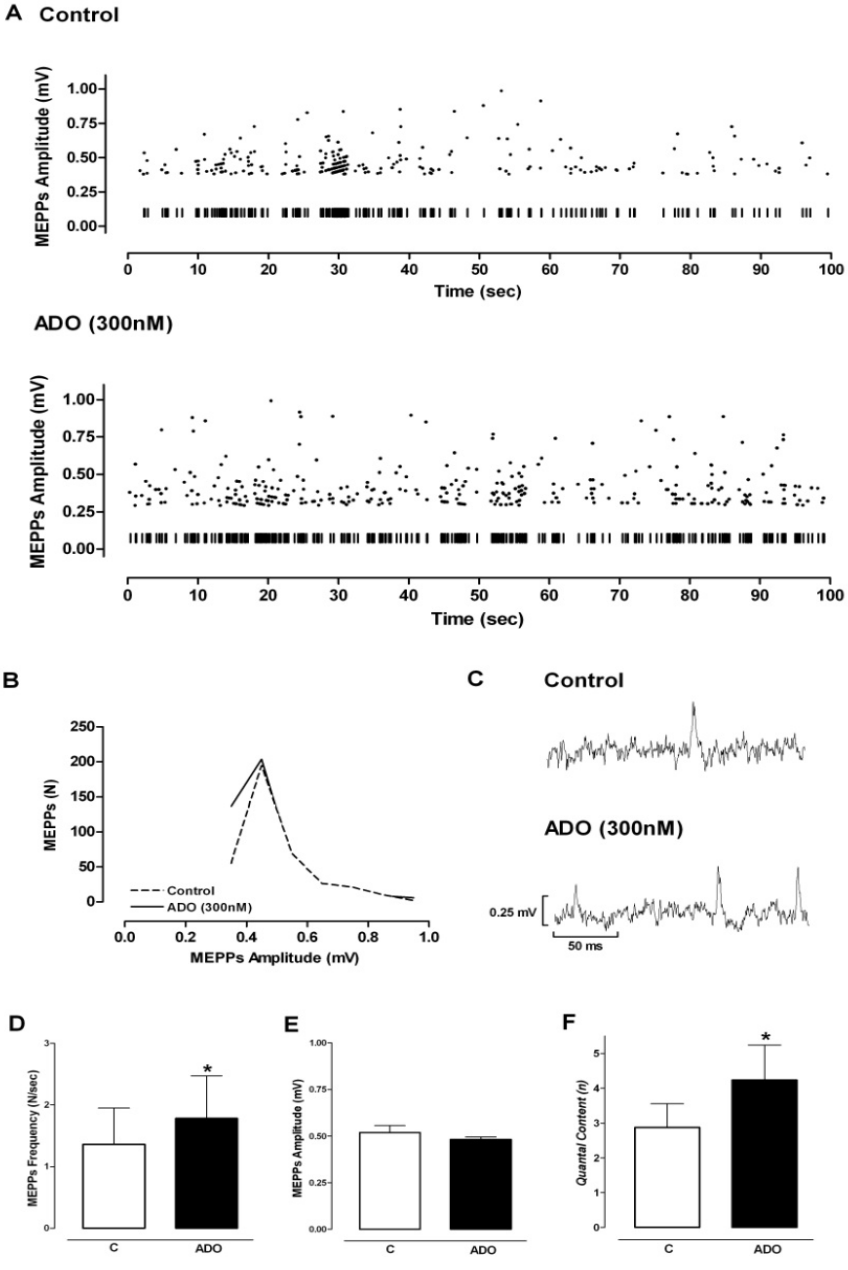

A Control
MEPPs Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
0 10 20 30 40 50 60 70 80 90 100
Time (sec)
ADO (300nM)
MEPPs Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
0 10 20 30 40 50 60 70 80 90 100
Time (sec)
B
MEPPs (N)
250
200
150
100
50
0
Control
ADO (300nM)
0.0 0.2 0.4 0.6 0.8 1.0
MEPPs Amplitude (mV)
C
Control
ADO (300nM)
0.25 mV
50 ms
D
MEPPs Frequency (N/sec)
3
2
1
0
*
C
ADO
E
MEPPs Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
C
ADO
F
Quantal Content (n)
5
4
3
2
1
0
*
C
ADO

Figure 4.2.4 – **Adenosine, at a submicromolar concentration, enhances the frequency and amplitude of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs)**. In (A) are shown the spontaneous events recorded in gap free mode across 100 seconds before (control, upper panel) and 30 min after adenosine (ADO, 300nM) perfusion (lower panel) in one experiment. Each point in (A) represents the amplitude of a single MEPP and each vertical trace immediately above the ordinates, indicates the occurrence of a MEPP. The resting membrane potentials were -62 mV to -61mV throughout the experiment. In (B), the MEPP amplitude distribution recorded in the same experiment over the same period of time as in (A) is shown. The event detection was settled to signals with amplitude ranging from 0.35 to 1mV and minimal duration of 2 ms. In (C) are represented samples of MEPPs recordings. Panels (D to F) represent the averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of adenosine (300nM); (*P<0.05; Student´s t test). Perfusing solutions contained 19mM Mg^{2+}.

4.2.4 The effect of the stable adenosine analogue, 2-chloroadnosine, on neuromuscular transmission.

Since adenosine is subject to uptake and enzymatic inactivation, we decided to explore the action of the stable analogue, CADO, which is not a substrate for adenosine deaminase (e. g. Daly, 1982), and has low affinity for the adenosine uptake system (Jarvis et al., 1985). The concentrations of CADO required to obtain effects similar to those of adenosine were about 10 times lower. Indeed, as observed with 300nM adenosine, CADO (30nM) increased the average amplitude of EPPs (41 ± 7.8%, n=7, Fig. 4.2.5). As observed with ITU and adenosine, the effect remained for at least 20 min after starting the washing out of CADO (Fig. 5A).

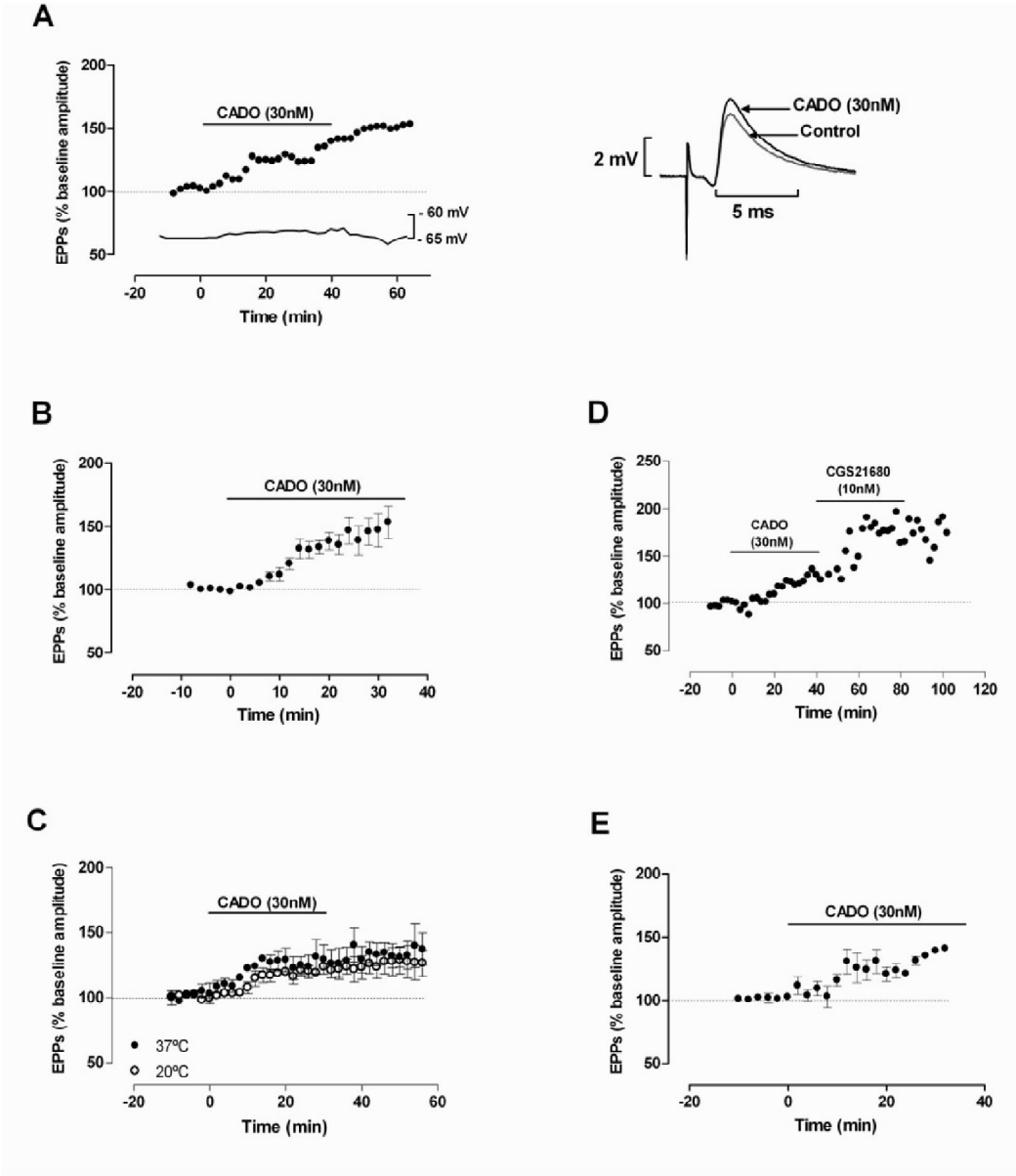
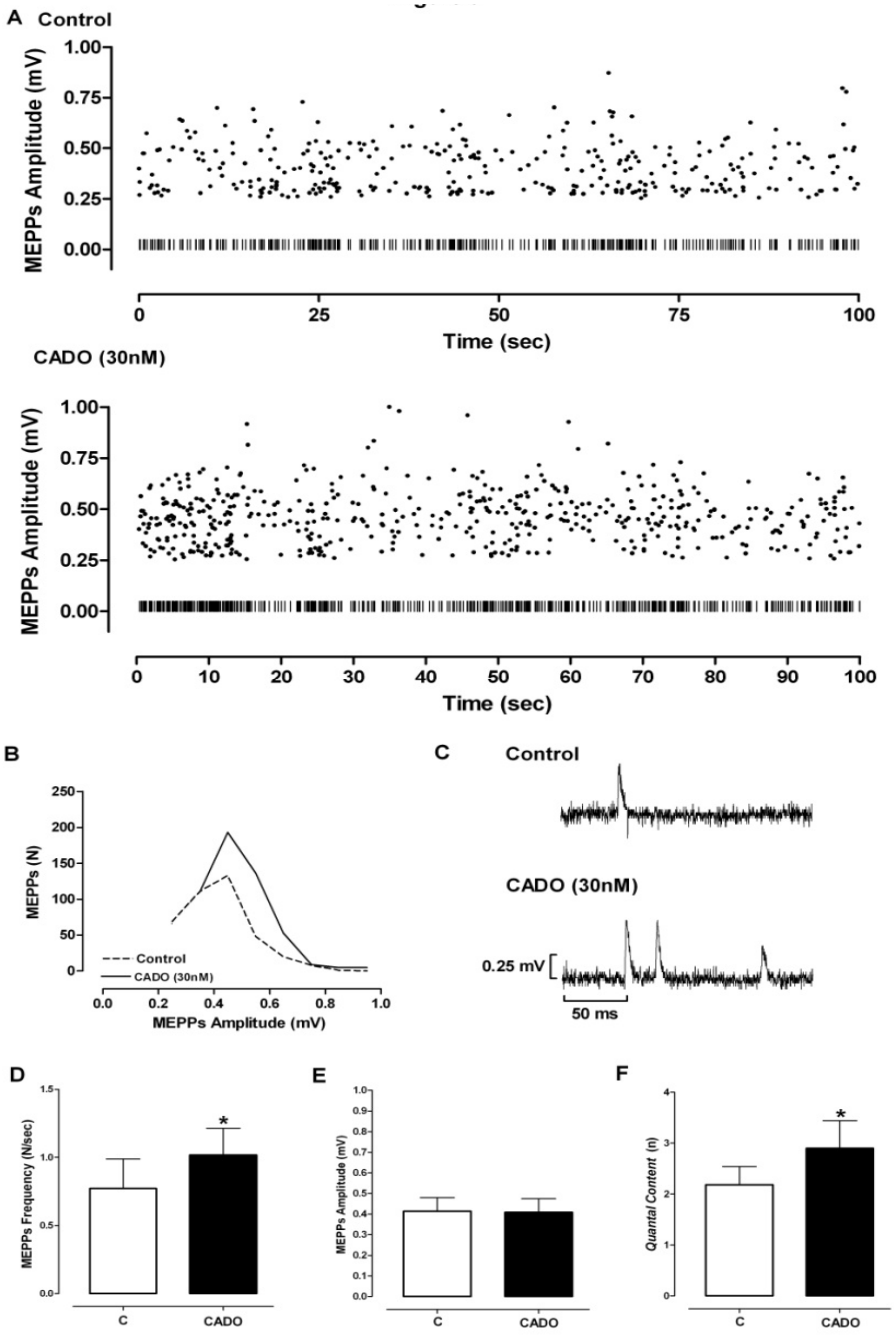

A
EPPs (% baseline amplitude)
CADO (30nM)
60 mV
65 mV
Time (min)
CADO (30nM)
Control
2 mV
5 ms
B
EPPs (% baseline amplitude)
CADO (30nM)
Time (min)
D
EPPs (% baseline amplitude)
CGS21680 (10nM)
CADO (30nM)
Time (min)
C
EPPs (% baseline amplitude)
CADO (30nM)
37ºC
20ºC
Time (min)
E
EPPs (% baseline amplitude)
CADO (30nM)
Time (min)

Figure 4.2.5. At submicromolar concentrations, 2-chloroadenosine (CADO) enhances the amplitude of evoked end-plate potentials (EPPs). In (A) is shown the time course of the changes in EPP amplitude and in the resting membrane potential caused by CADO (30nM) in one endplate paralyzed with tubocurarine (1.5 µM). In the lower panel in (A) are showed superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after addition of CADO (30nM). In B and E are shown the averaged changes in the amplitude of EPPs caused by CADO (30 nM) in experiments where muscle twitches were prevented by 1.5 µM tubocurarine (B) or 19 mM Mg^{2+} (E). In (C) is shown the time course of the averaged changes in EPP amplitude induced by CADO (30nM) in experiments performed at room temperature (o) and at a more physiological (37ºC) temperature (•); muscle twitches were prevented with tubocurarine (1.5 µM). In (D) is shown the effect of the selective adenosine A_{2A} receptor agonist when applied after the full excitatory effect of CADO (30nM); muscle twitches were prevented with tubocurarine (1.5 µM). Each point, in all panels, represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30nM): 3.7mV, n=1 (A); 2.4 ± 0.3 mV, n=7 (B); 2.0 ± 0.5 mV, n=3 (C); 2.0mV, n=1 (D); 1.1 ± 0.1 mV, n=3 (E) . Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -62 mV to -76 mV.

Most of the experiments now reported have been performed at room temperature (~22ºC) to minimize electrical noise and to increase biological viability, compatible with long lasting protocols. However, to evaluate if the unexpected long lasting excitatory effect of low concentrations of an adenosine receptor ligand, such as CADO, could be influenced by temperature, four experiments were performed at 36ºC and the results compared with those obtained in similar experiments performed at room temperature with similar periods of CADO application and washout. As

illustrated in Figure 4.2.5C, the facilitatory effect of CADO (30 nM) upon EPP amplitude was not influenced by the bath temperature, within the physiological limits.

Figure 4.2.6 – **2-Chloroadenosine, at a submicromolar concentration, enhances the frequency and amplitude of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs).** In (A) are shown the spontaneous events recorded in gap free mode across 100 seconds before (control, upper panel) and 30 min after 2-chloroadenosine (CADO, 30nM) perfusion (lower panel) in one experiment. Each point in (A) represents the amplitude of a single MEPP and each vertical trace immediately above the ordinates indicates the occurrence of a MEPP. The resting membrane potentials were -63 mV to -64.5mV throughout the experiment. In (B), the MEPP amplitude distribution recorded in the same experiment over the same period of time as in (A) is shown. The event detection was settled to signals with amplitude ranging from 0.35 to 1mV and minimal duration of 2 ms. In (C) are represented samples of MEPPs recordings. Panels (D to F) represent the averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of CADO (30nM); (*$P<0.05$; Student´s t test). Perfusing solutions contained 19mM Mg^{2+}.


A Control
MEPPs Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
0
25
50
75
100
Time (sec)
CADO (30nM)
MEPPs Amplitude (mV)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
80
90
100
Time (sec)
B
MEPPs (N)
250
200
150
100
50
0
Control
CADO (30nM)
0.0
0.2
0.4
0.6
0.8
1.0
MEPPs Amplitude (mV)
C
Control
CADO (30nM)
0.25 mV
50 ms
D
MEPPs Frequency (N/sec)
1.5
1.0
0.5
0.0
*
C
CADO
E
MEPPs Amplitude (mV)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
C
CADO
F
Quantal Content (n)
4
3
2
1
0
*
C
CADO

The excitatory effect of CADO (30nM) was observed either in the presence of tubocurarine (1.5µM; Fig. 4.2.5A, C), i.e. when the q.c. of EPPs is high, or in the presence of high Mg^{2+} (19mM; Fig. 4.2.5D), i.e. when quantal contents are low. CADO increased the frequency of MEPPs by 39 ± 12.2% (n=3; Fig. 4.2.6) without changing by more than 2% the average amplitude of MEPPs recorded in the same period. This facilitatory effect on asynchronous release was of similar magnitude in preparations where the motor nerve was stimulated once every 2 sec (39 ± 12.2%, n=3), or in the absence of stimulation (35 ± 3.2%, n=2). CADO (30nM) did not activate maximally the A_{2A} receptors, since the selective agonist of A_{2A} receptors, CGS 21680, when applied after the full effect of CADO, caused a further enhancement of the EPP amplitude (Fig. 4.2.5D).

Fig. 4.2.7A illustrates the concentration-response curve for the effects of CADO (10nM-10µM) on EPPs amplitude, which is noticeably biphasic, with excitatory effects at concentrations between 10nM and 100nM and the maximal excitatory effect being obtained with 30nM of CADO. At the concentration of 1mM, CADO did not affect the amplitude of EPPs, probably due to a balanced A_1/A_{2A} receptor activation, whereas at a higher concentration, CADO (10mM) decreased the amplitude of EPPs.

The A_{2A} receptor antagonist ZM241385 (50nM) prevented the excitatory effect of CADO (10 to 100nM, n=6) but did not influence the inhibition of a higher concentration of CADO (10mM) (Fig. 4.2.7A). Interestingly, in the presence of the A_{2A}R antagonist ZM241385 (50nM, Fig. 4.2.7B) there was a clear inhibitory effect of CADO at concentrations (30-300nM) that in the absence of ZM241385 (50nM) caused a facilitation of neuromuscular transmission. Furthermore, in the presence of the A_{2A} receptor

antagonist, there was a clear inhibition of EPPs caused by 1µM CADO. This indicates that at these concentrations the agonist is acting upon both A_1 and A_{2A} receptors.

As shown in Fig. 4.2.7 A and C, the A_1R antagonist DPCPX (10nM) at a concentration only 20 times higher than its Ki value for A_1 receptors in the same preparation (Sebastião et al., 1990) and well below its Ki value for A_{2A} receptors (see Jacobson et al., 1992), greatly attenuated both the excitatory and the inhibitory effects of CADO (10nM to 10µM).

Previous studies on the inhibitory effect of adenosine or CADO on EPPs were performed in adult rats. To allow comparisons and to evaluate whether the now described excitatory action also occurs in adult rats, we tested the effect of CADO (30nM) in 12-16 weeks old rats and, as shown in figure 4.2.8, it increased the amplitude of EPPs by 11 ± 1.0% ($P<0.05$, n=6). This effect was smaller ($P<0.05$) than the one observed in infant rats but it can also be attributed to A_{2A} receptor activation since it was prevented by the selective A_{2A} receptor antagonist, ZM241385 (50nM, Figure 4.2.8B). As it occurred in infant rats, the excitatory action of 30nM CADO was also prevented by the A_1R antagonist, DPCPX as well as by the A_{2A} receptor antagonist ZM241385 (50nM, Figure 4.2.8).

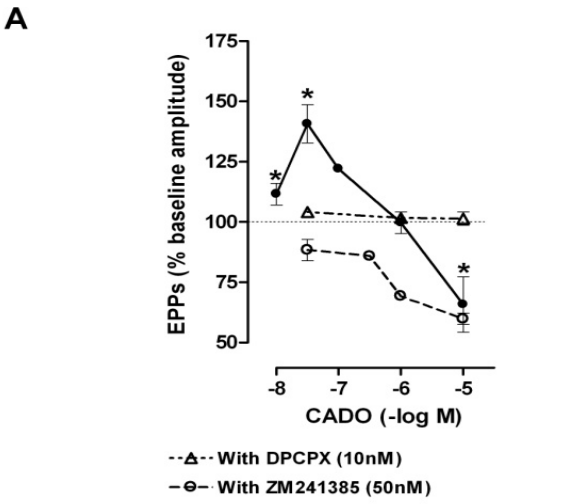

A
EPPs (% baseline amplitude)
175
150
125
100
75
50
-8
-7
-6
-5
CADO (-log M)
With DPCPX (10nM)
With ZM241385 (50nM)
B
EPPs (% baseline amplitude)
ZM 241385 (50nM)
CADO (μM)
0.03
0.30
10.0
140
120
100
80
60
40
-20
0
20
40
60
80
100
Time (min)
C
EPPs (% baseline amplitude)
DPCPX (10nM)
CADO (μM)
0.01
0.03
0.10
0.30
1.00
10.0
150
100
50
-30
0
30
60
90
120
150
180
Time (min)

Figure 4.2.7. Biphasic influence of CADO on endplate potentials (EPPs) amplitude. In (A) is shown the concentration-response curve for the effect of chloroadenosine (CADO) on the amplitude of EPPs. Each point is the average ± SEM of results obtained in 3-5 non cumulative experiments, except for CADO 100nM (●) where n=1, and for CADO 300 nM (o), which was applied after 30 nM CADO. *P<0.05 (Student´s t test) as compared with absence of CADO (100%). In (B) is shown the time course of the changes in EPP amplitude induced by CADO (30nM - 10µM) in one experiment where it was applied in the presence of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50nM); note that under this condition submicromolar concentrations of CADO caused an inhibition of EPPs. In (C), is shown the time course changes in EPP amplitude caused by CADO applied in the presence of the selective antagonist of A_1 receptors, DPCPX (10nM); note that under this condition CADO caused little or no effect on EPP amplitude. Averaged changes in the EPP amplitude caused by 30nM or 10µM of CADO in the presence of 10nM of DPCPX (∆) or 50nM of ZM241385 (○) are shown in (A). Each point, in (B) and (C), represents the averaged amplitude of 60 successive EPPs recorded from one experiment. In all panel, 100% in the ordinates represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO: 2.29 ± 0.1mV, n=19 (A, ●); 2.21 ± 0.3mV, n=14 (A, ○); 2.43 ± 0.4mV; n=12 (A, ∆); 2.4mV, n=1 (B); 2.1mV, n=1 (C). Perfusion solutions contained tubocurarine (1.5µM).

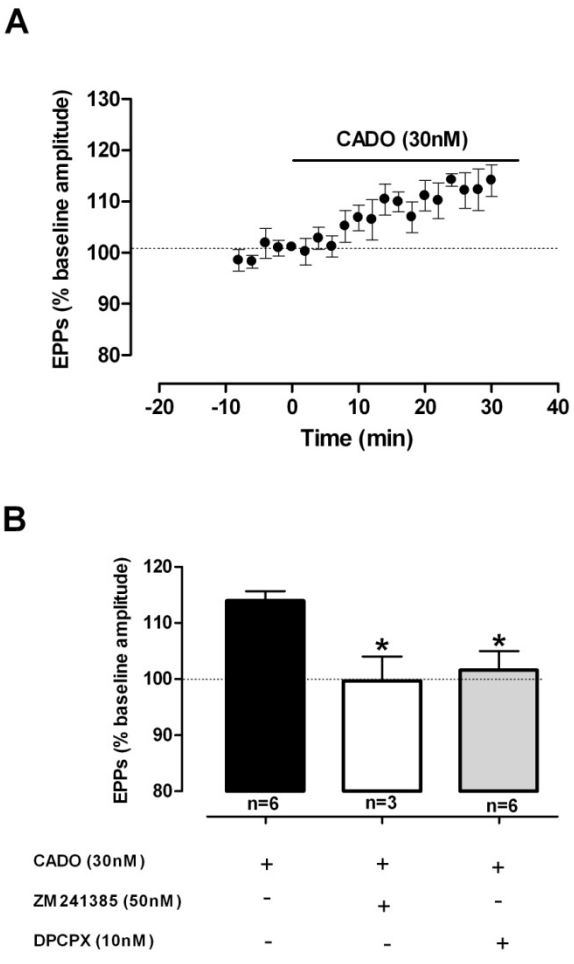

A
EPPs (% baseline amplitude)
130
120
110
100
90
80
CADO (30nM)
-20
-10
0
10
20
30
40
Time (min)
B
EPPs (% baseline amplitude)
120
110
100
90
80
*
*
n=6
n=3
n=6
CADO (30nM)
+
+
+
ZM 241385 (50nM)
-
+
-
DPCPX (10nM)
-
-
+

Figure 4.2.8. 2-Chloroadenosine (CADO, 30nM) also enhances the amplitude of evoked end-plate potentials (EPPs) in adult (12-16 weeks old) rats. In (A) is shown the time course of the changes in EPP amplitude induced by CADO (30nM); each point represents the averaged amplitude of 60 successive EPPs. Panel (B) shows the averaged changes in EPP amplitude induced by CADO (30nM) under the experimental conditions indicated below each column. Note that the increase in EPPs amplitude caused by CADO (30nM) alone (first column from left) was prevented by the selective adenosine A_{2A} receptor antagonist, ZM 241385 (50nM) (second column), and by the selective adenosine A_1 receptor antagonist, DPCPX (10nM) (third column),*P<0.05 (ANOVA one way analysis of variance followed by Tukeys multiple comparisons test) as compared with first column. 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30nM) and was 2.3 ± 0.4mV, n=6 (A and first column in B); 2.9 ± 1.3mV, n=4 (second column in B) and 2.3 ± 1.1mV, n=6 (third column in B). Resting membrane potential in all experiments at time zero (i.e. before drug addition) ranged from -62 mV to -75 mV. Perfusion Solutions contained 19mM Mg^{2+}.

4.2.5 Discussion

The main findings of the present chapter were that submicromolar concentrations of adenosine or CADO increase the amplitude of EPPs. These effects were clearly due to A_{2A} receptor, since they were antagonized by the selective A_{2A} receptor antagonist, ZM241385. The inhibitory effects, obtained with micromolar concentrations of adenosine or CADO, were related to activation of A_1 receptor and antagonized by the A_1R receptor antagonist, DPCPX.

The enhancement of neuromuscular transmission caused by adenosine or CADO results from an increase of the evoked release of ACh, since they increased the q.c. of EPPs without affecting the average amplitude of MEPPs recorded concomitantly. Even in the absence of electrical

stimulation, CADO increased the frequency of MEPPs, suggesting a facilitatory effect on spontaneous asynchronous release. Similar effects were observed either in experiments where the muscle twitches were prevented with tubocurarine (high q.c.) or where muscle twitches were prevented by high Mg^{2+} (low q.c.). So, the initial q.c. did not influence the facilitatory effect of adenosine and precludes the possibility that the high Mg^{2+}concentration influenced the action of adenosine or CADO, due to its ability to increase agonist binding (Yeung et al., 1985).

It is well established that adenosine kinase inhibitors, such as ITU, enhance extracellular adenosine, which activates Adenosine receptors (Kowaluk et al., 1998). The excitatory effect of ITU was similar to that observed with nanomolar concentrations of adenosine or CADO, and the excitatory effects of these drugs were antagonized by pre-incubation with the A_{2A} receptor antagonist, ZM241385, transforming those effects into inhibitory ones. This suggests that the influence of ITU, CADO and adenosine upon neuromuscular transmission results from a balance between activation of adenosine excitatory A_{2A} receptors and inhibitory A_1 receptors.

