Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6629–6633, July 1992
Biochemistry

Endosulfine, an endogenous peptidic ligand for the sulfonylurea receptor: Purification and partial characterization from ovine brain

(insulin release)

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Communicated by Viktor Mutt, March 4, 1992 (received for review January 1, 1992)

ABSTRACT **Antidiabetic sulfonylureas act through receptors coupled to ATP-dependent potassium channels. Using the binding of [^{3}H]glibenclamide, a highly potent sulfonylurea, to rat brain membranes to follow the purification procedure, we extracted from ovine brain, purified, and partially characterized two peptides that are endogenous ligands for the central nervous system sulfonylurea receptors. These peptides, referred to as α and β endosulfine, differ by their isoelectric points, the β form being more basic. Each form of endosulfine is recognized equally by the sulfonylurea receptors from the central nervous system and from insulin-secreting β cells. In the same concentration range that is active on the receptors, β endosulfine releases insulin from a β-cell line. Endosulfine is a good candidate for being implicated in the physiology of β cells and their disorders (e.g., type II diabetes) and in certain pathologies related to modifications of ion fluxes.**

The antidiabetic sulfonylureas (1) are widely used in the management of non-insulin-dependent diabetes mellitus (NIDDM), also referred to as type II diabetes (2). These drugs (3, 4) stimulate insulin secretion from the islets of Langerhans, which in diabetes display a decreased insulin content (5) and an impaired response to glucose (6). The sulfonylureas act at the cell surface of the β cell (7) through interaction with specific binding sites (or receptors) present in membranes from insulin-secreting cells or tissues (8–13). Receptors with similar characteristics were also observed in the central nervous system (CNS; refs. 10, 14, and 15) and in the myocardial tissue (16). The sulfonylurea receptor is closely associated to ATP-dependent potassium channels (17–19), also involved in glucose-induced insulin secretion (20). The existence of such a receptor led us to suggest that it represents a recognition site for an endogenous ligand (14) that would regulate physiological processes through mechanisms triggered, in a pharmacological context, by the sulfonylureas. Such an approach for the binding sites for morphine led to the discovery of the endorphines (21). In the CNS, the potential endogenous ligand is likely to be present near the receptor. We have shown that such a ligand exists in the rat brain (22). We describe the presence in and the purification from ovine brain of two peptides, representing most likely two forms of the same entity, for which we propose the name "endosulfine." Endosulfine is present in the brain of rat and sheep at similar concentrations—i.e., *ca* 0.1 pmol of glibenclamide equivalent per g of tissue—and in the rat pancreas. Highly purified endosulfine is like sulfonylureas in that, at concentrations where it interacts with the receptors, it is able to induce insulin release from βTC cells in culture.

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MATERIALS AND METHODS

Sulfonylureas. Glibenclamide was provided by the Guidotti laboratory. [^{3}H]Glibenclamide (40–50 Ci/mmol; 1 Ci = 37 GBq) was purchased from Hoechst Pharmaceuticals or Du-Pont.

Binding Studies with Labeled Sulfonylureas. Binding studies were conducted as described (14, 22) with membranes prepared from 200- to 300-g male Wistar rat cortex. Freshly prepared membranes (0.4 mg of protein per ml) were incubated at room temperature with 0.1 nM [^{3}H]glibenclamide/50 mM Tris·HCl, pH 7.5, in the presence or absence of extract, chromatographic fraction, or unlabeled sulfonylurea.

Tissue Extraction. Frozen ovine brains from Australia were obtained from S. A. Miko (Lattes, France). They were processed by using the heat coagulation/acid extraction method described for the isolation of gastrointestinal peptides (23).

Purification Methods. A 10 × 22 cm medium-pressure column (Pharmacia LKB) filled with SP-Sepharose Fast-Flow was connected to a model Prep 500A preparative chromatography system (Millipore–Waters). It was run at 80 ml/min with 10 mM ammonium acetate, pH 6.15/20% ethanol and eluted with a 50-min linear gradient from 10 mM to 500 mM acetate (solution A, 10 mM acetate/20% ethanol; solution B, 1 M acetate/20% ethanol).

Analytical cation-exchange chromatography was run at 1.5 ml/mn in 10 mM ammonium acetate, pH 6.0/30% acetonitrile on a 0.5 × 5 cm Mono-S column (Pharmacia) attached to an HPLC device. The chromatography was developed with a linear gradient of 10–500 mM acetate for 50 min (A, 10 mM acetate/20% ethanol; B, 1 M acetate/20% ethanol).

