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Inhibition of N-, P/Q- and other types of Ca^{2+} channels in rat hippocampal nerve terminals by the adenosine A_1 receptor

António F. Ambrósio [a], João O. Malva [a,b], Arsélio P. Carvalho [a], Caetana M. Carvalho [a,*]

[a] *Center for Neuroscience of Coimbra, Department of Zoology and Faculty of Medicine, University of Coimbra, 3000 Coimbra, Portugal*
[b] *Department of Biology, University of Minho, Braga, Portugal*

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Abstract

The effects of the adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA), on both the increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and on the release of endogenous glutamate in rat hippocampal synaptosomes were studied. The inhibitory effect of CPA on the increase in $[Ca^{2+}]_i$ stimulated with 4-aminopyridine was neutralized by the adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). The inhibitory effect of CPA was greater in synaptosomes from the CA1 subregion than in whole hippocampal synaptosomes. The inhibitory effects of both CPA and of the Ca^{2+} channel blockers, ω-conotoxin GVIA, ω-conotoxin MVIIC or ω-conotoxin GVIA plus ω-conotoxin MVIIC, were greater than those caused by the Ca^{2+} channel blockers. The release of endogenous glutamate was inhibited by 41% by CPA. The inhibition observed when CPA and ω-conotoxin GVIA or CPA and ω-conotoxin MVIIC were present was also greater than the inhibition by the Ca^{2+} channel blockers alone. The presence of both ω-conotoxin GVIA and ω-conotoxin MVIIC did not completely inhibit the release of glutamate, and CPA significantly enhanced this inhibition. The membrane potential and the accumulation of [^{3}H]tetraphenylphosphonium of polarized or depolarized synaptosomes was not affected by CPA, suggesting that adenosine did not increase potassium conductances. The present results suggest that, in hippocampal glutamatergic nerve terminals, adenosine A_1 receptor activation partly inhibits P/Q- and other non-identified types of Ca^{2+} channels.

Keywords: Glutamate release; Ca^{2+} concentration ($[Ca^{2+}]_i$), intracellular free; N^6-Cyclopentyladenosine (CPA); 4-Aminopyridine

1. Introduction

Adenosine is a neuromodulator of the central nervous system (CNS) that exerts its inhibitory effects by the activation of adenosine A_1 receptors (Ribeiro, 1995). However, the mechanisms which couple the activation of adenosine A_1 receptors to the inhibition of neurotransmitter release are not well understood. It has been argued that adenosine probably acts through several mechanisms, namely inhibition of Ca^{2+} currents, activation of K^+ currents, or even inhibition of the exocytotic machinery, and the importance of such mechanisms may vary among nerve terminals, animal species, with age and the mechanism of stimulation (Fredholm and Dunwiddie, 1988; Ribeiro, 1995).

Adenosine decreases the entry of $^{45}Ca^{2+}$ into synaptosomes (Wu et al., 1982; Gonçalves et al., 1991), and decreases Ca^{2+} currents in various cell types (Dolphin et al., 1986; MacDonald et al., 1986; Scholz and Miller, 1991). The inhibitory effect of adenosine may also result from an increase in K^+ conductances (Okada and Ozawa, 1980; Trussel and Jackson, 1985; Gerber et al., 1989; Zoltay and Cooper, 1990), which causes hyperpolarization of the membrane potential.

Glutamate is the most abundant excitatory neurotransmitter in the brain that can be released from an exocytotic pool (Nicholls et al., 1987), and adenosine inhibits glutamate release (Poli et al., 1991, 1993; Barrie and Nicholls, 1993), probably by inhibiting Ca^{2+} channels. Various lines of evidence suggest that adenosine reduces Ca^{2+} currents primarily by inhibiting N-type Ca^{2+} channels (Mogul et al., 1993; Yawo and Chuhma, 1993; Mynlieff and Beam, 1994; Wu and Saggau, 1994). Nevertheless, Wu and Saggau (1994) found that the inhibition produced by adenosine in presynaptic Ca^{2+} transients in hippocampal slices

* Corresponding author. Tel.: 351-39-25127; fax: 351-39-22776; e-mail: cmcarvalho@gemini.ci.uc.pt.

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is due, not only to inhibition of ω-conotoxin GVIA-sensitive channels, but also to unidentified Ca^{2+} channels, probably including Q-type Ca^{2+} channels.

In the hippocampus, an area with specific vulnerability to ischemia (Schreiber and Baudry, 1995), there is a very high density of adenosine A_1 receptors unevenly distributed in the various hippocampal subregions (Lee et al., 1983; Fastbom et al., 1987; Tetzlaff et al., 1987). Since adenosine depresses glutamatergic neurotransmission, it is important to understand the mechanisms underlying the actions of adenosine and its effects on Ca^{2+} influx and on glutamate release. In the present work we investigated whether the inhibitory effects of adenosine in hippocampal nerve terminals were exerted mainly by inhibition of Ca^{2+} channels or by activation of K^+ channels. The results indicate that the inhibition of Ca^{2+} channels is the main mechanism involved and we further investigated which type(s) of Ca^{2+} channels coupled to the exocytotic release of glutamate were inhibited. A preliminary account of part of this work has been presented (Carvalho et al., 1996).

2. Materials and methods

2.1. Materials

Adenosine deaminase type VI (EC 3.5.4.4) and L-glutamic acid dehydrogenase type II (EC 1.4.1.3) were purchased from Sigma Chemical, St. Louis, MO. ω-Conotoxin GVIA was obtained from Peninsula Laboratories, Belmont, CA. ω-Conotoxin MVIIC was from Peptide Institute, Osaka. The acetoxymethyl ester of Indo-1 (Indo-1/AM) was purchased from Molecular Probes, Eugene, OR. N^6-cyclopentyladenosine (CPA) and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from RBI-Research Biochemicals International, Natick, MA. Ionomycin and fatty acid free bovine serum albumin were from Calbiochem–Boehringer, San Diego. All other reagents were from Sigma Chemical, St. Louis, MO, or from Merck–Schuchardt, Germany.