The reversibility of A_1R-mediated inhibition of neuromuscular transmission, together with absence of DPCPX effect on neuromuscular transmission, suggests that at the neuromuscular junction of infant rats, A_1 receptors are not tonically activated by endogenous adenosine. In contrast, it was not possible to wash out the A_{2A} receptors-mediated effects, which renders it difficult to evaluate if A_{2A} receptors were tonically influenced by endogenous adenosine. For slowly reversible actions, one would not expect that removing the endogenous ligand or preventing its binding to the receptor with a competitive antagonist added in the presence of the endogenous

agonist, would result in a rapid decrease in response. Since enhancement of extracellular adenosine with ITU, or A_{2A} receptors activation with the selective agonist, CGS21680, facilitates neuromuscular transmission, one may infer that the levels of extracellular adenosine under basal conditions should be low and not enough to fully saturate A_{2A} receptors.

The facilitatory effects of adenosine, CADO or even ITU were not washed out, which was unexpected because dissociation of adenosine or CADO from its receptor is known to be fast. Adenosine is metabolically unstable, being taken up by cells and substrate for several enzymes. However, reversibility of the ligand/receptor interaction or inactivation of the ligand (as it occurs with adenosine) does not necessarily imply reversibility of the effect triggered by receptor activation. A_{2A} receptors at phrenic motor nerve endings are positively coupled to AC/cAMP/PKA transducing system (Correia-de-Sá and Ribeiro, 1994). Therefore, PKA dependent phosphorylation and its consequence upon neurotransmitter release may influence synaptic transmission, although the receptors might no longer be activated by the ligand. The possibility of a long-lasting A_{2A} receptors activation is reinforced by the observation that the facilitatory action of ITU was not reversed by adding ADA after the full effect of ITU. As expected, the effect of ITU was delayed by ADA, if added before, and antagonized by pre-incubation with an A_{2A} receptor antagonist. It appears that once triggered by A_{2A} receptor activation, the ongoing intracellular mechanisms cause a long-lasting increase in synaptic strength, which may share some mechanisms (e.g. cAMP/PKA-dependent phosphorylation events) with plasticity phenomena at synapses of the central nervous system.

Blockade of A_{2A} receptors unmasked the inhibitory actions of low CADO concentrations but did not exacerbate the inhibitory action of a high concentration.. Desensitization of A_{2A} receptors cannot account for this apparent lack of effect, since the selective A_{2A} receptor agonist, CGS21680, was still able to facilitate neuromuscular transmission when applied in the presence of adenosine (25mM). It appears that as the concentration of CADO increases (nanomolar to micromolar), there is a shift from a preponderant A_{2A} receptor activation towards an apparent exclusive A_1 receptor activation. This suggests that at the motor nerve endings of infant rats, the A_{2A} receptor has higher affinity and/or higher expression levels than the A_1 receptor. Once activated, A_{2A} receptor might inhibit the actions of A_1 receptors at low A_1 receptor occupancy. With high concentrations of adenosine, A_1 receptors would become widely activated, leading to a predominance of the inhibitory effect. Whether these results fit into an $A_1R/A_{2A}R$ heterodimerization model (see Franco et al., 2008) with a main influence of the A_{2A} binding site at low receptor occupancy, and a main influence of the A_1 binding site at high occupancy conditions, could not be directly assessed in the present work. Whatever the molecular mechanisms behind the present results, they challenge the previous concept on predominant A_1 receptor activation at low agonist concentrations and A_{2A} receptor activation at higher concentrations of adenosine (see Correia de Sá and Ribeiro, 1996; see also Franco et al., 2008).

In the present chapter, the A_{2A} receptor-mediated facilitation of neuromuscular transmission was antagonized not only by an A_{2A} receptor selective antagonist but also by an A_1 receptor selective antagonist; this contrasts with the A_1 receptor mediated inhibition of neuromuscular

transmission, which was prevented by the A_1 receptor antagonist but not by A_{2A} receptor antagonist. The inhibitory effect of the A_{2A} receptor agonist, CGS21680, in micromolar concentrations at both the hippocampus (Lupica et al., 1990) and neuromuscular junction (Correia-de-Sá et al., 1991), is probably due to A_1 receptor activation. However, the presently observed excitatory effects seen with low nanomolar concentrations of CGS21680, are well bellow its Ki for A_1 receptor (see Jacboson et al., 1992). The observation that DPCPX displaces the specific binding of low nanomolar concentrations of CGS21680 at hippocampal membranes (Cunha et al., 1996) led to the suggestion that A_{2A} receptor at the hippocampus and striatum differ slightly in pharmacological characteristics. Also, the finding that facilitation of hippocampal synaptic transmission by a low nanomolar concentration of CGS21680 requires co-activation of A_1 receptor led to the proposal that the A_{2A} receptor-mediated facilitation of transmission results from attenuation of the tonic A_1 receptor-mediated inhibition (Lopes et al., 2002). Indeed, in the hippocampus, synaptic transmission is under intense tonic inhibition by adenosine that activates A_1 receptors. This was not observed with the neuromuscular junction (present work), where the A_1 receptor antagonist, DPCPX, did not affect transmission. Another explanation for the loss of A_{2A} receptor-mediated effects upon A_1 receptor blockade is that A_{2A} receptors and A_1 receptors form heteromers, as it has it happens at glutamatergic terminals (Ciruela et al., 2006). So, blockade of the A_1 receptor could induce a conformational change in the heteromer that does not allow either A_{2A} receptor agonist binding or effective coupling to the A_{2A} receptor transducing system. Indeed, heteromerization of G-protein coupled receptors may affect receptor functioning (Franco et al., 2008), since monomers and heteromers can have distinct pharmacological and signaling

properties. In addition, $A_1R/A_{2A}R$ cross talk through the intracellular transducing pathways that are downstream to receptor binding (Sebastião and Ribeiro, 2000) might also explain the need for A_1 receptor function to trigger A_{2A} receptor-mediated response at the motor nerve endings. These two possibilities are not mutually exclusive.

Previous work (Correia-de-Sá and Ribeiro,1996), also performed at the rat diaphragm neuromuscular junction, demonstrated predominance of the tonic A_{2A} receptor-mediated facilitatory effect on [^{3}H]ACh release, if high frequency stimulation (50 pulses/s) is used. Since ATP is released together with ACh (e.g. Silinsky, 1975) and is quickly degraded extracellularly to adenosine (Cunha and Sebastião, 1991), extracellular accumulation of considerable amounts of adenosine is expected due to high frequency neuronal firing. Adenosine derived from high frequency-induced ATP release preferentially activates A_{2A} receptors (Cunha et al., 1996). As we now report, at the neuromuscular junction of infant rats the concentration of adenosine that accumulates extracellularly due to adenosine kinase inhibition. We also showed that lower concentrations of adenosine are required to observe excitatory instead of inhibitory effects. This high sensitivity of A_{2A} receptors, along with the consistent inhibitory effect of micromolar concentrations of the nucleoside, explains why previous studies using concentrations of adenosine above 1 µM did not detect facilitatory actions of adenosine on EPPs (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Age related differences cannot account for the results obtained, since in adult rats the facilitatory effect of CADO (30nM) predominates over the inhibitory effect.

In summary, the results described in this chapter clearly demonstrate that both A_1 and A_{2A} receptors at the motor nerve endings

of infant rats can be activated by low extracellular concentrations of adenosine whereas at nanomolar concentrations, activation of excitatory A_{2A} receptors predominates. This challenges previous ideas that the predominant action of adenosine at the neuromuscular junction was inhibitory at low frequencies of neuronal firing (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Facilitatory actions upon spontaneous and evoked ACh release under low neuronal firing may have particular relevance at developing neuromuscular junctions, where subtle changes in synaptic levels of ACh and, therefore, of nicotinic receptor activation, might influence synaptic stabilisation.

4.3 THE EFFECT OF AGEING ON NEUROMUSCULAR TRANSMISSION

4.3.1 Rationale

The neuromuscular junction has been reported to undergo significant periods of remodeling during early development and ageing. While the remodelling of neuromuscular junction during embryogenesis is required for successful development, the remodelling during advanced age often results in changes that impair neuromuscular transmission (Delbono, 2003). The caractherization of neuromuscular transmission upon ageing was first done by Kelly (1978). Other studies were published in the eighties about this topic (for e.g. Standaert and Booher, 1981; Kelly and Robins, 1983; Banker et al., 1983), although, the results reported by that time were not consensual in what concerns to the effect of ageing on neuromuscular transmission, probably because the authors used animals from different strains with different ages. Muscle-fiber preparations, and the stimulating protocols also differed among studies. Besides that, it is well stablished that age affects differently neuromuscular transmission of different muscles, being the phrenic-nerve diaphragm the less affected (Standaert and Booher, 1981). In spite of being accepted that age affects neuromuscular transmission, differences related to the strain and animals age, tissue preparations and the stimulating conditions that were used in previous works make it difficult a clearcut-comparision of the results. Therefore, we decided to investigate the effect of ageing on neuromuscular transmission at low frequency stimulation in different ages: from infant to aged animals. Low frequency stimulation was used to evaluate changes on neuromuscular transmission per se without the influence of another variable, the fatigue of either the pre or post-synaptic component.

4.3.2 Passive electrophysiological properties

As it can be seen from Table 4.I, the mean resting membrane potential remained unchanged upon ageing as it was previously reported by Kelly (1978).

Table 4.I – Effect of ageing on muscle fiber resting membrane potential

Age group	Age (w.o.)	Mean RMP (mV) ± SE	n
Infant	3 - 4	-68,1 ± 0,74	28
Young adult	12 – 16	-69,7 ± 1,51	23
Older adult	36 – 40	-70,2 ± 1,12	29
Aged	70 - 80	-69,1 ± 1,59	21

The resting membrane potential (RMP) recorded in the different aged groups did not vary between the different age groups (P>0.05; ANOVA one way analysis of variance).

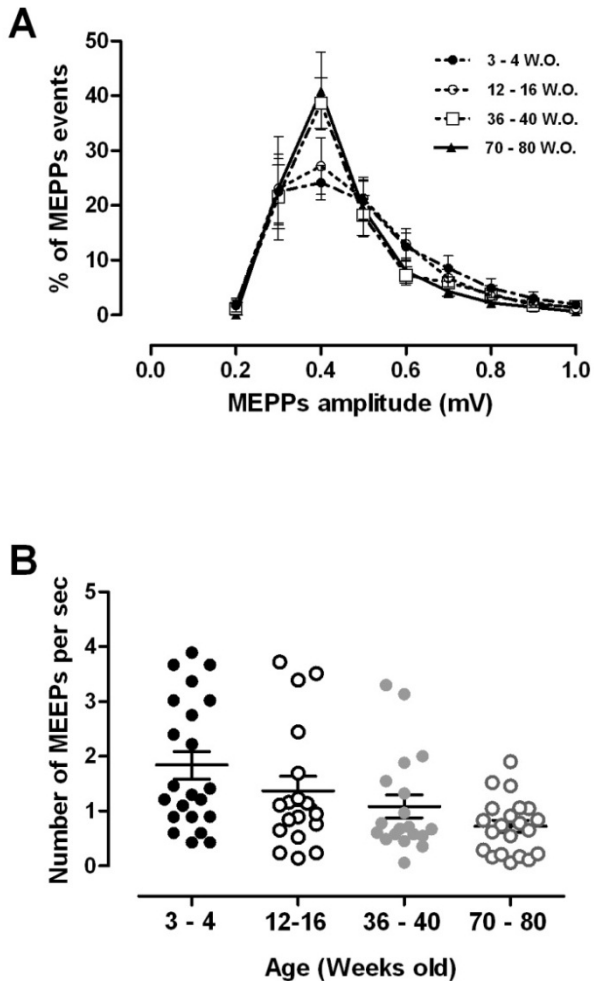
4.3.3 Spontaneous transmitter release

As summarized in table 4.II and illustrated in Fig. 4.3.1, the mean amplitude of MEPPs did not statistically vary between the different age groups (P>0.05).

There was a monotonous decrease in the frequency of MEPPs from 1,86 ± 0,26 events per sec (n=22, infant rats) to 0,72 ± 0,11 events per sec (n=21, aged rats), which represents a reduction in the frequency of MEPPs by about 61% in the aged animals as compared with infant rats (Table 4.II, Fig. 4.3.1). GMEPPs are spontaneous events with more than 1 mV of amplitude (Figure 4.3.2) and they were shown to have high incidence during development (Bennett and Pettigrew, 1974; Blackshaw and Warner, 1976;

Kullberg et al., 1977), in muscles innervated by regenerating (Miledi, 1960; Koenig and Pecot-Dechavassine, 1971; Bennet et al., 1973; Colmeus et al., 1982) or degenerating (Birks et al. 1960) nerve terminals, and in mice with motor endplate disease (Weinstein,1980). Accordingly, the frequency of GMEPPs was lower in young adult and older adult animals when compared with infant or aged animals (Table 4.II, Figure 4.3.2). The ratio between the frequency of GMEPPs and the frequency of MEPPs was calculated to investigate in which groups the probabiblity of GMEPPs was higher. As indicated in table 4.II, this value was similar in infant, young adult and older adult rats. Interestingly in aged animals the ratio between the frequency of GMEPPs and the frequency of MEPPs was significantly increased when campared with the other age groups, suggesting that, though the frequency of MEPPs is reduced in aged animals, the GMEPPs events occur more often than in the other groups (see Table 4.II).

Figure 4.3.1 – The frequency, but not the amplitude, of MEPPs diminuished with age. In (A) is shown the mean MEPP amplitude distribution recorded in in infant (●), young adult (○), older adult (□) or aged (▲) rats. In (B) is shown the average frequency of MEPPs in infant (●), young adult (○), older adult (●) or aged (○) rats. Each point in (B) represents the mean number of spontaneous events (MEPPs) per experiment, recorded in gap free mode across 100 seconds. The event detection was settled to signals with amplitude ranging from 0.35 to 1mV and minimal duration of 2 ms. The black lines represent the average frequency of MEPPs per group. Perfusing solutions contained 19mM Mg^{2+}.


A
% of MEPPs events
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
MEPPs amplitude (mV)
3 - 4 W.O.
12 - 16 W.O.
36 - 40 W.O.
70 - 80 W.O.
B
Number of MEEPs per sec
5
4
3
2
1
0
3 - 4
12-16
36 - 40
70 - 80
Age (Weeks old)

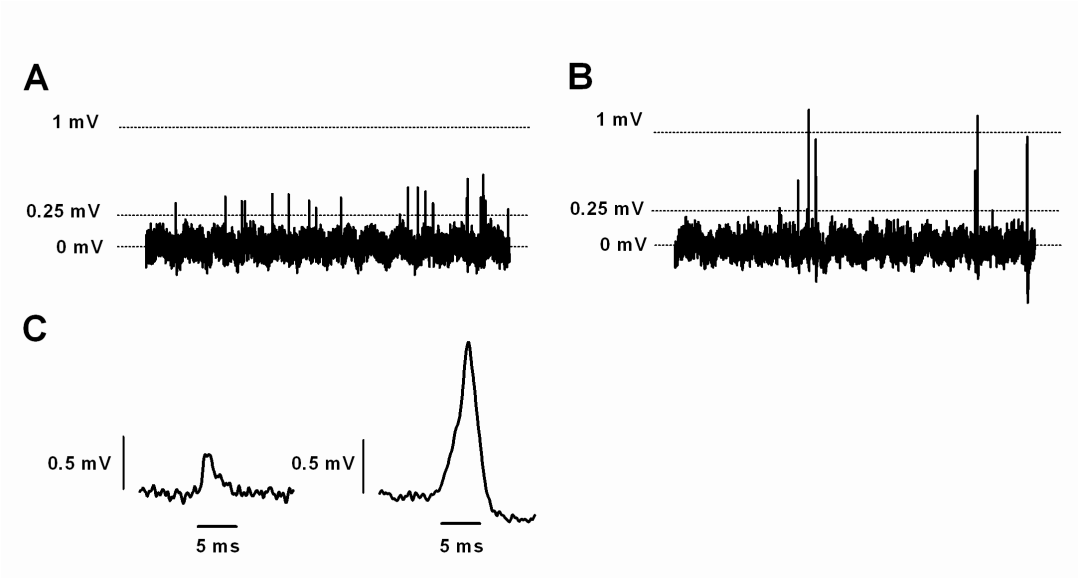


Figure 4.3.2 – Effects of ageing upon the spontaneous transmitter release. In (A) and (B) are shown the spontaneous events recorded in gap free mode across 10 seconds in young adult (A) and aged (B) rats. In (C) are shown traces of MEPPs (left side) and GMEPPs (right side). The lines in (A) and (B) indicate the threshold for detection of spontaneous events, which was set at the range of 0.25 mV – 1 mV of amplitude for MEPPs, whereas for detection of GMEPPs was set at the level of 1 mV. Perfusing solutions contained 19mM Mg^{2+}.

Table 4.II – Effect of ageing on spontaneous endplate potentials (MEPPs and EPPs).

Age group	MEPPs Frequency (s^{-1}) ± SE	GMEPPs Frequency (s^{-1}) ± SE	GMEEPs_Fr/ MEPPs_Fr	MEPPs Amplitude (mV) ± SE	n
Infant	1,86 ± 0,26	0,06 ± 0,01	0,03 ± 0,01	0,45 ± 0,02	22
Young adult	1,37 ± 0,27	0,03 ± 0,01	0,02 ± 0,01	0,41 ± 0,02	18
Older adult	1,09 ± 0,21	0,03 ± 0,01	0,03 ± 0,01	0,43 ± 0,02	19
Aged	0,72 ± 0,11*	0,05 ± 0,01	0,07 ± 0,02#	0,38 ± 0,02	21

*The mean frequency of MEPPs recorded in aged rats was significantly different (*P<0.05; ANOVA one way analysis of variance followed by Tuckey multiple comparisons test) from the mean frequency of MEPPs recorded in the other age groups. #The ratio between the number of GMEPPs within 1s (GMEPPs_Fr) the number of MEPPs within 1s in aged rats was significantly different (*P<0.05; ANOVA one way analysis of variance followed by Tuckey multiple comparisons test) from the value obtained in young adult rats.

4.3.4 Evoked transmitter release

The mean amplitude of EPPs recorded in infant, young adult and older adult rats did not differ significantly between groups (Table 4.III and Fig. 4.3.2). In aged rats, however, the mean amplitude of EPPs was reduced for about 35% (2,71 ± 0,28 mV, n=22, infant rats to 1,76 ± 0,30 mV, n=21, aged rats).

Accordingly, the quantal content of EPPs was also similar in the first three animal groups, whereas in aged rats it was decreased by around 23,1%: 6,02 ± 0,74 mV (n=22, infant rats) to 4,63 ± 1,17 mV (n=21, aged rats).

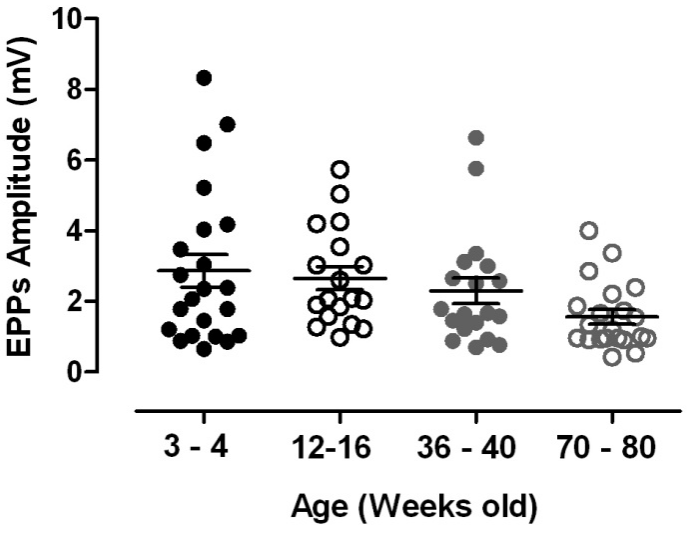


Figure 4.3.3 – Effects of ageing upon the mean amplitude of EPPs. In the figure is shown the mean EPPs amplitude distribution recorded in infant (●), young adult (○), older adult (●) or aged (○) rats. Each point represents the mean number of evokeds events (EPPs) per experiment, recorded in episodic mode across 2 min (60 sweeps). The black lines represent the average amplitude of MEPPs per group. Perfusing solutions contained 19mM Mg^{2+}.

Table 4. III – Effect of ageing on evoked neuromuscular transmission

Age group	EPPs Amplitude (mV) ± SE	EPPs Quantal Content ± SE	n
Infant	2,71 ± 0,28	6,02 ± 0,74	22
Young adult	2,56 ± 0,27	6,24 ± 0,75	18
Older adult	2,60 ± 0,37	6,04 ± 0,87	19
Aged	1,76 ± 0,30*	4,63 ± 1,17*	21

*The mean amplitude and quantal content of EPPs recorded in aged rats was significantly different (*P<0.05; ANOVA one way analysis of variance followed by Tuckey multiple comparisons test) from the mean amplitude and quantal content of EPPs recorded in the other age groups.

To evaluate the efficiency of neuromuscular transmission we analised the number of fibers where nerve stimulation failed to evoke EPPs, at least one time in a 2 min interval (60 consecutive stimulations). As documented in table 4.IV and illustrated in Figure 4.3.3, the number of fibers that presented EPP failures within 60 consecutive EPPs was different between groups. The percentage of failures was the lowest in young adult rats, indicating that the neuromuscular transmission is more efficient in this age group. Infant and aged rats have high percentage of EPP failures, 50% and 38%, respectively.

Table 4.IV – Effect of aged on evoked endplate potential failures

Age group	% Fibers with EPPs failures	Mean number of EPPs faillures per fiber ± SE	n
Infant	50	3,81 ± 1,29	22
Young adult	21	2,75 ± 0,47	18
Older adult	32	2,33 ± 0,46	19
Aged	38	1,68 ± 0,57*	21

*The mean number of EPPs failures per fiber recorded in aged rats was significantly different (*P<0.05; ANOVA one way analysis of variance followed by Tuckey multiple comparisons test) from the mean number of EPPs failures per fiber recorded in the other age groups.

4.3.5 Discussion

The main finding of the results of the present chapter was that neuromuscular transmission at the rat diaphragm remains unchanged until elderly. By that time the neuromuscular transmission became compromised. Accordingly, from a morphological point of view it is known that neuromuscular junctions are stable over long period of time, but when animals reach the age of 12-15 months, a small percentage of neuromuscular junctions starts to display some loss of motor terminal branches, an effect that is progressively more marked beyond 18-20 months of age (Balice-Gordon, 1997).

As shown in this chapter both, spontaneous and evoked neuromuscular transmission, are affected by ageing. The decrease of the mean amplitude of EPPs upon ageing is probably related to pre-synaptic events, since the average amplitude of the MEPPs did not changed significantly. In fact, the quantal content of EPPs was significantly decreased indicating that aged animals release less ACh than adult animals when

stimulated supramaximally, a result in agreement with the neurochemical finding that old rats have less ACh per terminal than mature rats (Smith and Weiler, 1987).

The raise in the ratio GMEPPs frequency/MEPPs frequency observed in aged animals is probably a consequence of age-related changes in the neuromuscular junction since it is known that GMEPPs have high incidence in muscles innervated by regenerating (Molgó and Thesleff, 1982) or degenerating (Urherwood et al., 1968) nerve terminals and in mice with motor endplate disease (Weinstein, 1980). Unlike the frequency of MEPPs, the frequency of GMEPPs is neither affected by nerve terminal depolarization, nor by changes in extracellular calcium concentration (Colméus et al., 1982; Thesleff et al., 1983). Furthermore, GMEPPs do not contribute to evoked monoquantal EPPs (Thesleff et al., 1983). GMEPPs are caused by a synchronized release of quanta of ACh triggered by Ca^{2+} release from intracellular stores (Liley, 1957; Lupa et al., 1986 and Gundersen, 1990). The increase of this multiquanta spontaneous release, nonsyncronized with presynaptic action potential might be a compensatory mechanism to the high incidence of EPP failures on aged animals.

Age-related changes at the neuromuscular junction can also be associated with alterations in axonal transport, which are known to be dramatically decreased (Gutmann and Hanzlikova, 1973), and might affect parameters such as availability of trophic factors as well as movement of organelles such as mitochondria, that are critical for neuronal survival. It is known that disruptions of mitochondria and their transport have been implicated in a wide variety of neurological diseases (Chan, 2006) and that If neurotrophin signaling is interrupted or neurotrophin expression is

suppressed, ACh receptors are dispersed, the neuromuscular junction is fragmented and affected muscles show increased fatigue (Gonzalez et al., 1999). Accordingly, ageing muscle fibers exhibit less specific force and similar endurance and recovery from fatigue than those from young-adult or middle-aged animals (González and Delbono, 2001a, 2001b).

Post-synaptic changes may not be excluded, though less significant than changes observed in the frequency of MEPPs; the mean amplitude of these spontaneous events in aged rats was slightly decreased. This effect might be related to an increase in the diameter of the fiber, since it has been found that the diameter of the fiber increases with ageing until a plateau level is reached, and it is known that MEPP amplitude is inversely related to muscle fiber diameter in mice (Rowe and Goldspink, 1969) and humans (Moore et al., 1971). On the other hand some authors showed that the synthesis and release of acetylcholine are reduced in aged animals (Gibson and Peterson, 1981; Pedata et al., 1983) and that there is a significant decline in high affinity choline uptake (Sherman and Friedman, 1990) in the brain of aged animals, which would cause a diminishment in the mean amplitude of MEPPs.

In summary, the results described in this chapter indicate that the synaptic transmission at the rat diaphragm neuromuscular junction remains stable through lifetime becoming compromised only in elderly. Future studies addressing age-related changes in the regulation and maintenance of motor nerve terminals and other neuromuscular junction components will allow the development of therapeutic strategies to attenuate, prevent, or ultimately reverse age-related muscle wasting and weakness.

4.4 NEUROMUSCULAR TRANSMISSION MODULATION BY ADENOSINE UPON AGEING

4.4.1 Rationale

It is well established that adenosine decreases the amplitude and the quantal content of evoked endplate potentials (EPPs) at the neuromuscular junctions from several species, including adult rats (Ginsborg and Hirst, 1972) and frogs (Ribeiro & Walker, 1975). It is also known that in the same motor nerve terminal, both inhibitory adenosine A_1 receptors and excitatory adenosine A_{2A} receptors coexist, modulating the evoked release of acetylcholine (Correia-de-Sá et al., 1991). The way in which adenosine is able to achieve a balance in the control of neurotransmission depends on the extracellular concentration of the nucleoside, which subsequently depends on extracellular adenosine generation and inactivation (via cellular uptake and/or extracellular deamination) (Sebastião and Ribeiro, 1988). At the rat neuromuscular junction extracellular adenosine can be originated by transport-mediated release (Cunha and Sebastião, 1993), in parallel with its formation from released adenosine triphosphate (ATP) (Smith, 1991). Recently, we described that at the motor nerve endings of infant rats low extracellular concentrations of adenosine activate both A_{2A} and A_1 receptors, but activation of facilitatory A_{2A} receptors predominates over inhibitory A_1 receptors (Pousinha et al., 2010).

In the central nervous system, the neuromodulatory action of adenosine and the expression of its membrane receptors change with age (see Sebastião and Ribeiro, 2009, Diógenes et al., 2011). So, it was considered of interest to investigate the role of adenosine at the neuromuscular junction, as a function of age, looking at the relative

importance of adenosine A_1 receptors vis a vis adenosine A_{2A} receptors. The results now reported show that excitatory A_{2A} receptor functioning at the motor nerve terminal decreases with ageing even disappearing in aged rats, whereas inhibitory A_1 receptor action is preserved throughout ageing. These age-dependent changes in the balance of excitatory over inhibitory neuromodulation may therefore contribute to the age-related decline in neuromuscular transmission.

