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Binding of K_{ATP} channel modulators in rat cardiac membranes

Cornelia Löffler-Walz & [1]Ulrich Quast

Department of Pharmacology, Medical Faculty, University of Tübingen, Wilhelmstr. 56, D-72074 Tübingen, Germany

1 The binding of [^{3}H]-P1075, a potent opener of adenosine-5′-triphosphate-(ATP)-sensitive K^+ channels, was studied in a crude heart membrane preparation of the rat, at 37°C.

2 Binding required MgATP. In the presence of an ATP-regenerating system, MgATP supported [^{3}H]-P1075 binding with an EC_{50} value of 100 μM and a Hill coefficient of 1.4.

3 In saturation experiments [^{3}H]-P1075 binding was homogeneous with a K_D value of 6 ± 1 nM and a binding capacity (B_{max}) of 33 ± 3 fmol mg^{-1} protein.

4 Upon addition of an excess of unlabelled P1075, the [^{3}H]-P1075-receptor complex dissociated in a mono-exponential manner with a dissociation rate constant of 0.13 ± 0.01 min^{-1}. If a bi-molecular association mechanism was assumed, the dependence of the association kinetics on label concentration gave an association rate constant of 0.030 ± 0.003 nM^{-1} min^{-1}. From the kinetic experiments the K_D value was calculated as 4.7 ± 0.6 nM.

5 Openers of the ATP-sensitive K^+ channel belonging to different structural classes inhibited specific [^{3}H]-P1075 binding in a monophasic manner to completion; an exception was minoxidil sulphate where maximum inhibition was 68%. The potencies of the openers in this assay agree with published values obtained in rat cardiocytes and are on average 3.5 times lower than those determined in rat aorta.

6 Sulphonylureas, such as glibenclamide and glibornuride and the sulphonylurea-related carboxylate, AZ-DF 265, inhibited [^{3}H]-P1075 binding with biphasic inhibition curves. The high affinity component comprised about 60% of the curves with the IC_{50} value of glibenclamide being $\approx$90 nM; affinities for the low affinity component were in the μM concentration range. The fluorescein derivative, phloxine B, showed a monophasic inhibition curve with an IC_{50} value of 6 μM, a maximum inhibition of 94% and a Hill coefficient of 1.5.

7 It is concluded that binding studies with [^{3}H]-P1075 are feasible in rat heart membranes in the presence of MgATP and of an ATP-regenerating system. The pharmacological profile of the [^{3}H]-P1075 binding sites in the cardiac preparation, which probably contains sulphonylurea receptors (SURs) from cardiac myocytes (SUR_{2A}) and vascular smooth muscle cells (SUR_{2B}), differs from that expected for SUR_{2A} and SUR_{2B}.

Keywords: K_{ATP} channel opener binding; [^{3}H]-P1075; glibenclamide; sulphonylurea receptor; rat heart membranes

Introduction

Adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K_{ATP} channels) form a class of K^+ channels gated by intracellular nucleotides, with ATP acting as an inhibitor and MgADP as an activator. These channels are modulated by synthetic drugs such as the sulphonylureas like glibenclamide as inhibitors and the K_{ATP} channel openers as activators (Ashcroft & Ashcroft, 1990; Quast, 1992; Edwards & Weston, 1993). Recently, the K_{ATP} channels in several tissues have been shown to be a complex of pore forming (α-) subunits (which belong to the class of inwardly rectifying K^+ channels) and of sulphonylurea receptors (SURs) as β-subunits (Inagaki *et al*., 1995; 1996; Sakura *et al*., 1995; Isomoto *et al*., 1996; Yamada *et al*., 1997; Clement *et al*., 1997). The SUR contains binding sites for sulphonylureas and nucleotides (Aguilar-Bryan *et al*., 1995) and confers on the complex the sensitivity for the sulphonylureas, the openers and the activating nucleotides (Inagaki *et al*., 1995; 1996; Sakura *et al*., 1995; Isomoto *et al*., 1996; Ämmälä *et al*., 1996; Yamada *et al*., 1997), whereas the inhibitory nucleotide site is localized on the α-subunit of the channel (Tucker *et al*., 1997).

The K_{ATP} channel openers are most potent in smooth muscle and the heart (Quast, 1992; Edwards & Weston, 1993). However, only few binding studies with the openers have been performed in cardiovascular preparations (Bray & Quast, 1992; Quast *et al*., 1993; Löffler & Quast, 1997; preliminary reports: Dickinson *et al*., 1993; 1996; Lemoine *et al*., 1996). By use of the opener, [^{3}H]-P1075, a compound structurally related to pinacidil, and the inhibitor [^{3}H]-glibenclamide, high affinity binding sites for these compounds have been identified in rings of rat aorta. The pharmacological profile of these sites was very similar to that found in functional studies in this vessel (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 al*., 1993; Löffler & Quast, 1997). Further progress has been hampered by the fact that no binding was detected in membrane fractions prepared from several rat tissues, even after the addition of ATP (Quast *et al*., 1993). Hence, a recent preliminary report describing binding of [^{3}H]-P1075 to membrane preparations from the rat brain, canine heart and rabbit skeletal muscle in the presence of nucleoside tri- and diphosphates (Dickinson *et al*., 1996) was surprising. This study led us to search for optimal conditions for [^{3}H]-P1075 binding in rat heart membranes and to characterize the pharmacological properties of these binding sites. The heart was chosen since cardiac myocytes are a rich source of K_{ATP} channels (Davies *et al*., 1991) and heart can be homogenized more easily than vascular tissue.