2.2. Preparation of synaptosomes

A partially purified synaptosomal fraction (P_2) was isolated from hippocampi or from hippocampal subregions (CA1, CA3 and dentate gyrus) of male Wistar rats (2 month-old), essentially as described previously for brain cortex (McMahon et al., 1992), with some modifications (Malva et al., 1996). In short, the hippocampi were homogenized in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, and centrifuged at $3{,}000\,g_{max}$ for 2 min. The pellet obtained was resuspended, followed by sedimentation at the same speed. The combined supernatants were spun for 12 min at $14{,}600\,g_{max}$, and a P_2 pellet was obtained. The upper, whiter, layer of this pellet, containing essentially synaptosomes, was resuspended in the sucrose medium. The protein concentration was determined by the Biuret method (Layne, 1957). Slices, 800 μm thick, were prepared for the isolation of synaptosomes from hippocampal subregions (CA1, CA3 and dentate gyrus). In each slice, the fimbria and the subiculum were separated from the rest of each slice, under stereomicroscopic observation. CA3 sub-slices were obtained by separation from CA1 and from dentate gyrus areas. Separation of CA1 and dentate gyrus was performed through the hippocampal sulcus. The separated sub-slices were homogenized in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, transferred to Eppendorf tubes and centrifuged as described for the isolation of whole hippocampal synaptosomes. The whole P_2 pellet of each subregion was resuspended in buffered sucrose medium.

For the membrane potential measurements, the synaptosomes were stored in suspension on ice, whereas for the $[Ca^{2+}]_i$ or glutamate release measurements the synaptosomes were stored as drained pellets, containing 0.75 mg or 1 mg of protein, respectively. All experiments were performed within 3 h of the isolation of synaptosomes.

2.3. $[Ca^{2+}]_i$ measurements

The measurement of $[Ca^{2+}]_i$ was essentially as described previously (Duarte et al., 1991), with some modifications. Synaptosomes (2.6 mg/ml) were incubated with 3 μM Indo-1/AM in incubation medium (132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 1.2 mM H_3PO_4, 10 mM glucose and 10 mM HEPES-Na, pH 7.4, in addition to 0.1% fatty acid-free bovine serum albumin for 20 min at 25°C, followed by 10 min at 35°C. The non-hydrolyzed probe was removed by pelleting the synaptosomes at $15{,}800\,g_{max}$ for 15 s. The fluorescence of Indo-1-loaded synaptosomes was monitored at 30°C, using a computer-assisted Spex Fluoromax spectrofluorometer, at two emission wavelengths of 410 nm and 485 nm, and with an excitation wavelength of 335 nm, using 5 nm slits. The maximal fluorescence ratio was obtained with 2.5 μM ionomycin, at 500 s, and the minimal ratio was determined with 10 mM EGTA solution (pH 10) at 600 s. The fluorescence intensities were automatically converted into $[Ca^{2+}]_i$ values by using the calibration equation for double emission wavelength measurements, and taking the dissociation constant of the Indo-1/Ca^{2+} complex as 250 nM (Grynkiewicz et al., 1985). In all experiments, the synaptosomes were pre-incubated for 300 s before stimulation, in the presence or absence of drugs.

2.4. Glutamate release

The release of endogenous glutamate was followed using a continuous fluorimetric assay as previously described (Nicholls et al., 1987), with some modifications (Malva et al., 1996). Synaptosomes (1 mg protein) were incubated for 20 min at 30°C in the following medium (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 0.1 $CaCl_2$,

10 glucose, 10 HEPES-Na, at pH 7.4, with 0.1% fatty acid-free bovine serum albumin. After this period, synaptosomes were centrifuged at 15,800 g_{max}, and resuspended in 1 ml of the same medium, without bovine serum albumin and with 1 mM $CaCl_2$ or with 200 nM free Ca^{2+} (Vásquez et al., 1994). The suspension was transferred to a stirred cuvette at 37°C, followed by the addition of 1 mM NADP and 50 U of purified glutamate dehydrogenase. Fluorescence was measured by using a Perkin Elmer model LS-5B luminescence spectrometer at the excitation and emission wavelengths of 340 nm and 460 nm, respectively, with excitation and emission slits of 5 nm and 10 nm, respectively. The data were collected at 0.5 s intervals and the quantitation of glutamate release was performed at the end of each experiment by adding 5 nmol of L-glutamate.

2.5. Measurement of membrane potentials with a tetraphenylphosphonium (TPP^+)-selective electrode

The plasma membrane potentials of synaptosomes were estimated from the accumulation of TPP^+ into the nerve terminals by using a TPP^+-selective electrode with a KCl reference electrode as described previously (Aiuchi et al., 1989). Synaptosomes (0.5 mg) were added to 1 ml of assay medium (132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES-Na, pH 7.4) containing 10 μM TPP^+, at 25°C. The difference in potential between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously. The plasma membrane potentials (E_m) were determined by using the formula developed by Aiuchi et al. (1989):

$$E_m = -\frac{RT}{F}\ln\left\{1 + \frac{([TPP^+]_{o,k} - [TPP^+]_o)(V_o + v)}{[TPP^+]_o[V_s + V_{mit}\cdot\exp(-(F\cdot E_{mit})/(RT))]}\right\}$$

where $R = 8.31$ J mol^{-1} K^{-1}, $T = (273.15 + t)$K, $F =$ 96485 C mol^{-1}, $[TPP^+]_o$ and $[TPP^+]_{o,k}$, are external concentration of TPP^+ in the assay medium in the presence and in the absence of a K^+ gradient, respectively. V_o, v, V_s, V_{mit} are the medium, synaptosomal, cytosolic synaptosomal and intramitochondrial volumes, respectively. For V_s, V_{mit} and E_{mit} we used 3 μl/mg protein, 80 nl/mg protein and -160 mV, respectively (Aiuchi et al., 1989).

