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Transducing system operated by adenosine A_{2A} receptors to facilitate acetylcholine release in the rat hippocampus

Nelson Rebola, Catarina R. Oliveira, Rodrigo A. Cunha*

Center for Neurosciences of Coimbra, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal

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Abstract

Although molecular biology studies indicate the presence of adenosine A_{2A} receptors in the rat hippocampus, the pharmacological characterization of adenosine A_{2A} receptor binding and of its putative facilitatory effects has revealed features essentially different from these found for adenosine A_{2A} receptors in most preparations. We now confirmed that activation of adenosine A_{2A} receptors with 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680, 1-30 nM) or 2-hexynyl-5′-*N*-ethylcarboxamidoadenosine (HENECA, 3–100 nM) facilitated the veratridine-evoked [^{3}H]acetylcholine release from hippocampal synaptosomes with a maximal effect of 14 ± 2% and 16 ± 2%, respectively. These effects were prevented by the adenosine A_{2A} receptor antagonists, 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385, 20 nM) and 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261, 20 nM), but not by the adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 20 nM). Adenosine A_{2A} receptors may activate adenylate cyclase and protein kinase A since CGS 21680 (10 nM) facilitation of [^{3}H]acetylcholine release was occluded by 8-bromo-cAMP (0.5 mM) and forskolin (10 μM) and prevented by H-89 (1 μM), but unaffected by phorbol-12,13-didecanoate (250 nM) or bisindolylmaleimide I (1 μM). The existence of adenosine A_{2A} receptors in hippocampal nerve terminals was further confirmed by a Western blot immunoreactivity qualitatively identical to that found in the striatum. This constitutes the first pharmacological identification of canonical adenosine A_{2A} receptors coupling to the expected cAMP/protein kinase A pathway in the hippocampus with the expected immunoreactivity.
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Keywords: Adenosine; A_{2A} receptor; Hippocampus; Acetylcholine release; Nerve terminal; Synaptosome

1. Introduction

Adenosine can either decrease or facilitate the evoked release of neurotransmitters in the nervous system by activating inhibitory adenosine A_1 or facilitatory adenosine A_{2A} receptors, respectively (Cunha, 2001). As occurs for the modulation of glutamate (Ambrósio et al., 1997; Lopes et al., 2002) and serotonin release (Okada et al., 2001), the modulation of acetylcholine release by adenosine in the hippocampus is also controlled by both adenosine A_1 and A_{2A} receptors (Cunha et al., 1994a; Jin and Fredholm, 1997). The presence of adenosine A_{2A} receptors in the hippocampus has been confirmed using molecular biology studies, like polymerase chain reaction (PCR), in situ hybridization (Cunha et al., 1994b) and Northern blot analysis (Peterfreund et al., 1996), but binding studies have revealed an atypical binding pattern (Cunha et al., 1996) and G protein coupling (Cunha et al., 1999) of these putative adenosine A_{2A} receptors. Some functional peculiarities of the facilitatory effects of the prototypical adenosine A_{2A} receptor agonist, CGS 21680 (Fredholm et al., 1994), include the strict dependency on co-activation of adenosine A_1 receptors in the control of glutamate release (Lopes et al., 2002), the atypical pharmacology in the control of GABA release (Cunha and Ribeiro, 2000a) and the coupling to protein kinase C in the control of excitatory synaptic transmission (Cunha and Ribeiro, 2000b; Lopes et al., 2002). Thus, none of the neurotransmitter systems controlled by putative adenosine A_{2A} receptors has yet proven to obey to a clear and typical adenosine A_{2A} receptor pharmacology and to be coupled to the postulated cAMP/protein kinase A transducing pathway (Fredholm et al., 1994).

We now report that the control by adenosine A_{2A} receptors of acetylcholine release from hippocampal nerve

* Corresponding author. Tel.: +351-239-820190; fax: +351-239-822776.

E-mail address: racunha@clix.pt (R.A. Cunha).

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terminals obeys to the expected pharmacology for adenosine A_{2A} receptors (antagonism by ZM 241385 and SCH 58261 but not by DPCPX) and requires the activation of the adenylate cyclase/cAMP/protein kinase A pathway rather than the protein kinase C transducing pathway.

