Target sites of anthelmintics

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SUMMARY

This paper reviews sites of action of anthelmintic drugs including: (1) levamisole and pyrantel, which act as agonists at nicotinic acetylcholine receptors of nematodes; (2) the avermectins, which potentiate or gate the opening of glutamate-gated chloride channels found only in invertebrates; (3) piperazine, which acts as an agonist at GABA gated chloride channels on nematode muscle; (4) praziquantel, which increases the permeability of trematode tegument to calcium and results in contraction of the parasite muscle; (5) the benzimidazoles, like thiabendazole, which bind selectively to parasite β-tubulin and prevents microtubule formation; (6) the proton ionophores, like closantel, which uncouple oxidative phosphorylation; (7) diamphenethide and clorsulon, which selectively inhibit glucose metabolism of *Fasciola* and; (8) diethylcarbamazine, which appears to interfere with arachidonic acid metabolism of filarial parasites and host. The review concludes with brief comments on the development of anthelmintics in the future.

Key words: acetylcholine receptor, levamisole, pyrantel, piperazine, ivermectin, praziquantel, benzimidazoles, closantel, diamphenethide, clorsulon, diethylcarbamazine.

INTRODUCTION

Anthelmintic drugs are used to control, prevent and treat nematode and trematode parasite infestations in both humans and domestic animals. They will continue to be used until effective vaccines are produced and/or hygiene standards have been improved. As anthelmintic drugs have been used continuously for parasite control, resistance has gradually developed and is now found in a number of parasite species against most of the anthelmintics (Prichard, 1994). This resistance occurs more frequently when the same anthelmintic agent has been used intensively and without good hygiene standards to assist control. If resistance to a particular anthelmintic occurs, it is likely that an anthelmintic that has the same mode of action will also be ineffective (cross-resistance). One reason for developing an understanding of the mode of action of anthelmintics is to enable selection of other anthelmintics that will be effective for therapeutic purposes. Another reason for pursuing the mode of action of anthelmintics is that we may be able to understand how parasites develop resistance to the anthelmintics.

There are two major modes of action of anthelmintics. There are the drugs that act on parasite membrane ion-channels and which usually have a more rapid therapeutic effect; the other group acts more slowly on a range of 'biochemical' target sites found in parasites. Table 1 summarizes the target site of anthelmintics that act on membrane ion-channels. The target ion-channels include: the excitatory nicotinic acetylcholine receptor on muscle of nematodes; the inhibitory γ-aminobutyric acid (GABA) receptor channel also present on nematode muscle; and the glutamate-gated Cl^- channel. Table 2 summarizes the modes of action of anthelmintics that act at more 'biochemical' target sites.

The target site (enzyme or ion-channel) of the anthelmintic may be present in the host animal as well as the parasite; it is usually pharmacologically distinct in the parasite in order to permit selective drug action. For example, the benzimidazole anthelmintics bind selectively to nematode β-tubulin to effect their action. β-tubulin is present in the host animal as well but is sufficiently different in its three dimensional protein structure in the mammalian host so that benzimidazole anthelmintics only bind to the nematode β-tubulin molecule. Other anthelmintics act on targets only present in parasites. The avermectins bind to a glutamate-gated Cl^- ion-channel that has only been found in certain invertebrates including nematodes. This permits the very selective toxic action of avermectins against nematode parasites without harming the host animal. Anthelmintics acting on a target protein only present in parasites are often favoured because of advantages of greater safety and selectivity.

This introductory review covers sites of actions of anthelmintic agents including the nicotinic acetylcholine channel of nematodes, the GABA channel, the glutamate-gated Cl^- channel and biochemical sites of action including β-tubulin and glycolytic enzymes. The review concludes with a brief comment on the future development of anthelmintics.

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Table 1. Ion-channel target sites of anthelmintic drugs

Target site (and parasite group)	Generic drug name
Nicotinic acetylcholine receptor (in nematodes) (Martin, 1993; Robertson & Martin, 1993; Evans & Martin, 1996)	Levamisole, butamisole, pyrantel, morantel, bephenium, thenium, methyridine
GABA receptors (in large intestinal nematodes) (Martin, 1985)	Piperazine
GluCl receptor (in nematodes and insect parasites) (Cully *et al*. 1996; Martin, 1996)	Ivermectin, abamectin, doramectin, moxidectin
Membrane calcium permeability (in cestodes and trematodes) (Redman *et al*. 1996)	Praziquantel

Table 2. Anthelmintic target sites other than ion-channels

Target site (and parasite group)	Generic drug name
β-tubulin (in nematodes)	Thiabendazole, cambendazole, oxibendazole, albendazole, albendazole sulphoxide
β-tubulin (in nematodes, cestodes and trematodes) (Roos *et al*. 1995)	Fenbendazole, oxfendazole, mebendazole, flubendazole, febantel, netobimin, thiophanate, triclabendazole
Proton ionophores (concentrated and effective against blood feeders: flukes, *Haemonchus contortus*, *Oestrus ovis*) (McKellar & Kinabo, 1991)	Closantel, rafoxanide, oxyclozanide, brotianide, nitroxynil, niclopholan, hexachlorophene, dibromosalan, niclosamide
Malate metabolism (in immature *Fasciola*) (Edwards *et al*. 1981*a*, *b*)	Diamphenethide
Phosphoglycerate kinase and mutase (in *Fasciola*) (Schulman *et al*. 1982*a*)	Clorsulon
Arachidonic acid metabolism and innate immunity of host (effective against filaria) (Maizels & Denham, 1992)	Diethylcarbamazine

Readers interested in details of the dosages and administration, uses (spectrum of action) and host toxicity, in humans are referred to the recent comprehensive text of Martindale (1996) and for equivalent data for animals are referred to the Compendium of Data Sheets for Veterinary Products (NOAH, 1997).

NICOTINIC ACETYLCHOLINE RECEPTORS

Somatic muscle cells of nematodes possess both synaptic and extrasynaptic nicotinic acetylcholine receptors. This has been shown, for example, by the experiments of Martin (1982) and Harrow & Gration (1985). These electrophysiological experiments involved the use of micropipettes placed intracellularly in muscle cells of the large nematode parasite of the pig, *Ascaris suum*. They showed that application of acetylcholine and the anthelmintics, levamisole, pyrantel and morantel (Fig. 1) resulted in the depolarization and increase in input conductance of the muscle membrane to sodium and potassium. The affects of acetylcholine were antagonized by the nicotinic antagonist tubocurarine. Subsequently patch-clamp studies, (Robertson & Martin, 1993; Robertson *et al*. 1994; Dale & Martin, 1995; Evans & Martin, 1996) have shown that the nicotinic anthelmintics open non-selective cation channels. Each channel is characterized by a particular conductance but the conductance varies between channels recorded from different patches in the range 19–60 pS. The mean open-time of the channels varies with the anthelmintic but is in the range of 0·5 ms to 2·5 ms. Thus with these electrophysiological experiments it has been possible to establish that the nicotinic anthelmintics have an action on a receptor with properties similar to but not identical with the nicotinic receptors in mammalian and vertebrate species. There must be pharmacological differences between the nicotinic receptors of nematodes and those of their host since levamisole is a selective agent producing depolarization and spastic paralysis of the nematode without a significant action on the host

