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MAINTENANCE OF THE K^+ ACTIVITY GRADIENT IN INSECT MUSCLE COMPARED IN DIPTERA AND LEPIDOPTERA: CONTRIBUTIONS OF METABOLIC AND EXCHANGER MECHANISMS

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Summary

Using a comparative approach, the mechanisms involved in maintenance of the transmembrane K^+ activity gradients in the larval body-wall muscles of two insects, *Phormia terraenovae* (Diptera) and *Spodoptera exigua* (Lepidoptera), have been investigated. Double-barrelled K^+-selective microelectrodes were used to obtain simultaneous measurements of intracellular K^+ activity and membrane potential, whilst ordinary microelectrodes were used to monitor input resistance. By application of a variety of general metabolic blockers, the K^+ gradients in both *P. terraenovae* and *S. exigua* muscle were found to be maintained, at least in part, by a metabolic component. Differences in sensitivity to dinitrophenol of the two insects suggested that the ATP-dependence of maintenance of the K^+ gradient was significantly higher in *P. terraenovae* than in *S. exigua*. Vanadate sensitivity suggested that both insects possess P-type ATPases. The K^+ activity gradient in *P. terraenovae* muscles was also found to be ouabain-sensitive, indicating the involvement of a Na^+/K^+-ATPase. In contrast, the K^+ gradient in *S. exigua* muscles proved to be totally insensitive to ouabain but sensitive to amiloride. Application of the H^+/K^+-ATPase-specific inhibitor SCH 28080 suggested the presence of an H^+/K^+ pump similar to the mammalian gastric H^+/K^+-ATPase in the lepidopteran muscles. *P. terraenovae* muscles, however, were found to be totally insensitive to this inhibitor. Using the anion (Cl^-)-dependent transport inhibitors bumetanide and SITS (4-acetamide-4-isothiocyanostilbene-2,2-disulphonic acid), *P. terraenovae* muscles were shown not to possess a Cl^--dependent K^+ transport mechanism. In contrast, a bumetanide-sensitive K^+/Cl^- cotransporter was likely to be involved in maintenance of the K^+ gradient in *S. exigua* muscle. An additional SITS-sensitive Cl^-/HCO_3^- exchanger could also have some indirect involvement in K^+ maintenance through regulation of the inward Cl^- gradient. The results are integrated in two ionic models, one for each insect, which could account for the bulk of K^+ transport in the body-wall muscles of these insects.

Key words: K^+ activity gradient, insect muscle, metabolic ion pumps, P-type ATPase, Na^+/K^+-ATPase, H^+/K^+-ATPase, K^+/Cl^- cotransport, $Na^+/K^+/Cl^-$ cotransport, *Phormia terraenovae*, *Spodoptera exigua*.

Introduction

Throughout the insect orders, the extracellular ion levels and transmembrane ion concentration gradients have been shown to vary greatly (see Djamgoz, 1987, for a review). These differences in extracellular ion levels are of profound importance to insect muscles since, unlike the central nervous system, there is no significant 'blood–brain barrier' surrounding these cells (Treherne and Pichon, 1972; Treherne and Schofield, 1981; Treherne, 1985).

Earlier studies on dipteran muscle have shown the magnitudes and directions of the prevailing ion gradients to be 'conventional' (Gupta *et al.* 1980; Dawson and Djamgoz, 1988). Thus, the K^+ gradient is outwardly directed, the equilibrium potential for K^+, E_K, being considerably more negative than resting membrane potential, E_m. The Na^+ gradient is inwardly directed, the Na^+ equilibrium potential, E_{Na}, being more positive than E_m, and Cl^- is in passive equilibrium across the membrane (Jan and Jan, 1976; Djamgoz and Dawson, 1988). Furthermore, studies of resting membrane electrogenesis in dipteran muscle have shown that E_m can be fully accounted for in terms of the partial permeabilities of Na^+ and K^+ (Jan and Jan, 1976; Djamgoz and Dawson, 1988). Preliminary studies in larval and prepupal dipterans (Djamgoz, 1986; Henon and Ikeda, 1981) also suggested that a ouabain-sensitive metabolic mechanism, presumably a Na^+/K^+-ATPase, was present in skeletal muscle.