[1] Author for correspondence.

Methods

Membrane preparation

Male Sprague-Dawley rats (200–350 g) were killed by stunning and decapitation, the hearts quickly removed and placed in ice-cold buffer containing (in mM): NaCl 139, KCl 5, $MgCl_2$ 1.2, HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid) 5 and EGTA (ethylene glycol-bis-(2-aminoethylether)-N,N,N′,N′-tetra-acetic acid) 1 at pH 7.4. The atria were removed and the ventricles minced in 3 vol g^{-1} wet weight of hypotonic buffer containing HEPES (10 mM), EGTA (1 mM), phenylmethylsulphonyl fluoride (PMSF; 0.2 mM), pepstatin A (0.2 μM), leupeptin (10 μM) and soybean trypsin inhibitor (10 μg ml^{-1}). The material was homogenized by three 10 s burst at 12000 rpm with a polytron homogenizer (probe PT-DA 3007/2) and the resulting homogenate passed through a stainless steel sieve of mesh 320 μm, to remove connective tissue and larger coronary vessels. The homogenate was then centrifuged for 45 min at $10^5 \times g$ and the resulting pellet suspended in 15–20 volumes of ice-cold HEPES-buffer (in mM: NaCl 139, KCl 5, $MgCl_2$ 2 and HEPES 20 (pH 7.4)) to a protein concentration of $\approx$50 mg ml^{-1} and frozen at -80°C. Protein concentration was determined according to Lowry *et al.* (1951) with bovine serum albumin as the standard.

Equilibrium binding experiments with [^{3}H]-P1075

For saturation experiments, the membrane preparation (0.8–0.9 mg ml^{-1}) was incubated with [^{3}H]-P1075 (0.7–23 nM) in a total volume of 1.1 ml at 37°C and pH 7.4 for 30 min in an incubation buffer containing (in mM): NaCl 139, KCl 5, $MgCl_2$ 25, $CaCl_2$ 1.25 and HEPES 20; the buffer was supplemented with creatine phosphokinase (50 u ml^{-1}), creatine phosphate (20 mM) and Na_2ATP (3 mM). The high concentration of Mg^{2+} was necessary to account for Mg^{2+} binding of creatine phosphate (equilibrium dissociation constant $\approx$25 mM, Dawson *et al.*, 1986). Incubation was stopped by diluting 0.3 ml aliquots (in triplicate) in 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/C filters. Filters were washed twice with 8 ml of ice-cold quench solution and counted for ^{3}H in the presence of 3 ml of scintillant (Ultima Gold; Packard). In the concentration range from 0.5 to 5 μM, [^{3}H]-P1075 was used in undiluted form; for higher concentrations it was diluted 1 : 10 with unlabelled P1075. Nonspecific binding (B_{NS}) was determined in the presence of 10 μM unlabelled P1075.

B_{NS} was proportional to the free label concentration, L, and was fitted to the equation, $B_{NS} = a \times L$, 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,

$$B_{tot} = B_{max} \times L \times (L + K_D)^{-1} + a \times L \qquad (1)$$

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.

Inhibition of [^{3}H]-P1075 binding was studied in the presence of [^{3}H]-P1075 (1 nM) and the inhibitor (I) of interest as described above. B_{tot} was 7.5 ± 0.2 fmol mg^{-1} protein ($n = 53$) and B_{NS} amounted to 35 ± 1% of B_{tot}. B_S in the presence of inhibitor I was normalized to % of B_S in the absence of I and fitted to the Law of Mass Action, taking into account two binding components:

$$B_S = 100 - A_1 \times I \times (IC_{50,1} + I)^{-1} - A_2 \times I \times (IC_{50,2} + I)^{-1} \qquad (2)$$

Here, A_1 and A_2 denote the extents of inhibition at saturation (amplitudes) of the two components and $IC_{50,1}$ and $IC_{50,2}$ represent the midpoints. Since the label concentration, L = 1 nM, was of the same order of magnitude as the K_D value of P1075 (= 6 nM), the IC_{50} values for the inhibitors deviated from the respective inhibition constant K_i by 17% according to the Cheng-Prusoff equation (Tallarida, 1995). Alternatively, data were fitted to the Hill equation,

$$B_S = 100 - A^{n_H} \times I^{n_H} \times (IC_{50}{}^{n_H} + I^{n_H})^{-1} \qquad (3)$$

where n_H is the Hill coefficient.

Kinetic experiments

To measure the association kinetics, membranes (0.5–0.6 mg ml^{-1} final concentration) were added to the incubation buffer supplemented with [^{3}H]-P1075 (1–6 nM), ATP (3 mM) and the ATP-regenerating system at 37°C. Aliquots (500 μl) were withdrawn at different time points for separation of bound and free ligand as described above. In some cases, pH was measured in the absence of radiolabel with a KC1/AgCl electrode connected to a pH-metre (WTW, Weilheim, Germany). Nonspecific binding, determined in the presence of P1075 (10 μM), did not change with time. Since the label concentration (L) was in large excess over the concentrations of binding sites the data were fitted to a single exponential as function of time (t),

$$B_S = B_{eq} \times [1 - \exp(-k_{app} \times t)] \text{ with } k_{app} = k_{on} \times L + k_{off} \qquad (4)$$

where B_{eq} denotes the concentration of the receptor-label complex at equilibrium and k_{app} the apparent rate constant of association which depends on the rate constants of association and dissociation (k_{on}, k_{off}) and on the concentration of L as indicated above (Tallarida, 1995).

