Peripherally administered L-PIA is known to cross the blood–brain barrier and produce behavioural changes including anti-convulsant effects and a depression of locomotor activity [17–19], the latter response being noted also in the present study. It is therefore possible that the observed increase of clonidine binding may be related to that reported in the vas deferens, but that either the change of clonidine receptor number occurs more slowly than in the vas, or that a larger change is required of a subpopulation of clonidine sites (e.g. postjunctional) because of the predominance of an unchanged subgroup (e.g. prejunctional). The problems of interpretation however, are much greater in chronic experiments, and it is not yet possible to eliminate other explanations of the present observation. For example, since purines, including L-PIA, are potent inhibitors of transmitter release [20], it is possible that the chronic suppression of presynaptic release by L-PIA has resulted secondarily in an up-regulation of α_2 receptors.

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Inhibition by benzodiazepines and β-carbolines of brief (5 seconds) synaptosomal accumulation of [^{3}H]-adenosine

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Adenosine is probably an important regulator of neuronal function in both the peripheral and central nervous systems [1, 2], and it has been proposed that a variety of compounds may owe at least part of their pharmacological activity to an effect on the uptake and thus inactivation of adenosine [3]. Much interest has centred on the benzodiazepines, which suppress significantly the uptake of adenosine into CNS preparations at therapeutically relevant concentrations [4–6]. However, it has been pointed out that with the incubation times usually used in such studies, the influence of metabolism by adenosine deaminase and adenosine kinase [7] and the loss of adenosine by efflux may reduce the reliability of the results as a true reflection of uptake [8, 9]. Effects on uptake attributed to compounds might for example be due to changes of metabolic processes, or, conversely, the potency of substances as uptake inhibitors may be under-estimated because the uptake process is not rate-limiting under the conditions of the assay. The present study was therefore undertaken to examine the effects of benzodiazepines on the uptake of adenosine into cerebral synaptosomes after an incubation period of only 5 seconds [10]. We have adopted the term "brief" accumulation to refer to this short time scale. A range of benzodiazepine related compounds have been tested, in order to assess the relevance of any observed effects to the behavioural actions of these substances.

Cerebral synaptosomes were prepared from male Wistar rats weighing 150–200 g as previously described [11, 12]. Briefly, animals were killed by stunning and cervical dislocation, and the cerebral cortex was dissected out and homogenised at 800 r.p.m. using a Potter Teflon/glass homogeniser on ice. All subsequent processing was performed on ice or at 2°. The homogenate was centrifuged at 900 *g* for 10 min. The 900 *g* supernatant was layered directly onto 1.2 M sucrose and centrifuged at 160,000 *g* for 15 min. The 0.32 M/0.8 M sucrose interphase was collected, diluted 1:3 with 0.32 M sucrose and layered onto 0.8 M sucrose. A further 15 min centrifugation at 16,000 *g* yielded the synaptosomal preparation (pelleted) which was resuspended in 0.32 M sucrose to form a suspension of 100–150 mg initial wet weight ml^{-1}.

For the brief accumulation of ^{3}H-adenosine into the synaptosomes a 250 μl aliquot of the synaptosomal suspension was added to oxygenated Krebs–Henseleit buffer containing test compounds (total volume = 2 ml) and preincubated for 1 hr at 25°. ^{3}H-Adenosine (1 μM, specific activity 1.04 TBq/mmol, Amersham International) was also maintained at 25°. A 250 μl aliquot of the radiolabelled solution and the synaptosomal preparation was then simultaneously transferred to the well of a filtration unit (millipore 12-port filtration system housing Whatman GF/B glass fibre filters) connected to a valved vacuum. After

4 sec, the vacuum valve was opened filtering almost instantaneously the mixture on the filter bed, and 1 sec later the filter was washed with 2 × 10 ml of buffer containing 1% glutaraldehyde [10]. Radioactivity of the filters was determined by scintillation spectroscopy.

The benzodiazepines used included Ro 15-1788 (ethyl-8-fluro-5,6-dihydro-5-methyl-6-oxo-4H-imidazol-1,5a–1,4-benzodiazepine-3-carboxylate) and Ro 05-4864 (7-chloro-1-methyl-5-(4-chloro-phenyl)-3H-1,4-benzodiazepine-2(1H-one)).

The three conventional benzodiazepines tested, diazepam, clonazepam and flurazepam proved to have IC_{50} values for inhibiting the brief accumulation of adenosine comparable with those reported previously for studies involving longer incubation times. The benzodiazepine "antagonist" Ro 15-1788 [13], and the peripheral benzodiazepine site ligand Ro 05-4864 also depressed the brief uptake with potencies of the same order as the "agonists" (Table 1).

