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Mg^{2+} and ATP dependence of K_{ATP} channel modulator binding to the recombinant sulphonylurea receptor, SUR2B

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1 The binding of modulators of the ATP-sensitive K^+ channel (K_{ATP} channel) to the murine sulphonylurea receptor, SUR2B, was investigated. SUR2B, a proposed subunit of the vascular K_{ATP} channel, was expressed in HEK 293 cells and binding assays were performed in membranes at 37°C using the tritiated K_{ATP} channel opener, [^{3}H]-P1075.

2 Binding of [^{3}H]-P1075 required the presence of Mg^{2+} and ATP. MgATP activated binding with EC_{50} values of 10 and 3 μM at free Mg^{2+} concentrations of 3 μM and 1 mM, respectively. At 1 mM Mg^{2+}, binding was lower than at 3 μM Mg^{2+}.

3 [^{3}H]-P1075 saturation binding experiments, performed at 3 mM ATP and free Mg^{2+} concentrations of 3 μM and 1 mM, gave K_D values of 1.8 and 3.4 nM and B_{MAX} values of 876 and 698 fmol mg^{-1}, respectively.

4 In competition experiments, openers inhibited [^{3}H]-P1075 binding with potencies similar to those determined in rings of rat aorta.

5 Glibenclamide inhibited [^{3}H]-P1075 binding with K_i values of 0.35 and 2.4 μM at 3 μM and 1 mM free Mg^{2+}, respectively. Glibenclamide enhanced the dissociation of the [^{3}H]-P1075-SUR2B complex suggesting a negative allosteric coupling between the binding sites for P1075 and the sulphonylureas.

6 It is concluded that an MgATP site on SUR2B with μM affinity must be occupied to allow opener binding whereas Mg^{2+} concentrations $\geqslant 10$ μM decrease the affinities for openers and glibenclamide. The properties of the [^{3}H]-P1075 site strongly suggest that SUR2B represents the drug receptor of the openers in vascular smooth muscle.

Keywords: Vascular sulphonylurea receptor SUR2B; K_{ATP} channel openers; [^{3}H]-P1075 binding; levcromakalim; minoxidil sulphate; aprikalim; diazoxide; glibenclamide; AZ-DF 265; MgATP dependence

Introduction

Adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K_{ATP} channels) are a class of weakly inwardly rectifying K^+ channels. They are gated by intracellular nucleotides with nucleoside triphosphates (NTPs) like ATP acting as inhibitors and nucleoside diphosphates (NDPs) like MgADP as activators. Found in many cells like pancreatic β-cells, many neurons and in all types of muscle cells, they link the membrane potential to the metabolic state of the cell as reflected by the levels of NTPs and NDPs (Ashcroft & Ashcroft, 1990; Edwards & Weston, 1993; Quast, 1996). These channels are closed by the antidiabetic sulphonylureas (SUs) like glibenclamide which induce insulin secretion. The K_{ATP} channel openers, a chemically heterogeneous class of compounds including levcromakalim, pinacidil, aprikalim, minoxidil sulphate and diazoxide, activate the channel. The openers are most potent in vascular muscle and their dominant effect *in vivo* is hypotension (Quast, 1992; Edwards & Weston, 1994).

Recently, the K_{ATP} channels in several tissues have been shown to be a heteromeric complex of pore forming subunits, which belong to the class of inwardly rectifying K^+ channels Kir 6.x, and of sulphonylurea binding subunits (sulphonylurea receptors, SURs) (Inagaki *et al*., 1995; 1996; Sakura *et al*., 1995; Isomoto *et al*., 1996; Yamada *et al*., 1997; Clement *et al*., 1997). SURs are members of the ATP binding cassette proteins and contain binding sites for sulphonylureas and nucleotides (Aguilar-Bryan *et al*., 1995; Inagaki *et al*., 1996; Isomoto *et al*., 1996; Tusnády *et al*., 1997). Electrophysiological studies on recombinant K_{ATP} channels have shown that the SUR confers on the channel complex the sensitivity for the sulphonylureas, the openers and the activating nucleotides and that it accounts for the major pharmacological differences found between the K_{ATP} channels in different tissues (Inagaki *et al*., 1995, 1996; Sakura *et al*., 1995; Isomoto *et al*., 1996; Ämmälä *et al*., 1996; Yamada *et al*., 1997; Okuyama *et al*., 1998). The channel of the pancreatic β-cell contains SUR1 (Inagaki *et al*., 1995; Sakura *et al*., 1995); current evidence suggests that the (major) SUR in heart and skeletal muscle is SUR2A (Inagaki *et al*., 1996) and that in smooth muscle is SUR2B (Isomoto *et al*., 1996; Yamada *et al*., 1997). From these electrophysiological studies it seems clear that the binding site(s) of the openers must be located on SUR. However, direct evidence in form of binding studies with recombinant SURs is not yet available with the exception of a recent preliminary report in which binding of openers to SUR2B expressed in COS7 cells was shown (Dörschner *et al*., 1998).

Using the opener [^{3}H]-P1075, a compound structurally related to pinacidil, and the inhibitor [^{3}H]-glibenclamide, high affinity binding sites for these radioligands have been identified in rings of rat aorta. The pharmacological profile of these sites was very similar to that found in functional studies (Bray & Quast, 1992; Quast *et al*., 1993; Löffler & Quast, 1997). Binding disappeared after metabolic inhibition concomitantly with the ATP content in the preparation, suggesting that the presence of ATP was necessary for binding to occur (Quast *et*

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al., 1993; Löffler & Quast, 1997). Work in membrane preparations from various sources has meanwhile shown that the presence of MgATP or of Mg^{2+} complexes with other NTPs and NDPs is indeed a prerequisite for [^{3}H]-P1075 binding (Dickinson *et al*., 1997; Dörschner *et al*., 1998; Löffler-Walz & Quast, 1998a).