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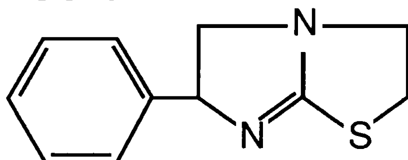

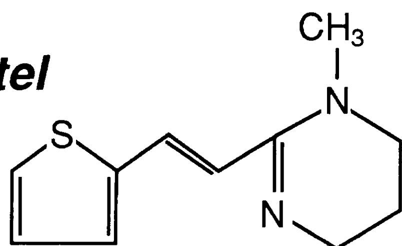

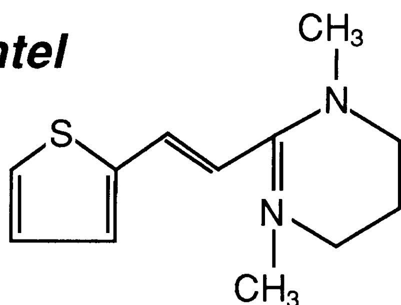

Fig. 1. The chemical structure of some nicotinic anthelmintics.

muscle. Thus the detailed pharmacology of the nematode nicotinic receptor must differ significantly from that of the host nicotinic receptors.

Structure of the nicotinic acetylcholine receptor ion-channel (nAChR)

The torpedo electric organ nicotinic receptor has been studied most extensively (Changeau, Devillers-Thiery & Chemouilli, 1996) because of its high concentration on the electric organ. It is a useful model for the nicotinic receptors of nematodes as well as higher species. The receptor-operated trans-membrane ion-channel is made up of 5 subunits arranged around a central ion-channel like the staves of a barrel. Each subunit is made up of between 437–501 amino acids (Fig. 2). The five subunits of *Torpedo* are: 2α, 1β, 1γ and 1δ, (Fig. 3). The α-subunits of all nicotinic receptors, including nematodes, are believed to contain the ligand binding site and are characterized by the presence of two adjacent cysteines at positions 192 and 193 coupled by -SS-bonds (Fig. 2). Fig. 3A shows that each subunit of the channel is formed from an extracellular loop at the *N*-terminal, four lipophilic α-helical units, M1, M2, M3 and M4 and a short *C*-terminal section. There is also an intracellular loop between M3 and M4. The pore of the ion channel is lined by the M2 sections. The pore of the ion channel formed by the M2 regions forms three negatively charged rings (Fig. 4). There is an extracellular ring lined by glutamate and aspartate which are negatively charged amino acids. There is a middle ring which is narrower and believed to form the selectivity filter of the ion-channel. It too is lined by negatively charge amino acids. This time just glutamate is involved. On the more intracellular region of the pore, another ring is formed, known as the cytoplasmic ring. It also contains negatively charged amino acids. The middle negatively-charged ring forms a narrow selectivity filter, passing only positively charged cations through the ion pore. The nicotinic acetylcholine receptor thus is able to conduct sodium, potassium and to a lesser extent, calcium through the pore. Large cationic structures like local anaesthetics, or indeed the anthelmintics like levamisole, pyrantel and morantel are too large to pass through the channel. They may pass through the extracellular ring but become blocked at the middle ring. Once they reach this site they are capable of producing channel block. Voltage-sensitive channel block by the anthelmintics, levamisole (Robertson & Martin, 1993), pyrantel (Robertson *et al.* 1994), oxantel (Dale & Martin, 1995) and morantel (Evans & Martin, 1996), has been described for the nicotinic receptors of the nematode *Ascaris suum*. These anthelmintics are agonists and antagonists of the nematode *nAChR*.

Vertebrate neuronal nicotinic receptors may be composed of a pentameric structure like that of the *Torpedo* nicotinic receptor but it is believed that only α and β-subunits combine to produce the ion channel (McGehee & Role, 1995). However, there are currently 7 different types of neuronal α-subunit and 3 different β-subunits, each of which may combine in a variety of ways to produce a large number of subtypes of *nAChR*s (McGehee & Role, 1995). It is known that injection of cRNA encoding α- and β-subunits into *Xenopus* oocytes will produce functional nicotinic channels of the neuronal type. These channels have been studied with the patch-clamp technique and it has been shown that they are characterized by heterogeneity (Papke & Heineman, 1996; Papke *et al.* 1996). The channels recorded from in each patch may be separated into subtypes which have different conductances and mean open-times. The subtype may be produced by: variations of the stoichiometry of the subunits of the channel ($\alpha\beta\alpha\beta\beta$ *vs.* $\alpha\beta\alpha\beta\alpha$); variations in the subunit composition ($\alpha\beta_2\alpha\beta_2\beta_2$ *vs.* $\alpha\beta_3\alpha\beta_3\beta_3$ – the subscript here refers to the different β subunit structure not the stoichiometry); or variations in the arrangement of the pentameric channel (i.e. $\alpha\alpha\beta\beta\beta$ *vs.* $\alpha\beta\alpha\beta\beta$).

Studies on the soil nematode, *Caenorhabditis elegans*, have shown that three genes: *lev-1*, *unc-38*

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ACH1

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                    1          11          21    *    31          41
MSVCTLLISC  AILAAPTLGS  LQERRLYEDL  MRNYNNLERP  VANHSEPVTV  HLKVALQQII
         51          61          71    *    81          91         101
DVDEKNQVVY  VNAWLDYTWN  DYNLVWDKAE  YGNITDVRFP  AGKIWKPDVL  LYNSVDTNFD
                              S---  ----------  S
        111         121                                 151        161
STYQTNMIVY  STGLVHWVPP  GIFKISCKID  IQWFPFDEQK  CFFKFGSWTY  DGYKLDLQPA
                                    SS                      ----------
        171         181         191         201         211
TGGFDISEYI  SNGEWALPLT  TVERNEKFYD  CCPEPYPDVH  FYLHMRRRTL  YYGFNLIMPC
-------M1-  --          ----------  -M2-----
                    241                     261         272     ------
ILTTLMTLLG  FTLPPDAGEK  ITLQITVLLS  ICFFLSIVSE  MSPPTSEAVP  LLGIFFTCCM
-----M3---  ----
                    301         311         321         331        341
IVVTASTVFT  VYVLNLHYRT  PETHDMGPWT  RNLLLYWIPW  ILRMKRPGHN  LTYASLPSLF
        351         361         371         381         391        401
STKPNRHSES  LIRNIKDNEH  SLSRANSFDA  DCRLNQYIMT  QSVSNGLTSL  GSIPSTMISS
                                                              --------
        411         421         431         441         451
NGTTTDVSQQ  ATLLILHRIY  HELKIVTKRM  IEGDKEEQAC  NNWKFAAMVV  DRLCLYVFTI
----M4----  ---
                    481
FIIVSTIGIF  WSAPYLVA
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UNC-38