2.6. Accumulation of [^{3}H]TPP^+

Synaptosomes (0.2 mg of protein) were incubated in the following medium: 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES-Na^+, pH 7.4, for 10 min, at 30°C. At this period, the same volume of a [^{3}H]TPP^+ solution (stock solution 1.3 nCi/nmol) prepared in the incubation medium (final concentration 2.5 nM) was added to the synaptosomal suspension. After 5 min, the time necessary to reach the maximum accumulation of [^{3}H]TPP^+, samples of synaptosomal suspension containing 0.1 mg of protein were filtered under vacuum, through glass fiber filters Whatman GF/B, and washed twice in cold (0–4°C) incubation medium.

The radioactivity in each filter was determined by liquid scintillation spectrometry, after 24 h equilibration in 5 ml of scintillation fluid (Universol, ICN), in a scintillation counter provided with a quenching dpm correction curve.

2.7. Analysis of adenine nucleotides

After 10 min incubation of synaptosomes (1 mg) in the following medium: 132 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 100 μM $CaCl_2$, 1.2 mM H_3PO_4, 10 mM glucose,

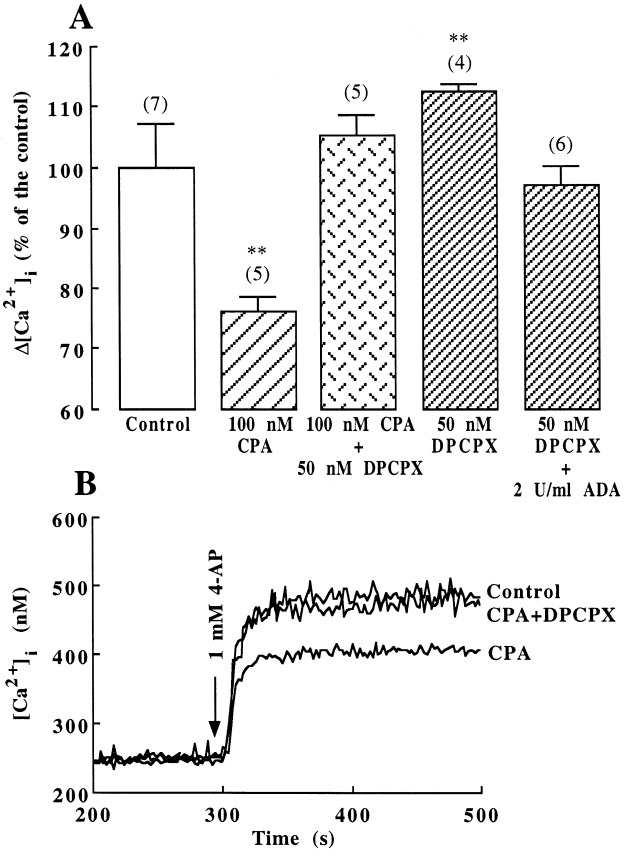

Fig. 1. Modulation of the increase in $[Ca^{2+}]_i$ evoked by 4-aminopyridine (4-AP), due to activation of adenosine A_1 receptor. (A) CPA (100 nM) inhibited the increase in $[Ca^{2+}]_i$ by about 24%. The adenosine A_1 receptor antagonist, DPCPX, produced a slight enhancement of the response, and reversed the effect of CPA. With adenosine deaminase (ADA) present, DPCPX did not affect the increase in $[Ca^{2+}]_i$. In each case, synaptosomes were stimulated with 1 mM 4-aminopyridine, 300 s after the beginning of each experiment. In (B), each trace shows the result of a representative experiment in the presence of ADA (2 U/ml). Stimulation with 1 mM 4-aminopyridine increased $[Ca^{2+}]_i$ by about 230 nM. Statistical significance was determined by ANOVA. The data are means ± S.D. of the number of independent experiments indicated above each bar. $^{**}P < 0.01$, significantly different from the control (Dunnett's post test).

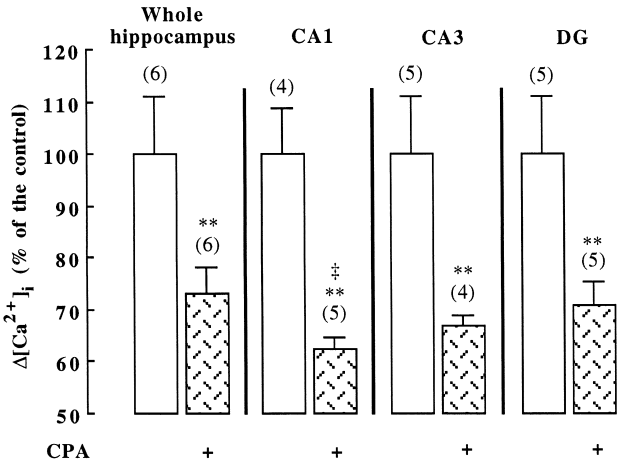

Fig. 2. Modulation of $[Ca^{2+}]_i$ increase in synaptosomes obtained from the whole hippocampus, or from the hippocampal subregions, CA1, CA3 or dentate gyrus (DG), upon activation of the adenosine A_1 receptor by CPA. The inhibition by CPA of the $[Ca^{2+}]_i$ increase induced by 4-aminopyridine (4-AP, 1 mM) in CA1 hippocampal synaptosomes was significantly greater than that in whole hippocampal synaptosomes. The differences between CA1, CA3 and DG are not significant. The experiments were performed in the presence of ADA (2 U/ml). Statistical significance was determined by ANOVA. The data are means ± S.D. of the number of independent experiments indicated above each bar. $^{**}P < 0.01$, significantly different from the control (Dunnett's post test). $^{‡}P < 0.05$, significantly different from whole hippocampus (Bonferroni's post test).

10 mM HEPES-Na, pH 7.4, and 0.1% fatty acid-free bovine serum albumin, at 30°C, the same volume of 1.2 M perchloric acid was added to the synaptosomal suspension, in ice. The synaptosomes were then centrifuged for 1 min at 15,800 g_{max} and the supernatants so obtained were neutralized with 3 M KOH in 1.5 M Tris and centrifuged at 15,800 g_{max} for 1 min. The resulting supernatants were assayed for adenine nucleotides (ATP, ADP and AMP) by separation in a reverse-phase high performance liquid chromatography, as described by Stocchi et al. (1985). The chromatographic apparatus used was a Beckman System Gold, consisting of a 126 Binary Pump Model and a 166 Variable UV detector. The column used was a Lichrospher 100RP-18 (5 μm) from Merck (Germany). Isocratic elution with 100 mM KH_2PO_4 buffer, at pH 6.5, and 1% methanol was performed at a flow rate of 1 ml/min. The adenine nucleotides (ATP, ADP and AMP) were detected at 254 nm for 6 min.

