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Research report

Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus

Nelson Rebola, Paulo C. Pinheiro, Catarina R. Oliveira, João O. Malva, Rodrigo A. Cunha*

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

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Abstract

Adenosine is a neuromodulator in the CNS that mainly acts through pre- and postsynaptic A_1 receptors to inhibit the release of excitatory neurotransmitters and NMDA receptor function. This might result from a highly localized distribution of A_1 receptors in the active zone and postsynaptic density of CNS synapses that we now investigated in the rat hippocampus. The binding density of the selective A_1 receptor antagonist, [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX), was enriched in membranes from Percoll-purified nerve terminals ($B_{max}=1839\pm52$ fmol/mg protein) compared to total membranes from the hippocampus ($B_{max}=984\pm31$ fmol/mg protein), the same occurring with A_1 receptor immunoreactivity. [^{3}H]DPCPX binding occurred mainly to the plasma membrane rather than to intracellular sites, since the binding of the membrane permeable A_1 receptor ligand [^{3}H]DPCPX to intact hippocampal nerve terminals ($B_{max}=1901\pm192$ fmol/mg protein) was markedly reduced ($B_{max}=321\pm30$ fmol/mg protein) by the membrane impermeable adenosine receptor antagonist, 8-sulfophenyltheophilline (25 μM). Further subcellular fractionation of hippocampal nerve terminals revealed that A_1 receptor immunoreactivity was strategically located in the active zone of presynaptic nerve terminals, as expected to understand the efficiency of A_1 receptors to depress neurotransmitter release. A_1 Receptors were also present in nerve terminals outside the active zone in accordance with the existence of a presynaptic A_1 receptor reserve. Finally, A_1 receptor immunoreactivity was evident in the postsynaptic density together with NMDA receptor subunits 1, 2A and 2B and with N-and P/Q-type calcium channel immunoreactivity, emphasizing the importance of A_1 receptors in the control of dendritic integration.

Theme: Neurotransmitters, modulators, transporters and receptors

Topic: Regional localization of receptors and transmitters

Keywords: Adenosine; A_1 receptor; Hippocampus; Nerve terminal; Active zone; Postsynaptic density; Synaptosome; NMDA receptor; Calcium channel

1. Introduction

Adenosine is a neuromodulator in the nervous system that mainly acts through inhibitory A_1 receptors [13]. Adenosine attenuates neuronal activity by different mechanisms, namely through a presynaptic inhibition of neurotransmitter release, through a postsynaptic control of neurotransmitter responsiveness or through neuronal hyperpolarization [4]. The study of adenosine neuromodulation in the hippocampus, one of the brain regions with the highest density of these metabotropic A_1 receptors (e.g. [15]), has been instructive to conclude that the A_1 receptor-mediated inhibition of synaptic transmission essentially depends on the inhibition of glutamate release by presynaptic A_1 receptors (e.g. [36,47]). Recent results, based on the use of A_1 receptor knockout and heterozygous animals, have confirmed that the efficiency of adenosine modulation of synaptic transmission is highly dependent on the number of A_1 receptors [23]. Thus, it is expected that there might be a higher density of A_1 receptors in nerve terminals explaining why A_1 receptor

Abbreviations: DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; 8-SPT, 8-sulfophenyltheophilline; TBS–T, Tris-buffered saline with 0.1% Tween 20; XAC, {4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl}xanthine

*Corresponding author. Tel.: +351-239-820-190; fax: +351-239-822-776.

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

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modulation of neurotransmitter release is the predominant mechanism of A_1 receptor inhibition of neuronal activity. Also, to understand the high stimulus-to-stimulus efficiency of A_1 receptors to depress glutamate release in the hippocampus [29], it is expected that A_1 receptors are particularly abundant in the active zone of hippocampal nerve terminals, although most metabotropic receptor that control neurotransmitter release have an higher abundance perisynaptically, i.e outside the active zone (e.g. [45,49]). This possible localization of A_1 receptors in the active zone would also facilitate understanding of how A_1 receptors control neurotransmitter release through an inhibition of N-type voltage sensitive calcium channels [40,51,52] or through a decrease in the sensitivity for calcium of the release apparatus [2,11,39,43], since these two purported molecular targets of A_1 receptors are mostly confined to the active zone of nerve terminals (e.g. [21]).

In contrast to the control of synaptic transmission by adenosine, the neuroprotective role of adenosine depends on a combined action of presynaptic A_1 receptor-mediated inhibition of glutamate release together with a postsynaptic A_1 receptor-mediated control of NMDA receptor function (reviewed in [8]). Thus, it is also expected that A_1 receptors might be co-localized with NMDA receptors in the postsynaptic density to understand the striking ability of A_1 receptors to act as low-pass filters for NMDA receptor activation [7,24].

Therefore, we anticipate that the distribution of A_1 receptors might be heterogeneous in hippocampal nerve terminals and synapses, in a manner similar to the previously reported heterogeneity of extracellular metabolism of adenosine, where channeling mechanisms were proposed to occur [6,14]. We now compare the binding density of A_1 receptors in different membrane fractions from the rat hippocampus and took advantage of a recently described ultrapurification method of the pre- and postsynaptic components of the active zone [34] to determine that A_1 receptors are indeed heterogeneously distributed within nerve terminals and synapses in the rat hippocampus.