Here we report on [^{3}H]-P1075 binding in membranes derived from HEK 293 cells (human embryonic kidney 293 cells; Graham *et al*., 1977), stably transfected with murine SUR2B (mSUR2B; Isomoto *et al*., 1996). The results provide evidence on SUR2B for an activatory MgATP site of high affinity and an inhibitory Mg^{2+} site of lower affinity. The data also show that expression of SUR2B alone reproduces the binding properties reported earlier for [^{3}H]-P1075 binding in rat aortic rings (Bray & Quast, 1992; Quast *et al*., 1993).

Methods

Cell culture and transfection

HEK 293 cells were a kind gift of Drs T. Krüger and J.E. Schultz, University of Tübingen. Cells were cultured in plastic dishes with a diameter of 9.4 cm at 37°C in a humidified atmosphere with 95% air and 5% CO_2 in Minimum Essential Medium (MEM) containing glutamine and supplemented with 10% foetal bovine serum and 20 μg ml^{-1} gentamycin. At 60–80% confluence (10–16 million cells per dish), cells were either rinsed off the dish with fresh medium and subcultured at a split ratio of 1:4 or transfected with the pcDNA 3.1 vector containing the coding sequence of mSUR2B (Isomoto *et al*., 1996; GenBank accession number D86038) using lipofectAMINE and Opti-MEM (Life Technologies) according to the manufacturer's instruction. Good results were obtained by preparing lipofectAMINE-DNA complexes with 4 μg DNA and 25 μl lipofectAMINE. Cells were allowed to express transfected DNA for 48 h. Control experiments were performed by omitting either DNA or lipofectAMINE.

Isolation of stably transfected cells was achieved by rinsing the culture dishes 2 days after transfection with fresh medium and diluting the suspension of detached cells 1:20 (vol:vol) into medium containing 700 μg ml^{-1} geneticin; 2 months later, geneticin was reduced to 450 μg ml^{-1} and, after an additional 2 months, to 300 μg ml^{-1}.

Preparation of membranes

Membranes from control and from stably transfected cells were prepared at a confluence of 70–80% (13–16 million cells per dish); in case of transient transfection cells were harvested 2 days after transfection at 85–95% confluence (17–20 million cells). Cells from 5–10 culture dishes were suspended by rinsing with medium and centrifuged for 6 min at 500 *g* at 4°C. The pelleted cells were lysed by addition of 20–40 ml of ice-cold hypotonic buffer containing (in mM): HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]), 10; EGTA (ethylene glycol-bis-(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 1 at pH 7.4. Cell rupture was assured by microscopy and the lysate centrifuged at 10^5 *g* and 4°C for 60 min. The resulting membrane pellet was resuspended in a buffer containing (in mM) HEPES, 3; KCl, 5; NaCl 139; $MgCl_2$ 0 or 1 (see below) at pH 7.4 and 4°C at a protein concentration of $\approx$0.7 mg ml^{-1} and frozen at −80°C. Protein concentration was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

Binding experiments with [^{3}H]-P1075

Membranes were thawed and homogenized with a polytron homogenizer for 2×5 s at 10^4 r.p.m. at 4°C. In initial experiments, membranes from transiently transfected cells (protein 20–50 μg ml^{-1}) were incubated with ^{3}[H]-P1075 (1.8 nM) at 4°C for 60 min or at 37°C for 30 min in a total volume of 1 ml at pH 7.4 in an incubation buffer containing (in mM) NaCl, 139; KCl, 5; $MgCl_2$, 2.2; HEPES 5 and supplemented with Na_2ATP, 1. Incubation was stopped by diluting 0.3 ml aliquots in triplicate into 8 ml of ice-cold quench solution (50 mM Tris, 154 mM NaCl, pH 7.4). Bound and free ligand were separated by rapid filtration under vacuum over Whatman GF/B filters. Filters were washed twice with 8 ml of ice-cold quench solution and counted for ^{3}H in the presence of 6 ml of scintillant (Ultima Gold; Packard). Nonspecific binding was determined in the presence of 10 μM unlabelled P1075.

Experiments with membranes from stably transfected cells were performed at 37°C with an incubation time of 30 min. The concentrations of $MgCl_2$, EDTA (ethylenediaminetetraacetic acid) and Na_2ATP were adjusted as indicated under Results. Free Mg^{2+} concentrations in the presence of varying concentrations of ATP (added as Na_2ATP) and EDTA were calculated using a programme written by Drs T. Suzuki (Australian National University, Canberra, Australia) and U. Russ (University of Tübingen, Germany) using the pK values and enthalpies of the MgATP and MgEDTA complexes compiled by Smith & Martell (1989). The pharmacological properties of SUR2B were studied at 3 mM Na_2ATP in the presence of low Mg^{2+} (1 mM EDTA, 0.92 mM $MgCl_2$; free Mg^{2+} = 3 μM) or high Mg^{2+} (0 mM EDTA, 3.8 mM $MgCl_2$; free Mg^{2+} = 1.0 mM).

Dissociation was initiated by addition of P1075 (10 μM) in the absence or presence of glibenclamide to the receptor-radioligand complex at equilibrium after incubation of the membranes with [^{3}H]-P1075 (1.5 nM) at 37°C for 30 min. 0.3 ml aliquots were withdrawn at different times to follow the dissociation kinetics.