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                         191         201
     .....NSRV  AKRRAKNYPS  CCPQSAYIDV  TYYLQL...
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Fig. 2. Amino-acid sequence of two α-subunits from *C. elegans*. Top: Amino acid sequence of an nAChR α-subunit (ACH1_CAEEL) from *C. elegans* determined from the gene sequence (Ballivet *et al*. 1996). During the synthesis of the receptor the signal sequence (*italics*) is cut off. The four putative membrane-spanning helical units M1, M2, M3 and M4 are indicated. The asterisk indicates an asparagine residue that is one site of high glycosylation. The region YDCC (tyrosine, aspartate, cysteine, cysteine) is the putative agonist binding site. The M2 region forms the putative pore region of the ion-channel. Bottom: Partial sequence of *unc-38* (Fleming *et al*. 1993 EMBL Accession Number: X98600) the α-subunit of *C. elegans* adult muscle receptor. Note that an extra amino acid proline separates the tyrosine (189) and the two cysteine (192, 193) amino acids. Deletion of the extra amino acid of *unc-38* (one from between tyrosine and the double cysteines) may remove the selective binding of levamisole and make the receptor like a mammalian receptor.

and *unc-29* of the 10–11 genes involved in levamisole resistance of this nematode (Table 3) encode the subunits of nicotinic ion-channels (Lewis *et al*. 1980; Fleming *et al*. 1997). Evidence for this is based on the homology of the *lev-1*, *unc-38* and *unc-29* amino acid sequences and the fact that when co-expressed in *Xenopus* oocytes they will produce functional channels gated by levamisole. Pairwise intranuclear injection of cDNA for *unc-38* along with *lev-1* is capable of producing functional channels (Fleming *et al*. 1997). Similarly, pairwise injection of *unc-38* with *unc-29* will also produce functional channels. This implies that the nicotinic receptor of *C. elegans* and perhaps that of nematodes could be comprised of varying combinations of subunits. An illustration of this varying structure which may make up the nicotinic channel is shown in Fig. 5: even in this illustration the number of possible arrangements is limited by maintaining the *unc-38* subunits, required for agonist binding, fixed in number and position. It is pointed out that other α subunits are also recognized in *C. elegans* including the subunit *ACh1* (also referred to as CE21, Ballivet *et al*. 1996) and have been cloned and expressed. Interestingly *ACh1* will express as a homo-oligomer in *Xenopus* oocytes and produce channels sensitive to nicotine but not levamisole. One explanation for the difference in the levamisole-sensitivity of channels produced with *unc-38* subunits in contrast to channels with *ACh1* subunits may relate to the addition of and extra amino acid at the putative agonist binding site between tyrosine (189) and cysteine (192) in *unc-38* which is not present in the levamisole insensitive *ACh1* subunit (Fig. 2).

It is believed that *unc-38* is required for the ligand-binding site. Evidence for this is based on the homology of *unc-38* with the α-subunit of torpedo and the fact that exclusion of *unc-38* from the pairwise cRNA injections into *Xenopus* oocytes results in the failure of expression.

It is known that the precise pharmacological profile of neuronal nicotinic receptors of vertebrates can vary with the molecular structure of the ion channel (Covernton *et al*. 1996). It varies with the molecular structure of the α-subunit and is also influenced by the β-subunit structure. It has been suggested that the agonist binding sites on nicotinic acetylcholine receptors may actually be on the interface between the α and β subunits (Karlin & Akabas, 1995). Thus in nematodes with the *unc-38*,

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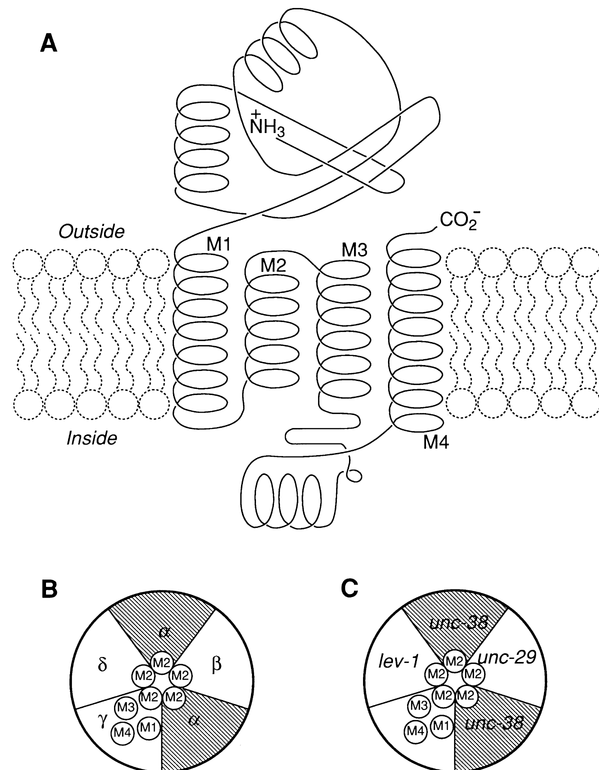

Fig. 3. The structure of the nicotinic channel. A: Diagram of the putative arrangement of the amino acid sequence in the membrane with the 4 α-helices M1, M2, M3 and M4 form transmembrane segments. There is a cytoplasmic loop between M3 and M4. B: The subunit arrangement of the *Torpedo* electric organ nicotinic receptor. Note that the M2 region forms the lining of the channel pore. C: A possible arrangement of the subunits *unc-38*, *unc-29*, *lev-1* that may make up the levamisole receptor. (Derived from Noda *et al.* 1983).

unc-29 and *lev-1* subunits, one could produce a variety of combinations of *unc-38* with *unc-29* and *lev-1* to produce the *nAChR* subtypes. Each of these particular combinations of ion channel will have slightly different pharmacological profiles and biophysical kinetics. It may be possible to separate out the properties of some of the individual combinations biophysically since they may have different conductance levels and mean open times.

We have examined the nicotinic receptor on *A. suum* muscle at the single channel level, using acetylcholine, levamisole, pyrantel and morantel as the agonists (Pennington & Martin, 1990; Robertson & Martin, 1993; Robertson *et al.* 1994; Evans & Martin, 1996). In all of these single channel studies, we have found that there is considerable variation in the conductance level of the channel observed between patch recordings. The conductance levels of the single channel currents have ranged between 15 pS at the lowest extreme up to 60 pS at the highest extreme. In some individual patch experiments it has been clear that more than one conductance level is present. The origin of this variability may be explained if we have different subunit combinations, some producing small conductance levels and others producing higher conductance levels. There are many other causes of variability. Our source of *Ascaris* varies dramatically and could easily influence the particular observations because factors like diet may alter the biophysical properties of the lipid membrane in which the ion channels site. To reduce this possibility, we have now conducted experiments on a smaller nematode, *Oesophagostomum dentatum* that may be infected from laboratory-maintained strains and produced in pigs under more controlled conditions. Our experiments on the nicotinic receptors of *O. dentatum* activated by levamisole also showed great variability. Fig. 6 shows an example of a channel recording at -75 mV where there are at least two amplitude current levels present. This implies that in this patch there are at least two different sub-types of nicotinic receptor, each may have a slightly different pharmacology, one channel type may be more resistant to opening by levamisole than the other. We have seen evidence suggesting that there are up to 4 nicotinic receptor subtypes present in *O. dentatum* (Martin *et al.* 1997). This heterogeneity may facilitate the development of anthelmintic resistance to nicotinic anthelmintics and perhaps other anthelmintics that act on membrane ion channels.