The commonly used inhibitors of nucleoside uptake, dipyridamole and hexobendine were highly effective inhibitors in the present system, being several orders of magnitude more potent than the benzodiazepines, and achieving almost total suppression of uptake at concentrations in the low micromolar range. The mean IC_{50}value of dipyridamole in the present system was 7.6×10^{-8} M and of hexobendine was 8.5×10^{-8} M (Table 1).

The β-carboline compounds tested were also effective inhibitors of the brief uptake system, the most potent substance being the β-carboline 3-carboxylate ethyl ester, which was more active than any of the benzodiazepine-related molecules, with an IC_{50} of only 1.7 μM (Table 1).

The potency of the benzodiazepine "agonists" tested here is sufficiently similar to that seen in studies employing longer incubation times [4–6, 11, 12] to suggest that the primary action of these compounds in most studies of nucleoside uptake has been on the transport process. This would be consistent with the finding that benzodiazepines displace nitrobenzylthioinosine from the nucleoside transporter [14, 15]. In contrast, dipyridamole and hexobendine appear to be appreciably more active in the present brief uptake system than in many previously examined uptake protocols using tissue from the rat and employing longer time scales [16]. The presently observed IC_{50} value for dipyridamole is comparable with that reported for guinea-pig synaptosomes [17].

The present results using the benzodiazepine "antagonist" Ro 15-1788 [13] and the peripheral site ligand Ro 05-4864 support the findings of an earlier study in which these compounds were found to share the uptake inhibitory activity of the central site "agonists" [11, 12]. This therefore supports the view that inhibition of nucleoside uptake is unrelated to the behavioural sedative and anxiolytic actions of the benzodiazepine series of compounds, actions not shared by Ro 15-1788 and Ro 05-4864.

Finally, the present work has revealed a potent action of some β-carboline derivatives on the brief accumulation of adenosine. These compounds are known to interact with benzodiazepine binding sites [18, 19]. In view of the activity of both benzodiazepines and β-carbolines on purine uptake, and particularly the high potency of β-carboline 3-carboxylate ethyl ester, one of the most potent agents displacing diazepam binding [18], it is an interesting possibility that some of the reported actions of β-carbolines [20–25] and their interactions with benzodiazepines [19, 21, 24, 25] might involve the nucleoside transporter.

In summary, in order to examine the effects on adenosine uptake into synaptosomes without the complications arising from changes of adenosine metabolism or efflux, a system has been used in which accumulation is studied after an incubation time of only 5 sec. The results indicate that benzodiazepines as well as the antagonist Ro 15-1788 and the peripheral site ligand Ro 05-4864 can inhibit this "brief" uptake. The classical inhibitors dipyridamole and hexobendine were found to be more potent than in earlier studies in rat tissue, and β-carboline derivatives which interact with benzodiazepine sites were also found to be effective inhibitors. The β-carboline 3-carboxylate ethyl ester is the most potent of these, with an IC_{50} of 1.7 μM.

Table 1. Inhibition of brief accumulation of ^{3}H-adenosine by benzodiazepine binding site ligands

Benzodiazepine binding site ligand	IC_{50} for the inhibition of brief accumulation of ^{3}H-adenosine (M)
"Agonists"	
Clonazepam	$1.6 \pm 0.4 \times 10^{-5}$[a]
Diazepam	$6.9 \pm 0.6 \times 10^{-5}$
Flurazepam	$1.2 \pm 0.4 \times 10^{-4}$
"Antagonist"	
Ro 15-1788	$9.6 \pm 4.3 \times 10^{-5}$
β-Carbolines	
Ethyl-β-carboline 3-carboxylate	$1.7 \pm 0.8 \times 10^{-6}$
Methyl-β-carboline 3-carboxylate	$4.0 \pm 2.0 \times 10^{-5}$
Propyl-β-corboline 3-carboxylate	$1.6 \pm 0.5 \times 10^{-4}$*
"Peripheral" type ligand	
Ro 05-4864	$1.4 \pm 0.2 \times 10^{-4}$
Classical inhibitors	
Dipyridamole	$7.6 \pm 2.8 \times 10^{-8}$
Hexobendine	$8.5 \pm 3.1 \times 10^{-8}$

[a] Mean + S.E.M. IC_{50} values for the inhibition of brief accumulation of ^{3}H-adenosine were obtained by linear regression analysis of log probit plots (N = 3–5 independent experiments performed in triplicate).