Data analysis

Data are expressed as means ± s.e.mean. With the exception of the saturation experiments (see below), concentration dependencies were analysed by fitting the logistic form of the Hill equation,

$$y = b + (a - b) * (1 + 10^{n*(px-pK)})^{-1}. \quad (1)$$

Here b denotes the starting level of the curve, a the level at saturation so that a−b represents the extent of the effect (amplitude); n ($=n_H$) is the Hill coefficient, x the concentration of the compound under study and K the midpoint of the curve with px = −logx and pK = −logK. The dependence of the midpoint of an inhibition curve (IC_{50} value) on the concentration of the radioligand, L, was calculated according to the Cheng & Prusoff (1973) equation,

$$IC_{50} = K_i * (1 + L/K_D), \quad (2)$$

where K_i is the inhibition constant and K_D the equilibrium dissociation constant of the radioligand.

In the saturation experiments, nonspecific binding (B_{NS}) was proportional to the free label concentration, L, and was fitted to the equation, $B_{NS} = a*L$, where a denotes the proportionality constant. Total binding (B_{TOT}) was then analysed as the sum of specific and nonspecific binding and was fitted to the equation,

$$B_{TOT} = B_{MAX} * L * (L + K_D)^{-1} + a * L, \quad (3)$$

to estimate the values of the equilibrium dissociation constant (K_D) and the maximum concentration of binding sites (B_{MAX}, fmol mg^{-1} protein) by the method of least squares.

For analysis of the dissociation kinetics, the equation of a monoexponential decay,

$$B = 100 * \exp(-k_{off} * t), \quad (4)$$

was fitted to the data; here k_{off} denotes the dissociation rate constant.

Fits of the equations were performed according to the method of least squares using the FigP programme (Biosoft, Cambridge, U.K.). Errors in the parameters derived from the fit to a single curve were estimated using the univariate approximation (Draper & Smith, 1981) and assuming that amplitudes and pK values are normally distributed. In the text, pK ± s.e.mean or K values with the 95% confidence interval in parentheses are given. pK values and amplitudes between two groups (low and high Mg^{2+}) were compared by the Student's *t*-test.

Drugs and solutions

[^{3}H]-P1075 (specific activity 121 Ci $mmol^{-1}$) was purchased from Amersham Buchler (Braunschweig, Germany). The reagents and media used for cell culture and transfection were from Life Technologies (Eggenstein, Germany); Na_2ATP was from Boehringer Mannheim (Mannheim, Germany) and EDTA from Fluka (Deisenhofen, Germany). The following drugs were kind gifts of the pharmaceutical companies given in parentheses: aprikalim (Rhône-Poulenc Rorer, Paris, France), AZ-DF 265 (4-[[N-α-phenyl-2-piperidino-benzyl)carbamoyl]methyl] benzoic acid; Thomae (Biberach, Germany), diazoxide (Essex Pharma, München, Germany), levcromakalim (SmithKline-Beecham, Harlow, U.K.), P1075 (N-cyano-N′-(1,1-dimethylpropyl)-N″-3-pyridylguanidine; Leo Pharmaceuticals, Ballerup, Denmark). Minoxidil sulphate was synthesized by Dr W.P. Manley (Novartis, Basel, Switzerland). Glibenclamide was from Sigma (Deisenhofen, Germany). K_{ATP} channel modulators were dissolved in ethanol and dimethyl sulphoxide (1 : 1) and further diluted with the same solvent or with incubation buffer; the final solvent concentration in the assays was always below 0.3%.

Results

Binding of [^{3}H]-P1075 in membranes from transiently transfected cells

Initial experiments were performed at 0°C in the presence of ATP (1 mM) and Mg^{2+} (2.2 mM). At a [^{3}H]-P1075 concentration of 1.8 nM, specific binding was 145 ± 16 fmol mg^{-1} and nonspecific binding amounted to 15 ± 2% of total binding ($n = 13$). At 37°C, specific binding was reduced to 73 ± 7% of that at 0°C and nonspecific binding increased to 27 ± 2% of total binding ($n = 7$). No specific binding was observed in membranes from control cells or from HEK cells exposed to lipofectAMINE or DNA alone. Binding activity was sensitive to freezing-thawing cycles, each cycle leading to a loss of 55 ± 9% ($n = 5$). Further experiments were performed at 37°C in membranes from stably transfected cells which gave 2–3 times higher specific binding and lower nonspecific binding (⩽10% of total binding).

Dependence of [^{3}H]-P1075 binding on ATP and Mg^{2+}

Binding of [^{3}H]-P1075 to membranes from HEK cells transfected with SUR2B required the presence of both Mg^{2+} and ATP (Figure 1a). In the presence of a high concentration of free Mg^{2+} (1 mM), ATP increased binding of [^{3}H]-P1075 (1.5 nM) with an EC_{50} value (95% confidence interval in parentheses) of 3.6 (3.0,4.6) μM and a Hill coefficient ≈1 to a level of ≈210 fmol mg^{-1}; at high ATP concentrations (>3 mM), slight inhibition was observed. At low free Mg^{2+} (3 μM), the ATP activation curve was shifted to higher concentrations (EC_{50} = 320 (186,537) μM) and reached a maximum level twice as high as that obtained at high Mg^{2+}. At the high ATP concentration of 30 mM, a sudden drop in binding was observed. Figure 1b shows a replot of the data as a function of the (calculated) MgATP concentration. The EC_{50} value of the curve in the presence of 1 mM Mg^{2+} remained unchanged (3.3 μM) since in the concentration region of activation, Mg^{2+} is in large excess over ATP and [MgATP] ≈ [ATP]. The activation curve in the presence of