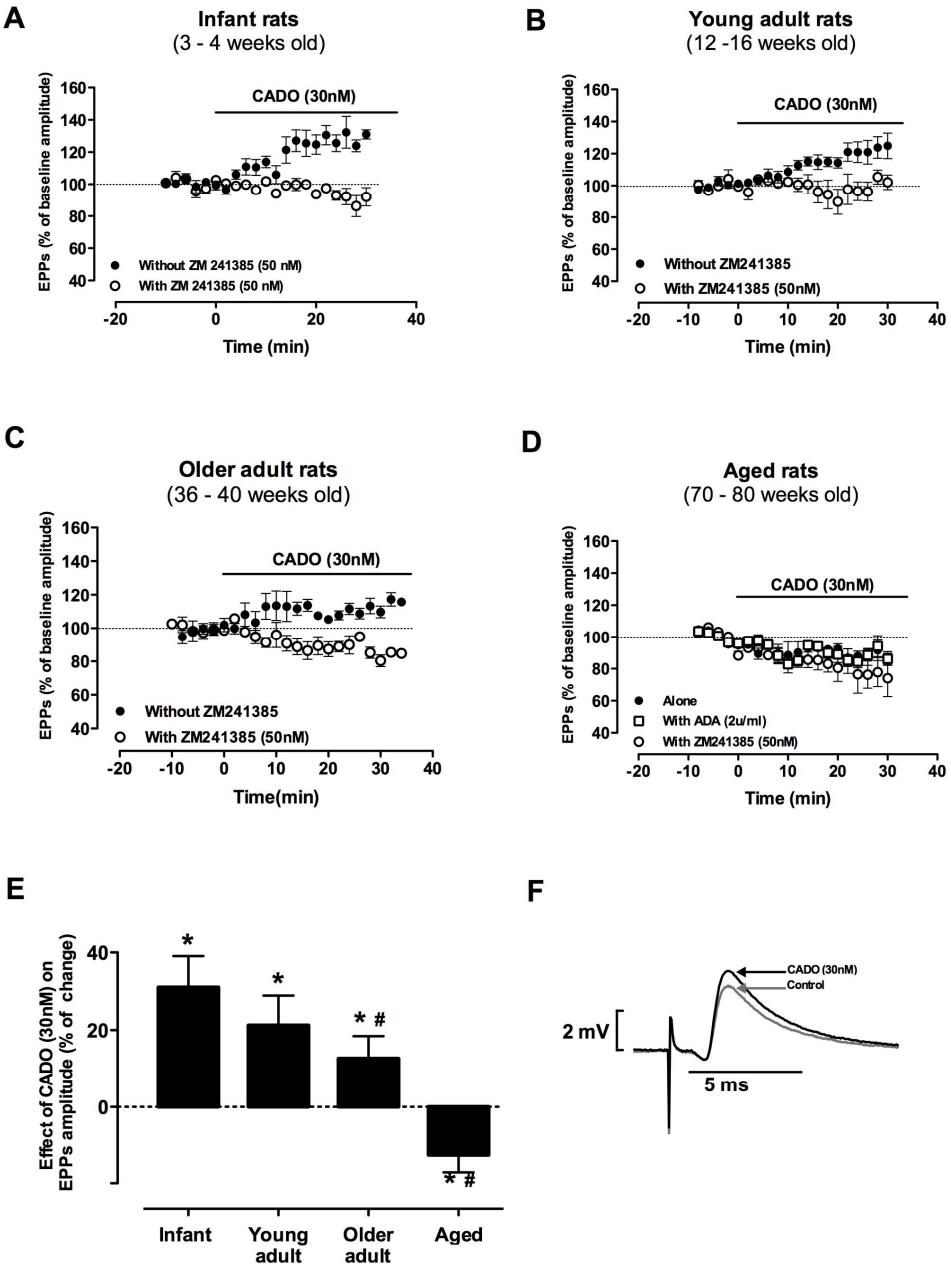
4.4.2 Relative role of adenosine A_1 and A_{2A} receptors upon ageing

To evaluate the relative importance of adenosine A_1 receptors *vis a vis* adenosine A_{2A} receptors, we decided to explore first the action of the stable analogue, CADO, which is not a substrate for adenosine deaminase (e. g. Daly, 1982), and has a low affinity for the adenosine uptake system (Jarvis et al., 1985). In innervated diaphragms from infant rats, and in accordance with what has been previously reported (Pousinha et al., 2010), CADO (30nM) increased EPP amplitude, but as illustrated in Figure 4.4.1, the magnitude of this excitatory effect decreased with age, turning into an inhibitory effect in aged rats. The effect caused by CADO (30nM) on the average amplitude of EPPs in all groups studied (% change: 31 ± 4,7%, n=5, infant rats, Figure 4.4.1A, E; 21 ± 7,6%, n=5, young adult rats, Figure 4.4.1B, E; 13 ± 5,7 %, n=5, older rats, Figure 4.4.1C, E, -12 ± 4,3%, n=3, aged rats, Figure 4.4.1D, E) was statistically significant ($P<0.05$ when compared with absence of CADO (pre-control) in the same experiments, Student t-test, Figure 4.4.1E). The effect in innervated diaphragms from older adults or aged rats was also significantly different from that in infant rats ($P<0.05$; ANOVA one-way analysis of variance followed by Tukey´s multiple comparisons test). Interestingly, when CADO

(30nM) was applied in the presence of the selective A_{2A} receptor antagonist, ZM 241385 (50nM, Poucher et al., 1995), it caused inhibition of EPP amplitude (Figure 4.4.1A-D), which was more evident in innervated diaphragms from older adult (-12 ± 2,4%, n=3; Figure 4.4.1C) or aged rats (-24 ± 10,2%, n=3; Figure 4.4.1D). On the other hand, in aged rats, the effect of CADO (30nM) in the absence or presence of the A_{2A} receptor antagonist, ZM 241385 (50nM), was not significantly different ($P<0.05$; Student t-test). Taken together, these results suggest that until midlife there is a predominant activation of A_{2A} receptors, which is lost upon ageing. Accordingly, and as shown in Figure 4.4.2, when a selective A_1 receptor agonist, CPA (100nM, Williams et al., 1986), was applied after the full excitatory effect of an A_{2A} selective agonist, CGS 21680 (10nM, Jarvis et al., 1989), the A_1 receptor agonist could not revert the excitatory influence of the selective A_{2A} receptor agonist. On the contrary, when CGS 21680 (10nM), the selective A_{2A} receptor agonist, was applied after the full inhibitory effect of CPA (100nM), neuromuscular transmission was increased, so that EPP amplitude nearly reverted towards pre-control levels (Figure 4.4.2B). Thus, activation of A_{2A} receptors was able to counteract the inhibition caused by activation of A_1 receptors, whereas A_1 receptor activation was not able to revert the A_{2A} receptor mediated excitatory action, further supporting the idea that in young animals the excitatory influence predominates over the inhibitory one.

To exclude the hypothesis that in aged rats the A_{2A} receptors are occupied by endogenous adenosine hampering its activation by an exogenous ligand, we evaluated the effect of CADO (30nM) after removing endogenous adenosine with adenosine deaminase (ADA, 2U/ml), an enzyme that converts adenosine into inosine (see Arch and Newsholme, 1978).

Figure 4.4.1. At submicromolar concentrations, 2-chloroadenosine (CADO) enhances the amplitude of evoked end-plate potentials (EPPs) in infant, young adult and older adult but not in aged rats. Panels A to D show the averaged changes in the amplitude of EPPs caused by the stable A1/A2A receptor agonist CADO (30 nM) in the absence (•) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM), in infant (A), young adult (B), older adult (C) or aged (D) rats. Panel D also shows that in aged rats the effect of CADO is not affected by the presence of adenosine deaminase (ADA, 2 U/ml, □), which was used to remove any putative occupation of receptors with the endogenous ligand, adenosine. The adenosine receptor antagonist was added to the preparations at least 45min before addition of the agonist; ADA was added at least 30min before the agonist. The horizontal bars in panels A to D indicate the period of agonist perfusion. Each point, in panels A to D, represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30nM): 2.4 ± 0.6mV, n=8 (infant rats); 3.0 ± 0.5mV, n=8 (young adult rats); 2.9 ± 1.0mV, n=8 (older adult rats); 1.5 ± 0.3mV, n=10 (aged rats). Panel E represents a bar chart that summarizes the effect of CADO (30nM) on the amplitude of EPPs in all groups studied; *$P<0.05$ (Student t- test), as compared with 0% (EPP amplitude before CADO in the same experiment; $^{\#}P<0.05$ (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) as compared with the effect of CADO in infant rats. Panel F shows representative traces of averaged EPPs recorded from one endplate (young adult rat), immediately before (control) and 30 min after addition of CADO (30 nM). Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -61 to -76 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.


A
Infant rats
(3 - 4 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
Without ZM 241385 (50 nM)
With ZM 241385 (50 nM)
Time (min)
B
Young adult rats
(12 -16 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
Without ZM241385
With ZM241385 (50nM)
Time (min)
C
Older adult rats
(36 - 40 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
Without ZM241385
With ZM241385 (50nM)
Time(min)
D
Aged rats
(70 - 80 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
Alone
With ADA (2u/ml)
With ZM241385 (50nM)
Time (min)
E
Effect of CADO (30nM) on EPPs amplitude (% of change)
Infant
Young adult
Older adult
Aged
F
CADO (30nM)
Control
2 mV
5 ms

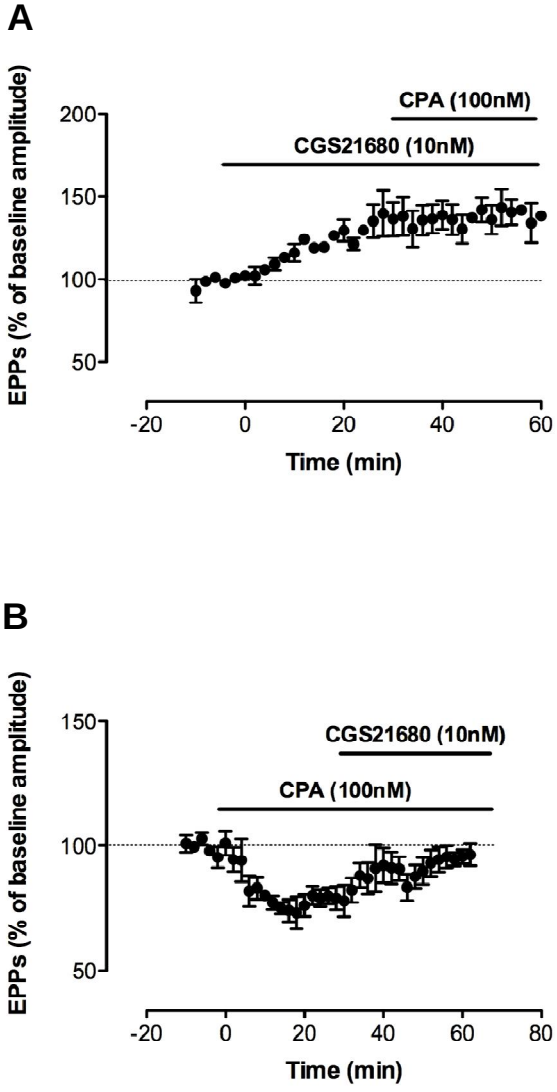


Figure 4.4.2. The A_{2A} receptor-mediated facilitatory action upon neuromuscular transmission is not reverted by co-application of an A_1 receptor mediated agonist. Averaged time course changes in the amplitude of EPPs caused by (A) CPA (100 nM) in the presence of the selective A_{2A} receptor agonist, CGS21680 (10nM) and by (B) CGS21680 (10nM) in the presence of the selective A_1 receptor agonist, CPA (100nM) in young adult rat endplates, as shown. The horizontal bars indicate the periods of drug perfusion. Each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of each selective agonist. (A: 2.8 ± 0.4 mV, n=3 and B: 2.6 ± 0.8 mV, n=3). Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -61 to -72 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

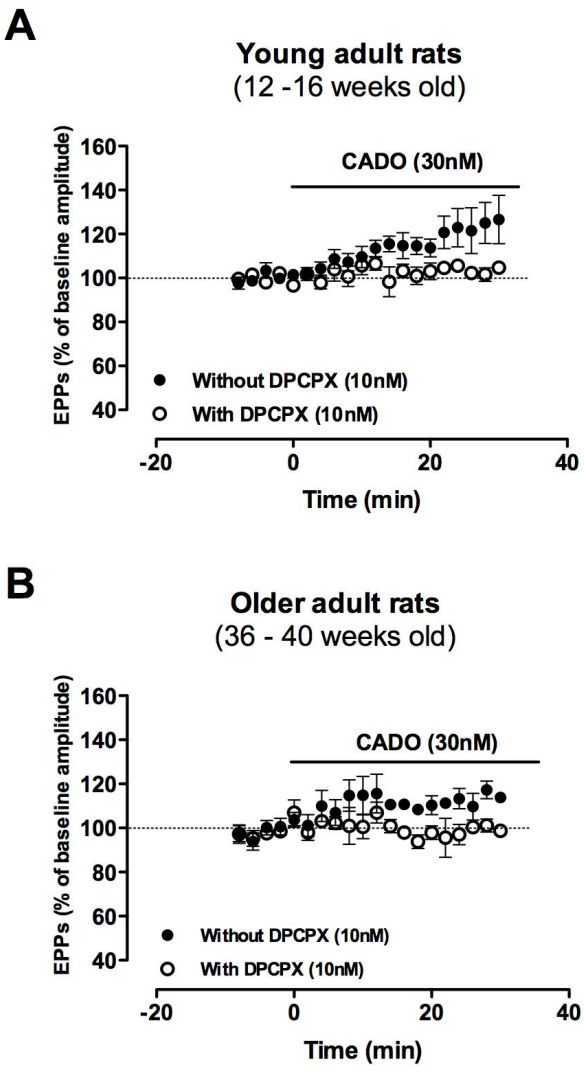
By itself, ADA (2U/ml) was devoid of effect on neuromuscular transmission (data not shown), suggesting that in these experimental conditions the level of extracellular adenosine is probably low. Furthermore, in the presence of ADA (2U/ml), the effect of CADO (30nM) on neuromuscular transmission in aged rats (Figure 4.4.1D) was not significantly different ($P>0.05$, n=3, student t- test) from what was observed when applied alone.

We previously reported (Pousinha et al., 2010) that the excitatory actions of adenosine at the neuromuscular junction of infant rats require tonic activation of adenosine A_1 receptors. Thus, we investigated if the excitatory effect of CADO (30nM) observed in young adult and older adult rats was also prevented by the A_1 receptor antagonist, DPCPX (10nM). This antagonist was used at a concentration only 20 times higher than its Ki value for A_1 receptors in the same preparation (Sebastião et al., 1990) and well below its Ki value for A_{2A} receptors (see Jacobson et al., 1992), but as illustrated in Figure 4.4.3, it fully prevented the facilitatory action of CADO (30nM) in young adult rats (Figure 4.4.3A) and older adult rats (Figure 4.4.3B) ($P<0.05$, n=3, student t-test).

Figure 4.4.4 summarizes the results from experiments where MEPPs and EPPs were recorded simultaneously, to evaluate changes in the quantal content of EPPs. Accordingly to what was previously described for infant rats (Pousinha et al., 2010), CADO (30nM) induced an increase in the frequency of MEPPs in young adult and older adult rats (Figure 4.4.4A). This effect on the frequency of MEPPs was not accompanied by changes in its mean amplitude (Figure 4.4.4B), indicating that the excitatory effect of CADO (30nM) is presynaptic, enhancing the release of the transmitter. Indeed, the quantal content of EPPs was significantly increased ($P<0.05$, n=5, Student´s t test) by

CADO (30nM) in infant, young adult and older adult rats (Figure 4.4.4C). In aged rats, CADO (30nM) induced the opposite effect, causing a decrease in the frequency of MEPPs (Figure 4.4.4A) without affecting their mean amplitude, indicating that in this group of animals, the inhibitory action of CADO (30nM) is still exerted at the presynaptic level.

Figure 4.4.3. The facilitatory effect of CADO (30nM) is prevented by the selective A_1 receptor antagonist. Averaged time course changes in the amplitude of EPPs caused by CADO (30 nM) in the absence (•) or in the presence (o) of the selective A_1 receptor antagonist, DPCPX (10nM), in young adult (A) and older adult (B) rat endplates as shown. The adenosine receptor antagonist was added to the preparations at least 45min before addition of the agonist. The horizontal bars indicate the period of agonist perfusion. Each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of 30 nM CADO (A: 3.1 ± 0.5mV, n=8 and B: 2.5 ± 0.6mV, n=8). Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -62 to -73 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.


A
Young adult rats
(12 -16 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
160
140
120
100
80
60
40
Without DPCPX (10nM)
With DPCPX (10nM)
-20
0
20
40
Time (min)
B
Older adult rats
(36 - 40 weeks old)
CADO (30nM)
EPPs (% of baseline amplitude)
160
140
120
100
80
60
40
Without DPCPX (10nM)
With DPCPX (10nM)
-20
0
20
40
Time (min)

4.4.3 Age-dependent changes in balanced A_1/A_{2A} receptor activation by endogenously released adenosine

Since both A_1 and A_{2A} receptors are present at the motor nerve endings, therefore being prone to be activated by the endogenous ligand, the next series of experiments was designed to assess if the A_1/A_{2A} activation balance could be influenced by age. To do so, extracellular levels of adenosine were increased by using the adenosine kinase inhibitor, 5´-iodotubericidin (ITU, 10µM). We previously showed (Pousinha et al., 2010) that the enhanced the neuromuscular transmission of infant rats, while being virtually devoid of effect on the resting membrane potential of the muscle fibre, an effect attributed to activation of A_{2A} receptors by released adenosine. We therefore evaluated if this excitatory effect was maintained upon ageing.

Figure 4.4.5 illustrates the effect of ITU (10µM) on the amplitude of EPPs in all groups studied. As shown, ITU (10µM) increased (P<0.05, n=5, Student´s t test) the mean amplitude of EPPs in infant (Figure 4.4.5A, E), young adult (Figure 4.4.5B, E) and older adult (Figure 4.4.5C, E) rats. However, in aged rats, ITU (10µM) inhibited neuromuscular transmission, decreasing (P<0.05, n=4) the mean amplitude of EPPs (Figure 4.4.5D, E). Comparison among the different groups (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) confirmed that the effect of ITU (10µM) in aged rats was significantly different (P<0.05, n=4, Student´s t test) from that in infant rats. As expected, when ITU (10µM) was perfused in the presence of the A_{2A} receptor selective antagonist, ZM241385, the excitatory effect was no longer evident (Figure 4.4.5).

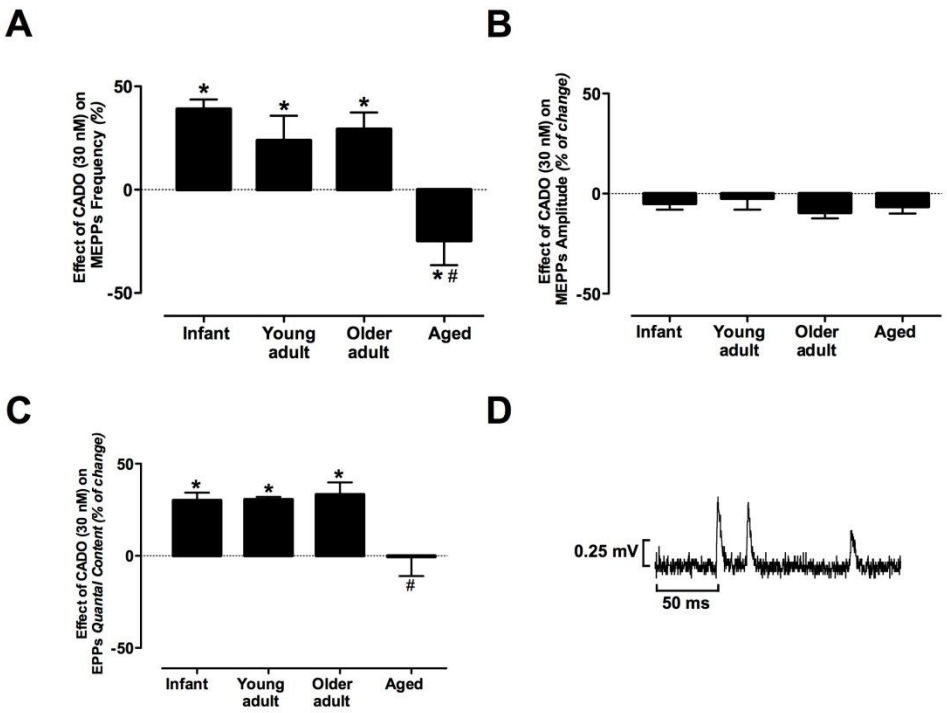


Figure 4.4.4 – At submicromolar concentrations, 2-chloroadenosine (CADO) enhances both the frequency of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs) in infant, young adult and older adult rats, but not in aged rats. Panels (A to C) represent the effect of CADO (30 nM) on the averaged frequency (A) and amplitude (B) of MEPPs, as well as quantal content (C) of EPPs in infant, young adult, older adult and aged rats. In the ordinates, 0% represents the averaged number of events per 100 sec (A), averaged amplitude recorded for 100 consecutive MEPPs (B) and the quantal content calculated immediately before addition of CADO (30nM). The effect induced by CADO (30nM) on the frequency of MEPPs and on the quantal content of EPPs in infant, young adult and older adult rats was significantly different from the control. *P<0.05 (Student´s t test) as compared with 0% (control waves before CADO); $^{\#}$P<0.05 (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) as compared with the effect of CADO in infant rats. Panel D illustrates a representative sample of MEPPs recordings. Perfusion solutions contained 19mM Mg^{2+}.

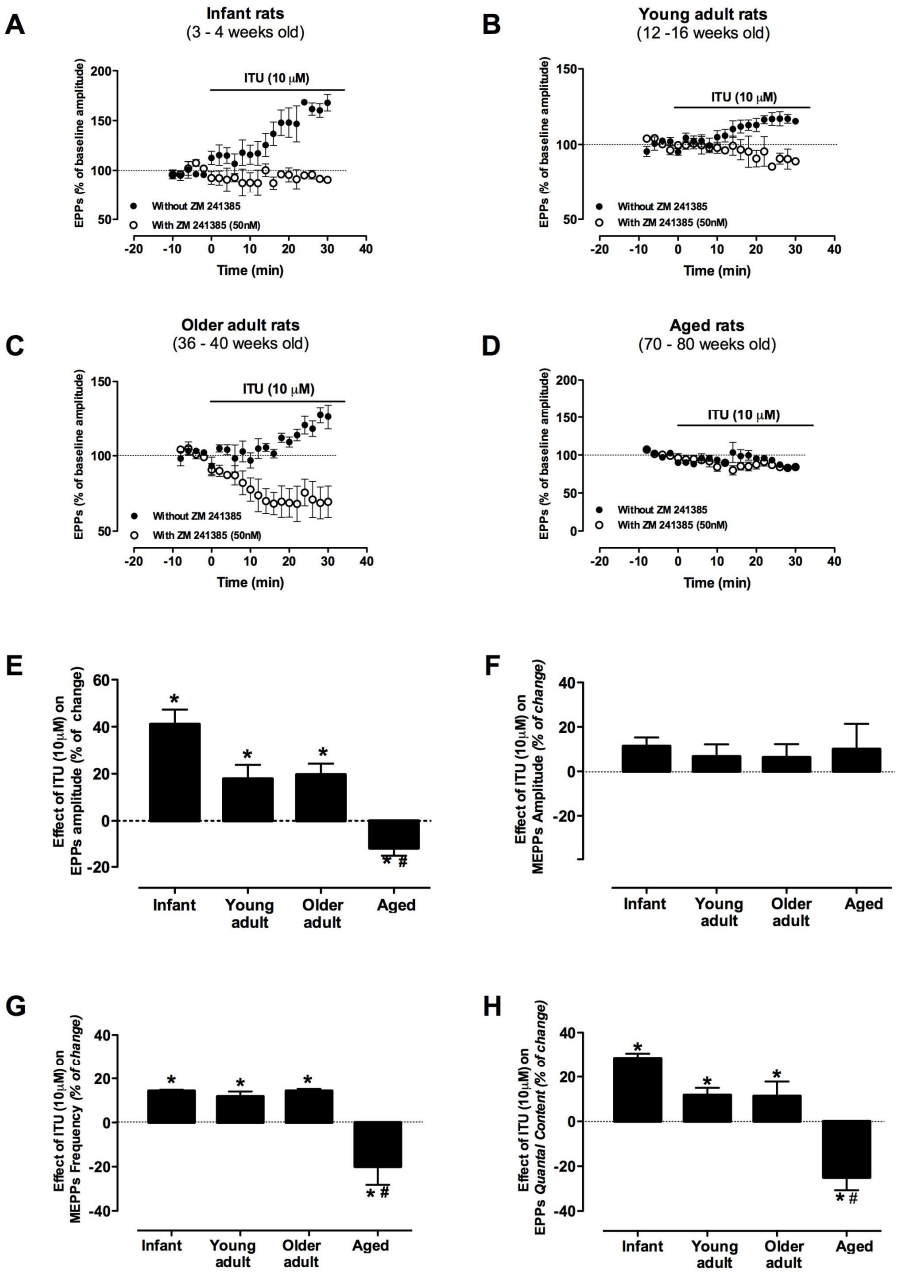

A
Infant rats
(3 - 4 weeks old)
B
Young adult rats
(12 -16 weeks old)
C
Older adult rats
(36 - 40 weeks old)
D
Aged rats
(70 - 80 weeks old)
ITU (10 μM)
EPPs (% of baseline amplitude)
Without ZM 241385
With ZM 241385 (50nM)
Time (min)
E
Effect of ITU (10μM) on EPPs amplitude (% of change)
F
Effect of ITU (10μM) on MEPPs Amplitude (% of change)
G
Effect of ITU (10μM) on MEPPs Frequency (% of change)
H
Effect of ITU (10μM) on EPPs Quantal Content (% of change)
Infant
Young adult
Older adult
Aged

Figure 4.4.5. The adenosine kinase inhibitor, ITU, enhances the amplitude of evoked end-plate potentials (EPPs), the frequency of miniature end-plate potentials (MEPPs) and the quantal content of end-plate potentials (EPPs) in infant, young adult and older adult rats, but not in aged rats. Panels A to D show the averaged changes in the amplitude of EPPs caused by ITU (10μM) in the absence (•) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM), in infant (A), young adult (B), older adult (C) or aged (D) rats The adenosine receptor antagonist was added to the preparations at least 45min before addition of ITU. The horizontal bars represent the period of ITU perfusion; each point, represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of ITU (10μM): 3.2 ± 0.5mV, n=8 (infant rats); 2.0 ± 0.4mV, n=8 (young adult rats); 1.6 ± 0.4mV, n=8 (older adult rats); 1.8 ± 0.3mV, n=7 (aged rats). Panels E to H represent a bar chart that summarizes the effect of ITU (10 μM) on the average amplitude of EPPs (E), on the average amplitude (F) and frequency (G) of MEPPs, as well as quantal content (G) of EPPs in all groups studied. In the ordinates 0% represents the averaged EPP amplitude recorded for 10 min immediately before addition of ITU (10 μM) (E), averaged amplitude recorded for 100 consecutive MEPPs (F), averaged number of events per 100 sec (G) and the quantal content calculated immediately before addition of ITU (10μM). The effect induced by ITU (10 μM) on the averaged amplitude of EPPs, frequency of MEPPs and on the quantal content of EPPs in infant, young adult and older adult rats was significantly different from the control. *P<0.05 (Student´s t test) as compared with 0% (control waves before ITU); $^{\#}$P<0.05 (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) as compared with the effect of ITU in infant rats. Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -60 to -77 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

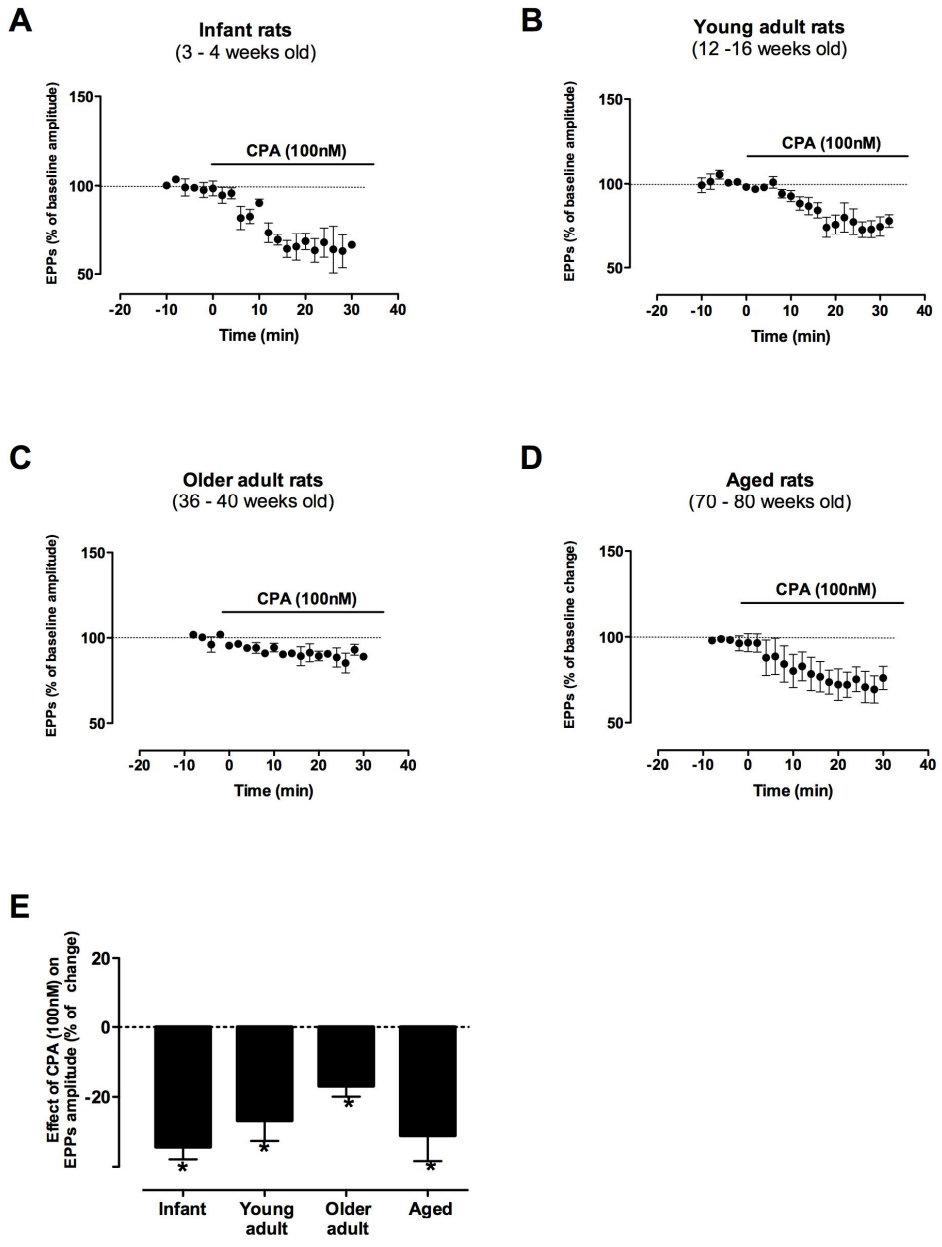

A
Infant rats
(3 - 4 weeks old)
EPPs (% of baseline amplitude)
150
100
50
CPA (100nM)
-20 -10 0 10 20 30 40
Time (min)
B
Young adult rats
(12 -16 weeks old)
EPPs (% of baseline amplitude)
150
100
50
CPA (100nM)
-20 -10 0 10 20 30 40
Time (min)
C
Older adult rats
(36 - 40 weeks old)
EPPs (% of baseline amplitude)
150
100
50
CPA (100nM)
-20 -10 0 10 20 30 40
Time (min)
D
Aged rats
(70 - 80 weeks old)
EPPs (% of baseline change)
150
100
50
CPA (100nM)
-20 -10 0 10 20 30 40
Time (min)
E
Effect of CPA (100nM) on EPPs amplitude (% of change)
20
0
-20
*
*
*
*
Infant
Young adult
Older adult
Aged