GABA AGONIST: PIPERAZINE

Piperazine is a heterocyclic ring without a carboxyl group. Despite this, it acts as a simple GABA agonist and gates open GABA receptors on the somatic muscle of nematodes (Del Castillo, De Mello & Morales, 1964; Corbett & Goose, 1971; Martin, 1985). It increases the Cl^- conductance of the muscle membrane leading to an increase in the membrane potential (a hyperpolarization) and a reduction in excitability. This leads to a relaxation of the body muscle and flaccid paralysis. Piperazine is effective against large intestinal nematodes and is potentiated by the presence of a high pCO$_2$. The CO_2 may interact with the heterocylic ring of piperazine and substitute for the carboxyl group of GABA.

AVERMECTINS

The avermectin anthelmintics (see Fig. 7) include ivermectin, abamectin, doramectin, and moxidectin, Their mode of action is to increase the Cl^- permeability of nerve and muscle membrane of invertebrates but the identity of the target ion-channel has been controversial (see Arena, 1994, for a review). The mode of action does not appear to be via GABA-gated Cl^- channels. Expression cloning experiments using *Xenopus* oocytes have suggested an action of avermectins on a glutamate-gated Cl^- (GluCl) channel (Cully *et al.* 1994). The channel is presumably a pentomer like that of the nicotinic

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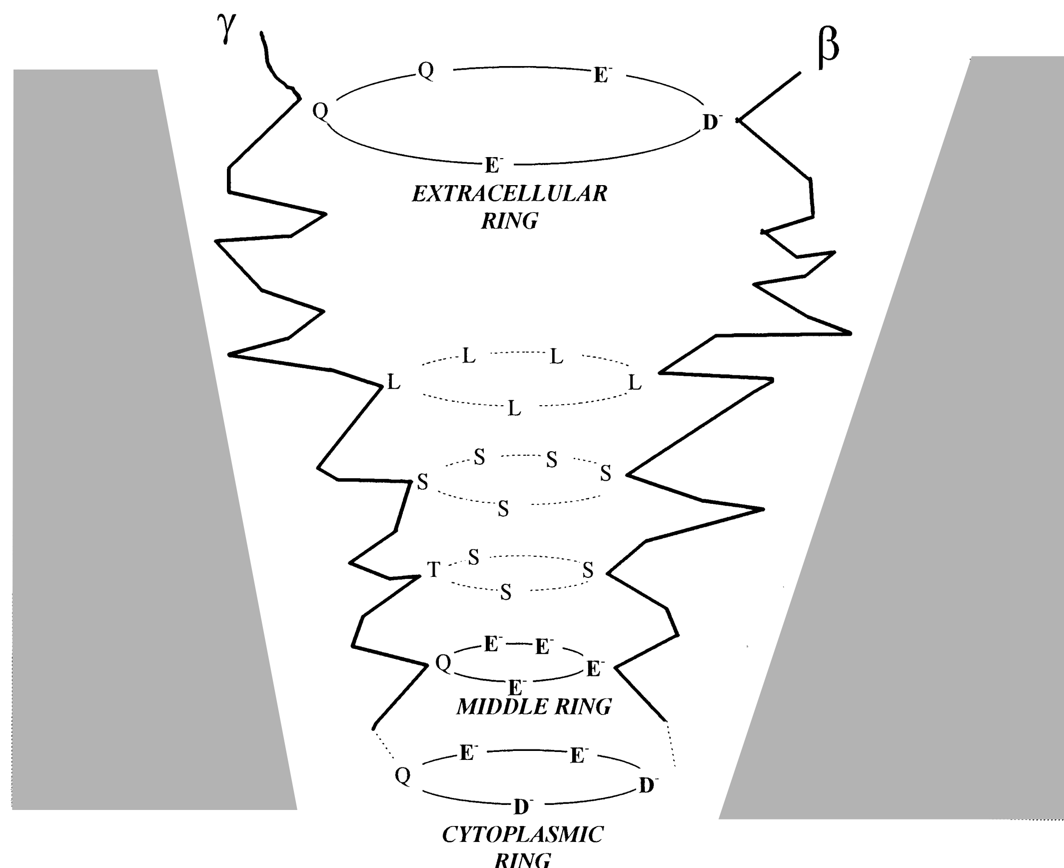

Fig. 4. The structure of the *Torpedo* nicotinic (derived from Revah *et al*. 1990). The organization of the pore of the nicotinic ion-channel showing: the presence of the outer extracellular ring lined with glutamate and aspartate amino acids; the middle ring which appears to act as a selectivity filter and is lined with glutamate amino acids forming a charged negative ring; the inner cytoplasmic ring lined with aspartate and glutamate amino acids.

Table 3. Genes responsible for levamisole resistance in *C. elegans* (Fleming *et al*. 1996)

Gene	Chromosome	Product
unc-38	I	α-subunit
unc-29	I	Non α-subunit
lev-1	IV	Non α-subunit
unc-74	I	Receptor expression
unc-50	III	Receptor expression
lev-8	X	Receptor expression
lev-9	X	Receptor expression
lev-10	I	Receptor expression
lev-11	I	Muscle specific Contraction defect
unc-22	IV	Muscle specific Contraction defect

Other *nAChR* subunit genes have been identified in the *C. elegans* genome. These subunits have not yet been demonstrated to be involved in levamisole resistance. They include: *ach1*, *ach2*, *ach3*, *deg-3*, *t09a5.3* and *ZC504*, *FO3F8.2*.

receptor but the equivalent of the M2 region which forms the ion channel pore of the *nAChR* channels is lined with positively charged sites so that the channel is selectively permeable to anions (Cl^-). The pentomer may be comprised of GluCl-α subunits which contain the glutamate binding site and the GluCl-β subunit that contains the ivermectin binding site.

The locations of the glutamate-gated chloride channel in nematodes were not determined by the expression studies of Culley *et al*. (1994). Molecular experiments using the *lac-Z* marker showed that the GluCl β-subunit of the glutamate channel was expressed in the pharyngeal muscle of *C. elegans* (Laughton, Wolstenholme & Lunt, 1995). The location of the GluCl-α subunit was not determined. As a result of these experiments, Martin (1996) used a two micro-electrode current clamp technique on the pharyngeal muscle of *A. suum*. These experiments demonstrated that the parasite possesses glutamate receptors that gate chloride channels and that this channel is potentiated by ivermectin analogues.