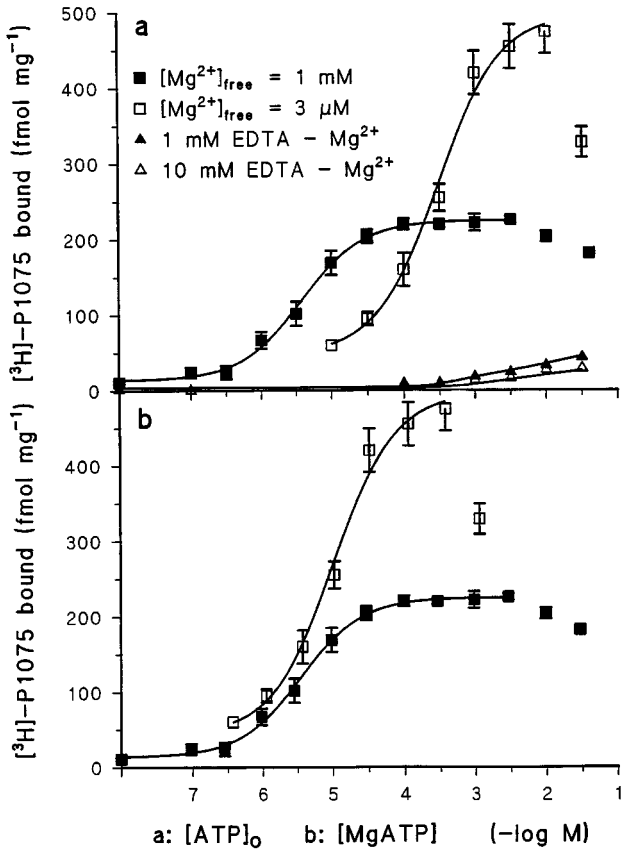

Figure 1 Dependence of [^{3}H]-P1075 binding to SUR2B on ATP concentration. (a) Specific binding (fmol mg^{-1} protein) of [^{3}H]-P1075 (1.5 nM) is shown as a function of ATP concentration ($[ATP]_0$) at free Mg^{2+} concentrations of 1 mM, 3 μM and '0' (no Mg^{2+} added, 1 and 10 mM EDTA). Data are means ± s.e.mean from four experiments. The solid curves show the fit of the Hill equation to the data yielding the following parameters at 1 mM/3 μM Mg^{2+}: pEC_{50} values: 5.43 ± 0.04/3.50 ± 0.07; amplitudes (fmol mg^{-1}): 212 ± 6/450 ± 19; Hill coefficients 0.99 ± 0.08/1.21 ± 0.28. To assure constant free Mg^{2+} concentrations at varying [Na_2ATP], different amounts of $MgCl_2$ were added as calculated using the programme mentioned in Methods; the incubation solution with 3 μM free Mg^{2+} contained 1 mM EDTA. (b) Replot of the data as a function of [MgATP]: The fit of the Hill equation gave pEC_{50} values of 5.48 ± 0.03 and 5.00 ± 0.09 for the curves at 1 mM and 3 μM Mg^{2+}, respectively; Hill coefficients were close to unity.

3 μM Mg^{2+} was, however, shifted leftward to an EC_{50} value for MgATP of 10 (6,18) μM.

In further experiments, the dependence of [^{3}H]-P1075 binding on the free Mg^{2+} concentration was measured at constant ATP (Figure 2a). At 10 mM ATP, Mg^{2+} activated binding with an EC_{50} value of 0.19 (0.12,0.30) μM and $n_H = 1$. At $[Mg^{2+}] \geqslant 10$ μM, inhibition occurred with an EC_{50} value of 70 (33,150) μM, reducing binding by 50%. In the presence of low ATP (0.3 mM), the activation curve of Mg^{2+} was described by an EC_{50} value of 0.7 (0.5,0.9) μM and a maximum about 60% that reached at 10 mM ATP. At high free Mg^{2+} concentrations (>0.3 mM), binding was again inhibited; the final level was the same as that obtained with 10 mM ATP. When plotted as a function of [MgATP] (Figure 2b), the ascending limb of the curves gave EC_{50} values of 2.6 (2.0,3.3) and 23 (12,45) μM for MgATP at 0.3 and 10 mM ATP, respectively. The inhibitory part of the curves was presented by a sharp drop or was greatly compressed into less than one concentration decade.

In the nominal absence of free Mg^{2+} (no Mg^{2+} added, 1 mM EDTA), ATP concentrations $\geqslant$1 mM elicited a low degree of binding; in the presence of 10 mM EDTA binding was further reduced (Figure 1a). This suggested that the incubation solution contained a trace contamination of Mg^{2+} and that, despite the presence of EDTA, at high ATP concentrations sufficient MgATP was formed to activate [^{3}H]-P1075 binding to a small degree.

Further experiments were conducted at an ATP concentration of 3 mM in the presence of 3 μM or 1 mM free Mg^{2+}, respectively; the corresponding MgATP concentrations are 0.11 and 2.8 mM. Figure 1a shows that under these conditions, binding is close to or at the maximum for low and high Mg^{2+}.