2. Materials and methods

2.1. Materials

Adenosine deaminase (type VI, 2000 U/ml), 8-(*p*-sulfophenyl)theophylline (8-SPT) and D-myo-inositol 1,4,5-triphosphate were from Sigma (Reagente 5, Portugal) and {4-[(2-aminoethyl)amino]carbonylmethyloxyphenyl}xanthine (XAC) was from Research Biochemicals (Reagente 5, Portugal). [^{3}H]1,3-Dipropyl-8-cyclopentyladenosine ([^{3}H]DPCPX, specific activity 109.0 Ci/mmol) and D-[^{3}H]inositol 1,4,5-triphosphate (20.0 Ci/mmol) were from DuPont NEN (Anagene, Portugal), rabbit purified IgG anti-adenosine A_1 receptor antibody (1.8 mg/ml) was from Affinity Bioreagents (Golden, USA), rabbit anti-calcium channel α1B subunit antibody (4 mg/ml) and rabbit anti-calcium channel α1A subunit antibody (2 mg/ml) were from Alomone Labs. (Jerusalem, Israel), rabbit anti-NMDA receptor subunit 1 antibody (0.1 mg/ml) was from Chemicon (Temecula, CA, USA), rabbit anti-NMDA receptor subunit 2A antibody (0.2 mg/ml) was from Molecular Probes (Leiden, The Netherlands), rabbit anti-NMDA receptor subunit 2B antibody (0.25 mg/ml) was from Transduction Labs. (Lexington, USA), ω-conotoxin GVIA was from Alomone Labs., (3-[^{125}I]-iodotyrosyl 22) ω-conotoxin GVIA (2000 Ci/mmol) was from Amersham (Buckinghamshire, UK). All other reagents were of the highest purity available.

Male Wistar rats (6–8 weeks old, 140–160 g) were used throughout this study and were handled according with the EU guidelines for use of experimental animals, the rats being anesthesized under halothane atmosphere before being sacrificed by decapitation.

2.2. Binding studies of A_1 receptors

Membranes from the whole hippocampus or from Percoll-purified hippocampal synaptosomes were prepared as previously described [25]. Briefly, the two hippocampi from one rat were homogenized at 4 °C in 10 volumes of a sucrose solution (0.32 M), containing 50 mM Tris–HCl, 2 mM EGTA and 1 mM dithiothreitol, pH 7.6. This mixture was centrifuged at 3000 *g* for 10 min at 4 °C, the supernatant collected and centrifuged at 14 000 *g* for 20 min at 4 °C. The pellet was again resuspended in 10 ml of sucrose solution and divided into two samples, both of which were again centrifuged at 14 000 *g* for 20 min at 4 °C. The pellet of one of the samples was resuspended in an incubation buffer containing 25 mM Tris, 2 mM $MgCl_2$, pH 7.4, and corresponded to the total hippocampal membranes. The pellet of the other sample was resuspended in 1 ml of a 45% v/v Percoll solution made up in a Krebs–Ringer solution (composition 140 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM EDTA, 10 mM glucose, pH 7.4). After centrifugation at 14 000 *g* for 2 min at 4 °C, the top layer was removed (synaptosomal fraction), washed in 1 ml of Krebs–Ringer solution and resuspended in 10 ml of the incubation buffer. This mixture was centrifuged at 14 000 *g* for 20 min at 4 °C and the pellet was resuspended in the incubation buffer and corresponded to the membranes of Percoll-purified nerve terminals.

Both membrane fractions were first incubated with 2 U/ml adenosine deaminase for 30 min at 37 °C to remove endogenous adenosine. The suspensions were then centrifuged for 20 min at 14 000 *g* and the pellet resuspended in the incubation buffer with 2 U/ml of adenosine deaminase. Binding of [^{3}H]DPCPX was for 2 h at 37 °C, with 162–214 μg of membrane protein in a final volume of 300

μl in the incubation buffer, as previously described [25]. Specific binding was determined by subtraction of the nonspecific binding that was measured in the presence of 2 μM of XAC and represented <15% of total binding. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 10 ml of the incubation buffer, kept at 4 °C. The filters were then placed in scintillation vials and 5 ml of scintillation liquid (Scintran Cocktail T, Wallac, Pharmacia-Portugal) were added. Radioactivity bound to the filters was determined after 12 h with an efficiency of 55–60% for 2 min. All binding assays were performed in triplicate. Membrane protein was determined according to Peterson [33].

When comparing the binding of [^{3}H[DPCPX in intact hippocampal synaptosomes in the absence and in the presence of 8-SPT, the Percoll-purified synaptosomes were resuspended in Krebs–HEPES solution (124 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose buffered with 25 mM HEPES, pH 7.4) plus 2 U/ml of adenosine deaminase to remove endogenous adenosine and the binding assays were performed as described for membranes preparations, except that the Krebs–HEPES solution was used as incubation and washing solutions (see [25]).

The data were initially processed in Microsoft EXCEL software to determine the average specific binding and then fitted by nonlinear regression using the Raphson–Newton method, performed with the GRAPHPAD INPLOT software package. An *F*-test ($P>0.05$) was used to determine whether the competition curves were fitted best by one or two independent binding sites.

