OPISTHORCHIS VIVERRINI: EFFECT OF PRAZIQUANTEL ON THE ADULT TEGUMENT

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Abstract. Ultrastructural changes of the tegument of adult liver flukes, *Opisthorchis viverrini*, after *in vitro* incubation in Minimal Essential Medium containing 0, 0.1, 1.0 and 10.0 μg/ml of anthelminthic praziquantel for 5, 15, 30, 45 and 60 minutes were investigated by scanning (SEM) and transmission (TEM) electron microscopy. SEM observations showed that the surface damage was composed of blebbing due to the swelling of microvilli, followed later by the disruption of these structures to form lesions that caused the erosion and desquamation of the surface. Sensory papillae, by contrast, appeared relatively unaffected. The surface changes could be observed at all doses but the extent of damage increased with increasing duration of incubation and concentration of the drug. The ventral as well as the dorsal surfaces exhibited similar change, whereas the anterior part tended to be damaged less than the posterior part. Under TEM observations, the earliest sign of changes was the depolymerization of the microtrabecular network in scattered foci, which resulted in the formation of non-membrane-bound vacuoles under microvilli. The basal infoldings also became dilated, and some turned into membrane-bound vacuoles in the basal zone. Subsequently, microvilli became enlarged, and eventually formed blebs that later rupture to form lesion spots as observed in the SEM. Finally, the microtrabecular network in all regions broke down, creating vacuoles of various sizes throughout the tegument, leading to its total disintegration and detachment. The sequence of morphological changes was generally similar at all doses; however, the changes occurred faster at the higher doses and the longer incubation times. In addition, at the longer durations myofilaments in most muscle cells also became depolymerized, while microtubules were unchanged by the drug. Therefore, it is possible that praziquantel, through its induction of Ca^{2+} influx, causes depolymerization of the microtrabecular network that leads to the vacuolization, swelling, blebbing, and eventually the disruption and detachment of the tegument, and the breakdown of myofilaments in the muscle cells.

INTRODUCTION

Praziquantel (PZQ) is a broadly effective trematocide and cestocide (Andrews *et al*, 1983) that has been widely used because of its low toxicity and mild side effects. In clinical, field and experimental trials, PZQ is very effective against *Opisthorchis viverrini* (Bunnag and Harinasuta, 1980; Tawatsin *et al*, 1984; Saowakontha *et al*, 1993). The damaging effects of PZQ on the tegument have been studied in various species of trematodes, such as *Clonorchis sinensis* (Kim *et al*, 1982; Mehlhorn *et al*, 1983), *Schistosoma mansoni* (Becker *et al*, 1980; Mehlhorn *et al*, 1981; Shaw and Erasmus, 1983a, b; Sobhon and Upatham, 1990), *S. japonicum* (Xiao *et al*, 1982; Mehlhorn *et al*, 1983; Sobhon and Upatham, 1990) and *S. mekongi* (Sobhon and Upatham, 1990). Reports on detailed morphological changes, particularly on the fine structure of the tegument, as induced by PZQ in *O. viverrini* are still limited (Mehlhorn *et al*, 1983; Sirisinha *et al*, 1984). Such detailed studies may help to elucidate the mode of action of this drug, especially with regard to how it acts on the tegumental structure. The major aim of the present study is, therefore, to observe the sequence of changes in the tegument of adult *O. viverrini* after *in vitro* exposure to various concentrations of PZQ using both scanning (SEM) and transmission (TEM) electron microscopy.

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MATERIALS AND METHODS

Encysted metacercariae of *O. viverrini* used for infecting hamsters were obtained from naturally infected cyprinoid fishes collected from Khon Kaen

province in the northeastern part of Thailand. They were separated from minced and partially trypsinized fish muscle as previously described (Apinhasmit *et al*, 1993). Each adult golden Syrian hamster was infected by gastric intubation with 50 metacercariae. At the end of the twelfth-week post infection, adult flukes were collected as reported previously (Tuti *et al*, 1982). The adult parasites were incubated immediately in Minimal Essential Medium (MEM) containing PZQ (Bayer Co, West Germany) at the final concentrations of 0.1, 1.0 and 10.0 µg/ml at 37°C. In the control group PZQ was omitted from the incubating medium. At 5, 15, 30, 45, and 60 minutes samples were collected and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, overnight, at 4°C. They were then processed for SEM and TEM, using conventional methods.

