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ATP/ADP Binding Sites Are Present in the Sulfonylurea Binding Protein Associated with Brain ATP-Sensitive K^+ Channels†

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ABSTRACT: Covalent labeling of nucleotide binding sites of the purified sulfonylurea receptor has been carried out with α-^{32}P-labeled oxidized ATP. The main part of ^{32}P incorporation is in the 145-kDa glycoprotein that has been previously shown to be the sulfonylurea binding protein (Bernardi et al., 1988). ATP and ADP protect against this covalent labeling with $K_{0.5}$ values of 100 μM and 500 μM, respectively. Nonhydrolyzable analogs of ATP also inhibit ^{32}P incorporation. Interactions between nucleotide binding sites and sulfonylurea binding sites have then been observed. AMP-PNP, a nonhydrolyzable analog of ATP, produces a small inhibition of [^{3}H]glibenclamide binding (20-25%) which was not influenced by Mg^{2+}. Conversely, ADP, which also produced a small inhibition (20%) in the absence of Mg^{2+}, produced a large inhibition (~80%) in the presence of Mg^{2+}. This inhibitory effect of the ADP-Mg^{2+} complex was observed with a $K_{0.5}$ value of 100 ± 40 μM. All the results taken together indicate that ATP and ADP-Mg^{2+} binding sites that control the activity of K_{ATP} channels are both present on the same subunit that bears the receptors for antidiabetic sulfonylureas.

ATP-sensitive K^+ channels (K_{ATP} channels) have now been identified in different cell types. They are key channels in β-cells in relation with glucose-induced insulin secretion (Petersen & Dunne, 1989). They are regulated by both ATP and ADP (Dunne & Petersen, 1986; Dunne et al., 1988; Kakei et al., 1986; Ribalet & Ciani, 1987). ATP inhibits channel activity; ADP in the presence of Mg^{2+} releases the inhibitory effect of ATP (Rorsman & Trube, 1990). They are present in skeletal muscle (Spruce et al., 1985) and in cardiac cells (Fosset et al., 1988; Noma, 1983) in which they are opened in fatigue (Castle & Haylett, 1987) or ischemic conditions (Fosset et al., 1988) and in smooth muscle cells (Davies et al., 1991). They have been identified in neuronal cells (Ashford et al., 1988, 1990; Politi & Rogawski, 1991). They are present both in cell bodies and in nerve terminals (Alzheimer et al., 1989; Miller, 1990; Politi et al., 1989) where they are involved in glucose-regulated neurotransmitter release (Amoroso et al., 1990).

K_{ATP} channels are blocked by antidiabetic sulfonylureas and particularly potently by glibenclamide (Schmid-Antomarchi et al., 1987; Sturgess et al., 1985). They are also the target of K^+ channel openers such as diazoxide, cromakalim, pinacidil, RP49356, nicorandil, or minoxidil sulfate (Dunne et al.,

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1989; Edwards & Weston, 1990; Escande et al., 1988; Quast & Cook, 1989; Schmid-Antomarchi et al., 1990).

The brain sulfonylurea binding protein has been previously affinity labeled and isolated in a pure form (Bernardi et al., 1988). This 145–150-kDa glycoprotein is thought to be an important component of the K_{ATP} channel. A similar binding component has been identified in β-cells (Aguilar-Bryan et al., 1990; De Weille et al., 1989; Kramer et al., 1988). However, this protein has not yet been reconstituted as an active K_{ATP} channel, and it is not known whether it contains ATP/ADP regulatory sites and structural components of ion permeation. The purpose of this work is to show that ATP/ADP binding sites are part of the glycoprotein which bears the antidiabetic sulfonylurea binding site.

Experimental Procedures

Materials. [^{3}H]Glibenclamide (50 Ci/mmol), glibenclamide, and tolbutamide were from Laboratoires Hoechst, Paris. 5′-[α-^{32}P]ATP[1] (410 Ci/mmol) was from Amersham, Paris. Glipizide was from Laboratoires Pfizer, Paris; cromakalim was from Beecham Pharmaceuticals, Great Britain; and diazoxide was from Laboratoire Cetrane, Paris. Nucleotides, sodium metaperiodate, sodium cyanoborohydride, dithiothreitol, 2-mercaptoethanol, and imidazole were from Sigma, France.