Dissociation was initiated by addition of P1075 (10 μM) to the receptor-label complex at equilibrium, after incubation of the microsomal preparation with [^{3}H]-P1075 (1 nM) at 37°C for 30 min. Aliquots were then withdrawn to follow the dissociation kinetics which were fitted to the equation of exponential decay,

$$B_S = B_{eq} \times \exp(-k_{off} \times t) \qquad (5)$$

with B_{eq} and k_{off} defined as above.

Calculations and statistics

Results are expressed as mean $\pm$ s.e.mean. Fits to the equations given in the text were performed according to the method of least squares by use of the FigP programme (Biosoft, Cambridge, U.K.). Errors in the parameters derived from the fit to a single curve were estimated by use of the univariate approximation (Draper & Smith, 1981). The fits to different binding models (one versus two components or versus the Hill equation) were compared according to the *F*-test (extra sum of squares principle) or by the 'minimum Akaike information criterion estimation' (Quast & Mählmann, 1982). In calculations involving two mean values with s.e., propagation of errors was taken into account according to Bevington (1969).

Drugs and solutions

[^{3}H]-P1075 (specific activity 116 Ci mmol^{-1}) was purchased from Amersham Buchler (Braunschweig, 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] benzoid acid; Thomae, Biberach, Germany), diazoxide (Essex Pharma, München, Germany), glibornuride (Hoffman-La Roche, Basel, Switzerland), levcromakalim (SmithKline-Beecham, Harlow, UK), nicorandil (Merck, Darmstadt, Germany), P1075 (N-cyano-N′-(1,1-dimethylpropyl)-N″-3-pyridylguanidine; Leo Pharmaceuticals, Ballerup, Denmark), rilmakalim (HOE234) and its antipode, S880050 (Hoechst, Frankfurt, Germany). Minoxidil sulphate and the enantiomers of pinacidil were synthesized by Dr W.P. Manley (Sandoz, Basel, Switzerland). Glibenclamide, phloxine B, creatine phosphokinase, creatine phosphate, leupeptin, peptstatin A and soybean trypsin inhibitor were from Sigma (Deisenhofen, Germany), PMSF from Fluka (Neu-Ulm, 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

Figure 1 illustrates the kinetics of [^{3}H]-P1075 binding to rat cardiac membranes at 37°C under three different conditions. Since binding of [^{3}H]-P1075 requires the presence of mM concentrations of ATP (rat aortic rings: Quast *et al*., 1993; membrane preparations: Dickinson *et al*., 1996), experiments were initially performed in the presence of 3 mM ATP and 2 mM Mg^{2+}. Under these conditions, binding initially increased but started to decline after 20 min; simultaneously, pH decreased from 7.4 to 6.5 (Figure 1, condition 1). In the presence of ATP and absence of Mg^{2+} (Mg^{2+} free buffer containing 1 mM EDTA), no specific binding was detected (not shown). Augmentation of the buffering capacity by increasing the HEPES concentration from 5 to 20 mM in the presence of MgATP reduced the decrease in pH and improved the stability of the binding slightly (Figure 1, condition 2). The decrease in binding and pH was tentatively attributed to a nucleotidase activity present in the preparation; ATP hydrolysis is known to liberate one proton per molecule of ATP (Stryer, 1988). In the additional presence of an ATP-regenerating system (creatine phosphokinase, 50 u ml^{-1}; creatine phosphate, 20 mM; Mg^{2+}, 25 mM), binding and pH were stable for up to 60 min (Figure 1, condition 3); pH-stabilization was achieved since synthesis of ATP from ADP and creatine phosphate consumes one proton per cycle (Stryer, 1988). A series of experiments (not shown) indicated that the concentrations used for the ATP-regenerating system, which are similar to those employed by Gopalakrishnan *et al*. (1991), were optimal. Hence, the conditions of trace 3 were used in this study if not otherwise stated.

The dependence of [^{3}H]-P1075 binding on MgATP concentration and pH was studied in the presence of the ATP-regenerating system. Figure 2 shows that MgATP supported [^{3}H]-P1075 binding with a pEC_{50} value of 4.02 ± 0.02 and a Hill coefficient of 1.4 ± 0.1. Lowering pH from 7.4 to 7.0 and 6.5 (i.e. to the steady state values reached in conditions 1 and 2 in Figure 1) did not affect specific [^{3}H]-P1075 binding (data not shown). Additional experiments