RESULTS

SEM and TEM observations of the tegument in the control groups

Under SEM and TEM the tegument of parasites after various incubation times showed only minimal signs of microvillus swelling and blebbing, and most of the tegument still retained normal topography and ultrastructure as shown in Fig 1A-D, and as reported previously by our group (Apinhasmit *et al*, 1993, 1994).

SEM observations of the tegument surface of the adult flukes after treatment with 0.1 µg/ml of PZQ

During the entire periods of incubation the anterior part of the tegument was the least affected in comparison to other parts of the parasite. At 5 minutes it showed only a limited number of patches of swollen microvilli that turned into various-sized "blebs" among the normal-looking microvilli and sensory papillae (Fig 2A); a few blebs appeared disrupted. The areas bearing blebs increased progressively if the incubation period was prolonged from 30 to 60 minutes (Fig 2B). In the middle part, between 5 to 30 minutes incubations, the blebs appeared on wider areas and many were disrupted (Fig 2C). After 45 to 60 minutes incubations, the surface began to show numerous lesion spots as a result of an increasing number of disrupted blebs, and some parts of the tegument were sloughed off (Fig 2D). In the posterior part, the surface underwent similar damage as in the middle part, but showed more disrupted blebs over wider areas even after 5 minutes incubation (Fig 2E). At 60 minutes incubation, most of the surface became disorganized and some areas began to slough off, thus exposing the underlying basal lamina and the musculature (Fig 2F).

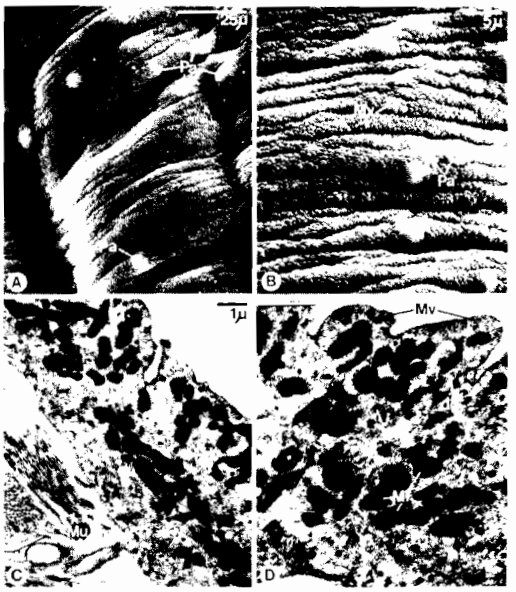

Fig 1–SEM and TEM micrographs of the tegument of adult *O. viverrini* in the control groups, incubated in MEM for 60 minutes.

A) A low-power SEM micrograph showing the corrugated surface of the tegument and numerous sensory papillae (Pa) located along the lateral border.

B) An enlargement of the area in A exhibiting the tegumental surface which is covered with numerous stubby microvilli (Mv) and sensory papilla (Pa).

C) A low-power TEM micrgraph demonstrating the tegument that is formed by fusion of the processes of the tegumental cells located underneath two layers of muscle (Mu). The muscle cells contain numerous thick and thin myofilaments.

D) An enlarged view of the area in C illustrating numerous tegumental granules, light (Ig) and dense (dg) granules, and mitochondria (Mi) scattered among the fine network of microtrabeculae, and microvilli (Mv).