In contrast, the ion gradients in lepidopteran muscle are

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'unconventional' (Djamgoz, 1986, 1987; Djamgoz and Dawson, 1989). Thus, although the K^+ gradient is outwardly directed, E_K is generally found to be more positive than E_m. An extremely weak, sometimes reversed, Na^+ gradient exists, E_{Na} also being more positive than E_m. The Cl^- gradient is inwardly directed, resulting in the Cl^- equilibrium potential, E_{Cl}, being more negative than E_m. In the study of Dawson *et al.* (1989) on resting membrane electrogenesis in *Chilo partellus* muscle, 85 % of the E_m could be accounted for by the K^+, Na^+ and Cl^- permeabilities. The remaining 15 % was proposed to be maintained by an electrogenic metabolic pump, although the possible involvement of other mechanisms, e.g. H^+ and Ca^{2+} gradients, was not dismissed. Preliminary evidence suggested that a ouabain-sensitive mechanism was not present in lepidopteran muscle (Rheuben, 1972; Dawson *et al.* 1989) and this would be consistent with the prevailing ionic gradients, which are not conducive to operation of a 'classical' Na^+/K^+-ATPase (Djamgoz, 1986).

Thus, to date, our understanding of the nature and function of metabolic ion pumps/exchangers in insect muscle is limited. Since K^+ is the principal ion involved in resting membrane electrogenesis in both Diptera and Lepidoptera, we have used a comparative approach to investigate the transport mechanisms involved in maintenance of the K^+ activity gradients in the ventral body-wall muscle fibres of the larvae of two insects, *Phormia terraenovae* (Diptera) and *Spodoptera exigua* (Lepidoptera). Double-barrelled K^+-selective microelectrodes (KSMs) were used to obtain simultaneous measurements of membrane potential (E_m) and intracellular K^+ activity (aK_i), whilst ordinary microelectrodes (OMs) were used to monitor input resistance (R_i), following application of several general metabolic blockers and ion transport inhibitors to the muscles of these two insects. A comparison of the possible ATP-dependence of maintenance of the K^+ gradients in the two insects was obtained by the use of general metabolic blockers. By using specific inhibitors of P-type ATPases and Na^+-dependent transport, it was possible to elucidate the nature of the ATP-dependent K^+ transporters (pumps) in each insect. In addition, the possible role of anion (Cl^-)-dependent transport mechanisms in K^+ activity gradient maintenance was also assessed by the use of the inhibitors bumetanide and SITS. The results suggested models which could account for the bulk of K^+ transport in the two insects.

Materials and methods

The preparations

Experiments were carried out on final-instar larvae of the dipteran *Phormia terrraenovae* (Robineau-Desvoidy) (reared at Imperial College, Silwood Park) and the lepidopteran *Spodoptera exigua* (Hubner) (supplied by Zeneca, Jealott's Hill). All preparations were dissected dorso-ventrally under saline. *P. terraenovae* preparations were bathed in Rice's saline (Finlayson and Osborne, 1970) containing (in mmol l^{-1}): NaCl, 173; KCl, 13.5; $MgCl_2$, 1.0; $NaHCO_3$, 1.2; NaH_2PO_4, 0.8; $CaCl_2$, 0.9; glucose, 55.5; pH 7.0. *S. exigua* preparations were bathed in modified Weevers saline (Weevers, 1966) containing (in mmol l^{-1}): NaCl, 12; KCl, 30.0; $MgCl_2$, 18.0; $NaHCO_3$, 1.5; NaH_2PO_4, 1.5; $CaCl_2 \cdot 2H_2O$, 2.0; sucrose, 152; pH 6.8. Preparations were allowed to equilibrate at room temperature (18–20 °C) for 30 min prior to microelectrode impalements.

