Ionic mechanisms underlying spontaneous muscle contractions in the liver fluke, Fasciola hepatica

M. K. GRAHAM, J. G. McGEOWN, AND I. FAIRWEATHER

The Queen's University of Belfast, Belfast BT9 7BL, United Kingdom

Graham, M. K., J. G. McGeown, and I. Fairweather. Ionic mechanisms underlying spontaneous muscle contractions in the liver fluke, Fasciola hepatica. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R374–R383, 1999.—Spontaneous contractions of liver fluke muscle were abolished in Ca2+-free saline and by 100 µM nifedipine and reduced by 5 mM cadmium chloride, suggesting that they are dependent on extracellular Ca2+. Caffeine (5 mM) significantly increased contraction amplitude and frequency. Ryanodine (100 µM) failed to block the caffeine response but significantly reduced spontaneous contraction frequency, suggesting that intracellular stores have a functional role. Cyclic nucleotides, including cAMP and cGMP, were without effect. However, 8-bromoadenosine 3',5'-cyclic monophosphate significantly increased spontaneous contractions, which implies that cAMP has a regulatory function in motility. Caffeine, however, produced no measurable increase in cAMP. The caffeine effect was inhibited by cadmium chloride and nifedipine, whereas IBMX-induced increases in amplitude were reduced by cadmium chloride. Thus caffeine and cAMP appear capable of opening plasma membrane Ca2+ channels, but the involvement of cAMP in caffeine responses has not been proved.

extracellular Ca2+; intracellular stores; ryanodine receptor; adenosine 3',5'-cyclic monophosphate

FASCIOLA HEPATICA is a parasitic flatworm (platyhelminth) belonging to the Class Trematoda (flukes). The study of flatworms is necessary, not only because of the medical and economic significance of the parasitic forms, but also because they are a link between lower and higher invertebrates. They are cephalized and bilaterally symmetrical and are the first metazoan group to possess a centralized nervous system (9). Thus they are useful phylogenetic subjects in the study of the evolution of systems in higher animal groups, including mammals.

The muscle of flatworms is nonstriated and consists essentially of two layers that act antagonistically to each other. Beneath the outer tegument there is a circular layer, underneath which there is a relatively thick layer of longitudinal muscle (see review, Ref. 40). Liver fluke muscle is composed of both thick and thin myofilaments (20). Both actin (46) and paramyosin (20), characteristic of invertebrate smooth muscle, have been localized in Fasciola muscle. Muscle contraction plays an important part in many aspects of the worm’s physiology, including motility. However, our knowledge of the musculature of the liver fluke is limited mainly to ultrastructural studies and there is little information concerning excitation-contraction (EC) coupling mechanisms.

In mammals, the rise in the intracellular Ca2+ concentration ([Ca2+]i) required to initiate muscle contraction is achieved either by the entry of extracellular Ca2+ through ion channels in the plasma membrane or by the release of intracellular Ca2+ stores (18). One mechanism of calcium release from intracellular stores is through ryanodine receptor (RyR)-regulated channels located in the sarcoplasmic reticulum (SR) membrane. RyRs are regulated by Ca2+ itself in a process known as Ca2+-induced Ca2+ release (8, 16). RyRs can also be modulated by the plant alkaloid ryanodine (11), and, depending on the cell type and concentration of ryanodine, the channels are either locked open, resulting in the depletion of stored Ca2+ (11), or locked shut, preventing Ca2+ release from the store (31). Three genes have been found to encode RyRs, with ryr-1, ryr-2, and ryr-3 occurring predominantly in skeletal, cardiac, and brain tissue, respectively, although they are frequently coexpressed within the same cell type (30). Both ryr-2 and ryr-3 isoforms are expressed in smooth muscle (see review, Ref. 47). The RyR in stomach smooth muscle cells from the toad Bufo marinus has homology with the canine cardiac receptor (53). The excitatory agonist caffeine appears to act on RyRs, increasing their sensitivity to Ca2+. This potentiates ryanodine-sensitive Ca2+ release in insect photoreceptors (50), single cultured guinea pig myenteric neurons (25), guinea pig ureter (4) and ileal smooth muscle (26), rat arteries (1, 45), and porcine tracheal smooth muscle (22). Ryanodine also inhibits caffeine-induced contractions in isolated frog atrial trabeculae (49) and crab and lobster striated muscle (27). RyRs have been identified in many invertebrates, including insects, crustaceans, and molluscs (see review, Ref. 47). A RyR identified in the roundworm (nematode) Caenorhabditis elegans has greater homology with the mammalian cardiac RyR (yr-2) than with the mammalian skeletal isoform (yr-1) (43). It does not appear to be essential for EC coupling but regulates body-wall muscle contraction by amplifying a calcium transient (36). Immunoreactivity to mammalian yr-2 has also been located in the cirrus gland, the lateral nerve cord, and in the muscle of the sub tegument, gut, and oral and ventral suckers of Fasciola (21), but the functional significance of these observations has not been established. One object of the present study was to test whether ryanodine-sensitive Ca2+ stores contribute to caffeine-induced excitation, as in studies per-
formed on mammalian smooth muscle (1, 4, 22, 26, 45). The investigation was extended to consider the ionic mechanisms underlying spontaneous contractility in the liver fluke F. hepatica. The role of extracellular Ca$^{2+}$ was examined by testing the effect of a number of mammalian Ca$^{2+}$ channel blockers. The contribution of Ca$^{2+}$ release from internal stores was also investigated, especially ryanodine-sensitive release. The information gained adds significantly to the limited amount known concerning control of parasite movement. Because mobility is required for feeding, reproduction, and maintenance of the worm in the host's bile duct, it is an ideal target for chemotherapy. However, an understanding of excitation and EC coupling is required before possible differences (for example, in receptor types and ion channels) between the parasite and the host can be manipulated in the design of novel anthelmintics.