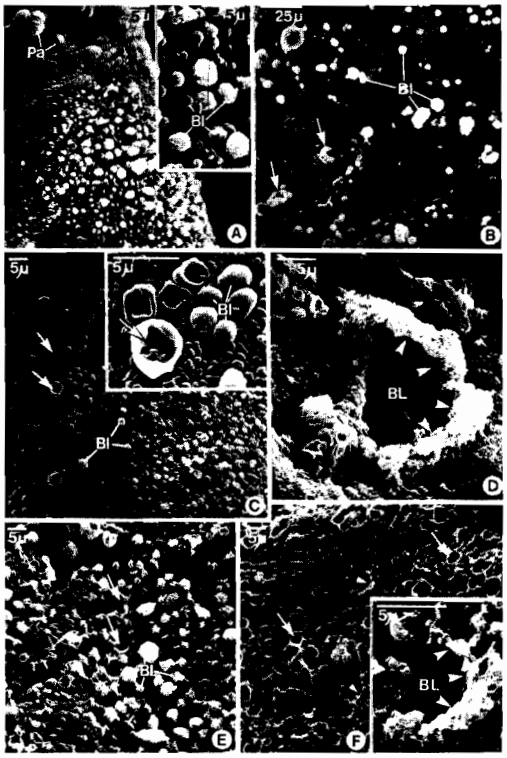

Fig 2–SEM micrographs of the tegumental surface of adult flukes incubated in MEM containing 0.1 μg/ml of PZQ.

A&B) At 5 minutes (A), most of the anterior part of the tegument still appears normal with sensory papillae (Pa) located along its lateral border; however, various-sized blebs (Bl) appear in groups scattering among an otherwise normal surface. After 30 minutes (B), more extensive areas of the same part are covered with blebs (Bl), some of which are disrupted (arrows).

C&D) The middle part of the tegument after 30 minutes (C) exhibits numerous blebs (Bl), and some are disrupted (arrows). At 60 minutes (D), few areas in the same part are sloughed off (arrow heads), exposing the underlying basal lamina (BL).

E&F) At 5 minutes (E) and 60 minutes (F) wide areas of the posterior part of the tegument are covered with blebs (Bl), most of which are disrupted (arrows). After 60 minutes, many areas lose their tegument (arrow heads) and were eroded down to the level of the underlying basal lamina (BL).

SEM observations after treatment with 1.0 μg/ml of PZQ

In the anterior part, after 5 to 15 minutes, various-sized blebs became more numerous and scattered over wider areas when compared to the lower dosage (Fig 3A). Lesion spots and sloughing of the tegument also appeared in many areas. After 30 to 60 minutes, the surface started to lose its normal appearance (Fig 3B), and the majority of parasites lost the greater part of their tegument through sloughing, thus revealing the underlying basal lamina (Fig 3B). Within the same time intervals more pronounced changes were generally observed in the tegument of the middle and the posterior parts (Figs 3C, D) than in the anterior part (Figs 3A, B).

SEM observations after treatment with 10.0 μg/ml of PZQ

As early as 5 minutes incubation, the greater part of the tegument lost its normal surface appearance (Figs 3E, G), and by 60 minutes practically almost all parts of the surface showed blebbing, disruption and shedding down to the levels of the basal lamina or muscle layers (Fig 3F).

TEM observations of the tegumental ultrastructure after treatment with 0.1 μg/ml of PZQ

The earliest sign of change which occurred in the tegument at 5 minutes incubation was the depolymerization of the microtrabecular network in the middle as well as the apical zones of the tegument's cytoplasm, which resulted in the formation of non-membrane-bound vacuoles (Figs 4A, B). If the depolmerization occurred near the tips of microvilli they would be distended and expanded to form blebs (Fig 4C) as visualized in the SEM. Some blebs were disrupted and their breakdown content was lost (Fig 4C). In the basal zone, infoldings of the inner membrane were also dilated, so much that some turned into membrane-bound vacuoles (Fig 4B). Most mitochondria in the tegument still exhibited normal appearance (Figs 4A-D). The subtegumental muscle remained intact and contained thick and thin myofilaments (Figs 4A, B). As in SEM observations, the tegumental damage was not uniform, some parts of the tegument appeared less affected than the others. After 30 minutes incubation, large areas of the microtrabecular network in all zones of the tegument were depolymerized, which gave rise to various-sized vacuoles throughout the width of the tegument (Figs 4D, E). In some areas, the inner plasma membrane was swollen and partially detached from the underlying basal lamina and muscle (Fig

