Significance of the sulfonylurea receptor (SUR) as the target of diflubenzuron in chitin synthesis inhibition in *Drosophila melanogaster* and *Blattella germanica*

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**Abstract**

Diflubenzuron (DIMILIN®) is a powerful insecticidal chemical which has been known for many years to inhibit chitin synthesis in vivo in insects and related arthropod species. However, its action mechanism has remained unresolved partly because of its inaction on any of the enzymes involved in chitin synthesis in vitro. Based on our previous work (Diflubenzuron affects gamma-thioGTP stimulated Ca2+ transport in vitro in intracellular vesicles from the integument of the newly molted American cockroach, *Periplaneta americana* L. Insect Biochem. Mol. Biol. 24 (1994) 1009) showing that diflubenzuron inhibits Ca2+ uptake by vesicles obtained from the integument of American cockroach, *Periplaneta americana* (L.), in vitro, we tested the hypothesis that the action site of diflubenzuron is an ABC (ATP binding cassette) transporter, probably a sulfonylurea-sensitive transporter. Glibenclamide, one of the most commonly used sulfonylureas for type II diabetes treatment, was the positive control. When given to immature insects, glibenclamide clearly caused toxicity, with symptoms indicating molting abnormality comparable to diflubenzuron. Its LD50 (0.472 μg/nymph) was approximately 2.8 times the value obtained for diflubenzuron (0.17 μg/nymph, topical) in German cockroach, *Blattella germanica* (L.). However, in terms of the inhibitory activities on chitin synthesis, in isolated integuments glibenclamide showed an identical potency to diflubenzuron in *B. germanica* nymphs. A competitive binding assay with [3H]-glibenclamide and unlabeled diflubenzuron clearly established that the latter is capable of competitively displacing the former radioligand. The K_D values observed for vesicles prepared from fruit fly larvae, *Drosophila melanogaster* M., were 44.9 nM for glibenclamide and 65.0 nM for diflubenzuron, respectively. Furthermore, glibenclamide was found to affect Ca2+ uptake by isolated cuticular vesicles from *B. germanica* in a manner very similar to diflubenzuron. These results support our conclusion that the sulfonylurea receptor (SUR) is the target of diflubenzuron in inhibition of chitin synthesis in these two insect species.

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**Keywords:** Diflubenzuron; Sulfonylurea receptor (SUR); Binding site; Glibenclamide; Chitin synthesis

1. Introduction

It has been well established that diflubenzuron (DFB) inhibits chitin synthesis in vivo and that such action constitutes the main insecticidal mechanism of this compound (Marks et al., 1982; Verloop and Ferrell, 1977). However, the precise molecular mechanism of its action has not been elucidated. The major problem in answering this question has been that so far neither DFB nor any other benzyolurea-type chemicals have shown inhibitory properties toward any of the enzymes associated with chitin synthesis when tested in vitro (Cohen, 2001). Furthermore, DFB does not inhibit chitin synthesis in yeast (Marks et al., 1982), unlike other chitin synthesis inhibitors such as polyoxin D (Endo et al., 1970), which is effective in inhibiting chitin synthesis both in insects and yeast. Clearly DFB-type chemicals act on an arthropod-specific cuticular component that is vital to chitin synthesis in vivo.

Apparently this component contributes to chitin synthesis only in intact integument cells, since it has been shown by Nakagawa et al. (1993) in isolated...
American cockroach (*Periplaneta americana* (L.)) integuments that [$^3$H]-N-acetylglucosamine incorporation into chitin is inhibited by DFB when each piece of integument is large enough (i.e. 1/8 of abdominal integument from the last instar nymph). Homogenization or further chopping of the integument into smaller pieces completely abolishes the inhibitory action of DFB. Furthermore, such action of DFB is completely abolished by simultaneous administration of valinomycin, a potassium ionophore. Subsequently Nakagawa and Matsumura (1994) produced the first evidence that DFB, at low concentrations (µM), can cause definite biochemical effects in vitro under cell free conditions: when it is applied to a vesicle preparation from the integuments of newly molted American cockroaches, it significantly inhibits $^{45}$Ca$^{2+}$ uptake. Such $^{45}$Ca$^{2+}$ uptake was stimulated by both ATP and gamma-thio-GTP and was inhibited by certain ionophores such as valinomycin (K$^+$ ionophore) and A23187 (Ca$^{2+}$ ionophore), but not much by monensin (Na$^+$ ionophore). Judging by the observation that sonication totally destroys Ca$^{2+}$ uptake as well as the action of DFB, it is clear that the presence of intact vesicle structure is needed in these actions of DFB.