**MATERIALS AND METHODS**

Tissue preparation for muscle strip physiology. Spontaneously active isolated muscle strips (~10 mm long and 3 mm wide) were obtained from adult liver flukes (F. hepatica) recovered from the bile ducts of experimentally infected laboratory rats. This was achieved by cutting whole flukes horizontally below the ventral sucker, trimming the posterior and sides, and slicing the resultant tissue longitudinally in two. The strips were suspended vertically in organ baths maintained at 37°C, and isometric tension was recorded as described previously (13). Drugs were continuously superfused and washed out with fresh Hédon-Fleig saline buffered with 10 mM HEPES and adjusted to pH 7.4 using 2 M NaOH. Stable activity was observed under control conditions for at least 20 min before drug application.

Measurement of cAMP. Two muscle strips were obtained per liver fluke as described above. One of the strips was incubated in Hédon-Fleig saline (to measure basal cAMP levels), while the other (from the same fluke) was incubated in saline containing 5 mM caffeine, in a final volume of 4 ml, at 37°C for 10 min. The strips were then immersed in liquid nitrogen, placed in eppendord tubes, and maintained at -20°C until the cAMP assay was carried out. cAMP was extracted by homogenizing the frozen tissue strips in cold 6% (wt/vol) TCA at 2–8°C. Tissue homogenates were then spun at 2,000 g for 15 min at 4°C. Pellets were retained for protein determination, while the TCA was removed from the supernatant by repeated washing in water-saturated diethyl ether. The aqueous extract remaining was dried in a freeze-dry vacuum (Hettvac, VR-1 model), dissolved in 1 ml of essay buffer (0.05 M acetic acid buffer, pH 5.8, containing 0.02% (vol/vol) bovine serum albumin and 0.01% (vol/vol) preservative) and measured using an enzyme-linked immunosorbant assay (Biotrak cAMP enzyme immunoassay system, Amer sham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK). Protein was measured by the method of Markwel et al. (34) using bovine serum albumin as standard.

Solutions and chemicals. Hédon-Fleig saline contained the following components (in mM): 120.7 NaCl, 4 KCl, 1.9 MgSO$_4$, 7H$_2$O, 0.9 CaCl$_2$·2H$_2$O, 18.5 NaHCO$_3$, 10 HEPES, and 15 d-glucose, pH 7.4. Calcium-free saline was prepared by omitting Ca$^{2+}$ from Hédon-Fleig saline and using double-distilled water. EGTA (5 mM) was also included to further reduce the free Ca$^{2+}$ concentration.