2.8. Data analysis

The data are expressed as means ± S.D. (in Fig. 4B we used S.E.M.) for the indicated number of experiments performed with different preparations. Statistical significance was determined by using an analysis of variance (ANOVA) followed by Dunnett's ($^{**}P < 0.01$, $^{*}P < 0.05$) or Bonferroni's ($^{‡‡‡}P < 0.001$, $^{‡‡}P < 0.01$, $^{‡}P < 0.05$) multiple comparisons post tests, as indicated in the figure legends.

3. Results

3.1. Modulation of the increase in $[Ca^{2+}]_i$ by adenosine A_1 receptor activation in rat hippocampal synaptosomes

The energetic status of the synaptosomal preparation utilized in this study was evaluated by determining the ATP/ADP ratio and the synaptosomal membrane potentials (E_m), as well as the basal levels of $[Ca^{2+}]_i$. The value for the ATP/ADP ratio obtained was 4.86 ± 0.63 (ATP, 3.77 ± 0.54 nmol/mg protein; ADP, 0.78 ± 0.09

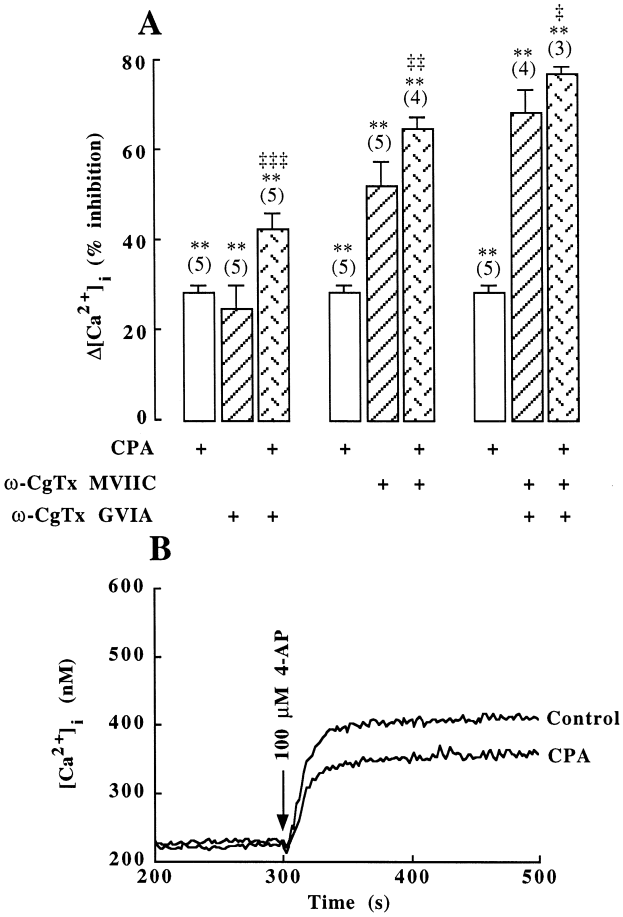

Fig. 3. Comparison of the inhibitory effect of CPA and of Ca^{2+} channel blockers on the increase in $[Ca^{2+}]_i$ induced by 100 μM 4-aminopyridine (4-AP). (A) Quantification of the inhibitory effects caused by CPA and/or Ca^{2+} channel blockers, ω-conotoxin GVIA (ω-CgTx GVIA) and ω-conotoxin MVIIC (ω-CgTx MVIIC). (B) Traces of the results of representative experiments in the presence of 2 U/ml ADA, showing that stimulation with 100 μM 4-aminopyridine increased basal levels of $[Ca^{2+}]_i$ by about 160 nM and that CPA inhibited this $[Ca^{2+}]_i$ signal to about 125 nM. The Ca^{2+} channel blockers were present during loading of synaptosomes with Indo-1/AM and during the experiments. Statistical significance was evaluated by ANOVA. The number of independent experiments is indicated above each bar. The data are presented as means ± S.D. $^{**}P < 0.01$, significantly different from the control (0% inhibition – Dunnett's post test). $^{‡}P < 0.05$, $^{‡‡}P < 0.01$, $^{‡‡‡}P < 0.001$, significantly different from ω-conotoxin MVIIC plus ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-conotoxin GVIA, respectively (Bonferroni's post test).

nmol/mg protein). Under non-depolarizing basal conditions, the calculated value for E_m was -81.5 ± 3.2 mV. The basal level of $[Ca^{2+}]_i$ was about 260 nM. The values obtained for these parameters (ATP/ADP, E_m and basal $[Ca^{2+}]_i$) were comparable to those reported by other groups for synaptosomal preparations (Kauppinen et al., 1988; Tibbs et al., 1989).

The increase in $[Ca^{2+}]_i$ in synaptosomes stimulated with 1 mM 4-aminopyridine was reduced by the presence of 100 nM CPA to $76.2 \pm 2.4\%$ of the control (Fig. 1A and B). Higher concentrations of CPA did not further inhibit the increase in $[Ca^{2+}]_i$ in response to 4-aminopyridine (not shown). Conversely, when the nerve terminals were stimulated in the presence of 50 nM DPCPX, an adenosine A_1 receptor antagonist, we observed an enhancement of the $[Ca^{2+}]_i$ response to 4-aminopyridine of about 12% over the control. This effect was not observed if the synaptosomes were previously treated with adenosine deaminase (2 U/ml). This observation suggests a tonic effect of endogenously leaked adenosine on the adenosine A_1 receptors. When both the agonist (CPA) and the antagonist (DPCPX) were present in the assay medium, before stimulation with 4-aminopyridine, we did not observe any effect of CPA, indicating a specific effect of the agonist on adenosine A_1 receptors. In subsequent experiments, adenosine deaminase was always present to eliminate the effects of endogenous adenosine.