Fig. 8 shows the effect of application of glutamate on the pharyngeal muscle of *Ascaris* which is to produce a brief hyperpolarization and a reversible increase in conductance of the muscle. It also shows the effect of the application of milbemycin, an avermectin. Recent molecular experiments with *C. elegans* on an ivermectin resistance strain (*avr-15*)

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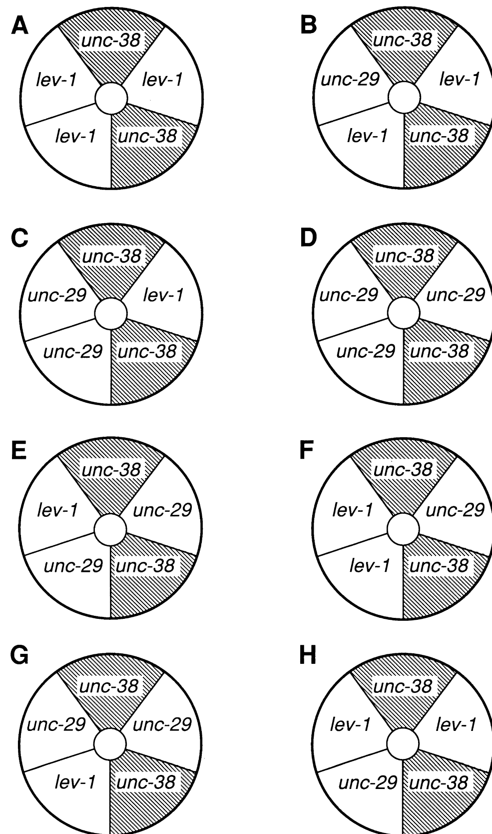

Fig. 5. Diagram of some possible subunit arrangments of *unc-38*, *unc-29* and *lev-1* subunits forming the nematode nAChR channel sensitive to levamisole. The *unc-38* is equivalent to the α subunit of vertebrate nicotinic channels. The *unc-29* and *lev-1* subunits are equivalent to vertebrate β subunits. The diagram shows that there are up to 8 different combinations possible if two *unc-38* subunits have to be maintained in the same relative position. If the conductance of the channel were altered only by the number of *unc-29* or *lev-1* subunits present in this scheme then 4 channel types would be distinguished on a basis of channel conductance. The diagram also illustrates that if the binding of the agonist molecule is at the interface of the *unc-38* subunit and an adjacent molecule then the binding of the agonist (including levamisole) will depend on subunit composition (derived from Martin *et al*. 1997).

suggests that it is a GluCl-α_2 subunit that is present in the pharyngeal muscle of *C. elegans*, not the GluCl-α_1 subunit (review, Cully *et al*. 1997). The location function and expression of the GluCl-α_1 remains to be determined. It does not appear to be present in the pharynx. The distribution of this subunit may help to determine other sites of action of the ivermectins.

CALCIUM PERMEABILITY: PRAZIQUANTEL

Praziquantel is used for the control of schistosomiasis and cestode infections. It has little action against nematode parasites. Its mode of action has been reviewed recently (Redman *et al*. 1996). Application of praziquantel to *Schistosoma mansoni* results in a slow depolarization of the tegument. Associated with the depolarization is an increase of influx of calcium (Fetterer, Pax & Bennett, 1980; Mehlhorn *et al*. 1981; Wolde-Mussie *et al*. 1982; Thompson, Pax & Bennett, 1984). The mode of entry of calcium is not known but Fig. 9 illustrates some possible routes. It is, however, known that its activity may be blocked by high concentrations of magnesium, lanthanum, nickel, and cobalt. The site of action of praziquantel appears to be the tegument of *Schistosoma* as application of praziquantel to isolated body muscle cells from *Schistosoma* has little effect. It is known that application of praziquantel to a muscle preparation from snails mimics the application of caffeine. In fact, prior application of caffeine to this snail muscle preparation pre-empts and abolishes the effect of praziquantel in low calcium extracellular solutions (Gardner & Brezden, 1984).

Thus it appears that the site of action of praziquantel may involve the same site as caffeine. Caffeine is known to act on a calcium-induced calcium release (CICR) channel in the sarcoplasmic reticulum of muscle. This channel releases a flood of calcium into the cytoplasm from the sarcoplasmic reticulum and is triggered by a smaller rise in cytoplasmic calcium produced through voltage-activated calcium channels in the cell (sarcolemma) membrane. The CICR channel of sarcoplasmic reticulum has a high conductance of several hundred pS and has some similarity to the large conductance channel in the tegument of *Schistosoma* (Day, Bennett & Pax, 1992).

It is also known that following the application of praziquantel through *S. mansoni* antigens are exposed that were not previously available to the host. As a result of this exposure, presumably by damage to the outer bilayer of the double bilayer of the tegument, host inflammatory cells move in to attack the schistosome. The exposure of the antigen may be a secondary effect of the elevated calcium since maintained calcium levels not only elevate the exposure of antigens but there is a blebbing of the schistosome membrane (Redman *et al*. 1996).

Most of the mode of action studies on praziquantel have involved *S. mansoni*. Less is known about the direct effect of praziquantel on cestode parasites. It is presumed the mode of action on both groups of parasite is the same.

BENZIMIDAZOLES: β-TUBULIN

The benzimidazole group of anthelmintics including thiabendazole, mebendazole and fenbendazole, are broad-spectrum anthelmintics which have an action against gastrointestinal nematodes and in some cases, at a higher concentration, actions against trematodes. Their mode of action is now known to involve binding to β-tubulin. β-tubulin along with

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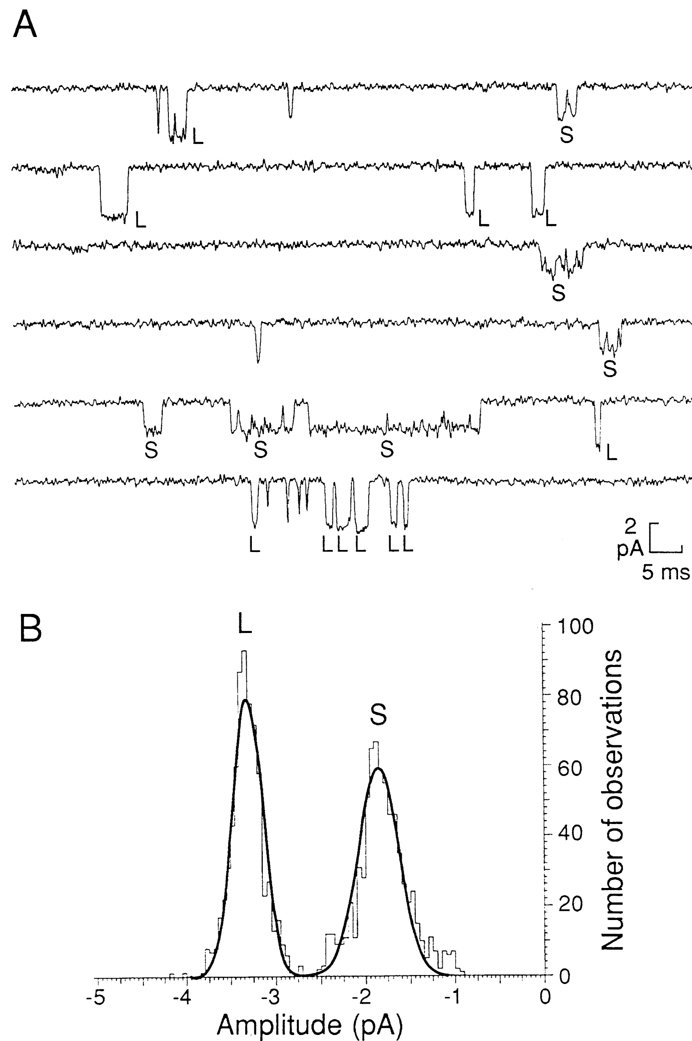

Fig. 6. A: Cell-attached patch recording at -75 mV showing a small (S) and larger (L) nAChR channel present. B: Amplitude histogram of open-channel amplitudes. Smaller conductance channel: mean current $= -1{\cdot}8$ pA $\pm 0{\cdot}2$ (24·8 pS) labelled S in Fig. 5A. Larger conductance channel: mean current $= -3{\cdot}3 \pm 0{\cdot}2$ pA (43·8 pS) labelled L in Fig. 5A). Histograms of the amplitudes of the open-channel currents were fitted with Gaussian distributions. 49% of the events were to the smaller amplitude, 51% were to the larger amplitude.