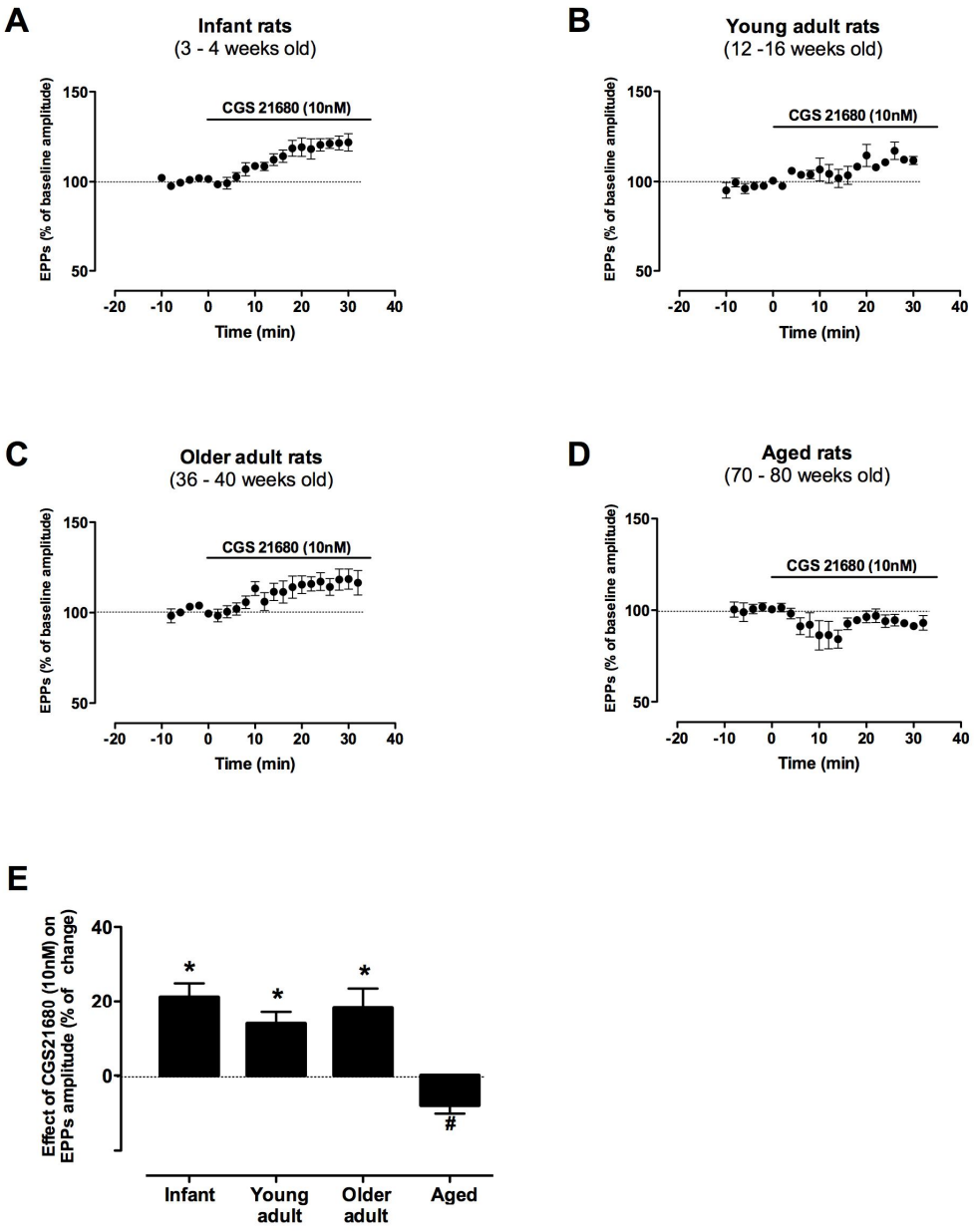
Figure 4.4.6. A_1 receptor activation decreases the average amplitude of EPPs in all groups studied. Panels A to D show the averaged changes in the amplitude of EPPs caused by the A1 receptor agonist CPA (100 nM) in infant (A), young adult (B), older adult (C) or aged (D) rats; the horizontal bars indicate the period of drugs perfusion; each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CPA (100nM 1.6 ± 0.3mV, n=5 (infant rats); 1.4 ± 0.3mV, n=5 (young adult rats); 3.4 ± 0.5mV, n=4 (older adult rats); 2.4 ± 1.2mV, n=4 (aged rats). Panel E represents a bar chart that summarizes the effect of CPA (100nM) on the amplitude of EPPs in all groups studied; *$P<0.05$ (Student t- test), as compared with 0% (EPP amplitude before CPA (100 nM) in the same experiment; $^{\#}P<0.05$ (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) as compared with the effect of CPA (100 nM) in infant rats. Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -60 to -70 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

Interestingly, in older adult rats the excitatory effect of ITU (10µM) turned into an inhibitory one (-29 ± 10.8%, n=4, Figure 4.4.5C, $P<0.05$, Student´s t test), indicating at this age group both A_1 and A_{2A} receptors can be hit by endogenously released adenosine. This inhibitory effect can be related to A_1 receptor activation by released adenosine, since, as expected, it was prevented by the adenosine A_1 receptor selective antagonist, DPCPX (10nM, n=3).

Accordingly to what was observed with CADO (30nM), ITU (10µM) induced an increase in the frequency of MEPPs in infant, young adult and older adult rats (Figure 4.4.5G). This effect was not accompanied by changes in the mean amplitude of MEPPs (Figure 4.4.5F) and therefore, ITU (10µM) significantly ($P<0.05$, n=4-5, Student´s t test) increased the quantal content of

EPPs (Figure 4.4.5H) in those age groups. Interestingly, in aged animals, ITU (10µM) decreased the frequency of MEPPs (-20 ± 6,3%, n=3; Figure 4.4.5G) without affecting its mean amplitude (Figure 5F), decreasing the quantal content of EPPs (Figure 4.4.5H); these inhibitory actions were prevented by the selective A_1 receptor antagonist DPCPX (10nM). One may therefore conclude that under situations of enhanced release of endogenous adenosine, due to inhibition of adenosine kinase, adenosine-mediated modulation of neuromuscular transmission also shifts from excitatory to inhibitory upon ageing.

Figure 4.4.7. The selective A_{2A} receptor agonist, CGS21680, increases the average amplitude of EPPs in infant, young adult and older adult rats, but not in aged rats. Panels A to D show the averaged changes in the amplitude of EPPs caused by the A_{2A} receptor agonist CGS21680 (10 nM) in infant (A), young adult (B), older adult (C) or aged (D) rats; the horizontal bar represents the period of agonist perfusion; each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before starting CGS21680 (10nM) perfusion, which was 2.6 ± 0.6mV, n=5 (infant rats); 2.0 ± 0.5mV, n=5 (young adult rats); 2.6 ± 0.4mV, n=4 (older adult rats); 1.5 ± 0.3mV, n=4 (aged rats). Panel E represents a bar chart that summarizes the effect of CGS21680 (10nM) on the amplitude of EPPs in all groups studied; *$P<0.05$ (Student t- test), as compared with 0% (EPP amplitude before CPA (100 nM) in the same experiment; $^{\#}P<0.05$ (ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test) as compared with the effect of CPA (100 nM) in infant rats. Resting membrane potential in all experiments at time zero (i.e. before adding the test drug) ranged from -63 to -74 mV. Perfusion solutions contained 19mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.


A
Infant rats
(3 - 4 weeks old)
CGS 21680 (10nM)
EPPs (% of baseline amplitude)
Time (min)
B
Young adult rats
(12 -16 weeks old)
CGS 21680 (10nM)
EPPs (% of baseline amplitude)
Time (min)
C
Older adult rats
(36 - 40 weeks old)
CGS 21680 (10nM)
EPPs (% of baseline amplitude)
Time (min)
D
Aged rats
(70 - 80 weeks old)
CGS 21680 (10nM)
EPPs (% of baseline amplitude)
Time (min)
E
Effect of CGS21680 (10nM) on EPPs amplitude (% of change)
Infant
Young adult
Older adult
Aged

4.4.4 Age-dependent influence of inhibitory receptors

To evaluate if the inhibitory action of adenosine on neuromuscular transmission was affected by ageing, we studied the effect of the selective adenosine A_1 receptor agonist, CPA, upon ageing (Figure 4.4.6). As expected, CPA (100nM) decreased the mean amplitude of EPPs in all groups studied (*P*<0.05, when compared with absence of CPA (pre-control) in the same experiments, n=4-5, Student´s t test). Among groups, there were no statistically significant differences (P>0.05; ANOVA one-way analysis of variance followed by Tuckey´s multiple comparisons test). This monotonous effect of CPA (100nM) suggests that the inhibitory role of adenosine A_1 receptors on neuromuscular transmission is maintained upon ageing.

4.4.5 Age-dependent influence of facilitatory receptors

As illustrated in Figure 4.4.7, the selective A_{2A} receptor agonist, CGS 21680 (10nM), increased (P<0.05, n=4-5, Student´s t test) the amplitude of EPPs in infant (Figure 4.4.7A, E), young adult (Figure 4.4.7B, E) and older adult (Figure 4.4.7C, E) rats. Unexpectedly, in aged rats (Figure 4.4.7D, E) CGS 21680 (10nM) failed (P>0.05, n=4) to enhance neuromuscular transmission suggesting the absence of functional adenosine A_{2A} receptors in that age. The absence of an excitatory effect in aged rats could be related to full occupancy of the receptors by endogenously released adenosine. To exclude this hypothesis we tested the effect of CGS 21680 (10nM) in the presence of adenosine deaminase, (ADA, 2U/ml). By itself, ADA (2U/ml) did not significantly modify the amplitude of EPPs (-12 ± 4,6 mV, n=3), indicating that the level of extracellular adenosine was low. When CGS 21680 (10nM) was applied 30 min after ADA (2U/ml) addition, the mean amplitude of EPPs

remained unchanged (-5 ± 0,8%, n=3), suggesting that the absence of the excitatory effect is not related to maximum receptor occupancy by the endogenous ligand.

4.4.6 Discussion

The main finding in the present chapter was that ageing influences adenosine A_1 and A_{2A} receptors at the rat diaphragm neuromuscular junction, in a different manner. The magnitude of the excitatory effects on neuromuscular transmission caused by endogenously released adenosine or by a mixed A_1/A_{2A} receptor agonist tends to decrease upon ageing, disappearing in aged rats, where only A_1 receptor-mediated inhibition was evident. Considering independent activation of A_1 or A_{2A} receptors by selective ligands, the magnitude of the A_1 receptor-mediated inhibition is kept about constant throughout age; in contrast, the magnitude of A_{2A} receptor-mediated facilitation is about the same up to middle aged (older adult rats), but is absent at the neuromuscular junction of aged rats. Therefore, it appears that upon ageing there is a gradual tendency for preponderance of inhibition, over excitation, when both receptors are simultaneously activated, in spite of proper A_1 and A_{2A} receptor functioning up to middle age.

Neuromuscular junctions differ in quantal content, safety factor and adaptation to repetitive activation (see Rowley et al., 2007; Ermilov et al., 2007). Whether such differences might influence the presently reported effects of adenosine upon ageing, needs to be explored at the light of the results obtained by Rowley et al (2007) and Ermilov et al (2007). However, in a previous work (see Ginsborg and Hirst, 1972) the initial quantal content of EPPs (either being low, in experiments where muscle contractions were

paralysed by high magnesium, or high in the experiments where muscle contractions were paralysed by tubocurarine) did not influence the inhibitory effects of adenosine at the rat diaphragm neuromuscular junction. How adenosine influences safety factor variability in nerve terminals innervating different fiber types (see Ermilov et al., 2007) cannot be answered in the present work and awaits further investigation.

It is well established that adenosine kinase inhibitors, such as ITU, increase extracellular adenosine availability. In the present work, the excitatory effect of low concentrations of CADO and ITU, observed in infant rats (Pousinha et al., 2010), tended to decrease upon ageing, even disappearing in aged rats (70–80 weeks old). The enhancement of neuromuscular transmission caused by the adenosine kinase inhibitor, ITU, as well as that caused by the mixed A_1/A_{2A} receptor agonist, CADO, should be attributed to adenosine A_{2A} receptor activation since they were antagonized by pre-incubation with the selective A_{2A} receptor antagonist ZM241385. A_{2A} receptor blockade transformed the excitatory effects of those drugs into inhibitory ones. This suggests that the influence of ITU and CADO upon neuromuscular transmission results from a balance between activation of adenosine excitatory A_{2A} and inhibitory A_1 receptors. The facilitation of neuromuscular transmission caused by ITU or CADO should be attributed to a presynaptic action, i.e. to an increase in the release of acetylcholine from the phrenic-nerve endings, since these drugs increased the quantal content of endplate potentials without affecting the average amplitude of MEPPs recorded concomitantly.

The failure of nanomolar concentrations of CADO and ITU to increase neuromuscular transmission in aged rats, suggests that those animals lost the

ability to activate A_{2A} receptors. This hypothesis is supported by (1) the observation that the effect of those drugs was not changed in preparations pre-incubated with the selective A_{2A} receptor antagonist, ZM241385, suggesting that only A_1 receptors become activated together with (2) the absence of excitatory effect of the A_{2A} receptor agonist, CGS 21680, when applied to aged rats. Full occupancy of A_{2A} receptors by high levels of endogenous adenosine cannot account for this lack of effect, since when these drugs were applied in the presence of adenosine deaminase, their effect on neuromuscular transmission was not changed. One possible explanation for the absence of adenosine excitatory effects could be a decrease in the number of A_{2A} receptors or/and to a decrease in the affinity of the receptor to its ligand. Nerve endings represent only a minor proportion of the tissue content in a nerve-muscle preparation, which precludes the use of receptor quantitative or semi-quantitative assays, such as ligand binding studies or Western Blot analysis. These approaches have been done in brain tissue, showing that the number of A_{2A} receptors, their affinity to ligands, and their coupling to G protein is decreased in striatal membranes of aged rats (24 months) when compared to young adult animals (3 months) (Lopes et al., 1999). Also, the expression of A_{2A} mRNA is decreased in striatum of aged rats (Schiffmann and Vanderhaeghen, 1993) and the excitatory effect of the A_{2A} receptor agonist, CGS 21680, on the spontaneous outflow of glutamate and aspartate in striatum was lost in aged rats (Corsi et al., 1999). So, in what concerns A_{2A} receptor-mediated neuromodulation, the neuromuscular junction appears to behave more likely the striatum, in clear contrast with what happens in the hippocampus, where A_{2A} receptor-mediated excitatory tonus is increased upon ageing (Cunha et al., 1995; Lopes et al., 1999). Interestingly,

the motor inhibitory adenosinergic tone is increased in aged animals with respect to young animals (Popoli et al., 1998), which can be attributed, at least in part, to a loss of excitatory tonus at the neuromuscular junction (present report) and striatum (Schiffmann and Vanderhaeghen, 1993; Corsi et al., 1999; Lopes et al., 1999).

As observed in infant rats (Pousinha et al., 2010), the A_{2A} receptor-mediated facilitation of neuromuscular transmission was antagonized, not only by the selective A_{2A} receptor antagonist, as also, by the A_1 selective receptor antagonist, suggesting that the interaction between both receptors is maintained upon ageing. Two possibilities that are not mutually exclusive can be advanced: (1) the formation of A_{2A} and A_1 receptors heteromers, as it has been shown to occur at glutamatergic nerve terminals (Ciruela et al., 2006) and astrocytes (Cristovão-Ferreira et al., 2011). In these conditions blockade of the A_1 receptor molecule could induce a conformational change in the heteromer that does not allow either A_{2A} agonist binding or effective coupling to the A_{2A} transducing system. Indeed, heteromerization of G-protein coupled receptors may affect several aspects of receptor functioning (see Franco et al., 2008), so that monomers and heteromers can have distinct pharmacological and signaling properties. Therefore, age-dependent changes in the proportion of adenosine receptors in an heteromeric or monomeric form may, at least in part, be responsible for the lost of excitatory effects in aged rats. (2) An A_1/A_{2A} receptor cross talk through intracellular transducing pathways downstream to receptor binding (see Sebastião and Ribeiro, 2000), which would also lead to the need of A_1 receptor function to trigger an A_{2A} receptor-mediated response.

Several authors reported morphological and physiological changes that are related to the decline of the neuromuscular system with ageing, as for example, loss of motor neurons, altered neurotransmitter release, disruption of post-synaptic acetylcholine receptor organization, denervation and reinnervation of muscle fibers, and motor unit remodelling (see Delbono, 2003). In rat diaphragm, the quantal content was reported to increase during the first 9 months of life and then to decline (Kelly, 1978). The reduction in A_{2A} receptors effects in aged rats might be one of the causes of the age-related changes in the neuromuscular transmission, since these receptors are known to influence the action of several neurotransmitters/ neuromodulators/ receptors/ transporters at the nerve terminal (see Sebastião and Ribeiro, 2009). For instance, A_{2A} receptors at motor nerve terminals trigger the action of brain derived neurotrophic factor (BDNF) (Pousinha et al., 2006), which enhances transmitter release at developing endplates (Boulanger and Poo, 1999), improves neuromuscular transmission in the adult rat diaphragm (Mantilla et al., 2004) and facilitates synaptic efficacy by increasing presynaptic depolarization at the neuromuscular junction (see Huang and Reichardt, 2001). BDNF is also important for maintenance of acetylcholine receptor clustering in the endplate (Belluardo et al., 2001; Gonzalez et al., 1999). Therefore, the decrease of A_{2A} receptors upon ageing can compromise BDNF actions that occur at the neuromuscular junction. Interestingly, studies performed on adenosine receptors KO mice revealed that A_{2A} receptors play much more important roles than A_1 receptors in regulating locomotor activity (Huang et al., 2005), since both A_{2A} receptor KO and double A_1-A_{2A} receptors KO mice present lower locomotion activity and consume less oxygen than their wild type controls and apparently unaltered in A_1 receptor KO mice.

Though those changes are usually regarded as a result of changes in basal ganglia control of motor activity, A_{2A} receptor changes at the neuromuscular junction may also play a role.

In conclusion, the results described in this chapter clearly document that ageing influences adenosine A_1 and A_{2A} receptors, at the motor nerve terminals, in a different manner. The actions of A_{2A} receptors decrease with ageing, even disappearing in aged rats. It remains to be clarified what are the consequences of the absence of these receptors for fine-tune of motor control and whether this relates to the age-associated decline in the neuromuscular control.

5. FINAL CONCLUSION

The main goal of the present thesis was to investigate how transmission at the neuromuscular junction is influenced by ageing and how interactions between the neuromodulator adenosine and the neurotrophic factor BDNF influence neuromuscular transmission in young ages. Considering that there is a crosstalk between adenosine A_{2A} receptors and BDNF TrkB receptors in the brain (Diógenes et al., 2004), and since the neuromuscular junction is a very simple ´synapse`(see Introduction) we decided also to explore this aspect. The main conclusions are that: (a) there is a cross-talk between adenosine A_{2A} receptors and TrkB receptors at the neuromuscular junction, where A_{2A} receptors trigger the excitatory action of BDNF on neuromuscular transmission, through a mechanism that involves PLCγ; (b) there is a predominance of excitatory adenosine effects at the motor nerve endings of infant rats; (c) the synaptic transmission at the rat diaphragm neuromuscular junction remains stable through lifetime becoming compromised only in eldery; (d)ageing influences adenosine A_1 and A_{2A} receptors, at the motor nerve terminals, in a different manner. The actions of A_{2A} receptors decrease with ageing, even disappearing in aged rats, whereas the actions of A_1 receptors remain unchanged upon ageing.

Taken together, the results described in this thesis suggest that adenosine A_{2A} receptors play a crucial role in the maintenance of neuromuscular transmission, exerting its effects directly on neuromuscular transmission or by modulating other molecules/receptors effects, like BDNF. The absence of adenosine A_{2A} receptors effects in aged animals might be one of the causes of the neuromuscular transmission impairment.

For example, amyotrophic lateral sclerosis (ALS) is a progressive and lethal neurodegenerative pathology characterized by a selective degeneration of cerebral cortex, brainstem and spinal cord motorneurons, leading to muscle deterioration and ultimately respiratory failure (see Turner & Talbot 2008 for review). It has been suggested that ALS is a “dying back” axonopathy (Pun et al. 2006), and several electro-diagnostic observations indicate severe impairment of neuromuscular transmission in patients with ALS (Denys and Norris 1979). The question of whether ALS impairments result primarily from disease-induced direct changes into the cholinergic motor nerve terminal or muscle cell, so to say, the neuromuscular junction, or if it is mainly a consequence of central motor nerve degeneration may lack a unique answer, since different cell types may orchestrate motor neuron death. *Sera* from ALS patients enhance the frequency of miniature end plate potentials (MEPPS) without changing its average amplitude or its time course, and without changing the endplate membrane resting potential (Uchitel et al. 1988, Appel et al. 1991, O´Shaughnessy et al. 1998). Besides spontaneous asynchronous release there is also an increase in the evoked release of acetylcholine (ACh) evaluated through the quantal content of evoked end plate potentials (EPPS) (O´Shaughnessy et al. 1998). No post-synaptic changes were detected and the post-synaptic region was structurally intact (Maselli et al. 1993). The action of ALS sera is mimicked by activation of pre-synaptic adenosine A_{2A} receptors at the motor nerve endings, which facilitate ACh release (Correia-de-Sá et al. 1991) through mobilization of the intracellular calcium pools (thapsigargin-sensitive calcium stores) as well as through and/or P and L-type channels (Correia-de-Sá et al. 2000).

Neurotrophic factors are well-recognized neuroprotectors but there are many difficulties in clinical applications in particular with respect to side effects and adverse pharmacokinetic properties. So far, clinical trials with BDNF have failed to rescue the degenerating motorneurons. However, previous studies have shown that motoneurons from ALS patients are not deprived of neurotrophic support; on the contrary it seems that BDNF levels and other neurotrophins are up regulated in the motoneurons of ALS patients. Thus, it is possible that the affected motoneurons change their responsiveness towards neurotrophins since TrkB receptors in the spinal cord of ALS patients are much less phosphorylated on tyrosine residues than the controls, which may be involved in the enhancement of neurotrophin-induced aptotic events. It is possible that TrkB receptors change their affinity to BDNF preventing its action. This raises new questions about the implications of BDNF and the cross talk between neurotrophic factor receptors (TrkB) and adenosine A_{2A} receptors at motor nerve endings in ALS, in particular if we take into account that the excitatory effects mediated by adenosine A_{2A} receptors disappear in aged rats and that age-related changes detected at the neuromuscular junction are similar to the detrimental changes observed in ALS models.

By finding out if ALS models lack the excitatory adenosine A_{2A} receptors effect (like we observed in aged rats) and if it is so, to investigate when and why it happens, would provide knowledge about the disease and therefore, the development of novel and potential therapeutical approaches to treat this disease.

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8. APPENDICES

Original articles published with the scientific content of the present thesis.

I. **Pousinha PA**, Diógenes MJ, Ribeiro JA and Sebastião AM (2006) Triggering of BDNF facilitatory action on neuromuscular transmission by adenosine A_{2A} receptors. Neurosci Letters, 404:143-147.

II. **Pousinha PA**, Correia AM Sebastião AM, Ribeiro JA (2010) Predominance of adenosine excitatory over inhibitory effects on transmission at the rat neuromuscular junction of infant rats. Journal of Pharmacology and Experimental Therapeutics, 332: 1-11.

III. **Pousinha PA**, Correia AM, Sebastião AM, Ribeiro JA (2012) Neuromuscular transmission modulation by adenosine upon aging. Neurobiology of Aging. (in the press).

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Triggering of BDNF facilitatory action on neuromuscular transmission by adenosine A_{2A} receptors

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Abstract

Motor nerve terminals possess adenosine A_{2A} receptors and brain derived neurotrophic factor (BDNF) TrkB receptors. In the present work we evaluated how BDNF actions on neuromuscular transmission would be influenced by adenosine A_{2A} receptors activation. BDNF (20–100 ng/ml) on its own was devoid of effect on evoked endplate potentials (EPPs) recorded intracellularly from rat innervated diaphragms paralysed with tubocurarine. However, when BDNF was applied 45 min after a brief (2 min) depolarizing KCl (10 mM) pulse or when the adenosine A_{2A} receptors were activated with CGS 21680 (10 nM), BDNF (20 ng/ml) increased EPPs amplitude without influencing the resting membrane potential of the muscle fibre. The action of BDNF was prevented by the adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM) as well as by the TrkB receptor phosphorylation inhibitor, K252a (200 nM). The PKA inhibitor, H-89 (1 μM), prevented the excitatory effect of CGS 21680 (10 nM) on EPPs as well as prevented its ability to trigger a BDNF effect. The PLCγ inhibitor, U73122 (5 μM), did not prevent the excitatory action of CGS 21680 (10 nM) on neuromuscular transmission, but abolished the action of BDNF in the presence of the A_{2A} receptor agonist. The results suggest the following sequence of events in what concerns cooperativity between A_{2A} receptors and TrkB receptors at the neuromuscular junction: A_{2A} receptor activates the PKA pathway, which promotes the action of BDNF through TrkB receptors coupled to PLCγ, leading to enhancement of neuromuscular transmission.

Keywords: Adenosine; Adenosine A_{2A} receptor; BDNF; TrkB receptor; Neuromuscular junction; Neuromuscular transmission

Brain derived neurotrophic factor (BDNF) facilitates neuromuscular transmission at developing neuromuscular junctions [5], an action regulated by cyclic AMP [6]. In the rat hippocampus the effect of this neurotrophin is enhanced when adenosine A_{2A} receptors are activated [11]. Since, adenosine A_{2A} receptors are present in the rat motor nerve terminals [2], facilitate neuromuscular transmission [10] and are coupled to cyclic AMP [9], it is of interest to investigate if A_{2A} receptors could influence the action of BDNF on neuromuscular transmission at the rat neuromuscular junction. The mechanisms involved in the potential interplay between adenosine A_{2A} receptors and BDNF were also investigated.

The experiments were performed on isolated preparations of the phrenic nerve-diaphragm from male 3–4-weeks-old wistar rats from Harlan Interfauna Iberia, SL (Barcelona). The animals, handled according to European Community guidelines and Portuguese Law on animal care, were killed by decapitation under halothane anaesthesia. A strip of the left hemidiaphragm was isolated together with the phrenic nerve and was mounted in a 3 ml Perspex chamber through which the solutions flowed continuously at a rate of 3 ml/min. The bathing solution contained (mM) NaCl 117, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.2; was continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4) and kept at room temperature (22–25 °C). Muscle twitches were prevented by a submaximal concentration (1.5 μM) of tubocurarine.

