

Glibenclamide and meglitinide block the transport of low molecular weight solutes into malaria-infected erythrocytes

Kiaran Kirk^a, Heather A. Horner^a, Dominique J. Spillett^b and Barry C. Elford^b

^aUniversity Laboratory of Physiology, Parks Road, Oxford, OX1 3PT, UK and ^bInstitute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK

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Following infection by the malaria parasite, human erythrocytes show increased uptake of a wide variety of low molecular weight solutes via pathways with functional characteristics different from those of the transporters of normal erythrocytes. In this study glibenclamide and meglitinide were shown to inhibit the induced transport of a sugar alcohol (sorbitol), an amino acid (threonine), an inorganic anion (Cl^-) and an organic cation (choline) into human erythrocytes infected in vitro with *Plasmodium falciparum*. The results are consistent with the hypothesis that a diverse range of substrates enter malaria-infected cells via common pathways which have features in common with Cl^- channels in other cell types. Glibenclamide and meglitinide were also shown to inhibit the in vitro growth of the intracellular parasite which would suggest that these pathways may be a viable chemotherapeutic target.

Erythrocyte; Malaria; Induced transport, Cl^- channel, Glibenclamide; K^+ -ATP channel

1. INTRODUCTION

The sulfonyl urea glibenclamide and the structurally related compounds meglitinide and tolbutamide stimulate the release of insulin from pancreatic β -cells and have been used in the treatment of diabetes. Their hypoglycaemia-inducing action has been attributed to inhibition of ATP-sensitive K^+ (K^+ -ATP) channels in the β -cell plasma membrane and they also inhibit K^+ -ATP channels in other cell-types [1,2]. Amongst cation-selective channels glibenclamide and its various analogues appear to be fairly specific for K^+ -ATP channels. However glibenclamide was shown some years ago to inhibit Cl^- conductance in rabbit kidney [3] and more recently it has been identified as a potent inhibitor of chloride currents through CFTR (the cystic fibrosis transmembrane conductance regulator), a cAMP-activated Cl^- channel, mutations in which cause the disease cystic fibrosis [4].

Following infection by the malaria parasite, human erythrocytes show increased permeability to a wide variety of low molecular weight solutes (reviewed in [5–7]).

The increased permeability is mediated by pathways with characteristics unlike those of the transporters of normal erythrocytes but showing functional similarities to chloride channels in other cell-types [8]. As expected for channels, and in contrast to conventional carriers [9,10], the parasite-induced pathways show no tendency to saturate with increasing substrate concentration (up to millimolar levels) and do not discriminate between stereoisomers. They are anion-selective with a high permeability to Cl^- , and, like Cl^- channels elsewhere [11] they have a significant permeability to cations, the magnitude of which depends on the nature of the counter-anion present (Kirk, K., Elford, B.C., Horner, H.A., Ellory, J.C. and Newbold, C.I., submitted). The pathways are inhibited by the anion channel blockers niflumate, furosemide and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) [8], and the IC_{50} value for inhibition of malaria-induced choline transport by NPPB falls within the range of those reported previously for Cl^- channels in other tissues [3,12–14].

Following on from the recent identification of glibenclamide as a potent inhibitor of the CFTR Cl^- channel [4] we have, in this study, tested the effect of glibenclamide, meglitinide and tolbutamide on the transport of a variety of low molecular weight solutes into malaria-infected cells and on the in vitro growth of the malaria parasite. The results have implications not only for the possible identity and chemotherapeutic potential of the malaria-induced pathways, but also for the use of glibenclamide and its analogues as tools for the diagnosis of K^+ -ATP channel involvement in physiological processes.

Correspondence address: K. Kirk, University Laboratory of Physiology, Parks Road, Oxford, OX1 3PT, UK. Fax: (44) (865) 272469.

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DMSO, dimethyl sulfoxide; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid.

2. MATERIALS AND METHODS

2.1. Materials

[¹⁴C]Choline Cl and Na³⁶Cl were from Amersham International. Plasmagel was a gift from Laboratoire Roger Bellon, Neuilly sur Seine, France. The sulfonyl ureas and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were from Sigma. 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was a generous gift from Prof. R. Greger (Physiologisches Institut der Albert-Ludwigs-Universität, Freiburg, Germany). The chemical structures of glibenclamide, meglitinide and tolbutamide are shown in Fig. 1.

