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An endogenous ligand for the central sulfonylurea receptor

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An endogenous ligand for the rat central sulfonylurea receptor has been evidenced in the rat central nervous system. The characteristics of this ligand (extractibility, non-dialysability, chromatographic behaviour on different media, sensitivity to proteases) indicate that it is a neutral to slightly basic peptide.

Hypoglycemic sulfonylurea; Central receptor; Endogenous ligand; Peptide

1. INTRODUCTION

The mode of action of hypoglycemic sulfonylureas, a class of drugs widely used for the management of non-insulino-dependent diabetes mellitus [1], is now widely accepted as being the interaction with specific receptors present at the cell surface, both at the peripheral [2,3] and the central [4,5] levels. A receptor for a natural regulatory molecule (such as a hormone or a neuro-regulator) is composed of a binding site, recognizing specifically the ligand and the related compounds, linked to an effector system (such as adenylate cyclase, phospholipase C, ionic pump, or ionic channel). As far as the sulfonylurea receptor is concerned, it has been clearly established that the binding site is specific for the different hypoglycemic sulfonylureas with respective affinities in close relation with their in vivo biological activities [2–5]. Furthermore, an ionic channel is suspected to be the effector system linked to the sulfonylurea binding site, although some discrepancies appeared in the literature concerning its exact type, either an ATP-dependent K^+ [6,7], a Ca^{2+}-dependent K^+ [8] or a voltage-dependent Ca^{2+} [9] channel. Thus, the sulfonylurea receptor displays all the characteristics of a biologically relevant receptor for a still unknown endogenous ligand [5]. The presence of sulfonylurea receptors in the rat central nervous system [4,5] prompted us to search for the possible presence in this tissue of an endogenous ligand for this receptor.

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2. MATERIALS AND METHODS

2.1. *Sulfonylureas*

Glipizide, 1-cyclohexyl-3-[(*p*-[2-(5-methylpyrazinecarboxamido)ethyl]phenyl)sulfonyl]urea, was a generous gift from Farmitalia Carlo Erba, Milan, Italy. Glibenclamide, 1-[(*p*-[2-(5-chloro-*o*-anisamido)ethyl]phenyl)sulfonyl]-3-cyclohexylurea, from Laboratori Guidotti SPA was kindly donated by Centre de Recherche Pierre Fabre, Castres, France. Both are highly potent second generation sulfonylureas, widely used in the management of non-insulino-dependent diabetes mellitus.

2.2. *Binding assay*

This was performed as described previously [5]. Membranes were prepared from rat cerebral cortex by homogenization in a motor-driven glass-teflon Potter-Braun in 10 mM Tris-HCl, pH 7.5 (10 up and down strokes at 1000 rpm), followed by differential centrifugation: after a 600 × *g* centrifugation at 4°C for 10 min, the supernatant was spun down at 46000 × *g* at the same temperature for 30 min. The resulting pellet was washed twice in 50 mM Tris-HCl, pH 7.5, and recentrifuged. Membranes were incubated at 0.4 mg membrane protein/ml for 60 min at room temperature with 0.1 nM [^{3}H]Glibenclamide (40 Ci/mmol, from Hoechst, FRG) or with 2.5 nM [^{3}H]Glipizide (21 Ci/mmol, a generous gift from Carlo Erba, Italy) in 1 ml of 50 mM Tris-HCl, pH 7.5, and, whenever necessary, unlabelled sulfonylurea at adequate concentrations

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or the biological samples. Separation of bound from free sulfonylurea was performed by filtration on GF/B glass fiber filters (Whatman, England) under vacuum. The filters were quickly washed 3 times with 5 ml incubation buffer at 0°C before ^3H counting in a scintillation medium (ACS II from Amersham, England). For each experiment, a standard curve was drawn from data obtained with unlabelled sulfonylurea and the sulfonylurea equivalent in each biological sample (crude extract or chromatographic fraction) was calculated by reference to the standard curve. Only fresh membranes were used throughout.