* Value obtained by extrapolation of log probit plots.

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Inhibition of phorbol ester-stimulated chemiluminescence and superoxide production in human neutrophils by fructose 1,6-diphosphate

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Fructose, 1,6-diphosphate (FDP) has been shown to influence cellular responses by interacting with several types of cell membranes and by regulating the glycolytic pathway of cells, with consequent ATP production, despite its inability to cross the cell membranes [1, 2]. Our previous experiments have demonstrated the effectiveness of FDP in preventing mast cell histamine release, induced by several agents [3, 4], and the ability of the drug to increase the ATP content, both in stimulated and unstimulated cells. The aim of the present experiment was to investigate the action of FDP on neutrophil oxidative metabolism in order to obtain further insight into the possible role of FDP in inflammatory processes, in the light of the stimulatory effects of the drug on the phagocytic activity of RES [5]. The generation of reactive oxygen radicals by human neutrophils plays an essential role in host defense, because together with their antimicrobial properties, they are capable of destroying a wide variety of biological targets [6]. These oxygen derivatives can also, in turn, modulate the inflammatory response, with host tissue destruction. The neutrophil oxidative metabolism can be detected through the chemiluminescent response (CL), arising from activated cells, which is an index of both the generation and the biochemical processes mediated by oxygen radicals [7]. Moreover in digitonin stimulated neutrophils activation is an energy-requiring process, which is linked to ongoing ATP synthesis [8]. On the other hand it has also been suggested that ATP might be a physiological regulator of the catalytic activity of the enzyme responsible for O_2^- production [9]. For this reason, investigations were also made into the effects of FDP, a glycolysis stimulator [1], on ATP intracellular variations, to test whether the drug could influence the neutrophil oxidative burst, by interfering with energy metabolism.

Materials and methods

Ferricytochrome C (type VI), bovine superoxide dismutase, phorbol myristate acetate (PMA), xanthine, xanthine-oxidase and luminol were obtained from Sigma (Saint Louis, MO). Kits for the bioluminescent assay for ATP were purchased from Packard (Milano, Italy). Fructose 1,6-diphosphate (Esafosfina) was kindly supplied by Biomedica Foscama. Eagle modified Dulbecco medium for chemiluminescence (MEM), phosphate-buffered saline (PBS) were from Boehringer Biochemia (Milano, Italy). Fycoll-Hypaque was obtained from Flow Lab. (Milano, Italy).

Cell isolation. Human neutrophils were isolated from heparinized peripheral blood of healthy adult donors and purified by one-step density gradient centrifugation on Ficoll-Hypaque following the method of Ferrante *et al.* [10].

Chemiluminescence. The luminol amplified chemiluminescent response induced by PMA stimulation of the cells was used to monitor neutrophil oxidative metabolism, after challenging the cell suspension (2×10^5 cells/ml) with 0.1 μg/ml of PMA in the presence or the absence of different concentrations of fructose 1,6-diphosphate (0.2 mM, 1 mM, 5 mM). A Packard Picolite Luminometer, thermostatted at 37°, operating in the dark was used to measure CL. Results are expressed as counts per minute. Statistical evaluation of the integrated area underlying the CL curves, was performed by Student's *t*-test for paired data.

O_2^- *assay.* Superoxide production was determined as SOD-inhibitable cytochrome *c* reduction at 37° in 1 ml cell suspension containing 10^6 neutrophils, 0.1 μg/ml PMA, FDP (0.2 mM, 1 mM, 5 mM), as described by Cohen and Chovaniec *et al.* [8]. The cell free superoxide generating system, xanthine–xanthine oxidase, was used to test whether FDP could act as O_2^- scavenger, following the method of Fridovich [11].

ATP determination. Samples for ATP determination were incubated for 5 min with FDP (0.2–5 mM) at 37°, before challenging the cells suspension with 0.1 μg/ml PMA. The cells were further incubated for 10 min and ATP intracellular levels were measured after extraction with Pico Ex S, using the bioluminescent luciferine–luciferase reaction.

Results and discussion

Incubation of neutrophils with FDP for 10 min before stimulation with PMA markedly affected the cell oxidative metabolism, as revealed by the degree of inhibition exerted on the luminol-amplified chemiluminescence. The inhibition was dose-dependent over the range 0.2 mM–5 mM, being maximal at the highest FDP concentration (80% inhibition) as shown in Fig. 1. The effect of FDP on the CL suggests that, possibly, the generation and efflux of a wide variety of reaction oxygen species is inhibited. Since