K^+-selective microelectrodes

Double-barrelled K^+-selective microelectrodes (KSMs) were constructed and used as described previously (Djamgoz and Dawson, 1986; Fitzgerald and Djamgoz, 1995). Lengths of borosilicate glass tubing (1.0 and 1.5 mm o.d.) were glued together with Araldite (Ciba-Geigy), twisted through 180 ° in the middle in a vertical electrode puller (Narashige) and then pulled. The inside of the larger (active) barrel was silanised by exposing the opening to dimethyldichlorosilane vapour (Sigma). The tip of this barrel was filled with a column (approximately 2 mm in length) of a K^+-selective, low-impedance valinomycin-based neutral ion carrier (Fluka Chemicals) (Ammann *et al.* 1987). The active barrel was backfilled with 0.5 mmol l^{-1} KCl and the filamented 'reference barrel' was filled with 5 mmol l^{-1} LiCl. KSMs were calibrated in a series of saline solutions of varying K^+ activity (using equimolar Na^+ as the substitute). K^+ activity refers to the effective concentration representing the freely mobile (i.e. 'active') K^+ which is available to contribute to the generation of E_K. Calibration curves had slopes of 50–58 mV for a tenfold change in K^+ activity. K^+ activity measurements were accepted only if the calibrations of the KSM before and after the experiment were within ±5 mV of each other at every point.

Ordinary microelectrodes

Ordinary microelectrodes were pulled from borosilicate glass capillaries (1 mm o.d., 0.6 mm i.d.) on a vertical puller. They were filled with 5 mmol l^{-1} LiCl and had tip resistances in the range 6–10 MΩ. The microelectrodes were connected to a microprobe system (WPI M701) with an inbuilt bridge circuit for current injection which was used for measurement of cell input resistance.

Inhibitor solutions

The following general metabolic blockers were used: dinitrophenol (DNP), potassium cyanide (KCN), sodium azide and rotenone. In addition, the P-type ATPase inhibitor vanadate, the Na^+/K^+-ATPase-specific inhibitor ouabain, the Na^+-dependent transport inhibitor amiloride, and SCH 28080, an H^+/K^+-ATPase-specific inhibitor, were used. The anion (Cl^-)-dependent transport inhibitors bumetanide and SITS were also used. Micromolar concentrations of bumetanide inhibit $Na^+/K^+/Cl^-$ cotransport, whilst at millimolar concentrations there is evidence that K^+/Cl^- cotransport is inhibited by this drug (Lauf, 1985; Bernhardt *et al.* 1988; Geck and Heinz, 1986). 4-Acetamide-4-isothiocyanostilbene-2,2-disulphonic acid (SITS) is an inhibitor of a number of Cl^- transport mechanisms such as Cl^-/HCO_3^- exchange (Thomas, 1984; Jennings, 1992), Na^+-dependent Cl^-/HCO_3^- exchange

(Thomas, 1977; Boron and Russell, 1983) and Na^+/HCO_3^- cotransport (Boron and Boulpaep, 1983; Deitmer and Szatkowski, 1990). Unless otherwise stated, all drugs were used at a concentration of 1 mmol l^{-1}, dissolved in saline. Both SCH 28080 and bumetanide were dissolved in 1 % (v/v) ethanol saline solution. At 1 %, ethanol was found to have no effect on any of the parameters measured (data not shown). With the exception of SCH 28080, which was a gift from Schering Pharmaceuticals USA, all drugs were obtained from Sigma.

Inhibitor sensitivity tests

For each inhibitor, sensitivity tests were conducted to ensure that the addition of a particular inhibitor to the saline did not cause any interference with the response of the KSMs to K^+ activity (aK) (Fitzgerald and Djamgoz, 1995). KSMs were first calibrated in 'normal' saline solutions and then in solutions containing the inhibitor. The concentration of inhibitor (interferent) was kept constant (the same as that used in experiments) throughout the series of calibration solutions. Between three and five electrodes were calibrated per inhibitor to obtain an average curve.