2.3. *Tissue extractions*

Two types of extraction were performed. One consisted in taking as the extract the supernatant of the membrane preparation. This corresponds to a mild extraction method using a homogenization in a hypotonic medium, which is known to disrupt the nerve terminals and to liberate their content (in particular the neurotransmitters) in the medium. The second consisted of a classical heat-coagulation/acid extraction method [10] widely used for peptide isolation. Briefly, the tissues were immersed for 3 min in boiling water and allowed to cool down. The tissues were then homogenized with a rotary blade apparatus in 1 M CH_3COOH (5 ml/g of tissue) and lyophilized or run directly on a chromatography column.

2.4. *Purification methods*

High-performance liquid chromatography (HPLC) was performed with an apparatus consisting of two solvent delivery systems (6000A, Waters Associates, France) controlled by a gradient controller (720 from Waters) and a U6K injector (Waters) fitted with reverse-phase columns (Lichrosorb RP-8 from Merck, FRG, Nucleosil CN from Société Française Chromato Colonne, France, or μC-18 from Waters). Gradient elution was conducted as indicated in the legend to the respective figure. Fractions were collected in glass tubes with a fraction collector (1220 Isco, USA) and lyophilized after evaporation of the organic solvent under vacuum (Speed-Vac® , Savant Instruments, USA). Sep-Pak separations were performed by running the extracts on C-18 reverse-phase cartridges (Sep-Pak® from Waters) previously equilibrated in 1% CF_3COOH buffered with diethylamine, pH 2.5 (TFA-DEA, [11]), and eluted with TFA-DEA/CH_3CN (20:80). The purified extract was either lyophilized or run on HPLC after evaporation of CH_3CN under vacuum.

Ion-exchange separations were performed on S-Sepharose® fast-flow from Pharmacia (Sweden), a strong cation-exchange medium, or Q-Sepharose® fast-flow, a strong anion-exchange medium. Both media were packed in laboratory-made polypropylene mini-columns (1 cm I.D., 3 cm gel bed) fitted with polypropylene frits and operated by syringe pressure at a flow rate of 5 ml/min. The columns were equilibrated with 0.1% TFA/CH_3CN (80:20), pH 2.0, and eluted with the same buffer containing NaCl (for cation-exchange chromatography) or equilibrated with 10 mM NH_4HCO_3/CH_3CN (80:20), pH 8.0, and eluted with 0.5 M NH_4HCO_3/CH_3CN (80:20), pH 8.0 (for anion-exchange chromatography). In both cases, the fractions were desalted on Sep-Pak before lyophilization.

2.5. *Enzymatic digestions*

Enzymatic digestion was performed on Tris extracts from 15 rat cortices which were concentrated on Sep-Pak (see 2.4) and lyophilized. Papain (EC 3.4.22.2, Boehringer-Mannheim, FRG), preincubated for 30 min at 25°C, was used at 40 μg/ml (final concentration) in 0.1 M NH_4HCO_3, pH 8.0, containing 0.1 mM $CaCl_2$ for 120 min at 37°C. Pepsin (EC 3.4.23.1, Boehringer-Mannheim, FRG) was used at 20 μg/ml in 1 mM HCl for 120 min at 25°C. Phospholipase A_2 (EC 3.1.1.4, Sigma, USA) was used in 0.5 M Tris-HCl, pH 7.5, at 100 U/ml (final concentration) for 120 min at 37°C. Controls were incubated in parallel in the absence of enzyme. Reactions were ended by degrading the enzymes at 100°C for 5 min (boiling water bath). The activity of the controls and of the experimental tubes was tested in the binding assay.

2.6. *Miscellaneous*

200–300 g male Wistar rats (Iffa-Credo, France), fed on standard laboratory chow, were used throughout. Protein concentrations were measured according to Bradford [12] with the Bio-Rad protein assay kit (Bio-Rad, USA). Dialysis was performed in 24 Å porosity Visking cellulose tubing (Union Carbide, USA) against distilled water for 18 h at 4°C under continuous stirring. CH_3CN was from SDS (France), TFA from Pierce (USA), other chemicals were from Merck (FRG).