2. Materials and methods

2.1. Drugs and solutions

8-Bromoadenosine-3′:5′-cyclic monophosphate (8-bromo-cAMP), hemicholinium-3, 7ß-deacetyl-7ß-(γ-*N*-methylpiperazino)butyrylforskolin (forskolin), veratridine and 1,9-dideoxyforskolin were from Sigma; 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), phorbol-12,13-didecanoate and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were from RBI; H-89 and bisindolylmaleimide were from Calbiochem; 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) was from Tocris; and [methyl-^{3}H]choline (specific activity 41.3 Ci/mmol) was from Amersham. Rolipram [4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone (4-RS)] was a gift of Schering; 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4 triazolo[1,5-c]pyrimidine (SCH 58261) was provided by E. Ongini (Schering Plough, Milan); 2-hexynyl-5′-*N*-ethylcarboxamidoadenosine (HENECA) was offered by G. Cristalli (Univ. Camerino, Italy); and goat purified IgG anti-adenosine A_{2A} receptor antibody (200 μg/ml) was from Santa Cruz Biotechnology-Europe. All other reagents were of the highest purity available.

Forskolin was made up to a 20 mM stock solution in ethanol. Dideoxyforskolin was made up to a 20 mM and rolipram in a 50 mM stock solution in dimethylsulfoxide. CGS 21680, HENECA, ZM 241385, SCH 58261, phorbol-12,13-didecanoate, H-89 and bisindolylmaleimide were made up into 5 mM stocks in dimethylsulfoxide. DPCPX was made up into a 5 mM stock in 99% dimethylsulfoxide–1% NaOH (1 M) (v/v). Aqueous dilution of these stock solutions was made daily.

2.2. [^{3}H]acetylcholine release from nerve terminals

The release of [^{3}H]acetylcholine from a synaptosomal fraction from the rat hippocampus was carried out as previously described (Cunha et al., 1995) using male Wistar rats (130–140 g) decapitated after anesthesia under halothane atmosphere, following the European Union guidelines for handling experimental animals. Briefly, the synaptosomes were equilibrated for 10 min at 37 °C, loaded with [^{3}H]choline (10 μCi/ml, 0.125 μM), layered over Whatman GF/C filters into 90-μl chambers and superfused at a flow rate of 0.6 ml/min with a gassed (95% O_2 and 5% CO_2) Krebs solution of the following composition: NaCl 125 mM, KCl 3 mM, NaH_2PO_4 1 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 1.5 mM, $MgSO_4$ 1.2 mM, glucose 10 mM and hemicholinium-3 0.01 mM (see Cunha et al., 1995). After a 30-min washout period, the synaptosomes were stimulated with veratridine (10 μM) for 2 min, at 6 and 26 min after starting sample collection (S_1 and S_2). At the end of the experiments, the filters were removed from the chambers to determine the amount of tritium retained by the synaptosomes. At least 12 h after addition of 5 ml of scintillation cocktail (Scintran T, BDH) to each of the 2-min effluent samples and to the filters, scintillation counting was performed with a 55–60% efficiency during 2 min. The fractional release of tritium was expressed in terms of percentage of total radioactivity present in the tissue at the time of sample collection. The release of tritium evoked by each veratridine pulse, i.e. the evoked release, was calculated by integration of the area of the peak upon subtraction of the estimated basal tritium outflow from the total outflow of tritium due to stimulation. To measure [^{3}H]actylcholine in the total tritium outflow, [^{3}H]acetylcholine was separated using a cation exchanger, tetraphenylboron, after phosphorylation of [^{3}H]choline, as previously described (Cunha et al., 1994a).

When the effect of drugs on the release of acetylcholine was investigated, these drugs were added to the superfusion medium 6 min before S_2, i.e. 20 min after starting sample collection, and remained in the bath up to the end of the experiment. The effect of these drugs on the evoked release of acetylcholine was expressed by alterations of the ratio between the evoked release due to second stimulation period and the evoked release due to the first stimulation period (S_2/S_1 ratio). This ratio is remarkably constant in between experiments, in contrast to absolute values of S_1 that vary in between experiments, precluding a reliable analysis of this parameter. When we evaluated the modifications of the effect of these drugs by modifiers, the modifiers were applied 15 min before starting sample collection and hence were present during S_1 and S_2. When present during S_1 and S_2, none of the modifiers (ZM 241385, SCH 58261, DPCPX, 8-Br-cAMP, forskolin, dideoxyforskolin, rolipram, H-89, bisindolylmaleimide or phorbol-12,13-didecanoate), significantly altered (P>0.05) the S_2/S_1 as compared to the S_2/S_1 ratio obtained in the absence of the modifiers (data not shown).

The values are presented as mean ± S.E.M. The percentage effect of drugs was calculated in each individual experiment and the S.E.M. is relative to the variance of this percentage effect. To test the significance of the effect of drugs versus control, a paired Student's *t* test was used. When making comparisons from a different set of experiments with control, a one-way analysis of variance (ANOVA) was used, followed by Dunnett's test. $P < 0.05$ was considered to represent a significant difference.