Chemicals used were caffeine, cadmium chloride, nifedipine, ryanodine, cyclopiazonic acid (CPA), 3-isobutyl-1-methylxanthine (IBMX), forskolin, and 8-bromoadenosine 3',5'-cyclic monophosphate (8-BrCAMP). All drugs were obtained from Sigma (Poole, Dorset, UK) with the exception of cadmium chloride (The British Drug Houses, Poole, Dorset, UK). Caffeine was soluble in Hédon-Fleig saline. Cadmium chloride forms a precipitate with sulfates and carbonates, whereas the magnesium sulfate and sodium hydrogen carbonate in the saline were replaced by equimolar concentrations of magnesium chloride and sodium chloride, respectively, in the experiments involving cadmium chloride.

A stock solution of ryanodine (1 x 10$^{-2}$ M) was prepared in absolute methanol. The final dilutions of solvent for the 10 and 100 µM ryanodine experiments were 0.1% and 1% (vol/vol), respectively, neither of which had an effect on spontaneous motility. The stock solution for the 1 µM nifedipine experiments was prepared in absolute ethanol. The final dilution of ethanol was 0.01% (vol/vol), and this concentration had no effect on muscle strip motility. However, when preparing a 100 µM nifedipine solution from ethanol-dissolved stock, the vehicle at its final concentration (0.1%, vol/vol) had an excitatory effect on motility. Therefore, a stock solution of nifedipine dissolved in DMSO was used, because at the final concentration of 0.1% (vol/vol) this vehicle had no effect on spontaneous activity. The remaining drugs, CPA, IBMX, forskolin, and 8-BrCAMP were initially dissolved in DMSO before addition to the saline. The final dilutions of vehicle (0.05–0.1%, vol/vol) had no effect on the movement of the muscle strips. Nifedipine and forskolin are readily soluble in DMSO but form a slight precipitate when subsequently added to Hédon-Fleig saline. The actual concentration of these drugs would thus have been <100 µM.

Data analysis and presentation. Each experiment involving the measurement of isometric tension was repeated at least five times, using strips of tissue from separate flukes. Contraction amplitude and frequency were analyzed separately and only contractions of an amplitude ≥0.5 mN were counted.

Calcium-free saline and the drugs cadmium chloride, nifedipine, ryanodine, and 8-BrCAMP were tested for their effects on spontaneous activity. The mean amplitude and frequency of contractions ± SE were plotted for 3-min time periods over a 15-min control period and a number of drug-exposure periods. A paired Student's t-test was used to compare the mean values in the presence of a drug with those during the last 3 min of the control period.

Experiments examining the effect of cadmium chloride and nifedipine on the tissue's response to a 3-min application of caffeine were completed. Graphs illustrated the mean caffeine-induced increases (compared with the preceding 3-min period) in frequency and amplitude before and after exposure to 5 mM cadmium chloride or 100 µM nifedipine. The magnitudes of the two caffeine responses were compared using a single factor, repeated measures ANOVA and Fisher's protected least significant difference test. The effect of cadmium chloride on a 5-min exposure to IBMX was also performed and analyzed in the same way.

Muscle strips were superfused with forskolin for a short period (5 min). The graph showed the mean amplitude and frequency of contractions of the 5-min period immediately preceding the forskolin addition and the 5 min period during forskolin exposure. Mean values before and during the forskolin addition were compared using a paired Student's t-test.

Basal cAMP levels were compared with the levels of cAMP after exposure to 5 mM caffeine using an unpaired Student's t-test.

For all experiments, differences in means were accepted as statistically significant at the 95% level.
RESULTS

Spontaneous activity. All tissue strips showed spontaneous, phasic activity (Fig. 1). This activity had a mean amplitude of 2.36 ± 0.06 mN and a mean frequency of 10.45 ± 0.19 contractions/3 min (or 3.48 ± 0.06 contractions/min; n = 40).

Ca\(^{2+}\)-free saline. Superfusion with Ca\(^{2+}\)-free saline temporarily increased contraction frequency, but spontaneous activity was reduced and eventually abolished after 45 min exposure to Ca\(^{2+}\)-free saline (Fig. 1). Frequency and amplitude were reduced from 10.8 ± 2.63 contractions/3 min and 1.21 ± 0.15 mN to 0, respectively (n = 5, P < 0.01; Student’s t-test).

Ca\(^{2+}\) channel blockers. The possible role for the entry of extracellular Ca\(^{2+}\) was examined by testing the Ca\(^{2+}\) channel blockers cadmium chloride and nifedipine. In mammals, the inorganic compound cadmium chloride acts as a nonspecific blocker (48). Spontaneous motility (n = 6) and the contractile response to caffeine (n = 8) were examined using 5 mM cadmium chloride. It reduced the frequency and amplitude of contractions of all six muscle strips and totally blocked motility in three muscle strips after 15-min exposure (Fig. 2).

