MODIFICATION OF PURINERGIC SIGNALING IN THE HIPPOCAMPUS OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Abstract—Diabetic encephalopathy is a recognized complication of untreated diabetes resulting in a progressive cognitive impairment accompanied by modification of hippocampal function. The purinergic system is a promising novel target to control diabetic encephalopathy since it might simultaneously control hippocampal synaptic plasticity and glucose handling. We now tested whether streptozotocin-induced diabetes led to a modification of extracellular ATP homeostasis and density of membrane ATP (P2) receptors in the hippocampus, a brain structure involved in learning and memory. The extracellular levels of ATP, evaluated in the cerebrospinal fluid, were reduced by 60.4±17.0% in diabetic rats. Likewise, the evoked release of ATP as well as its extracellular catabolism was also decreased in hippocampal nerve terminals of diabetic rats by 52.8±10.9% and 38.7±6.5%, respectively. Western blot analysis showed that the density of several P2 receptors (P2X3,5,7 and P2Y2,6,11) was decreased in hippocampal nerve terminals. This indicates that the synaptic ATP signaling is globally depressed in diabetic rats, which may contribute for diabetes-associated decrease of synaptic plasticity. In contrast, the density of P2 receptors (P2X1,2,5,6,7 and P2Y6) but not P2Y2 increased in whole hippocampal membranes, suggesting an adaptation of non-synaptic P2 receptors to sense decreased levels of extracellular ATP in diabetic rats, which might be aimed at preserving the non-synaptic purinergic signaling. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: ATP, P2 receptors, ecto-nucleotidases, hippocampus, diabetes, streptozotocin.

Diabetes mellitus is a metabolic disease characterized by an impaired glucose homeostasis, which is caused by deficient production or action of insulin. Diabetic conditions often include central neuropathic modifications, namely decreased cognitive performance (e.g. Cox et al., 2005; Gispen and Biessels, 2000) accompanied by modifications of hippocampal morphology and plasticity (e.g. Convit et al., 2003; Trudeau et al., 2004). This diabetes-induced dysfunction of synaptic plasticity is likely to be due to a perturbed efficiency of the release of neurotransmitters, as gauged by the reduction of neurotransmitter release (Guyot et al., 2001; Yamato et al., 2004) and change in presynaptic proteins associated with vesicular release of neurotransmitters upon diabetes in cortical preparations (Duarte et al., 2006). Thus, one possible strategy to correct this diabetes-induced modification of synaptic efficiency might be to target presynaptic neuromodulation systems. One promising candidate is the ATP modulation system since it has the simultaneous potential to control neurotransmitter release (Rodrigues et al., 2005), synaptic plasticity (e.g. Pankratov et al., 2002), a purported neurophysiological trait of learning and memory (e.g. Lynch, 2004), and can also control glucose utilization (e.g. Solini et al., 2003) and insulin release (e.g. Léon et al., 2005).

ATP is released by most cells, namely from neurons in an exocytotic manner (North and Verkhratsky, 2006), and extracellular ATP regulates a variety of cellular processes through activation of ATP receptors (P2Rs), which include ionotropic (P2X1-7) and metabotropic (P2Y1-15) receptors (Abbracchio et al., 2006; Khakh and North, 2006). Extracellular ATP regulates key physiological functions such as neurotransmitter release (Rodrigues et al., 2005), synaptic plasticity phenomena (e.g. Almeida et al., 2003) and glucose homeostasis, namely through the modulation of insulin secretion (e.g. Léon et al., 2005), hepatic glucose metabolism and release (Buxton et al., 1986; Haussinger et al., 1987), and glucose transport in several cell types (Solini et al., 2003; Fisher et al., 1999; Kim et al., 2002). However, extracellular ATP is a double-edge sword signaling system since it is also a danger signal (di Virgilio, 2000), and P2R blockade was shown to afford neuroprotection against metabolic insults (Cavaliere et al., 2001a,b), ischemic conditions (e.g. Lammer et al., 2006) and glutamate toxicity (reviewed in Franke et al., 2006).