2.2. Cell culture

Human erythrocytes (type O) infected with the ITO4 line of *Plasmodium falciparum* [15] were cultured under 1% O₂, 3% CO₂, 96% N₂ in RPMI 1640 culture medium (Gibco, Glasgow, UK) supplemented with HEPES (40 mM), glucose (10 mM), glutamine (2 mM), gentamicin sulphate (25 mg/l) and serum (10% v/v, pooled from different donors, Blood Transfusion Service, Oxford and Bristol). All experiments were carried out using synchronised suspensions of trophozoite-infected cells (approximately 35 h post-invasion, 40–90% parasitaemia), prepared using a combination of sorbitol haemolysis [16] and gelatin (Plasmagel) flotation [17] as described elsewhere [18].

Cell concentrations were determined using either a Coulter counter (Model Zm) or an improved Neubauer counting chamber. Parasitaemia was estimated from methanol-fixed Giemsa-stained smears. In experiments comparing uninfected cells with malaria-infected material, erythrocytes (from the same donor) were incubated in parallel with the infected red cell cultures under identical conditions for at least 24 h prior to the experiment.

2.3. Iso-osmotic haemolysis experiments

The enhanced permeability of the malaria-infected erythrocyte membrane to the sugar alcohol, D-sorbitol, and the amino acid, L-threonine, was studied using a simple haemolysis method [19]. Iso-osmotic solutions of sorbitol and threonine were prepared by dissolving the compounds to a concentration of 300 mM in H₂O containing 10 mM HEPES (pH 7.4). Haemolysis time courses were commenced by the addition of malaria-infected cells (suspended at a haematocrit of approximately 10% in normal growth medium) to the different solutions of interest (pre-warmed to 37°C) to give a final haematocrit of approximately 0.5%. Aliquots of the suspension were sampled at regular intervals into microcentrifuge tubes containing ice-cold, hypertonic sucrose solution (400 mM, 0.5 ml). The tubes were centrifuged (10,000 × g, 15 s) and the amount of haemoglobin in the supernatant solution (and hence the degree of haemolysis) was estimated from the absorbance at 540 nm.

Normal uninfected erythrocytes remained stable in the iso-osmotic solutions for the period of the experiments (30–60 min). Haemolysis may therefore be attributed to the net influx of the compounds into the infected cells via the malaria-induced transport pathways.

2.4. Cl[−] influx measurements

Time courses for Cl[−] influx were measured at room temperature in cells washed (× 4) in HEPES-buffered, serum-free saline (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 5 mM glucose) then treated with DIDS, a potent inhibitor of the band 3 anion exchanger. For each time course 6 microcentrifuge tubes were prepared, each containing a lower oil phase of 0.2 ml of dibutyl phthalate and an upper aqueous phase of 0.15 ml of saline which contained DIDS (10 μM), ³⁶Cl[−] (0.6 μCi/ml) and, where appropriate, glibenclamide, meglitinide or tolbutamide. At predetermined intervals, an aliquot of cell suspension (0.15 ml, preincubated for 10 min at room temperature with 10 μM DIDS) was dispensed into each tube in turn to give a final cell concentration of around 1 × 10⁸ cells/ml. Immediately following the addition of cells to the final tube in the series the samples were centrifuged (10,000 × g, 30 s) to sediment the cells below the oil and thereby terminate the flux. The time taken between starting the centrifuge and termination of the flux was taken as 2 s [20]. Following sedimentation of the cells below

the oil, the aqueous supernatant solution was removed by aspiration then the radioactivity remaining on the walls of the tube was removed by rinsing the tubes four times with water. The dibutyl phthalate was aspirated then the cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml) and deproteinised by the addition of 5% w/v trichloroacetic acid (0.5 ml), followed by centrifugation (10,000 × g, 10 min). The amount of radiolabel was measured using a β-scintillation counter.