Pharmacological properties of the [^{3}H]-P1075 binding sites

Figure 3 shows a saturation binding experiment with [^{3}H]-P1075 at low and high Mg^{2+}. From three experiments similar to that illustrated in Figure 3, K_D values of 1.8 (1.4,2.3) and 3.4 (2.6,4.6) nM were obtained in the presence of low and high Mg^{2+}, respectively ($P<0.05$); the corresponding B_{MAX} values were 876 ± 25 and 698 ± 18 fmol mg^{-1} ($P<0.01$).

The binding of several K_{ATP} channel openers and inhibitors to SUR2B was studied in competition experiments with [^{3}H]-P1075 (1.5 nM) at low and high Mg^{2+}; the level of control binding under the two conditions differed up to 2 fold (Figure 4). The results are summarized in Table 1 and some competition curves are illustrated in Figure 4. The openers P1075, levcromakalim, aprikalim and diazoxide inhibited [^{3}H]-P1075 binding completely with Hill coefficient 1 whereas minoxidil sulphate inhibited [^{3}H]-P1075 binding only to 80 ± 3 and 73 ± 2% at low and high Mg^{2+}, respectively (Figure 4). The K_i values of P1075, levcromakalim and aprikalim were slightly higher at low Mg^{2+}; those of minoxidil sulphate and diazoxide did not differ under the two conditions. In addition, the K_i values for P1075 at low and high Mg^{2+} were identical with the respective K_D values obtained in the saturation binding experiments (Table 1).

Glibenclamide and the benzoic acid sulphonylurea analogue, AZ-DF 265, inhibited [^{3}H]-P1075 binding completely and in a monophasic manner (Table 1, Figure 4). The K_i values of glibenclamide in the presence of low and high Mg^{2+} were

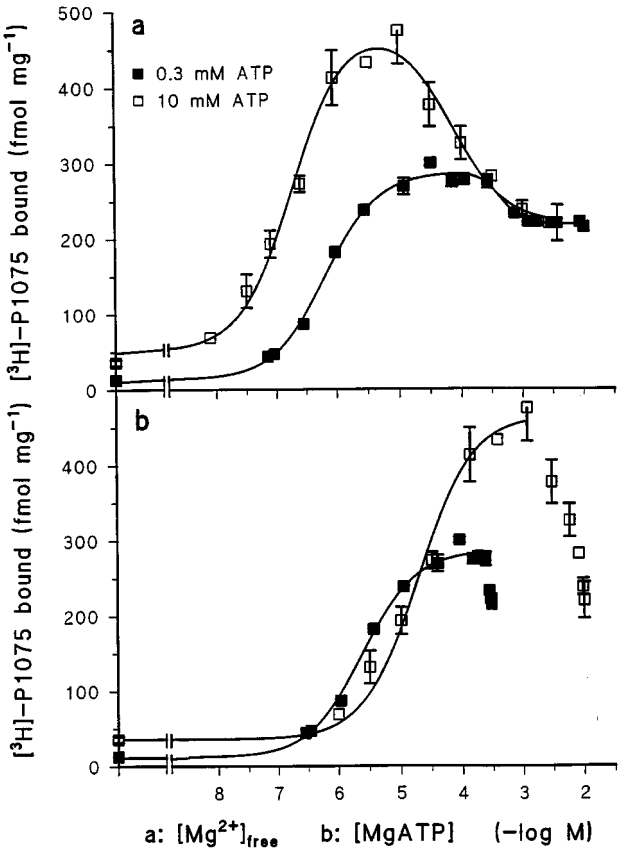

Figure 2 Dependence of [^{3}H]-P1075 binding on free Mg^{2+} concentration in the presence of 10 and 0.3 mM ATP. Experiments were performed as indicated in Figure 1; $n=4$. (a) The Hill equation with two components was fitted to the data giving for the ascending part of the curves (10 mM/0.3 mM ATP): pEC_{50}: 6.73 ± 0.09/6.18 ± 0.06; amplitudes (fmol mg^{-1}): 434 ± 22/275 ± 8; n_H was fixed to 1. For the descending limbs, the parameters were pEC_{50}: 4.15 ± 0.18/3.36 ± 0.09; amplitudes: 265 ± 21/70 ± 6; n_H: 0.9 ± 0.4/2.4 ± 0.9. (b) Representation of the data as a function of [MgATP]; the pEC_{50} values of the ascending part were 4.63 ± 0.10/5.60 ± 0.06 for the curves at 10 and 0.3 mM ATP, respectively; $n_H \approx 1$.

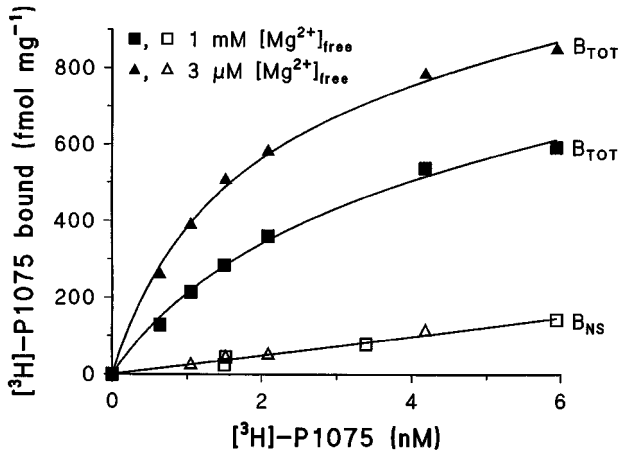

Figure 3 Saturation experiments with [^{3}H]-P1075. [ATP] was 3 mM and free [Mg^{2+}] was 3 μM and 1 mM. Nonspecific binding (B_{NS}, open symbols) was determined in the presence of 10 μM P1075 and increased proportionally to the radioligand concentration. The slope (a in equation (3) of Methods) was 24 fmol mg^{-1} nM^{-1}, irrespective of the free Mg^{2+} concentration. Analysis of total binding (B_{TOT}, closed symbols) according to equation (3) gave K_D values of 1.5 and 2.6 nM and B_{MAX} values of 911 and 672 fmol mg^{-1}, respectively for specific [^{3}H]-P1075 binding at 3 μM and 1 mM free Mg^{2+}. Similar experiments were performed three times spanning a radioligand concentration range from 0.5 to 12 nM.