2.3. Binding studies of N-type calcium channels and IP3 receptors

Hippocampal synaptosomes were prepared as described above and resuspended in 1 ml of an incubation solution containing 50 mM Tris, 150 mM NaCl, 3 mM KCl, 2 mM EGTA and 0.1% bovine serum albumin, pH 7.4. This solution was then separated in two aliquots and each was diluted to a final volume of 2.2 ml. To one of the aliquots (intact synaptosomes) 44 μl of incubation solution was added, whereas to the other aliquot (permeabilized synaptosomes) 44 μl of Triton X-100 were added. Binding of [^{3}H]inositol 1,4,5-triphosphate (IP3) or of [^{125}I]ω-conotoxin GVIA were for 1 h at 37 °C, with 188–243 μg of membrane protein (of either intact or permeabilized synaptosomes) in a final volume of 200 μl in the incubation solution, as previously described [3,41]. Specific binding was determined by subtraction of the nonspecific binding that was measured in the presence of either 1 μM of IP3 or 100 nM ω-conotoxin GVIA and represented <31% of total binding. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fiber filters, followed by washing of the filters and reaction tubes with 4 ml of the incubation solution, kept at 4 °C.

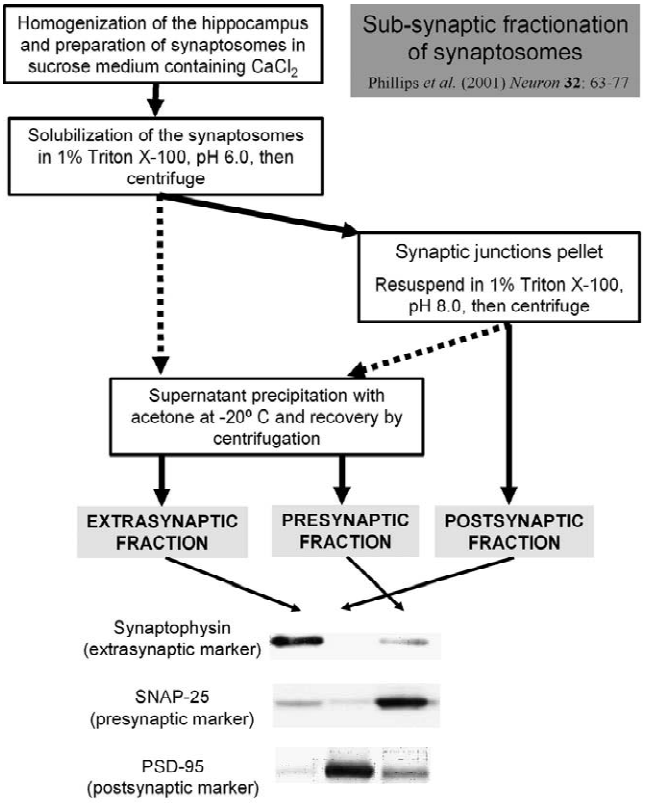

Fig. 1. Schematic representation of the approach used to separate the presynaptic, postsynaptic and extrasynaptic fractions of nerve terminals and its validation. Typical Western blot analysis of the relative immunoreactive (measured densitometrically, assuming 100% as the sum of the densitometric values of the three samples per gel) of SNAP-25 (a presynaptic active zone marker), PSD-95 (a marker of postsynaptic densities) and synaptophysin (a marker of extrasynaptic membranes since it is located in synaptic vesicles) that validates the efficiency of the separation in the three fractions derived from nerve terminals.

2.4. Separation of the presynaptic, postsynaptic and extrasynaptic fractions from nerve terminals

The separation of the presynaptic, postsynaptic and nonsynaptic fractions was carried out as previously described [34,35]. The overall strategy is presented in Fig. 1, which also displays the separation by Western blot analysis of markers of the different fractions that validate this separation protocol (see [35]). Briefly, synaptosomes were diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2× solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) pelleted (40 000 *g* for 30 min at 4 °C). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with six

volumes of acetone at −20 °C and recovered by centrifugation (18 000 *g* for 30 min at −15 °C). The synaptic junctions pellet was washed in solubilization buffer (pH 6.0) and resuspended in 10 volumes of a second solubilization buffer (1% Triton X-100, 20 mM Tris but at pH 8.0). This increase in pH allows the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the postsynaptic density (see [34]). Hence, the active zone is solubilized whereas the postsynaptic density is essentially preserved because the amount of detergent is not enough for solubilization [34]. After incubation for 30 min on ice with mild agitation, the mixture is centrifuged and the supernatant (presynaptic fraction) processed as described for the extrasynaptic fraction. The protease inhibitor, phenylmethylsulfonyl fluoride (1 mM, from Sigma) was added to the suspension in all extraction steps. The pellets from the supernatants and the final insoluble pellet (postsynaptic fraction) were solubilized in 5% SDS, the protein concentration determined by the bicinchoninic acid protein assay and the samples were added to an equal volume of 2× SDS–PAGE sample buffer [62.5 mM Tris–HCl, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol] prior to freezing at −20 °C. As shown in Fig. 1, this fractionation procedure allows an effective separation (over 90% efficiency) of markers of the presynaptic (e.g. SNAP25), postsynaptic (e.g. PSD-95) and nonsynaptic (e.g. synaptophysin) fractions, as evaluated by Western blot [35]. Thus, this technique, which has also been validated by electron microscopy analysis [34], presents the potential advantage of overcoming eventual difficulties of accessibility of antibodies to epitopes located in the synapse that may be hindered by the dense protein matrix that holds the synapse together, which is disrupted on solubilization of the different components.