The amount of radiolabel trapped in the extracellular space within the cell pellets was estimated from ice-cold samples containing NPPB (0.1 mM, [8]) which were centrifuged immediately following the addition of DIDS-treated cell suspension (0.15 ml) to the ³⁶Cl[−] solution (0.15 ml).

2.5. Choline influx measurements

The unidirectional influx of choline into erythrocytes at 37°C was estimated from the uptake of [¹⁴C]choline during a short (5 min) incubation period that fell within the initial, approximately linear portion of the [¹⁴C]choline influx time course. Unless specified otherwise, infected cells were washed four times in HEPES-buffered serum-free saline (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 5 mM glucose) at 37°C, then resuspended and combined with the appropriate reagent solutions in microcentrifuge tubes. The tubes were transferred to a 37°C water bath and allowed at least 10 min to temperature-equilibrate. Fluxes were started by the addition of [¹⁴C]choline, together with unlabelled substrate to give a final activity of 1 μCi ml^{−1} and a final extracellular choline concentration of 1 mM. The final sample volume was 0.5 ml and the final cell concentration was typically 2 × 10⁸ RBC ml^{−1}.

Choline influx was terminated by transferring aliquots (0.11 ml) of the suspension to microcentrifuge tubes containing 0.8 ml of ice-cold 'stopping solution' (isotonic saline supplemented with 0.1 mM furosemide; [8]) layered over 0.25 ml of dibutyl phthalate. The tubes were centrifuged immediately (10,000 × g, 30 s) to sediment the cells below the oil, then the supernatant solution was removed by aspiration and the samples processed for β-scintillation counting as described above.

The amount of [¹⁴C]choline trapped in the extracellular space within the cell pellets was estimated from aliquots (0.11 ml) taken from samples containing the transport inhibitors identified in this study, immediately following the addition of radiolabel. These were transferred to stopping solutions (0.8 ml) layered over dibutyl phthalate (0.25 ml) and processed as described above.

In experiments to test the reversibility of the effect of glibenclamide on choline influx, cells in HEPES-buffered saline were incubated in the presence or absence of the inhibitor for 10 min then washed (× 4) by repeated centrifugation and resuspension in HEPES-buffered. In experiments to test the effect of serum (10% v/v) on glibenclamide-inhibition cells were washed (× 4) in RPMI then resuspended in the same solution in the presence or absence of serum (10% v/v). Glibenclamide was added at a concentration of 0.1 mM and the samples were incubated for 10 min at 37°C before measuring choline influx.

2.6. In vitro growth assays

The antimalarial activity of glibenclamide, meglitinide and tolbutamide was tested using [³H]hypoxanthine incorporation as a marker for parasite growth [21,22]. Serial dilutions of the inhibitors in DMSO (0.4 μl) were dispensed into 96-well culture plates, to which were then added tightly synchronised cultures of ring-stage infected erythrocytes (200 μl at 2% haematocrit and 1–3% parasitaemia in growth medium). [³H]hypoxanthine was added at an activity of 2.5 μCi/ml (spec. act. 5 mCi/mmol). The plates were maintained in a gas-tight box at 37°C for 18–24 h so that labelling occurred within a single cycle of maturation, then harvested using an LKB harvester. A flat-bed Betaplate scintillation system was used to measure incorporated radioactivity [22].