The HPLC device, from Millipore–Waters, consisted of two model 6000A solvent delivery systems controlled by a model 720 controller and a model U6K injector. UV detection was carried out by one or two model 440 detectors and a model CR-4A Chromatopac integrator (Shimadzu, Japan). The columns were from the Société Française Chromato Colonne. Packings, solvents, and conditions are described in the legends to the figures. One-minute fractions were collected in glass tubes and, after concentration *in vacuo*, were run on the next column or were lyophilized.

Enzymatic Digestions. They were conducted with papain and pepsin as described (22) on active fractions from the first reversed-phase HPLC. After inactivation of the enzyme by boiling, the sample was chromatographed on a reversed-phase CN column, and the activity was measured in the fractions. Control incubations were run in the absence of enzyme.

Cell Culture and Studies on Insulin Release. The RIN m5F cell line (24, 25) derives from a rat insulinoma. The βTC cell

Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; CNS, central nervous system.

line (26, 27) was obtained by using a transgenic approach. Both cell lines were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and supplemented with 10% (vol/vol) fetal calf serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Cell membranes were prepared from both types of cells by described methods (28). For studying insulin release, βTC cells (5×10^5 cells per well) were plated into 12-well Corning plates in 1-ml DMEM containing 5.5 mM glucose. This medium was changed 18 hr before the experiments. On the day of the experiment, the cells were washed twice with Krebs–Ringer bicarbonate solution containing 20 mM Hepes and 0.1% bovine serum albumin (basal buffer) and preincubated for two periods of 30 min separated by a change in the medium. The cells were incubated for 60 min at 37°C in basal buffer supplemented with 0.44 mM glucose and the sample to be tested. The glucose concentration was chosen from the dose–response curve (not shown) that corresponded to 30–40% of the maximal insulin release. Insulin was measured in the medium by radioimmunoassay as described (29).

RESULTS

Evidence for the Presence and Characterization of Endosulfine in Ovine Brain. Fractions from a C_8 reversed-phase HPLC run of a crude extract of ovine brain contain a molecule that interacts with the sulfonylurea receptors present in rat brain membranes (Fig. 1*A*). The main peak (fractions 13–18), referred to as crude endosulfine, was run on a C_{18} column (Fig. 1*B*), leading to several peaks of activity including a main one (fractions 40 and 41). A run of these fractions on a CN column resulted in two perfectly separated peaks of activity (Fig. 1*C*). When comparing these data to those obtained with rat brain extracts (23), the following observations may be made: (*i*) from both aliphatic bonded phases, rat (22) and ovine (see Fig. 1 *A* and *B*) endosulfines were eluted as a main peak; (*ii*) rat and ovine endosulfines displayed slightly different retention times in these systems (see Fig. 1 *A* and *B*); and (*iii*) both rat (22) and ovine (Fig. 1*C*) crude endosulfines were separated by the CN column in two forms, which we call α and β endosulfine. It has been suggested (22) that the two molecular forms of endosulfine are separated on the CN column by an ion-exchange effect (22). Analysis of each peak on a cation-exchange HPLC system (Fig. 2) confirmed this hypothesis: α endosulfine, the less retarded peak on CN, was also less retarded on the Mono-S column than was β endosulfine (Fig. 2), which, accordingly, must be considered to be the more basic form.

The peptidic nature of ovine endosulfine was evidenced by its sensitivity to proteases as follows: incubating crude endosulfine with pepsin or papain led to 97% and 86% inactivations, respectively (not shown).