Oxidation of ATP. 5′-[α-^{32}P]ATP was oxidized as described by Clertant and Cuzin (1982). A total of 1 nmol of radiolabeled ATP was dried under vacuum and then dissolved in 100 μL of 1 mM HCl. Oxidation was started by addition of 10 nmol of sodium metaperiodate. After 30 min of incubation at 20 °C in the dark, the reaction was stopped by addition of 50% glycerol, and the pH was raised to 7.5 with 20 μL of 500 mM triethylamine at pH 7.5. The product was used immediately. The oxidation yield and the purity of 5′-[α-^{32}P]Ox-ATP were checked by thin-layer chromatography on poly(ethylenimine) cellulose sheets using a 1 M formate/1 M LiCl solvent (Clertant & Cuzin, 1982).

Purification of Sulfonylurea Receptor. The binding protein was purified from pig brain as previously described by Bernardi et al. (1988). The purification procedure involves a chromatography step on a ADP–agarose column which was prepared according to Lamed et al. (1973). Purified fractions were treated with 2% 2-mercaptoethanol for 30 min at room temperature and then were dialyzed for 12 h at 4 °C against 1 L of 25 mM imidazole hydrochloride at pH 7.5.

Covalent Labeling of Purified Sulfonylurea Binding Protein. Covalent labeling was performed by incubating 0.5–5 pmol of the purified receptor with 10–100 pmol of oxidized 5′-[α-^{32}P]ATP (5′-[α-^{32}P]Ox-ATP) in 100 μL of 25 mM imidazole/HCl buffer at pH 7.5 in the presence of 50–500 pmol of sodium cyanoborohydride to reduce the Schiff base formed between 5′-[α-^{32}P]Ox-ATP and the sulfonylurea receptor. The reaction was carried out at 4 °C for 6 h and then 1 mL of acetone at −20 °C was added to each sample. After 30 min at 4 °C, samples were centrifuged at 12000*g* for 15 min, and pellets were washed twice with cold acetone and dissolved for 20 min at 56 °C in a denaturing buffer containing 75 mM Tris-HCl at pH 7.5, 2% sodium dodecyl sulfate, 7.5% glycerol, 2% 2-mercaptoethanol. Samples were analyzed according to Laemmli (1970) on an 8% isocratic acrylamide resolving gel. After being stained with Coomassie Blue R-250 and destained with 10% acetic acid, gels were dried prior to autoradiography for 4–10 days at −70 °C on Kodak X-Omat-AR5 film with a Du Pont Cronex Hi-Plus intensifying screen. Autoradiographies were quantified using a Hoefer GS300 scanning densitometer.

The purified sulfonylurea binding protein was incubated for 1 h at 4 °C in a total volume of 500 μL with 1 nM [^{3}H]glibenclamide and various concentrations of ATP, ADP, AMP, or other ligands altering K_{ATP} channel activity in the presence of 10 mM dithiothreitol in a 50 mM 3-(*N*-morpholino)-propanesulfonic acid buffer at pH 7.5. Aliquots of 200 μL were loaded onto 5 mL of Sephadex G-50 medium columns equilibrated with 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 20 mM imidazole hydrochloride at pH 7.5. Bound radioactivity was eluted by 2.4 mL of 0.5 M NaCl and counted in Aquassure scintillation liquid (New England Nuclear).

Results and Discussion

ATP and ADP are important regulators of K_{ATP} channels. The mechanism by which they regulate this important class of channels has been particularly well studied in β-pancreatic cells [see, for example, Rorsman and Trube (1990)] and in cardiac cells (Lederer & Nichols, 1989). ATP is an inhibitor of K_{ATP} channel activity whereas ADP tends to release the channel from ATP blockade. In fact, the channel is regulated by the ATP/ADP ratio (Dunne & Petersen, 1986; Dunne et al., 1988; Kakei et al., 1986; Lederer & Nichols, 1989; Ribalet & Ciani, 1987) which is an exquisite way for the cell membrane to sense variations in cellular energy metabolism. It was not really known until recently whether ATP and ADP binding sites were identical or distinct. However, recent work with fluorescein derivatives, which are known to bind to nucleotide sites in (Na^+,K^+)- and Ca^{2+}-ATPases, has shown that two different types of sites are in charge of the inhibitory or activatory effects of ATP and ADP, respectively (De Weille et al., 1992), in K_{ATP} channels from insulinoma cells.