α-tubulin polymerizes to form microtubule structures inside the cells of nematodes and the host animal (Stryer, 1995).

The mode of action of the benzimidazoles started to be understood when it was realized that mebendazole given to *Ascaris* produced damage to the intestinal cells of the parasite. It was found that there was a loss of cytoplasmic tubules of both the intestinal cells and teguments of cestodes and nematodes (Van den Bossche & De Nollin, 1973; van den Bossche, Roshette & Horig, 1982). This was associated with a loss of transport of secretory vesicles and the failure of the intestinal cells to take up glucose. Thus following application of mebendazole, the parasite starved. It was found in *Ascaris* that mebendazole bound to cytoplasmic proteins that had a molecular weight of 50 kDa and 100 kDa which are monomers and dimers of tubulin. It was found that the benzimidazoles anthelmintics competed for the binding site on β-tubulin with colchicine, a substance known to block cell division in the metaphase (Sangster, Prichard & Lacey, 1985; Lacey & Gill, 1994). Microtubules serve a variety of intracellular functions including transport of cytoplasmic secretory vesicles (Stryer, 1995).

Structure and function of microtubules

Microtubules are formed in a dynamic process by the combination of two 450 amino acid proteins that are known as α-tubulin and β-tubulin (Stryer, 1995). The formation of the microtubules involves the polymerization of tubulin at one end, known as the positive pole, and the depolymerization at the other end, known as the negative pole. Microtubules that form are made up of thirteen tubulin molecule rings (6 α-tubulin plus 7 β-tubulin, alternating with 7 α-tubulin plus 6 β-tubulin rings). There are a number of factors which encourage polymerization and these factors include GTP, magnesium and an increase in

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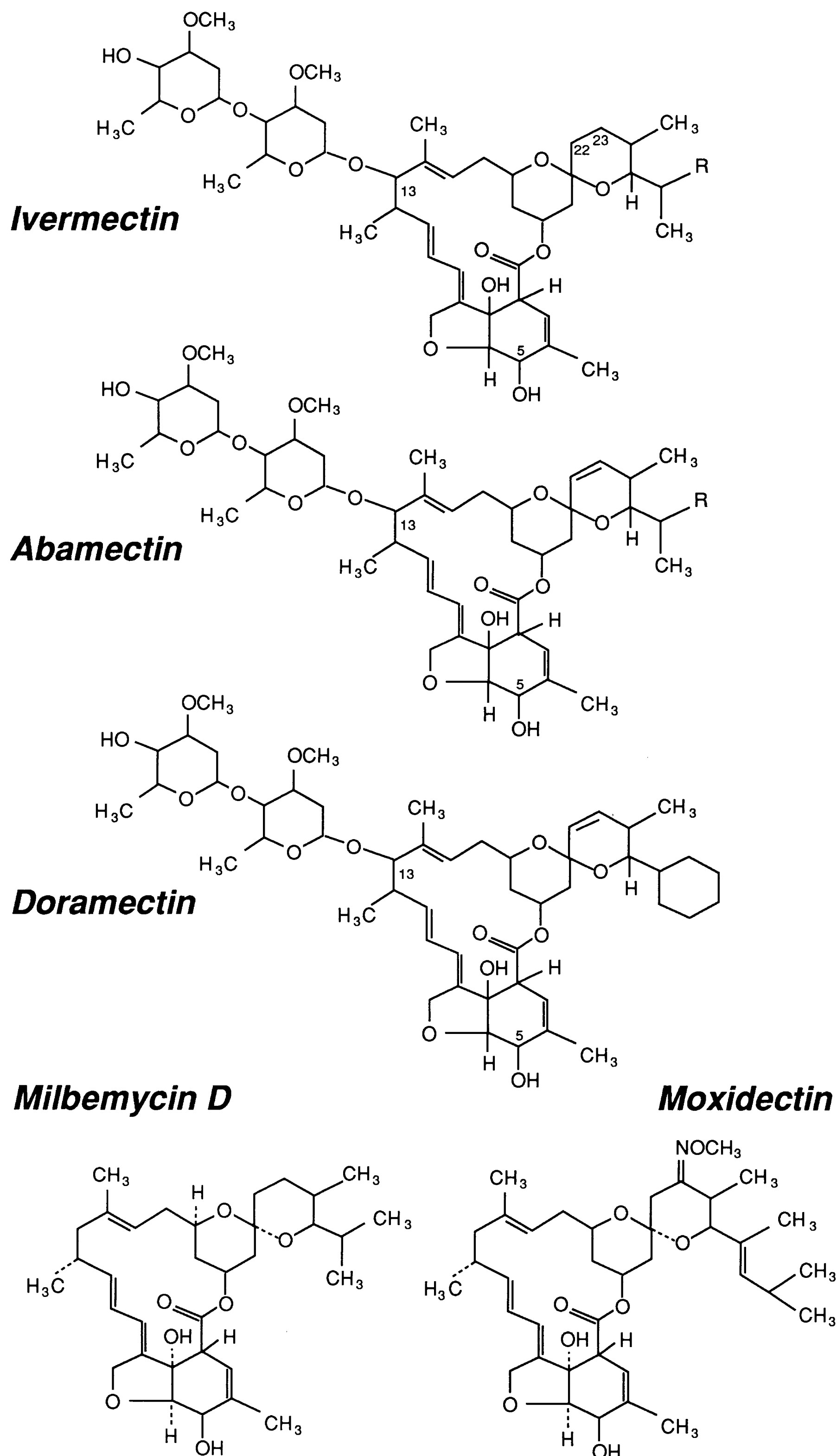

Fig. 7. The chemical structures of the avermectin anthelmintics.

temperature: a fall in temperature to 4 °C or the presence of calcium or calmodulin favours depolymerization. The formation of microtubules can be prevented or inhibited by substances that bind to the positive pole (the leading edge) of polymerization. This process is known as capping and it can be achieved by colchicine, vinblastine, vincristine, or the benzimidazoles, which do this by binding to β-tubulin molecules.