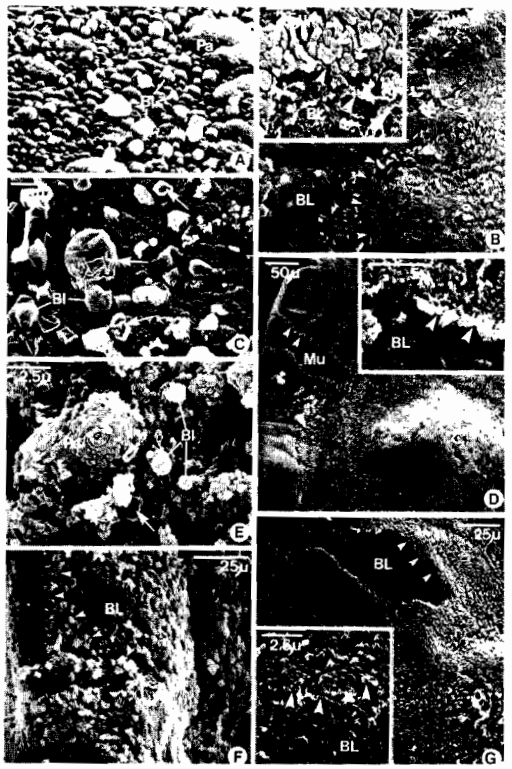

Fig 3–SEM micrographs of the tegumental surface of adult flukes incubated in MEM containing 1.0 and 10.0 μg/ml of PZQ.

A) After incubation in 1.0 μg/ml of PZQ for 5 minutes, a large area of the anterior part of the tegument exhibits blebs (Bl) while sensory papillae (Pa) appear normal.

B) After 60 minutes incubation in 1.0 μg/ml of PZQ, the anterior tegumental surface is sloughed (arrow heads) down to the level of underlying basal lamina (BL).

C) After 5 minutes incubation in 1.0 μg/ml of PZQ, the posterior tegument exhibits numerous blebs (Bl), and most of them are disrupted (arrows).

D) After 60 minutes incubation in 1.0 μg/ml of PZQ, extensive area of the posterior part is covered with partially broken down tegument (arrow heads). Some areas are sloughed off to the level of basal lamina (BL, in inset) or muscle (Mu).

E&F) After 5 minutes (E) and 60 minutes (F) incubations in 10.0 μg/ml of PZQ, most of the anterior tegument has lost the normal appearance. Wide areas show blebbing (Bl), shedding and sloughing of the surface (arrow heads), exposing the basal lamina (BL); however, the sensory papillae (Pa) appear to be less affected than the rest of the tegument.

G) After 5 minutes incubation in 10.0 μg/ml of PZQ, the posterior tegument shows total disorganization and breakdown. In some areas, the tegument is eroded (arrow heads) down to the level of basal lamina (BL).

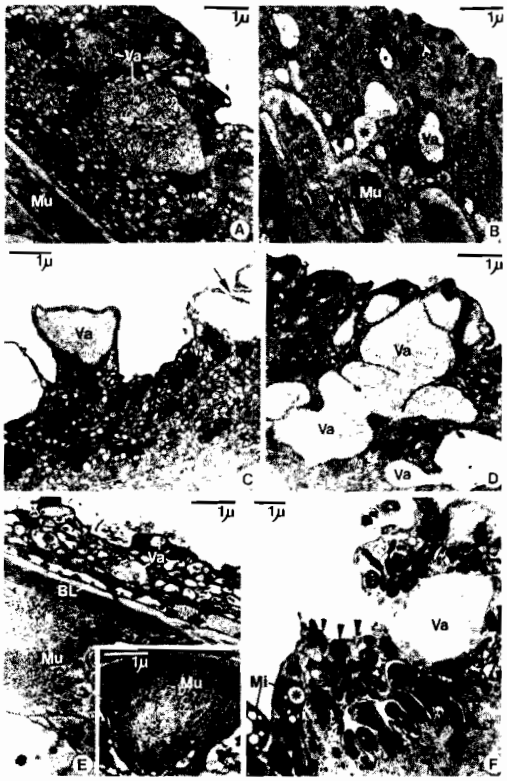

Fig 4–TEM micrographs of the adult tegument incubated in MEM containing 0.1 μg/ml of PZQ.