The ion transport characteristics of these cuticular vesicles are similar to those of some ATP-binding cassette (ABC) transporters. The ABC transporter superfamily proteins carry out many critical functions in diverse biological systems (Bryan and Aguilar-Bryan, 1999; Liss and Roeper, 2001; Ueda et al., 1999), including chemical transport, ion channels, drug pumping (for drug resistance), receptors, and signal transduction. One prominent member of the superfamily whose functions resemble those of cuticular vesicles is the sulfonyleurea receptor (SUR), a structure that contains ABC proteins (Bryan and Aguilar-Bryan, 1999). The SUR forms a heteromer with two transmembrane-spanning potassium channel subunits (Kir6) to form ATP-sensitive potassium channels (Liss and Roeper, 2001). The SUR-K$^+$-channel is affected by glibenclamide (also called glybenclamide or glyburide), one of the most commonly used sulfonurea agents for stimulating insulin release from pancreatic β cells as a therapeutic drug for type II diabetes patients (Gribble et al., 1998). Glibenclamide at nM concentrations leads to an influx of Ca$^{2+}$ ions, which triggers insulin release to the extracellular space.

Recently, it was reported by Nasonkin et al. (1999) that there is a novel SUR showing characteristics of the ABC transporter family in *Drosophila*, designated DSUR. This gene product most closely resembles those of SUR1 of human and SUR1 and SUR2 of rat, based on DNA structure as well as glibenclamide-affected ion-channel functions when expressed in *Xenopus* oocytes. Based on such a breakthrough and the knowledge gained from the previous work in our own laboratory regarding DFB-sensitive cuticular vesicles, we hypothesized that those DFB-sensitive cuticular vesicles could contain SUR-type transporters that impact chitin synthesis.

We tested this hypothesis in two insect species, fruit fly, *Drosophila melanogaster*, (to relate to the work of Nasonkin et al., 1999) and German cockroach, *Blatella germanica*, (to relate to our previous work) and obtained the results supporting our hypothesis which are being reported in this paper.

### 2. Materials and methods

#### 2.1. Chemicals

Two of the compounds tested, diflubenzuron (DIMILIN®) (N-[(4-chlorophenyl) amino]carbonyl]-2,6-difluoro-benzamide) and dechloro-diflubenzuron, were from our stock as reported previously (Nakagawa and Matsumura, 1994). A reagent grade sample of glibenclamide (EUGLUCON®) (N$^1$-[β-(2-methoxy-5-chlorobenzoyl amino) ethyl]benzen-sulphonyl]-N$^2$-cyclohexylurea) was purchased from Sigma Chemical Co. [$^3$H]-glibenclamide was purchased from Perkin Elmer Life Sciences, Inc. (Boston, MA) at a specific activity of 50.0 Ci/mmol, [$^{14}$C]-N-acetyl-d-1-glucosamine was from Sigma-Aldrich Co. at specific activity of 45 mCi/mmol, and $^{45}$CaCl$_2$ (37 MBq/mg Ca) was from ICN Biomedicals (Irvine, CA). All other biochemical reagents were of the highest purity grade from Sigma Chemical Co.

#### 2.2. Insect cultures

An insecticide-susceptible laboratory strain (CSMA) of the German cockroach, *B. germanica* (Scott and Matsumura, 1983) was used in this study. The cockroach colony was fed Purina dog chow and water ad libitum and maintained in cheesecloth-covered plastic containers with Tree Tanglefoot (Tanglefoot Co., Grand Rapids, MI) coating the rim. The colony was maintained at 27 ± 2°C, 60 ± 5% relative humidity, and 12:12 h (L:D). An old laboratory wild type strain (Canton-S) of *D. melanogaster*, kindly provided by Dr K.C. Burtis (Division of Biological Sciences, University of California, Davis), was used for comparative purposes. The strain was maintained on standard *Drosophila* medium (Formula 4-24R, Carolina Biological Supply Co., Burlington, NC) at 25 ± 2°C, 60 ± 5% relative humidity, with a 12 h night/day period. From that colony, we have established our stock culture for this study.
2.3. Bioassays

2.3.1. German cockroaches

Assays were performed using cockroach last-instar nymphs. Serial dilutions of each test compound were prepared in dimethylsulfoxide (DMSO): ethanol (1:1) on the basis of wt/vol (AI) and expressed as μg/μl. Topical application was performed by delivering a 1.0 μl drop of test compound to the first three abdominal sternites, using a 5 μl Hamilton gas-tight syringe. Five to six concentrations were chosen to give >0 and <100% mortality. Two to three replicates of 10 cockroaches were tested per concentration for each mortality test. Control cockroaches received only solvent mixture. Mortality was recorded after 24–72 h. Insects not responding to touch or remaining ventral side up when turned were considered dead. Symptoms of anti-molting were also observed in each assay.