2.3. Western blot analysis of A_{2A} receptors

The analysis of adenosine A_{2A} receptor immunoreactivity was carried out in whole membranes of the striatum and

in membranes from a Percoll-purified synaptosomal fraction of the hippocampus, prepared as previously described (e.g. Cunha et al., 1996). After determining the amount of protein (Spector, 1978), each sample was diluted with two volumes of a solution containing 8 M urea, 100 mM dithiothreitol, 2% (w/v) sodium dodecyl sulfate and 375 mM Tris–HCl pH 6.8 and incubated for 2 h at 37 °C. These diluted samples and the pre-stained molecular weight markers (Amersham) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% with a 4% concentrating gel) under reducing conditions (Laemmeli, 1970) and electro-transferred to polyvinylidene difluoride membranes (0.45 μm from Amersham). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline, pH 7.6 containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with the anti-adenosine A_{2A} receptor antibody (1:100 dilution). After four washing periods for 10 min with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase-conjugated anti-goat secondary antibody (1:2000 dilution from Calbiochem) in TBS-T containing 1% milk during 90 min at room temperature. After five 10-min washes in TBS-T 0.5% milk, the membranes were incubated with Enhanced Chemi-Fluorescent during 5 min and then analysed with a Storm (Molecular Devices).

3. Results

3.1. Pharmacological characterization of facilitatory adenosine A_{2A} receptors in hippocampal cholinergic nerve terminals

The two selective adenosine A_{2A} receptor agonists, CGS 21680 and HENECA (1–100 nM) (Fredholm et al., 1994), facilitated the evoked release of acetylcholine from hippocampal nerve terminals (Fig. 1A), as previously described (Cunha et al., 1995, 1997). The concentration-dependent curve for the facilitation of the evoked release of acetylcholine was similar for the two agonists, with increasing effects with increasing applied concentrations of the agonist, CGS 21680 being slightly more potent than HENECA (Fig. 1A). Also, for both agonists, there was a decreased facilitation of the evoked release of acetylcholine at the highest concentrations of the agonists tested that may either result from an adenosine A_{2A} receptor-induced desensitization (see Cunha, 2001 for discussion) or from a loss of selectivity towards inhibitory adenosine A_1 receptors (see, e.g. Lupica et al., 1990).

Previous studies by our group had already tentatively characterized this facilitatory effect of acetylcholine release by CGS 21680 and HENECA as being mediated by adenosine A_{2A} receptors based on the efficiency of the adenosine A_{2A} receptor antagonists, CSC (8-(3-chlorostyryl)caffeine) and of ZM 241385, to prevent these effects (Cunha et al., 1995, 1997). However, some studies have cast doubts on the

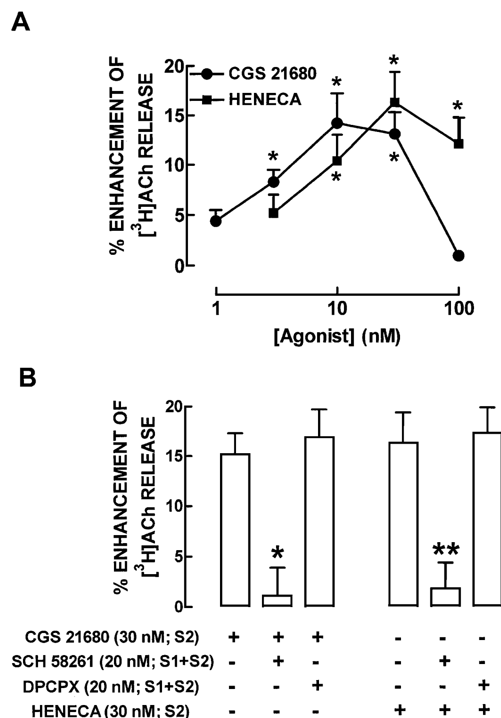

Fig. 1. Concentration-dependent facilitation by the adenosine A_{2A} receptor agonists, CGS 21680 and HENECA, of the evoked release of [^{3}H]acetylcholine ([^{3}H]ACh) from rat hippocampal synaptosomes (A), and comparison of the efficiency of the adenosine A_1 and A_{2A} receptor antagonists, DPCPX and SCH 58261, to antagonize this adenosine A_{2A} receptor-mediated facilitation of [^{3}H]ACh release (B). The superfused nerve terminals (142 ± 8 μg protein per chamber) were stimulated twice (S_1 and S_2) with veratridine (10 μM). The evoked tritium release was Ca^{2+}-dependent (74%) and mainly constituted by [^{3}H]ACh (for details see Cunha et al., 1997), and was assumed to represent exocytotic ACh release. HENECA or CGS21680 were added 7 min before S_2 and remained in the bath up to the end of the experiment. The facilitatory effects of HENECA or CGS21680 were calculated from the alteration of the S_2/S_1 ratio compared with the S_2/S_1 ratio in the absence of these adenosine A_{2A} receptor agonists. *$P<0.05$ compared with 0%. In (B), the drugs were present either during S2 (added 7 min before S2) or in S1 and S2 (added 15 min before starting sample collection) as indicated by the symbols below each column. SCH 58261 (20 nM) or DPCPX (20 nM) when present during S_1 and S_2 did not modify the S_2/S_1 ratio compared with control. *$P<0.05$ compared with the effect of CGS 21680 (30 nM, first bar from the left), **$P<0.05$ compared with the effect of HENECA (30 nM, forth column from the left). The results are mean ± S.E.M. of three to four experiments in (A) and (B).