determined to 0.35 and 2.4 μM, respectively, giving a 7 fold difference between the two states; for AZ-DF 265, this difference was 3 fold (Table 1). In order to gain some insight into the interaction between the sites for glibenclamide and [^{3}H]-P1075, the dissociation kinetics of the SUR2B-[^{3}H]-P1075 complex were measured in the absence and presence of glibenclamide. Figure 5 shows that addition of unlabelled P1075 (10 μM) to the radioactive complex induced monophasic dissociation kinetics. In four experiments, the dissociation rate constant (k_{off}) was determined to 0.08 ± 0.01 min^{-1}, corresponding to a half-time of 8 min. When glibenclamide was given in addition to P1075, k_{off} was increased concentration-dependently (Figure 5). In the presence of low Mg^{2+}, k_{off} in the absence of glibenclamide was 0.18 ± 0.01 min^{-1} (half-time 4 min); again, glibenclamide speeded up dissociation (not illustrated). The time resolution of the filtration assay allowed measurements up to 30 μM glibenclamide; in this concentration range, k_{off} increased linearly with the glibenclamide concentration at low and high Mg^{2+} (Figure 5, inset).

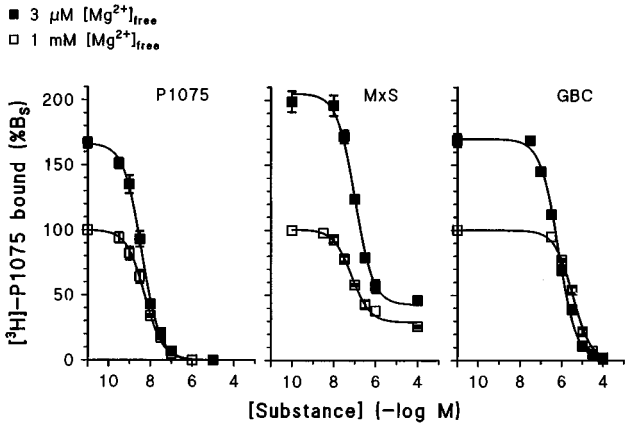

Figure 4 Competition between [^{3}H]-P1075 and selected K_{ATP} channel modulators in the presence of low and high Mg^{2+}. MxS denotes minoxidil sulphate and GBC glibenclamide. Binding is normalized with respect to the level of specific binding at high Mg^{2+} in the absence of competitor ($B_S = 203 \pm 8$ fmol mg^{-1}; $n = 15$). Data are means $\pm$ s.e.mean from four experiments. The mean pK_i values determined from the fits to the individual inhibition curves are listed in Table 1. Inhibition of [^{3}H]-P1075 binding was studied in the presence of [^{3}H]-P1075 (1.5 nM) and the inhibitor of interest at 3 mM ATP and free Mg^{2+} concentrations of 3 μM and 1 mM: B_{NS} amounted to 7 ± 1% and 13 ± 1% of B_{TOT} at low and high free Mg^{2+}, respectively.

Discussion

MgATP dependence of binding

The data in Figures 1 and 2 show that both Mg^{2+} and ATP were necessary to obtain binding of [^{3}H]-P1075. Experiments in which the ATP concentration was varied at two widely separated free Mg^{2+} concentrations gave EC_{50} values for ATP which differed 100 fold; however, when the data were plotted as a function of [MgATP], EC_{50} values of 3.6 and 10 μM were obtained. This strongly suggests that MgATP was the major activating species and that ATP or Mg^{2+} played only a modulatory role. The SURs have two nucleotide binding folds