Purification of Ovine Endosulfine. To analyze further the endosulfine properties, the purification procedure was scaled up to several kilograms of ovine brain and was modified to handle medium-to-large-scale extracts. A preparative cation-exchange chromatography allowed a preliminary separation of two forms of endosulfine (Fig. 3), called peak I and peak II. After two successive preparative reversed-phase HPLC on C_8 and C_{18} columns (not shown), each peak was run on a semipreparative CN column (Fig. 4), giving a main peak with the retention times of α and β endosulfine, respectively (see Fig. 1). Surprisingly, the most retarded peak (peak II) from the first ion-exchange column was eluted first from the CN column, with the retention time of α endosulfine. Conversely, the less retarded peak on the SP column (peak I) obviously corresponded to β endosulfine (Fig. 4). An artifact occurring during the first chromatography is a likely explanation for this observation. α and β endosulfine were purified further by a run on an analytical CN column. α-Endosulfine activity was eluted from this column as a wide and obviously heterogeneous peak, as evidenced by the UV absorbance profile and the presence of several protein bands in polyacrylamide gel electrophoresis (not shown). In contrast, β endosulfine appeared at a much higher degree of purity. Thus, the activity was eluted together with a small and symmetrical UV peak, both at 214 and 280 nm (Fig. 5). These observations prompted us to test β endosulfine on insulin secretion (see below).

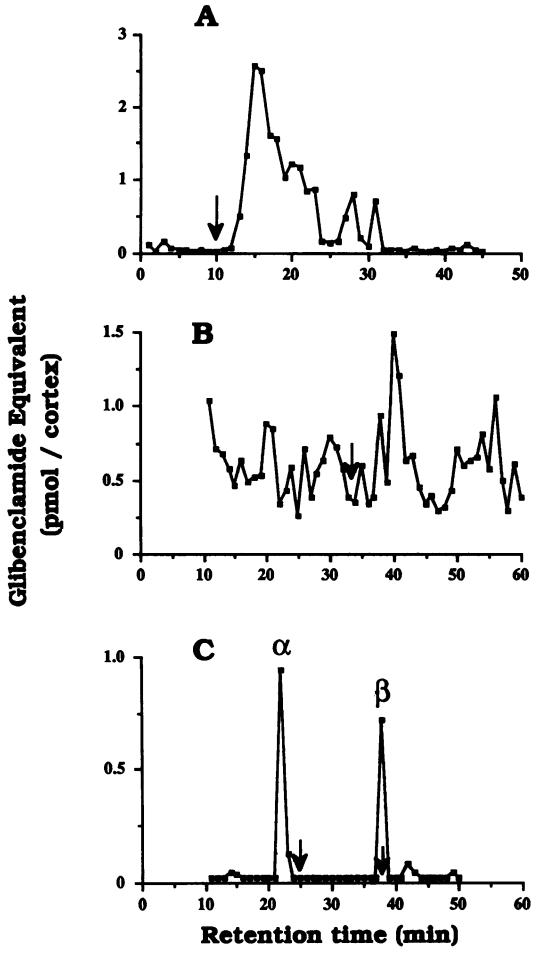

FIG. 1. Series of three HPLC steps in separating α and β endosulfines from an extract of 350 g of sheep brain. (*A*) The first step used a 1.0 i.d. × 25 cm column filled with Lichrosorb RP-8 solid phase and equilibrated with 0.4 M CH_3COONH_4, pH 7.0/3% (vol/vol) propanol. The column was eluted with a linear gradient of 20–60% CH_3CN (solution A, acetate/propanol; solution B, 100% CH_3CN) in 50 min at 2 ml/min. Fractions 13–18 were loaded on the next column. (*B*) The second step used a 1.0-cm i.d. × 25 cm reverse-phase C_{18} column equilibrated with 1% CF_3COOH adjusted to pH 2.5 with diethylamine and eluted with a linear gradient of 10–50% CH_3CN (A, aqueous solvent; B, 100% CH_3CN) in 60 min at 6 ml/min. Fractions 40 and 41 were loaded on the next column. (*C*) The third step used a 0.46 i.d. × 25 cm reverse-phase CN column equilibrated with 0.1% CF_3COOH and eluted with a linear gradient of 10–50% CH_3CN (A, 0.1% CF_3COOH; B, 100% CH_3CN) in 60 min at 1.5 ml/min. The arrow indicates the retention times of rat endosulfine under the same conditions (22).