3. RESULTS

Fig.1 shows that a concentrated Tris extract from rat cortex was able to inhibit in a dose-dependent manner the [^3H]Glipizide binding on membranes from rat cerebral cortex. The dilution curves for the standard and the extract were parallel, indicating a similar behaviour at the receptor level of the drug and of the active component present in the extract. It may be calculated from these data that 1 g of rat cortex contains 1.5 pmol Glipizide equivalent of active component. According to the respective affinity of Glipizide and Glibenclamide for the central sulfonylurea receptor [5], this corresponds to ~0.1 pmol Glibenclamide equivalent/g tissue (wet wt). It must be noticed that, if we disregard the difference in sulfonylurea equivalent, the experiments on biological samples gave identical results, whichever Glipizide or Glibenclamide was used as the tracer or the standard (not shown). The activity present in the Tris extract was not lost after dialysis (table 1), indicating that the active component has a molecular mass high enough to be retained by the 24 Å porosity membrane. This rules out the possibility that small molecules such as ions may have interfered with the sulfonylurea binding. Running the concentrated Tris extract on anion- and cation-exchange media (table 1) indicated that

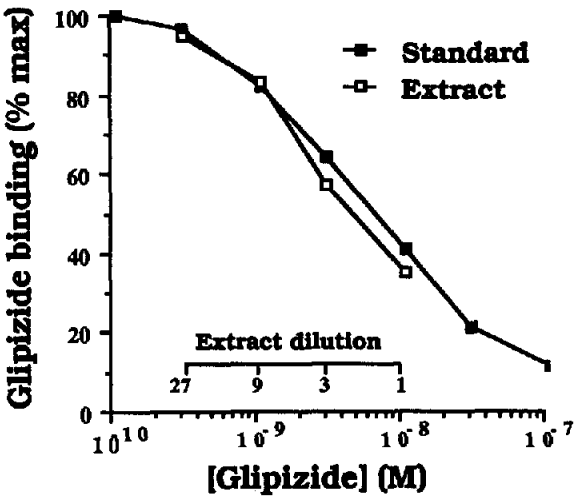

Fig.1. Effects of unlabelled Glipizide (standard) and of dilutions of a Sep-Pak concentrated Tris extract from rat cerebral cortex (extract) on the binding of [^{3}H]Glipizide to membranes prepared from rat cerebral cortex. The highest dilution point (27) was placed on the standard curve and the other points placed according to their respective dilution. Similar results were observed in 5 repeated experiments.

Table 1

Effect of a concentrated Tris extract of rat cerebral cortex on the binding of [^{3}H]Glipizide to membranes prepared from rat cerebral cortex after the following experiments

Experiment	Activity (%)
(1) Dialysis	control 100 dialysed 92
(2) Cation exchange (pH 2)	void 6 eluate 94
(3) Anion exchange (pH 8)	void 72 eluate 28

(1) Before (control) or after (dialyzed) dialysis in 24 Å porosity Visking membrane. (2) Run on S-Sepharose fast-flow in 0.1% TFA/CH_3CN, 80:20 (cation exchange, pH 2). Void, void volume (not retained by the column); eluate, fraction eluted in 0.1% TFA/CH_3CN, 80:20 containing 1 M NaCl. (3) Run on Q-Sepharose fast-flow in 10 mM NH_4HCO_3/CH_3CN, 80:20 (anion exchange, pH 8). Void, void volume (not retained by the column); eluate, 0.5 M NH_4HCO_3/CH_3CN, 80:20. Data are expressed as activity measured by the binding assay as % of the control (dialysis) or as % of the total activity recovered (cation- and anion-exchange chromatography). Similar results were obtained in three repeated experiments (dialysis) or two experiments (chromatographies)

the active molecule is not retained by an anion exchanger at pH 8, but is retained at pH 2 on a cation exchanger. It may be eluted from this cation exchanger at a salt concentration of 0.25 M (not shown). Fig.2 provides direct evidence of the peptidic nature of the active molecule. Indeed, incubating the concentrated Tris extract with proteases such as pepsin or papain resulted, under the conditions used, in the loss of 60 to 82% of the activity (fig.2). In contrast, no degradation of the activity was observed after incubation with phospholipase A_2 (fig.2). Together, these data suggest that the active molecule is a peptide displaying a neutral to slightly basic isoelectric point.