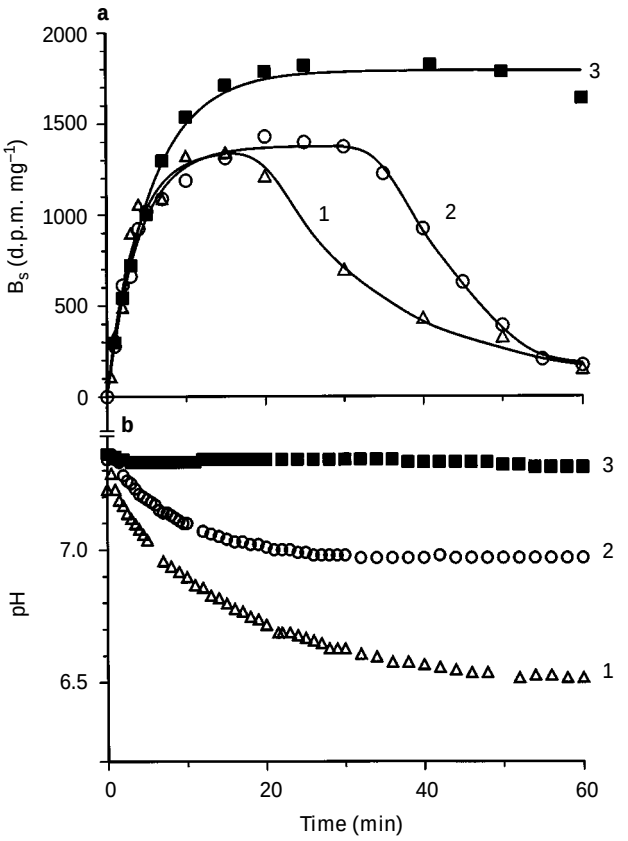

Figure 1 Kinetics of specific [^{3}H]-P1075 binding (B_S) and changes in pH in rat cardiac membranes at 37°C. Experiments were started by addition of membranes to incubation buffer in the presence of: (1) 3 mM Na_2ATP, 2 mM $MgCl_2$, 5 mM HEPES; (2) 3 mM Na_2ATP, 2 mM $MgCl_2$, 20 mM HEPES; (3) 3 mM Na_2ATP + 25 mM $MgCl_2$, 20 mM HEPES, 50 u ml^{-1} creatine phosphokinase, 20 mM creatine phosphate. Representative traces are shown; experiments were repeated 3 to 6 times. (a) Binding kinetics (final [^{3}H]-P1075 concentration 1 nM). Nonspecific binding, determined in the presence of 10 μM P1075, was 30–35% of total binding and was independent of time or condition. The fit of the association kinetics in trace (3) to an exponential function (equation 4) gave a rate constant, k_{app} of 0.17 ± 0.01 min^{-1}. (b) Changes in pH following addition of membranes as measured in separate experiments under conditions 1–3.

showed that binding was stable at 4°C in the presence of mM concentrations of MgATP alone (data not shown).

Attempts to fractionate the crude microsomal preparation by differential centrifugation, according to the initial steps of van Alstyne *et al*. (1980) or Jones *et al*. (1979), were not successful; these procedures neither improved specific binding of [^{3}H]-P1075 nor clarified whether or not binding was localized in mitochondrial or plasmalemmal compartments (not shown).

Figure 3 shows total and nonspecific binding of [^{3}H]-P1075 to rat cardiac membranes at equilibrium as a function of the free label concentration. Specific binding, calculated as the difference between total and nonspecific binding, was consistent with a single class of non-interacting sites with $K_D = 6.0 \pm 1.1$ nM and $B_{max} = 33 \pm 3$ fmol mg^{-1} protein. The kinetics of association and dissociation are illustrated in Figure 4. Association, measured at a label concentration of 1.3 nM, was monoexponential with a rate constant, k_{app}, of 0.22 ± 0.01 min^{-1}. After equilibrium was reached, dissociation of the complex was initiated by addition of 10 μM P1075. The dissociation kinetics again followed a single exponential with a

rate constant of dissociation, $k_{off} = 0.12 \pm 0.01$ min^{-1}. From 7 such experiments, the mean value of k_{off} was determined to be 0.13 ± 0.01 min^{-1}, corresponding to a half-time of the complex of 5 min.

In further experiments the dependence of the association kinetics on the label concentration, L, was measured in the range of 1 to 6 nM; at higher [^{3}H]-P1075 concentrations the kinetics were too fast to be followed. In all cases, the association kinetics were monoexponential (not shown). The dependence of the rate constants, k_{app} on L is shown in the inset in Figure 4. In the case of a bimolecular reaction mechanism, k_{app} would increase linearly with L, with the slope of the line giving the second order rate constant, k_{on}, and the ordinate intercept k_{off} (equation 4 in Methods; Tallarida, 1995). From the fit of this equation to the data, k_{on} was determined to be 0.030 ± 0.003 nM^{-1} min^{-1} and k_{off} to be 0.14 ± 0.01 min^{-1}; the quotient k_{off}/k_{on} then gave a K_D value of 4.7 ± 0.6 nM, which is in good agreement with the results of the saturation equilibrium experiments in Figure 1.

Figure 5 illustrates the inhibition of specific [^{3}H]-P1075 binding in rat cardiac membranes by K_{ATP} channel modulators. Of the different structural families of the openers (Edwards & Weston, 1994) we tested P1075 and the enantiomers of pinacidil (cyanoguanidines), levcromakalim, rilmakalim and its antipode (benzopyrans), aprikalim (a thioformamide), minoxidil sulphate (a pyrimidine), nicorandil (a pyridine) and diazoxide (a benzothiadiazine, chemically related to the thiazide diuretics). All openers inhibited specific [^{3}H]-P1075 binding in a monophasic manner and with a Hill slope of 1. Inhibition at saturation was complete or close to 100% with the exception of minoxidil sulphate, where maximum inhibition was only 68% (Figure 5, Table 1). Inhibition was stereoselective with an almost 200 fold difference in potency between rilmakalim and its distomer; for the enantiomers of pinacidil the potency ratio was only 13.