With the exception of DNP, none of the inhibitors used had a significant effect on the response of the KSMs to K^+ activity. DNP, however, behaved as a 'classical' interferent, adding significantly to the K^+ activity recorded by a KSM in solutions of low aK but having no effect on the K^+ activity recorded in solutions of high (greater than 97 mmol l^{-1}) aK. Correction of the recorded values of intracellular K^+ activity (aK$_i$) for DNP interference was achieved by the method outlined in Fitzgerald and Djamgoz (1995). These corrected values of aK$_i$ are referred to throughout the paper.

Evaluation of inhibitor effects

We have evaluated whether active K^+ transport was inhibited by a given inhibitor as follows. As outlined in the Introduction, previous studies have shown that E_m in dipteran muscle is maintained principally by a strong outward K^+ gradient, which generates an E_K considerably more negative than E_m. An inward Na^+ gradient generates an E_{Na} more positive than E_m, whilst Cl^- is in passive equilibrium and thus makes no contribution to E_m (Jan and Jan, 1976; Dawson and Djamgoz, 1988; Djamgoz and Dawson, 1988). Importantly, in all these studies, the partial permeabilities of K^+ and Na^+ were shown to account fully for resting membrane electrogenesis in dipteran body-wall muscle. Thus, any active K^+ transporter present is unlikely to contribute any additional electrogenic component to the E_m, but rather seems likely to be responsible for maintenance of the Na^+ and K^+ gradients. From this, it follows that inhibition of an active K^+ transport mechanism will reduce aK$_i$ and hence the driving force for K^+ efflux. As E_K then depolarises, so E_m will also depolarise. However, as E_m depolarises, it is also possible that voltage-dependent K^+ and/or Na^+ channels, known to occur in insect muscle, will in turn open and exert their effects on E_m (Salkoff and Wyman, 1983; Miller and Usherwood, 1990). In the following summary of inhibitor effects, we have therefore assumed that, if a significant decrease in aK$_i$ was due to inhibition of an active K^+ transporter, the E_K–E_m difference would change in the same direction as the corresponding E_m. However, if there was a change in membrane conductance, E_K–E_m could change differently depending on the relative changes in Na^+ and K^+ conductances, G_{Na} and G_K. Such changes in conductance could also be reflected as a change in R_i.

As with *Phormia terraenovae*, it has been assumed that inhibition of active K^+ transport in *Spodoptera exigua* muscle will cause a significant reduction of aK$_i$ coupled with E_K–E_m remaining constant or changing in the same direction. Where E_K–E_m changes differently and/or R_i changes, we have assumed that ionic conductance has also changed. Interpretation of these changes in E_K–E_m and/or R_i is based on the findings of other studies which have shown that lepidopteran muscles have 'unconventional' transmembrane ion gradients. Thus, although an inward K^+ gradient persists, E_K tends, in general, to be more positive than the corresponding E_m. The Na^+ gradient is extremely weak, sometimes even reversed, with E_{Na} being slightly more positive than E_m. The Cl^- gradient is inwardly directed, with E_{Cl} consistently found to be more negative than E_m (Djamgoz and Dawson, 1989).

Experimental procedure

Time-course experiments

KSMs were used to monitor the time courses of the changes in aK$_i$ and E_m (and calculated E_K) for up to 60 min during application of a given inhibitor. Initial recordings of E_m and aK$_i$ were made from several cells bathed in normal saline over an initial period of 10 min, designated time T_0. The bathing solution was then changed for a saline + inhibitor solution, and further recordings from individual cells were made repeatedly by impaling as many cells as possible for a period of up to 55–70 min. This latter period was subdivided into approximately 10 min intervals (designated time T_{10} to T_{60}). In this way, 7–10 cells on average could be sampled during each time interval, in a given preparation. In control experiments, the same procedure was followed, except that, after T_0, the solution was changed to normal saline, rather than saline + inhibitor. The total number of preparations was between three and eight per treatment per species. Values of E_K were calculated from the Nernst equation. Data were pooled from the total number of preparations in a given treatment. Values of E_m, aK$_i$ and E_K–E_m are presented in Tables 1 and 2 as means ± standard error of the mean (S.E.M.). All experiments were carried out at room temperature (18–20 °C).