The concentration dependence of ATP and ADP effects on K_{ATP} channels has not been studied in detail in neuronal cells, but detailed data are available for β-cell and cardiac cell K_{ATP} channels (Ashcroft, 1988; Dunne & Petersen, 1986; Dunne et al., 1988; Kakei et al., 1986; Lederer & Nichols, 1989; Ribalet & Ciani, 1987). ATP blockade of K_{ATP} channels does not require ATP hydrolysis. Nonhydrolyzable derivatives of ATP such as ATP-γ-S and AMP–PNP also inhibit K_{ATP} channel activity (Dunne & Petersen, 1986; Dunne et al., 1988; Findlay, 1988; Ohno-Shosaku et al., 1987). ATP inhibition of K_{ATP} channel activity has been measured on excised membrane patches of β-cells and cardiac cells. $K_{0.5}$ values for the inhibitory effect are 20–100 μM for β-cells and 500 μM for cardiac cells (Dunne & Petersen, 1991).

ADP activation of cardiac K_{ATP} channels which had previously been inhibited by ATP has been observed with $K_{0.5}$ values of 50–100 μM (Lederer & Nichols, 1989).

Covalent labeling of nucleotide binding sites to the sulfonylurea binding protein has been obtained using a strategy that has previously proven to be successful for labeling the ATP/ADP binding site of the (Na^+,K^+)ATPase (Ponzio et al., 1983). The strategy of labeling used for this ion transport enzyme is indicated in Figure 1. Aldehyde functions of 5′-[α-^{32}P]Ox-ATP form a Schiff base with the ε-amino group of lysine residues situated within or in the vicinity of the nucleotide binding site, and this Schiff base is further reduced by cyanoborohydride. Figure 1 also shows that 5′-[α-^{32}P]-Ox-ATP in the presence of cyanoborohydride associates covalently with the purified sulfonylurea receptor. Covalent labeling reaches a maximum level after 3 h of incubation. The main part of ^{32}P incorporation is in the 145-kDa glycoprotein

[1] Abbreviations: ATP, adenosine 5′-triphosphate; Ox-ATP, oxidized ATP.