The apparent peptidic nature of the active molecule led us to run the concentrated Tris extract on an HPLC system [11] set up for purifying peptides. A single peak of activity was found (not shown), indicating that reverse-phase HPLC may be used for isolating the active peptide. On the other hand, the concentrated Tris extract was not suitable for this purpose, and the possibility of extracting the active peptide with more classical methods [10] was tested. It was found that very similar results were obtained with either method (not shown). Fig.3 shows three purification steps of a batch of 100 g of rat cerebral cortex extracted according to [10] with slight modifications. In the first step (C-8 column run at pH 7, fig.3A), a single peak of activity was observed at an acetonitrile concentration of 28.5%. The corresponding fractions were pooled and run on the next column. In

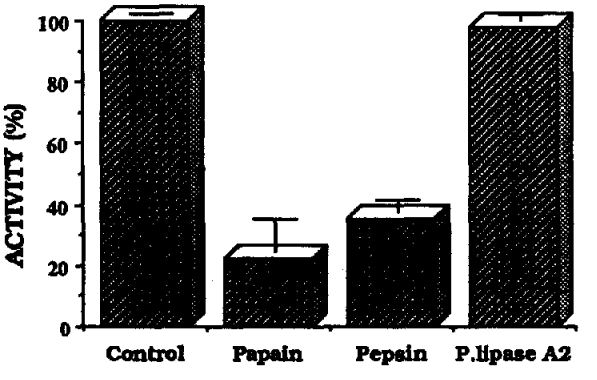

Fig.2. Effects of a concentrated Tris extract on the binding of [^{3}H]Glipizide to membranes prepared from rat cerebral cortex either before (control) or after enzymatic digestions by papain, pepsin or phospholipase A_2. Data are expressed as % of control (incubated without enzyme). Data are the means ± SE of 3 repeated experiments.