2.3.2. Drosophila

Last-instar larvae of Drosophila were collected from our stock culture for bioassays. The tested compounds were mixed with the standard Drosophila food medium. Each test concentration was thoroughly mixed with 4 g of diet in a 20 ml glass container. Forty larvae were introduced into each container and kept until adult emergence. Two replicates were used for each test concentration. Diet treated with only solvent (DMSO–ethanol) served as control treatment. Adult emergence was counted for each concentration, and percentage unemerged calculated and used as a criterion for the biological activity of the tested compounds.

2.3.3. Data analysis

The data obtained from the dose-mortality (cockroach assay) and concentration-mortality (unemerged adults in the Drosophila assay) experiments were analyzed by the standard probit analysis (Finney, 1971) using SYSTAT 10.2.01 to obtain the estimates of slope and LD50 (or EC50) values. Control mortality never exceeded 10%. In all cases, data were corrected for control mortality using Abbott’s (1925) formula.

2.4. Chitin synthesis assay in situ

Following the method described by Ishii and Matsumura (1992), newly molted (white to brownish cuticular appearance) fourth-instar German cockroach nymphs (approximately 0.05–0.06 g weight) were always used for this assay. The insects were collected just before each experiment and chilled on ice. Their abdominal integuments were collected mostly free from fat bodies and rinsed in 50 mM phosphate buffer, pH 7.2, containing 128 mM Na+, 11 mM K+, and 89 mM Cl−. They were cut along the longitude line, blotted, and each abdomen half placed in a 10 × 75 mm glass test tube containing 500 μl buffer. Test compound (diflubenzuron or glibenclamide) dissolved in 1 μl of solvent (DMSO:ethanol, 1:1) was added. For each test, at least three replicates were made. After preincubation for 30 min at 30 °C, 5 μl of [14C]-N-acetylglucosamine (0.05 μCi diluted with cold NAGA) was added to make a final concentration of 10−5 M. The mixture was incubated for 2–3 h at 30 °C, then the reaction stopped by addition of 1 ml 50% KOH and boiling for 1 h. The KOH-treated digest was neutralized with 3 ml of 4 N HCl and filtered through a glass filter (Whatman GF/F, diameter = 25 mm). The filter was successively washed with 3 ml distilled water, 3 ml 95% ethanol and 3 ml distilled water. The radioactivity remaining on each filter was counted using a liquid scintillation counter, and 14C dpm values were computed.

45Ca uptake by isolated vesicles from the abdominal integuments of the newly molted German cockroach nymphs was measured using the technique of Nakagawa and Matsumura (1994) with some minor modifications. The integuments were cut into small pieces and homogenized using a precisely fit glass–glass Potter-Elvehjem homogenizer in isotonic MES–sucrose buffer (10 mM MES containing 250 mM sucrose and 2.5 mM MgSO4, adjusted to pH 6.6 with NaOH). The homogenate was centrifuged for 15 min at 1000g. The supernatant was further centrifuged at 10,000g for 20 min and the precipitate suspended in the same buffer. The reaction was initiated by the addition of 20 μl of this preparation to 180 μl of isotonic MES–sucrose buffer containing glibenclamide (added in 2 μl of a 1:1 mixture of DMSO–ethanol to make a final concentration of 2 μM) plus a diagnostic agent (ATP [1 mM], NaCl [100 mM], or KCl [100 mM]) and 45CaCl2 (1 μM; 0.02 μCi), and the mixture was incubated for 5 min at room temperature. The control preparation received exactly the same amount of DMSO–ethanol solvent only. After incubation, the sample was diluted with 4 ml of ice-cold 150 mM KCl solution and filtered immediately through a 0.45 μm Millipore filter (25 mm diameter) followed by washing with 4 ml of ice-cold 150 mM KCl solution. The filter was air-dried and the radioactivity counted by liquid scintillation counter.