Interaction of Ovine Endosulfine with CNS Receptors and β-Cell Receptors. At this stage of characterization, it was of particular interest to determine whether the molecules that interact with the CNS receptors also recognize receptors from the pancreatic β cells. Indeed, both types of receptors display an identical pharmacology when sulfonylureas are used as test agents (10), whereas the use of the natural ligand might reveal the existence of receptor subclasses. Furthermore, endosulfine also exists in the rat pancreas under similar

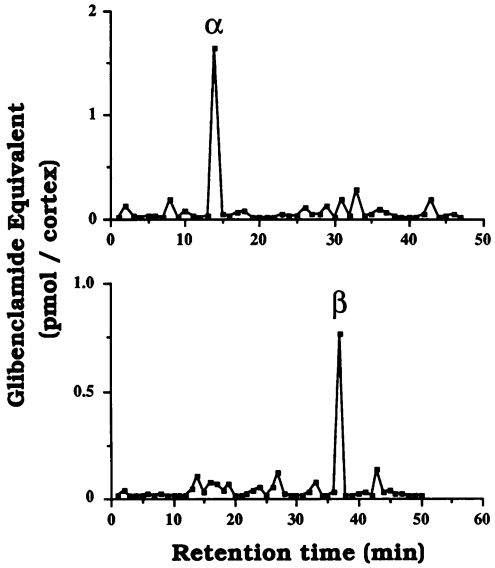

FIG. 2. Cation-exchange chromatography on a Mono-S column of α and β endosulfines obtained in the Fig. 1*C* experiment. The column equilibrated with 10 mM CH_3COONH_4/100% CH_3CN, 70:30 (vol/vol), pH 6.0, was eluted with a linear gradient of 10–500 mM CH_3COONH_4/100% CH_3CN, 70:30 (vol/vol), pH 6.0 in 50 min at 1.5 ml/min.

molecular forms (30), increasing the probability that the CNS forms of ligand recognize the β-cell receptors. This is indeed the case: the analysis of α and β endosulfines by the binding test performed both with rat brain membranes (CNS receptor; Fig. 6, *Upper*) and with membranes prepared from insulin-secreting RIN m5F cells (β-cell receptor; Fig. 6, *Lower*) clearly indicated that both α and β endosulfines are equally recognized by both types of receptors.

Stimulation of Insulin Secretion by β Endosulfine. The preceding data led us to study a possible insulinotropic effect of the ligand. Preliminary trials with RIN m5F cells indicated that this model is not suitable for studying insulin release in response to glucose or sulfonylureas. We used βTC cells, which display a good response to glucose and sulfonylureas (27), after having established that the receptors present in membranes from this cell line, from rat cortex, and from RIN m5F cells display the same behavior towards glibenclamide, allowing direct comparisons (Fig. 7).

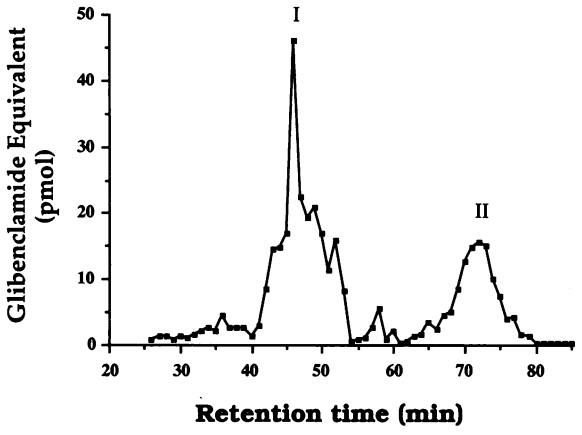

FIG. 3. First step of the endosulfine purification from 1.5 kg of sheep brain extract. A 10 i.d. × 22 cm column filled with SP-Sepharose Fast-Flow® cation-exchange gel equilibrated with 10 mM CH_3COONH_4, pH 6.15/20% (vol/vol) ethanol was eluted with a linear gradient of 10–500 mM CH_3COONH_4, pH 6.15/20% ethanol in 50 min at 80 ml/min. Aliquots (5 ml) were taken for the binding assay.

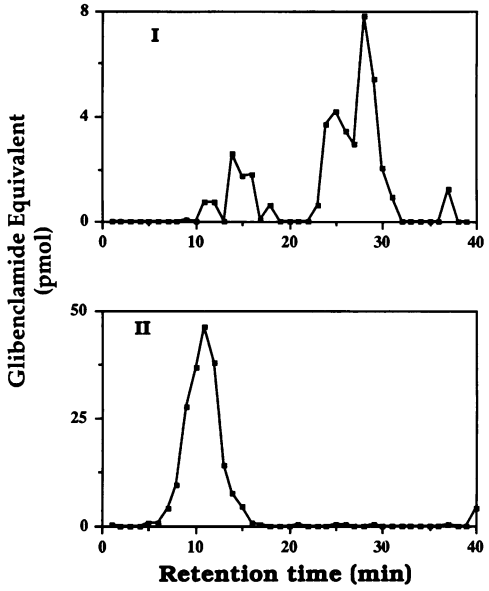

FIG. 4. Step 4 of α- and β-endosulfine purification. A 1.0 i.d. × 25 cm reverse-phase CN column equilibrated with 0.1% CF_3COOH was eluted with a linear gradient of 10–50% CH_3CN (A, 0.1% CF_3COOH; B, 100% CH_3CN) in 40 min at 6 ml/min. Aliquots of 20% were taken off and analyzed in the binding assay.