Input resistance measurements

In order to determine whether any change in membrane conductance occurred following application of inhibitors, ordinary microelectrodes (OMs) and a bridge balance amplifier were used to record input resistance (R_i) from individual muscle cells in both insects. Initial recordings of E_m and R_i were made from between seven and nine cells over a period of 10 min (time T_0) in preparations bathed in normal saline. The bathing medium was then changed for a saline + inhibitor

solution, and the preparation was left for a further 60 min, after which additional recordings of E_m and R_i were made for approximately 10 min. The timing of this latter set of measurements was designated T_{60}. Thus, values of both E_m and R_i were obtained from cells both before (T_0) and after (T_{60}) application of an inhibitor. In control treatments, the solution change after T_0 was to normal saline rather than to saline + inhibitor. Between four and seven preparations were used per treatment per insect. Consequently, between 19 and 51 cells were sampled in each time interval (T_0 and T_{60}). Importantly, the E_m data obtained using OMs were not significantly different from the data obtained using KSMs (data not shown). Data were only accepted if there had been no change in the resistance of either the OM or the cell as a result of the impalement of the cell. Data are presented in Tables 1 and 2 as means ± S.E.M.

Since all the drugs used in this study would ultimately result in poisoning and cell death, we have assumed that changes in R_i due to uncoupling effects will be inherent. We have therefore neglected uncoupling in our subsequent discussion of inhibitor effects. Experiments were conducted at room temperature (18–20 °C).

Statistics

Student's *t*-tests (using 95 % confidence limits) were carried out on values of aK$_i$, E_m, the E_K–E_m difference and R_i obtained at time T_0 *versus* T_{60}. These essential quantitative data are presented in Tables 1 and 2 as means ± S.E.M., with the results of Student's *t*-tests given as follows; $*P<0.05$, $**P<0.01$, $***P<0.001$.

Results

Effects of inhibitors on Phormia terraenovae *muscles*

Traces from a typical experiment showing recordings of aK$_i$ and E_m in *Phormia terraenovae* muscles, and the effect of an inhibitor (1 mmol l^{-1} ouabain), are shown in Fig. 1. The upper trace shows the membrane potential (E_m), whilst the lower trace shows the recording of intracellular K^+ activity (aK$_i$). Quantitative data for both species are summarised in Tables 1 and 2. The percentage changes in aK$_i$ and E_m between T_0 and T_{60} $[(T_{60}-T_0)/T_0]$ are presented below as ΔaK$_i$ and ΔE_m, respectively. It should be noted that, where E_m depolarised, E_m has been presented as a positive value. Negative values of E_m denote a hyperpolarisation of E_m.

General metabolic blockers

Of the four metabolic blockers tested, only azide was ineffective on aK$_i$ (Table 1; see Fig. 2). DNP, rotenone and KCN all reduced aK$_i$ and caused depolarisation of E_m. The protonophore DNP, which uncouples phosphorylation by dissipating the mitochondrial proton gradient, had by far the most marked effects on both aK$_i$ (ΔaK$_i$ −68 %, Fig. 3A) and E_m (ΔE_m 54 %). The E_K–E_m difference closely followed the changes in E_K, which ultimately depolarised to a greater extent than E_m. If aK$_i$ had decreased as the result of a selective increase in G_K, a hyperpolarisation of E_m would have been expected. To explain the lack of change in R_i, therefore, it follows that an equal and opposite increase in the conductance of some other ion, presumably Na^+, must also have occurred. Consequently, the observed decrease in aK$_i$ was probably due largely to inhibition of an ATP-dependent K^+ transporter, although possible involvement of ion channel(s) could not be ruled out.