2.5. Western blot analysis

The analysis of A_1 receptor immunoreactivity was first carried out in total membranes and in membranes from a Percoll-purified synaptosomal fraction, prepared as described above. The relative A_1 receptor immunoreactivity was then evaluated in the purified presynaptic and postsynaptic components of the active zone as well as in the extrasynaptic fraction of rat hippocampal nerve terminals, prepared as described above. The samples, diluted in SDS–PAGE sample buffer, and the prestained molecular weight markers (Biorad) were separated by SDS–PAGE (13%) under reducing conditions and electrotransferred to polyvinylidene difluoride (PVDF) membranes (0.45 μm, from Amersham). After blocking for 2 h at room temperature with 5% low-fat 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 antibodies against either adenosine A_1 receptor (1:1000 dilution), α1B subunit of N-type calcium channels (1:200 dilution), α1A subunit of P-type calcium channels (1:500 dilution), NMDA receptor subunit 1 (1:400 dilution), NMDA receptor subunit 2A (1:800 dilution) or NMDA receptor subunit 2B (1:800 dilution). After four washing periods for 10 min with TBS-T containing 0.5% low fat milk, the membranes were incubated with the alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:10 000–1:20 000 dilution from Amersham) in TBS–T containing 1% low fat milk for 90 min at room temperature. After five 10-min washes in TBS–T containing 0.5% low fat milk, the membranes were incubated with ECF for 5 min and then analyzed densitometrically with a Storm (Molecular Dynamics).

2.6. Statistics

The values presented are mean±S.E.M. of *n* experiments. Significance was considered at $P \leq 0.05$ using the paired Student's *t* test.

3. Results

Previous studies have documented the presence of presynaptic A_1 receptors [9,12,18,46] but it is not known if there was an enrichment of A_1 receptors in nerve terminals. In fact, an immunohistochemical study of A_1 receptor distribution in the rat hippocampus has concluded that A_1 receptors were mostly located in axons rather than in nerve terminals [44]. We now investigated this question by comparing the A_1 receptor binding density and immunoreactivity in total hippocampal membranes versus membranes from hippocampal nerve terminals. The selective A_1 receptor antagonist, DPCPX, bound to total hippocampal membranes with a B_{max} of 984±31 fmol/mg protein and a K_D of 0.96 nM (95% confidence interval: 0.70–1.22 nM, $n=5$). As illustrated in Fig. 2A, there was a higher ($P<0.05$) density of binding of [^{3}H]DPCPX in membranes derived from a Percoll-purified synaptosomal fraction (B_{max} of 1839±52 fmol/mg protein) without change in K_D (0.89 nM, 95% confidence interval: 0.52–1.25 nM, $n=5$), suggesting that A_1 receptors are enriched in nerve terminals of the rat hippocampus. This was confirmed by using Western blot analysis of A_1 receptor immunoreactivity. As shown in Fig. 2B, the A_1 receptor immunoreactivity was more intense (150±17% greater, $n=3$, $P<0.05$) in membranes from hippocampal nerve terminals than in total hippocampal membranes.

Although it has previously been observed that A_1 receptors are present in nerve terminals [9,12,18,46], the subcellular synaptic localization of A_1 receptors has not yet had direct experimental support. A critical issue to support the two main hypothesis proposed to understand the efficiency of A_1 receptors to inhibit neurotransmitter release (i.e. inhibition of N-type voltage sensitive calcium