The sulphonylureas glibenclamide and glibornuride and the benzoic acid analogue, AZ-DF 265 (Garrino & Henquin, 1988) showed flat inhibition curves with Hill coefficients of 0.57, 0.85 and 0.78 (Figure 5, fits not illustrated). Statistical analysis with both the F-test or the Akaike criterion (Quast & Mählmann, 1982) showed that for all three compounds the fit to two independent classes of sites (equation 2 of Methods) was significantly superior. These fits (Figure 5b) gave a high affinity component of approximately 60% of specific [^{3}H]-P1075 binding; the IC_{50} value of high affinity glibenclamide binding was 88 ± 18 nM (Table 1). Phloxine B, a fluorescein derivative, has been shown to inhibit [^{3}H]-glibenclamide binding in HIT T15 insulinoma cells with IC_{50} values of 2 to 3 μM (de Weille *et al.*, 1992; Schwanstecher *et al.*, 1995). Figure 5b shows that

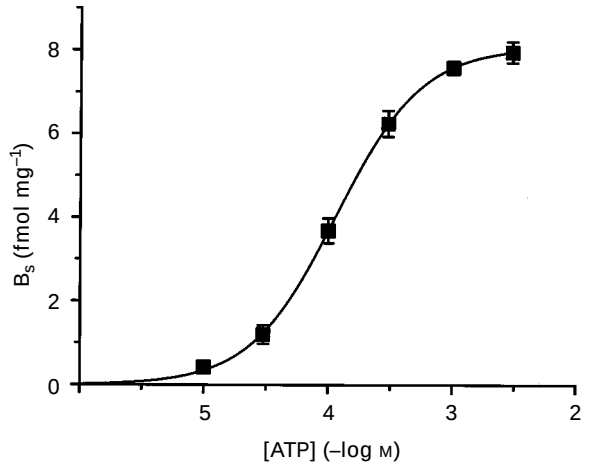

Figure 2 Dependence of specific [^{3}H]-P1075 binding (B_S) on ATP concentration in the presence of the ATP-regenerating system and 25 mM Mg^{2+}. ATP was added as the Na^+ salt; [^{3}H]-P1075 concentration was 1 nM. Specific binding at maximum was 7.8 ± 0.2 fmol mg^{-1} protein ($n = 4$).

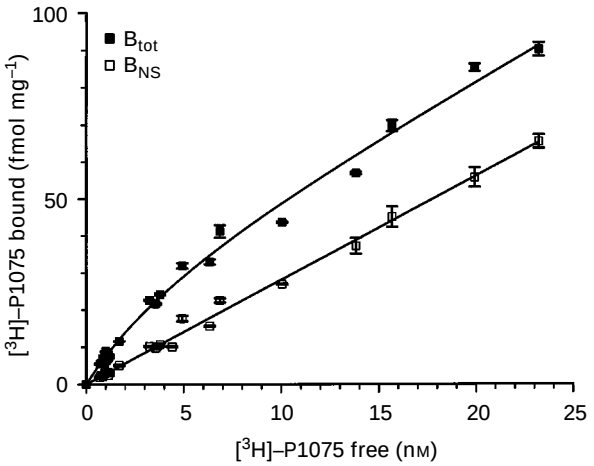

Figure 3 Saturation of [^{3}H]-P1075 binding in rat cardiac membranes. Total (B_{tot}) and nonspecific binding (B_{NS}) are shown as a function of the free label concentration. B_{NS} increased linearly with [^{3}H]-P1075 concentration with a slope of 2.81 ± 0.03 fmol mg^{-1} nM^{-1}. B_{tot}, fitted as the sum of B_{NS} + specific binding to a single class of non-interacting sites (equation 1) gave for specific binding $K_D = 6.0 \pm 1.1$ nM and $B_{max} = 33 \pm 3$ fmol mg^{-1} protein. Data were pooled from 3 experiments; error bars show the variation of the triplicate determinations.

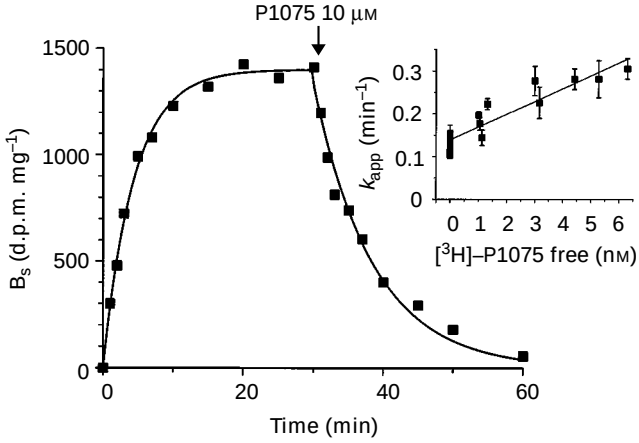

Figure 4 Kinetics of [^{3}H]-P1075 binding. Association was initiated by adding membrane protein (0.5 mg ml^{-1} final concentration) to [^{3}H]-P1075 (1.3 nM) in standard buffer containing 3 mM ATP and the ATP-regenerating system. The association kinetics followed a single exponential with a rate constant, k_{app}, of 0.22 ± 0.01 min^{-1}. Dissociation was induced by adding P1075 (10 μM at 30 min). Dissociation kinetics followed an exponential time course; the fit of the data to equation 5 gave for the rate constant of dissociation, k_{off}, the value of 0.12 ± 0.01 min^{-1}. The trace is representative of 4 experiments. Insert: dependence of k_{app} on the label concentration, L. Individual data points ($n = 15$) were determined from experiments similar to those in Figure 1a (trace 3) and Figure 4. The fit of equation 4 to the data gave an association rate constant, k_{on}, of 0.030 ± 0.003 nM^{-1} min^{-1} and an ordinate intercept (k_{off}) of 0.14 ± 0.01 min^{-1}.

phloxine B inhibited [^{3}H]-P1075 binding in a monophasic manner with an IC_{50} value of 6 μM and a Hill coefficient of 1.5.