A&B) At 5 minutes, the depolymerization of microtrabecular network occurs at random to form non-membrane-bound vacuoles (Va), while some basal infoldings are swollen to form membrane-bound vacuoles (stars). The underlying muscle cells (Mu) appears normal.

C) At 5 minutes, some microvilli with non-membrane-bound vacuoles (Va) inside are enlarged into blebs (Bl); some blebs are disrupted and lose their content (arrows).

D&E) At 30 minutes, numerous vacuoles (Va) occur in all zones of the tegument. Most parts of the inner plasma membrane are detached from the underlying basal lamina (BL). The subtegumental muscle cells (Mu) show depolymerization of thick myofilaments in their central area where only thin filaments remain.

F) At 60 minutes, the tegument contains blebs with vacuoles (Va) inside them. The apical part of some areas are disrupted (arrow heads). Mitochondria (Mi) also become aggregated in the blebs, and some are vacuolated (asterisk).

4E). Muscle cells underneath the tegument also exhibited limited depolymerization of myofilaments, especially the thick filaments within the core of each myofiber (Fig 4E). At 60 minutes incubation, the tegument showed extensive vacuolization and blebbing; and in some areas blebs were disrupted, resulting in the detachment of the apical part of the tegument (Fig 4F). Mitochondria in the tegument were swollen and became rounder than normal, and some of them were vacuolized (Fig 4F).

TEM observations after treatment with 1.0 μg/ml of PZQ

Tegumental damage was essentially similar but more pronounced than that described after incubation in 0.1 μg/ml of PZQ. At 5 minutes incubation, the vacuolization appeared throughout the width of the tegument while blebs were formed all over the surface; and most of the basal infoldings were extremely dilated. Mitochondria inside the tegument were swollen and appeared rounder than normal. The muscle cells also showed increased depolymerization of the myofilaments. After 30 minutes, the tegument became even more vacuolized and the surface was practically covered with blebs. Mitochondria were vacuolized and their membranes disrupted. The muscle underneath the tegument exhibited extensive depolymerization of both thick and thin filaments throughout the whole cell. After 60 minutes, numerous vacuoles were distributed throughout the tegument (Figs 5A, B). Eventually, the tegument became totally disrupted and detached from the parasites' body; the erosion exposed the basal lamina or even the muscle layer (Figs 5A, B). The changes in tegumental mitochondria and subtegumental muscle appeared similar to that described at 30 minutes incubation (Figs 5A, B).

TEM observations after treatment with 10.0 μg/ml of PZQ

Morphological changes were similar to the lower dosages; however, the damages occurred

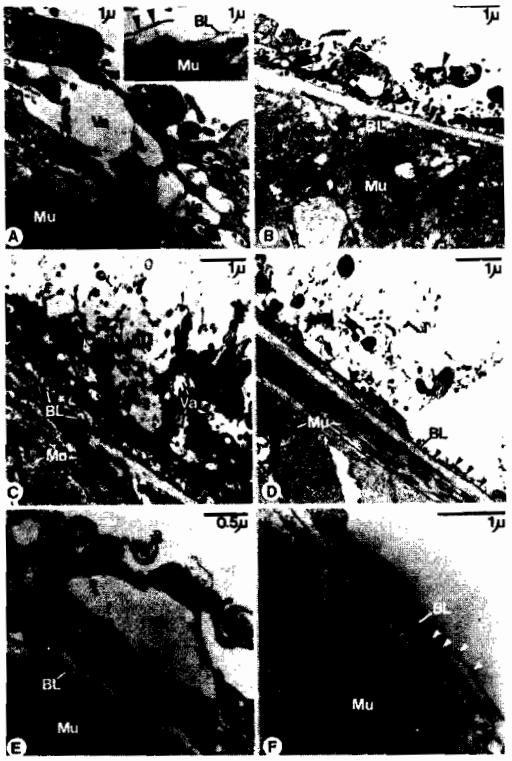

Fig 5–TEM micrographs of the adult tegument incubated in MEM containing 1.0 and 10.0 μg/ml of PZQ.