real selectivity of CSC and of ZM 241385 (Cunha et al., 1996; de Mendonça and Ribeiro, 1994; Lindström et al., 1996; Lopes et al., 1999a) towards adenosine A_1 receptor-mediated responses. Thus, we decided to test the ability of SCH 58261 to antagonize the CGS 21680- and HENECA-induced facilitation of acetylcholine release, since this compound has repeatedly been shown to behave as a selective antagonist of adenosine A_{2A} receptors (Cunha et al., 1999; Fredholm et al., 1998; Lindström et al., 1996). As illustrated in Fig. 1B, the facilitatory effects of either CGS 21680 (30 nM) or HENECA (30 nM) were nearly abolished in the presence of SCH 58261 (20 nM) and were virtually

unaffected by the adenosine A_1 receptor antagonist, DPCPX (20 nM).

So far, we have only been able to show that activation of adenosine A_{2A} receptors with selective adenosine A_{2A} receptor agonists triggered a facilitation of acetylcholine release with a pharmacology compatible with the activation of adenosine A_{2A} receptors. We then sought to demonstrate that a facilitatory response could be obtained with non-selective adenosine receptor ligands. It is difficult to use adenosine itself to carry out a pharmacological characterization of adenosine receptor-mediated effects since adenosine is rapidly taken up and metabolized by virtually all eukaryotic cell types. Thus, we tested the effect of the closest chemical analogue of adenosine, 2-chloroadenosine, which is less prone to uptake and metabolism and activates all adenosine receptor subtypes (Fredholm et al., 1994). As illustrated in Fig. 2A, 2-chloroadenosine (10 μM) inhibited the evoked release of acetylcholine, in accordance with previous observations in hippocampal slices (Cunha et al., 1994a). This inhibitory effect was abolished by the adenosine A_1 receptor selective antagonist, DPCPX (20 nM), indicating that it is an adenosine A_1 receptor-mediated effect. In the presence of DPCPX (20 nM), we now observed that 2-chloroadenosine facilitated the evoked release of acetylcholine (Fig. 2B). This facilitatory effect was prevented by SCH 58261 (20 nM), indicating that it is mediated by facilitatory adenosine A_{2A} receptors (Fig. 2C). These results show that the evoked release of acetylcholine is under the control of both inhibitory A_1 and facilitatory A_{2A} receptors, and that the exogenous addition of a non-selective adenosine receptor agonist mainly leads to the activation of inhibitory adenosine A_1 rather than of facilitatory adenosine A_{2A} receptors.