Previous studies have already indicated diabetes-induced changes of the efficiency of P2R in peripheral tissues. Thus, the ATP-driven modulation of glucose transport, proposed to rely on P2YR activation (Fischer et al., 1999; Kim et al., 2002), was found to be impaired in fibroblasts of type 2 diabetic individuals (Solini et al., 2003) and changes in pancreatic P2Rs were reported to occur in an experimental model of type 1 diabetes (Coutinho-Silva et al., 2003). Also, an enhanced P2X7R activity was associated with diabetes-induced vascular damage (Solini et al., 2004) and retinopathy (Sugiyama et al., 2004) and the P2X7R gene emerges as a candidate susceptibility gene for non-obese diabetes (Elliott and Higgins, 2004). This prompts the hypothesis that diabetes may also cause modifications of the purinergic system in the brain, which may lead to an impairment of the physiological actions operated

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by ATP through the activation of P2Rs, such as long-term potentiation (LTP), that may underlie diabetes-induced cognitive impairment. The present study was designed to investigate if the purinergic signaling, namely extracellular ATP homeostasis and the density of different P2Rs, is altered in the hippocampus of streptozotocin (STZ)-treated rats, an animal model of type 1 diabetes, which displays learning deficits (e.g. Biessels et al., 1996).

**EXPERIMENTAL PROCEDURES**

**Reagents**

The antibodies against P2X1 (generated against residues 382–399 of rat P2X1 and used at a 1:500 dilution from a 0.6 mg/ml stock), P2X4 (generated against residues 370–388 of rat P2X4 and used at a 1:1000 dilution from a 0.2 mg/ml stock), P2X5 (generated against residues 357–359 of rat P2X5 and used at a 1:500 dilution from a 0.2 mg/ml stock), P2X6 (generated against an internal sequence of rat P2X6 and used at a 1:200 dilution from a 0.2 mg/ml stock), P2Y1 (generated against an internal sequence of human P2Y1 and used at a 1:500 dilution from a 0.6 mg/ml stock) and P2Y2 receptors (generated against residues 227–244 of human P2Y2 and used at a 1:500 dilution from a 0.2 mg/ml stock) were from Alomone Laboratories (Jerusalem, Israel); the antibodies against P2Y11 receptor antibody (generated against the third cytoplasmic loop of human P2Y11 and used at a 1:500 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-goat IgG secondary alkaline phosphatase-tagged antibody, were purchased from Amersham USA; anti-P2Y2 receptor antibody (generated against residues 576–595 of rat P2X7 and used at a 1:5000 dilution from a 0.3 mg/ml stock), P2Y2 (generated against residues 227–244 of rat P2Y2 and used at a 1:5000 dilution), P2Y1 (generated against an internal sequence of human P2Y1 and used at a 1:500 dilution from a 0.3 mg/ml stock) and P2Y2 receptors (generated against residues 337–350 of rat P2Y2 and used at a 1:1000 dilution from a 0.3 mg/ml stock) were from Alomone Laboratories (Jerusalem, Israel); the antibodies against P2X2 (generated against residues 356–471 of human P2X2 and used at a 1:500 dilution from a 0.2 mg/ml stock), P2X5 (generated against residues 356–455 of rat P2X5 and used at a 1:200 dilution from a 0.2 mg/ml stock), P2X6 (generated against residues 351–431 of human P2X6 and used at a 1:200 dilution from a 0.2 mg/ml stock), P2Y1 (generated against an internal sequence of human P2Y1, and used at a 1:500 dilution from a 0.2 mg/ml stock) and P2Y2 receptors (generated against an internal sequence of human P2Y2 and used at 1:500 dilution from a 0.2 mg/ml stock), as well as the anti-goat IgG secondary alkaline phosphatase-tagged antibody, were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-P2Y11 receptor antibody (generated against the third cytoplasmic loop of human P2Y11, and used at 1:500 dilution from a 0.3 mg/ml stock) was from Zymed (Medell, Portugal); anti-α-tubulin antibody was from Sigma (Sintra, Portugal); alkaline phosphatase–tagged anti-mouse IgG and anti-rabbit IgG secondary antibodies for Western blot were purchased from Amersham Biosciences (Carnaxide, Portugal). KH2PO4 was purchased from VWR International (Carnaxide, Portugal). STZ and all the other common use reagents were from Sigma.