Fig. 8 shows a dose–response curve for insulin release from βTC cells in response to glibenclamide at a glucose concentration of 0.44 mM, which, because of the known hypersensitivity to glucose of this cell line (27), triggered 30–40% of the maximal glucose-induced insulin release. It may be seen that a maximal 15-fold response may be obtained over a 2-orders-of-magnitude range of glibenclamide concentration.

The possible insulinotropic effect of β endosulfine was tested by analyzing the effect of the three fractions corresponding to the peak of activity detected by binding experiments. Two different batches of β endosulfine, which displayed a very low UV absorbance indicating a high degree of homogeneity (see Fig. 5), were tested. Fig. 9 shows the result of one of these experiments. It may be seen that a peak of insulin-releasing activity corresponded to the peak of binding activity and that there was a good quantitative correlation between the glibenclamide equivalent for binding and that for insulin release. The second experiment gave very similar results (not shown). This strongly suggests that the molecule eluted in the active peak is insulinotropic at concentrations

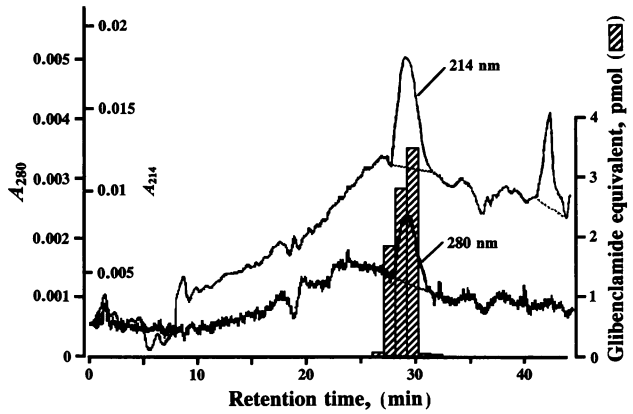

FIG. 5. β endosulfine from the preparative CN column (see Fig. 4) was run on a 0.46 × 25 cm CN column. Gradient conditions were as in Fig. 4. The eluate was monitored by UV absorbance at both 214 and 280 nm and by the binding assay (hatched bars).

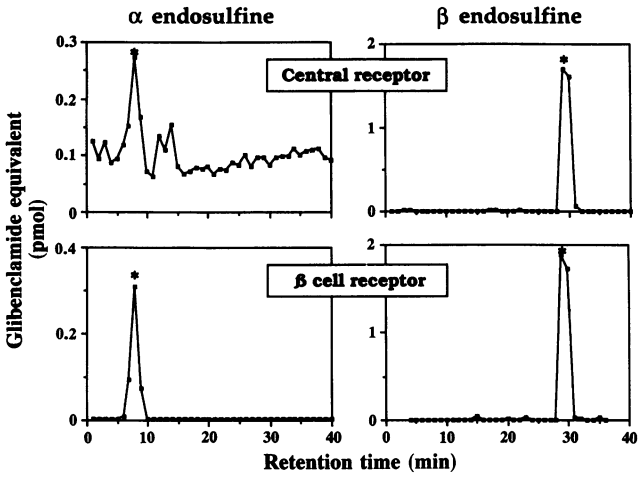

FIG. 6. Effect of α and β endosulfine on [^{3}H]glibenclamide binding to rat CNS receptors and β-cell receptors. α and β endosulfines obtained after the CN column (Fig. 4) were run again on an analytical CN column (Fig. 5). Aliquots were tested in the binding of [^{3}H]glibenclamide to rat brain membranes (*Upper*) and to RIN m5F cell membranes (*Lower*).

that are active on the receptors. Thus, β endosulfine appears to display on pancreatic β cells biological characteristics that are very similar to that of glibenclamide.