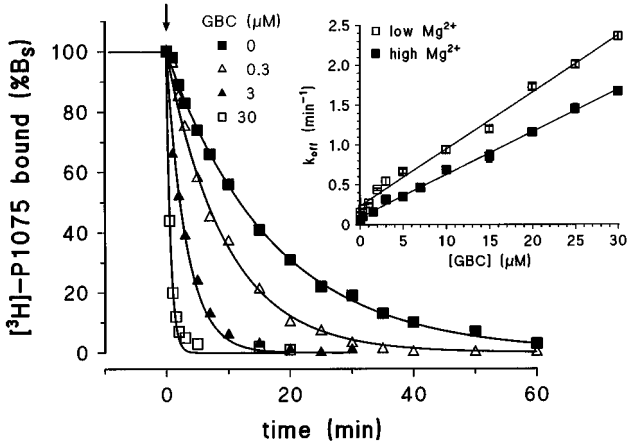

Figure 5 Dissociation kinetics of the SUR2B-[^{3}H]-P1075 complex in the absence and presence of glibenclamide. Membranes were incubated with [^{3}H]-P1075 (1.5 nM) in the presence of 3 mM ATP and 1 mM free Mg^{2+} at 37°C for 30 min; then (see arrow), dissociation was initiated by addition of P1075 (10 μM) together with glibenclamide (GBC) at the concentrations indicated. Data were normalized with respect to specific binding (B_S) at the beginning of dissociation (100% corresponds to 148 ± 12 fmol mg^{-1}; $n = 8$). The equation of monoexponential decay was fitted to the data and yielded the following values for the rate constant of dissociation (k_{off}, min^{-1}) at the glibenclamide concentrations given in parentheses: 0.06 ± 0.03 (0); 0.10 ± 0.01 (0.3 μM); 0.31 ± 0.02 (3 μM); 1.7 ± 0.1 (30 μM). Representative experiments are shown; the errors are derived from the fit. Inset: Dependence of k_{off} on glibenclamide concentration ([GBC]) at low and high Mg^{2+}. The fit of a straight line to the data gave ordinate intercepts of $0.23 \pm 0.03/0.09 \pm 0.01$ min^{-1} and slopes of $0.071 \pm 0.001/0.053 \pm 0.001$ min^{-1} μM^{-1} at low and high Mg^{2+}, respectively.

Table 1. Inhibition of [^{3}H]-P1075 binding by K_{ATP} channel modulators[1]

	SUR2B		*Rat aortic strips*[2]
K_{ATP} channel modulator	*pK_i (3 μM [Mg^{2+}]$_{free}$)*	*pK_i (1 mM [Mg^{2+}]$_{free}$)*	*pK*
P1075	8.72 ± 0.10*	8.48 ± 0.07	8.54 ± 0.03
Minoxidil sulphate[3]	7.20 ± 0.05	7.28 ± 0.02	7.45 ± 0.10
Levcromakalim	7.12 ± 0.02*	6.95 ± 0.03	7.33 ± 0.06
Aprikalim	6.73 ± 0.03*	6.49 ± 0.08	7.05 ± 0.05
Diazoxide	5.05 ± 0.03	5.11 ± 0.06	4.66 ± 0.03
Glibenclamide	6.46 ± 0.05**	5.62 ± 0.05	6.36 ± 0.04
AZ-DF 265	5.70 ± 0.06**	5.20 ± 0.05	5.95 ± 0.03

[1]Experiments were performed in the presence of 1.5 nM [^{3}H]-P1075, 3 mM ATP and 3 μM or 1 mM free Mg^{2+} as shown in Figure 4. The Hill equation (see Methods) was fitted to individual competition curves yielding pIC_{50}, amplitudes (≈ 100% with the exception of minoxidil sulphate, see below) and Hill coefficients n_H (≈ 1). pIC_{50} values were corrected for the presence of the radiolabel according to the equation of Cheng & Prusoff (1973) which, on the log scale, corresponded to the addition of 0.26 or 0.16 at low and high Mg^{2+} to give the pK_i values listed. [2]pK_i values for inhibition of [^{3}H]-P1075 binding to rat aortic strips (Quast *et al.*, 1993). [3]Minoxidil sulphate inhibited [^{3}H]-P1075 binding to 80 ± 3 and 73 ± 2% of the control binding at low and high Mg^{2+}, respectively. *, ** denotes the significance levels ($P < 0.05$, $P < 0.01$) of differences between pK_i values under the two conditions.

located intracellularly (Aguilar-Bryan *et al*., 1995; Tusnády *et al*., 1997) and, theoretically, binding of MgATP to either one of these or to both could be important for [^{3}H]-P1075 binding. The activation curves had Hill coefficients of 1; this suggests that the two binding folds, if they both participated in the effect, should have similar affinity for MgATP and that there should be no cooperativity between them.

The critical dependence of [^{3}H]-P1075 binding on MgATP has also been established recently in membranes from COS7 cells transfected with SUR2B (Dörschner *et al*., 1998) and in membranes prepared from rabbit skeletal muscle (Dickinson *et al*., 1997) and from rat cardiac ventricle (Löffler-Walz & Quast, 1998a). EC_{50} values of 30 and 100 μM were found for ATP in the presence of high Mg^{2+} and of an ATP-regenerating system in heart (Dickinson *et al*., 1997) and skeletal muscle membranes (Löffler-Walz & Quast, 1998a); according to present evidence, the native K_{ATP} channel in these tissues contains SUR2A (Inagaki *et al*., 1996; Okuyama *et al*., 1998). Since ATP activated SUR2B binding in the presence of high Mg^{2+} with an EC_{50} value of 3.6 μM (Figure 1a), one concludes that SUR2B is considerably (8–28 times) more sensitive to MgATP than SUR2A. The mechanism by which MgATP enables SUR to bind [^{3}H]-P1075 has not been investigated here but it may be related to ATP hydrolysis by SUR since only hydrolysable nucleotides activate binding (Dickinson *et al*., 1997; Dörschner *et al*., 1998).

Mg^{2+} dependence and pharmacological properties of the [^{3}H]-P1075 binding sites

Increasing Mg^{2+} concentrations induced an inhibition of [^{3}H]-P1075 binding which depended in magnitude and EC_{50} value on the ATP concentration present; at saturation, the same final level of binding was reached (Figure 2). The concentration dependence of this effect suggested that free Mg^{2+} rather than MgATP was the inhibitory species; however, it may well be that additional factors like the ADP formed by the ATPase activity of SUR2B and other proteins in the preparation may play a role. The ATP concentration in smooth muscle is 2–3 mM (Butler & Davies, 1980) and free Mg^{2+} is between 0.5 and 1 mM (London, 1991); hence, inhibition will be essentially complete under physiological conditions. Saturation binding experiments performed at 3 mM ATP and in the presence of 3 μM and 1 mM free Mg^{2+} showed that occupation of the inhibitory site reduced the affinity of [^{3}H]-P1075 about 2 fold and the number of available sites by 20%. These results suggest that there are two different states of the protein according to the Mg^{2+} concentration present.