Evoked endplate potentials (EPPs) were recorded with intracellular electrodes (10–20 MΩ resistance) filled with KCl (3 M) inserted in the motor endplate. The reference electrode was an Ag–AgCl pellet. The nerve was stimulated supramaximally (rectangular pulses of 20 μs duration applied once every 2 s) through a suction electrode. Individual EPPs, as well as the resting membrane potential of the muscle fibre, were continuously monitored. Throughout the experiment, 60 consecutive EPPs were averaged and stored on a personal computer with the LTP programme [1]. The resting membrane potential was continu-

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ously monitored and the averaged values stored every 2 min in a second channel of the computer.

Drugs used: brain-derived neurotrophic factor, was a gift by Regeneron Pharmaceuticals; 2-*p*-(2-carboxyethyl) phenethylamino]-5′-*N*-ethylcarboxamido adenosine hydrochloride (CGS 21680), *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89) and *d*-tubocurarine chloride were from Sigma-RBI; 4-(2-[7-amino-2-(2-furly)[1,2,4]triazolo[2,3-a][1,3,5]triazin5ylamino]ethyl)phenol (ZM-241385) and 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) were from Tocris Cookson; K252a was from Calbiochem; BDNF was supplied as a 1.0 mg/ml stock solution in 150 mM NaCl, 10 mM sodium phosphate buffer and 0.004% Tween-20. CGS 21680, ZM 241385 and H-89 were made up in 5 mM stock solutions in DMSO. K252a was made up in a 1 mM stock solution in DMSO. U73122 was made up in a 5 mM stock solution in DMSO. Aliquots of these stock solutions were kept frozen at −20 °C until use. The maximum concentration of DMSO applied to the preparations (0.02% (v/v)) was devoid of effect on EPPs.

The data are expressed as mean ± S.E.M. from *n* experiments. To allow comparisons between different experiments the EPP amplitude was normalised, taking as 100% the averaged values obtained during 10 min immediately before applying the test drug. The significance of the differences between means was evaluated by Student's *t*-test, when only two means were compared, or by one-way ANOVA followed by the Bonferroni test whenever multiple comparisons were made. Values of $P < 0.05$ were considered to represent statistically significant differences.

BDNF (20–100 ng/ml, $n = 4$), when applied alone at the rat diaphragm neuromuscular junction, did not influence the amplitude of EPPs within 60 min of its application (Fig. 1A). To evaluate if a BDNF action could be triggered by depolarization, the depolarizing conditions used were inspired by those that proved to trigger an excitatory action of BDNF on hippocampal synaptic transmission [11]; so, the bathing solution was changed for 2 min by a solution where 10 mM KCl had been added, therefore the potassium concentration in the bath was transiently shifted to 15 mM. As illustrated in Fig. 1D, the membrane potential was transiently depolarized during the

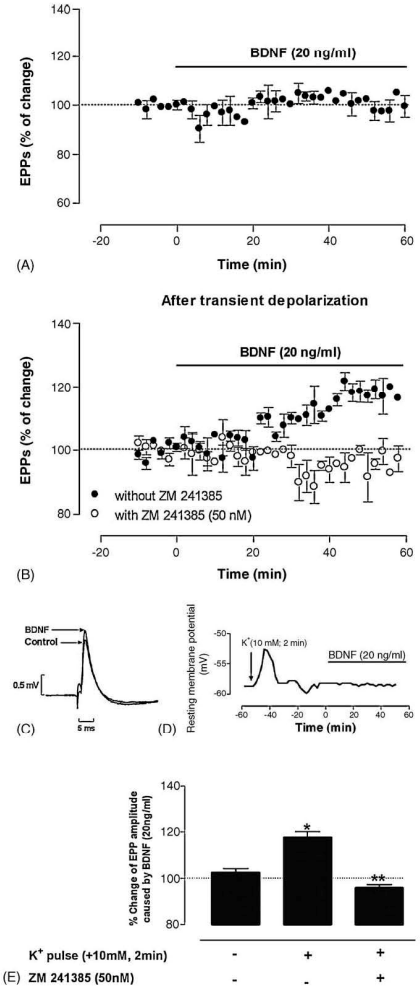

Fig. 1. Depolarization triggers an excitatory action of BDNF on transmission at the rat innervated diaphragm, an action requiring co-activation of adenosine A_{2A} receptors. In (A) and (B) are shown the time course of the changes in EPP amplitude induced by BDNF (20 ng/ml) when applied to preparations that had not been pre-depolarized (A) or to preparations that had been depolarized with 10 mM KCl for 2 min 46 min before BDNF application (B) in the absence (●) or in the presence (○) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM). Panel (C) shows superimposed traces of averaged e.p.ps recorded from one experiment immediately before (control) and 60 min after addition of BDNF (20 ng/ml). In panel (D) are shown the time course of changes in the resting membrane potential recorded from the same experiment as in (C); the application of the K^+ pulse is indicated by the arrow and that of BDNF by the horizontal bar. Panel (E) shows the averaged increase in EPP amplitude induced by BDNF (20 ng/ml) under the experimental conditions indicated below each column. The change of e.p.ps amplitude caused by BDNF in pre-depolarized preparations (middle column) was significantly different ($^*P < 0.05$; ANOVA one-way analysis of variance followed by Bonferroni multiple comparisons test) from the effect of BDNF applied alone (left column) as well as from the effect of BDNF when applied in pre-depolarized preparations superfused with ZM 241385 (right column, $^{**}P < 0.05$). 100% in A, B and E represents the averaged amplitude of EPPS recorded for 10 min immediately before starting BDNF perfusion, which was 1.4 ± 0.1 mV, $n = 4$ (A, and E left column), 1.4 ± 0.2 mV, $n = 3$ (B ●, and E middle column) and 1.5 ± 0.2 mV, $n = 3$ (B ○, and E right column). Resting membrane potential in all experiments at time zero ranged from −52 to −65 mV.

increase of KCl in the bath and returned to its resting level within 20 min after returning to the normal KCl concentration (5 mM) in the bath. The EPP amplitude values changed during the period of membrane depolarization but a stable value could be again recorded within 30 min after returning to the normal bathing solution. BDNF (20 ng/ml) was added 46 min after the K^+ pulse, therefore after the resting membrane potential and the amplitude of the EPPs became stable again. When applied to innervated diaphragm preparations under these experimental conditions, the neurotrophin caused an increase in EPP amplitude ($18.0 \pm 2.6\%$ increase, $n = 3$, $P < 0.05$; Fig. 1B and E), while being virtually devoid of effect on the resting membrane potential of the muscle fibre (Fig. 1D). As also illustrated in Fig. 1, the enhancement caused by BDNF on EPP amplitude in preparations that had been pre-depolarized by the K^+ pulse was fully prevented ($n = 3$) when A_{2A} adenosine receptors were blocked with ZM 241385, at a concentration (50 nM) that selectively antagonizes A_{2A} receptors in the rat [23] and that by itself was virtually devoid of effect on EPPs ($n = 3$).

To further evaluate the interplay between adenosine A_{2A} receptors and the action of BDNF at the neuromuscular junction, we investigated if the agonist, CGS 21680 at a concentration (10 nM) selective for A_{2A} receptors [15] could trigger a BDNF action even in preparations that had not been pre-depolarized by K^+. CGS 21680 was added to the preparation at least 40 min before BDNF application and caused an increase in EPPs amplitude of $23.0 \pm 4.4\%$ ($n = 7$; Fig. 2A). CGS 21680 (10 nM) was not causing maximum enhancement of neuromuscular transmission since in one experiment it was observed that excitatory effect of CGS 21680 (50 nM) on EPP amplitude was about twice of that observed in the same endplate with CGS 21680 (10 nM). As illustrated in Fig. 2, when BDNF (20 ng/ml) was applied after the full effect of CGS 21680 (10 nM) had been observed, it induced a further enhancement ($18.0 \pm 1.6\%$, $n = 5$, $P < 0.05$) of the EPP amplitude. Both the effect of CGS 21680 (10 nM) as well as the effect of BDNF in the presence of CGS 21680 were prevented by the A_{2A} receptor antagonist, ZM 241385 (50 nM, $n = 3$) (Fig. 2). The enhancement caused by BDNF on EPP amplitude most probably involves Trk phosphorylation since it was

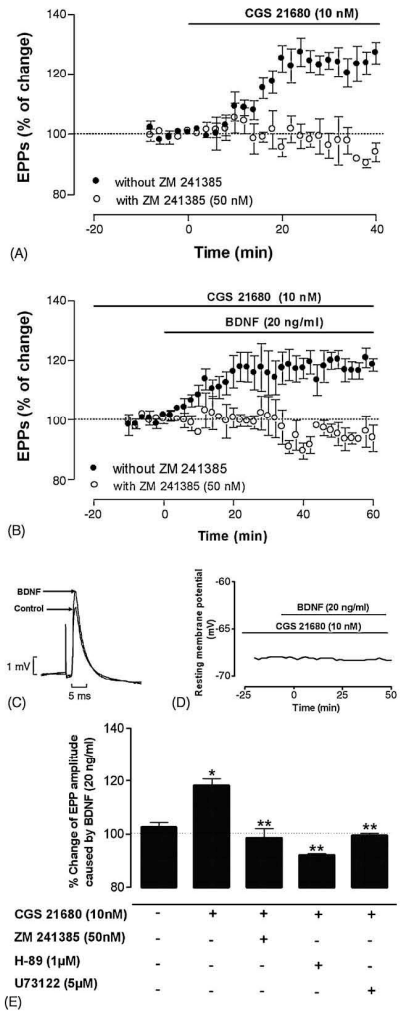

Fig. 2. The excitatory effect of BDNF on EPPs amplitude at the rat diaphragm neuromuscular junction requires PLCγ, through a mechanism that involves A_{2A} receptor and PKA activation. In (A) and (B) are shown the time course of the changes in EPP amplitude induced by CGS 21680 (10 nM) and BDNF (20 ng/ml), respectively, when applied to preparations in the absence (●) or in the presence (○) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM). Panel (C) shows superimposed traces of averaged EPPs recorded from one experiment immediately before (control) and 60 min after addition of BDNF (20 ng/ml). In panel (D) are shown the time course of changes in the resting membrane potential recorded from the same experiment as in (C). Panel (E) shows the averaged increase in EPP amplitude induced by BDNF (20 ng/ml) under the experimental conditions indicated below each column. The change of EPPs amplitude caused by BDNF in the presence of CGS 21680 (10 nM) (second column) was significantly different ($^*P < 0.05$; ANOVA one-way analysis of variance followed by Bonferroni multiple comparisons test) from the effect of BDNF applied alone (first column), an effect prevented ($^{**}P < 0.05$) by: the selective adenosine A_{2A} receptor antagonist, ZM 241385 (50 nM) (third column); the PKAs inhibitor, H-89 (1 μM) (fourth column) or the PLCγ blocker, U73122 (5 μM) (last column). 100% in (A) represents the averaged EPP amplitude recorded for 10 min immediately before addition of CGS 21680 (10 nM) and in (B) to the averaged EPP amplitude recorded for 10 min immediately before addition of BDNF (20 ng/ml) and was 2.2 ± 0.4 mV, $n = 7$ (A), 2.9 ± 0.5 mV; $n = 5$ (B ●, and E second column), 2.3 ± 0.5 mV, $n = 3$ (B ○, and E third column), 1.4 ± 0.1 mV, $n = 4$ (E first column), 3.6 ± 0.2 mV, $n = 3$ (E fourth column) and 1.7 ± 0.5 mV, $n = 3$ (E fifth column). Resting membrane potential in all experiments at time zero ranged from −58 to −71 mV.

abolished (% change caused by BDNF: $-6.0 \pm 5.7\%$, $n = 3$) in the presence of the inhibitor of Trk phosphorylation, K252a (200 nM) [3], which was added to the perfusion solution approximately 25 min after CGS 21680 (10 nM) and 15 min before BDNF (20 ng/ml). BDNF preferentially activates TrkB receptors [19], which are present at the neuromuscular junction [14]. So, the presently observed effect of BDNF is likely due to TrkB receptor activation. K252a (200 nM) did not influence ($P > 0.05$) the excitatory effect of CGS 21680 (10 nM) on EPP amplitude either when applied before ($n = 3$) or after ($n = 3$) the A_{2A} receptor agonist.

Adenosine A_{2A} receptors in most preparations, including the rat neuromuscular junction [9] are coupled to adenylate cyclase. We therefore evaluated how a PKA inhibitor, H-89 [7], would influence the actions of CGS 21680 and BDNF on neuromuscular transmission. H-89 (1 μM) was added 30 min before CGS 21680 (10 nM) and prevented ($P < 0.05$) the excitatory effect of the A_{2A} receptor agonist on EPP amplitude (% change caused by CGS 21680 in the presence of H-89: $3.0 \pm 3.2\%$, $n = 3$). As illustrated in Fig. 2E, the enhancement of EPP amplitude caused by BDNF in the presence of CGS 21680 (10 nM) was also prevented ($P < 0.05$, $n = 3$) by H-89 (1 μM); indeed, in the presence of H-89 + CGS 21680, BDNF even slightly decreased (% change: $-8 \pm 0.4\%$, $n = 3$, $P < 0.05$) rather than increased EPP amplitude.

The phosphorylated TrkB receptor serve as protein interaction sites for adapter protein SCH, phosphotidylinositol-3 kinase (PI-3K) and phospholipase C-γ (PLCγ) [28]. BDNF slow actions, i.e. related to its influence on gene expression and leading to neuronal survival, are probably mediated through SHC [20] and the PI-3K [12]. In contrast, BDNF fast actions are usually related to the recruitment of PLCγ, which induces the cleavage of phosphotidylinositol-4,5-biphosphate to generate inositoltriphosphate (IP3) and diacylglycerol (DAG), therefore inducing the release of Ca^{2+} from internal stores and promoting protein kinase C (PKC) activation [16]. In the present work the PLCγ inhibitor, U73122 (5 μM) [4], added 20 min before CGS 21680 (10 nM), did not influence ($P > 0.05$) the excitatory action of the A_{2A} receptor agonist on EPP amplitude (% change caused by CGS 21680 in the presence of U73122: $26.0 \pm 9.2\%$, $n = 3$), but abolished ($P < 0.05$, $n = 3$) the enhancement of EPP amplitude caused by BDNF (10 ng/ml) in the presence of CGS 21680 (10 nM) (Fig. 2E).

The present results clearly show that pre-depolarizing conditions and A_{2A} receptor activation trigger an excitatory action of BDNF on neuromuscular transmission at the rat innervated diaphragm. The facilitation of the excitatory effect of BDNF caused by depolarization can result from the following mechanisms: (1) recruitment of TrkB receptors to the plasma membrane since in cultures of spinal motor neurons it was reported that within minutes of depolarization, there is a rapid recruitment of TrkB receptors to the plasma membrane by translocation from intracellular stores [21]; (2) increase in cyclic AMP levels, since it was demonstrated that the catalytic unit of adenylate cyclase is sensitive to the membrane potential, being active under depolarization condition [24]; it is also known that cyclic AMP can induce expression of mRNA for TrkB receptors and neurotrophin in primary astroglial cultures [8]; (3) increase in extracellular adenosine levels [17], which in turn can activate A_{2A} membrane receptors and induce an increase in cyclic AMP levels. Depolarization triggers adenosine release [22] and we found that the action of BDNF after a pre-depolarizing pulse was prevented when adenosine A_{2A} receptors are blocked. This suggests that adenosine is a link between depolarization and BDNF actions at the neuromuscular junction. Corroborating this hypothesis is our finding that in the absence of pre-depolarizing conditions, A_{2A} receptor activation with a selective agonist can also trigger the action of BDNF on neuromuscular transmission.

TrkB receptors in cultured hippocampal neurons can be activated by A_{2A} receptor agonists even in the presence of BDNF [18]. This transactivation of TrkB receptors is rather slow being evident only after 2 h of incubation with CGS 21680. The triggering of the excitatory action of BDNF by CGS 21680 observed in the present work occurs faster (within 45 min after CGS 21680 application). Furthermore, the excitatory action of CGS 21680 by itself on EPPs was not influenced by K252a, indicating that CGS 21680 does not facilitate neuromuscular transmission by inducing transactivation of TrkB receptors. A common feature between our results and those by Lee and Chao [18] is that in both cases there is a cross-talk between adenosine A_{2A} receptors and TrkB receptors.

Extracellular adenosine mediates two independent actions on neuromuscular transmission: a fast and easily washable inhibitory action, mediated by A_1 receptors [13,26,27]; and a slower A_{2A} receptor mediated enhancement of EPPs, which as pointed out previously [10] is hardly reversible. The reason for this slower and sustained adenosine A_{2A} receptor mediated action might be the transducing system operated by the receptor, which involves cyclic AMP formation and PKA activation [9] with subsequent protein phosphorylation, whereas in the case of adenosine A_1 receptors, in mammalian nerve terminals, it involves a fast inhibition of calcium entry [25,29]. It is therefore conceivable that the consequences of adenosine A_{2A} receptor activation extend far beyond receptor activation and adenosine removal from the synaptic cleft, whereas the consequences of A_1 receptor activation vanish shortly after dissociation of adenosine from the receptor.

Since BDNF actions are facilitated by cyclic AMP [6,11] and we observed that PKA inhibition prevents both the action of the A_{2A} receptor agonist as well as the effect of BDNF on neuromuscular transmission, one can suggest that after depolarization there is an increase of the extracellular adenosine levels, which activates A_{2A} receptors coupled to adenylate cyclase leading to increase in cyclic AMP formation, followed by PKA activation, which finally facilitates the BDNF action through TrkB receptors. Whether PKA operates by inducing recruitment of TrkB receptors to the plasma membrane [21] or by promoting the ability of TrkB receptors to recruit PLCγ, and consequently to mobilize calcium leading to enhancement of neuromuscular transmission, requires further investigation. The presently observed action of BDNF on neuromuscular transmission most probably involves PLCγ since it was prevented by a PLCγ inhibitor. Interestingly, the PLCγ inhibitor did not influence the excitatory action of the A_{2A} receptor agonist, CGS

21680 on neuromuscular transmission, indicating that PLCγ is not required for the ability of A_{2A} receptors to enhance neuromuscular transmission but is required for the facilitatory action of BDNF.

In conclusion, there is a cross-talk between adenosine A_{2A} receptors and TrkB receptors at the neuromuscular junction, where A_{2A} receptors trigger the excitatory action of BDNF on neuromuscular transmission. This cross-talk involves the cyclic AMP / PKA transducing mechanism, required for the action of A_{2A} receptors and PLCγ, required for the action of BDNF.

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Predominance of Adenosine Excitatory over Inhibitory Effects on Transmission at the Neuromuscular Junction of Infant Rats

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ABSTRACT

Adenosine-induced modulation of neuromuscular transmission in young (3–4-week-old) rats was evaluated. Inhibition of adenosine kinase with iodotubercidin (ITU; 10 μM), which is known to induce adenosine release, enhanced the amplitude of evoked end-plate potentials (EPPs) recorded from innervated diaphragm muscle fibers. This facilitatory effect was transformed into an inhibitory one upon blockade of adenosine A_{2A} receptors with 4-(2-[7-amino-2-(2-furly)[1,2,4]triazolo[2,3-a][1,3,5]triazin5ylamino] ethyl) phenol (ZM 241385) (50 nM); further blockade of adenosine A_1 receptors with the selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 10 nM) abolished that inhibition. Adenosine or 2-chloroadenosine (CADO), at submicromolar concentrations, increased the amplitude and the quantal content of EPPs, whereas at low micromolar concentrations they decreased EPP amplitude. Blockade of A_1 receptors with DPCPX (10 nM) prevented both excitatory and inhibitory effects, whereas blockade of A_{2A} receptors with ZM241385 (50 nM) prevented only the excitatory effects. DPCPX and ZM241385 also prevented the excitatory effect of the selective A_{2A} receptor agonist 2-[*p*-(2-carboxyethyl) phenethylamino]-5′-*N*-ethylcarboxamido adenosine hydrochloride (CGS 21680; 10 nM). CADO (30 nM) also increased neuromuscular transmission in adult (12–16-week-old) rats. It is suggested that at the motor nerve endings, low extracellular concentrations of adenosine activate both A_{2A} and A_1 receptors, but activation of A_{2A} receptors predominates over A_1 receptors; the activity of A_{2A} receptors might, however, require coactivation of A_1 receptors. This facilitatory action of low concentrations of extracellular adenosine upon acetylcholine release may be particularly relevant at developing neuromuscular junctions, where subtle changes in synaptic levels of acetylcholine might influence synaptic stabilization.

It is well established that adenosine decreases the amplitude and the quantal content (q.c.) of evoked endplate potentials (EPPs) at the neuromuscular junctions (NMJ) of adult rats (Ginsborg and Hirst, 1972) and frogs (Ribeiro and Walker, 1975). It is also known that in the same motor nerve ending, both inhibitory adenosine A_1 receptors (A_1Rs) and excitatory adenosine A_{2A} receptors (A_{2A}Rs) coexist, modulating the evoked release of acetylcholine (Correia-de-Sá et al., 1991). The way in which adenosine is able to achieve a balance in the control of neurotransmission depends on the extracellular concentration of the nucleoside, which subsequently depends on extracellular adenosine generation and inactivation (via cellular uptake and/or extracellular deamination) (Sebastião and Ribeiro, 1988). At the rat NMJ, extracellular adenosine can be originated by transport-mediated release (Cunha and Sebastião, 1993), in parallel with its formation from released ATP (Smith, 1991).

Adenosine kinase is an intracellular enzyme that catabolizes adenosine to AMP; therefore, its inhibition leads to enhancement of adenosine release into the extracellular space (Arch and Newsholme, 1978). While exploring the action of endogenous adenosine on neuromuscular transmission (NMT) by using the adenosine kinase inhibitor (AKI) 5′-iodotubericidin (ITU), we observed, as reported in the present work, that instead of inhibition, ITU greatly facilitated NMT. In addition to this effect of ITU, it seemed,

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ABBREVIATIONS: q.c., quantal content; EPP, evoked end-plate potential; NMJ, neuromuscular junction; A_1R, adenosine A_1 receptor; A_{2A}R, adenosine A_{2A} receptor; AR, adenosine receptor; AK, adenosine kinase; NMT, neuromuscular transmission; AKI, adenosine kinase inhibitor; ITU, 5′-iodotubericidin; CADO, 2-chloroadenosine; MEPP, miniature end-plate potential; ADA, adenosine deaminase; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; AICAR, 5-aminoimidazole-4-carboxamide-1-β-*d*-ribofuranoside; ZM 241385, 4-(2-[7-amino-2-(2-furly)[1,2,4]triazolo[2,3-a][1,3,5]triazin5ylamino] ethyl) phenol; CGS 21680, 2-[*p*-(2-carboxyethyl) phenethylamino]-5′-*N*-ethylcarboxamido adenosine hydrochloride; AMPK, AMP-activated protein kinase; ADO, adenosine.

therefore, of interest to reevaluate the excitatory effect of adenosine at the rat NMJ, by using the endogenous ligand adenosine and the stable adenosine analog 2-chloroadenosine (CADO). CADO has approximately the same affinity as the endogenous ligand for adenosine receptors (Ribeiro and Sebastião, 1985), with the advantage of not being metabolized, allowing to evaluate its action at defined concentrations. A brief report on some of the results has been presented previously (Pousinha et al., 2007).

Materials and Methods

The experiments were performed on isolated preparations of the phrenic nerve-diaphragm from male (3–4-week-old) Wistar rats obtained from Harlan Interfauna Iberia, SL (Barcelona, Spain). In some experiments and as mentioned, 12- to 16-week-old rats were used. The animals, handled according to European Community guidelines and Portuguese law on animal care, were killed by decapitation under halothane anesthesia. A strip of the left hemidiaphragm was isolated together with the phrenic nerve and was mounted in a 3-ml Perspex chamber through which the solutions flowed continuously at a rate of 3 ml/min via a roller pump. Except otherwise indicated, the solutions were at room temperature (22–23°C). In some experiments, the perfusion solutions were warmed up to the physiological temperature (36–37°C). The bath volume was kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from one flask to another. This involved a minimum of disturbance to the preparation and allowed prolonged recording from the same fiber with many solution changes. The change-over times in the figures of this article indicate the times at which the inlet tube of the pump was transferred to a new solution. Whenever testing the effect of a drug in the presence of an adenosine receptor (AR) antagonist, superfusion of the antagonist started at least 45 min before addition of another drug to the solution. In all cases, the superfusion of the drugs was only initiated when the recordings were considered stable by online analysis of the results.

EPPs and miniature end-plate potentials (MEPPs) were recorded in the conventional way (Fatt and Katz, 1951), with intracellular electrodes filled with KCl (3 M) and 10- to 20-MΩ resistance, inserted in the motor end-plate. The reference electrode was a silver-silver chloride pellet. The nerve was stimulated supramaximally (rectangular pulses of 20-μs duration applied once every 2 s) through a suction electrode. Individual EPPs, as well as the resting membrane potential of the muscle fiber, were continuously monitored. Muscle fibers with a resting membrane potential less negative than −60 mV were rejected. Throughout the experiment, EPPs and the resting membrane potential were continuously monitored and digitally stored on a personal computer with the Clampex program (pCLAMP 10; Axon Instruments, Foster City, CA). The results were later analyzed off-line, and each 60 consecutive EPPs were averaged. MEPPs were sampled and stored before the application of the drugs at 16 and 30 min and in some experiments 46 min, after starting drug perfusion. Later, the samples were analyzed off-line. MEPPs were detected by an event detection protocol. The threshold for detection of MEPPs was set at the level of 0.35-mV amplitude and 2-ms duration to prevent the contamination of the signal with electric noise. Mean MEPP frequency was measured by counting the number of MEPPs acquired in gap free mode for 100-s periods, at the following times: −10 min (before adding the drug), 0 min (immediately before addition), and 30 min after starting drug perfusion. The values were only considered for posterior analysis whenever MEPP frequency at −10 and 0 min did not differ by more than 5%. The mean amplitude of MEPPs was calculated considering the average of the mean amplitude of 100 consecutive MEPPs. In the calculation of the mean amplitude of the MEPPs, the occasional "giants" (>1 mV) were neglected, because it has been shown that they do not contribute to the components of evoked release (Menrath and Blackman, 1970). A change in MEPP frequency that was not accompanied by a change in MEPP amplitude was interpreted as a change in spontaneous transmitter release (Katz, 1969). Quantal contents were estimated as the ratio of the average evoked response to the average amplitude of the MEPPs recorded during the same period.

The bathing solution contained 117 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 11 mM glucose, 1.5 mM $CaCl_2$, and 1.2 mM $MgCl_2$. It was continuously gassed with 95% O_2 and 5% CO_2, pH 7.4, and kept at room temperature (22–25°C). Muscle twitches were prevented by a submaximal concentration (1.5 μM) of tubocurarine, except where otherwise indicated. In experiments designed to record MEPPs, the muscle twitches were prevented by increasing the bath concentration of Mg^{2+} to 19 mM.

Drugs. CADO; adenosine deaminase (ADA) (type VI, 1803 $U \cdot ml^{-1}$; EC 3.5.44); CGS 21680, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX); ITU; and *d*-tubocurarine chloride were obtained from Sigma/RBI (Natick, MA). ZM241385 and 5-aminoimidazole-4-carboxamide-1-β-*d*-ribofuranoside (AICAR) were supplied by Tocris Bioscience (Ellisville, MO). Stock solutions (5 mM) of CGS 21680, DPCPX, ZM241385, and ITU were prepared in dimethyl sulfoxide. Aliquots of these stock solutions were kept frozen at −20°C until used. The stock solution of ITU was kept in the dark to prevent photodecomposition. The maximal concentration of dimethyl sulfoxide applied to the preparations [0.02% (v/v)] was devoid of effect on EPPs.

The data are expressed as mean ± S.E.M. from n experiments. To allow comparisons between different experiments, the EPP amplitude, as well as the MEPP frequency or amplitude and the quantum content, were normalized, with 100% determined as the averaged values obtained during 10 min immediately before applying the test drug. The significance of the differences between means was evaluated by Student's t test, when only two means were compared, or by one-way analysis of variance followed by the Tukey's test whenever multiple comparisons were made. Values of $P < 0.05$ were considered to represent statistically significant differences.

Results

Endogenous Adenosine

Effects of 5′-Iodotubericidin. Figure 1 illustrates the effect of the AKI ITU (10 μM) on the amplitude of EPPs. As shown, 40 min after its application, ITU enhanced the amplitude of EPPs by 25 ± 6.0% (n = 4; Fig. 1B), whereas it was virtually devoid of effect on the resting membrane potential of the muscle fiber (Fig. 1A). This effect was sustained during the period of application of ITU and EPPs amplitude did not return to the baseline within 30 min after reperfusion with the normal bathing solution (Fig. 1A). At lower concentration (1 μM) ITU caused a smaller (15 ± 1.8%; n = 3) increase in the amplitude of EPPs.