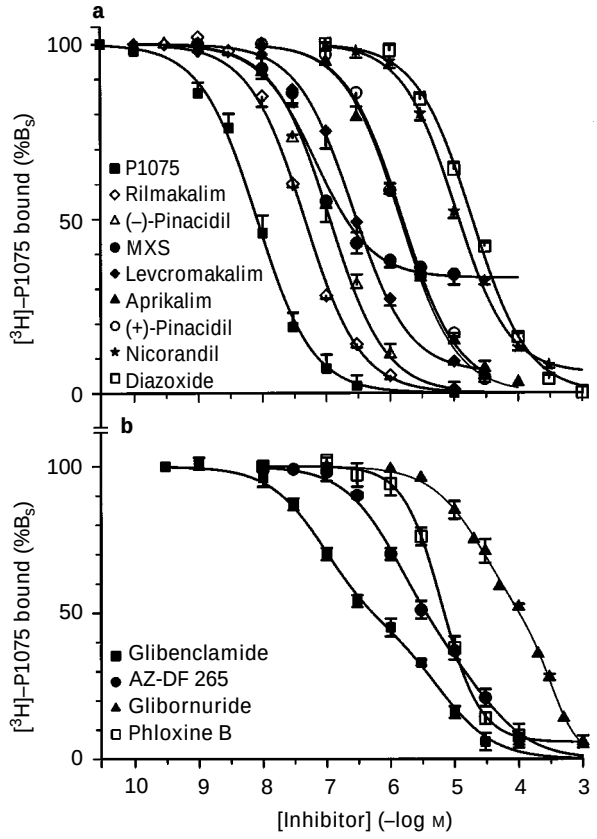

Figure 5 Inhibition of specific [^{3}H]-P1075 binding (1 nM) by selected K_{ATP} channel modulators. (a) Openers. (−)- and (+)-pinacidil denote the two enantiomers of pinacidil; MXS, minoxidil sulphate. (b) Sulphonylureas, AZ-DF 265 and phloxine B. Data are means from 4 experiments; vertical lines show s.e.mean. Individual inhibition curves were fitted assuming competition for one or two classes of binding sites with Hill slope 1; the exceptions were phoxine B (Hill coefficient 1.4±0.1) and glibornuride, where a Hill coefficient of 2 was necessary to fit the low affinity component of the inhibition curve. The fitting parameters are given in Table 1; B_{tot} was 7.5±0.2 fmol mg^{-1} and B_{NS} was 35% of B_{tot} (n=53).

Discussion

Requirements for [^{3}H]-P1075 binding

This study presents evidence for specific binding of K_{ATP} channel modulators in cardiac microsomes. At 37°C, mM concentrations of MgATP and an ATP-regenerating system were required to obtain stable binding, suggesting considerable nucleotidase activity in the preparation (see also Dickinson *et al.*, 1996). Such nucleotidase activity may explain why the earlier search for [^{3}H]-P1075 binding in membranes from various rat tissues was unsuccessful despite the addition of ATP to the incubation medium (Quast *et al.*, 1993). The results show that the hydrolysis of ATP abolishes binding, whereas the accompanying change in pH (from 7.4 to 6.5) is not important. In the presence of the ATP-regenerating system and high concentrations of Mg^{2+}, ATP supported [^{3}H]-P1075 binding in a positive cooperative manner (Hill coefficient 1.4) and with an EC_{50} value of 100 μM. The requirement for Mg^{2+} suggests that the nucleotide binding site which regulates opener binding is different from the site which mediates inhibition of the channel by ATP; at this inhibitory site, which is localized on the α-subunit of the channel (Tucker *et al.*, 1997) ATP and MgATP are of similar potency (Nichols & Lederer, 1991).

Interaction of [^{3}H]-P1075 with the drug receptor

Saturation binding experiments suggested the existence of a homogeneous class of non-interacting binding sites for P1075 in the cardiac membrane preparation with values for K_D (6 nM) and B_{max} (33 fmol mg^{-1} protein) similar to those in intact rings of rat aorta and the rat aortic cell line, A10 (K_D: 6.1 and 5.5 nM; B_{max}: 78 and 150 fmol mg^{-1} protein; Quast *et al.*, 1993; Russ *et al.*, 1997). The kinetic experiments suggested that the association of [^{3}H]-P1075 to its receptor followed a bimolecular mechanism: firstly, the apparent rate constant determined in the association measurements increased linearly with the concentration of radiolabel and, secondly, the rate constants for association and dissociation gave a K_D value similar to that obtained in the equilibrium saturation experiments. The rate constants are in excellent agreement