3. RESULTS

The effect of glibenclamide, meglitinide and tolbu-

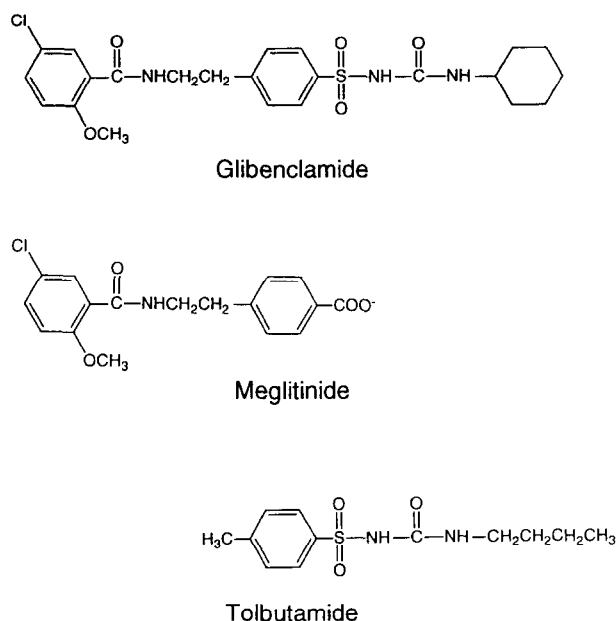


Fig. 1. Chemical structures of glibenclamide, meglitinide and tolbutamide. Tolbutamide consists of much of the sulfonyl urea portion of glibenclamide, while meglitinide is equivalent to the non-sulfonyl urea portion of the molecule.

tamide on the transport of the sugar alcohol D-sorbitol and the amino acid L-threonine into malaria-infected cells is illustrated in Fig. 2. Glibenclamide (0.1 mM) caused a dramatic decrease in the rate of haemolysis in both the sorbitol and threonine solutions, consistent with it having blocked the influx of both compounds into the infected cells. Meglitinide (0.1 mM) was less effective at slowing haemolysis in the two solutions, while tolbutamide (0.1 mM) was without significant effect.

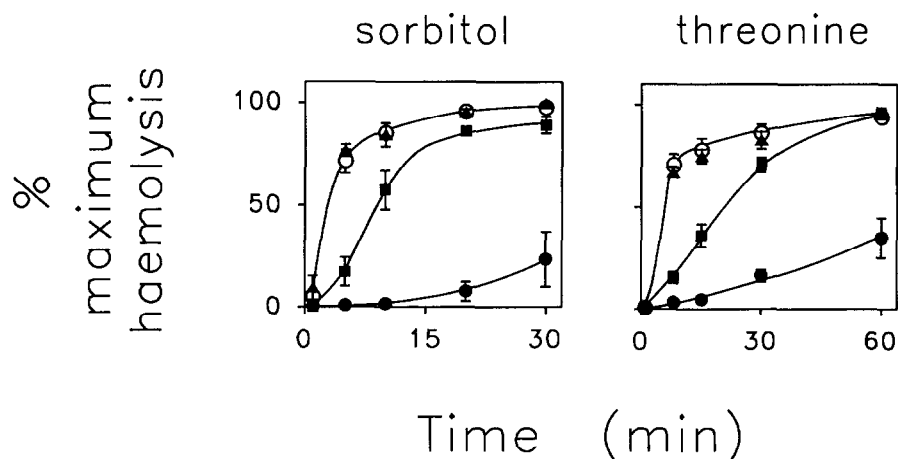


Fig. 2. Time courses for haemolysis of malaria-infected erythrocytes suspended in iso-osmotic solutions of sorbitol and threonine in the absence (○) or presence of glibenclamide (0.1 mM, ●), meglitinide (0.1 mM, ■) or tolbutamide (0.1 mM, ▲). The data were averaged from three different experiments using cells from different donors and are shown $\pm$ S.E.M. In similar experiments with normal uninfected erythrocytes the cells remained stable in the iso-osmotic solutions for ≥ 1 h. Haemolysis of infected cells may therefore be attributed to parasite-induced modification of the host cells.

Fig. 3 shows time courses for the transport of Cl^- into infected and uninfected cells. In uninfected erythrocytes, pretreated with 10 μM DIDS there was negligible uptake of Cl^- over 21 s. In contrast, Cl^- was transported rapidly into DIDS-treated infected cells and equilibrated within a few seconds. The influx of Cl^- into infected cells was slowed by glibenclamide (0.5 mM) and, to a lesser extent, by meglitinide (0.5 mM). Tolbutamide (0.5 mM) had little effect.

The inhibitory properties of glibenclamide, meglitinide and tolbutamide were investigated in more detail using the monovalent organic cation choline as a test substrate. Choline is transported into normal human erythrocytes via a saturable carrier with a K_m of around 10 μM [23,24]. Transport of choline via the malaria-induced pathways is non-saturable [25–27] and in the experiments reported here choline was therefore presented at an extracellular concentration of 1 mM in order to saturate and thereby minimise the relative contribution of the native transporter to the total influx. Under these conditions the unidirectional influx of choline measured in trophozoite-infected cell suspensions (45–85% parasitaemia, $n = 8$) was 117 ± 15 ($\pm$ S.E.M.) $\mu\text{mol}/(10^{12}$ RBC·h), compared with a value of 4.2 ± 0.5 $\mu\text{mol}/(10^{12}$ RBC·h) in uninfected cells from the same donors; in almost all experiments over 95% of the choline influx measured in infected cell suspensions was parasite-induced.