To evaluate whether this excitatory effect could be attributed to activation of A_{2A}Rs by released adenosine, ITU (10 μM) was perfused in the presence of the A_{2A}R-selective antagonist ZM241385 (Poucher et al., 1995). In these conditions, the excitatory effect of ITU was transformed into an inhibitory effect (−28 ± 2.4%; n = 3; Fig. 1C), which can be related with A_1R activation by released adenosine, because it was prevented by the adenosine A_1R-selective antagonist DPCPX (10 nM). Surprisingly, in the presence of DPCPX, ITU (10 μM) failed to enhance NMT (1 ± 1.8%; n = 6; Fig. 1D), indicating a possible interplay between high affinity A_1Rs and A_{2A}Rs. To further evaluate the interaction between these ARs, we investigated whether the A_1Rs antagonist DPCPX (10 nM) could prevent the effect of the A_{2A}R-selective agonist CGS 21680 (Jarvis et al., 1989). As illustrated in the

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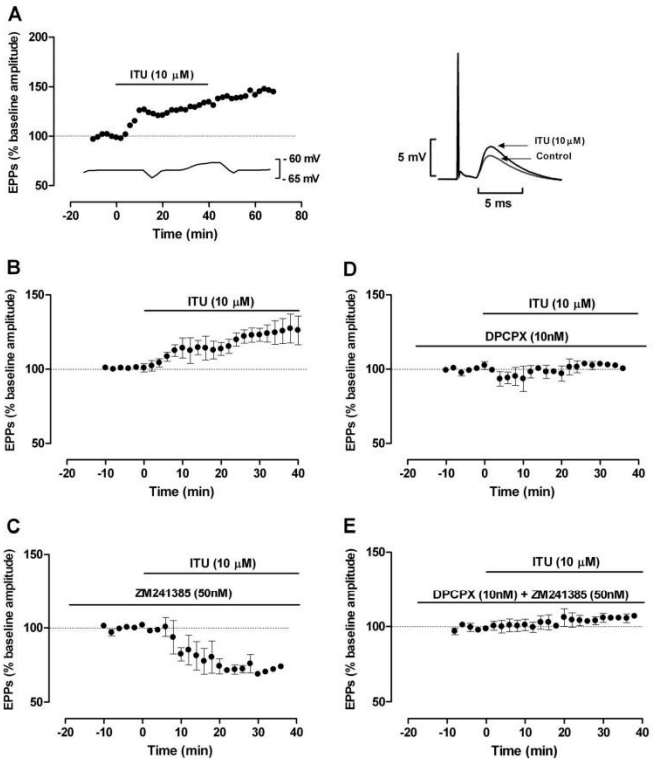

Fig. 1. Adenosine kinase inhibition influence the amplitude of evoked EPPs recorded from a muscle fiber of the rat diaphragm. A, time course of the changes in EPP amplitude and in the resting membrane potential caused by the adenosine kinase inhibitor ITU (10 μM), in one experiment. Bottom, superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ITU (10 μM). B, averaged change in the amplitude of EPPs caused by ITU (10 μM) in four experiments. C to E, averaged changes in the amplitude of EPPs caused by ITU (10 μM), when it was applied in the presence of the selective antagonist of adenosine A_{2A} receptors ZM 241385 (50 nM) (C), in the presence of the selective antagonist of adenosine A_1 receptors DPCPX (10 nM) (D), and in the presence of the coadministered selective antagonist of adenosine A_1 receptors DPCPX (10 nM) with the selective antagonist of adenosine A_{2A} receptors ZM 241385 (50 nM) (E). In the ordinates, 100% represents the averaged amplitude of EPPs recorded for 10 min immediately before starting ITU perfusion: 3.2 mV, $n = 1$ (A); 3.9 ± 0.7 mV, $n = 4$ (B); 3.7 ± 1.2 mV, $n = 3$ (C); 3.2 ± 1.4 mV, $n = 4$ (D); and 1.3 ± 0.2 mV, $n = 3$ (E). Each point represents the averaged amplitude of 60 successive EPPs. The horizontal bars indicate the period of drugs perfusion. Resting membrane potential in all experiments at time 0 (i.e., before ITU addition) ranged from −60 to −72 mV. Solutions contained a submaximal concentration (1.5 μM) of tubocurarine, which decreased EPP amplitude below threshold of action potential generation therefore preventing muscle twitches in response to nerve stimulation.

Fig. 2, this agonist enhanced EPP amplitude (23 ± 4.4%; $n = 7$), an effect abolished (0 ± 3.2%; $n = 6$) by the A_1R antagonist DPCPX. As expected, the $A_{2A}R$ antagonist ZM241385 (50 nM) also abolished the facilitatory action of CGS 21680 (10 nM) on EPP amplitude.

To further confirm that the action of ITU on NMT are due to changes in the extracellular levels of adenosine, experiments were performed in the presence of both adenosine A_1R and $A_{2A}R$ antagonists DPCPX (10 nM) and ZM241385 (50 nM), which were added together to the preparations before addition of ITU (10 μM). Under these conditions, and as expected, ITU did not modify the amplitude of EPPs (Fig. 1E).

The above-described effects of ITU upon NMT suggest that by enhancing extracellular adenosine it is possible to induce a long-lasting facilitation of NMT due to $A_{2A}R$ activation. This long-lasting facilitatory effect is not reverted by inactivation of extracellular adenosine with ADA (2 U/ml), an enzyme that converts adenosine into inosine (Arch and Newsholme, 1978); thus, in four experiments where ADA (2 U/ml) was perfused after the full excitatory effect of ITU (10 μM), the amplitude of EPPs did not decrease toward pre-ITU levels. However, when ADA (2 U/ml) was applied before ITU (10 μM), a time lag of approximately 10 min occurred before any facilitation of NMT started to be observed. After this time lag, the amplitude of EPPs started to increase probably because the rate of adenosine production started to be faster than the rate of adenosine deamination by the concentration of ADA used (2 U/ml). Higher concentrations of ADA were not tried to avoid the risk of nonenzymatically related actions (Cunha et al., 1996a).

When applied alone, ADA (2 U/ml) caused only a slight decrease in the amplitude of EPPs (−6.8 ± 1.8%; $n = 5$; $P < 0.05$). The selective $A_{2A}R$ antagonist ZM241385 (50 nM) or the selective A_1R antagonist DPCPX (10 nM) did not modify the amplitude of EPPs (3 ± 6.7%, $n = 5$; and 0 ± 5.5%, $n = 7$, respectively).

ITU has been reported to inhibit AMP-activated protein kinase (AMPK) (Musi et al., 2001). To evaluate whether inhibition of this enzyme could mimic the effect of ITU on NMT, we tested the AMPK activator, 5-aminoimidazole-4-

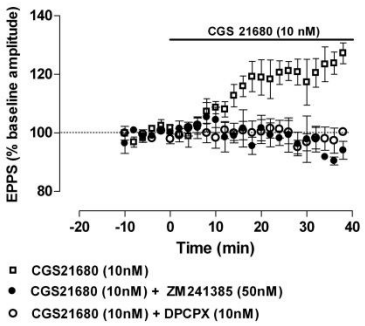

Fig. 2. The selective A_{2A} receptor agonist enhances the amplitude of EPPs, an action blocked by either A_1 or A_{2A} receptor-selective antagonists. The time course of the changes in EPP amplitude induced by the A_{2A} receptor agonist CGS 21680 (10 nM) when applied to preparations in the absence (□) or in the presence (○) of the selective adenosine A_1 receptor antagonist DPCPX (10 nM), or in the presence (●) of the selective adenosine A_{2A} receptor antagonist ZM241385 (50 nM) is shown. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CGS 21680 (10 nM): 2.2 ± 0.4 mV, $n = 7$ (□); 2.5 ± 0.6 mV, $n = 4$ (○); and 1.6 ± 0.5 mV, $n = 6$ (○). Resting membrane potential in all experiments at time 0 (i.e., before CGS 21680 addition) ranged from −60 to −72 mV. Perfusing solutions contained tubocurarine (1.5 μM).

carboxamide-1-β-*d*-ribofuranoside (AICAR). However, this compound, even at a lower concentration (300 μM) than that usually used to activate AMPK (1 mM; Giri et al., 2004), caused a marked hyperpolarization (by approximately 10 mV); therefore, any changes in EPP amplitude were masked by this postsynaptic effect. When used at 1 mM, AICAR caused a similar (~10 mV) membrane hyperpolarization. Because this marked hyperpolarization was not mimicked by any adenosine receptor agonist or by ITU, no further protocols were designed with AICAR. Membrane hyperpolarization caused by submillimolar concentrations of AICAR is probably due to changes in membrane K^+ conductance (Klein et al., 2009).

Facilitation by Exogenous Adenosine

As seen in Fig. 3, adenosine (300 nM) enhanced NMT by 38 ± 6.8% ($n = 5$), an effect that was not diminished in the next 30 min after stopping adenosine perfusion (Fig. 3A). This excitatory effect of a submicromolar concentration of adenosine was consistently observed (Fig. 3B) and was antagonized by the selective A_{2A}R antagonist ZM241385 (50 nM; Fig. 3C). When concentrations of adenosine above 10 μM were applied to the preparations, the amplitude of EPPs clearly decreased, in accordance with what has been described previously (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). To evaluate whether adenosine A_{2A}Rs were fully activated in the presence of adenosine concentrations that induced a decrease in

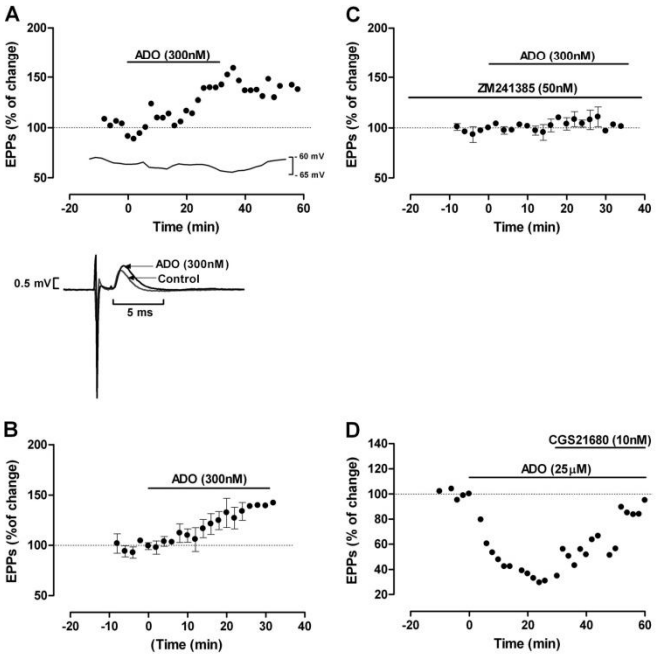

Fig. 3. At submicromolar concentrations, adenosine enhances the amplitude of evoked EPPs. A to C, time course of the changes in EPP amplitude induced by ADO (300 nM) when applied to preparations in the absence (A and B) or in the presence (C) of the selective adenosine A_{2A} receptor antagonist ZM 241385 (50 nM). A, time course of the changes in EPP amplitude and in the resting membrane potential caused by ADO (300 nM) in one experiment. Bottom, superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after the addition of ADO (300 nM). D, effect of the selective agonist of adenosine A_{2A} receptor CGS 21680 when applied in the presence of a concentration of ADO (25 μM), which markedly inhibited the amplitude of EPPs. Each point represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of the drugs indicated above the bars: 0.8 mV, $n = 1$ (A); 1.7 ± 0.3 mV, $n = 5$ (B); 3.4 ± 0.8 mV, $n = 3$ (C); and 3.0 mV, $n = 1$ (D). Resting membrane potential in all experiments at time 0 (i.e., before drug addition) ranged from −63 to −72 mV. Perfusing solutions contained 19 mM Mg^{2+}, which prevented muscle action potentials and twitches in response to nerve stimulation.

the amplitude of EPPs, we applied the selective $A_{2A}R$ agonist CGS 21680 to the bath solution after the full inhibitory effect of adenosine (25 μM) was observed. In these conditions, CGS 21680 induced an increase in the amplitude of EPPs (Fig. 3D), indicating that adenosine (25 μM) might not be able to completely activate $A_{2A}Rs$ or that CGS 21680 is more efficient than adenosine itself to attenuate A_1R functioning. Because CGS 21680 has higher selectivity and affinity for $A_{2A}Rs$ than adenosine itself, it is plausible that it more efficiently attenuates A_1R-mediated inhibition (Cunha et al., 1994) than the endogenous agonist adenosine, which activates both A_1 and A_{2A} receptors. Thus, A_{2A} receptors at the neuromuscular junction seem to start to be activated by very submicromolar adenosine concentrations, but maximal recruitment of A_{2A} receptors by adenosine seems to be achieved only with very high adenosine concentrations, which hardly occur under physiological conditions. Indeed, under physiological conditions neuromodulatory receptors are usually not fully recruited by their endogenous ligands.

Figure 4 summarizes the results from experiments where MEPPs and EPPs were recorded simultaneously with Mg^{2+} (19 mM)-paralyzed preparations, to evaluate changes in the q.c. of EPPs. Adenosine (300 nM) increased the frequency of MEPPs ($n = 3$) by 40 ± 2.5%, without changing its average amplitude. The q.c. of EPPs in the presence of adenosine (300 nM) was increased by 55 ± 9.7% ($n = 3$). This facilitation caused by submicromolar concentrations of adenosine contrasts with the inhibitory action of adenosine at micromolar concentrations that inhibited both the MEPP frequencies and q.c. of EPPs (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975).

Effect of CADO

Because adenosine is subject to uptake and enzymatic inactivation, we decided to explore the action of the stable analog CADO, which is not a substrate for adenosine deaminase (e.g., Daly, 1982) and has low affinity for the adenosine uptake system (Jarvis et al., 1985). The concentrations of CADO required to obtain effects similar to those of adenosine were approximately 10 times lower. Indeed, as observed with 300 nM adenosine, CADO (30 nM) increased the average amplitude of EPPs (41 ± 7.8%; $n = 7$, Fig. 5). As observed with ITU and adenosine, the effect remained for at least 20 min after starting the washing out CADO (Fig. 5A).

Most of the experiments now reported have been performed at room temperature (~22°C) to minimize electrical noise and to increase biological viability, compatible with long-lasting protocols. However, to evaluate whether the unexpected long-lasting excitatory effect of low concentrations of an AR ligand, such as CADO, could be influenced by temperature, four experiments were performed at 36°C, and the results compared with those obtained in similar experiments performed at room temperature with similar periods of CADO application and washout. As illustrated in Fig. 5C, the facilitatory effect of CADO (30 nM) upon EPP amplitude was not influenced by the bath temperature, within the physiological limits.

The excitatory effect of CADO (30 nM) was observed either in the presence of tubocurarine (1.5 μM; Fig. 5, A and C), that is, when the q.c. of EPPs is high, or in the presence of high Mg^{2+} (19 mM; Fig. 5D), that is, when quantal contents are low. CADO increased the frequency of MEPPs by 39 ± 12.2% ($n = 3$; Fig. 6) without changing by more than 2% the average amplitude of MEPPs recorded in the same period. This facilitatory effect on asynchronous release was of similar magnitude in preparations where the motor nerve was stimulated once every 2 s (39 ± 12.2%; $n = 3$), or in the absence of stimulation (35 ± 3.2%; $n = 2$). CADO (30 nM) did not activate maximally the $A_{2A}Rs$, because the selective agonist of $A_{2A}Rs$, CGS 21680, when applied after the full effect of CADO, caused a further enhancement of the EPP amplitude (Fig. 5D).

Figure 7A illustrates the concentration-response curve for the effects of CADO (10 nM–10 μM) on amplitude of EPPs, which is noticeably biphasic, with excitatory effects at concentrations between 10 and 100 nM and the maximal excitatory effect being obtained with 30 nM CADO. At the concentration of 1 μM, CADO did not affect the amplitude of EPPs, probably due to a balanced A_1/A_{2A} receptor activation, whereas at a higher concentration, CADO (10 μM) decreased the amplitude of EPPs.

The $A_{2A}R$ antagonist ZM241385 (50 nM) prevented the excitatory effect of CADO (10–100 nM; $n = 6$) but did not influence the inhibition of a higher concentration of CADO (10 μM) (Fig. 7A). It is interesting that in the presence of the $A_{2A}R$ antagonist ZM241385 (50 nM; Fig. 7B), there was a clear inhibitory effect of CADO at concentrations (30–300 nM) that in the absence of ZM241385 (50 nM) caused a facilitation of NMT. Furthermore, in the presence of the $A_{2A}R$ antagonist, there was a clear inhibition of EPPs caused by 1 μM CADO. This indicates that at these concentrations the agonist is acting upon both A_1 and A_{2A} receptors.

As shown in Fig. 7, A and C, the A_1R antagonist DPCPX (10 nM) at a concentration only 20 times higher than its K_i value for A_1Rs in the same preparation (Sebastião et al., 1990) and well below its K_i value for $A_{2A}Rs$ (Jacobson et al., 1992), greatly attenuated both the excitatory and the inhibitory effects of CADO (10 nM–10 μM).

Previous studies on the inhibitory effect of adenosine or CADO on EPPs were performed in adult rats. To allow comparisons and to evaluate whether the now described excitatory action also occurs in adult rats, we tested the effect of CADO (30 nM) in 12- to 16-week-old rats and, as shown in Fig. 8, it increased the amplitude of EPPs by 11 ± 1.0% ($P < 0.05$; $n = 6$). This effect was smaller ($P < 0.05$) than the one observed in infant rats, but it can also be attributed to $A_{2A}R$ activation because it was prevented by the selective $A_{2A}R$ antagonist ZM241385 (50 nM; Fig. 8B). As it occurred in infant rats, the excitatory action of 30 nM CADO was also prevented by the A_1R antagonist DPCPX as well as by the $A_{2A}R$ antagonist ZM241385 (50 nM; Fig. 8).

Discussion

The main findings of the present work were that submicromolar concentrations of adenosine or CADO increase the amplitude of EPPs. These effects were clearly due to $A_{2A}R$, because they were antagonized by the selective $A_{2A}R$ antagonist ZM241385. The inhibitory effects, obtained with micromolar concentrations of adenosine or CADO, were related to activation of A_1R and antagonized by the A_1R receptor antagonist DPCPX.

The enhancement of NMT caused by adenosine or CADO results from an increase of the evoked release of acetylcho-

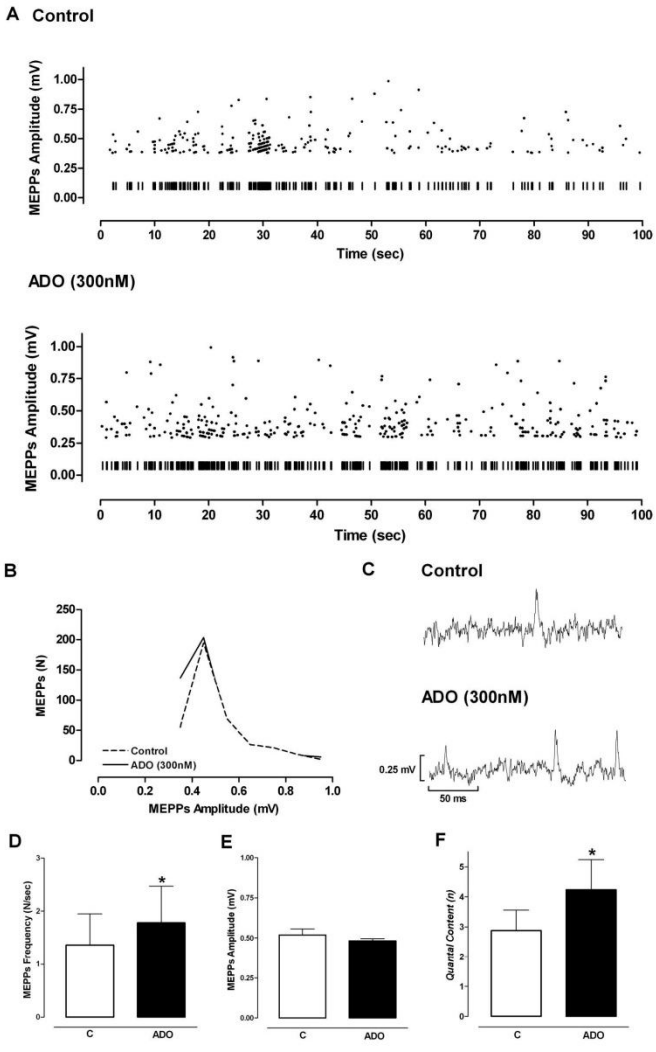

Fig. 4. Adenosine, at a submicromolar concentration, enhances the frequency and amplitude of MEPPs and the quantal content of EPPs. A, spontaneous events recorded in gap free mode across 100 *s* before (control, top) and 30 min after ADO (300 nM) perfusion (bottom) in one experiment. Each point in A represents the amplitude of a single MEPP, and each vertical trace immediately above the ordinates indicates the occurrence of a MEPP. The resting membrane potentials were −62 to −61 mV throughout the experiment. B, MEPP amplitude distribution recorded in the same experiment over the same period of time as shown in A. The event detection was settled to signals with amplitude ranging from 0.35 to 1 mV and minimal duration of 2 ms. C, representative samples of MEPPs recordings. D to F, averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of adenosine (300 nM) (*, $P < 0.05$, Student's *t* test). Perfusing solutions contained 19 mM Mg^{2+}.

line, because they increased the q.c. of EPPs without affecting the average amplitude of MEPPs recorded concomitantly. Even in the absence of electrical stimulation, CADO increased the frequency of MEPPs, suggesting a facilitatory effect on spontaneous asynchronous release. Similar effects were observed either in experiments where the muscle twitches were prevented with tubocurarine (high q.c.) or where muscle twitches were prevented by high Mg^{2+} (low q.c.). So, the initial q.c. did not influence the facilitatory effect of adenosine and precludes the possibility that the high Mg^{2+} concentration influenced the action of adenosine or CADO, due to its ability to increase agonist binding (Yeung et al., 1985).

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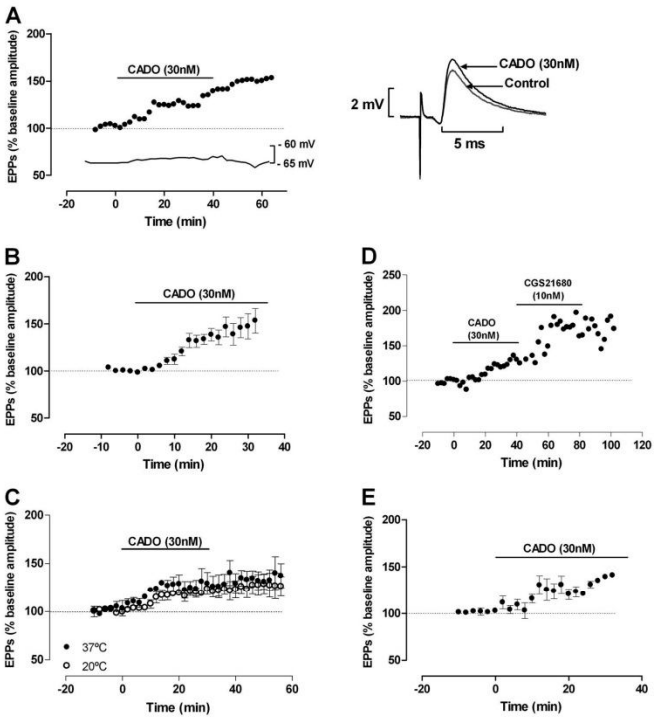

Fig. 5. At submicromolar concentrations, CADO enhances the amplitude of evoked EPPs. A, time course of the changes in EPP amplitude and in the resting membrane potential caused by CADO (30 nM) in one endplate paralyzed with tubocurarine (1.5 μM). Bottom, superimposed traces of averaged EPPs recorded from the same experiment, immediately before (control) and 30 min after addition of CADO (30 nM). B and E, averaged changes in the amplitude of EPPs caused by CADO (30 nM) in experiments where muscle twitches were prevented by 1.5 μM tubocurarine (B) or 19 mM Mg^{2+} (E). C, time course of the averaged changes in EPP amplitude induced by CADO (30 nM) in experiments performed at room temperature (○) and at a more physiological (37°C) temperature (●); muscle twitches were prevented with tubocurarine (1.5 μM). D, effect of the selective adenosine A_{2A} receptor agonist when applied after the full excitatory effect of CADO (30 nM); muscle twitches were prevented with tubocurarine (1.5 μM). Each point, in all panels, represents the averaged amplitude of 60 successive EPPs. In the ordinates, 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30 nM): 3.7 mV, n = 1 (A); 2.4 ± 0.3 mV, n = 7 (B); 2.0 ± 0.5 mV, n = 3 (C); 2.0 mV, n = 1 (D); and 1.1 ± 0.1 mV, n = 3 (E). Resting membrane potential in all experiments at time 0 (i.e., before drug addition) ranged from −62 to −76 mV.

It is well established that AKIs, such as ITU, enhance extracellular adenosine, which activates ARs (Kowaluk et al., 1998). The excitatory effect of ITU was similar to that observed with nanomolar concentrations of adenosine or CADO, and the excitatory effects of these drugs were antagonized by preincubation with the A_{2A}R antagonist ZM241385, transforming those effects into inhibitory ones. This suggests that the influence of ITU, CADO, and adenosine upon NMT results from a balance between activation of adenosine excitatory A_{2A}Rs and inhibitory A_1Rs.

The reversibility of A_1R-mediated inhibition of NMT, together with absence of DPCPX effect on NMT, suggests that at the NMJ of infant rats, A_1Rs are not tonically activated by endogenous adenosine. In contrast, it was not possible to wash out the A_{2A}R-mediated effects, which renders it difficult to evaluate whether A_{2A}Rs were tonically influenced by endogenous adenosine. For slowly reversible actions, one would not expect that removing the endogenous ligand or preventing its binding to the receptor with a competitive antagonist added in the presence of the endogenous agonist, would result in a rapid decrease in response. Because enhancement of extracellular adenosine with ITU, or A_{2A}R activation with the selective agonist CGS 21680, facilitates NMT, one may infer that the levels of extracellular adenosine under basal conditions should be low and not enough to fully saturate A_{2A}R.

The facilitatory effects of adenosine, CADO, or even ITU were not washed out, which was unexpected because dissociation of adenosine or CADO from its receptor is known to be fast. Adenosine is metabolically unstable, being taken up by cells and substrate for several enzymes. However, reversibility of the ligand/receptor interaction or inactivation of the ligand (as it occurs with adenosine) does not necessarily imply reversibility of the effect triggered by receptor activation. A_{2A}Rs at phrenic motor nerve endings are positively coupled to adenylate cyclase/cAMP/protein kinase A transducing system (Correia-de-Sá and Ribeiro, 1994). Therefore, PKA-dependent phosphorylation and its consequence upon neurotransmitter release may influence synaptic transmission, although the receptors might no longer be activated by the ligand. The possibility of a long-lasting A_{2A}R activation is reinforced by the observation that the facilitatory action of ITU was not reversed by adding ADA after the full effect of ITU. As expected, the effect of ITU was delayed by ADA, if added before, and antagonized by preincubation with an A_{2A}R antagonist. It seems that once triggered by A_{2A}R acti-

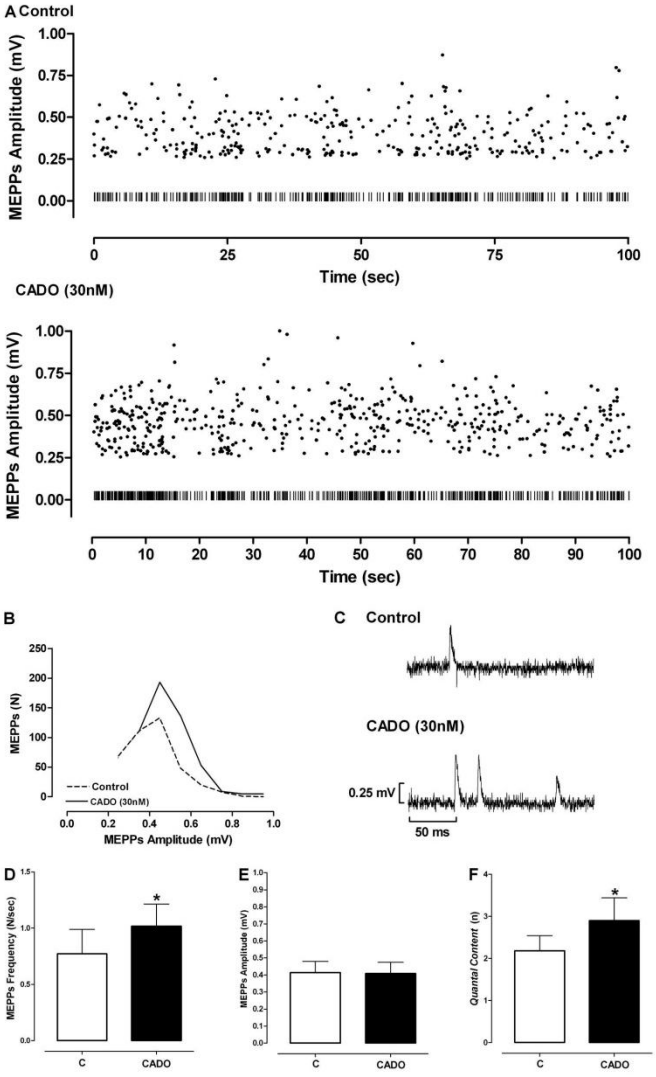

Fig. 6. 2-Chloroadenosine, at a submicromolar concentration, enhances the frequency and amplitude of MEPPs and the quantal content of EPPs. A, spontaneous events recorded in gap free mode across 100 s before (control, top) and 30 min after CADO (30 nM) perfusion (bottom) in one experiment. Each point in A represents the amplitude of a single MEPP, and each vertical trace immediately above the ordinates indicates the occurrence of a MEPP. The resting membrane potentials were from −63 to −64.5 mV throughout the experiment. B, MEPP amplitude distribution recorded in the same experiment over the same period of time as shown in A. The event detection was settled to signals with amplitude ranging from 0.35 to 1 mV and minimal duration of 2 ms. C, representative samples of MEPPs recordings. D to F, averaged frequency (D) and amplitude (E) of MEPPs, as well as quantal content (F) of EPPs in the absence (open bars) or in the presence (filled bars) of CADO (30 nM) (*, $P < 0.05$; Student's t test). Perfusing solutions contained 19 mM Mg^{2+}.

vation, the ongoing intracellular mechanisms cause a long-lasting increase in synaptic strength, which may share some mechanisms (e.g., cAMP/protein kinase A-dependent phosphorylation events) with plasticity phenomena at synapses of the central nervous system.

