imparted antagonistic properties, as determined by nicotine-induced prostration. A good correlation was observed between the nicotinic receptor binding characteristics of the *N*-alkylcarbamyl esters and their efficacy in producing prostration and seizures in rodents.

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Semicarbazide-sensitive amine oxidase activity (SSAO) of rat epididymal white adipose tissue

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In recent years the presence of amine oxidase (AO) activities, resistant to the irreversible mitochondrial monoamine oxidase inhibitors (MAO, flavin dependent amine oxygen oxidoreductase EC 1.3.4), have been described in many mammalian tissues [1]. To study this activity tissue homogenates are usually exposed to a millimolar concentration of an irreversible MAO inhibitor, such as pargyline. In these conditions, any remaining AO activity measured with benzylamine as substrate and inhibited by semicarbazide is conventionally referred to as "semicarbazide-sensitive amine oxidase activity (SSAO)".

Barrand and Callingham [2], described the SSAO of rat brown adipose tissue (BAT) and more recently the presence of a SSAO activity was also found in cultured white and brown preadipocytes transformed by lipogenic agents [3]. These findings might reinforce the hypothesis that SSAO could play a physiological role related to some BAT metabolic functions. Because BAT represents only a small fraction of the total adipose tissue disseminated in the body, our aim was to investigate on the presence of this enzyme in white adipose tissue (WAT).

Materials and Methods

WAT was obtained from epididymal fat pads of adult male Wistar (150–300 g) rats (Morini, S. Polo D'Elsa, Italy) killed by cervical dislocation. About 300 mg of minced tissue was washed in cold saline solution and then homogenized in 3 mL of ice-cold 1 mM potassium phosphate buffer containing 0.25 M sucrose (sucrose–phosphate buffer) pH 7.8. Mature adipocytes were prepared according to Rodbell [4]. The purified cells were resuspended and homogenized in ice-cold sucrose buffer, pH 7.8, to get a final protein concentration of 0.3–0.5 mg/mL [5].

SSAO activity was assayed radiochemically according to Buffoni and Ignesti [6], in the condition of MAO inhibition by 1 mM pargyline. Unless otherwise stated labelled substrates (50 μL) were reacted with the enzyme for 5 min.

When unlabelled amines were screened as possible substrates enzymatic activity was measured spectrophotometrically [6, 7] in the presence of 1 mM pargyline.

White adipocyte homogenates were then processed for subcellular fractionating. The supernatant from a first sedimentation at 600 $g \times 20$ min was again centrifuged at 12,000 $g \times 30$ min. After this the resulting supernatant was then spun at 105,000 g for 60 min. All procedures were run at 4°. Each pellet was washed in cold sucrose–phosphate buffer and resuspended in 2 mL of the same ice-cold buffer and assayed radiochemically for SSAO, for 5′-nucleotidase [8], and for cytochrome *c* oxidase [9].

Moreover, some "sandwich" immunoassays were carried out using pure pig plasma benzylamine oxidase (BAO), prepared according to Dixon and Purdom [10], and the 12,000 g pellet from white adipocytes as antigens and a rabbit serum challenged with pure BAO [11]. Assays were performed according to Ref. 12.

Antigens were coated on 96 multiwell plates and a 1:5000 dilution of the anti-BAO serum and 1:3000 dilution of a goat anti-rabbit Ig–peroxidase conjugate were used (Biorad). Extinction at 392 nm was always subtracted for the respective control value. [7-^{14}C]Benzylamine hydrochloride (57 mCi/mmol) was purchased from ICN Chemical Radioisotope Division (Irvine, CA, U.S.A.), β-[ethyl-1-^{14}C]phenylethylamine hydrochloride (50 mCi/mmol) from New England Nuclear (Boston, MA, U.S.A.). All the other reagents were analytical grade products.