Previous work indicated that the density of adenosine A_1 receptors varies among hippocampal subregions (Lee et al., 1983). Thus, we compared the modulatory effect of CPA on the increase of $[Ca^{2+}]_i$ in whole hippocampal synaptosomes and in synaptosomes isolated from hippocampal CA1, CA3 and dentate gyrus subregions. When synaptosomes isolated from the whole hippocampus, or from the three subregions (CA1, CA3 and dentate gyrus) were stimulated with 1 mM 4-aminopyridine, the increase in $[Ca^{2+}]_i$ was inhibited by CPA to $73.1 \pm 5.1\%$, $62.4 \pm 2.2\%$, $66.8 \pm 2.0\%$ or $70.9 \pm 4.4\%$ of the control, respectively (Fig. 2). The inhibition obtained in CA1 synaptosomes was significantly greater than that obtained in the whole hippocampal synaptosomes ($^{\ddagger}P < 0.05$).

3.2. Characterization of Ca^{2+} channel type(s) affected by the activation of adenosine A_1 receptors

We used 4-aminopyridine as the stimulatory agent to depolarize the hippocampal synaptosomes, with a subsequent increase in $[Ca^{2+}]_i$, which may be coupled to the exocytotic release of endogenous glutamate. Thus, in all subsequent experiments we used a sub-maximal concentration of 4-aminopyridine (100 μM), which, in our experience, causes an essentially Ca^{2+}-dependent release of neurotransmitters (Carvalho et al., 1995; Ambrósio et al., 1996). The contribution of each Ca^{2+} channel type to the increase in $[Ca^{2+}]_i$, due to 4-aminopyridine (100 μM) stimulation, was determined in the presence of saturating concentrations of two Ca^{2+} channel blockers (Malva et al., 1995). Thus, ω-conotoxin GVIA (500 nM; N-type blocker), or ω-conotoxin MVIIC (500 nM; P/Q-type blocker), inhibited the 4-aminopyridine-induced increase in $[Ca^{2+}]_i$ by $24.6 \pm 5.2\%$ or $52.2 \pm 5.4\%$, respectively (Fig. 3A, middle bar of first and second group of bars). When both blockers were present, the inhibition was $68.5 \pm 5.0\%$ (Fig. 3A, middle bar of third group of bars).

We then determined which of these type(s) of Ca^{2+} channels were affected by the adenosine A_1 receptor agonist, CPA, by measuring its effect in the absence or in the presence of the Ca^{2+} channel blockers. CPA alone reduced the increase in the $[Ca^{2+}]_i$ due to 4-aminopyridine by $28.2 \pm 1.7\%$ (Fig. 3, first bar in each group). In the presence of both 100 nM CPA and either 500 nM ω-conotoxin GVIA or 500 nM ω-conotoxin MVIIC, the inhibition of the Ca^{2+} signal was significantly increased to $42.4 \pm 3.6\%$ ($^{\ddagger\ddagger\ddagger}P < 0.001$) or $64.8 \pm 2.7\%$ ($^{\ddagger\ddagger}P < 0.01$), respectively (Fig. 3, third bar in each group), as compared to the inhibition caused by either ω-conotoxin GVIA or ω-conotoxin MVIIC. These results seem to indicate that non-N-type and non-P/Q-type Ca^{2+} channels, respectively, may be inhibited by adenosine A_1 receptor activation. In Fig. 3, we can also see that after blockade of the N- and P/Q-type channels there was still a fraction of the $[Ca^{2+}]_i$ signal which was partially inhibited by 100 nM CPA. Thus, in the presence of CPA, ω-conotoxin GVIA

Table 1
Effects of CPA on membrane potential and on the accumulation of $[^3H]TPP^+$ in hippocampal synaptosomes

	E_m (mV)[a]		$[^3H]TPP^+$ accumulated[b] (pmol/mg protein)	
	—	CPA	—	CPA
Control	-81.54 ± 3.23 ($n = 11$)	-80.65 ± 1.29 ($n = 4$)	2.91 ± 0.36 ($n = 8$)	3.30 ± 0.63 ($n = 4$)
100 μM 4-aminopyridine	-80.01 ± 2.99 ($n = 4$)	-80.70 ± 2.69 ($n = 5$)	3.01 ± 0.73 ($n = 4$)	2.96 ± 0.69 ($n = 4$)
1 mM 4-aminopyridine	$-74.81 \pm 2.35^*$ ($n = 3$)	ND	2.43 ± 0.33 ($n = 4$)	2.39 ± 0.34 ($n = 4$)

[a] Membrane potential (E_m) was estimated with an electrode sensitive to TPP^+, by using the equation developed by Aiuchi et al. (1989). The results represent means ± S.D. of the number of experiments indicated.
[b] The accumulation of $[^3H]TPP^+$ was determined as described in Section 2. CPA (100 nM) was added at 5 min of incubation and 4-aminopyridine (100 μM or 1 mM) at 9.5 min. The results represent means ± S.D. of the number of experiments indicated, in triplicate. Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post test.
$^*P < 0.05$.
ND: not determined.

and ω-conotoxin MVIIC, the inhibitory effect was 77.2 ± 1.6%, as compared to 68.5 ± 5.0%, in the absence of CPA.

3.3. Effect of adenosine A_1 receptor activation on synaptosomal membrane potential

In order to look for possible effects of CPA on K^+ channels we also investigated the effect of CPA on the membrane potential of hippocampal synaptosomes, estimated by the method of Aiuchi et al. (1989). Table 1 shows that the activation of adenosine A_1 receptors with CPA did not significantly alter the membrane potential of hippocampal synaptosomes maintained under basal conditions. It is also shown that CPA did not cause any hyperpolarization in synaptosomes depolarized with 100 μM 4-aminopyridine. Since this method may not detect slight effects on membrane potential, we also studied the effect of CPA on the accumulation of [^{3}H]TPP^+ by the nerve terminals (Table 1). Under non-depolarizing conditions, hippocampal synaptosomes accumulated 2.91 ± 0.36 pmol [^{3}H]TPP^+/mg protein and, in the presence of CPA, the accumulation was slightly greater (3.30 ± 0.63 pmol/mg protein), but not significantly different ($P > 0.05$) from the control. It can also be seen in Table 1 that CPA did not cause any effect on the accumulation of [^{3}H]TPP^+ in synaptosomes depolarized with 4-aminopyridine (100 μM or 1 mM). These results suggest that the activation of adenosine A_1 receptors did not cause significant membrane hyperpolarization in hippocampal synaptosomes.