Thus we know that the mode of action of benzimidazole anthelmintics is to bind to nematode β-tubulin more selectively than the host microtubule β-tubulin. The onset of benzimidazole anthelmintic action is therefore slower than that of the anthel-

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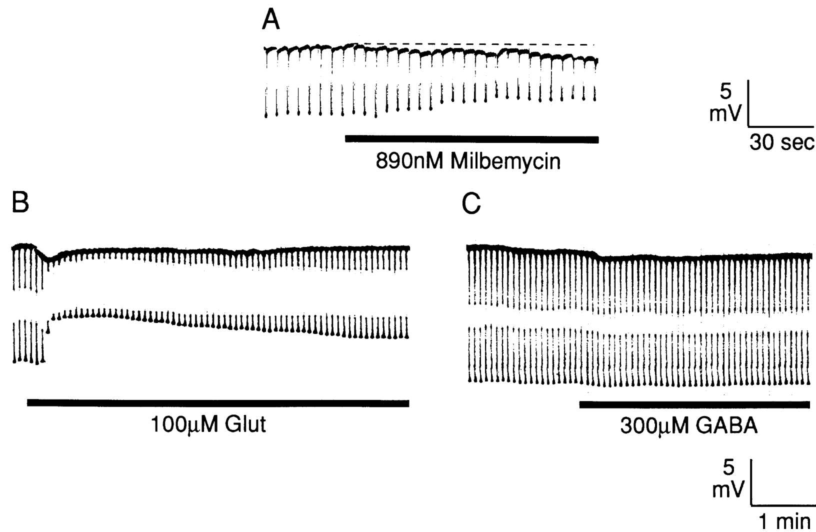

Fig. 8. A: Effect of 890 nM milbemycin D on the membrane potential and input conductance. Milbemycin slowly produced an increase in input conductance that was not reversed on washing (not shown). B: Effect of application of 100 μM L-glutamate on membrane potential and input conductance. Different preparation from B. Glutamate (applied during horizontal bar) produced a transient small hyperpolarization of 1 mV associated with an input conductance change from 157 μS to a peak of 429 μS (ΔG: 272 μS) desensitizing to 231 μS (ΔG: 74 μS) after 4 minutes. C: After washing the preparation the input conductance of the pharynx returned towards control levels (142 μS) and the effect of 300 μM GABA was tested without effect (applied during the horizontal bar). The lack of effect of GABA was not due to desensitization because subsequent application of 100 μM L-glutamate increased the input conductance again (not shown) (derived from Martin *et al*. 1995).

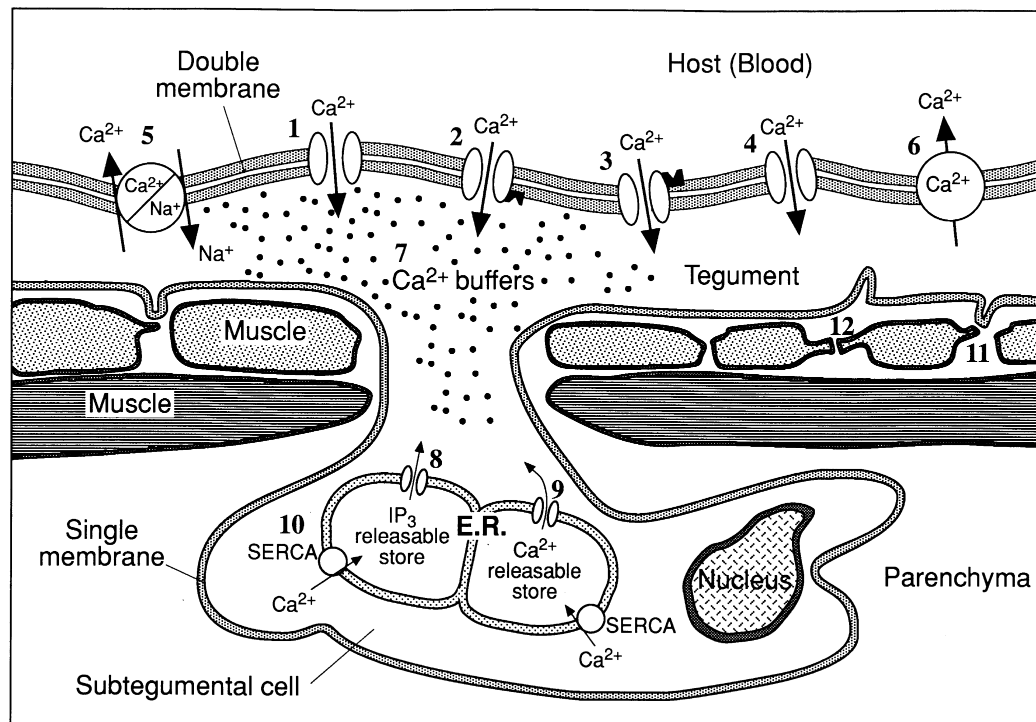

Fig. 9. Diagram of the structure of the body wall of *Schistosoma mansoni* showing the possible sites of action of praziquantel and mechanisms of Ca^{2+} transport into and out of the tegument. 1: voltage-activated Ca^{2+} channel. 2: Intracellular messenger activated Ca^{2+} channel. 3: Extracellular receptor operated Ca^{2+} channel. 4: Non-selective cation channel also allowing entry of Ca^{2+}. 5: Na^+/Ca^{2+} exchanger. 6: Ca^{2+} ATPase pumping out Ca^{2+}. 7: Intrategumental Ca^{2+} buffers. 8: IP_3 releasable store from the sarcoplasmic reticulum. 9: Ca^{2+}-induced Ca^{2+} release channel (CICR channel). 10: Ca^{2+} ATPase pump: sarcoplasmic endoplasmic reticulum Ca^{2+} (SERCA). 11: Electrical junction between muscle cell and tegument. 12: Electrical junctions between muscle cells. If praziquantel acts like caffeine it would act on site 9 (derived from Redman *et al*. 1996).

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mintics that work directly on ion-channels. Benzimidazoles must produce starvation of the nematode by intestinal disruption and/or inhibition of egg production.

In *Haemonchus contortus*, two types of β-tubulin have been recognized: isotype 1 and isotype 2 (Roos, Kwa & Grant, 1995). These two isotypes have separate genes and have a number of alleles: there are up to six alleles for isotype 1 and up to 12 alleles for isotype 2. The function of the specific isotopes is not known in nematodes. Roos *et al*. (1995) has shown that there is a reduction in the number of isotype alleles for β-tubulin during the appearance of benzimidazole resistance. Observations made on the appearance of resistance of *H. contortus* showed that there was a progressive loss of alleles for isotype 1 and a total loss of alleles for isotype 2. Resistance may be explained by loss of susceptible phenotypes of the β-tubulin and the survival of resistance phenotypes. It is known that benzimidazole resistance in fungi is associated with the appearance of a different form of β-tubulin (Fujimura *et al*. 1992). It is characterized by the appearance of a tyrosine instead of phenylalanine in position 200 on the β-tubulin chain. Unfortunately, mammalian β-tubulins also have tyrosine at position 200 (Lewis, Lee & Cowen, 1985) so it is unlikely that benzimidazole resistance may be overcome by changes in the chemistry of the drug since the fungal benzimidazole has learned to mimic the host β-tubulin. If such a phenomenon occurs in nematode parasites, it would result in the inability of the benzimidazoles to exert a selective toxic effect.