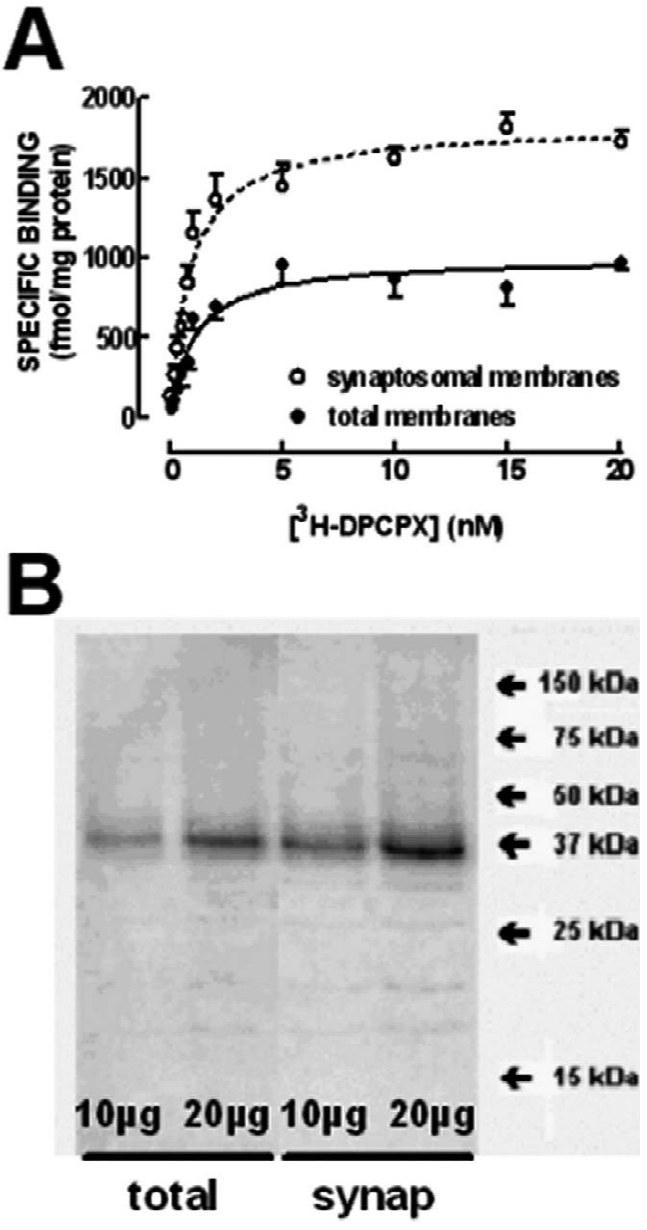

Fig. 2. Enrichment of A_1 receptors in hippocampal nerve terminals. (A) Saturation binding curves of the selective A_1 receptor antagonist [^{3}H]DPCPX to total hippocampal membranes (filled symbols and filled lines) and from hippocampal Percoll-purified nerve terminals (open symbols, dashed lines). The ordinates represent the specific binding of [^{3}H]DPCPX obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC, from total binding. The points are mean±S.E.M. of five experiments and the curves were generated from the average binding parameters. (B) Western blot (representative of three similar blots from different animals) comparing the A_1 receptor immunoreactivity, corresponding to the 36-kDa band, in total hippocampal membranes (total membranes, first two lanes from the left) and from hippocampal Percoll-purified nerve terminals (synap, third and fourth lane from the left), applied in different quantities (10 or 20 μg of protein) to the SDS–PAGE gel. In this particular Western blot, the densitometric intensity of immunoreactivity corresponding to the identified as A_1 receptor was 152 and 114% greater in synaptosomal membranes than in total membranes using 10 and 20 μg protein, respectively.

channels or the decrease in the calcium sensitivity of the release apparatus) is to know if the presynaptic A_1 receptors are located in the active zone of nerve terminals, where both voltage-sensitive calcium channels and the exocytotic machinery are clustered [16,21]. To answer this question, we took advantage of a recently described method that allows the separation of the presynaptic and postsynaptic components of the active zone with an over 90% efficiency, as evaluated by the clear separation of the immunoreactivity of markers of the postsynaptic density (PSD95 and NMDA receptor 1 subunit) and of the presynaptic active zone (syntaxin and SNAP25) (see [34,35] and Fig. 1). As shown in Fig. 3A, there was a clear A_1 receptor immunoreactivity in the presynaptic component of the active zone of hippocampal nerve terminals (39.1±3.6% of total immunoreactivity, $n=4$). The blot in Fig. 3A (representative of blots obtained in four separations from different groups of rats) also shows that A_1 receptor immunoreactivity is also abundant in the postsynaptic component of the active zone (35.9±2.1% of total immunoreactivity, $n=4$), and is also present in the extrasynaptic fraction of nerve terminals (25.0±4.0% of total immunoreactivity, $n=4$). When quantifying the amount of protein recovered in each of these three subsynaptic fractions, it was found that the amount of protein recovered in the active zone fraction represented 21.8±2.9%, in the postsynaptic density fraction 46.0±4.4% and in the presynaptic nonactive zone 32.2±2.2% ($n=4$). Therefore, the observation that the active zone fraction has the lower protein recovery and the higher specific A_1 receptor immunoreactivity (normalized per mg of protein) further suggests that A_1 receptors are enriched in the active zone of hippocampal nerve terminals.

We further investigated the subcellular location of one of the purported targets of A_1 receptors, N-type voltage sensitive calcium channels [40,51,52]. As illustrated in Fig. 3B, the immunoreactivity of α1B subunit that is characteristic of N-type calcium channels is located presynaptically in the active zone, although it is also located in the postsynaptic density where it is apparently more abundant in accordance with its role in controlling postsynaptic excitability and dendritic synaptic plasticity [10,31]. Interestingly, the same occurs with the α1A subunit that is characteristic of P-type calcium channels (Fig. 3C), further supporting that N- or P/Q- types of voltage sensitive calcium channels are not good presynaptic markers (e.g. [20]). In contrast, we confirmed that the more abundant NMDA receptor subunits for which selective antibodies are commercially available (1, 2A and 2B) are highly enriched in the postsynaptic density fraction (Fig. 3D–F).