3.4. Modulation of glutamate release by activation of adenosine A_1 receptors

In these experiments, we selected the glutamatergic hippocampal synaptosomes by following the exocytotic release of endogenous glutamate due to stimulation with 4-aminopyridine. In the presence of low external free [Ca^{2+}] (200 nM), 4-aminopyridine (100 μM) did not cause any significant release of endogenous glutamate (Fig. 4B), as shown previously for striatal synaptosomes (Ambrósio et al., 1996). Conversely, when 1 mM Ca^{2+} was present, the synaptosomes released about 4 nmol/mg protein/5 min of endogenous glutamate, and this release was inhibited by 41% by activation of the adenosine A_1 receptor with CPA (Fig. 4).

We then studied the contribution of ω-conotoxin GVIA- and ω-conotoxin MVIIC-sensitive Ca^{2+} channels to the release of glutamate, and investigated the possible modulation of these channels by CPA, as determined from the effect on Ca^{2+}-dependent glutamate release. The N-type blocker, ω-conotoxin GVIA (500 nM), inhibited the release of glutamate (Fig. 4) by only 16.4 ± 5.1%, but ω-conotoxin MVIIC (500 nM) inhibited a larger fraction of the glutamate released (48.8 ± 5.5%). The release decreased by 75.5 ± 4.9% in the presence of both ω-conotoxin GVIA and ω-conotoxin MVIIC. This shows that the two inhibitors did not completely block the release of glutamate, suggesting that other non-identified Ca^{2+} channel(s) contribute to Ca^{2+} entry into glutamatergic nerve terminals, coupled to the exocytotic release of glutamate.

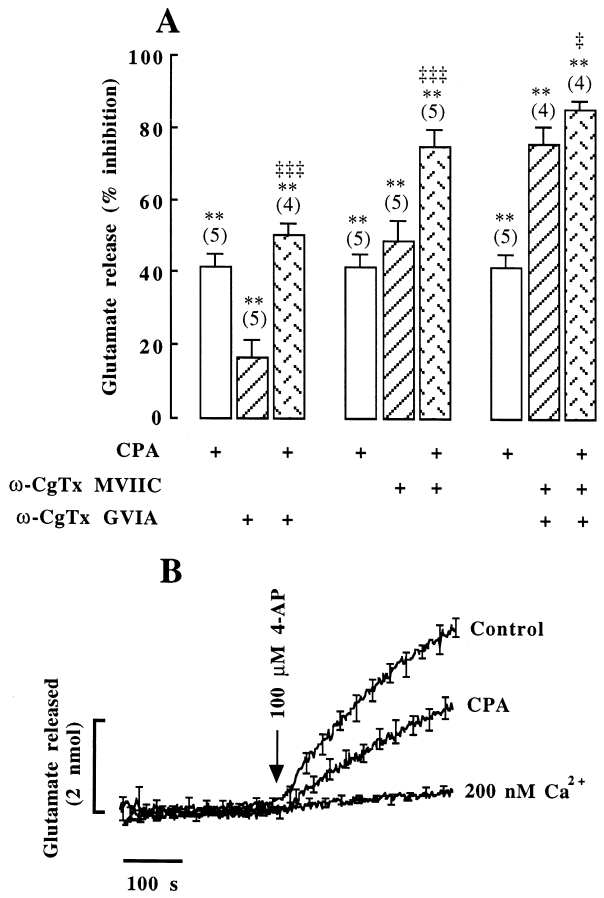

Fig. 4. Modulation by CPA of Ca^{2+} channels coupled to endogenous glutamate release in hippocampal glutamatergic nerve terminals. (A) Synaptosomes were depolarized with 100 μM 4-aminopyridine (4-AP) in the presence of 100 nM CPA and/or Ca^{2+} channel blockers, ω-conotoxin MVIIC (ω-CgTx MVIIC) and/or ω-conotoxin GVIA (ω-CgTx GVIA). The Ca^{2+} channel blockers were present in the pre-incubation period and in the cuvette during the glutamate release experiments. (B) Traces showing the release of glutamate induced by 100 μM 4-aminopyridine. Each trace represents the means ± S.E.M. (every 30 s) of at least four independent experiments. The experiments showing the exocytotic release of glutamate were performed with 1 mM Ca^{2+} present in the medium. Only the experiments concerning the lower trace were performed with 200 nM free Ca^{2+} in the medium. In part A, the number of independent experiments is indicated above each bar. The results are the means ± S.D. $^{**}P < 0.01$, significantly different from the control (0% inhibition – Dunnett's post test). $^{‡}P < 0.05$, significantly different from ω-conotoxin MVIIC plus ω-conotoxin GVIA, $^{‡‡‡}P < 0.001$, significantly different from ω-conotoxin MVIIC or ω-conotoxin GVIA (Bonferroni's post test).

also significantly different ($^{\ddagger\ddagger\ddagger}P < 0.001$) from that obtained with ω-conotoxin MVIIC alone, suggesting that CPA may modulate non-P/Q-type Ca^{2+} channels. Furthermore, the inhibitory effects of CPA and ω-conotoxin MVIIC show some overlap, suggesting that CPA also modulates P/Q-type Ca^{2+} channels involved in the release of glutamate. When both Ca^{2+} channel blockers were present, CPA was still capable of significantly increasing the inhibition due to both blockers ($85.1 \pm 2.5\%$ of inhibition), suggesting that other type(s) of Ca^{2+} channel(s) present in glutamatergic nerve terminals may be modulated when adenosine A_1 receptors are activated.