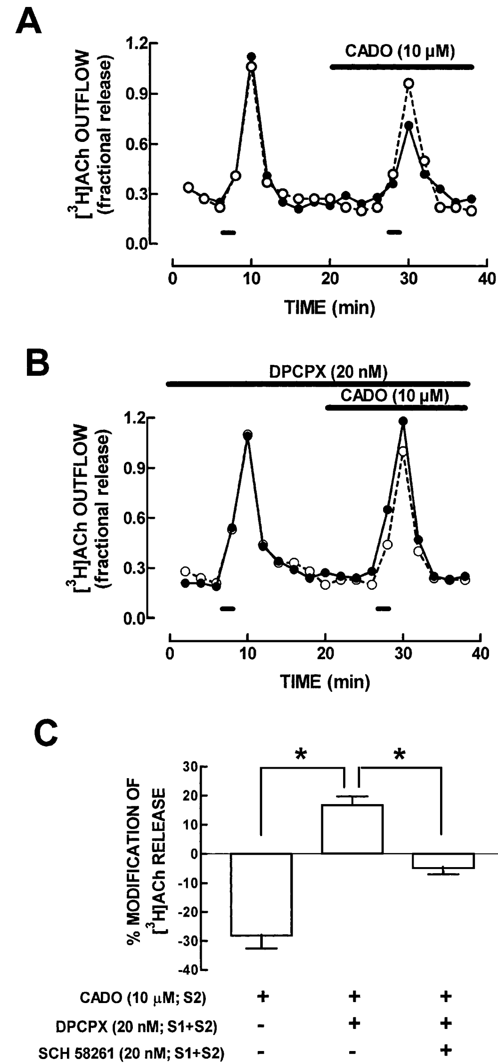

Fig. 2. Inhibition by 2-chloroadenosine (CADO, the closest chemical analogue of adenosine) of the evoked release of acetylcholine (ACh) from rat hippocampal nerve terminals (A), and reversal of this effect upon blockade of adenosine A_1 receptors with DPCPX (B) that is prevented by the adenosine A_{2A} receptor antagonist, SCH 58261 (C). In A and B are shown the time course of [^{3}H]ACh release, measured as the fractional release that is expressed in terms of the percentage of total radioactivity present in the preparation at the beginning of the collection of each sample. The preparation was challenged with two periods of stimulation with 10 μM veratridine (S_1 and S_2), as indicated by the bars above the abscissa. The open symbols represent [^{3}H]ACh release from a control chamber, to which no drug was added in (A) or that were in the presence of DPCPX (20 nM) from 15 min before starting sample collection onwards in (B), and the filled symbols represent [^{3}H]ACh release of the test chamber, to which CADO (10 μM) was added through the superfusate, as indicated by the upper bar. In (C) are shown the average modulatory effects of CADO (10 μM) in the absence or in the presence of DPCPX (20 nM) and of SCH 58261 (20 nM) as indicated by the symbols below each bar. $^*P<0.05$. The results are mean ± S.E.M. of four experiments.

3.2. Transducing system operated by facilitatory adenosine A_{2A} receptors in hippocampal cholinergic nerve terminals

Classically, adenosine A_{2A} receptors are recognized as being coupled to the adenylate cyclase/cAMP transducing pathway (Fredholm et al., 1994). However, several evidences indicate that some facilitatory effects triggered by the prototypical adenosine A_{2A} receptor agonist, CGS 21680, in low nanomolar concentrations, are dependent on protein kinase C activation (Cunha and Ribeiro, 2000a,b; Lopes et al., 2002). We first tested the effect of the direct activation of protein kinase A and of protein kinase C on acetylcholine release. The direct activation of protein kinase C with its direct cell-permeable activator phorbol-12,13-didecanoate (250 nM) facilitated the evoked release of acetylcholine by 13.1 ± 2.2% ($n=4$). As illustrated in Fig. 3, the protein kinase C inhibitor, bisindolylmaleimide (1 μM), significantly attenuated this facilitatory effect of phorbol-12,13-didecanoate, indicating that it was indeed due to protein kinase C activation ($n=4$). The direct activation of protein kinase A with its direct cell-permeable activator 8-bromo-cyclic AMP (8-Br-cAMP, 0.5 mM) facilitated the evoked release of acetylcholine by 21.2 ± 3.4% ($n=4$). As illustrated in Fig. 3, the protein kinase A inhibitor, H-89 (1 μM), completely prevented this facilitatory effect of 8-Br-cAMP, indicating that it was indeed due to protein kinase A

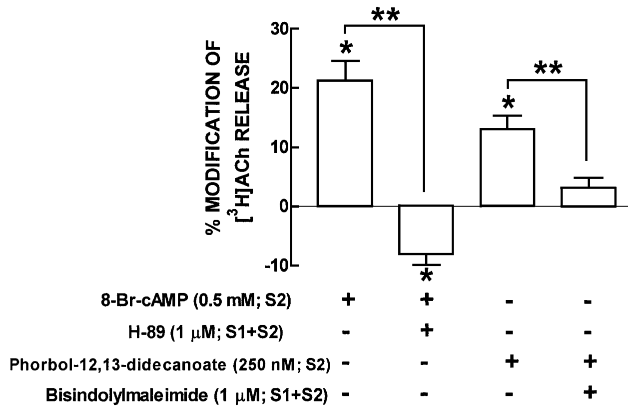

Fig. 3. Ability of drugs interfering with the protein kinase A or protein kinase C pathways to affect the evoked release of [^{3}H]acetylcholine ([^{3}H]ACh) from rat hippocampal nerve terminals. The superfused nerve terminals (142 ± 8 μg protein per chamber) were stimulated twice (S_1 and S_2) with veratridine (10 μM). The bar graph shows the ability of the membrane-permeable cAMP analogue, 8-bromo-cAMP (0.5 mM), to facilitate [^{3}H]ACh release, an effect reverted by the protein kinase A inhibitor, H-89 (1 μM), and the ability of the protein kinase C activator, phorbol-12,13-didecanoate (250 nM), to facilitate [^{3}H]ACh release, an effect attenuated by the protein kinase C inhibitor, bisindolylmaleimide (1 μM). The drugs were present either during S2 (added 7 min before S2) or in S1 and S2 (added 15 min before starting sample collection) as indicated by the symbols below each column. H-89 (1 μM) or bisindolylmaleimide (1 μM) when present during S_1 and S_2 did not modify the S_2/S_1 ratio compared with control. *$P<0.05$ compared with 0%; **$P<0.05$. The results are mean ± S.E.M. of four experiments.