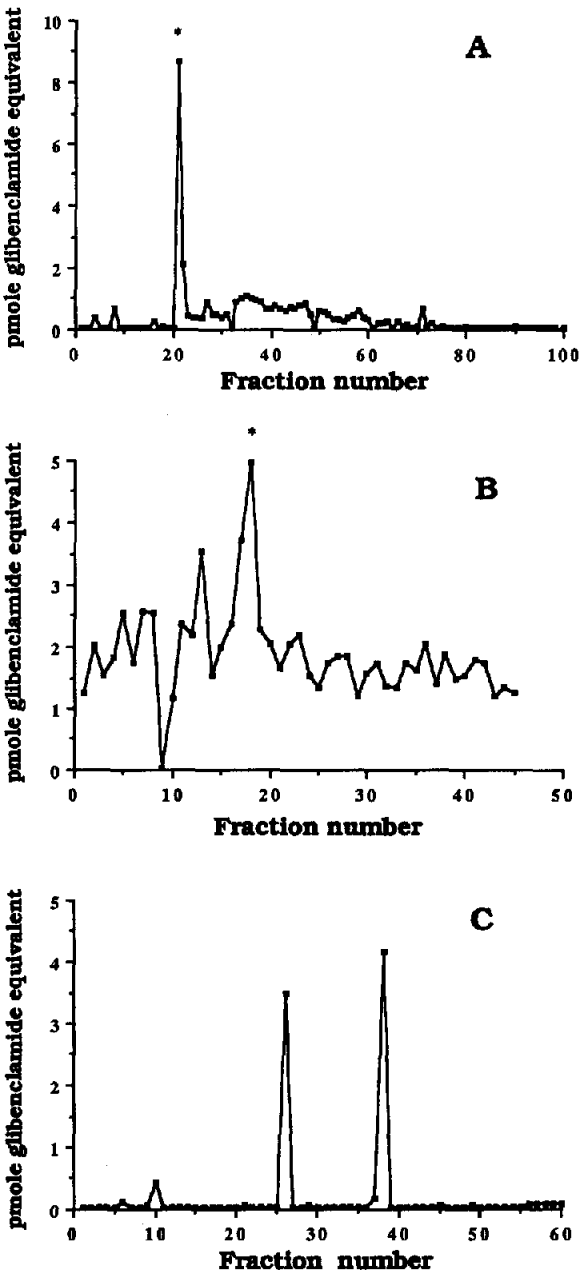

Fig.3. (A) High-performance liquid chromatography (HPLC) of an extract obtained from 100 rat cortices by the heat coagulation-acid extraction method [10] on a 1.0 I.D. × 30 cm column filled with Lichrosorb RP-8 and equilibrated with 0.4 M CH_3COONH_4, pH 7.0, containing 3% propanol. The chromatography was developed by a linear gradient of 20–60% CH_3CN in 50 min at 2 ml/min. The fractions corresponding to the peak of activity indicated by the star was loaded on the next column (B). (B) HPLC of the fractions (*) from C-8 (A) on a 1.0 × 30 cm μC-18 column equilibrated with 1% TFA-DEA [11]/CH_3CN (80:20) and eluted with a linear gradient of 20–50% CH_3CN in 45 min at 6 ml/min. The peak of activity indicated by the star was loaded on the next column (C). (C) HPLC on a 0.39 × 30 cm Nucleosil CN column equilibrated with 0.1% TFA/CH_3CN (90:10) of the fraction (*) in B. Elution was performed with a linear gradient of 10–50% in 60 min at 1.5 ml/min. 0.5- (A) or 1-min fractions (B or C) were collected, lyophilized after evaporation of the organic solvent and tested in the binding assay.

the second chromatography (C-18 column run at pH 2.5, fig.3B), a main peak of activity was visible, eluting at 32%. This peak, run on a CN column at pH 2, was resolved into two peaks of activity (fig.3C). This indicates either that a partial molecular modification occurred between step 2 and step 3, or, more probably, that two molecular forms existed in the original extract, forms which were separated from each other by the charged cyano functional group of the CN column but not by the aliphatic functional groups of the C-8 or C-18 columns. It must be noticed that the column yields were 79% and 88% for the C-18 and the CN steps and that the UV absorbance at all wavelengths of the active fractions declines strongly at each step to reach a very low level at the last one. This indicates that the purification factor was high from the original extract to the CN step.

4. DISCUSSION

The hypoglycemic sulfonylureas, a family of drugs of particular interest in the management of diabetes mellitus [1], display specific membrane-bound receptors both at the level of the pancreatic B-cells [2,3] and in the central nervous system [4,5], a receptor which is linked to an ionic channel effector [6–9]. This led us to suggest that this biologically relevant receptor is probably the natural recognition system of an endogenous ligand [5]. In the present study, we have focused our attention on the rat central sulfonylurea receptor and thus on the possible presence of an endogenous ligand nearby the receptor, i.e. in the rat central nervous system. We show that an endogenous ligand is indeed present on the same side of the blood-brain barrier as the receptor itself and that this ligand is peptidic in nature, displaying

physico-chemical characteristics found in many regulatory peptides. The arguments for these assessments are as follows: (i) the ligand is found in the supernatant of the membrane preparation from the rat cerebral cortex, suggesting that it has been liberated by the disruption of the neuronal structure through the mild homogenization in a hypotonic medium, known to break the cell membrane, in particular the neuroregulatory molecule-containing nerve endings; (ii) the ligand is not dialysable, indicating a molecular size compatible with its suspected peptidic nature; (iii) a method widely used to obtain an extract enriched in regulatory peptides [10] yielded a solution containing the ligand at a concentration (per gram of tissue) similar to that obtained with the first type of extraction; (iv) the ligand binds to reverse-phase cartridges and may be eluted from them under conditions that are classically used for peptides [11]; (v) the ligand may be purified by reverse-phase HPLC under conditions as in (iv); (vi) the isoelectric point is neutral to slightly basic as assessed by the ion-exchange experiments; (vii) the peptidic nature of the ligand is definitely substantiated by the disappearance of the activity after incubation with proteases.

This peptidic nature of the endogenous ligand for the sulfonylurea receptor is reminiscent to that of ligands for other drug receptors such as endorphines for the morphine receptor [13] or diazepam binding inhibitor/endozepine for the benzodiazepine receptor [14,15] as well as ligands for sites of other psychotropic drugs [16].

The presence in the rat central nervous system of a biologically relevant receptor and the existence nearby of an endogenous ligand for this receptor open up a new era of research on the complex regulatory processes occurring at the central level. The isolation and chemical characterization of the peptide which binds to the sulfonylurea receptor will also shed a new light on the mode of action of this class of drugs used every day by millions of people.

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