Glibenclamide, meglitinide and tolbutamide inhibited the influx of choline into malaria-infected erythrocytes with the same rank order of potency as that seen for the other substrates tested. Dose-response curves are shown in Fig. 4A. The IC_{50} values for glibenclamide, meglitinide and tolbutamide (i.e. the concentrations at which the inhibitory effects were half maximal) were 11 ± 2 , 52 ± 6 and > 500 μM , respectively ($n = 4$). The inhibi-

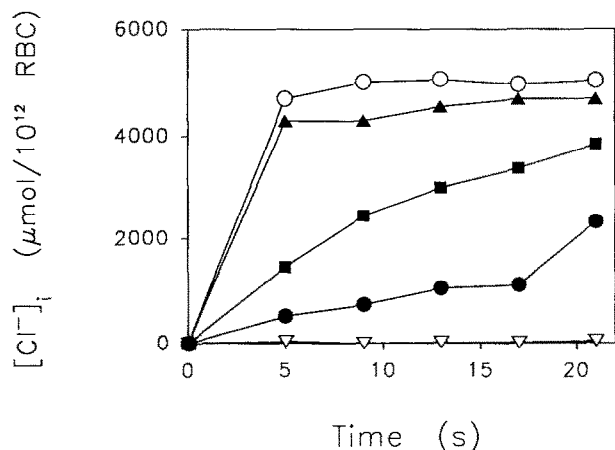


Fig. 3. Time courses for influx of Cl^- in uninfected erythrocytes ($\square$) and in a synchronised trophozoite-infected cell suspension (84% parasitaemia), either in the absence of induced transport inhibitors ($\circ$) or in the presence of glibenclamide (0.5 mM, $\bullet$), meglitinide (0.5 mM, $\blacksquare$) or tolbutamide (0.5 mM, $\blacktriangle$). All samples contained 10 μM DIDS. The data is from one of two similar experiments on cells from different donors.

tion of malaria-induced choline transport by glibenclamide was fully reversible; when infected cells were preincubated for 10 min with glibenclamide then washed ($\times 3$) by centrifugation and resuspended in glibenclamide-free saline the choline flux was the same as that in cells not previously exposed to the inhibitor (Fig. 4B). Glibenclamide inhibition was attenuated by the presence of serum (10% v/v) in the extracellular medium, which would suggest that serum components compete with the transport pathways for binding the inhibitor (Fig. 4B).

If, as has been postulated [28,29], the malaria-induced permeation pathways play an important role in the development of the intracellular parasite it might be expected that the transport inhibitors identified here should inhibit parasite growth. We therefore tested the

effect of the three compounds of interest on the incorporation of [^3H]hypoxanthine by the parasite over an 18–24 h period (commencing with cells synchronised at the ring stage of maturation). In our initial growth assays in which cells were grown in the presence of 10% v/v human serum the inhibitors were without significant effect. However, when the serum concentration was reduced to 2% (the minimum required to support parasite growth in the short term) glibenclamide and meglitinide, but not tolbutamide, were found to inhibit [^3H]hypoxanthine incorporation (Fig. 5). The IC_{50} value for glibenclamide was 39 ± 6 ($\pm$ S.E.M., $n = 3$) μM while that of meglitinide was approximately 5-fold higher.