Overall, contraction frequency was initially increased, but then gradually decreased. Cadmium chloride reduced the frequency from a mean predrug level of 8.5 ± 0.5 contractions/3 min to 3.0 ± 1.44 contractions/3 min after 15 min exposure (n = 6, P < 0.05; Student’s t-test). Amplitude was reduced from 1.95 ± 0.28 to 0.59 ± 0.19 mN (P < 0.01; Student’s t-test). These effects were reversible on washout of the cadmium chloride.

Caffeine (5 mM) had an excitatory effect (Fig. 3): it increased frequency from 9.83 ± 0.54 to 17.32 ± 0.49 contractions/3 min after 15 min exposure (n = 41, P < 0.01; Student’s t-test). Amplitude was also raised, from 2.22 ± 0.16 to 4.84 ± 0.28 mN (P < 0.01; Student’s t-test). There was no difference in the response of the tissue to repeated caffeine applications under control conditions. Cadmium chloride (5 mM) blocked the caffeine response in six of eight tissue strips tested (Fig. 3).
The mean increase in frequency was 10.5 ± 1.18 contractions/3 min before cadmium chloride, but only 0.88 ± 0.72 contractions/3 min after 15 min exposure to the Ca²⁺ blocking agent (P < 0.01; ANOVA). The mean increase in amplitude decreased from 3.22 ± 0.41 to 0.84 ± 0.22 mN (P < 0.01; ANOVA). In summary, the use of compounds that block the entry of Ca²⁺ into the cell inhibit both spontaneous activity and the contractile response to caffeine.

Intracellular stores. The plant alkaloid ryanodine was used in an attempt to block Ca²⁺ release from ryanodine-sensitive stores. Ryanodine at a concentration of 100 µM significantly reduced the frequency of spontaneous contractions from 11.0 ± 1.57 to 4.0 ± 0.68/3 min (n = 6; P < 0.01; Student’s t-test), but had no significant effect on contraction amplitude when exposed for 30 min (Fig. 6). Ryanodine had no significant

mean increase in frequency was 10.5 ± 1.18 contractions/3 min before cadmium chloride, but only 0.88 ± 0.72 contractions/3 min after 15 min exposure to the Ca²⁺ blocking agent (P < 0.01; ANOVA). The mean increase in amplitude changed from 2.17 ± 0.42 to 0.01 ± 0.16 mN (P < 0.01; ANOVA).

The dihydropyridine nifedipine is a blocker that is relatively specific for L-type Ca²⁺ channels (12). The effects of 1 and 100 µM nifedipine were tested on spontaneous motility and the caffeine response. The lower concentration of nifedipine (1 µM) had no effect on spontaneous motility or caffeine-induced excitation even after superfusion for 1 h (n = 8). However, the higher concentration of 100 µM had an inhibitory effect (Fig. 4). The mean control values for frequency and amplitude were 11.0 ± 0.9 contractions/3 min and 2.1 ± 0.4 mN, respectively, and 100 µM nifedipine completely blocked spontaneous contractions after 15 min superfusion in all of the muscle strips (n = 6).

The higher concentration of nifedipine (100 µM) also inhibited the response to 5 mM caffeine (Fig. 5). The mean caffeine-induced increase in frequency fell from 6.63 ± 0.82 contractions/3 min initially to 2.88 ± 0.67 contractions/3 min after superfusion with nifedipine (n = 8; P < 0.01; ANOVA), while the mean increase in amplitude decreased from 3.22 ± 0.41 to 0.84 ± 0.22 mN (P < 0.01; ANOVA). In summary, the use of compounds that block the entry of Ca²⁺ into the cell inhibit both spontaneous activity and the contractile response to caffeine.

Intracellular stores. The plant alkaloid ryanodine was used in an attempt to block Ca²⁺ release from ryanodine-sensitive stores. Ryanodine at a concentration of 100 µM significantly reduced the frequency of spontaneous contractions from 11.0 ± 1.57 to 4.0 ± 0.68/3 min (n = 6; P < 0.01; Student’s t-test), but had no significant effect on contraction amplitude when exposed for 30 min (Fig. 6). Ryanodine had no significant
effect on the mechanical response to 5 mM caffeine (n = 6). Due to the use-dependent effect of ryanodine (49), where it may fail to block an initial caffeine addition but will successfully block subsequent caffeine applications, additional experiments were performed to see if a second caffeine addition could be blocked after reexposure to 100 µM ryanodine. However, ryanodine still failed to block the caffeine response.