**Animals**

Male Wistar rats (8-weeks old, 150–170 g, obtained from Harlan Ibérica, Barcelona, Spain) were used throughout study and were handled according with the EU guidelines for the use of experimental animals (86/609/EEC), the rats being anesthetized under halothane atmosphere before being killed by decapitation. The experiments were performed with special care for minimizing the number of animals used and their suffering.

We used a well-studied and validated model of type 1 diabetes mellitus, which is based on the administration of STZ (e.g. Rees and Alcolado, 2005). STZ (65 mg/kg, prepared in sodium citrate buffer 10 mM, pH 4.5) was administered by i.p. injection and induced sustained levels of blood glucose above 250 mg/dl after 3 days onwards (Duarte et al., 2006, 2007), as measured by the glucose oxidase method using a glucometer (Elite, Bayer, Portugal). The rats were maintained for 30 days with food and water ad libitum and all the analyses were carried out 30 days after STZ treatment. The control rats were age-matched untreated rats maintained in the same conditions. Table 1 summarizes body weight and glycemia of both control and diabetic rats. Since STZ is not known to cross the blood–brain barrier and has to be directly injected into the brain to cause direct effects in the brain parenchyma (e.g. Lester-Coll et al., 2006), it is assumed that the modifications caused by STZ mainly result from its ability to induce a type 1 diabetic state (e.g. Rees and Alcolado, 2005).

**Cerebrospinal fluid (CSF) sampling and ATP quantification**

Rats were anesthetized with sodium thiopental (40 mg/kg, i.p.) and the CSF was drawn (40–60 μl per rat) by direct puncture of the cisterna magna with a tuberculin syringe (27 gauge × 13 mm length), and immediately stored at −80 °C until ATP quantification. These samples and standard solutions of ATP (10⁻⁹ to 10⁻¹² M) were placed in wells of a white 96-well microplate to determine ATP levels using the luciferin-luciferase luminometric assay (Cunha et al., 1996). Briefly, 50 μl of luciferin–luciferase solution (FLAAM kit from Sigma, resuspended in 5 ml) was added to 25 μl of sample (diluted 1/5) and the luminescence produced was quantified in an LMax II²³⁴ luminescent luminometer (Molecular Devices, Union City, USA).

**Preparation of purified nerve terminals and hippocampal membranes**

Membranes from the whole hippocampus or from Percoll-purified hippocampal synaptosomes were prepared as previously described (Duarte et al., 2006). Briefly, the two hippocampi from one rat were homogenized at 4 °C in sucrose–Hepes buffer (composition 0.32 M sucrose, 1 mM EDTA, 10 mM Hepes, 1 mg/ml bovine serum albumin, pH 7.4). The resulting homogenate was centrifuged at 100,000 × g for 10 min at 4 °C, the supernatant collected and centrifuged at 14,000 × g for 12 min at 4 °C. The pellet was resuspended in 1 ml of a 45% (v/v) Percoll solution made up in Krebs–Hepes solution (composition in mM: 140 NaCl, 5 KCl, 10 Hepes, 1 EDTA, 5 glucose, pH 7.4). After centrifugation at 21,000 × g for 2 min at 4 °C, the top layer (synaptosomal fraction) was removed, washed and resuspended in Krebs–Hepes solution. For total membrane preparation, a portion of the supernatant of the first centrifugation was taken, resuspended in a solution of 50 mM Tris and 10 mM MgCl₂ (pH 7.4), centrifuged at 28,000 × g for 1 hour at 4 °C.

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>Glycemia (mg/dl)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
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<tr>
<td>Before treatment</td>
<td>254.7±13.2</td>
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<tr>
<td>3 days after treatment</td>
<td>n.d.</td>
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<tr>
<td>1 month after treatment</td>
<td>345.7±14.8**</td>
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*n=18 for each condition; n.d., not determined.

* P<0.01, different from control.

** P<0.01, different from before treatment.
for 20 min at 4 °C, and the resulting pellet resuspended in a Krebs–Hepes solution. An aliquot of each membrane preparation was saved for protein quantification using the bicinchoninic acid method (kit from Pierce Biotechnology, Rockford, USA).