Results and Discussion

The presence of an AO activity which is resistant to

Table 1. The presence of SSAO activity in rat epididymal WAT

Source of enzyme	Activity (nmol/mg of protein/min)
WAT	1.4 ± 0.3
Adipocytes	2.5 ± 0.1
WAT + semicarbazide	0
Adipocytes + semicarbazide	0

Results are means ± SE of four experiments, run in duplicate. Crude homogenates of WAT (0.1 mL) and of isolated adipocytes (0.1 mL) were preincubated for 30 min at 37° in 0.06 M sodium phosphate buffer, pH 7.4, together with 1 mM pargyline. In this case reactions were run for a further 30 min after the addition (50 μL) of 1.66 mM [^{14}C]benzylamine as substrate. Reactions were stopped by the addition of 0.1 mL of 3 N HCl and the radioactive metabolites extracted in 1 mL of ethylacetate. An aliquot of the extract (500 μL) was then counted in 15 mL of scintillation liquid (Packard). Quench correction was obtained by a channel ratio method. Semicarbazide, 0.1 mM, was preincubated for 30 min together with 1 mM pargyline before the addition of benzylamine. To minimize enzyme retroinhibition by the reaction products the incubation time of 5 min was then chosen.

pargyline but sensitive to semicarbazide was found in rat WAT (Table 1). Separation of fat cells from other stromal components results in an increase of this SSAO specific activity as compared to that found in the original WAT. This finding confirms that this SSAO is concentrated in adipocytes and probably located on cell membranes (Fig. 1) from where it can be solubilized using Triton X-100 at 0.08% (w/v), a concentration which did not affect enzyme activity. At this detergent concentration the 73% ± 10 (mean ± SE of four experiments run in duplicate) of the enzymatic activity was recovered in the 105,000 *g* supernatant.

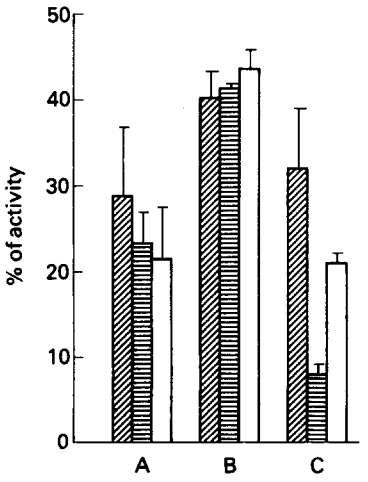

Fig. 1. Subcellular fractioning of SSAO, 5′-nucleotidase, cytochrome *c* oxidase activities from rat white adipocytes. Results are means ± SE of four experiments run in duplicate. SSAO was assayed radiochemically as described in Materials and Methods using 50 μM [^{14}C]benzylamine. SSAO in the cytosol fraction was 1.7% ± 0.3 of the total activity. (A) Pellet at 600 *g*. (B) Pellet at 12,000 *g*. (C) Pellet at 105,000 *g*. (□) 5′-nucleotidase; (▤) SSAO; (▨) cytochrome *c* oxidase. Each percentage of enzymatic activity was calculated by taking the activity measured in the original homogenate as 100%.

This SSAO oxidizes benzylamine, β-phenylethylamine, histamine and β-aminoproprionitrile [13] (Table 2). The affinity for benzylamine is high and its oxidation is competitively inhibited Table 3 by 5×10^{-4} M of histamine (K_i calculated according to Dixon [14] was 400 μM). In addition β-aminoproprionitrile at 1 mM concentration produced a 70% ± 2.0 (mean ± SE of four experiments run in duplicate) of enzyme inhibition that is reverted by dialysis against 0.06 M sodium phosphate buffer, pH 7.4, for 24 hr at 4°. White adipocyte SSAO is then completely inhibited by 0.1 mM concentration of hydralazine, α-aminoguanidine, cuprizone (IC_{50} 5.88×10^{-6} M ± 0.31, mean ± SE of four experiments run in duplicate) and of 3,5-ethoxy 4-amino methyl pyridine, a novel SSAO inhibitor [15].

We also found that the 12,000 *g* pellet from white adipocyte homogenates is immunologicall recognized by an anti-BAO serum (Fig. 2). Because of the high specificity of the method used and because of the high concentration of SSAO in this fraction, we hypothesize that SSAO from

Table 2. Substrates for white adipocyte SSAO

Substrates	Activity (nmol/mg of protein/min)
Benzylamine	4.0 ± 0.5
Putrescine	0
β-Phenylethylamine	2.7 ± 0.9
Histamine	5.6 ± 0.9
Spermine	0
5-OH-Tryptamine	0
β-Aminoproprionitrile	2.3 ± 0.5

Results are means ± SE of four experiments run in duplicate. Enzymatic activity was assayed spectrophotometrically. 0.1 mL of white adipocyte homogenate was preincubated in 1 mL 0.06 M sodium phosphate buffer, pH 7.4, containing peroxidase (0.2 units/mL), *o*-dianisidine (0.25 mg/mL), pargyline (1 mM), for 30 min at 37°. Substrates at 1 mM concentration were then added and the reactions run for a further 30 min in the same condition of temperature. Extinction at 540 nm was measured after the addition of 2 mL of sulfuric acid to stop the reaction.