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

	Rat heart microsomes		*Rat aortic strips*[a]		*Rat cardiomyocytes*[b]
K_{ATP} channel modulator	*pIC_{50}*	*A* (%B_S)	*pIC_{50}*	*A* (%B_S)	*pIC_{50}*
P1075	8.08±0.04	100	8.54±0.03	100	8.28±0.06
Rilmakalim	7.34±0.04	100	–	–	7.74±0.10
(−)-Pinacidil	6.97±0.05	100	7.66±0.06	100	7.38±0.06
Minoxidil sulphate	7.13±0.08	68±2	7.45±0.10	88	6.60±0.11
Levcromakalim	6.56±0.03	94±1	7.33±0.06	88	6.85±0.07
Aprikalim	5.86±0.05	97±1	6.75±0.05	100	5.77±0.09
(+)-Pinacidil	5.85±0.04	100	6.27±0.16	100	6.12±0.06
S880050[c]	5.07±0.06	100	–	–	–
Nicorandil	4.97±0.04	94±2	5.31±0.06	100	4.99±0.07
Diazoxide	4.66±0.05	100	4.66±0.03	100	–
Glibenclamide[d]	7.06±0.09	60±4	6.36±0.04	100	–
Glibornuride[d]	4.50±0.09	63±6	4.43±0.10	100	–
AZ-DF 265[d]	5.89±0.11	62±9	5.95±0.03	100	–
Phloxine B	5.20±0.02	94±2	≈3[e]	>50	–

pIC_{50} = −log IC_{50} (M); A denotes the maximum extent of inhibition in % of specific binding (B_S) in the absence of inhibitor. [a] Data from Quast *et al.* (1993). [b] Data from Lemoine *et al.* (1996). [c] S880050 denotes the distomer of rilmakalim. [d] High affinity component only; pIC_{50} values of the low affinity components were 5.23, 3.50 and 4.56 for glibenclamide, glibornuride and AZ-DF 265, respectively. [e] Baumlin & Quast, unpublished data. The corresponding fits are shown in Figure 5 with the exception of the data for S880050 which were omitted for clarity.

with those for [^{3}H]-P1075 binding to rat glomeruli; in the glomerular preparation, this binding is localized on the remnants of afferent arterioles attached to the glomeruli and has the pharmacological properties of the vascular receptor (Metzger & Quast, 1996).

Evidence for heterogeneity of the [^{3}H]-P1075 binding sites

Most openers competed with [^{3}H]-P1075 with regular inhibition curves extrapolating to complete inhibition at saturation; this is compatible with a direct competition for a homogeneous class of binding sites. However, the fact that minoxidil sulphate only inhibited [^{3}H]-P1075 binding by 68% indicated heterogeneity of the opener binding sites. Two explanations are possible; either minoxidil sulphate binds to only a fraction of the P1075 sites, or it binds to a class of site which is different from, but negatively allosterically coupled to the P1075 site. Further experiments showed that minoxidil sulphate, up to 100 μM, did not enhance the rate of dissociation of the [^{3}H]-P1075-receptor complex (not illustrated). This renders the negative allosteric model unlikely and favours the possibility that the preparation contains two classes of (non-interacting) binding site with a ratio of 2 : 1; both classes of site have very similar affinities for all openers tested, with the exception of minoxidil sulphate which does not bind to the second class.

Heterogeneity of the P1075 sites was also evident from the biphasic competition curves obtained with the sulphonylurea inhibitors and with AZ-DF 265. With [^{3}H]-glibenclamide as the radiolabel, heterogeneity of the glibenclamide sites has been found in membranes from rat heart (Gopalakrishnan *et al*., 1991) and in rings from rat aorta (Löffler & Quast, 1997). However, inhibition of [^{3}H]-P1075 binding by sulphonylureas in vascular smooth muscle cells has always been found to be monophasic, resulting in either complete (rat aorta, Quast *et al*., 1993) or partial inhibition (afferent arteriole: 90% (Metzger & Quast, 1996); A10 cells: 75% (Russ *et al*., 1997)). To our knowledge, biphasic inhibition of [^{3}H]-P1075 binding by sulphonylureas has not previously been demonstrated. It is intriguing that the high affinity component of the sulphonylurea inhibition curves amounts to about 60% of specific [^{3}H]-P1075 binding. This is close to the amplitude of the minoxidil sulphate inhibition curve (68%), thus supporting the evidence for two classes of receptors with an approximate ratio of 2 : 1.

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

The heart is a highly vascularized organ and cardiomyocytes make up only 33% of the total number of cells in the heart, the remaining cells being endothelial cells, pericytes, fibroblasts, smooth muscle cells and macrophages (Nag, 1980). Hence it is likely that membrane preparations from native tissue will contain plasmalemmal contributions from these different cells types (Tomlins *et al*., 1986). It is known that cardiomyocytes, vascular myocytes and endothelial cells are endowed with K_{ATP} channels (for review see Ashcroft & Ashcroft, 1990; Quast, 1992; Edwards & Weston, 1993); in addition, a mitochondrial K_{ATP} channel has been described which is activated by K_{ATP} channel openers and inhibited by glibenclamide (Paucek *et al*., 1992; Garlid *et al*., 1996; Szewczyk *et al*., 1997). In principle, all of these components could contribute to the observed [^{3}H]-P1075 binding. Considering first the pharmacological profile of the K_{ATP} channel in rat liver mitochondria, one notes that the potencies obtained for activation of this channel by cromakalim (6.3 μM) and diazoxide (2.3 μM) (Garlid *et al*., 1996) are incompatible with the values found here in rat cardiac membranes. Similarly, it seems unlikely that the sulphonylurea binding site present in mitochondria (Paucek *et al*., 1992; Szewczyk *et al*., 1997) contributes much to the low affinity sites seen in the [^{3}H]-P1075 binding assay, since the mitochondrial sites, when labelled with [^{3}H]- glibenclamide, do not recognize the openers (Szewczyk *et al*., 1997).