The competition experiments between [^{3}H]-P1075 and (unlabelled) K_{ATP} channel modulators were also performed in the low (3 μM) and the high (quasiphysiological, 1 mM) Mg^{2+} state of the SUR. The K_i values of the openers showed only minor (<2) or no differences between the two states. These K_i values, obtained for mSUR2B, were in excellent agreement with those determined for inhibition of [^{3}H]-P1075 binding in rings of rat aorta (Bray & Quast, 1992; Quast *et al*., 1993). Since representatives of the major classes of K_{ATP} channel openers were tested, the agreement of opener potencies in the two systems allows the following tentative conclusions: First, SUR2B is indeed the functionally relevant drug receptor for the openers in vascular smooth muscle (Isomoto *et al*., 1996; Yamada *et al*., 1997; Dörschner *et al*., 1998). Second, regarding opener binding to SUR2B, there is little species difference between the mouse and the rat. Third, the interaction of SUR2B with Kir subunits is not required to give the 'correct' opener binding profile; experiments confirming this directly in HEK cells co-transfected with Kir 6.1 and SUR2B are in preparation.

Minoxidil sulphate did not inhibit [^{3}H]-P1075 binding completely. This has been observed also in smooth muscle cells from calf coronary artery (69% inhibition; Lemoine *et al*., 1996) and in A10 cells, a cell line derived from rat aorta (70 ± 2% Russ *et al*., 1997) but neither in rat aortic strips (88 ± 14%; Quast *et al*., 1993) nor in afferent arterioles from rat kidney (102 ± 6%; Metzger & Quast, 1996). The reason for these divergent results is not clear. One explanation is that the opener sites may be heterogeneous in some preparations and that minoxidil sulphate binds to most but not all of these sites whereas P1075 and other openers bind homogeneously to all of them. Earlier studies of opener-induced [$^{42}K^+$]- and [$^{86}Rb^+$]-efflux from rat aorta lend support to such a model (Bray & Quast, 1991). In our case only SUR2B is present; the heterogeneity of opener sites could then reflect differences in e.g. glycosylation or phosphorylation of the protein. Clearly, other explanations cannot be excluded and more work is necessary to distinguish between the different possibilities.

The inhibitors, glibenclamide and AZ-DF 265, discriminated more strongly between the low and the high Mg^{2+} state of the SUR than the openers; for glibenclamide, the difference in K_i values was 7 fold. Surprisingly enough, it is the K_i values at low (non-physiological) Mg^{2+} which correspond better to the values determined in rat aortic strips (Table 1). The determination of the affinity of glibenclamide in vascular smooth muscle cells is complicated by the fact that the binding sites of P1075 and glibenclamide appear to be allosterically coupled (Bray & Quast, 1992) and that the [^{3}H]-P1075 binding assay underestimates the affinity of glibenclamide by about 25 times (Löffler & Quast, 1997). Binding assays with [^{3}H]-glibenclamide yield the correct K_D value (Löffler & Quast, 1997); however, they require the presence of an intact cytoskeleton (Löffler-Walz & Quast, 1998b) and must therefore be conducted in intact cells. Such experiments are currently underway.

In high Mg^{2+}, the [^{3}H]-P1075-SUR2B complex dissociated with a half-time of 8 min, a value close to those determined in afferent arterioles (6 min; Metzger & Quast, 1996) and in rat aortic rings (19 min; Bray & Quast, 1992). Glibenclamide increased the rate constant of dissociation (k_{off}) at low and high Mg^{2+}. This effect of glibenclamide is best explained by the formation of a transient ternary complex between SUR, [^{3}H]-P1075 and glibenclamide and strongly suggests that the binding sites for the opener and the SU are negatively allosterically coupled (Bray & Quast, 1992). The linear dependence of k_{off} on glibenclamide concentration up to 30 μM, the highest concentration at which the kinetics could be followed, suggests that the [^{3}H]-P1075-SUR complex has a low affinity for glibenclamide ($K_D >> 30$ μM). That glibenclamide speeds up the dissociation of [^{3}H]-P1075 from its binding sites had been observed before in rat aortic strips (Bray & Quast, 1992) and in membranes from rabbit skeletal muscle (Dickinson *et al*., 1997) and rat heart (Löffler-Walz & Quast, unpublished observation); however this effect required glibenclamide concentrations higher than the K_i value of glibenclamide against P1075 binding. In membranes from the pancreatic β-cell line HIT, [^{3}H]-glibenclamide binding assays had suggested that the binding sites for the SU and pinacidil (structurally related to P1075) were coupled by negative allosterism (Schwanstecher *et al*., 1992). Hence, all SURs cloned to date (SUR1, SUR2A and SUR2B) have separate binding sites for SUs and pinacidil-type openers and these sites seem to be linked by negative allosterism.

In conclusion, this study has shown that MgATP in the μM concentration range is required for binding of the K_{ATP}

channel opener, [^{3}H]-P1075 to SUR2B and that physiological concentrations of Mg^{2+} depress binding. SUR2B, expressed alone, has the drug binding properties characteristic of the K_{ATP} channel in vascular smooth muscle. Using methods like mutational analysis it will now be possible to elucidate the localization and the precise mode of interaction of the different ligands (e.g. openers, SUs, MgATP, Mg^{2+}) which modulate the activity of this protein.

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