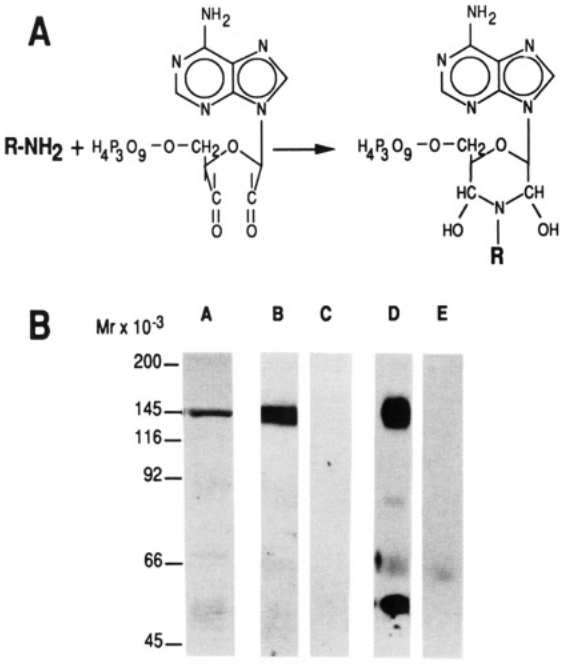

FIGURE 1: Affinity labeling of purified sulfonylurea receptor with [^{3}H]glibenclamide and with 5′-[α-^{32}P]Ox-ATP. (A) Typical chemical modification of the primary amino group by Ox-ATP. (B) Lane A: Analysis by silver staining of purified sulfonylurea receptor on a 8% isocratic SDS–polyacrylamide gel carried out under reducing conditions. Lane B: Autoradiographic pattern obtained after photoaffinity labeling of purified sulfonylurea receptor carried out with 1 nM [^{3}H]glibenclamide in a 1-mL solution containing 25 mM imidazole hydrochloride, pH 7.5, at 4 °C. Samples were irradiated by using a 8-W UV lamp (254 nm, Vilbert Lourmat) for 6 min at a distance of 20 cm at 4 °C. Samples were analyzed on an 8% SDS–polyacrylamide gel electrophoresis. Gels were treated for fluorography with 1 M sodium salicylate for 30 min, dried, and exposed to Kodak X-Omat-AR5 film with a Du Pont Cronex intensifying screen for 2 weeks at −70 °C. Lane C: Experiment as in lane B in the presence of 0.1 μM unlabeled glibenclamide. Lane D: Autoradiographic pattern obtained after affinity labeling of purified sulfonylurea receptor with [^{32}P]Ox-ATP. Lane E: Experiment as in lane D in the presence of 10 mM unlabeled Ox-ATP.

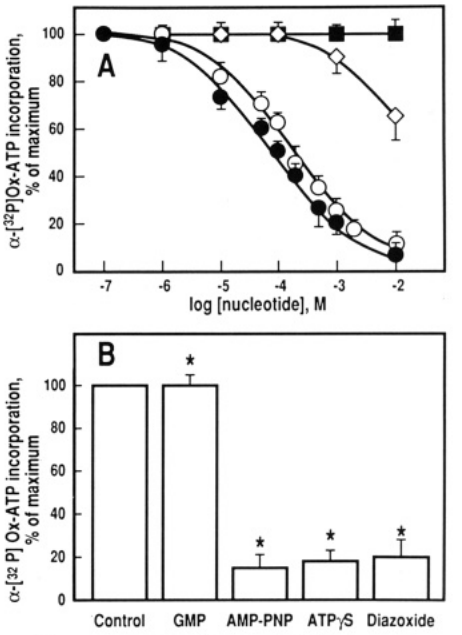

FIGURE 2: Effects of nucleotides and ATP-dependent potassium channel opener diazoxide on covalent labeling of purified sulfonylurea receptor with 5′-[α-^{32}P]Ox-ATP. (A) Dose–response inhibition curves of the covalent incorporation of 5′-[α-^{32}P]Ox-ATP in the presence of an increasing concentration of ATP (●), ADP (○), AMP (◇), and adenosine (■). (B) Effects of 1 mM GMP, 1 mM AMP–PNP, 1 mM ATP-γ-S, and 0.1 mM diazoxide on the covalent incorporation of 5′-[α-^{32}P]Ox-ATP. All experiments were carried out with the same batch of sulfonylurea binding protein. Signals were quantified by densitometry. Values represent ±SEM of eight samples and were normalized for comparison. 0% represent 10% of the total densitometer signal. For panel B, the asterisk indicates $P < 0.05$ versus control.

which has been previously shown to be the sulfonylurea binding protein (Bernardi et al., 1988). It is also the same protein that is photoaffinity labeled by [^{3}H]glibenclamide (Figure 1). Labeling by 5′-[α-^{32}P]Ox-ATP is completely protected by an excess of unlabeled ATP which indicates that the covalent incorporation does indeed occur on an ATP binding site. Three additional polypeptides are labeled by 5′-[α-^{32}P]Ox-ATP in a specific way, i.e., with protection by an excess of ATP. They have M_r values of 80 000, 64 000, and 50 000. The 50-kDa polypeptide is more labeled than the two others. It is not known whether these additional polypeptides are associated with the sulfonylurea binding protein to form the K_{ATP} channel, are contaminations, or are degradation products of the 145-kDa glycoprotein. We have observed that a mild tryptic digestion of the sulfonylurea binding protein first transforms the 145–150-kDa polypeptide in a shorter 62–65-kDa polypeptide.

The first important conclusion of these results is that the 145–150-kDa protein bears both the sulfonylurea binding site(s) and ATP binding sites.