4. DISCUSSION

In this study we tested the effect of glibenclamide, meglitinide and tolbutamide on the transport of a sugar alcohol, an amino acid, an inorganic anion and an organic cation via the permeation pathways that are induced in human erythrocytes following malaria infection. While not as potent as some of the transport inhibitors identified previously [8] glibenclamide and meglitinide were found to block the malaria-induced transport of all of the substrates tested, and in each case the relative inhibitory potency of the three reagents was the same - glibenclamide > meglitinide $\gg$ tolbutamide. This is, to our knowledge, the first demonstration of the inhibition by these widely used compounds of the transport of organic substrates. The data are consistent with the view that a diverse range of solutes enter infected cells via common pathways. Furthermore, they provide additional support for the hypothesis that these pathways have features in common with anion-selective channels in other cells. The sulfonyl ureas are best known as inhibitors of K^+ -ATP channels and are not widely recognised as Cl^- channel blockers. However, glibenclamide inhibits Cl^- conductance in the mammalian kidney [3] and has recently been identified as an

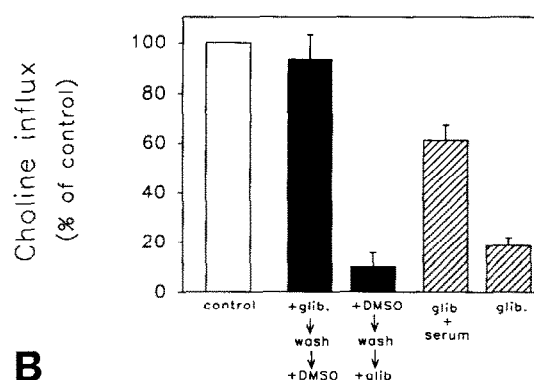
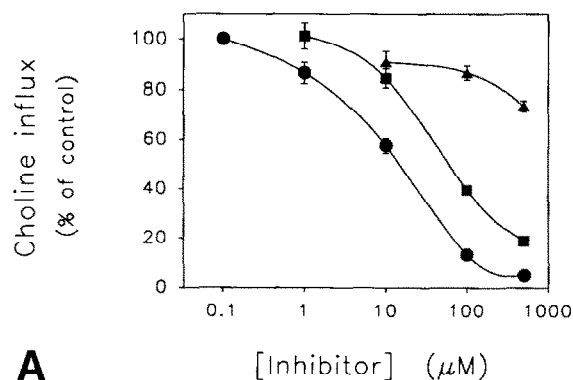


Fig. 4. (A) Dose-response curves for the effects of glibenclamide ($\bullet$), meglitinide ($\blacksquare$) and tolbutamide ($\blacktriangle$) on influx of choline into malaria-infected erythrocytes. (B) Reversibility (solid bars) and effect of serum (10% v/v; cross-hatched bars) on glibenclamide-inhibition of choline influx into malaria-infected erythrocytes. The extracellular choline concentration was 1 mM. Choline influx in the presence of inhibitor (or in cells exposed previously to inhibitor) is expressed as a percentage of that measured in cells treated in a similar manner in the absence of inhibitor. The data were averaged from at least three different experiments using cells from different donors and are shown $\pm$ S.E.M.

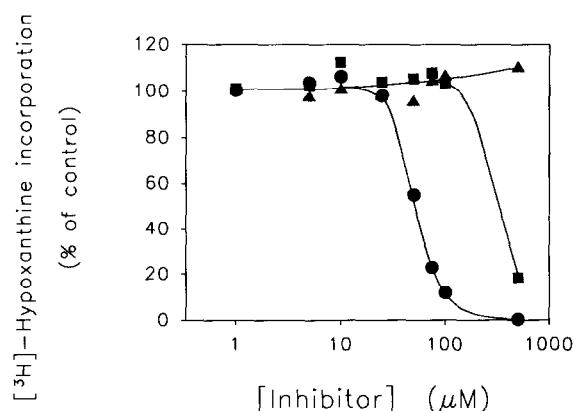


Fig. 5. Dose-response curves for the effects of glibenclamide, meglitinide and tolbutamide on incorporation of [^3H]hypoxanthine by malaria parasites over a 18 h period, commencing with sorbitol-synchronised ring-stage cultures. The growth medium contained 2% v/v serum. The data are from a single representative experiment; each point represents the average [^3H]hypoxanthine incorporation measured in 7 replicate wells of a 96-well plate. Standard deviations fell, in most cases, within the symbols and are not shown.