Rotenone and KCN, both well-known inhibitors of the mitochondrial electron transport chain, were considerably less effective in reducing aK$_i$ (Fig. 2). In the case of rotenone, E_K–E_m increased significantly since E_m depolarised to a greater extent than E_K (ΔaK$_i$ −19 %, ΔE_m 32 %). This implied either that G_K had decreased or that there had been an increase

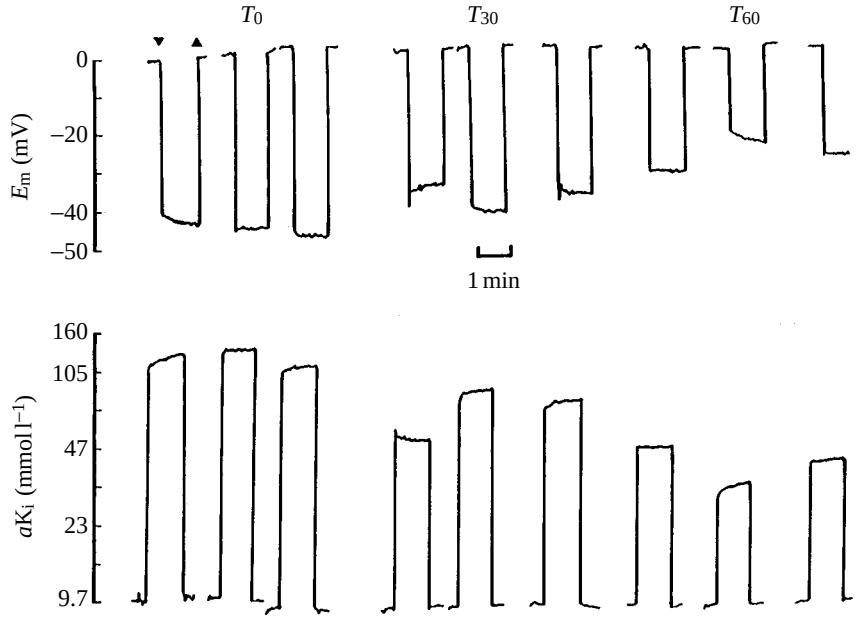

Fig. 1. Chart recording from a typical experiment showing recordings of membrane potential (E_m) and intracellular K^+ activity (aK$_i$) in *Phormia terraenovae* muscles, under control conditions (T_0) and following application of 1 mmol l^{-1} ouabain for 30 min (T_{30}) and 60 min (T_{60}). The upper trace shows E_m and the lower trace shows aK$_i$. The moments of impalement and withdrawal of the electrode from the cell are indicated by the downward- and upward-pointing arrowheads, respectively.

Table 1. *Effects of general metabolic blockers and ion transport inhibitors on* E_m, aK_i, E_K–E_m *and* R_i *in* Phormia terraenovae *muscle*