The last issue we addressed was if the A_1 receptor immunoreactivity found in nerve terminals outside synapses (the extrasynaptic fraction) mostly corresponded to A_1 receptors in intracellular compartments [38,48] or to A_1 receptors located in the plasma membrane outside the active zone [44,46]. In contrast to most adenosine A_1

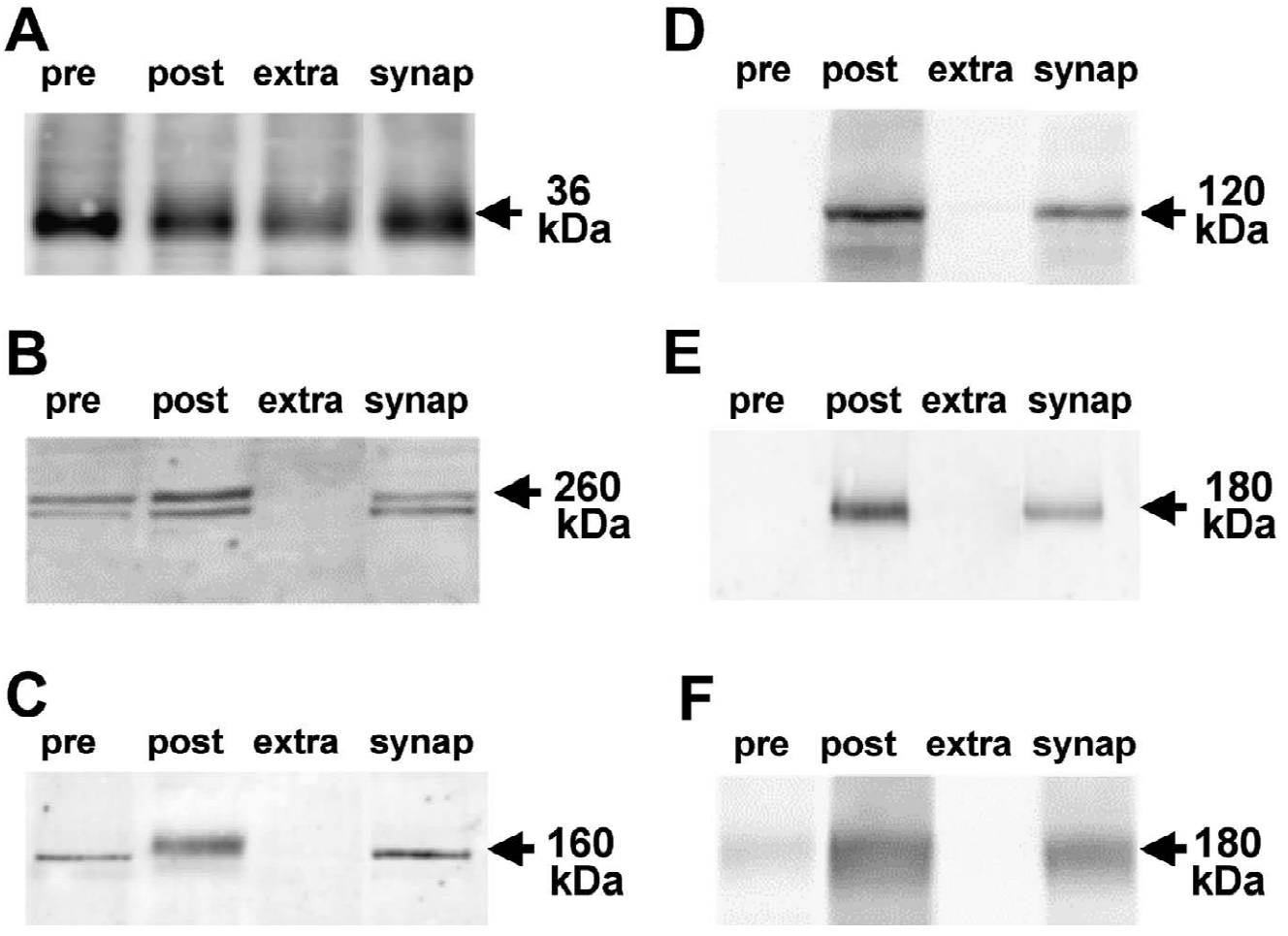

Fig. 3. Adenosine A_1 receptors are located in the active zone of hippocampal nerve terminals together with N-type voltage sensitive calcium channels as well as in the postsynaptic density together with NMDA receptors and with N- and P/Q-type voltage sensitive calcium channels. (A) Western blot (representative of four similar blots from different animals) comparing the A_1 receptor immunoreactivity, corresponding to the 36 kDa-band, in a fraction enriched in the presynaptic active zone (pre), in the postsynaptic density (post), in nerve terminals outside the active zone (extra) and in the initial synaptosomal fraction (synap) from where fractionation began. These fractions were obtained by pH fractionation, after solubilization, of purified hippocampal nerve terminals (whole) as described previously (Phillips et al. [34]), allowing an over 90% efficiency of separation, and 10 μg of protein of each fraction was applied to the SDS–PAGE gel. Likewise, both N-type (B, 100 μg protein per lane) and P-type calcium channels (C, 100 μg protein per lane) are located both in the active zone but mainly in the postsynaptic density, whereas the NMDA receptor subunits 1 (D, 25 μg protein per lane), 2A (E, 25 μg protein per lane) and 2B (F, 25 μg protein per lane) are nearly confined to the postsynaptic density. Each blot is representative of 2–4 blots from different animals with similar results.