DISCUSSION

Antidiabetic sulfonylureas are essential drugs for the management of NIDDM. They act essentially by stimulating insulin secretion from islets that have partially lost their sensitivity to glucose, the physiological stimulus (6). Effects of sulfonylureas on muscle, fat, and liver have also been described that, by facilitating insulin action, enhance the hypoglycemic properties of the drug (1). It is now well accepted that sulfonylureas act on their target tissues through an interaction with receptors present in β cells, insulin-secreting tumors, fat cells, heart, and the CNS. All of these receptors appear to have a very similar pharmacology. These receptors are linked to or form part of ATP-dependent potassium channels, which close upon interaction with sulfonylurea. The resulting depolarization of the membrane opens a voltage-gated Ca^{2+} channel, the consequent Ca^{2+} influx triggering insulin release (19). This series of events appears to be similar to that triggered by glucose, the mode of action of which has several steps in common with that of sulfonylureas. Some of these steps might be impaired in NIDDM.

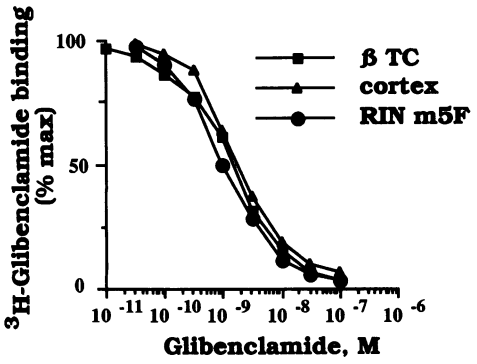

FIG. 7. Comparison of [^{3}H]glibenclamide binding to membranes prepared from rat cortex (▲), insulin-secreting RIN m5F cells (●), and βTC cells (■).

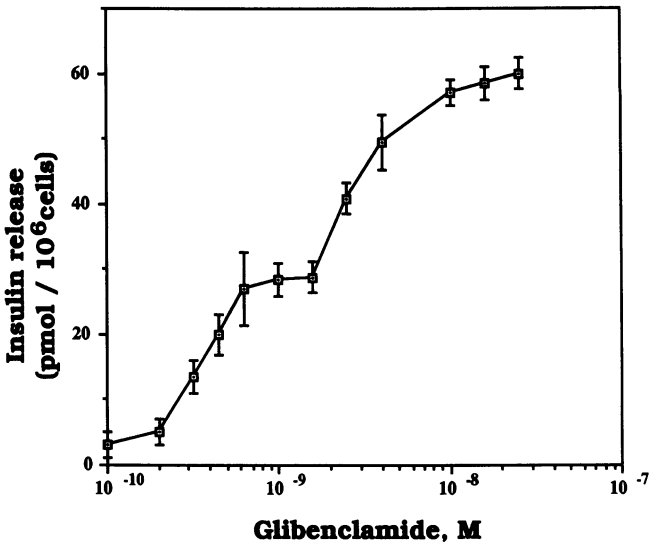

FIG. 8. Dose-related effect of glibenclamide on insulin release from βTC cells in culture. Incubations were performed at 0.44 mM glucose. Insulin released in the medium is expressed as pmol of insulin released per 10^6 cells during the incubation time. Data are means ± SEM of three experiments, each performed in triplicate.

The present data show that an endogenous peptide, which appears to exist under two molecular forms, is a natural ligand for the sulfonylurea receptor. We propose the name "endosulfine" for this peptide by analogy to the endorphines (21), endogenous peptidic ligands for the morphine receptor. Our data on insulin release obtained with highly purified β endosulfine strongly suggest that the consequence of the interaction of endosulfine with its receptor is the same series of events as that triggered by sulfonylureas, leading to insulin release.