Blockade of A_{2A}Rs unmasked the inhibitory actions of low CADO concentrations but did not exacerbate the inhibitory action of a high concentration. Desensitization of A_{2A}Rs cannot account for this apparent lack of effect, because the selective A_{2A}R agonist CGS 21680 was still able to facilitate

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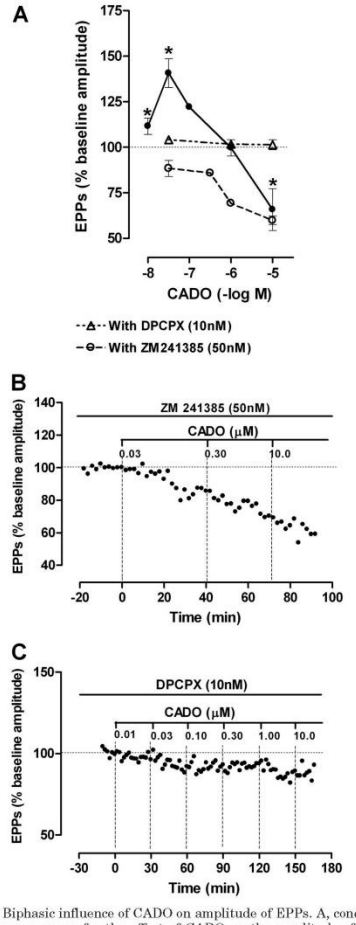

Fig. 7. Biphasic influence of CADO on amplitude of EPPs. A, concentration-response curve for the effect of CADO on the amplitude of EPPs. Each point is the average ± S.E.M. of results obtained in three to five noncumulative experiments, except for 100 nM CADO (●) where $n = 1$, and for 200 nM CADO (○), which was applied after 30 nM CADO. *, $P < 0.05$ (Student's t test) compared with absence of CADO (100%). B, time course of the changes in EPP amplitude induced by CADO (30 nM–10 μM) in one experiment where it was applied in the presence of the selective antagonist of adenosine A_{2A} receptors ZM241385 (50 nM); note that under this condition, submicromolar concentrations of CADO caused an inhibition of EPPs. C, time course changes in EPP amplitude caused by CADO applied in the presence of the selective antagonist of A_1 receptors DPCPX (10 nM); note that under this condition, CADO caused little or no effect on EPP amplitude. Averaged changes in the EPP amplitude caused by 30 nM or 10 μM CADO in the presence of 10 nM DPCPX (△) or 50 nM ZM241385 (○) are shown in A. Each point in B and C represents the averaged amplitude of 60 successive EPPs recorded from one experiment. In all panels, 100% in the ordinates represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO: 2.29 ± 0.1 mV, $n = 19$ (A; ●); 2.21 ± 0.3 mV, $n = 14$ (A; ○); 2.43 ± 0.4 mV, $n = 12$ (A; △); 2.4 mV, $n = 1$ (B); and 2.1 mV, $n = 1$ (C). Perfusion solutions contained tubocurarine (1.5 μM).

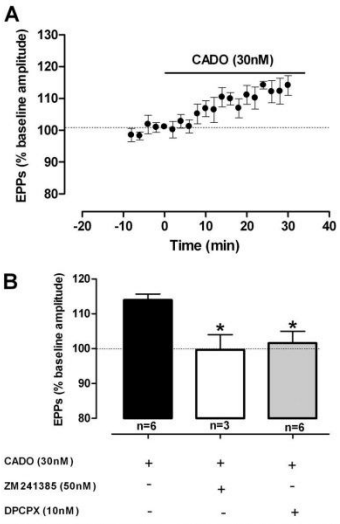

Fig. 8. CADO (30 nM) also enhances the amplitude of evoked EPPs in adult (12–16-week-old) rats. A, time course of the changes in EPP amplitude induced by CADO (30 nM); each point represents the averaged amplitude of 60 successive EPPs. B, averaged changes in EPP amplitude induced by CADO (30 nM) under the experimental conditions indicated below each column. Note that the increase in EPPs amplitude caused by CADO (30 nM) alone (first column from left) was prevented by the selective adenosine A_{2A} receptor antagonist ZM241385 (50 nM) (second column) and by the selective adenosine A_1 receptor antagonist DPCPX (10 nM) (third column) (*, $P < 0.05$ (one-way analysis of variance followed by Tukey's multiple comparisons test) compared with first column. The 100% represents the averaged EPP amplitude recorded for 10 min immediately before addition of CADO (30 nM) and was 2.3 ± 0.4 mV, $n = 6$ (A and first column in B); 2.9 ± 1.3 mV, $n = 4$ (second column in B); and 2.3 ± 1.1 mV, $n = 6$ (third column in B). Resting membrane potential in all experiments at time 0 (i.e., before drug addition) ranged from −62 to −75 mV. Perfusion Solutions contained 19 mM Mg^{2+}.

NMT when applied in the presence of adenosine (25 μM). It seems that as the concentration of CADO increases (nanomolar to micromolar), there is a shift from a preponderant $A_{2A}R$ activation toward an apparent exclusive A_1R activation. This suggests that at the motor nerve endings of infant rats, the $A_{2A}R$ has higher affinity and/or higher expression levels than the A_1R. Once activated, $A_{2A}R$ might inhibit the actions of A_1Rs at low A_1 receptor occupancy. With high concentrations of adenosine, A_1Rs would become widely activated, leading to a predominance of the inhibitory effect. Whether these results fit into an $A_1R/A_{2A}R$ heterodimerization model (Franco et al., 2008), with a main influence of the A_{2A} binding site at low receptor occupancy, and a main influence of the A_1 binding site at high occupancy conditions, could not be directly assessed in the present work. Whatever the molecular mechanisms behind the present results, they

challenge the previous concept on predominant A_1R activation at low agonist concentrations and $A_{2A}R$ activation at higher concentrations of adenosine (Correia-de-Sá and Ribeiro, 1996; Franco et al., 2008).

In the present work, the $A_{2A}R$-mediated facilitation of NMT was antagonized not only by an $A_{2A}R$-selective antagonist but also by an A_1R-selective antagonist; this contrasts with the A_1R mediated inhibition of NMT, which was prevented by the A_1R antagonist but not by $A_{2A}R$ antagonist. The inhibitory effect of the $A_{2A}R$ agonist CGS 21680 in micromolar concentrations at both the hippocampus (Lupica et al., 1990) and NMJ (Correia-de-Sá et al., 1991) is probably due to A_1R activation. However, the presently observed excitatory effects seen with low nanomolar concentrations of CGS 21680 are well below its K_i value for A_1R (Jacobson et al., 1992). The observation that DPCPX displaces the specific binding of low nanomolar concentrations of CGS 21680 at hippocampal membranes (Cunha et al., 1996b) led to the suggestion that $A_{2A}R$ at the hippocampus and striatum differ slightly in pharmacological characteristics. In addition, the finding that facilitation of hippocampal synaptic transmission by a low nanomolar concentration of CGS 21680 requires coactivation of A_1R led to the proposal that the $A_{2A}R$-mediated facilitation of transmission results from attenuation of the tonic A_1R-mediated inhibition (Lopes et al., 2002). Indeed, in the hippocampus, synaptic transmission is under intense tonic inhibition by adenosine that activates A_1Rs. This was not observed with the NMJ (this study), where the A_1R antagonist DPCPX did not affect transmission. Another explanation for the loss of $A_{2A}R$-mediated effects upon A_1R blockade is that $A_{2A}Rs$ and A_1Rs form heteromers, as it happens at glutamatergic terminals (Ciruela et al., 2006). So, blockade of the A_1R could induce a conformational change in the heteromer that does not allow either $A_{2A}R$ agonist binding or effective coupling to the $A_{2A}R$ transducing system. Indeed, heteromerization of G protein-coupled receptors may affect receptor functioning (Franco et al., 2008), because monomers and heteromers can have distinct pharmacological and signaling properties. In addition, $A_1R/A_{2A}R$ cross-talk through the intracellular transducing pathways that are downstream to receptor binding (Sebastião and Ribeiro, 2000) might also explain the need for A_1R function to trigger $A_{2A}R$-mediated response at the motor nerve endings. These two possibilities are not mutually exclusive.

Previous work (Correia-de-Sá and Ribeiro,1996), also performed at the rat diaphragm NMJ, demonstrated predominance of the tonic $A_{2A}R$-mediated facilitatory effect on [^{3}H]acetylcholine release, if high frequency stimulation (50 pulses/s) is used. Because ATP is released together with acetylcholine (Silinsky, 1975) and is quickly degraded extracellularly to adenosine (Cunha and Sebastião, 1991), extracellular accumulation of considerable amounts of adenosine is expected due to high frequency neuronal firing. Adenosine derived from high frequency-induced ATP release preferentially activates $A_{2A}Rs$ (Cunha et al., 1996a). As we now report, at the NMJ of infant rats the concentration of adenosine that accumulates extracellularly due to AKI. We also showed that lower concentrations of adenosine are required to observe excitatory instead of inhibitory effects. This high sensitivity of $A_{2A}R$, along with the consistent inhibitory effect of micromolar concentrations of the nucleoside, explains why previous studies using concentrations of adenosine above 1 μM did not detect facilitatory actions of adenosine on EPPs (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Age-related differences cannot account for the results obtained, because in adult rats the facilitatory effect of CADO (30 nM) predominates over the inhibitory effect.

In conclusion, the work now reported demonstrates that both A_1Rs and $A_{2A}Rs$ at the motor nerve endings of infant rats can be activated by low extracellular concentrations of adenosine whereas at nanomolar concentrations, activation of excitatory $A_{2A}Rs$ predominates. This challenges previous ideas that the predominant action of adenosine at this NMJ was inhibitory at low frequencies of neuronal firing (Ginsborg and Hirst, 1972; Ribeiro and Walker, 1975). Facilitatory actions upon spontaneous and evoked acetylcholine release under low neuronal firing may have particular relevance at developing NMJs, where subtle changes in synaptic levels of acetylcholine, and therefore of nicotinic receptor activation, might influence synaptic stabilization.

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Neuromuscular transmission modulation by adenosine upon aging

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Abstract

In infant rats adenosine A_{2A} receptor-mediated modulation of neuromuscular transmission predominates over A_1 receptor-mediated neuromodulation. We investigated whether aging affects this A_{2A}/A_1 receptor balance. Evoked (EPPs) and miniature end plate potentials (MEPPs) were recorded from single fibers of (weeks-old) infant (3–4), young adult (12–16), older (36–38), and aged (80–90) male rat-diaphragm. The non A_1/A_{2A} selective agonist, 2-chloroadenosine (CADO; 30 nM) and the adenosine kinase inhibitor, iodotubericidin (ITU; 10 μM) increased mean amplitude and quantal content of EPPs in infant, young adult, and older adult rats, but not in aged rats. The facilitatory effects were prevented by the A_{2A} receptor antagonist, ZM241385 (50 nM) and mimicked by the A_{2A} receptor agonist, CGS21680 (10 nM). The A_1 receptor agonist, 6-cyclopentyladenosine (CPA; 100 nM), decreased EPPs amplitude in all age groups. It is concluded that aging differently influences adenosine A_1 receptor and A_{2A} receptor-mediated presynaptic modulation of neuromuscular transmission, so that the facilitatory influence decreases upon aging, whereas the inhibitory influence remains unchanged in aged animals. The reduction of adenosine A_{2A} receptors upon aging may contribute to the age-related changes in neuromuscular function.

Keywords: Aging; Adenosine; Adenosine receptors; Neuromuscular junction; Neuromuscular transmission; EPPs and MEPPs

1. Introduction

It is well established that adenosine decreases the amplitude and the quantal content of evoked endplate potentials (EPPs) at the neuromuscular junctions from several species, including adult rats (Ginsborg and Hirst, 1972) and frogs (Ribeiro and Walker, 1975). It is also known that in the same motor nerve terminal, both inhibitory adenosine A_1 receptors and excitatory adenosine A_{2A} receptors coexist, modulating the evoked release of acetylcholine (Correia-de-Sá et al., 1991). The way in which adenosine is able to achieve a balance in the control of neurotransmission depends on the extracellular concentration of the nucleoside, which subsequently depends on extracellular adenosine generation and inactivation (via cellular uptake and/or extracellular deamination) (Sebastião and Ribeiro, 1988). At the rat neuromuscular junction extracellular adenosine can be originated by transport-mediated release (Cunha and Sebastião, 1993), in parallel with its formation from released adenosine triphosphate (ATP) (Smith, 1991). Recently, we described that at the motor nerve endings of infant rats low extracellular concentrations of adenosine activate both A_{2A} and A_1 receptors, but activation of facilitatory A_{2A} receptors predominates over inhibitory A_1 receptors (Pousinha et al., 2010).

In the central nervous system, the neuromodulatory action of adenosine and the expression of its membrane receptors change with age (see Diógenes et al., 2011; Sebastião and Ribeiro, 2009). So, it was considered of interest to investigate the role of adenosine at the neuromuscular junction, as a function of age, looking at the relative importance of adenosine A_1 receptors vis-a-vis adenosine A_{2A} receptors. The results now reported show that excitatory A_{2A} receptor functioning at the motor nerve terminal decreases with aging even disappearing in aged rats, whereas

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inhibitory A_1 receptor action is preserved throughout aging. These age-dependent changes in the balance of excitatory over inhibitory neuromodulation may therefore contribute to the age-related decline in neuromuscular transmission.

2. Methods

2.1. Animals

The experiments were performed on isolated preparations of the phrenic nerve-diaphragm from male Wistar rats obtained from Harlan Interfauna Iberia, SL (Barcelona). Four groups of rats were used: infant (3–4 weeks old), young adult (12–16 weeks old), older adult (36–40 weeks old), and aged (70–80 weeks old). The animals, handled according to European Community guidelines and Portuguese Law on animal care, were killed by decapitation under halothane anesthesia. A strip of the left hemidiaphragm was isolated together with the phrenic nerve and was mounted in a 3 mL Perspex chamber through which the solutions flowed continuously at a rate of 3 mL per minute via a roller pump. The solutions were at room temperature (22 °C–23 °C). The bath volume was kept constant by suction. Solutions were changed by transferring the inlet tube of the pump from 1 flask to another. This involved a minimum of disturbance to the preparation and allowed prolonged recording from the same fiber with many solution changes. The change-over times in the figures of this report indicate the times at which the inlet tube of the pump was transferred to a new solution. Whenever testing the effect of a drug in the presence of an adenosine receptor (AR) antagonist, superfusion of the antagonist started at least 45 minutes before addition of another drug to the solution. In all cases, the superfusion of the drugs was only initiated when the recordings were considered stable by online analysis of the results. When used, adenosine deaminase (ADA) was added to the preparations at least 30 minutes before addition of the adenosine receptor agonist in its presence.

2.2. Electrophysiology recordings

Evoked EPPs and miniature end-plate potentials (MEPPs) were recorded in the conventional way (Fatt and Katz, 1951), with intracellular electrodes filled with KCl (3 M) and 10–20 MΩ resistance, inserted in the motor end plate. The reference electrode was an Ag-AgCl pellet. The nerve was stimulated supramaximally (rectangular pulses of 20 μs duration applied once every 2 seconds) through a suction electrode. Individual EPPs, as well as the resting membrane potential of the muscle fiber, were continuously monitored. Muscle fibers with a resting membrane potential less negative than −60 mV, were rejected. Throughout the experiment, EPPs and the resting membrane potential were continuously monitored and digitally stored on a personal computer with the Clampex program (pCLAMP 10; Axon Instruments, Foster City, CA, USA). The results were later analyzed off-line and each 60 consecutive EPPs were averaged. The n used in statistical analyses was number of experiments, which corresponds to a single neuromuscular junction per preparation/animal. The neuromuscular junctions from which data that was considered for further analysis was selected by the following parameters: the stability of the resting membrane potential, which could not spontaneously vary by more than 5% while assaying a drug effect, the initial value of the membrane potential, which ranged between −60 and −80 mV, and the initial amplitude of EPPs, which ranged between 2 and 4 mV. MEPPs were sampled and stored before the application of the drugs at 16 and 30 minutes and in some experiments 46 minutes, after starting drug perfusion. Later, the samples were analyzed off-line. MEPPs were detected by an event detection protocol. The threshold for detection of MEPPs was set at the level of 0.25 mV of amplitude and 2 ms of duration to prevent the contamination of the signal with electric noise. Mean MEPP frequency was measured by counting the number of MEPPs acquired in gap free mode for 100-second periods, at the following times: −10 minutes (before adding the drug), 0 minutes (immediately before addition), and 30 minutes after starting drug perfusion. The values were only considered for posterior analysis whenever MEPP frequency at −10 minutes and 0 minutes did not differ by more than 5%. The mean amplitude of MEPPs was calculated considering the average of the mean amplitude of 100 consecutive MEPPs. In the calculation of the mean amplitude of the MEPPs, the occasional "giants" (above 1 mV) were neglected, because it has been shown that they do not contribute to the components of evoked release (Menrath and Blackman, 1970). A change in MEPP frequency that was not accompanied by a change in MEPP amplitude was interpreted as a change in spontaneous transmitter release (see Katz, 1969). Quantal contents were estimated as the ratio of the average evoked response to the average amplitude of the MEPPs recorded during the same period.

2.3. Drugs

The bathing solution contained (mM): NaCl 117, KCl 5, $NaHCO_3$ 25, NaH_2PO_4 1.2, glucose 11, $CaCl_2$ 2.5, $MgCl_2$ 1.2. It was continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4) and kept at room temperature (22 °C–25 °C). Muscle twitches were prevented by increasing the bath concentration of Mg^{2+} to 19 mM.

Drugs used were: adenosine, 2-chloroadenosine (CADO), ADA (type VI, 1803 U mL^{-1}, EC 3.5.44), 2-p-(2-carboxyethyl) phenethylamino]-5′-N-ethylcarboxamido adenosine hydrochloride (CGS-21680), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 5′-iodotubericidin (ITU), and d-tubocurarine chloride were obtained from Sigma-RBI (St. Louis, MO, USA); 4-(2-[7-amino-2-(2-furly)[1,2,4] triazolo[2,3-a][1,3,5] triazin5ylamino] ethyl) phenol (ZM241385) was supplied by Tocris Cooksonhem (Ballwin, MO, USA). Stock solutions (5 mM) of CGS21680, 6-cyclopentyladenosine (CPA), DPCPX, ZM241385, and ITU were prepared in dimethyl sulfoxide (DMSO). Aliquots of these stock solutions

were kept frozen at −20 °C until used. The stock solution of ITU was kept in the dark to prevent photodecomposition. The maximum concentration of DMSO applied to the preparations (0.02% vol/vol) was devoid of effect on EPPs.

2.4. Analysis of the data

The data are expressed as mean ± SEM from n experiments. To allow comparisons between different experiments, the EPP amplitude, as well as the MEPP frequency or amplitude and the quantum content, were normalized, with 100% determined as the averaged values obtained during 10 minutes immediately before applying the test drug. The significance of the differences between means was evaluated by Student t test, when only 2 means were compared, or by 1-way analysis of variance (ANOVA) followed by the Tukey's test whenever multiple comparisons were made. Values of $p < 0.05$ were considered to represent statistically significant differences.

Results

3.1. Relative role of adenosine A_1 and A_{2A} receptors upon aging

To evaluate the relative importance of adenosine A_1 receptors vis-a-vis adenosine A_{2A} receptors, we decided to explore first the action of the stable analogue, CADO, which is not a substrate for adenosine deaminase (e.g., Daly, 1982), and has a low affinity for the adenosine uptake system (Jarvis et al., 1989). In innervated diaphragms from infant rats, and in accordance with what has been previously reported (Pousinha et al., 2010), CADO (30 nM) increased EPP amplitude, but as illustrated in Fig. 1, the magnitude of this excitatory effect decreased with age, turning into an inhibitory effect in aged rats. The effect caused by CADO (30 nM) on the average amplitude of EPPs in all groups studied (% change: 31 ± 4.7%, $n = 5$, infant rats, Fig. 1A, E; 21 ± 7.6%, $n = 5$, young adult rats, Fig. 1B, E; 13 ± 5.7%, $n = 5$, older rats, Fig. 1C, E; −12 ± 4.3%, $n = 3$, aged rats, Fig. 1D, E) was statistically significant ($p < 0.05$ when compared with absence of CADO (precontrol) in the same experiments, Student t test, Fig. 1E). The effect in innervated diaphragms from older adults or aged rats was also significantly different from that in infant rats ($p < 0.05$; ANOVA 1-way analysis of variance followed by Tukey's multiple comparisons test). Interestingly, when CADO (30 nM) was applied in the presence of the selective A_{2A} receptor antagonist, ZM 241385 (50 nM, Poucher et al., 1995), it caused inhibition of EPP amplitude (Fig. 1A–D), which was more evident in innervated diaphragms from older adult (−12 ± 2.4%, $n = 3$; Fig. 1C) or aged rats (−24 ± 10.2%, $n = 3$; Fig. 1D). On the other hand, in aged rats, the effect of CADO (30 nM) in the absence or presence of the A_{2A} receptor antagonist, ZM 241385 (50 nM), was not significantly different ($p < 0.05$; Student t test). Taken together, these results suggest that until midlife there is a predominant activation of A_{2A} receptors, which is lost upon aging. Accordingly, and as shown in Fig. 2, when a selective A_1 receptor agonist, CPA (100 nM, Williams et al., 1986), was applied after the full excitatory effect of an A_{2A} selective agonist, CGS 21680 (10 nM, Jarvis et al., 1989), the A_1 receptor agonist could not revert the excitatory influence of the selective A_{2A} receptor agonist. On the contrary, when CGS 21680 (10 nM), the selective A_{2A} receptor agonist, was applied after the full inhibitory effect of CPA (100 nM), neuromuscular transmission was increased, so that EPP amplitude nearly reverted toward precontrol levels (Fig. 2B). Thus, activation of A_{2A} receptors was able to counteract the inhibition caused by activation of A_1 receptors, whereas A_1 receptor activation was not able to revert the A_{2A} receptor-mediated excitatory action, further supporting the idea that in young animals the excitatory influence predominates over the inhibitory one.

To exclude the hypothesis that in aged rats the A_{2A} receptors are occupied by endogenous adenosine hampering its activation by an exogenous ligand, we evaluated the effect of CADO (30 nM) after removing endogenous adenosine with ADA (2 U/mL), an enzyme that converts adenosine into inosine (see Arch and Newsholme, 1978). By itself, ADA (2 U/mL) was devoid of effect on neuromuscular transmission (data not shown), suggesting that in these experimental conditions the level of extracellular adenosine is probably low. Furthermore, in the presence of ADA (2 U/mL), the effect of CADO (30 nM) on neuromuscular transmission in aged rats (Fig. 1D) was not significantly different ($p > 0.05$, $n = 3$, Student t test) from what was observed when applied alone.

We previously reported (Pousinha et al., 2010) that the excitatory actions of adenosine at the neuromuscular junction of infant rats require tonic activation of adenosine A_1 receptors. Thus, we investigated if the excitatory effect of CADO (30 nM) observed in young adult and older adult rats was also prevented by the A_1 receptor antagonist, DPCPX (10 nM). This antagonist was used at a concentration only 20 times higher than its Ki value for A_1 receptors in the same preparation (Sebastião et al., 1990) and well below its Ki value for A_{2A} receptors (see Jacobson et al., 1992), but as illustrated in Fig. 3, it fully prevented the facilitatory action of CADO (30 nM) in young adult rats (Fig. 3A) and older adult rats (Fig. 3B) ($p < 0.05$, $n = 3$, Student t test).

Fig. 4 summarizes the results from experiments where MEPPs and EPPs were recorded simultaneously, to evaluate changes in the quantal content of EPPs. Accordingly to what was previously described for infant rats (Pousinha et al., 2010), CADO (30 nM) induced an increase in the frequency of MEPPs in young adult and older adult rats (Fig. 4A). This effect on the frequency of MEPPs was not accompanied by changes in its mean amplitude (Fig. 4B), indicating that the excitatory effect of CADO (30 nM) is presynaptic, enhancing the release of the transmitter. Indeed, the quantal content of EPPs was significantly increased ($p < 0.05$, $n = 5$, Student t test) by CADO (30 nM) in infant, young adult, and older adult

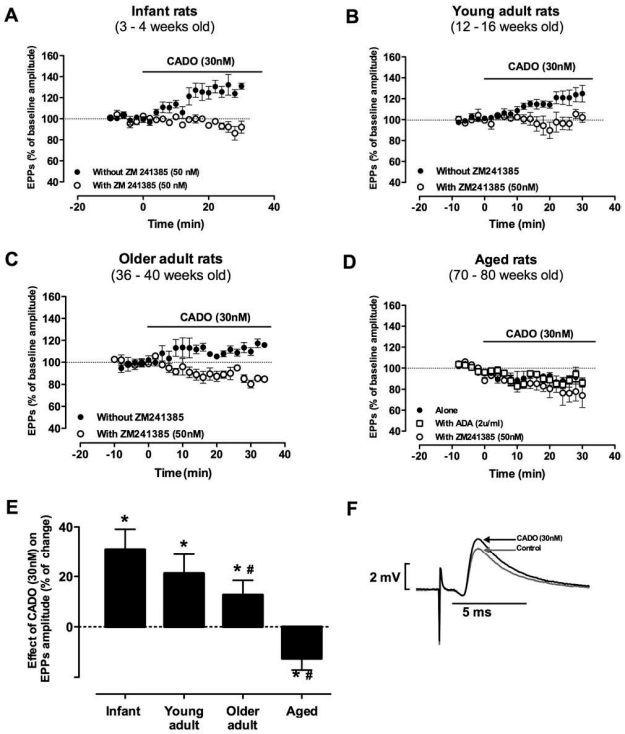

Fig. 1. At submicromolar concentrations, 2-chloroadenosine (CADO) enhances the amplitude of evoked end plate potentials (EPPs) in infant, young adult, and older adult, but not in aged rats. (A–D) The averaged changes in the amplitude of EPPs caused by the stable A_1/A_{2A} receptor agonist CADO (30 nM) in the absence (●) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM), in infant (A), young adult (B), older adult (C), or aged (D) rats. (D) In aged rats, the effect of CADO is not affected by the presence of adenosine deaminase (ADA; 2 U/mL, □), which was used to remove any putative occupation of receptors with the endogenous ligand, adenosine. The adenosine receptor antagonist was added to the preparations at least 45 minutes before addition of the agonist; ADA was added at least 30 minutes before the agonist. The horizontal bars in (A–D) indicate the period of agonist perfusion. Each point, in (A–D) represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minutes immediately before addition of CADO (30 nM): 2.4 ± 0.6 mV, $n = 8$ (infant rats); 3.0 ± 0.5 mV, $n = 8$ (young adult rats); 2.9 ± 1.0 mV, $n = 8$ (older adult rats); 1.5 ± 0.3 mV, $n = 10$ (aged rats). (E) Bar chart that summarizes the effect of CADO (30 nM) on the amplitude of EPPs in all groups studied; * $p < 0.05$ (Student t test), as compared with 0% (EPP amplitude before CADO in the same experiment); # $p < 0.05$ (1-way ANOVA followed by Tukey's multiple comparisons test) as compared with the effect of CADO in infant rats. (F) Representative traces of averaged EPPs recorded from 1 end plate (young adult rat), immediately before (control) and 30 minutes after addition of CADO (30 nM). Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −61 to −76 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

rats (Fig. 4C). In aged rats, CADO (30 nM) induced the opposite effect, causing a decrease in the frequency of MEPPs (Fig. 4A) without affecting their mean amplitude, indicating that in this group of animals, the inhibitory action of CADO (30 nM) is still exerted at the presynaptic level.