2.6. [3H]-Glibenclamide binding studies (competition assays)

The integuments from newly molted fourth-instar nymphs of German cockroach and the whole body of last-instar larvae of Drosophila were used to prepare the vesicles as described above. In both cases the integuments were homogenized in the same isotonic MES-sucrose buffer using a precisely fit glass–glass
2.7.1. Drosophila treatment

For quantitation of SUR mRNA expression, paired and unpaired experiments were performed with a limited test compound exposure time. For the paired experiment, *D. melanogaster* adults were anesthetized by chilling, and 10 females and 10 males each were incubated for 2 h in nursery flasks containing ~20 g medium. After adult removal, nursery flasks were incubated at room temperature for ~90 h, then the larvae harvested by flotation in 20% sucrose. Equal numbers of larvae from a given nursery flask went into paired treatment and control test flasks, with three replicate pairs. Test flasks were made by mixing the test compound or the same volume of solvent mixture (diflubenzuron or dechlorodiflubenzuron analogue) in a series of concentrations. Specific binding was determined by subtracting the value obtained from the addition of 1 μM unlabeled glibenclamide, and the dissociation constant (K_D) and the number of ligand binding sites per cell (B_max) were estimated using Prism 4.0 (GraphPad Software, Inc.).

2.7.2. RNA extraction

*Drosophila* larvae were blotted to remove RNAlater, homogenized in TRIzol® LS (Invitrogen) using a glass–glass Dounce homogenizer, and extracted for RNA following the Invitrogen protocol. Contaminating DNA was eliminated by RNase-free DNase I (Roche) digestion in the presence of RNAguard (Amersham) RNase inhibitor. RNA concentration was determined spectrophotometrically.

2.7.3. cDNA

For cDNA synthesis 1 μg RNA was denatured and hybridized with 320 pmol oligo-dT primer (15 bases long, MWG Biotech) in 14 μl water at 60 °C for 5 min. The hybridized RNA-primer was incubated with 1 μl RNAGuard plus reagents from the Qiagen Omniscript kit—1 μl reverse transcriptase, 10 nmol dNTPs, and 10X RT buffer—in a volume of 40 μl for 60 min at 37 °C, then for 10 min at 70 °C.

2.7.4. PCR

The cDNA was subjected to PCR amplification for the *Drosophila* sulfonylurea receptor. Two primer pairs were used (Table 1). We have not yet identified a primer set capable of amplifying SUR in German cockroach. For each primer pair, 2 μl cDNA was incubated with 16 pmol of each primer plus reagents from the Qiagen Taq DNA polymerase kit—2.5 units Taq polymerase, 5 μl Q solution, 10X PCR buffer—and 10 nmol dNTP mix (Qiagen), in a volume of 50 μl. Incubation conditions were 2 min at 94 °C, followed by cycling at 94 °C for 45 s, 53 °C for 60 s, and 72 °C for 44 cycles, and ending with 7 min at 72 °C. PCR products were run on a 1% agarose gel with 0.8 ppm ethidium bromide (BioRad) and a DNA ladder (Novagen) with fragments from 100 to 1000 bp at 100-bp increments. Bands were viewed under UV and the image recorded using AlphaImager 5.5 software (Alpha Innotech). Bands were excised, purified by spin column (Qiagen), and sequenced (University of California, Davis, Division of Biological Sciences Automated DNA Sequencing Facility).

All quantitative RT-PCR (qRT-PCR) assays were conducted using the Roche LightCycler®, the Qiagen QuantiTect® SYBR Green kit, and the second SUR primer pair. Conditions were a 10 min initial denaturation at 95 °C; cycling consisting of denaturation at 95 °C for 15 s, annealing at 59 °C for 20 s, extension at 72 °C for 10 s, and fluorescence data collection at the

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<th>Table 1</th>
<th>PCR primers used for <em>Drosophila melanogaster</em></th>
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<td>Forward primer</td>
<td>Reverse primer</td>
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<tr>
<td><strong>Pair 1</strong> (Rozen and Skaletsky, 2000)</td>
<td><strong>Pair 2</strong></td>
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<tr>
<td>AGA ACG GCA GTG</td>
<td>GGA TTC CCT GCT GTT</td>
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<td>GCA AGA</td>
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<td>ACT AT</td>
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<tr>
<td>Fragment size</td>
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end of a 5 s step at 82 °C; and a melting curve starting at a 64 °C annealing temperature. For normalization, beta-actin was amplified for each sample in the same run using primers for human beta-actin (Albino et al., 1991). RNase-free water in place of cDNA served as a negative control. The number of cycles needed to reach a threshold value, set arbitrarily within the log-linear phase of amplification for each run, was determined for each sample, and relative concentrations were calculated using the formula $c = 2^{-\Delta C}$, where $\Delta C$ is the number of cycles.

