Adenosine is released *per se* from cell, in physiological condition, and is contained in synaptic vesicles

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We have recently demonstrated (Melani et al., 2012) by the use of the microdialysis technique *in vivo,* that under physiological conditions, in the presence of the recently synthesized ecto-ATPase inhibitor PV4 (100 μmol/L) (Muller et al., 2006), the extracellular concentration of ATP increased significantly but the adenosine concentration was not altered. This demonstrates that, under physiological conditions, adenosine derives *per se* from cells and that it is not a product of extracellular ATP. To assess if adenosine outflow occurred through the equilibrative nucleoside transporter (ENT), the adenosine transporter inhibitor, dipyridamole (100 μmol/L), was added to PV4. In the presence of PV4 plus dipyridamole, adenosine outflow progressively increased indicating that adenosine is not transported out of cells by ENT but rather is transported into cells. Moreover, by immunoelectron microscopy, we have demonstrated the presence of the concentrative nucleoside transporter CNT2 on plasma and vesicle membranes isolated from the rat striatum (Melani et al., 2012).

Up to now, direct evidence that adenosine is contained in synaptic vesicles was lacking. Specific aim of this study was to investigate if adenosine is present in rat brain synaptic vesicle fractions.

Synaptic vesicles were isolated according to the procedure described by Ohsawa and Uchizono (1975) with some modifications. Brains obtained from male Wistar rats, were homogenized in 10% (w/vol) 0.32 M sucrose buffer. The homogenate was centrifuged at 1000 g for 10 min at 4°C. Pellet (P1) was discarded and supernatant (S1) was centrifuged at 11000 g for 30 min at 4°C. Supernatant (S2) was discarded and pellet (P2), containing the crude synaptosomal fraction (Barker et al., 1972), was recovered and osmotically shocked. This sample was layered over a five-layer discontinuous density gradient consisting of equal volumes of 0.05 M, 0.1 M, 0.15 M, 0.2 M and 0.3 M sucrose and ultracentrifugated. Eight synaptic vesicle fractions were recovered and ultracentrifuged at 105000 g for 20 min at 4°C. The pellet (P3) of each fraction was recovered and resuspended in 80 μl of Tris/HCl pH 7.4 buffe*r*. Part of the sample was used for quantification of synaptophysin that is a specific molecular marker of synaptic vesicles (Wiedenmann and Franke; 1985) by Western blotting analysi*s;* the other part of the sample was used for adenosine quantification in the P3 fractions by High Performance Liquid Chromatography (HPLC) analysis coupled to a fluorescence detector (minimum sensitivity 10 fentomoles). The immunoblot analysis of synaptophysin showed that fractions from 3 to 8 are rich in synaptophysin. Distribution of adenosine in the eight fractions is coherent with distribution of synaptophysin. The sum of adenosine found in fractions 3-8 is (mean±S.E.M) 2450.78±352.78 pmol/mg of tissue protein. This amount is comparable to the content of ATP (800 pmol/mg of protein) estimated in the synaptic vesicles of bovine brain by Zisapel and Zurgil (1979). We proved that adenosine measured was constitutive of vesicles, since that adenosine exogenously added to the crude synaptosomal pellet P2 preferentially distributed in fractions 1 and 2 and did not contaminate the vesicle pellet P3.

Information that adenosine is present in synaptic vesicle is consistent with the notion that adenosine stored in synaptic vesicles, is released under normoxic physiological conditions by an excitation-secretion mechanism, typical of neuronal cells.

Barker et al. (1972). *Biochem. J.* 130, 1063-1075.
Melani et al. (2012). *Exp Neurol.* 233, 193-204.
Muller et al. (2006). *Bioorg. Med. Chem. Lett.* 16, 5943-5947.
Ohsawa and Uchizono (1975). *Proc Jpn Acad.* 51, 202-207.
Wiedenmann and Franke (1985). *Cell* 41, 1017-1028.
Zisapel and Zurgil (1979). *Brain Res.* 178, 297-310.