Very recently, evidence for a K_{ATP} channel on endothelial cells of terminal arterioles in guinea-pig heart has been obtained; this channel is unusually sensitive to diazoxide (EC_{50} value for hyperpolarization of the cell $\approx$10 pM; Langheinrich & Daut, 1997). This indicates a very high affinity of diazoxide for the SUR from endothelial cells, which is not compatible with the IC_{50} value of 22 μM obtained here. One has to conclude that the endothelial SUreceptor does not make a significant contribution to the observed [^{3}H]-P1075 binding and that the SURs from cardiocytes and from vascular smooth muscle cells (according to current knowledge SUR_{2A} and SUR_{2B}, Inagaki *et al*., 1996; Isomoto *et al*., 1996; Chutkow *et al*., 1996) may be dominant.

Of the ten K_{ATP} channel openers tested here, eight have also been tested in binding assays in rat aortic rings (Quast *et al*., 1993) and in rat ventricular myoctes (Lemoine *et al*., 1996); these data are compiled in Table 1. Comparing the potencies of the openers in cardiac membranes and in cardiomyocytes gives an excellent correlation ($r = 0.96$) with a correlation line which coincides with the line of identity (not shown). Comparison of the data in cardiac membranes with those in rat aorta gives an even better correlation (0.98). Again, the correlation line has slope of 1, although it is shifted off the line of identity by 0.55 units (not shown). This indicates that these openers are, on average, 3.5 times more potent in rat aorta than in cardiac membranes. Hence, the limited data available suggest that the binding described here is primarily to membranes from ventricular myocytes. This proposal may be tested by [^{3}H]-P1075 binding assays with recombinant SUR_{2A}. However, the potencies determined in the binding assays are more than one order of magnitude higher than the potencies of openers in activating the cardiac K_{ATP} channel at physiological ATP levels (P1075: 35 μM (Xu *et al*., 1993); rilmakalim: 1 μM (Terzic *et al*., 1994; Krause *et al*., 1995); RP 49356, the racemate of which aprikalim is the eutomer: 400 μM (Thuringer & Escande, 1989)).

Unfortunately, data for binding of sulphonylureas to cardiocytes are not available. However, comparison of the high affinity component in rat cardiac membranes with the data in rat aorta shows that the potencies of glibornuride and AZ-DF 265 are identical in the two preparations, whereas glibenclamide is four times more potent in the heart. A 160 fold potency difference between the two tissues is found for phloxine B, which available evidence in insulinoma cells suggests to bind to the glibenclamide binding site of SUR_1 (Schwanstecher *et al*., 1995). These differences suggest again that the [^{3}H]-P1075 binding described here is not to the vascular smooth muscle component of the cardiac preparation.

Preliminary studies have suggested that, similar to rat aorta, the high affinity site for glibenclamide in the cardiac preparation is negatively allosterically coupled to the P1075 site (Löffler-Walz & Quast, unpublished observations); in rat aorta, the [^{3}H]-P1075 binding assay underestimates the true affinity of glibenclamide by about 25 times (Bray & Quast, 1992; Löffler & Quast, 1997). Keeping this in mind, it seems likely that the high affinity site for glibenclamide described here (IC_{50} value $\approx$90 nM) corresponds to the site which mediates the effects of glibenclamide in cardiocytes (low nM range;

Hamada *et al*., 1990; Findlay, 1992; Benz & Kohlhardt, 1994; Krause *et al*., 1995). The potency of glibenclamide binding in cardiac membranes is also higher than that found with recombinant SUR_{2A}, the putative β-subunit of the native K_{ATP} channels in cardiac and skeletal muscle; here, values of 350 and 630 nM have been obtained for glibenclamide in $^{86}Rb^+$ efflux experiments and [^{125}I]-glibenclamide binding studies, respectively (Inagaki *et al*., 1996). This may suggest the presence of additional, unidentified SURs in cardiocytes with nanomolar affinity for glibenclamide.

In conclusion, we have described here the requirements for and the properties of high affinity [^{3}H]-P1075 binding sites in rat cardiac membranes. The availability of a binding assay for openers in membranes may allow one to investigate the subcellular localization of the opener binding site, the modulation of binding by nucleotides and pH, and the coupling between the sites for openers and sulphonylureas. With regard to opener binding, the pharmacological characteristics of the [^{3}H]-P1075 binding sites described here agree quantitatively with published results obtained in rat cardiocytes. However, the inhibition of P1075 binding by sulphonylureas is not in agreement with the available data for SUR_{2A}. [^{3}H]-P1075 binding experiments with recombinant SURs are eagerly awaited to resolve these issues.

Note added in proof

During revision of this manuscript, a study on [^{3}H]-P1075 binding to membranes from rabbit skeletal muscle was published (Dickinson *et al*. (1977). *Mol. Pharmacol*., **52,** 473–481). The results are similar to ours.

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