**ATP release from hippocampal nerve terminals**

The measurement of ATP release from synaptosomes was adapted from Cunha et al. (1996). Synaptosomes were resuspended in calcium containing Krebs–Hepes solution (composition in mM: 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 2 CaCl₂, 26 Hepes, 10 glucose, pH 7.4). Aliquots of 240 μl of synaptosomes (0.21–0.36 mg of protein) were placed in wells of a white 96-well microplate to which 50 μl of luciferin–luciferase solution (FLAAM kit from Sigma, prepared in 5 ml of water) were added. The mixture was placed in the luminometer at 37 °C and the electrical signal generated by the photomultiplier recorded. After obtaining a stable baseline, 10 μl of the Krebs–Hepes solution containing KCl (to attain a final concentration of 20 mM) was automatically injected and the plate shaken for 2 s. The measurement of the photomultiplier signal restarted after 4 s, and the variation in signal recorded was used to estimate the evoked release of ATP by interpolation in a calibration curve of ATP standards. We confirmed that mechanical stimulation of the nerve terminals also triggered an outflow of ATP, but this displayed a slower time course and had an amplitude considerably lower than the K⁺-evoked release of ATP.

**ATP catabolism in hippocampal nerve terminals**

Synaptosomes (0.32–0.43 mg) were resuspended in 500 μl of Krebs–Hepes solution and incubated at 37 °C for at least 5 min of stabilization. At time zero, ATP (made up in Krebs–Hepes solution) was added to a final concentration of 10 μM and samples (75 μl) were collected every minute during the next 5 min of incubation. Each sample was spun down, and the supernatant immediately frozen in liquid N₂ and then at −80 °C until high performance liquid chromatography (HPLC) analysis (Cunha et al., 1998). Separation of adenine nucleotides was performed at room temperature using a reverse-phase column [LiChroCART 125×4 mm LiChrospher 100 RP-18 (5 μm) cartridge fitted into a ManuCART holder (Merck Darmstadt, Germany)], using a GOLD™ system (Beckman, UK) equipped with a UV detector set at 254 nm. The eluent was a 100 mM KH₂PO₄ solution with 1.2% methanol (pH 5.5) with a flow rate of 1.2 ml/min. The identification of the peaks was performed by comparison of relative retention times with standards and their quantification achieved by calculating the peak areas then converting to concentration values by calibration with known standards (0.1–10 μM). The activity of the ecto-enzymes responsible for the extracellular catabolism of ATP was defined as the rate of ATP degradation.

**Western blot analysis**

The determination of the density of P2 receptors was carried out by Western blot analysis, as previously described (Rodrigues et al., 2005). Briefly, each sample was diluted with five volumes of SDS-PAGE buffer containing 30% (v/v) glycerol, 0.6 M dithiothreitol, 10% (w/v) sodium dodecyl sulfate and 375 mM Tris–HCl pH 6.8, and boiled at 95 °C for 5 min. These diluted samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% separation gel with a 4% concentrating gel in the top) under reducing conditions, in two or three different protein concentrations, together with pre-stained molecular weight markers (Biorad, USA), and then electro-transferred to polyvinylidene difluoride membranes (0.45 μm, from Amersham Biosciences, UK). After blocking for 1 h at room temperature with 5% milk in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6), containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4 °C with the primary antibodies against P2 receptors. The selectivity of the tested antibodies against hippocampal P2 receptors has previously been validated (Rodrigues et al., 2005). After three 15 min washing periods with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase–conjugated anti-rabbit IgG or anti-goat IgG secondary antibody (dilution 1:1,000) in TBS-T containing 1% milk during 90 min at room temperature. After three 20 min washes in TBS-T with 0.5% milk the membranes were incubated with enhanced chemi-fluorescent substrate (Amersham Biosciences) and then analyzed with a VersaDoc 3000 system (Biorad).

The membranes were then re-probed and tested for α-tubulin immunoreactivity to confirm that similar amounts of protein were applied to the gels (Duarte et al., 2006). Briefly, the membranes were incubated at room temperature for 30 min with 40% (v/v) methanol and 1 h with 0.1 M glycine buffer pH 2.3, and then blocked as previously described before incubation with an anti-α-tubulin antibody (dilution 1:10,000) for 2 h at room temperature. The membranes were then washed, incubated with an anti-mouse IgG alkaline phosphatase-conjugated secondary antibody and analyzed as described above.