A&B) After incubation in 1.0 μg/ml of PZQ for 60 minutes, some areas of the tegument (A) show numerous vacuoles (Va) and detachment of the inner plasma membrane from the underlying basal lamina. Other areas (inset, B) the tegument is disrupted and detached (arrow heads) from the parasite's body, exposing the basal lamina (BL) and muscle (Mu). The myofilaments of the subtegumental muscle (Mu) are also extensively depolymerized, especially the thick ones.

C) At 5 minutes incubation in 10.0 μg/ml of PZQ, numerous vacuoles (Va) are formed throughout the tegument. The inner plasma membrane and basal infoldings are dilated and cause the detachment of the tegument from the basal lamina (BL).

D) At 5 minutes incubation in 10.0 μg/ml of PZQ, there are severe disruption of the apical part of the tegument in most areas, and the total detachment of the whole tegument in some (arrow heads), exposing the basal lamina (BL).

E&F) At 60 minutes incubation in 10.0 μg/ml of PZQ, the tegument exhibits vacuolization (Va) throughout its whole thickness, and the surface shedding covers a wide area. The inner plasma membrane is

detached from the underlying basal lamina (BL) and muscle (Mu). Some parts of the tegument are totally sloughed off (arrow heads), to the level of basal lamina (BL) or muscle cells (Mu). The thick myofilaments in the latter are completely depolymerized while some thin filaments still remain.

faster and covered a more extensive area. As early as 5 minutes incubation, vacuoles were formed throughout the tegument (Fig 5C). The surface of the tegument showed partial to total disruption in many areas, thereby revealing the basal lamina and muscle underneath (Fig 5D). However, most of the subtegumental muscle remained intact (Figs 5C, D). The tegumental changes after 30 to 60 minutes incubations were similar to 5 minutes, but the vacuolization, surface blebbing, surface shedding and disruption of the tegument were pervasive in most areas (Figs 5E, F). The subtegumental muscle showed extensive depolymerization of almost all thick filaments while the remaining were mostly thin type (Figs 5E, F).

DISCUSSION

Two striking phenomena elicited by the treatment with PZQ in both cestodes and trematodes were the instantaneous tetanic contraction of the musculature of the body wall, and the rapid vacuolization of the tegument (Andrews *et al*, 1983). The destructive effects of PZQ on the tegument have been clearly demonstrated in various species of helminths, such as *Hymenolepsis nana* and four other tapeworm species (Becker *et al*, 1981), *S. mansoni* (Becker *et al*, 1980; Mehlhorn *et al*, 1981; Shaw and Erasmus, 1983a, b), *S. japonicum* (Xiao *et al*, 1982; Mehlhorn *et al*, 1983; Sobhon and Upatham, 1990), *S. mekongi* (Sobhon and Upatham, 1990), and *C. sinensis* (Kim *et al*, 1982; Mehlhorn *et al*, 1983). By contrast, PZQ had less noticeable effect in *Paragonimus westermani*, a lung fluke with an exceptionally thick and condensed tegumental structure (Mehlhorn *et al*, 1983); and in *Fasciola hepatica*, a liver fluke, which possessed bundles of fortifying fibrils in the tegument (Becker *et al*, 1980). The effects of PZQ on adult *O. viverrini* both *in vitro* and *in vitro*, have been reported previously by using the SEM and TEM: the changes in the tegument consisted of vacuolization, bubbles formation that later disrupted to form crater-like lesions (Mehlhorn *et al*, 1983; Sirisinha *et al*, 1984). However, the ultrastructural details, particularly the damage of the tegument cytoskeleton, and the sequence of changes as related to varying dosages and incubation times have not yet been clearly defined. In the present study, ultrastructural observations showed that the tegument of *O. viverrini* was sensitive to the drug at all dosages, but the extent of the damage became progressive more severe with increasing duration of incubation and increasing drug concentration, particularly in the range of 1 to 10 μg/ml. These *in vitro* dosages were equivalent to the serum concentration of PZQ between 0.2-1.0 μg/ml, which in man is reached after the administration of therapeutic doses of 20-50 mg of PZQ/kg body weight (Leopold *et al*, 1978). Both the ventral and the dorsal surfaces of the parasites exhibited similar changes, but the anterior part tended to be damaged less than the posterior part. The differential effect of PZQ upon various regions of the flukes' surface was also reported in *H. nana* (Becker *et al*, 1981), and in *S. japonicum* and *S. mekongi* (Sobhon and Upatham, 1990). This might be related to physiological differences in different regions of the tegument (Shaw and Erasmus, 1983a). It should be noted; however, that there were also variations in individual resistance to the drug, and not all of them exhibited similar damage at the same duration of treatment.