Figure 2A shows the concentration dependence of the protective effects of ATP, ADP, and AMP against the covalent incorporation of 5′-[α-^{32}P]Ox-ATP into the 145-kDa glycoprotein. $K_{0.5}$ values for these inhibitions were found to be 100 μM and 250 μM for ATP and ADP, respectively. AMP had only a small protective effect at high concentrations (10 mM), and adenosine was without effect. Nonhydrolyzable or weakly hydrolyzable analogs of ATP such as AMP–PNP and ATP-γ-S also inhibit 5′-[α-^{32}P]Ox-ATP incorporation with $K_{0.5}$ values of about 500 μM (Figure 2B). Glibenclamide and tolbutamide, two sulfonylureas, had no effect at 10 μM (not shown).

ADP probably prevents 5′-[α-^{32}P]Ox-ATP incorporation (made in the absence of Mg^{2+}) by competitive interaction at the ATP binding site itself, since ADP in the absence of Mg^{2+} inhibits the K_{ATP} channel as ATP itself (Lederer & Nichols, 1989). $K_{0.5}$ values for ATP and ADP are similar to those found with patch–clamp techniques for the inhibition of cardiac K_{ATP} channel activity by these two nucleotides ($K_{0.5,ATP}$ = 25 μM and $K_{0.5,ADP}$ = 275 μM (Lederer & Nichols, 1989)).

Is there an interaction between ATP/ADP binding sites situated on the cytoplasmic side of the channel and the externally located sulfonylurea binding site(s)? Previous experiments have shown that ATP, ADP, and nonhydrolyzable analogs do indeed alter [^{3}H]glibenclamide binding to brain microsomes (Gopalakrishnan et al., 1991). The same observation had been made with insulinoma cells (Niki et al., 1990).

We first fully confirmed observations made with brain microsomes by Gopalakrishnan et al. (1991). We then decided to analyze whether interactions between nucleotide binding sites and sulfonylurea binding sites were preserved in the purified 145-kDa glycoprotein.

AMP–PNP, the nonhydrolyzable analog of ATP which blocks K_{ATP} channels as ATP itself (Ohno-Shosaku et al., 1987) only has a limited effect on [^{3}H]glibenclamide binding. A maximal inhibition of 20–25% has been observed with a $K_{0.5}$ value of 200 ± 100 μM (not shown), and this small inhibitory effect was not influenced by the presence of Mg^{2+} (in a 1:1 ratio with AMP–PNP). Effects observed with ADP are more interesting. ADP in the absence of Mg^{2+} (i.e., under conditions

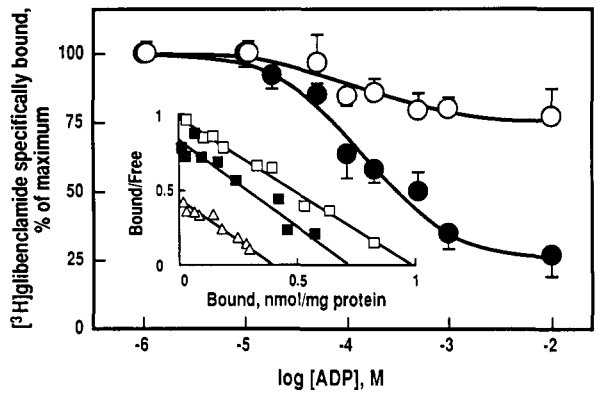

FIGURE 3: Effects of ADP on [^{3}H]glibenclamide binding to purified sulfonylurea receptor. ADP was added in the absence (○) or in the presence (●) of $MgCl_2$ (1 mol of ADP/1 mol of $MgCl_2$). Incubations were carried out in the presence of 10 mM DTT. pH values of each samples were adjusted to 7.5 with 0.1 N NaOH. Inset: Scatchard plots for the specific [^{3}H]glibenclamide binding in the absence of ADP (□), K_D = 1 nM and B_{max} = 1 nmol/mg of protein; in the presence of 0.1 mM ADP (■), K_D = 0.9 nM and B_{max} = 0.71 nmol/mg of protein; and in the presence of 1 mM ADP (△), K_D = 0.9 nM and B_{max} = 0.38 nmol/mg of protein. Incubations contain 10 mM DTT and $MgCl_2$ in ratio 1/1 with ADP. All experiments were carried out with the same batch of sulfonylurea binding protein. Values represent ±SEM of six samples and were normalized for comparison. 100% binding represents 3000 dpm, and 0% binding represents 160 dpm. Experiments were done in duplicate for the Scatchard.