3. Results

The initial mortality tests indicate that glibenclamide is a reasonably toxic chemical to both *B. germanica* (Fig. 1) and *Drosophila* (Fig. 2). While glibenclamide was definitely less toxic than diflubenzuron (DFB) in these species, its speed of action was faster in *B. germanica* at the dose level required to produce the same 48-h mortality as DFB. More importantly, the *B. germanica* nymphs thus affected by glibenclamide showed typical molting problems, some dying with old cuticles still attached and some with malformed adult features such as twisted wings and short abdomens (Fig. 3). Those symptoms are identical to those observed for DFB-affected *B. germanica* cockroaches. The difference in LD$_{50}$ (for *B. germanica*) or EC$_{50}$ (for *Drosophila*) values between DFB and glibenclamide treatment was species- or mode of treatment-dependent—only 2- to 3-fold for *B. germanica* but over 100-fold for *Drosophila*.

The assay of glibenclamide versus DFB chitin synthesis inhibition in isolated integuments of *B. germanica* incubated with $^{14}$C-N-acetylglucosamine clearly showed that glibenclamide is just as potent a chitin synthesis inhibitor as DFB in this in situ test system (Fig. 4). The IC$_{50}$ value of $10^{-8}$ M for both indicates
that glibenclamide is indeed a potent inhibitor when it is delivered on the exposed integument.

The assays of $^{45}$Ca$^{2+}$ uptake by cuticular vesicles isolated from $B$. germanica nymphs indicate that uptake is indeed stimulated by ATP (Fig. 5). However, even in the presence of such a stimulator, the inhibitory effect of glibenclamide was clearly recognizable. The action of glibenclamide on $^{45}$Ca$^{2+}$ uptake was similar to that confirmed previously of DFB in vesicles isolated from cuticular integuments of American cockroach nymphs (Nakagawa and Matsumura, 1994). Such action was reduced when the incubation buffer contained a high concentration of K$^+$, but reduction was much less when K$^+$ was replaced by the same concentration of Na$^+$, especially in the case of vesicles incubated with glibenclamide versus control.

The assays of competitive binding of [$^3$H]-glibenclamide in $B$. germanica cuticular vesicles indicated that all unlabeled compounds were capable of displacing the radioligand, but among them glibenclamide appeared to be most potent, followed by DFB, then dechloro-DFB (Fig. 6). The same experiment with Drosophila vesicles showed the same order of competitive potencies among the compounds (Fig. 7).

A repeat of the competitive binding assay in Drosophila at several concentrations around the $K_D$ values yielded a $K_D$ value for glibenclamide of 44.9 nM, compared to 64.9 nM for diflubenzuron (Fig. 8). The Scatchard plot analysis indicates that $B_{max}$ of glibenclamide binding is in the neighborhood of 50,830 fmol/mg protein, with $1/K_D = 0.021$ nM (Fig. 9), while the DFB $B_{max}$ value is on the order of 69,790

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Fig. 4. Inhibitory effects of varying concentrations of diflubenzuron and glibenclamide on chitin biosynthesis in situ measured by using $[^{14}$C]-N-acetylglucosamine (0.05 Ci) (final concentration = 0.5 μM) and isolated abdominal integuments from newly molted German cockroach, $Blattella$ germanica, nymphs. The integuments were preincubated with either tested compound and thereafter incubated with $[^{14}$C]-N-acetylglucosamine for 3 h, all at 30 °C. Values are means ± SEM. Means are not significantly different.

Fig. 5. Inhibitory action of glibenclamide (2 × 10$^{-6}$ M) and certain diagnostic agents on $^{45}$Ca$^{2+}$ uptake by vesicles prepared from cuticular integment of German cockroach, $Blattella$ germanica, nymphs. The reaction mixture was incubated 5 min in 10 mM MES–NaOH buffer (pH 6.6) containing 2.5 mM MgSO$_4$ and 250 mM sucrose. Values are the mean ± SEM of two or three experimental runs, and three tubes (replications) for each treatment. The bars marked with an asterisk indicate significant differences between glibenclamide and control (Student’s $t$-test, $P < 0.05$).