A further attempt to establish the role of intracellular stores was made using CPA, which prevents the replenishment of depleted internal Ca\(^{2+}\) stores by inhibiting the SR Ca\(^{2+}\)-ATPase pump (44). When 5 µM CPA was tested on the muscle strip preparations, it failed to have an effect on either spontaneous activity (n = 6) or the tissue's response to 5 mM caffeine (n = 6), even after superfusion for 1 h. Therefore, there is some evidence to indicate a role for intracellular Ca\(^{2+}\) stores in the motility of spontaneous contractions (using ryanodine), but not for the response to caffeine in F. hepatica.

Role of cAMP. Because ryanodine and CPA failed to inhibit the contractile response to caffeine, an alternative second messenger pathway for caffeine was investigated. In addition to sensitizing RyRs, caffeine can also act as a phosphodiesterase inhibitor (51). Phosphodiesterase is an enzyme that hydrolyzes cAMP to adenosine 5'-monophosphate (5'-AMP); thus an inhibitor of the enzyme will result in elevated cAMP levels. To test this hypothesis in Fasciola, a number of compounds were used that raise cAMP levels. The compound IBMX acts as a cAMP phosphodiesterase inhibitor. The effect of 100 µM IBMX (5 min) was tested on Fasciola muscle strips (Fig. 7). It had an excitatory effect, increasing both the frequency and amplitude of contractions. Frequency increased from 18.27 ± 1.03 to 33.36 ± 1.98 contractions/5 min (n = 11; P < 0.01; Student's t-test). Contraction amplitude was raised from 2.15 ± 0.20 to 3.95 ± 0.27 mN (P < 0.01; Student's t-test). This excitatory action was reversible and repeatable, with no significant difference between the magnitude of the two responses.

Fig. 5. Effect of 100 µM nifedipine on response to caffeine in a single muscle strip (A) and summarized data for amplitude and frequency before (filled bars) and after (open bars) exposure to nifedipine (n = 8; B). **Significant reduction in amplitude and frequency (P < 0.01).

Fig. 6. Effect of 100 µM ryanodine on muscle strip activity. A: tension record showing first 10 min of ryanodine exposure and also from 20 to 30 min. B: summary data for 3-min blocks for amplitude (□) and frequency (●) in 6 strips. Frequency was significantly reduced after 30 min exposure to ryanodine (P < 0.01).
Experiments also were completed to see if cadmium chloride (5 mM) could block the excitatory action of 100 µM IBMX (Fig. 7) in addition to inhibiting the tissue's response to 5 mM caffeine (Fig. 3). The mean increase in amplitude induced by IBMX was reduced from $1.56 \pm 0.19$ to $0.52 \pm 0.26$ mN after superfusion with cadmium chloride for 10 min ($n = 6$; $P < 0.05$; Student's $t$-test), whereas the corresponding rise in amplitude was from $3.51 \pm 0.37$ to $6.67 \pm 0.47$ mN ($P < 0.01$; Student's $t$-test).

The phosphodiesterase-resistant cAMP analog 8-BrcAMP was applied to the muscle strips (Fig. 9). The strips were superfused with a recirculating solution of 2 mM 8-BrcAMP. Both frequency and amplitude were significantly increased from $12 \pm 0.73$ to $19 \pm 1.66$ contractions/3 min ($n = 6$; $P < 0.01$; Student's $t$-test) and from $2.52 \pm 0.2$ to $3.42 \pm 0.38$ mN ($P < 0.05$; Student's $t$-test), respectively. These experiments would seem to establish that elevation of intracellular cAMP can stimulate the contractile activity of F. hepatica.

A direct measurement of cAMP was made after exposing muscle strips to 5 mM caffeine. Caffeine failed to significantly increase the cAMP levels after 10 min exposure. The mean basal level was $7.8 \pm 1.7$ pmol cAMP/mg protein, and the mean level after incubation in caffeine was $11.8 \pm 2.1$ pmol cAMP/mg protein ($n = 6$). The failure to demonstrate an elevation of cAMP in response to caffeine will be considered in the discussion.