4. Discussion

4.1. Modulation of the increase in $[Ca^{2+}]_i$ caused by 4-aminopyridine stimulation by adenosine A_1 receptor activation

In the present study we investigated two possible mechanisms (inhibition of Ca^{2+} channels or activation of K^+ channels) for the inhibition of the release of endogenous glutamate in hippocampal synaptosomes by adenosine A_1 receptor activation. The results obtained suggest that the reduction of the Ca^{2+} influx is the main mechanism involved. We also investigated the Ca^{2+} channel types that may be modulated by the activation of adenosine A_1 receptors.

Synaptosomes were stimulated with 4-aminopyridine, which reduces K^+ channel conductivity, leading to a transient opening of voltage sensitive calcium channels (Tibbs et al., 1989). This type of stimulus was preferred since it more closely approximates the electrical action potential generation in nerve terminals. We also stimulated the synaptosomes with KCl depolarization (15 mM and 30 mM), but the inhibition by CPA of the KCl-induced $[Ca^{2+}]_i$ signal was much smaller (about 12%, data not shown) and therefore more difficult to analyze.

In the hippocampal synaptosomal preparation, we observed that (1) endogenous adenosine tonically activates adenosine A_1 receptors and that (2) the effect of CPA was specific for the adenosine A_1 receptor, because DPCPX prevented the inhibitory effect caused by CPA (Fig. 1). We also determined that the inhibition by CPA of the increase in the $[Ca^{2+}]_i$ due to 4-aminopyridine was greater in hippocampal synaptosomes from the CA1 subregion (38%) than in whole hippocampal synaptosomes (27%). This seems to indicate that adenosine A_1 receptors are differently distributed in hippocampal nerve terminals, the nerve terminals from CA1 area being enriched in adenosine A_1 receptors. Another possible interpretation is that glutamatergic terminals form a higher percentage of the synaptosomes in the CA1 preparation. This correlates well with the results obtained by other investigators, who have reported that the CA1 subregion is enriched in adenosine A_1 receptors (Fastbom et al., 1987; Tetzlaff et al., 1987). These observations may also explain the greater protective effect of adenosine in the CA1 hippocampal subregion found in studies of hippocampal ischemia (Rudolphi et al., 1992).

We investigated the contribution of several Ca^{2+} channel types to the increase in $[Ca^{2+}]_i$ stimulated with 100 μM 4-aminopyridine, a sub-maximal concentration of 4-aminopyridine which is used to ensure that only the Ca^{2+}-dependent release of glutamate is elicited (Fig. 4). The inhibition caused by ω-conotoxin MVIIC was greater than that caused by ω-conotoxin GVIA, which indicates that P/Q-type Ca^{2+} channels play a major role in synaptic transmission, as previously shown (Luebke et al., 1993; Wheeler et al., 1994; Graham and Burgoyne, 1995). When both ω-conotoxin MVIIC and ω-conotoxin GVIA were present, the inhibition of $[Ca^{2+}]_i$ increase was not complete, suggesting that other subtype(s) of Ca^{2+} channel(s) remain to be characterized. Similar results were obtained in our laboratory by using KCl as a depolarizing agent (Malva et al., 1995). Nitrendipine (1 μM), that completely blocks L-type Ca^{2+} channels, had no effect in this preparation (not shown). R-type Ca^{2+} channels are expressed in hippocampus, but they are essentially localized in cell bodies (Yokoyama et al., 1995).

The observation that CPA enhanced by 17% the inhibition by ω-conotoxin GVIA of the increase in $[Ca^{2+}]_i$ (Fig. 3), suggests that CPA modulates ω-conotoxin GVIA-insensitive Ca^{2+} channels. Since ω-conotoxin MVIIC-sensitive Ca^{2+} channels constitute the major portion of channels present in these nerve terminals, they are good candidates for modulation by CPA. In addition, in the presence of CPA and ω-conotoxin MVIIC, the inhibition observed was significantly greater than the inhibition caused by ω-conotoxin MVIIC alone, suggesting that ω-conotoxin MVIIC-insensitive Ca^{2+} channels, for example N-type or non-identified-type Ca^{2+} channels, may be modulated.

The adenosine A_1 receptor agonist significantly enhanced (9%) the inhibition caused by ω-conotoxin GVIA plus ω-conotoxin MVIIC, suggesting that activation of adenosine A_1 receptors blocks the entry of Ca^{2+} through non-identified type(s) of channel(s). In contrast to the results previously obtained, considering that N-type Ca^{2+} channels are the main target of adenosine action (Mogul et al., 1993; Yawo and Chuhma, 1993; Wu and Saggau, 1994) our results seem to indicate that adenosine A_1 receptor activation affects several types of Ca^{2+} channels in hippocampal nerve terminals, namely N-type, P/Q-type and other non-identified type(s) of Ca^{2+} channel(s).

4.2. Possible modulatory effects of adenosine A_1 receptor activation on synaptosomal membrane potential

Although adenosine may activate potassium currents (Trussel and Jackson, 1985; Gerber et al., 1989; Thompson et al., 1992), in several studies adenosine failed to alter K^+

conductances (Dolphin et al., 1986; MacDonald et al., 1986; Barrie and Nicholls, 1993). In many cases, the hyperpolarization caused by adenosine or its agonists was observed in postsynaptic membranes (Okada and Ozawa, 1980; Gerber et al., 1989), although in some studies adenosine hyperpolarized presynaptic nerve terminals, such as the giant calyciform nerve terminals of chick ciliary ganglia (Bennett and Ho, 1991). In rat cortical synaptosomes, 2-chloroadenosine promotes the efflux of ^{86}Rb, suggesting hyperpolarization (Zoltay and Cooper, 1990). In olfactory cortex slices, adenosine induces a presynaptic action through the activation of 4-aminopyridine sensitive K^+ current (Scholfield and Steel, 1988).