Table 3. Kinetic constants for [^{14}C]benzylamine and [^{14}C]phenylethylamine: effect of histamine on [^{14}C]benzylamine oxidation

Substrates	K_m (μM)	V_{max} (nmol/mg of protein/min)
[^{14}C]Benzylamine	11.3 ± 1.7	15.3 ± 2.8
[^{14}C]Phenylethylamine	145.5 ± 29.3	1.3 ± 0.03
[^{14}C]Benzylamine + 5×10^{-4} M histamine	25.2 ± 5.2*	15.9 ± 5.7

Results are means ± SE of seven experiments run in duplicate calculated by a Lineweaver–Burk plot.

* Statistically different value ($P < 0.05$ Student's t-test) from [^{14}C]benzylamine K_m. Unlabelled histamine was added simultaneously with [^{14}C]benzylamine.

[^{14}C]Benzylamine was used at 166, 50, 10, 5 and 1 μM concentrations.

[^{14}C]Phenylethylamine was used at 1660, 166, 50, 10, 5 and 1 μM concentrations.

White adipocyte homogenates were preincubated for 30 min at 37° with 1 mM pargyline and the reaction stopped after 5 min from the addition of substrates. [^{14}C]Benzylamine at 1660 μM concentration, incubated for 5 min in the same conditions as above, produced a 50% ± 8 inhibition of white adipocytes SSAO.

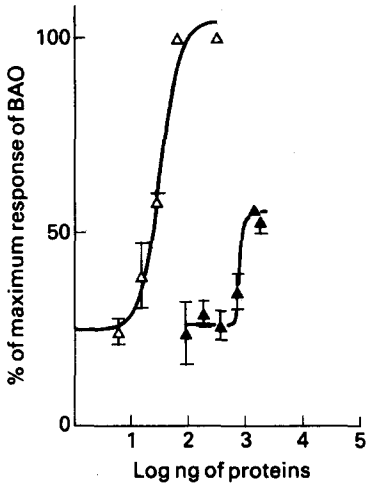

Fig. 2. Immunorecognition of the 12,000 g pellet from adipocyte by an anti-pig plasma BAO serum. Concentration-dependent immunorecognition between: (△) pure pig plasma BAO (295, 118, 59, 30, 15 and 9 ng); (▲) 12,000 g pellets from white adipocyte homogenates (1600, 1440, 721, 360, 180 and 90 ng). Antigens immobilized on solid phase were processed for immunoenzymatic assays (see Materials and Methods). Reactions were stopped within 5 min after the addition of o-phenylendiamine (Sigma) in citrate buffer. Extinction was then measured at 392 nm in a Titertek Multiscanner (Biorad). The maximal reaction developed from 1440 ng of the 12,000 g pellet was 50% of that obtained with a saturating concentration (59 ng) of pure BAO.

WAT could actually be recognized by the anti BAO serum.

In conclusion, the presence of a SSAO in WAT confirms that this enzyme is concentrated in adipose tissues where it is associated with cell type.

In summary, an amine oxidase activity distinguishable from MAO, which is inhibited by carbonyl reagents is present in rat epididymal WAT. This enzyme, referred to as semicarbazide-sensitive amine oxidase (SSAO), appears concentrated in adipose cells. Close homologies between WAT SSAO and the circulating plasma BAO are discussed.

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Amiodarone: biochemical evidence for its interaction with myocardial Na^+-K^+-ATPase in guinea pig microsomal preparations