Experiments also were completed to see if cadmium chloride (5 mM) could block the excitatory action of 100 µM IBMX (Fig. 7) in addition to inhibiting the tissue's response to 5 mM caffeine (Fig. 3). The mean increase in amplitude induced by IBMX was reduced from $1.56 \pm 0.19$ to $0.52 \pm 0.26$ mN after superfusion with cadmium chloride for 10 min ($n = 6$; $P < 0.05$; ANOVA). The mean increase in frequency was $14.0 \pm 2.05$ contractions/5 min before cadmium chloride superfusion and $7.5 \pm 3.96$ contractions/5 min afterward, but this change was not statistically significant. This reflects the high level of variation observed, because cadmium chloride completely blocked the IBMX response in some of the tissues, whereas in the remainder the amplitude was decreased but there were small contractions of high frequency.

The agent forskolin was also tested. One of its effects is to increase cAMP levels through activation of adenylate cyclase, a plasma membrane-bound enzyme that converts ATP into cAMP. Tissue strips were tested with a 5-min application of 100 µM forskolin (Fig. 8). It increased the amplitude and frequency of contractions, and this effect was repeatable. Frequency increased from $19.33 \pm 0.62$ to $27.5 \pm 1.06$ contractions/5 min ($n = 6$; $P < 0.01$; Student's $t$-test), whereas the corresponding rise in amplitude was from $3.51 \pm 0.37$ to $6.67 \pm 0.47$ mN ($P < 0.01$; Student's $t$-test).

The phosphodiesterase-resistant cAMP analog 8-BrcAMP was applied to the muscle strips (Fig. 9). The strips were superfused with a recirculating solution of 2 mM 8-BrcAMP. Both frequency and amplitude were significantly increased from $12 \pm 0.73$ to $19 \pm 1.66$ contractions/3 min ($n = 6$; $P < 0.01$; Student's $t$-test) and from $2.52 \pm 0.2$ to $3.42 \pm 0.38$ mN ($P < 0.05$; Student's $t$-test), respectively. These experiments would seem to establish that elevation of intracellular cAMP can stimulate the contractile activity of F. hepatica.

A direct measurement of cAMP was made after exposing muscle strips to 5 mM caffeine. Caffeine failed to significantly increase the cAMP levels after 10 min exposure. The mean basal level was $7.8 \pm 1.7$ pmol cAMP/mg protein, and the mean level after incubation in caffeine was $11.8 \pm 2.1$ pmol cAMP/mg protein ($n = 6$). The failure to demonstrate an elevation of cAMP in response to caffeine will be considered in the discussion.
The present investigation was concerned with the role of Ca\(^{2+}\) in the spontaneous contractions of liver fluke muscle strips. Both the entry of extracellular Ca\(^{2+}\) across the plasma membrane and release of intracellular Ca\(^{2+}\) stores were examined, particularly ryanodine-sensitive release. Comparison with the mechanisms involved in muscle contraction in mammals is important, as any differences may be targeted for the design of anthelmintics directed at the parasite but that have no detrimental effect on its definitive host (namely, sheep, cattle, and also man).

Contractions of muscle strips from *F. hepatica* were abolished in Ca\(^{2+}\)-free saline and reduced by the mammalian Ca\(^{2+}\) channel blockers cadmium chloride and nifedipine, which suggests that they require a source of extracellular Ca\(^{2+}\). In fact, contractions were completely abolished in the presence of 100 µM nifedipine, which implies that Ca\(^{2+}\) channels similar to mammalian L-type may be present in the fluke. However, the concentration used is ~100-fold higher than that required to block these channels in other tissues (28) and the effect of nifedipine may have been that of a nonspecific Ca\(^{2+}\) antagonist. Contractions in mammalian smooth muscle are dependent on extracellular Ca\(^{2+}\) (41) and are inhibited by Ca\(^{2+}\) channel blockers, whereas skeletal muscle is less sensitive to these drugs (24). The sensitivity of *Fasciola* to mammalian Ca\(^{2+}\) channel blockers indicates that its somatic muscle more closely resembles the mammalian smooth type. This is consistent with its ultrastructure (nonstriated) (40) and its similarities to invertebrate smooth muscle (20, 46). The slow undulatory movement of the worm is also more consistent with smooth-type fibers (42).