receptor antagonists, 8-sulfophenyltheophilline (8-SPT) is hydrophilic and does not easily cross cell membranes, as occurs with lipophilic A_1 receptor antagonists such as DPCPX or XAC. This makes 8-SPT an useful compound to test the relative amounts of A_1 receptors located in intracellular compartments compared to plasma membrane in intact preparations. Indeed, 8-SPT will occlude the binding of [^{3}H]DPCPX only to the plasma membrane, but is not expected to affect the binding of [^{3}H]DPCPX to intracellular A_1 receptors. Thus, we compared the binding of [^{3}H]DPCPX to intact Percoll-purified hippocampal nerve terminals in the absence and in the presence of 8-SPT. [^{3}H]DPCPX bound to intact nerve terminals from the rat hippocampus with a B_{max} of 1901±192 fmol/mg protein and a K_D of 4.44 nM (95% confidence interval: 2.94–5.94 nM, $n=5$). In the presence of 8-SPT (25 μM), there was a marked decrease of the density of binding of [^{3}H]DPCPX (B_{max} of 321±30 fmol/mg protein) without significant change in K_D (4.70 nM, 95% confidence interval: 2.56–6.83 nM, $n=5$) (Fig. 4). This suggests that most of the A_1 receptors in hippocampal nerve terminals might be located in the plasma membrane with only 15–20% of A_1 receptors located intracellularly. We checked that the tested concentration of 8-SPT is supramaximal, since 8-SPT (25 μM) virtually abolished the specific binding of [^{3}H]DPCPX (0.1–20 nM) to membranes from disrupted hippocampal nerve terminals ($n=2$, data not shown).

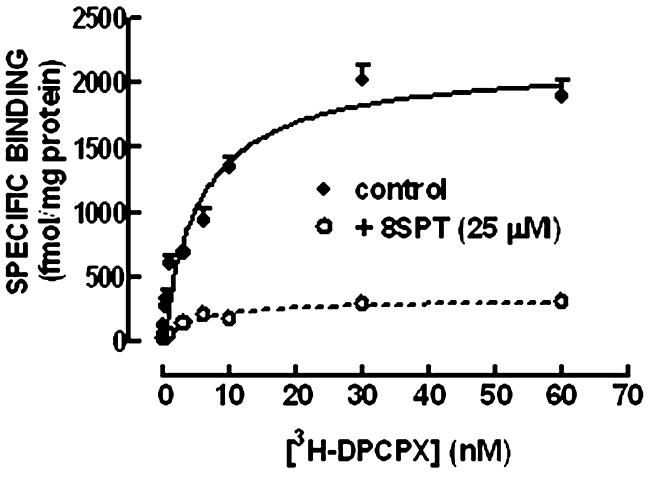

Fig. 4. Adenosine A_1 receptors are predominantly located in the plasma membrane of hippocampal nerve terminals. The membrane-permeable selective A_1 receptor antagonist [^{3}H]DPCPX binding to intact Percoll-purified nerve terminals in the presence (open symbols, dashed lines) of the water soluble adenosine A_1 receptor antagonist, 8-sulfophenyltheophilline (25 μM) is greatly reduced when compared to its absence (filled symbols, filled lines). The ordinates represent the specific binding of [^{3}H]DPCPX obtained on subtraction of the nonspecific binding, determined in the presence of 2 μM XAC, from total binding. The points are mean±S.E.M. of five experiments and the curves were generated from the average binding parameters.

An important issue to consider to validate our conclusion that A_1 receptors in nerve terminals are mostly located at the plasma membrane is whether the relation between intracellular and plasma membranes is maintained in purified synaptosomes. To address this issue, we studied the binding to IP3 receptors, that are known to be located in endoplasmatic reticulum membranes [27], and the binding to N-type calcium channels that are known to be essentially located in the plasma membrane [20]. We found that the specific binding of [^{3}H]IP3 (100 nM) in intact synaptosomes only represented 14±3% ($n=3$) of the specific binding of [^{3}H]IP3 in permeabilized synaptosomes. Preliminary experiments also found suggest that the specific binding of the selective N-type calcium channel ligand, [^{125}I]ω-conotoxin GVIA (100 pM) to permeabilized synaptosomes was not higher than that found in intact synaptosomes ($n=2$). These results suggest that the relation between the plasma and intracellular membranes might be generally preserved in synaptosomes.

4. Discussion

The main conclusions of the present work are that adenosine A_1 receptors are enriched in nerve terminals and are mainly located in synapses, both in the active zone and in the postsynaptic density in the rat hippocampus. The location of A_1 receptors in nerve terminals has already been reported by others [9,12,18,46], but no data were previously available on the relative enrichment of A_1 receptors in nerve terminals or on the subcellular synaptic location of A_1 receptors within nerve terminals, namely its localization in the active zone.