activation ($n=4$). In accordance with previous suggestions that high micromolar concentrations of 8-Br-cAMP might trigger the activation of inhibitory A_1 receptors (e.g. Dunwiddie and Hoffer, 1980; Brundege et al., 1997), it was observed that 8-Br-cAMP (0.5 mM) in the presence of H-89 (1 μM) now inhibited the evoked release of acetylcholine (Fig. 3).

We then tested if the activation or inhibition of the protein kinase A and protein kinase C pathways impacted on the ability of adenosine A_{2A} receptors to facilitate the evoked release of acetylcholine (Fig. 4). The activation of protein kinase A with 8-Br-cAMP (0.5 mM, $n=4$) or with forskolin (10 μM, $n=4$), but not with the inactive analogue 1,9-dideoxyforskolin (10 μM, $n=2$), blunted the ability of CGS 21680 (30 nM) to facilitate the evoked release of acetylcholine (Fig. 4A). Likewise, inhibition of protein kinase A activity with H-89 (1 μM, $n=4$) also attenuated the ability of CGS 21680 (30 nM) to facilitate the evoked release of acetylcholine (Fig. 4A). To further re-enforce our conclusion that adenosine A_{2A} receptor facilitation of acetylcholine release depends on the activation of the cAMP/protein kinase A pathway, we tested if the inhibition of the phosphodiesterases responsible for the catabolism of cAMP would enhance the effect of a sub-maximal concentration of CGS 21680. CGS 21680 (1 nM) facilitated the evoked release of acetylcholine by 4.4 ± 1.1% ($n=3$), but in the presence of the phosphodiesterase IV