PROTON IONOPHORES

The proton ionophores include the salicylanilides and substituted phenols (for a review see McKellar & Kinabo, 1991). They include closantel, rafoxanide, oxyclozanide and brotianide. They are a range of compounds that have anti-fluke action. Their mode of action is that of a proton ionophore which is usually called an oxidative phosphorylase uncoupler. Each of the salicylanilides or the substituted phenols contains a detachable proton group. The compounds are all very lipophilic so that they dissolve in the phospholipid membranes of cells and may shuttle across the membrane carrying protons. They do this particularly across the inner mitochondrial membrane and will reduce any proton gradient. Since a proton gradient is necessary for the production of ATP by mitochondria there is an inhibition of the energy production by the mitochondria following poisoning by the salicylanilide.

The selective mode of action of the series of compounds that act as oxidative phosphorylase uncouplers is believed to involve and to be associated with the very high plasma protein binding of these compounds which are effective therapeutically. The anthelmintics used in this way have a very long half-life of more than fourteen days. More than 95% of the salicylanilides are plasma protein bound. Thus the selective action of these anthelmintics may be explained by an action on blood-sucking parasites concentrating the anthelmintic in the parasite as it takes in high levels of plasma protein.

Pax & Bennett (1989) have examined the pH gradient across *S. mansoni* following treatment with salicylanilides. They were able to demonstrate an effect on the tegument of the parasite indicating that this may be a site of action for these anthelmintics. Thus mitochondria may not be the only site at which these compounds exert their effect.

MALATE METABOLISM

The compound diamphenethide is an agent that is effective against immature *Fasciola hepatica* in the liver but less effective against *Fasciola* in the bile ducts. Diamphenethide is deacetylated in the host liver to an active monoamine and diamine (Coles, 1976). The amine of diamphenethide has an action which produces an elevation of malate concentration in *Fasciola* (Edwards *et al*. 1981*b*). Malate is an intermediary break-down product of glucose in this parasite. It is known that dopamine, a putative neurotransmitter in *Fasciola*, has a protective effect against diamphenethide but the mode of action of a diamphenethide has yet to be defined in greaer detail (Edwards *et al*. 1981*a*).

PHOSPHOGLYCERATE KINASE AND MUTASE INHIBITION

Clorsulon is very similar in structure to 1,3-diphosphoglycerate and because of this it inhibits the enzymes phosphoglycerate kinase and phosphoglyceromutase of *Fasciola* (Schulman & Valentino, 1982; Schulman *et al*. 1982*a*). Thus it prevents the full breakdown of glucose by the Emden-Meyerhoff pathway and inhibits glucose utilization. The inhibition of the phosphoglycerate kinase was competitive with clorsulon inhibiting the binding of 3-phosphoglycerate and ATP to the kinase. Clorsulon thus has a dose-dependent inhibitory effect on immature and mature *Fasciola* (Schulman *et al*. 1982*b*).

ARACHIDONIC ACID METABOLISM AND INNATE IMMUNITY

Diethylcarbamazine is a piperazine derivative used in low doses as an anti-filarial drug. Although it has a good microfilaricidal action, it has limited macrofilaricidal action. Its action against some adult helminths requires nearly 10 times the prophylactic filarial dose and may involve another mode of action.

Although the structure of diethylcarbamazine might suggest it acts like piperazine, electro-

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physiological studies following bath application to *A. suum* have shown that it does not mimic the actions of piperazine (Martin, 1982). It appears that there is little direct action on filariae when diethylcarbamazine is added to isolated parasites. It has quite marked *in vivo* effects in experiments in contrast to its *in vitro* effects. Following intravenous injection it produces an anti-filarial action within about 4 minutes (Hawking & Laurie, 1949). In mice that have had their immune system impaired genetically (nude, athymic mice) which are infected with *Brugia pahangi* there is a marked reduction in microfilariae following administration of diethylcarbamazine (Vickery, Nayar & Tamplin, 1986). These experiments show that T-cells and T-dependent responses (IgG and IgE) are not involved.

It is known that diethylcarbamazine has an antagonistic action on the metabolism of arachidonic acid which is produced from the breakdown of cell membranes under the influence of phospholipase A_2 (Maizels & Denham, 1992). It is often said that 5-lipoxygenase is the enzyme which is inhibited by diethylcarbamazine. But it has also been suggested that synthesis of LTA_4 by LTA_4-synthase from 5-HPETE is blocked by diethylcarbamazine in mast cells (Mathews & Murphy, 1982; Razin *et al*. 1984). Diethylcarbamazine is also believed to block production of PGI_2 (prostacyclin) by inhibition of endothelial cyclo-oxygenase (Kanesa-Thasan, Douglas & Kazura, 1991). It does not appear to have any effect on the production of thromboxane (TXA_2). Microfilariae also produce their own PGI_2 and PGE_2 and these too are inhibited by diethylcarbamazine during treatment (Kanesa-Thasan *et al*. 1991). It seems then that diethylcarbamazine inhibits production of PGI_2 and PGE_2 by both endothelial cells and microfilariae. These two eicosanoids are involved in the control of blood vessel tone and the inhibition of neutrophil aggregation and granulocyte aggregation.

Thus a reasonable explanation for the action of diethylcarbamazine at low doses to prevent microfilariae is that it alters the metabolism of arachidonic acids in the host endothelial cells and microfilariae. As a result of this, there is constriction of the blood vessels and aggregation of the host granulocytes and host platelets. Thus it appears that diethylcarbamazine activates an innate immune response rather than an adaptive response (Maizels & Denham, 1992). This mode of action could explain why diethylcarbamazine does not have an effect *in vitro* against the microfilariae and is not effective in non-immune animals.

FUTURE DEVELOPMENT OF ANTHELMINTICS

Continued economic losses in animal production and human disease due to parasites are still of concern to industrial chemists looking for new anthelmintic agents. The intensive use of drugs for the control of nematode parasites has and will continue to lead to the development of resistance. New anthelminitics are needed for the future but the huge costs associated with the development on new compounds and the small economic size of the market for anthelmintics will make future development slow. The limited market size and concerns over resistance and residues are reflected in the decrease in patent applications. With increased knowledge of parasite physiology, particularly the nicotinic acetylcholine receptors, the GABA receptors and the glutamate-Cl^- channel as examples, a directed rational search for new leads should be possible. Surveying natural substances (plant extracts and antibiotics) is also in an early stage with only a small fraction of the microbial ecosystem having been surveyed. Recently, the dioxapyrrolomycins as lead compounds for AC303630 (Hunt, 1994), the anthelmintic cyclodepsipeptide PF1022A (Martin *et al*. 1996) and paraherquamides (Schaeffer *et al*. 1990) have been reported. These show the continuing potential of such an approach. There remains a demand for new potent broad-spectrum anthelmintics which are effective against resistant parasites, harmless to the environment and can be cost-effective. Anthelmintics with different parasite target sites are to be expected in the future.

ACKNOWLEDGEMENTS

R. J. M. is pleased to acknowledge the financial support of The Wellcome Trust who have supported my electrophysiological work on the nicotinic anthelmintics and the glutamate receptors of *Ascaris*. H.B. is pleased to acknowledge the support of the Danish National Research Foundation.

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https://doi.org/10.1017/S0031182097001029 Published online by Cambridge University Press