There have been few studies on the control of parasitic flatworm movement. Two studies reported in the literature involved the triclad ectoparasite *Bdelloura candida* (3) and the human blood fluke *Schistosoma mansoni* (7). Compounds were tested on isolated muscle cells, rather than muscle strips, which means that their effects were exerted on the muscle directly without any possible neuronal influence. However, many compounds that were effective on muscle strips also had an effect on the muscle cells themselves. In *Bdelloura*, the contraction of isolated muscle cells was entirely dependent on extracellular Ca\(^{2+}\), and an inward Ca\(^{2+}\) current has been identified under voltage-clamped conditions (3). Brain cells from this organism also exhibit an inward, voltage-dependent Ca\(^{2+}\) current that is partially blocked by cadmium (10 mM) and nifedipine (10 µM) (2). Comparison of *F. hepatica* with *S. mansoni* is of particular interest, because both are digenetic trematodes. Early work involving motility recordings from whole schistosome worms suggested that spontaneous contractions of the fluke are dependent on extracellular Ca\(^{2+}\), and an inward Ca\(^{2+}\) current has been identified under voltage-clamped conditions (3). Brain cells from this organism also exhibit an inward, voltage-dependent Ca\(^{2+}\) current that is partially blocked by cadmium (10 mM) and nifedipine (10 µM) (2). Comparison of *F. hepatica* with *S. mansoni* is of particular interest, because both are digenetic trematodes. Early work involving motility recordings from whole schistosome worms suggested that spontaneous contractions of the fluke are dependent on extracellular Ca\(^{2+}\), and an inward Ca\(^{2+}\) current has been identified under voltage-clamped conditions (3). Brain cells from this organism also exhibit an inward, voltage-dependent Ca\(^{2+}\) current that is partially blocked by cadmium (10 mM) and nifedipine (10 µM) (2). Comparison of *F. hepatica* with *S. mansoni* is of particular interest, because both are digenetic trematodes. Early work involving motility recordings from whole schistosome worms suggested that spontaneous contractions of the fluke are dependent on extracellular Ca\(^{2+}\), and an inward Ca\(^{2+}\) current has been identified under voltage-clamped conditions (3). Brain cells from this organism also exhibit an inward, voltage-dependent Ca\(^{2+}\) current that is partially blocked by cadmium (10 mM) and nifedipine (10 µM) (2).
saline (5). In this respect, the muscle is more similar to smooth than striated muscle in mammals. A voltage-sensitive Ca\(^{2+}\) current identified in somatic muscle cells from the nematode Ascaris suum has similarities to both mammalian L- and T-type Ca\(^{2+}\) currents (35). Although there are ultrastructural differences between flatworms and roundworms, it would appear that they share a requirement for extracellular Ca\(^{2+}\) for muscle contraction.

Ryanodine (10 µM) blocks muscle contraction in the roundworm Ascaris suum (21), and the RyR identified in the roundworm C. elegans appears to regulate body-wall muscle contraction (36). In the current experiments on F. hepatica, ryanodine reduced the frequency of spontaneous contractions. This reduction suggests that internal Ca\(^{2+}\) stores may have a role in the initiation of spontaneous contractions. The failure of CPA to have a similar effect would seem to be paradoxical in this context and suggests that at least some of the Ca\(^{2+}\) uptake into stores is not blocked by this agent. The demonstration of immunoreactivity to an RyR in F. hepatica (21) and the evidence that indicates that Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase activity is predominantly located in the endoplasmic reticulum in S. mansoni (6) further supports the idea that intracellular stores have a role to play in muscle contraction. To confirm their function, however, it would be necessary to measure the effects of ryanodine and CPA on [Ca\(^{2+}\)] directly, using ratiometric Ca\(^{2+}\) indicators (14).

Caffeine (5 mM) had an excitatory effect on isolated muscle strips from Fasciola. The failure of ryanodine (100 µM) and CPA (5 µM) to alter the tissue’s response to 5 mM caffeine suggests that caffeine may be exerting an effect independent of any action on Ca\(^{2+}\) stores. One of the known effects of caffeine is to raise cAMP levels by inhibition of cyclic nucleotide phosphodiesterase (51). The enzymes responsible for the synthesis of cAMP from ATP (adenylate cyclase) and its hydrolysis to 5’-AMP (cyclic 3’,5’-phosphodiesterase) have been identified in F. hepatica (32, 33) and S. mansoni (17). In addition, there are at least three cAMP-dependent protein kinases present in Fasciola, which have similar physical and kinetic properties to the mammalian enzyme (19). The presence of these enzymes suggests that cAMP has a signaling function in these organisms.