Fig. 6. Inhibition of total [$^3$H]-glibenclamide binding by glibenclamide, diflubenzuron, and dechloro-diflubenzuron in vesicles isolated from cuticular integment of the German cockroach, $Blattella$ germanica, nymphs. Values are the means of 2–4 independent experiments. Data are presented as the means ± SEM; three replications were used for each experiment.
fmol/mg protein, with $1/K_D = 0.015$ nM, very similar to glibenclamide (Fig. 9).

RT-PCR showed that $D_{SUR}$ is expressed in *Drosophila* larvae. Two different sets of primers gave PCR product fragments of the predicted size (Fig. 10), and DNA base analysis of PCR products confirmed that the sequences, corresponding to bases 2334 through 2492 and bases 2486 through 2783 of the $D_{SUR}$ cDNA,
are identical to those published by Nasonkin et al. (1999), except that base 2472 was C instead of G and base 2625 was A instead of G.

Quantitative RT-PCR values for SUR in paired treatments showed no consistent effect of either test compound on mRNA levels, while unpaired treatments showed large variability within each treatment with little variability between test compound and control for both compounds (data not shown). The product gave a single melting curve peak at 85.1 °C, indicating a clean PCR product.

4. Discussion

The primary objective of the current study has been to test our hypothesis that SUR protein is the major target of DFB in causing disruption of chitin synthesis in both B. germanica and D. melanogaster. To this end, we have shown that: (1) a typical sulfonylurea, glibenclamide, induces the same toxic end results as DFB, (2) glibenclamide disrupts chitin synthesis in isolated cuticular integuments at the same low concentration as DFB in both species, (3) glibenclamide suppresses 

\[ ^{45}\text{Ca}^2+ \] uptake by cuticular vesicles of B. germanica similar to DFB with P. americana, and (4) both compounds show high competitive affinity binding to the glibenclamide binding site. The specificity of DFB action on the binding site is supported by the observation that dechloro-DFB, the less active analog of DFB, showed a much lower binding affinity than DFB. While much more elaborate structure–activity relationship studies would be helpful in confirming this specificity, for the purpose of meeting the objective of this study the above evidence, along with the establishment of the low \( K_D \) value comparable to those determined in mammalian studies, supports our basic hypothesis. The fact that glibenclamide was used as the standard compound by Nasonkin et al. (1999) to demonstrate the functional identity of Drosophila SUR as a sulfonylurea-sensitive ATP-dependent K\(^+\)-channel when expressed in Xenopus oocytes also supports our conclusion.

Having established that all of the above evidence indeed supports our hypothesis, it may be prudent to indicate the topics we did not adequately address in the current study. The most important one is the functional relationship between SUR and chitin synthesis. According to Nasonkin et al. (1999), \( D_{\text{SUR}} \) is expressed during embryogenesis, particularly in the tracheal system, as shown by in situ hybridization. Other areas showing significant \( D_{\text{SUR}} \) expression are protein spiracles and salivary glands. While they mention that \( D_{\text{SUR}} \) is expressed throughout larval and adult stages, no data were presented to show whether it is expressed in the integument. Our current study shows that \( D_{\text{SUR}} \) protein is present in the isolated integuments, as evidenced by specific binding of \( ^{3}\text{H}\)-glibenclamide, but its function in assisting chitin synthesis is not yet explained. One possibility is that \( D_{\text{SUR}} \) acts as a transporter in vesicles carrying N-acetylglucosamine or its processed products, even though in the case of pancreatic \( \beta \)-cells glibenclamide is known to act on the plasma membrane SUR, not on vesicles.

The second important topic we did not address in the current study was the precise mechanism of action of DFB, particularly the action sequence of DFB and glibenclamide in blocking chitin synthesis. Judging by the observation of Nakagawa et al. (1993) that valinomycin, a specific potassium ionophore, also acts as a powerful chitin synthesis inhibitor in intact cockroach integument, it is probable that a glibenclamide-induced equalization of potassium ion concentrations between the outside and inside of the vesicles is the disruptive force in the chitin synthesis process. Accordingly, the main conclusion we can derive from these experiments is that based on the compounds’ affinity for SUR, their immediate action is likely to be direct binding to the receptor, altering its K\(^+\)-channel and leading to subsequent chitin synthesis inhibition (Nakagawa et al., 1993).