Treatment	E_m (mV)	aK_i (mmol l^{-1})	E_K–E_m (mV)	Cells/ preparations	R_i (MΩ)	Cells/ preparations
Control						
T_0	–46±0.7	120±4.9	–14±1.5	37/5	1.4±0.01	26/4
T_{60}	–47±0.7	114±3.2	–10±1.0	31/5	1.6±0.2	25/4
Dinitrophenol						
T_0	–41±1.0	114±6.0	–20±1.1	26/4	5.6±1.5	29/3
T_{60}	–19±0.6***	37±3.0**	–14±1.0**	21/4	6.1±1.0	24/3
KCN						
T_0	–48±0.6	122±4.8	–15±1.1	44/6	4.0±1.2	25/4
T_{60}	–40±0.7***	108±4.6*	–15±1.7	35/6	1.4±0.4**	30/4
Azide						
T_0	–47±0.7	123±3.7	–16±0.8	57/7	3.5±0.7	35/5
T_{60}	–34±1.1***	111±4.8	–26±1.3***	57/7	6.1±1.1	30/5
Rotenone						
T_0	–48±1.0	136±5.0	–18±0.9	49/7	2.9±0.9	22/3
T_{60}	–33±0.6***	110±3.0**	–28±0.8***	44/7	1.1±0.3**	21/3
Vanadate						
T_0	–46±0.8	152±8.6	–22±1.1	52/7	1.3±0.5	41/6
T_{60}	–31±0.9***	75±4.6***	–19±1.6	30/7	1.3±0.6	39/6
Ouabain						
T_0	–46±0.8	127±6.5	–16±1.1	57/7	5.1±0.9	41/6
T_{60}	–30±1.2***	49±2.6***	–9±1.0***	37/7	2.5±0.6**	43/6
Amiloride						
T_0	–46±0.6	123±7.8	–16±1.4	44/6	4.3±1.0	35/5
T_{60}	–41±1.2***	123±6.5	–22±2.0*	26/6	3.4±1.0**	37/5
SCH 28080						
T_0	–50±0.9	220±10.8	–28±1.1	30/7	4.0±0.8	20/3
T_{60}	–54±0.8**	201±11.7	–21±1.1***	29/7	4.0±1.0	25/3
Control						
T_0	–49±0.9	147±25	–18±1.6	38/5	1.4±0.1	26/4
T_{60}	–49±0.9	142±15	–18±0.8	38/5	1.6±0.2	25/4
Bumetanide (1 mmol l^{-1})						
T_0	–48±1.3	170±18	–24±0.8	23/3	2.3±0.4	24/3
T_{60}	–48±0.8	160±31	–22±2.5	23/3	2.5±0.6	24/3
SITS						
T_0	–48±3.1	164±21	–23±0.8	24/3	7.9±1.5	22/3
T_{60}	–48±1.3	158±29	–22±0.04	22/3	7.2±1.4	20/3

Data are presented as means ± S.E.M.
The results of Student *t*-tests between values at T_0 and T_{60} are given as follows; significant at *P<0.05, **P<0.01, ***P<0.001.

in conductance of some other ion(s) with an opposing equilibrium potential (e.g. G_{Na}). The observed significant decrease in R_i would suggest that the latter had occurred. On the basis of these assumptions, no more than 5 mV (the depolarisation of E_K) of the observed depolarisation of E_m could be due directly to inhibition of an ATP-dependent K^+ pump. KCN was slightly less effective than rotenone in reducing aK_i (ΔaK_i −12 %). Nonetheless, E_K–E_m remained constant, suggesting that the effects were largely due to inhibition of a metabolically driven K^+ transporter. The significant drop in R_i could possibly be explained by increases in voltage-dependent conductance as E_m depolarised (ΔE_m 17 %). Somewhat surprisingly, azide, which acts in exactly the same way as KCN, had no significant effect on aK_i (ΔaK_i −10 %) but depolarised E_m (ΔE_m 28 %), whilst the E_K–E_m difference increased owing to the greater depolarisation of E_m over E_K. This suggested that E_m may have depolarised as a result of an increase in G_{Na} and/or a decrease in G_K. Such equal and opposite conductance changes would explain the lack of effect on R_i. The reasons for the apparent lack of effect of azide on ATP-dependent K^+ transport, however, remain unclear (see Fitzgerald, 1992).