![Fig. 1. Extracellular ATP levels are modified in the hippocampus of STZ-treated rats. (A) ATP concentration in the CSF, measured with a luminometric assay, was lower in diabetic (filled bars) than control (open bars) rats (n=7, *P<0.05). (B) The K⁺-evoked release of ATP from hippocampal nerve terminals prepared from STZ-treated rats (filled symbols) was also lower when compared with controls (open symbols). In this experiment, KCl was added at time zero in a concentration of 20 mM, thus depolarizing the nerve terminals and triggering a vesicular release of ATP, which was quantified by luminometry. In (C) are presented the average data showing that the initial evoked release of ATP (measured 4 s after KCl addition) from hippocampal nerve terminals of diabetic (filled bars) was nearly half of that observed in hippocampal nerve terminals of control (open bars) rats (n=4, *P<0.05).](image-url)
Statistical analysis

Results are presented as mean ± S.E.M. values of n experiments, and significance was considered at P<0.05 using a Student’s t-test.

RESULTS

Modification of extracellular ATP concentration and metabolism

We first evaluated if the diabetic rats presented abnormal extracellular ATP levels in the brain. As shown in Fig. 1A, 1 month after STZ-induction of diabetes, the concentration of ATP in the CSF was less than half of that in control rats. The synaptic levels of ATP were also decreased in diabetic rats, as gauged by the reduction of the K⁺-induced evoked release of ATP from hippocampal nerve terminals (Fig. 1B, C).

We next investigated the rate of extracellular catabolism of ATP by following the consumption of ATP after its addition to a synaptosomal suspension (see Cunha, 2001). It was found that the rate of hydrolysis of extracellular ATP and the consequent formation of ADP was reduced in hippocampal nerve terminals derived from diabetic rats (Fig. 2A, B). This indicates that diabetes induces a reduction in the activity of membrane-bound ecto-enzymes involved in ATP catabolism (Fig. 2C). This global reduction of extracellular ATP homeostasis prompts the hypothesis that P2Rs in the brain of diabetic rats may face lower extracellular ATP levels than in control rats.

Modification of the density of P2 receptors in hippocampal membranes

Since ATP simultaneously fulfils a role as a synaptic modulator (North and Verkhratsky, 2006) and as a non-synaptic role as a neuron–glia messenger (Fields and Burnstock, 2006), we simultaneously evaluated if there was a modification of the density of P2Rs in synaptic membranes and in whole membranes of the hippocampus of diabetic rats. We use a Western blot analysis using antibodies that we were previously defined to be selective for hippocampal P2 receptors (Rodrigues et al., 2005). We always evaluated two or three different amounts of loaded protein (hippocampal nerve terminals or whole membranes) from the
hippocampus of control and diabetic rats in each gel to simultaneously evaluate the sensitivity of the Western assay. This was attempted for all the P2XR subunits and most P2YRs and all results were then expressed as average percentage of modification (density found in diabetic relative to its respective control) and summarized in the Fig. 3.

In hippocampal nerve terminal membranes of diabetic rats 1 month after STZ-treatment, there was a global trend toward a decrease of the immunoreactivity of P2Rs. The

![Graphs and images showing Western blots comparing the P2XR immunoreactivity in nerve terminal–enriched membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in synaptosomal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (n=6 for P2X1,2,3,5,7Rs and n=5 for P2X4,6Rs). * P<0.05, ** P<0.01, *** P<0.001 comparing the immunoreactivity in the two groups of animals.]

Fig. 4. Representative Western blots comparing the P2XR immunoreactivity in nerve terminal–enriched membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in synaptosomal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (n=6 for P2X1,2,3,5,7Rs and n=5 for P2X4,6Rs). * P<0.05, ** P<0.01, *** P<0.001 comparing the immunoreactivity in the two groups of animals.
density of the ionotropic receptors P2X3, P2X5 and P2X7 was decreased by 7.8 ± 1.6% (n = 6, P < 0.005), 7.5 ± 2.4% (n = 6, P < 0.03) and 13.7 ± 2.3% (n = 6, P < 0.0001), respectively (Fig. 4). Also the density of the metabotropic receptors P2Y2, P2Y6 and P2Y11 was reduced by 12.0 ± 3.0% (n = 8, P < 0.03), 16.7 ± 3.8% (n = 4, P < 0.005) and 7.5 ± 1.8% (n = 3, P < 0.005), respectively (Fig. 5).