effective inhibitor of CFTR, the cystic fibrosis Cl^- channel [4]. The IC_{50} found here for the inhibition by glibenclamide of malaria-induced choline transport ($11 \mu\text{M}$) is very similar to that reported for CFTR ($\sim 20 \mu\text{M}$); however glibenclamide inhibition of the malaria-induced pathway was readily reversible (Fig. 4B) whereas that of CFTR was reportedly not [4]. The pathways also differ somewhat in their susceptibility to tolbutamide which blocks CFTR with an IC_{50} of around $150 \mu\text{M}$ [4] but which has little effect on the malaria-induced pathways at concentrations below $500 \mu\text{M}$. The effect of meglitinide on the CFTR channel has not, to our knowledge, been reported.

Glibenclamide is the most potent inhibitor of the CFTR channel that has been identified to date. The same is not true of the malaria-induced pathway which is blocked by NPPB at sub-micromolar concentrations. NPPB does block CFTR, though relatively high concentrations are required [30]. The pharmacological similarities between CFTR and the malaria-induced pathways are therefore more qualitative than quantitative, which makes it seem unlikely that CFTR itself is responsible for the enhanced permeability of infected cells. Perhaps more likely is the possibility that the induced transport is mediated by a related protein, conceivably another member of the ABC (ATP binding cassette) superfamily. This is a large group of membrane proteins which show a varying degree of homology and which include numerous bacterial active transport systems, the human multidrug resistance pump, P-glycoprotein [31], and the products of the *P. falciparum* genes *pfmdr1* and *pfmdr2* [32,33]. In bacteria, members of this family are involved in the import of a wide variety of nutrients, whereas in eukaryotes they nor-

mally perform an export function [31]. Of the family members identified to date, only CFTR has been shown conclusively to function as a chloride channel; however recent evidence suggests that the same may be true of P-glycoprotein [34,35]. Whether other members of the family might also function as anion-selective channels, with properties similar to CFTR, remains an open question.

The rank order of potencies of the three inhibitors tested here on the malaria-induced pathways (glibenclamide > meglitinide > tolbutamide) is the same as that for the inhibition of K^+ -ATP channels [1]. The IC_{50} values for inhibition of malaria-induced choline influx are much higher than those for K^+ -ATP channels in β -cells [1] but they are within an order of magnitude of those for K^+ -ATP channels in muscle [2]. Quite apart from the question of whether this might reflect structural similarities between the parasite-induced pathways and K^+ -ATP channels (as has been suggested for CFTR; [36]), this has obvious implications for the use of glibenclamide and its analogues as tools for the diagnosis of K^+ -ATP channel involvement in physiological processes. Amongst cation-selective channels glibenclamide and its various analogues are apparently fairly specific for K^+ -ATP channels (reviewed in [37]); however, the malaria-induced pathways, although anion-selective, have a significant permeability to cations, including K^+ ($^{86}\text{Rb}^+$) [18]. Whatever their identity, pathways of this sort should therefore be considered before sulfonyl urea-sensitive K^+ transport may be attributed unambiguously to K^+ -ATP channels.

In the *in vitro* growth assays, parasites grown in medium containing 10% serum were almost unaffected by the three inhibitors of interest. However, when the serum concentration was reduced to 2% v/v, [^3H]hypoxanthine incorporation was inhibited by glibenclamide ($\text{IC}_{50} \approx 39 \mu\text{M}$) and, with lesser potency, meglitinide (Fig. 5). Tolbutamide had no effect, at concentrations up to 0.5 mM . The relative effects of glibenclamide, meglitinide and tolbutamide on [^3H]hypoxanthine incorporation were therefore the same as those for their inhibitory effects on the induced transport pathways. This is consistent with the induced pathways being the site of action for the antimalarial activity of these drugs. The IC_{50} values for inhibition by glibenclamide and meglitinide of parasite growth were somewhat higher than those for the inhibition of induced transport which was measured in cells exposed for short times in serum-free solution. However, these differences can be accounted for by binding of the inhibitors to serum components as it is clear from Fig. 4B that the presence of serum alleviates inhibition of the induced transport pathways. If inhibitors such as these are to be of chemotherapeutic value the challenge will be to identify reagents that will bind preferentially to the induced transport pathways in the presence of serum.

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