3.2. Age-dependent changes in balanced A_1/A_{2A} receptor activation by endogenously released adenosine

Because both A_1 and A_{2A} receptors are present at the motor nerve endings, therefore being prone to be activated

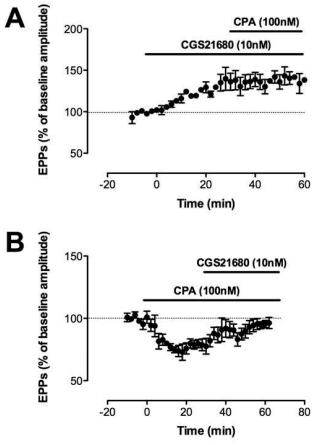

Fig. 2. The A_{2A} receptor-mediated facilitatory action upon neuromuscular transmission is not reverted by coapplication of an A_1 receptor-mediated agonist. Averaged time course changes in the amplitude of evoked end plate potentials (EPPs) caused by (A) 6-cyclopentyladenosine (CPA) (100 nM) in the presence of the selective A_{2A} receptor agonist, CGS21680 (10 nM) and by (B) CGS21680 (10 nM) in the presence of the selective A_1 receptor agonist, CPA (100 nM) in young adult rat end plates, as shown. The horizontal bars indicate the periods of drug perfusion. Each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minute immediately before addition of each selective agonist in (A), 2.8 ± 0.4 mV, $n = 3$; and (B), 2.6 ± 0.8 mV, $n = 3$. Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −61 to −72 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

by the endogenous ligand, the next series of experiments was designed to assess if the A_1/A_{2A} activation balance could be influenced by age. To do so, extracellular levels of adenosine were increased by using the adenosine kinase inhibitor, ITU (10 μM). We previously showed (Pousinha et al., 2010) that ITU (10 μM) enhanced the neuromuscular transmission of infant rats, while being virtually devoid of effect on the resting membrane potential of the muscle fiber, an effect attributed to activation of A_{2A} receptors by released adenosine. We therefore evaluated if this excitatory effect was maintained upon aging. Fig. 5 illustrates the effect of ITU (10 μM) on the amplitude of EPPs in all groups studied. As shown, ITU (10 μM) increased ($p < 0.05$, $n = 5$, Student t test) the mean amplitude of EPPs in infant (Fig. 5A, E), young adult (Fig. 5B, E), and older adult (Fig. 5C, E) rats. However, in aged rats, ITU (10 μM) inhibited neuromuscular transmission, decreasing ($p < 0.05$, $n = 4$) the mean amplitude of EPPs (Fig. 5D, E). Comparison among the different groups (ANOVA 1-way analysis of variance followed by Tukey's multiple comparisons test) confirmed that the effect of ITU (10 μM) in aged rats was significantly different ($p < 0.05$, $n = 4$, Student t test) from that in infant rats. As expected, when ITU (10 μM) was perfused in the presence of the A_{2A} receptor selective antagonist, ZM241385, the excitatory effect was no longer evident (Fig. 5). Interestingly, in older adult rats the excitatory effect of ITU (10 μM) turned into an inhibitory one (−29 ± 10.8%, $n = 4$, Fig. 5C; $p < 0.05$, Student t test), indicating that at this age group both A_1 and A_{2A} receptors can be hit by endogenously released adenosine.

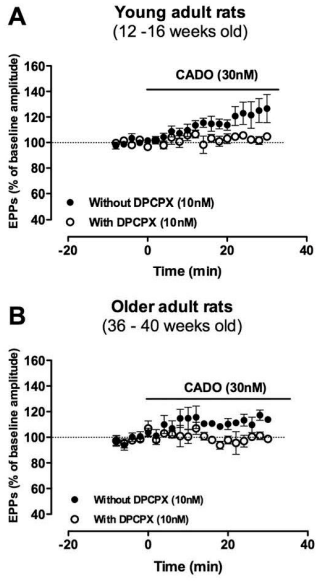

Fig. 3. The facilitatory effect of 2-chloroadenosine (CADO) (30 nM) is prevented by the selective A_1 receptor antagonist. Averaged time course changes in the amplitude of evoked end plate potentials (EPPs) caused by CADO (30 nM) in the absence (●) or in the presence (○) of the selective A_1 receptor antagonist, DPCPX (10 nM), in young adult (A) and older adult (B) rat end plates as shown. The adenosine receptor antagonist was added to the preparations at least 45 minutes before addition of the agonist. The horizontal bars indicate the period of agonist perfusion. Each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minutes immediately before addition of 30 nM CADO (A), 3.1 ± 0.5 mV, $n = 8$; and (B) 2.5 ± 0.6 mV, $n = 8$. Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −62 to −73 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

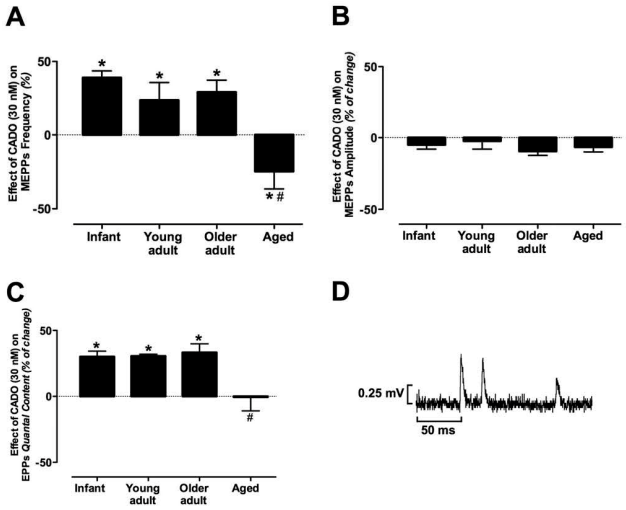

Fig. 4. At submicromolar concentrations, 2-chloroadenosine (CADO) enhances both the frequency of miniature end plate potentials (MEPPs) and the quantal content of end plate potentials (EPPs) in infant, young adult, and older adult rats, but not in aged rats. (A–C) The effect of CADO (30 nM) on the averaged frequency (A) and amplitude (B) of MEPPs, as well as quantal content (C) of EPPs in infant, young adult, older adult, and aged rats. In the ordinates, 0% represents the averaged number of events per 100 seconds (A), averaged amplitude recorded for 100 consecutive MEPPs (B), and the quantal content calculated immediately before addition of CADO (30 nM). The effect induced by CADO (30 nM) on the frequency of MEPPs and on the quantal content of EPPs in infant, young adult, and older adult rats was significantly different from the control. * $p < 0.05$ (Student t test) as compared with 0% (control waves before CADO); # $p < 0.05$ (1-way analysis of variance [ANOVA] followed by Tukey's multiple comparisons test) as compared with the effect of CADO in infant rats. (D) Representative sample of MEPPs recordings. Perfusion solutions contained 19 mM Mg^{2+}.

This inhibitory effect can be related to A_1 receptor activation by released adenosine, because, as expected, it was prevented by the adenosine A_1 receptor selective antagonist, DPCPX (10 nM, $n = 3$).

Accordingly to what was observed with CADO (30 nM), ITU (10 μM) induced an increase in the frequency of MEPPs in infant, young adult, and older adult rats (Fig. 5G). This effect was not accompanied by changes in the mean amplitude of MEPPs (Fig. 5F) and therefore, ITU (10 μM) significantly ($p < 0.05$, $n = 4$–5, Student t test) increased the quantal content of EPPs (Fig. 5H) in those age groups. Interestingly, in aged animals, ITU (10 μM) decreased the frequency of MEPPs ($-20 \pm 6.3\%$, $n = 3$; Fig. 5G) without affecting its mean amplitude (Fig. 5F), decreasing the quantal content of EPPs (Fig. 5H); these inhibitory actions were prevented by the selective A1 receptor antagonist DPCPX (10 nM). One may therefore conclude that under situations of enhanced release of endogenous adenosine, due to inhibition of adenosine kinase, adenosine-mediated modulation of neuromuscular transmission also shifts from excitatory to inhibitory upon aging.

3.3. Age-dependent influence of inhibitory receptors

To evaluate if the inhibitory action of adenosine on neuromuscular transmission was affected by aging, we studied the effect of the selective adenosine A_1 receptor agonist, CPA, upon aging (Fig. 6). As expected, CPA (100 nM) decreased the mean amplitude of EPPs in all groups studied ($p < 0.05$, when compared with absence of CPA [precontrol] in the same experiments, $n = 4$–5, Student t test). Among groups, there were no statistically significant differences ($p > 0.05$; 1-way ANOVA followed by Tukey's multiple comparisons test). This monotonous effect of CPA (100 nM) suggests that the inhibitory role of adenosine A_1 receptors on neuromuscular transmission is maintained upon aging.

3.4. Age-dependent influence of facilitatory receptors

As illustrated in Fig. 7, the selective A_{2A} receptor agonist, CGS 21680 (10 nM), increased ($p < 0.05$, $n = 4$–5, Student t test) the amplitude of EPPs in infant (Fig. 7A, E), young adult (Fig. 7B, E), and older adult (Fig. 7C, E) rats.

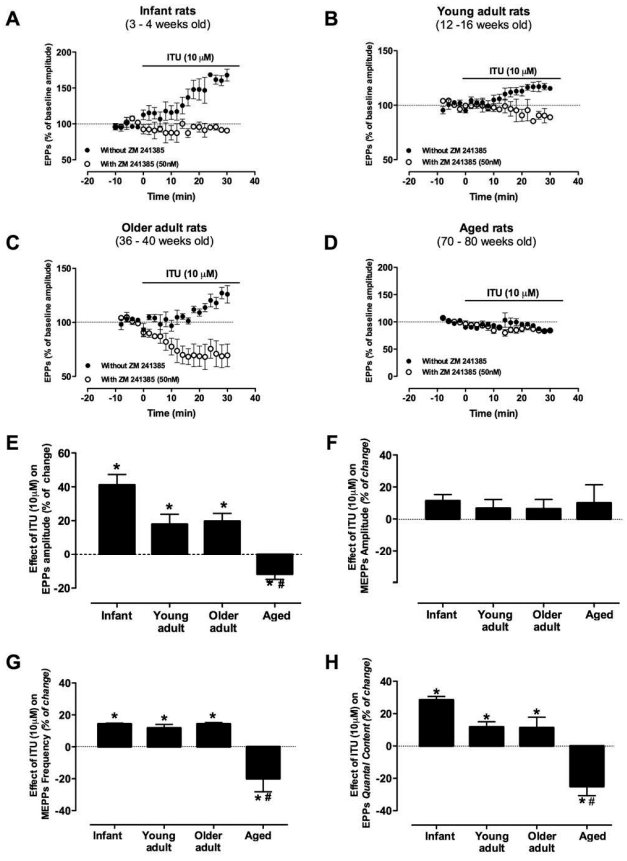

Fig. 5. The adenosine kinase inhibitor, iodotubericidin (ITU), enhances the amplitude of evoked end plate potentials (EPPs), the frequency of miniature end plate potentials (MEPPs) and the quantal content of EPPs in infant, young adult, and older adult rats, but not in aged rats. (A–D) Averaged changes in the amplitude of EPPs caused by ITU (10 μM) in the absence (●) or in the presence (o) of the selective antagonist of adenosine A_{2A} receptors, ZM 241385 (50 nM), in infant (A), young adult (B), older adult (C), or aged (D) rats The adenosine receptor antagonist was added to the preparations at least 45 minutes before addition of ITU. The horizontal bars represent the period of ITU perfusion; each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minutes immediately before addition of ITU (10 μM): 3.2 ± 0.5 mV, $n = 8$ (infant rats); 2.0 ± 0.4 mV, $n = 8$ (young adult rats); 1.6 ± 0.4 mV, $n = 8$ (older adult rats); 1.8 ± 0.3 mV, $n = 7$ (aged rats). (E–H) Bar chart that summarizes the effect of ITU (10 μM) on the average amplitude of EPPs (E), on the average amplitude (F) and frequency (G) of MEPPs, as well as quantal content (G) of EPPs in all groups studied. In the ordinates 0% represents the averaged EPP amplitude recorded for 10 minutes immediately before addition of ITU (10 μM) (E), averaged amplitude recorded for 100 consecutive MEPPs (F), averaged number of events per 100 sec (G), and the quantal content calculated immediately before addition of ITU (10 μM). The effect induced by ITU (10 μM) on the averaged amplitude of EPPs, frequency of MEPPs, and on the quantal content of EPPs in infant, young adult, and older adult rats was significantly different from the control. * $p < 0.05$ (Student t test) as compared with 0% (control waves before ITU); $^{\#}p < 0.05$ (1-way analysis of variance [ANOVA] followed by Tukey's multiple comparisons test) as compared with the effect of ITU in infant rats. Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −60 to −77 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

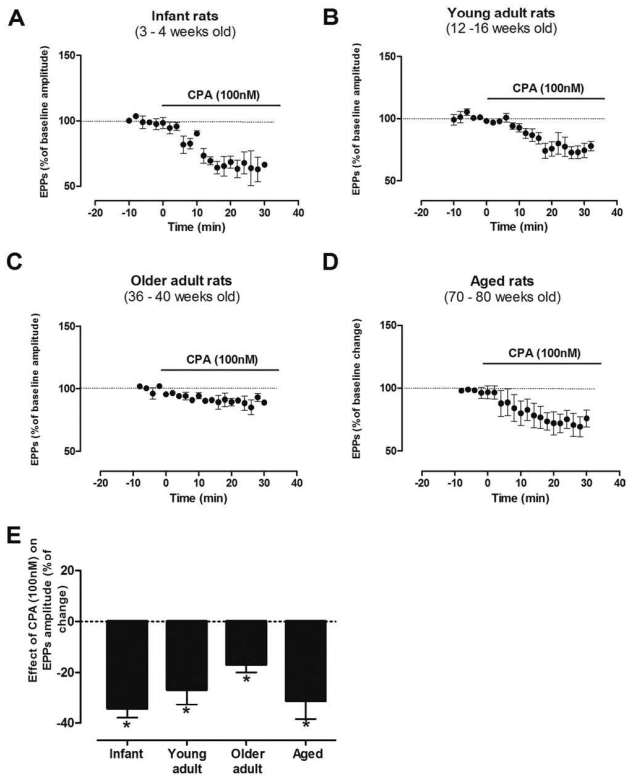

Fig. 6. A_1 receptor activation decreases the average amplitude of evoked end plate potentials (EPPs) in all groups studied. (A–D) Averaged changes in the amplitude of EPPs caused by the A_1 receptor agonist 6-cyclopentyladenosine (CPA) (100 nM) in infant (A), young adult (B), older adult (C), or aged (D) rats; the horizontal bars indicate the period of drugs perfusion; each point represents the averaged amplitude of 60 successive EPPs, in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minutes immediately before addition of CPA (100 nM, 1.6 ± 0.3 mV, $n = 5$ infant rats; 1.4 ± 0.3 mV, $n = 5$ young adult rats; 3.4 ± 0.5 mV, $n = 4$ older adult rats; 2.4 ± 1.2 mV, $n = 4$ aged rats. (E) Bar chart that summarizes the effect of CPA (100 nM) on the amplitude of EPPs in all groups studied; * $p < 0.05$ (Student t test), as compared with 0% (EPP amplitude before CPA [100 nM]) in the same experiment; # $p < 0.05$ (1-way analysis of variance [ANOVA] followed by Tukey's multiple comparisons test) as compared with the effect of CPA (100 nM) in infant rats. Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −60 to −70 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

Unexpectedly, in aged rats (Fig. 7D, E) CGS 21680 (10 nM) failed ($p > 0.05$, $n = 4$) to enhance neuromuscular transmission suggesting the absence of functional adenosine A_{2A} receptors in that age. The absence of an excitatory effect in aged rats could be related to full occupancy of the receptors by endogenously released adenosine. To exclude this hypothesis we tested the effect of CGS 21680 (10 nM) in the presence of ADA (2 U/mL). By itself, ADA (2 U/mL) did not significantly modify the amplitude of EPPs (−12 ± 4.6 mV, $n = 3$), indicating that the level of extracellular adenosine was low. When CGS 21680 (10 nM) was applied 30 minutes after ADA (2 U/mL) addition, the mean amplitude of EPPs remained unchanged (−5 ± 0.8%, $n = 3$), suggesting that the absence of the excitatory effect is not related to maximum receptor occupancy by the endogenous ligand.

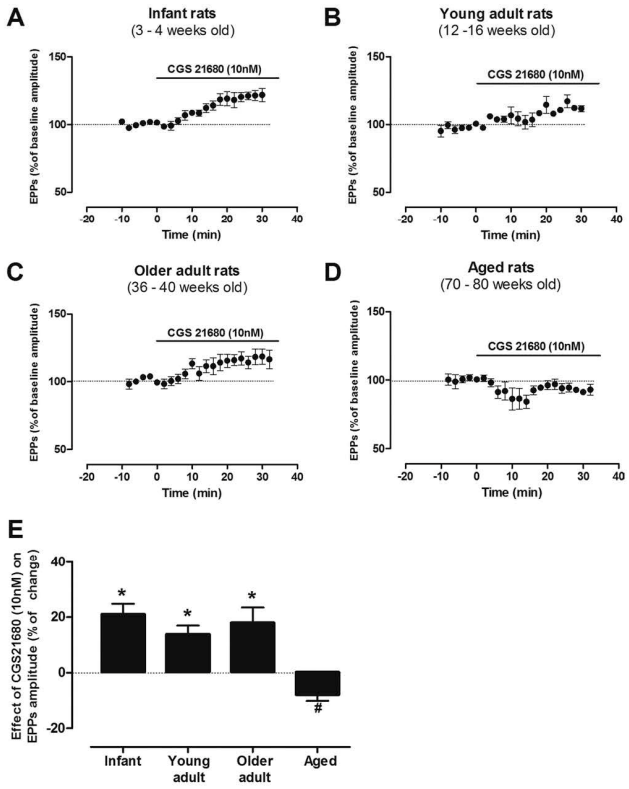

Fig. 7. The selective A_{2A} receptor agonist, CGS21680, increases the average amplitude of evoked end plate potentials (EPPs) in infant, young adult, and older adult rats, but not in aged rats. (A–D) Averaged changes in the amplitude of EPPs caused by the A_{2A} receptor agonist CGS21680 (10 nM) in infant (A), young adult (B), older adult (C), or aged (D) rats; the horizontal bar represents the period of agonist perfusion; each point represents the averaged amplitude of 60 successive EPPs; in the ordinates, 100% represents the averaged EPP amplitude recorded for 10 minutes immediately before starting CGS21680 (10 nM) perfusion, which was 2.6 ± 0.6 mV, $n = 5$ infant rats; 2.0 ± 0.5 mV, $n = 5$ young adult rats; 2.6 ± 0.4 mV, $n = 4$ older adult rats; 1.5 ± 0.3 mV, $n = 4$ aged rats. (E) Bar chart that summarizes the effect of CGS21680 (10 nM) on the amplitude of EPPs in all groups studied; * $p < 0.05$ (Student t test), as compared with 0% (EPP amplitude before 6-cyclopentyladenosine [CPA] 100 nM) in the same experiment; # $p < 0.05$ (1-way analysis of variance [ANOVA] followed by Tukey's multiple comparisons test) as compared with the effect of CPA (100 nM) in infant rats. Resting membrane potential in all experiments at time 0 (i.e., before adding the test drug) ranged from −63 to −74 mV. Perfusion solutions contained 19 mM Mg^{2+} which prevented muscle action potentials and twitches in response to nerve stimulation.

Discussion

The main finding in the present work was that age influences adenosine A_1 and A_{2A} receptors at the rat diaphragm neuromuscular junction, in a different manner. The magnitude of the excitatory effects on neuromuscular transmission caused by endogenously-released adenosine or by a mixed A_1/A_{2A} receptor agonist tends to decrease upon aging, disappearing in aged rats, where only A_1 receptor-mediated inhibition was evident. Considering independent activation of A_1 or A_{2A} receptors by selective ligands, the magnitude of the A_1 receptor-mediated inhibition is kept about constant throughout age; in contrast, the magnitude of A_{2A}

receptor-mediated facilitation is about the same up to middle-aged (older adult rats), but is absent at the neuromuscular junction of aged rats. Therefore, it appears that upon aging there is a gradual tendency for preponderance of inhibition, over excitation, when both receptors are simultaneously activated, despite proper A_1 and A_{2A} receptor functioning up to middle age.

Neuromuscular junctions differ in quantal content, safety factor, and adaptation to repetitive activation (see Ermilov et al., 2007; Rowley et al., 2007). Whether such differences might influence the presently reported effects of adenosine upon aging, needs to be explored at the light of the results obtained by Rowley et al. (2007) and Ermilov et al. (2007). However, in a previous work (see Ginsborg and Hirst, 1972) the initial quantal content of EPPs (either being low, in experiments where muscle contractions were paralyzed by high magnesium, or high in the experiments where muscle contractions were paralyzed by tubocurarine) did not influence the inhibitory effects of adenosine at the rat diaphragm neuromuscular junction. How adenosine influences safety factor variability in nerve terminals innervating different fiber types (see Ermilov et al., 2007) cannot be answered in the present work and awaits further investigation.

It is well established that adenosine kinase inhibitors, such as ITU, increase extracellular adenosine availability. In the present work, the excitatory effect of low concentrations of CADO and ITU, observed in infant rats (Pousinha et al., 2010), tended to decrease upon aging, even disappearing in aged rats (70–80 weeks old). The enhancement of neuromuscular transmission caused by the adenosine kinase inhibitor, ITU, as well as that caused by the mixed A_1/A_{2A} receptor agonist, CADO, should be attributed to adenosine A_{2A} receptor activation because they were antagonized by preincubation with the selective A_{2A} receptor antagonist ZM241385. A_{2A} receptor blockade transformed the excitatory effects of those drugs into inhibitory ones. This suggests that the influence of ITU and CADO upon neuromuscular transmission results from a balance between activation of adenosine excitatory A_{2A} and inhibitory A_1 receptors. The facilitation of neuromuscular transmission caused by ITU or CADO should be attributed to a presynaptic action, i.e., to an increase in the release of acetylcholine from the phrenic nerve endings, because these drugs increased the quantal content of end plate potentials without affecting the average amplitude of MEPPs recorded concomitantly.

The failure of nanomolar concentrations of CADO and ITU to increase neuromuscular transmission in aged rats, suggests that those animals lost the ability to activate A_{2A} receptors. This hypothesis is supported by (1) the observation that the effect of those drugs was not changed in preparations preincubated with the selective A_{2A} receptor antagonist, ZM241385, suggesting that only A_1 receptors become activated together with the absence of excitatory effect of the A_{2A} receptor agonist, CGS 21680, when applied to aged rats. (2) Full occupancy of A_{2A} receptors by high levels of endogenous adenosine cannot account for this lack of effect, because when these drugs were applied in the presence of adenosine deaminase, their effect on neuromuscular transmission was not changed. One possible explanation for the absence of adenosine excitatory effects could be a decrease in the number of A_{2A} receptors or/and to a decrease in the affinity of the receptor to its ligand. Nerve endings represent only a minor proportion of the tissue content in a nerve-muscle preparation, which precludes the use of receptor quantitative or semiquantitative assays, such as ligand binding studies or Western blot analysis. These approaches have been done in brain tissue, showing that the number of A_{2A} receptors, their affinity to ligands, and their coupling to G protein, is decreased in striatal membranes of aged rats (24 months) when compared with young adult animals (3 months) (Lopes et al., 1999). Also, the expression of A_{2A} messenger (m)RNA is decreased in striatum of aged rats (Schiffmann and Vanderhaeghen, 1993) and the excitatory effect of the A_{2A} receptor agonist, CGS 21680, on the spontaneous outflow of glutamate and aspartate in striatum was lost in aged rats (Corsi et al., 1999). So, in what concerns A_{2A} receptor-mediated neuromodulation, the neuromuscular junction appears to behave more likely the striatum, in clear contrast with what happens in the hippocampus, where A_{2A} receptor-mediated excitatory tonus is increased upon aging (Cunha et al., 1995; Lopes et al., 1999). Interestingly, the motor inhibitory adenosinergic tone is increased in aged animals with respect to young animals (Popoli et al., 1998), which can be attributed, at least in part, to a loss of excitatory tonus at the neuromuscular junction (present report) and striatum (Corsi et al., 1999; Lopes et al., 1999; Schiffmann and Vanderhaeghen, 1993).

As observed in infant rats (Pousinha et al., 2010), the A_{2A} receptor-mediated facilitation of neuromuscular transmission was antagonized, not only by the selective A_{2A} receptor antagonist, and also, by the A_1 selective receptor antagonist, suggesting that the interaction between both receptors is maintained upon aging. Two possibilities that are not mutually exclusive can be advanced: (1) the formation of A_{2A} and A_1 receptors heteromers, as it has been shown to occur at glutamatergic nerve terminals (Ciruela et al., 2006) and astrocytes (Cristóvão-Ferreira et al., 2011). In these conditions blockade of the A_1 receptor molecule could induce a conformational change in the heteromer that does not allow either A_{2A} agonist binding or effective coupling to the A_{2A} transducing system. Indeed, heteromerization of G protein coupled receptors may affect several aspects of receptor functioning (see Franco et al., 2008), so that monomers and heteromers can have distinct pharmacological and signaling properties. Therefore, age-dependent changes in the proportion of adenosine receptors in a heteromeric or monomeric form may, at least in part, be responsible for the lost of excitatory effects in aged rats(2). An A_1/A_{2A} receptor crosstalk through intracellular transducing pathways down-

stream to receptor binding (see Sebastião and Ribeiro, 2000), which would also lead to the need of A_1 receptor function to trigger an A_{2A} receptor-mediated response.

Several authors reported morphological and physiological changes that are related to the decline of the neuromuscular system with aging, as for example, loss of motor neurons, altered neurotransmitter release, disruption of postsynaptic acetylcholine receptor organization, denervation and reinnervation of muscle fibers, and motor unit remodeling (see Delbono, 2003). In rat diaphragm, the quantal content was reported to increase during the first 9 months of life and then to decline (Kelly, 1978). The reduction in A_{2A} receptors effects in aged rats might be one of the causes of the age-related changes in the neuromuscular transmission, because these receptors are known to influence the action of several neurotransmitters/neuromodulators/receptors/transporters at the nerve terminal (see Sebastião and Ribeiro, 2009). For instance, A_{2A} receptors at motor nerve terminals trigger the action of brain derived neurotrophic factor (BDNF) (Pousinha et al., 2006), which enhances transmitter release at developing end plates (Boulanger and Poo, 1999), improves neuromuscular transmission in the adult rat diaphragm (Mantilla et al., 2004) and facilitates synaptic efficacy by increasing presynaptic depolarization at the neuromuscular junction (see Huang and Reichardt, 2001). BDNF is also important for maintenance of acetylcholine receptor clustering in the end plate (Belluardo et al., 2001; Gonzalez et al., 1999). Therefore, the decrease of A_{2A} receptors upon aging can compromise BDNF actions that occur at the neuromuscular junction. Interestingly, studies performed on adenosine receptors knockout (KO) mice revealed that A_{2A} receptors play much more important roles than A_1 receptors in regulating locomotor activity (Huang et al., 2005), because both A_{2A} receptor KO and double A_1-A_{2A} receptors KO mice present lower locomotion activity and consume less oxygen than their wild type controls and apparently unaltered in A_1 receptor KO mice. Though those changes are usually regarded as a result of changes in basal ganglia control of motor activity, A_{2A} receptor changes at the neuromuscular junction may also play a role.

In conclusion, the work here reported clearly documents that aging influences adenosine A_1 and A_{2A} receptors, at the motor nerve terminals, in a different manner. The actions of A_{2A} receptors decrease with aging, even disappearing in aged rats. It remains to be clarified what are the consequences of the absence of these receptors for fine-tuning of motor control and whether this relates to the age-associated decline in neuromuscular control.

Disclosure statement

The authors disclose no conflicts of interest.

The animals were handled according to European Community guidelines and Portuguese Law on animal care.

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