In the present study, the cAMP potentiating compounds IBMX (100 µM), forskolin (100 µM), and 8-Br-cAMP (2 mM) mimicked the excitatory action of 5 mM caffeine in liver fluke muscle strips. Previously, forskolin has been shown to activate the serotonin (5-HT)-stimulated adenylate cyclase in Fasciola (29). It appears to act at the level of the enzyme and not through the 5-HT receptor, stimulating adenylate cyclase to over 35 times the basal enzyme level at a concentration of 300 µM, in cell-free fluke particles. In intact flukes, 100 µM forskolin increased the endogenous cAMP level to more than twice the level found in control flukes (29). In liver fluke heads, the phosphodiesterase inhibitor IBMX (1 mM) has been shown to increase the length of time and the amount by which cAMP levels are raised after exposure to 5-HT (32), whereas it increases endogenous cAMP in intact flukes at a concentration of 2 mM (29). As both forskolin and IBMX increased fluke motility at concentrations that raised cAMP levels in a previous study (29) it is likely that their excitatory action on the muscle strips in the present investigation is a result of elevated cAMP and not some other action. The present study failed to demonstrate an elevation in cAMP in response to caffeine, which suggests that caffeine does not inhibit phosphodiesterase activity in this tissue. However, it is not conclusive proof that caffeine does not affect cAMP in liver fluke muscle strips (see below).

In the present study, the excitatory effect of caffeine was inhibited by compounds that block Ca\(^{2+}\) influx through plasma membrane Ca\(^{2+}\) channels. Nifedipine (100 µM) significantly reduced the caffeine effect, and 5 mM cadmium chloride inhibited the tissue’s response to both caffeine (5 mM) and IBMX (100 µM). This suggests that caffeine and IBMX may be activating a Ca\(^{2+}\)-permeable channel in the plasma membrane of the liver fluke. Caffeine has been shown to affect Ca\(^{2+}\) channels in the plasma membrane of toad gastric smooth muscle cells. In 85% of the cells, caffeine activated a nonselective, Ca\(^{2+}\)-permeable cation channel, which could be blocked by gadolinium chloride, in addition to releasing Ca\(^{2+}\) from intracellular stores (15). It is possible that caffeine opens Ca\(^{2+}\) channels through a local rise in cAMP in the immediate vicinity of the channel. This rise would be small compared with total cAMP levels in the tissue and would remain undetected in the assay used to measure cAMP. In addition to opening Ca\(^{2+}\) channels, caffeine may be having a more direct effect by increasing the sensitivity of the contractile apparatus (myofilaments) in muscle to Ca\(^{2+}\) (37).

In conclusion, the evidence suggests that the muscle in F. hepatica has similarities to mammalian smooth muscle in terms of structure and dependence on extracellular Ca\(^{2+}\) for contractions. In the latter respect, Fasciola bears a resemblance not only to parasites of the same class (trematodes) but also to parasites from a different phylum, namely, the nematodes. This may be useful in the design of broad-spectrum antihelmintics. There is also evidence to suggest that cAMP may play a part in the regulation of fluke motility. Finally, caffeine does not act through ryanodine-sensitive stores and failed to significantly elevate cAMP in mixed tissue preparations, but caffeine and cAMP may be opening Ca\(^{2+}\) channels in the plasma membrane. Further work is required to confirm the mechanism of action of caffeine, the pharmacology of any channels activated by caffeine, and the role of intracellular Ca\(^{2+}\) stores. This will become possible when a technique for viable muscle cell isolation is achieved.

This research was supported by a postgraduate studentship from the Department of Agriculture for Northern Ireland (to M. K. Graham) and a research grant from the Wellcome Trust (to J. G. McGeown).

Address for reprint requests and other correspondence: I. Fairweather, School of Biology and Biochemistry, Medical Biology Centre, The Queen’s Univ. of Belfast, 97 Lisburn Rd., Belfast BT9 7BL, UK (E-mail: i.fairweather@qub.ac.uk).

Received 23 July 1998; accepted in final form 9 April 1999.
REFERENCES


8. Fabiato, A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. Am. J. Physiol. 245 (Cell Physiol. 14): C1–C14, 1983.