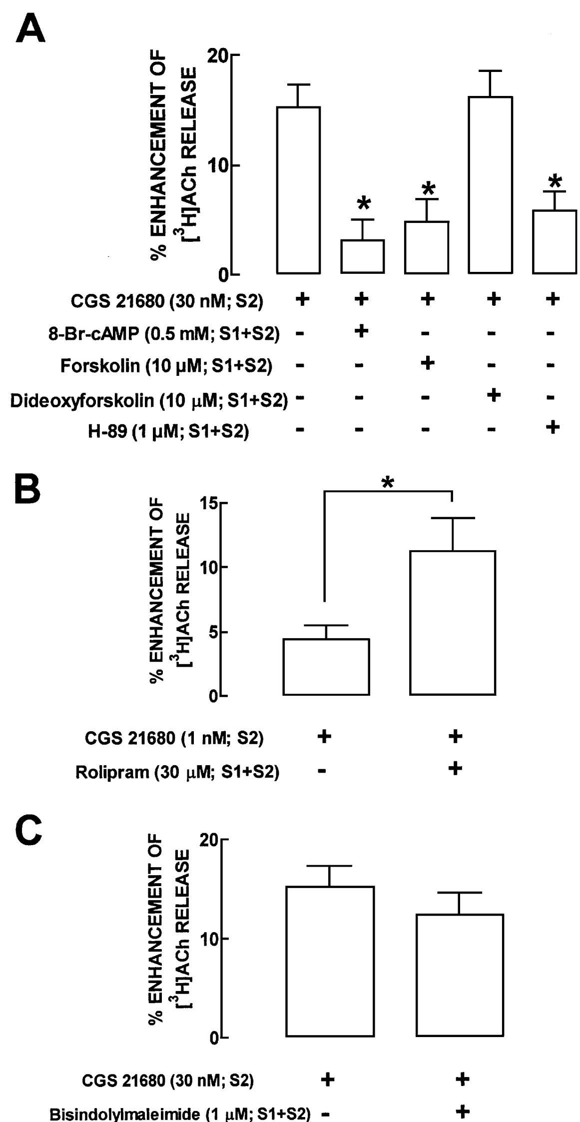

Fig. 4. Ability of drugs interfering with the protein kinase A pathway and inability of the drugs interfering with the protein kinase C pathway to modify the adenosine A_{2A} receptor-mediated facilitation of [^{3}H]acetylcholine ([^{3}H]ACh) from rat hippocampal nerve terminals. The superfused nerve terminals (142 ± 8 μg protein per chamber) were stimulated twice (S_1 and S_2) with veratridine (10 μM). The adenosine A_{2A} receptor agonist, CGS 21680 (30 nM), was present during S2 (added 7 min before S2) and the different tested modifiers were present during S1 and S2 (added 15 min before starting sample collection) as indicated by the symbols below each column. In (A) is shown the ability of the membrane-permeable cAMP analogue, 8-bromo-cAMP (0.5 mM), of the adenylate cyclase activator, forskolin (10 μM), but not of its inactive analogue, 1,9-dideoxyforskolin (10 μM), and of the protein kinase A inhibitor, H-89 (1 μM), to attenuate the CGS 21680-induced facilitation of [^{3}H]ACh release. The results are mean ± S.E.M. of two to four experiments. *$P<0.05$ when compared with the facilitatory effect of 30 nM CGS 21680 (first column from the left). In (B) is shown the ability of the phophodiesterase IV inhibitor, rolipram (30 μM), to potentiate the facilitatory effect of a lower concentration of CGS 21680 (1 nM) on evoked [^{3}H]ACh release. The results are mean ± S.E.M. of three to four experiments. *$P<0.05$. In (C) is shown the inability of the protein kinase C inhibitor, bisindolylmaleimide (1 μM) to modify the CGS 21680-induced facilitation of [^{3}H]ACh release. The results are mean ± S.E.M. of four experiments.

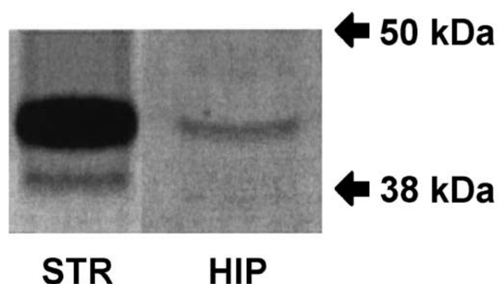

Fig. 5. Western blot identification of adenosine A_{2A} receptors, in membranes prepared from Percoll-purified nerve terminal membranes obtained from the rat hippocampus (HIP) and from whole membranes of the striatum (STR). The SDS-PAGE gel was loaded with 120-µg protein in each lane and is representative of four similar separations carried out with membranes prepared from different animals.

inhibitor, rolipram (30 µM, $n = 4$), CGS 21680 (1 nM) now facilitated the evoked release of ACh by $11.2 \pm 2.6\%$ (Fig. 4B). Finally, the observation that the protein kinase C inhibitor, bisindolylmaleimide (1 µM, $n = 4$), failed to significantly ($P < 0.05$) modify the ability of CGS 21680 (30 nM) to facilitate the evoked release of acetylcholine strongly suggests that the adenosine A_{2A} receptor-mediated facilitation of acetylcholine release might not depend on protein kinase C activation (Fig. 4C).

3.3. Immunological identification of adenosine A_{2A} receptors in hippocampal nerve terminals

The pharmacological characterisation of adenosine A_{2A} receptors has been mostly carried out in the striatum (Fredholm et al., 1994). To further re-enforce that hippocampal nerve terminals are indeed endowed with a molecular entity identical to striatal adenosine A_{2A} receptors, we investigated by Western blot analysis if the molecular entities recognized by adenosine A_{2A} receptor antibodies displayed a similar electrophoretic mobility in hippocampal and striatal membranes. In Fig. 5 is shown a Western blot, representative of four similar blots carried out in membranes from different animals, demonstrating the presence in hippocampal nerve terminal membranes of a band of similar apparent molecular weight (42 kDa) to that recognized in the striatum by adenosine A_{2A} receptor antibodies, albeit with a much lower intensity. Control experiments showed that the lack of addition of the primary antibody resulted in background fluorescence in the gel (data not shown).

4. Discussion

The present study presents evidences for the presence of adenosine A_{2A} receptors with pharmacological and biochemical features identical to these found for the prototypical adenosine A_{2A} receptors in the striatum. In fact, we identified by Western blot an adenosine A_{2A} receptor-like immunoreactivity apparently identical in the hippocampus and striatum. Furthermore, the control by adenosine A_{2A} receptors of acetylcholine release from hippocampal nerve terminals obeys the expected pharmacology for adenosine A_{2A} receptors (antagonism by ZM 241385 and SCH 58261 but not by DPCPX) and requires the activation of the adenylate cyclase/cAMP/protein kinase A pathway, the same biochemical transducing system activated by striatal adenosine A_{2A} receptors.