The mode of action of PZQ at the molecular level is still not well understood. Preliminary study in *O. viverrini*, indicated that PZQ enhanced the influx of Ca^{2+} into the adult flukes (Ruenwongsa *et al*, 1983). The magnitude of Ca^{2+} uptake in the presence of PZQ was dependent on the concentration of Ca^{2+}; and it was enhanced by Ca^{2+} ionophore A23187, but was competitively inhibited in the presence of Mg^{2+}. The stimulating effect of PZQ was suppressed by La^{3+}, vanadate and verapamil, which were known to interfere with Ca^{2+} transport across the membrane. It was also suggested that due to its high lipid solubility, PZQ could adversely interact with lipid components of the membrane Ca^{2+} channels in such a way that caused the influx of Ca^{2+} into the tegument (Jim and Triggle, 1979; Ruenwongsa *et al*, 1983; Harder *et al*, 1988). In schistosomes, an active Ca^{2+} transport mechanism has been suggested to occur at the muscle cell membranes (Mussie *et al*, 1982).

From our previous study, the tegumental cytoskeleton of *O. viverrini* was shown to consist of a microtrabecular network and microtubules; the

former plays a major role in stabilizing the membrane and providing a general support and maintenance of the shape of the tegument (Sobhon and Apinhasmit, 1995). TEM observations in the present study indicated that, sequentially, PZQ caused the depolymerization of microtrabecular network, that led to the vacuolization and the weakening of the tegument's structural integrity. The osmotic imbalance might then result in the swelling and blebbing of the surface as seen in the SEM. The disruption of blebs, surface lesions, and total sloughing of the tegument were the final sequel of changes in the tegument's cytoplasm. Furthermore, it was observed that PZQ also caused depolymerization of myofilaments in the muscle cells underneath the tegument, and that both thick and thin filaments were affected. The depolymerization of myofilaments could also be caused by PZQ induction of Ca^{2+} influx into muscle cells.

A high concentration of Ca^{2+} is known to cause the depolymerization of F-actin filaments in other eukaryotic cells, through the activation of the actin-severing factor (Weber and Osborn, 1985). In contrast, microtubules in the tegument and cell processes appeared unaffected, although it is known that increased Ca^{2+} concentration could also cause the depolymerization of microtubules *in vitro* and the inhibition of their repolymerization (Weisenberg, 1972; Rosenfeld *et al*, 1976). However, the level of Ca^{2+} that could cause depolymerization of microtubules in the tegument might have to be higher than that in the microtrabecular network.

The membrane-bound vacuoles arising from the swelling of basal infoldings have been observed in the basal zone of the tegument of *O. viverrini*. Such vacuoles have also been observed in the tegument of *S. mansoni* (Shaw and Erasmus, 1983b) and *F. hepatica* (Threadgold and Brennan, 1978) and were thought to occur in response to the change of osmolarity of the incubating medium. In *F. hepatica* such changes did not necessarily cause permanent damage, and the infoldings returning to their normal appearance within 1 hour, irrespective of the osmolarity of the culture medium. By contrast in *O. viverrini* the swelling of basal infoldings seemed irreversible, perhaps because PZQ also resulted in the increased water passage accompanying the Ca^{2+} influx into the tegument.

In PZQ treated *O. viverrini* mitochondria became vacuolized and assumed a rounder shape than normal, and their membrane was partially disrupted. Under normal circumstances, mitochondria in most eukaryotic cells deal with excess calcium ions by inducing their precipitation into large osmotically inactive particles. However, the sudden influx of Ca^{2+} caused by PZQ might be so massive that mitochondria could not cope. Similar finding was reported in *C. sinensis* treated with PZQ, where the swelling and degradation of mitochondria in the nerve bulbs of sensory papillae surrounding the sucker and excretory pore were observed (Kim *et al*, 1982).

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