In contrast, in whole hippocampal membranes (which include both neurons and mainly glia) of diabetic rats 1 month after STZ-treatment, there was a global trend toward an increase of the immunoreactivity of P2Rs. In fact, as illustrated in Fig. 6, there was an increase of all ionotropic P2Rs (with the exception of P2X3 and P2X7). There was an increased density of P2X3Rs (+12.8 ± 3.0%, n = 8, P < 0.005), P2X4Rs (+24.4 ± 4.9%, n = 8, P < 0.0001), P2X5Rs (+19.2 ± 2.1%, n = 5, P < 0.0001), P2X6Rs (+7.1 ± 2.4%, n = 5, P < 0.03) and P2X7Rs (+10.2 ± 2.6%, n = 6, P < 0.001) in whole membranes derived from rats 1 month after STZ treatment. With respect to metabotropic P2YRs in whole hippocampal membranes of diabetic hippocampus, we found that the density of P2Y2Rs was 19.3 ± 3.7% larger than control (n = 3, P < 0.001). In contrast, the density of the other P2YRs was not significantly modified (P > 0.05) compared with whole membranes from control rats, except for P2Y11Rs, whose density was decreased by 14.1 ± 4.7% (n = 7, P < 0.03) in diabetic rats (Fig. 7).

**DISCUSSION**

The main conclusion of this study is that there is a deregulation of P2 receptor-mediated signaling in the hippocampus of STZ-induced type 1 diabetic rats. We found that there was a decrease in the CSF levels of ATP in diabetic rats, together with a decrease of the evoked release of ATP in hippocampal nerve terminals, suggesting that P2Rs may be facing lower concentration of extracellular ATP. Also the extracellular metabolism of ATP is reduced in nerve terminals from the diabetic hippocampus, possibly due to decreased activity of ecto-nucleotidases from the ecto-ATPase family (Zimmermann, 2000). Interestingly, we found that there was an asymmetric global modification of the density of P2 receptors (P2Rs) in synapses and outside synapses in the hippocampus, in accordance with the double role of ATP as a synaptic modulator (North and Verkhratsky, 2006; Cunha and Ribeiro, 2000) and as an extra-synaptic neuron–glia messenger (Fields and Burnstock, 2006). In fact, in nerve terminal membranes, there was a global decrease of the density of P2Rs, whereas in whole hippocampal membranes there was a global trend for an increased density of P2Rs.
It is well established that the development of a diabetic condition is accompanied by the increased incidence of neurological complications, in particular cognitive dysfunction (Cox et al., 2005; Gispen and Biessels, 2000). The hippocampus is a brain region with a key role in the implementation of mnemonic traits, and deficits in hippocampal function prime cognitive dysfunction (Squire et al., 2004). In particular, deficits in hippocampal synaptic plasticity phenomena, namely of LTP, are considered a neurophysiological trait of memory dysfunction (Lynch, 2004). Accordingly, there is a parallel deficit of the induction and maintenance of LTP as well as of the performance in memory-related tasks in STZ-induced diabetic rats (Biessels et al., 1996). Interestingly, extracellular ATP, which is released in a frequency-dependent manner (Cunha et al., 1996), plays a role in the development of LTP-like changes of synaptic efficiency through the activation of P2Rs (e.g. Pankratov et al., 2002; Almeida et al., 2003). This might be due to combined effects of different P2Rs acting both presynaptically to control the release of glutamate (Rodrigues et al., 2005) and postsynaptically to facilitate the activation of ionotropic glutamate receptors, namely