Since we were not sure whether, in our preparation, the inhibitory effect induced by CPA on the $[Ca^{2+}]_i$ signal was the result of a direct action on Ca^{2+} channels or an indirect result of adenosine-activated potassium currents, we investigated whether CPA might be hyperpolarizing the membrane potential. We estimated the membrane potentials with a TPP^+-selective electrode in the presence of CPA, under resting and depolarizing conditions, and did not observe hyperpolarization in either case. We also measured $[^3H]TPP^+$ uptake, and under both conditions CPA also failed to induce an increase in the uptake of $[^3H]TPP^+$. These results agree with those of previous studies with guinea pig cortical synaptosomes (Barrie and Nicholls, 1993). In hippocampal slices in culture, adenosine acts pre- and postsynaptically at receptors that are not pharmacologically different. The postsynaptic action is mediated by activation of a K^+ current, but the presynaptic effect seems to be mediated by another mechanism, probably inhibition of Ca^{2+} currents (Thompson et al., 1992). A CPA-induced increase in K^+ currents could not be ruled out in the present experiments, although it is unlikely to constitute a major contribution to the reduction of Ca^{2+} currents in these nerve terminals. Very recently Wu and Saggau (1997), based on several lines of evidence, suggested that inhibition of presynaptic Ca^{2+} channels plays the major role in presynaptic inhibition caused by several neurotransmitters and by adenosine. Another hypothesis for the inhibitory effect of adenosine favors an action on a mechanism downstream of calcium entry (Silinsky, 1984). Nevertheless, this mechanism seems to be important only for presynaptic inhibition of spontaneous release (Wu and Saggau, 1997), but we cannot exclude it as a potential mechanism to account for the inhibition of elicited release.

4.3. Modulation of endogenous glutamate release by adenosine A_1 receptor activation

Adenosine inhibits the Ca^{2+}-dependent release of glutamate in rat hippocampal and goldfish brain synaptosomes (Poli et al., 1991, 1993; Barrie and Nicholls, 1993), cerebellar neurons (Dolphin and Prestwich, 1985) and slices of hippocampal CA1 area (Burke and Nadler, 1988). In the present work, we pharmacologically characterized the types of Ca^{2+} channels present in glutamatergic nerve terminals which might be affected by adenosine A_1 receptor activation. We cannot directly correlate the results of $[Ca^{2+}]_i$ measurements (heterogenous population of synaptosomes) with those for glutamate release (glutamatergic nerve terminals), but the Ca^{2+} measurements discussed in Section 4.1 permit determination of whether adenosine A_1 receptor activation inhibits the entry of Ca^{2+} into nerve terminals and which types of Ca^{2+} channels might be modulated.

The adenosine A_1 receptor agonist, CPA, reduced the Ca^{2+}-dependent release of glutamate by 41% (Fig. 4A). This observation clearly shows that inhibitory adenosine A_1 receptors are present in hippocampal glutamatergic nerve terminals. As expected (Luebke et al., 1993; Graham and Burgoyne, 1995), the N-type channels made a slight contribution (16%) to the exocytotic release of glutamate, whereas ω-conotoxin MVIIC inhibited the release of glutamate by 49%, which clearly shows that P/Q-type channels have a predominant role in glutamatergic synaptic transmission. The release of glutamate was not totally blocked when both N- and P/Q-type blockers were present, suggesting that other non-identified type(s) of Ca^{2+} channel(s) contribute to the exocytotic release of glutamate. This fraction resistant to blockade may be identical to the resistant channel that is involved in glutamate exocytosis in cortical synaptosomes (Turner and Dunlap, 1995). Considering that (1) N-type Ca^{2+} channels contribute only slightly to glutamatergic synaptic transmission and that (2) the effect of adenosine A_1 receptor activation on Ca^{2+}-dependent glutamate release is greater than the effect of ω-conotoxin GVIA, it is reasonable to speculate that other types of Ca^{2+} channels (for example P/Q-type) are probably modulated by adenosine. In addition, the fact that the inhibitory effect obtained when CPA and ω-conotoxin GVIA were present was much greater than the effect of ω-conotoxin GVIA alone, indicates that ω-conotoxin GVIA-insensitive Ca^{2+} channels may be modulated by adenosine A_1 receptors, probably the P/Q-type Ca^{2+} channels, which have an important role in the release of glutamate. In fact, the inhibitory effects of CPA and ω-conotoxin MVIIC overlap partially, indicating that P/Q-type Ca^{2+} channels present in glutamatergic nerve terminals may be modulated by adenosine A_1 receptor activation (Fig. 4). On the other hand, the inhibition caused by CPA plus ω-conotoxin MVIIC was significantly greater than that caused by ω-conotoxin MVIIC alone, indicating that other type(s) of Ca^{2+} channel(s), for example N-type or other unknown type(s), might be modulated. However, N-type Ca^{2+} channels do not have an important role in the release of endogenous glutamate suggest that N-type Ca^{2+} channels may not be significantly modulated through the activation of adenosine A_1 receptor. Finally, CPA significantly increased, by 10%, the inhibition of glutamate release when N- and P/Q-type Ca^{2+} channels were blocked, indicating that the remaining fraction of Ca^{2+}

channels may be another target for neuromodulation by adenosine.

In contrast to some results previously obtained, where N-type Ca^{2+} channels were the main target for the action of adenosine (Mogul et al., 1993; Yawo and Chuhma, 1993; Mynlieff and Beam, 1994; Wu and Saggau, 1994), our results seem to indicate that the activation of adenosine A_1 receptors modulates several types of Ca^{2+} channels in hippocampal nerve terminals, namely P/Q-, N- and other non-identified-type(s) of Ca^{2+} channels. Wu and Saggau (1994) speculated that, in hippocampal CA3–CA1 synapses, Q-type Ca^{2+} channels can also be modulated.

In the present work, we showed that adenosine A_1 receptor activation decreases both Ca^{2+} entry into hippocampal nerve terminals and Ca^{2+}-dependent glutamate release. The results also suggest that nerve terminals from the hippocampal CA1 subregion may be enriched in adenosine A_1 receptors, as compared to whole hippocampal synaptosomes. We also showed that adenosine A_1 receptor activation modulates several types of Ca^{2+} channels, and that in hippocampal glutamatergic nerve terminals, P/Q- and other non-identified-types of Ca^{2+} channel(s) may be targets for adenosine modulation.

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