The presence of A_1 receptors in hippocampal nerve terminals was expected based on the previous evidence that A_1 receptors efficiently inhibit intra-synaptosomal calcium transients [1,51] and the evoked release of glutamate [1], acetylcholine [5,22] or serotonin [32] from hippocampal preparations. One could also infer that the density of A_1 receptors in nerve terminals of the rat hippocampus might be higher than in other subcellular locations since electrophysiological studies concluded that the inhibitory effect of adenosine on synaptic transmission mostly results from a presynaptic A_1 receptor-mediated inhibition of glutamate release [36,47]. Accordingly, the present results provide a direct demonstration that A_1 receptors are enriched in nerve terminals when compared to total hippocampal membranes. This may be the basis for the predominant presynaptic effect of adenosine in the modulation of synaptic transmission since it has previously been shown in transgenic animals that the number of A_1 receptors is critical to define the efficiency of adenosine to modulate synaptic transmission [23]. Among presynaptic modulatory system, it is still largely unknown which are located in the active zone and which are located in the terminals outside the active zone. This knowledge is important since, although both types of modulatory systems can modulate the release of neurotransmitters, it is predicted that they may act in different conditions [45,49]. We now experimentally confirmed that A_1 receptors are enriched in the active zone of hippocampal nerve terminals. This aspect is crucial to support the two major hypotheses to explain A_1 receptor-mediated inhibition of neurotransmitter release (inhibition of voltage sensitive calcium channels and decreased sensitivity of the release machinery for calcium) that are based on the assumption that A_1 receptors are located in the active zone of nerve terminals [11,40,43,51], which, to the best of our knowledge, has not received direct experimental confirmation.

The present results also show a clear A_1 receptor immunoreactivity in the postsynaptic density, which displays a marked enrichment in NMDA receptor subunits 1, 2A and 2B immunoreactivities (see also [34,35]). It is interesting to note that the NMDA receptor function is under tight control of A_1 receptors [7,24,42], although only the control by A_1 receptors of perisynaptic NMDA receptors has so far received direct experimental support (e.g. [24]). Thus, the presently observed location of A_1 receptors in the postsynaptic density provides a molecular support for a role of A_1 receptors in controlling synaptic NMDA receptors that trigger synaptic plasticity phenomena. It was also observed that one of the purported molecular targets of A_1 receptor activation, N-type voltage sensitive calcium channels [11,40,51,52], are mainly lo-

cated in the postsynaptic density, although they are also located in the active zone where they regulate the release of neurotransmitters (e.g. [21]). Interestingly, it has been reported that A_1 receptors not only inhibit presynaptic N-type calcium channels but also control postsynaptic N-type calcium channels (e.g. [28,30]). Postsynaptic A_1 receptors are also known to control potassium channels [17,26,50], but since this A_1 receptor-mediated control of postsynaptic potassium conductances has little impact on basal synaptic transmission [36,47], it is not likely to occur in dendritic shafts, let alone in the postsynaptic density.

Our results also show that A_1 receptors are located in nerve terminals outside the active zone. It should be noted that the extrasynaptic fraction of nerve terminals potentially includes presynaptic plasma membrane not included in the active zone and intracellular membranes from presynaptic organelles, although the exact contribution of the different membranes has not been defined. Due to its heterogeneity, we decided to evaluate in intact nerve terminals if there was a considerable amount of A_1 receptors located in intracellular membranes. We first confirmed that the relation between the plasma and intracellular membranes is generally preserved in synaptosomes since binding to IP3 receptors only occurs on solubilization of synaptosomes and binding to N-type calcium channels is not increased on solubilization. It was found that A_1 receptors in nerve terminals might be essentially located in the plasma membrane rather than intracellularly. This favors the possibility that the A_1 receptor immunoreactivity found in the extrasynaptic fraction may essentially result from A_1 receptors located in the plasma membrane outside the active zone rather than located intracellularly. These extrasynaptic A_1 receptors may constitute a presynaptic A_1 receptor reserve [23,50] to control neurotransmitter release. Alternatively, these presynaptic A_1 receptors located in the plasma membrane outside the active zone may be involved in an indirect control of the functioning of nerve terminals, like the control of nerve terminal metabolism [19], the control of axonal growth and nerve terminal maturation [37] or in volume transmission [49].

Finally, it should also be noted that the present data support the view that there is a highly heterogeneous distribution of A_1 receptors in hippocampal nerve terminals since most of the presynaptic A_1 receptors are located in the synapses, both in the active zone and in the postsynaptic density, which only accounts for a minor volume in the CNS. This further adds to our previous observations indicating a heterogeneous extracellular metabolism of adenosine in hippocampal synapses [6,14]. This also re-enforces our previous idea [4] that adenosine neuromodulation involves a restricted geographical region in nerve terminals. This contention implies that overall measurements of adenosine receptor density or of extracellular concentrations of adenosine may grossly overlook punctual changes in adenosine neuromodulation that may be of major physiological relevance.

In conclusion, the parallel use of cell fractionation approaches and receptor binding and Western blot analysis has enabled us to assess the subcellular location of A_1 receptors in nerve terminals and synapses. We concluded that A_1 receptors are enriched in nerve terminals where they are mainly located in the plasma membrane. In particular, these A_1 receptors are located in the presynaptic component of the active zone, a strategic location to understand the efficient A_1 receptor-mediated inhibition of neurotransmitter release, and in the postsynaptic density, a key observation to understand the importance of A_1 receptors in the control of NMDA receptor firing and dendritic integration.

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