To obtain sufficient amounts of endosulfine for its structural determination, large-scale purification must be performed, as the peptide is present in low amounts in both rat and sheep brain [*ca.* 0.1 pmol/g (wet weight) of glibenclamide equivalent]. The affinity constant of glibenclamide for the receptor (*ca.* 1 nM) is close to that usually observed *in vitro*

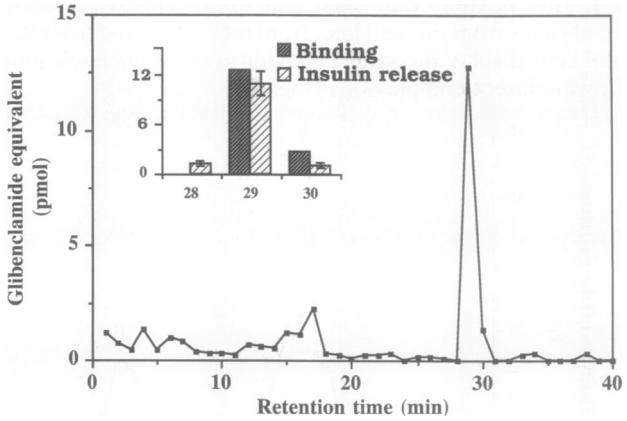

FIG. 9. Effect of highly purified β endosulfine (see Fig. 6) on insulin release from βTC cells. Analysis of the fractions by the binding assay is shown. The active fractions (28, 29, and 30) were tested on insulin release. (*Inset*) Glibenclamide equivalents in binding (dark hatched columns) and in insulin release (clear hatched columns) calculated from the glibenclamide dose–response curve of Fig. 8. Data on insulin release are means ± SEM of quadruplicate determinations. Significant differences ($P < 0.01$) exist between insulin released by fraction 29 as compared with that released by either fraction 28 or 30. Similar results were obtained in two repeated experiments on two different batches of β endosulfine.

for peptidic ligands, and the affinity of endosulfine is likely to be similar. Therefore, the real concentration of the peptide is probably close to that calculated by reference to glibenclamide, the most potent sulfonylurea.

In summary, what is known about endosulfine is as follows. (*i*) It is a peptide, since it is degraded by proteases. (*ii*) The molecular weight is >2000, since it does not pass through a dialysis membrane with a M_r 2000 cutoff (23). (*iii*) It is extracted by methods developed for peptides. (*iv*) Its distribution in the rat (30) fits rather well with that of the binding sites in the same animal species; thus, its localization in the central nervous system is relatively wide, although somehow preferential for particular regions (30) as observed for the binding sites (15). No significant amounts of endosulfine were noted in other peripheral tissues such as intestine, stomach, liver, or kidney (30), indicating that, if endosulfine is produced in these tissues, its abundance is less than in the CNS or the pancreas. (*v*) Its affinity for the CNS receptor is identical to that for the receptor present in the β cells, supporting further the idea that the pharmacology of the CNS receptors is very close, if not identical, to that of peripheral receptors.

Endosulfine displays many characteristics for being a natural regulator of insulin secretion through mechanisms that are crucial in the physiology of the islet of Langerhans. An additional possible role for endosulfine is the regulation of β-cell proliferation. Loubatières *et al.* (4) noted such an effect of early types of sulfonylurea. This effect was not confirmed by others (31) who used new-generation sulfonylureas. In a recent report (32), a stimulation by tolbutamide (a first-generation sulfonylurea) of β-cell proliferation was clearly demonstrated in addition to a β-cell cytotoxic effect of second-generation sulfonylureas, which might have hindered the intrinsic β-cell cytotrophic effect of sulfonylureas. This gave a plausible explanation for the discrepancies indicated above. Endosulfine, the natural ligand, is likely to be devoid of cytotoxic effect, resembling more tolbutamide from this point of view, and, therefore, might have a role in controlling the β-cell mass, a critical parameter in the diabetic pathogeny (2). Accordingly, a poor expression and/or secretion of endosulfine in NIDDM might be of importance in the processes leading to the diabetic pathology. On the other hand, its presence in the CNS at the vicinity of its receptors suggests that it may operate as a regulator of the K^+ flux between neurones and astrocytes. As this flux has a key role in the dramatic consequences of cerebral ischemia (15), future researches on CNS endosulfine might be of importance in the knowledge and the prevention of this pathology.

Therefore, the demonstration of the existence of endosulfine opens up a new avenue of research on both etiology and prevention of NIDDM and disorders of the cerebral circulation.

We thank Dr. Douglas Hanahan, University of California at San Francisco, and Drs. Bernadette Bréant and Corinne Lavergne, Institut National de la Santé et de la Recherche U55, Paris, for providing us with the βTC cells. We thank Dr. Beatrice Guardiola from the Institut de Recherche International Servier (IRIS) for her kind helpfulness. A.V.-V. acknowledges fellowship support from IRIS. This work was supported by the Fondation pour la Recherche Médicale.

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