Fig. 6. Representative Western blots comparing the P2XR immunoreactivity in total membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-α-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in total hippocampal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (n=8 for P2X1,2,3Rs, n=7 for P2X4R, n=5 for P2X5,6Rs and n=6 for P2X7R). * P<0.05, ** P<0.01, *** P<0.001 comparing the immunoreactivity in the two groups of animals.
NMDA receptors (Kloda et al., 2004; Ortinau et al., 2003). Thus, the currently observed lower release of ATP together with the global down-regulation of synaptic P2Rs in the hippocampus of diabetic rats raises the hypothesis that the deficit in synaptic ATP signaling may contribute to the memory dysfunction observed in diabetes. This down-regulation of ATP signaling in hippocampal synapses may also be an adaptive response to preserve nerve terminals. In fact, type 1 diabetes is associated and constitutes a risk factor for neurological conditions associated with dysfunction of synaptic transmission, which can eventually lead to idiopathic generalized seizures (e.g. McCorry et al., 2006). Convulsive episodes are precipitated by hypoglycemic episodes (see Jones and Davis, 2003) and are effective triggers for synaptic and neuronal damage (Pitkänen and Sutula, 2002). These hypoglycemic episodes cause an acute release of ATP (Juranyi et al., 1999), which plays a key role in triggering (e.g. Cavaliere et al., 2001a) and controlling the recovery (Aihara et al., 2002) of hypoglycemia-induced neuronal damage. Therefore, the decrease of synaptic ATP signaling can be viewed as an adaptive response to compensate for increased risk of P2R-mediated neurotoxicity in type 1 diabetes.

In contrast to what occurs in hippocampal synapses, we observed that there was a trend for an increase of the density of P2Rs in whole hippocampal membranes, which are mainly derived from extra-synaptic membranes given that synapses only represent \( \frac{1}{50} \) of hippocampal volume (Rusakov et al., 1998). Apart from its synaptic role in the control of synaptic transmission and plasticity, extracellular ATP also fulfills important signaling roles outside synapses, mainly in the communication between neurons and glia, which may also contribute for non-synaptic-mediated neurotransmission (reviewed in Fields and Burnstock, 2006). In astrocytes, which are the most abundant cellular elements in the brain, there is evidence that P2Rs can contribute to astrogliosis (Abbracchio and Verderio, 2006; Neary and Kang, 2005) and P2Rs actually protect astrocytes from damage (Shinozaki et al., 2005). This is particularly relevant in diabetic conditions since astrocytes are expected to be the main cellular element able to metabolize and resolve the increased extracellular levels of glucose (Pellerin and Magistretti, 2004). Hence, it seems logical to expect maintenance of this astrocytic ATP signaling in a diabetic condition and the increased P2R density in whole membranes may be an adaptive response to compensate for

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**Fig. 7.** Representative Western blots comparing the P2YR immunoreactivity in total membranes from the hippocampus of control rats and from rats treated with STZ, applied in different quantities to the SDS-PAGE gel as indicated above each lane. A re-probing of the membranes with an anti-\( \alpha \)-tubulin antibody, shown below each gel in each panel, confirmed that similar amounts of protein were added for each concentration of hippocampal membranes. The graphs below the gels show the average relative immunoreactivity of in total hippocampal membranes from control rats (open symbols) and from STZ-treated rats (filled symbols) according to the amount of protein applied to gel in different Western blot analysis using membranes from different groups of rats (\( n = 8 \) for P2Y1R, \( n = 7 \) for P2Y2R, \( n = 5 \) for P2Y4R, \( n = 3 \) for P2Y6R and \( n = 4 \) for P2Y11R). * \( P < 0.05 \) comparing the immunoreactivity in the two groups of animals.
the decreased ATP levels faced by these extra-synaptic P2Rs. Since it has been reported that there is an astrogliosis in type 1 diabetes (Saravia et al., 2002), it will be interesting to test if there will be an increased P2R-mediated signaling in astrocytes in the diabetic brain and whether this may be related to an increased ability to handle extracellular glucose.

CONCLUSION

In conclusion, the present study provides evidence showing that the ATP signaling system is compromised in the hippocampus of STZ-treated rats, an experimental model of type 1 diabetes mellitus. These modifications could lead to alterations in the modulation of neurotransmission and glutotransmission, which may contribute to the diabetes-induced progressive cognitive impairment, although the direct impact of such alterations on both neuronal and glial functions remains to be determined. In particular, the diversity of P2Rs and the currently observed different modification of the density of different receptors in this model of type 1 diabetes open the real possibility of selectively manipulating beneficial responses operated by particular P2Rs without exacerbating noxious responses mediated by other P2Rs.

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