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Amiodarone is a very potent class III antiarrhythmic drug used in the treatment of a broad spectrum of cardiac tachyarrhythmias [1]. The mechanism for its antiarrhythmic effect is probably based on its multiple actions, particularly its inhibition of the fast sodium channels [2], or slow calcium channels [3] or membrane stabilizing effects [4]. However, apart from its well-established antiarrhythmic effect, amiodarone also possesses a number of other properties such as its neurotoxicity [1], or the tendency to induce or enhance cardiac arrhythmias [1, 5, 6], which often causes serious clinical complications. While its inhibition of synaptosomal Na^+-K^+-ATPase has recently been discussed with regard to its neurological side effects [7], the mechanism of its proarrhythmic or arrhythmogenic actions remains to be elucidated. It is known however, that the inhibition of myocardial Na^+-K^+-ATPase (EC 3.6.1.3) is arrhythmogenic in character [8], and it appears to provide adequate explanation of the mechanism of digitalis-induced arrhythmias, for example [9]. Furthermore, it was recently postulated that malfunction of the electrogenic pump activity of the myocardial Na^+-K^+-ATPase may, in general, contribute towards the mechanism of such drug-induced arrhythmias [8]. However, only limited information is currently available in the literature pertaining to studies on interactions between antiarrhythmic agents and this enzyme system. In the present study, we therefore examined the actions of amiodarone on the Mg^{2+}-dependent ATP-hydrolysis by myocardial Na^+-K^+-ATPase, and its mode of interaction with ouabain in this system, in order to assess their potential relevance for some of its cardiac actions.

Materials and Methods

Myocardial Na^+-K^+-ATPase was prepared as described previously [10]. Accordingly, hearts isolated from guinea pigs of either sex weighing 0.6–1.1 kg were homogenized for 10 min in sucrose buffer to give a 15% suspension. The homogenate was centrifuged for 15 min at 14,000 *g* using an RC5C Centrifuge (Sorvall Instruments), and the supernatant was filtered with a Millipore SC 8.0 μm filter (Millipore Corporation, Bedford, MA, U.S.A.). The enzyme was then separated on a TSK Toyopearl HW-55F Gel column (Pierce Chemicals, U.S.A.) equilibrated with KCl buffer (KCl 500 mM, imidazole 10 mM, Na_2·EDTA 1 mM, pH 7.4). The fractions were collected using LKB-2211 Superrac and dialysed in Visking 20/32 dialysis membrane (Serva Finebiochemica, U.S.A.) three times, each time for at least 6 hr using twice 10 mM first, and then once 100 mM imidazole buffer, pH 7.4. All steps were carried out at 4°. Different drug concentrations were then pre-incubated with 10–12 μg protein for 20 min at 37° in 100 mM imidazole buffer containing (in mM) Mg^{2+} 5, Na^+ 100, K^+ 5 and Na_2·EDTA 1. The reaction was initiated by adding 2 mM ATP, and the liberated inorganic phosphate determined spectrophotometrically at 660 nm after 20 min by the method of Eibl and Lands [11]. The Na^+/K^+-stimulated ATPase activity was calculated as the difference between the total and the Mg^{2+}/Na^+-dependent activity.

Protein concentration was determined using Coomassie reagent (Pierce Chemicals). Drugs used were amiodarone (Sanofi) and ouabain (Fluka). All the other reagents were of analytical grade. Statistical significance was calculated by Student's *t*-test using the Statgraphics software package version 3.0 (Graphic Software Systems, Inc., 1988). Significance criteria refer to $P < 0.05$.

Results and Discussion

Both ouabain and amiodarone exhibited concentration-dependent inhibitory actions on myocardial Na^+-K^+-ATPase activity at the range of 0.05 to 100 μM and 0.65 to 90 μM, respectively (Fig. 1). The concentrations required to inhibit the enzyme activity by 50% (IC_{50} values) were 1.93 ± 0.27 μM for ouabain and 8.50 ± 1.87 μM for amiodarone. Thus, the inhibitory potency of amiodarone was comparable to that of ouabain, a specific inhibitor of the enzyme system [9]. The present results therefore demonstrate that amiodarone is a very potent inhibitor of the myocardial Na^+-K^+-ATPase activity.

As depicted in Fig. 2, incubation of the enzyme in presence of both ouabain and amiodarone produced additive effects at amiodarone concentrations below 1.0 μM. However, the combined effects became significantly less additive in character with increasing amiodarone concentrations above 1.0 μM. Thus for example, while single concentrations of ouabain inhibited the enzyme activity by approximately 55% at 2.5 μM or 65% at 5.0 μM, the addition of 2.5 μM ouabain reduced the amount of amiodarone required for total inhibition from 90 μM to approximately 50 μM. Similarly, about 40 μM of amiodarone were required to attain 100% inhibition in the presence of 5.0 μM ouabain. On the other hand, less than