Within the scope of the current study, there are a number of items that merit further discussion. The first is the difference in the physicochemical properties of these two study chemicals. The published log \( K_{\text{ow}} \) value for diflubenzuron is 3.89 (Tomlin, 1997). While we could not locate a reference specifying the corresponding value for glibenclamide, its theoretically calculated log \( K_{\text{ow}} \) value is 3.53 (Kasim et al., 2004), which is high among the group of similar medically used sulfonylureas (e.g. 2.44 for acetohexamide (Sangster, 1994a) and 2.69 for tolazamide (Sangster, 1994b)) but close to the DFB value. If this calculated log \( K_{\text{ow}} \) is similar to the real value, the difference in lipophilicity may contribute little to the susceptibility difference shown in the LD\(_{50}\) values.

Perhaps more importantly, there is a significant difference in water solubility between these two compounds: glibenclamide’s solubility is on the order of 3 mg/l at pH 6.0 and 30 mg/l at pH 7.8 (Wunderlich et al., 1999), approximately 40- to 400-fold higher, depending on pH, than the 0.08 mg/l solubility of diflubenzuron (Tomlin, 1997). It is known that many highly water soluble compounds do not readily penetrate the insect cuticle; therefore, it is reasonable to assume that externally applied diflubenzuron will penetrate into the insect body better than glibenclamide. This difference would pertain to the B. germanica nymphs, for which the LD\(_{50}\) values were obtained through topical application (i.e. administered on the outside of the cuticle) for both compounds. As would be expected, diflubenzuron showed higher toxicity than glibenclamide. The lack of difference in chitin synthesis
IC$_{50}$ values for isolated *B. germanica* integuments in buffer solution (Fig. 4) can be explained by the compounds’ access to the inner surface of the isolated integument.

It is not easy to explain, however, the large difference in LD$_{50}$ values observed in *Drosophila* larvae, which received both compounds through their growth medium. One possibility is that penetration of glibenclamide through the insect gut is limited. Another possibility is that metabolic degradation of glibenclamide took place either in the medium or in the *Drosophila* gut, but these are speculations.

Another important item for discussion is whether diflubenzuron’s limited water solubility affects its binding behavior to the receptor. Based on the published solubility value, its maximum concentration in water is approximately 257 nM under our experimental conditions. The binding data points in the current investigation were obtained below this solubility limit, except at the highest concentration tested, and no attempt was made to assess SUR binding values at concentrations higher than 1 μM for either compound. Thus, the $K_D$ and $B_{\text{max}}$ values obtained are not likely be skewed by this physicochemical limitation of diflubenzuron.

Another item worthy of discussion is the nature of “cuticular vesicles” and the possible location of SUR protein in our preparations. Our decision to call these centrifugally precipitated fractions “vesicles” comes from our observation that their response to diflubenzuron is abolished upon ultrasonication (Nakagawa and Matsumura, 1994), indicating that the maintenance of physical integrity is needed for diflubenzuron to influence their ion transporting activities. Having made this point clear, however, it must be emphasized that we still cannot eliminate the possibility that such vesicles do not include those purely made of plasma membrane. Since in pancreas glibenclamide acts on plasma membrane SUR, this point could become an important study subject in the future.

The last discussion item is the direction of K$^+$ flow in vesicles that are affected by these two chemicals. While we do not have firm evidence, if we were to hypothesize, the favored direction would be the outward transport of K$^+$ from the vesicles, which is coupled to Ca$^{2+}$ influx into the vesicles. The observation supporting this hypothesis is that an increase in the outside concentration of K$^+$ (Fig. 5) reduces the inhibitory action of glibenclamide on Ca$^{2+}$ uptake. Certainly, this work has raised a number of new questions which are very important in clarifying the roles of newly discovered SUR proteins.

In conclusion, we have obtained enough evidence to indicate that the insecticide DFB acts on the same target site on the SUR proteins as a typical sulfonylurea, glibenclamide, in *D. melanogaster* and *B. germanica* and that such action by these chemicals is the cause of their inhibitory effect on chitin synthesis. Much more effort will be needed to elucidate the functional role of SUR in normal development and maintenance of insects to fully understand the action mechanisms of benzoyleurea insecticides.

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**References**


receptor family member expressed in the embryonic *Drosophila* dorsal vessel and tracheal system. Journal of Biological Chemistry 274, 29420–29425.