The present results are particularly important in light of the several reports showing that the prototypical adenosine A_{2A} receptor agonist, CGS 21680, essentially binds to extra-striatal regions of the central nervous system to sites apparently different from adenosine A_{2A} receptors (Cunha et al., 1996, 1999; Johansson and Fredholm, 1995; Johansson et al., 1993; Lindström et al., 1996). The functional demonstration of pharmacologically well-defined adenosine A_{2A} receptors in hippocampal neurons extends previous demonstrations that adenosine A_{2A} receptors mRNA is present in the hippocampus (Cunha et al., 1994a; Dixon et al., 1996; Peterfreund et al., 1996), namely in neurons (Lopes et al., 2001), and constitutes a clear demonstration that adenosine A_{2A} receptors are not confined to the striatum. This had already been proposed in other reports (e.g. Barraco et al., 1995, 1996; Li and Henry, 1998; Okada et al., 2001; Phillis, 1998; Robertson and Edwards, 1998). However, the pharmacological and biochemical data presented in these studies do not allow distinguishing between typical striatal-like adenosine A_{2A} receptors and atypical receptors and/or binding sites recognized by adenosine A_{2A} receptor agonists.

It is interesting to note that the cholinergic system appears to display a well-conserved adenosine A_{2A} receptor-mediated facilitatory system in most preparations where this has been investigated. In fact, the presence of adenosine A_{2A} receptors has been observed in cholinergic nerve terminals of the striatum (Brown et al., 1990), in motor nerve terminals (Correia-de-Sá and Ribeiro, 1994) and in Torpedo electric organ nerve terminals (Satoh et al., 1997), although in the cerebral cortex the picture is less clear (cf. Broad and Fredholm, 1996; Phillis et al., 1993; Spignoli et al., 1984). As we now observed in the hippocampus, the facilitation of the evoked release of acetylcholine by adenosine A_{2A} receptors in all these systems is small in amplitude (lower than 30%), appears to be larger in amplitude in intact preparations than in purified nerve terminals (see Cunha et al., 1994a; Jin and Fredholm, 1997), but always involves the activation of the cAMP/protein kinase A pathway. Interestingly, we had previously reported that the density of adenosine A_{2A} receptor-like binding sites and the facilitatory effect of CGS 21680 on acetylcholine release were markedly enhanced in the hippocampus of aged rats (Lopes et al., 1999b). And in these aged animals, but not in young adult rats, we observed that CGS 21680 enhanced the levels of cAMP (Lopes et al., 1999b), as occurs in the striatum both in young adult and in aged rats. Also, in aged animals,

the control of hippocampal glutamatergic transmission is not significantly changed by blockade of adenosine A_1 receptors (Cunha and Ribeiro, 2001) whereas in young adult animals, the adenosine A_{2A} receptor-mediated facilitation of synaptic transmission is strictly dependent on tonic adenosine A_1 receptor activation (Lopes et al., 2002). Interestingly, whereas adenosine A_{2A} receptor facilitation of hippocampal excitatory synaptic inhibition is mediated by protein kinase C in young adult animals (Cunha and Ribeiro, 2000b), it mostly involves activation of protein kinase A in aged animals (Cunha and Ribeiro, 2001). Thus, it appears that when adenosine A_{2A} receptor-mediated effects involve protein kinase A, then these effects become independent of adenosine A_1 receptor function, as was presently confirmed for the adenosine A_{2A} receptor-mediated facilitation of the evoked release of acetylcholine from hippocampal nerve terminals. It should be noted that, although we could not find evidences for the participation of protein kinase C on adenosine A_{2A} receptor-mediated facilitation of acetylcholine release in hippocampal nerve terminals, we cannot exclude an eventual minor role of this transducing pathway when the protein kinase A pathway is blocked, as was reported to occur in striatal nerve terminals (Gubitz et al., 1996).

The possible physiological role of adenosine A_{2A} receptors in the control of acetylcholine release in the central nervous system is still ill-defined. In fact, with the experimental conditions used, the predominant effect of adenosine in cholinergic nerve terminals is to activate inhibitory adenosine A_1 receptors (e.g. Jackisch et al., 1984), as now confirmed by the clear inhibitory effect caused by the closest adenosine analogue, 2-chloroadenosine (see also Cunha et al., 1994a; Jin and Fredholm, 1997). Only when blocking adenosine A_1 receptors is it possible to reveal an adenosine A_{2A} receptor-mediated facilitation of acetylcholine release (see also Cunha et al., 1994a; Jin and Fredholm, 1997). At the motor nerve terminals of the phrenic nerve, the elegant work of the group of Correia-de-Sá et al. (1996) has provided clear evidences that the come into play of adenosine A_{2A} receptors depends on the frequency of stimulation, but this issue has not yet been tested in cholinergic pathways of the central nervous system.

In conclusion, the present demonstration of the presence of adenosine A_{2A} receptors in cholinergic nerve terminals of the rat hippocampus clearly shows that adenosine A_{2A} receptors are not confined to the basal ganglia in the central nervous system.

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