in which ADP suppresses K_{ATP} channel activity) only inhibits 20% of the total [^{3}H]glibenclamide binding (Figure 3). However, in the presence of Mg^{2+} which is known to be required for the activatory effect of ADP, i.e., the release of ATP inhibition of K_{ATP} channels by ADP (Findlay, 1987; Lederer and Nichols, 1989), which then works as the ADP–Mg^{2+} complex (Dunne & Petersen, 1991; Findlay, 1987), inhibition of [^{3}H]glibenclamide binding is drastically increased. The inhibitory effect of ADP–Mg^{2+} on [^{3}H]glibenclamide binding reaches about 80%, and the $K_{0.5}$ value for the ADP–Mg^{2+} complex is 100 ± 40 μM (Figure 3).

Scatchard plots of the inhibitory effect of ADP in the presence of Mg^{2+} indicate that the inhibition is of the noncompetitive type. ADP–Mg^{2+} reduces the maximal binding capacity for [^{3}H]glibenclamide without changing the K_d value (Figure 3, inset).

Nucleotide monophosphates such as AMP and GMP were without effect on [^{3}H]glibenclamide binding at concentrations of 10 mM. K_{ATP} channel openers such as cromakalim and diazoxide (1 mM) were also without effect on [^{3}H]glibenclamide binding (not shown).

All these results taken together indicate that ATP and ADP binding sites are situated in the same protein of 145 kDa which has been previously identified by purification and affinity labeling as the receptor of antidiabetic sulfonylureas. They also indicate that there is a negative allosteric interaction between ADP binding sites and sulfonylurea binding sites within this 145-kDa glycoprotein.

We have confirmed, with the purified sulfonylurea receptor, observations made by others with brain microsomes (Gopalakrishnan et al., 1991) indicating that inhibition of [^{3}H]-glibenclamide could only be observed in the presence of dithiothreitol. This indicates a probable requirement for nonoxidized thiol for nucleotide binding or/and for the allosteric interaction between nucleotide regulatory sites and sulfonylurea binding sites.

The negative interaction between ADP–Mg^{2+} binding sites and sulfonylurea binding sites is consistent with a number of recent functional studies. For example, it has been observed that, in cardiac cells, application of ADP–Mg^{2+} on the cytoplasmic surface leads to activation of K_{ATP} channels which then become more resistant to blockade by antidiabetic sulfonylureas (Venkatesh et al., 1991). Moreover, affinity labeling of the ADP binding site by the fluorescein derivative, eosin-5-maleimide, in K_{ATP} channels of insulinoma cells, leads to irreversible activation of K_{ATP} channels which can still be inhibited by ATP acting at the ATP binding site. However, the irreversibly activated K_{ATP} channel has then become largely insensitive to sulfonylureas (De Weille et al., 1992).

Another important class of drugs acting on K_{ATP} channels is that of K^+ channel openers (Edwards & Weston, 1990). Functional studies with electrophysiological techniques have previously shown that there is also an apparent allosteric interaction between sites for these K_{ATP} channel effectors and ATP/ADP binding sites (Dunne & Petersen, 1991; Lederer & Nichols, 1989; Tung & Kurachi, 1991). An increase of intracellular ATP concentration tends to decrease the effects of K^+ channel openers and vice versa (Fan et al., 1990; Ripoll et al., 1990; Takano & Noma, 1990; Thuringer & Escande, 1989; Tseng & Hoffman, 1990). This is particularly true for diazoxide action on β-cell K_{ATP} channels (Dunne et al., 1987). The observation made in Figure 2B that diazoxide prevents affinity labeling of the ATP binding site by 5′-[α-^{32}P]Ox-ATP suggests that the 145-kDa glycoprotein might also contain sites for K^+ channel openers. This would not be surprising since in other types of ion channels such as voltage-dependent Na^+ channels (Barhanin et al., 1983; Lazdunski et al., 1986) and Ca^{2+} channels (Hosey & Lazdunski, 1988), a single subunit contains all binding sites for activators and inhibitors. However, of course, more detailed studies have to be carried out to definitely prove this point.

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