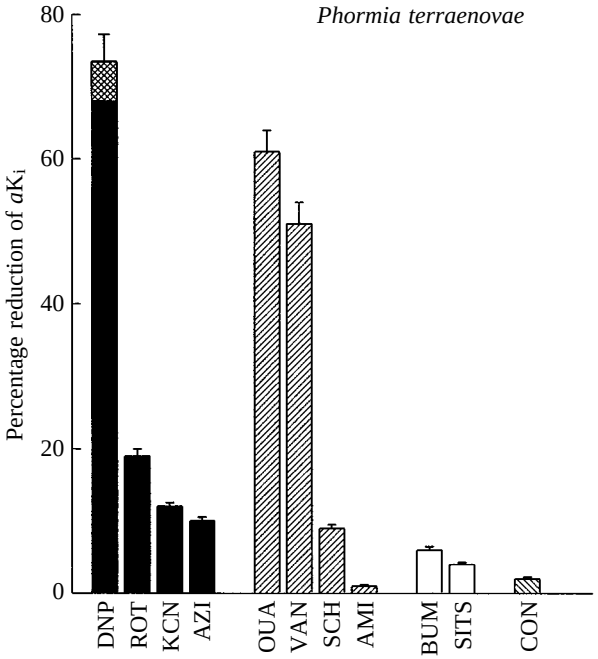

Fig. 2. Percentage reduction of intracellular K^+ activity (aK_i) following application of general metabolic blockers (solid bars), P-type ATPase/Na^+-dependent transport inhibitors (hatched bars) and anion (Cl^-)-dependent transport inhibitors (open bars) to *Phormia terraenovae* muscles for 60 min. The general blockers used were dinitrophenol (DNP), rotenone (ROT), potassium cyanide (KCN) and sodium azide (AZI). The P-type ATPase/Na^+-dependent transport inhibitors used were ouabain (OUA), vanadate (VAN), SCH 28080 (SCH) and amiloride (AMI). The anion (Cl^-)-dependent transport inhibitors used were bumetanide (BUM) and SITS. Error bars indicate S.E.M. DNP caused a 68 % inhibition over 60 min, and a 76 % inhibition between T_{10} and T_{60} (crosshatched bar), suggesting that a strongly ATP-dependent mechanism (ion pump) was involved in regulation of the K^+ gradient. The marked inhibition caused by ouabain (61 %) and vanadate (51 %) indicated that a P-type Na^+/K^+-ATPase is the principal mechanism involved in maintenance of the K^+ gradient in these muscles. The lack of significant inhibition by bumetanide and SITS showed that no Cl^--dependent mechanism is involved. CON, control. Values of *N* are given in Table 1.

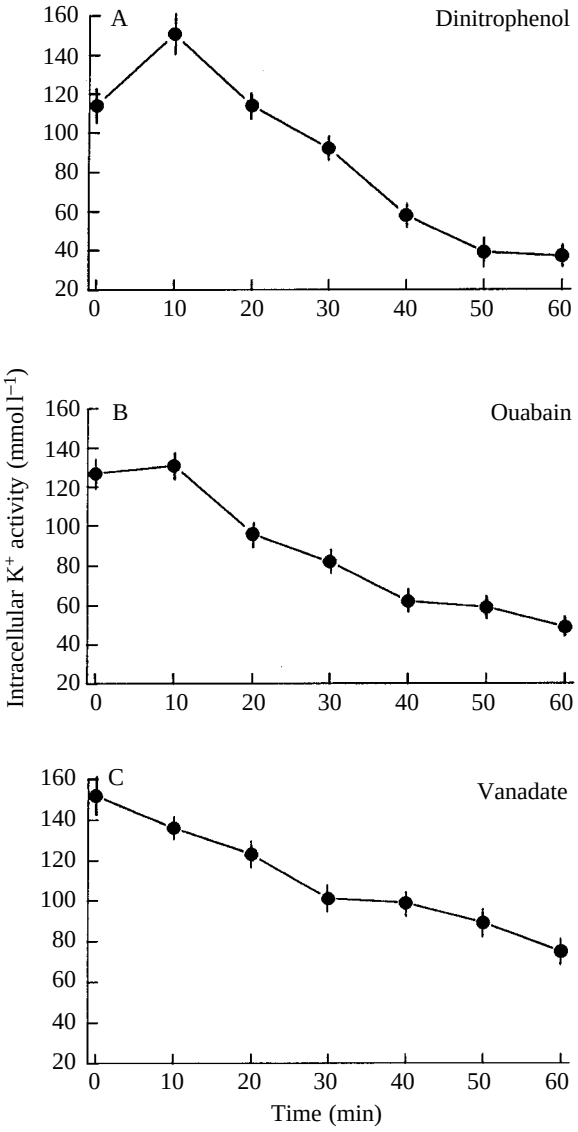

Fig. 3. Time courses showing the effects of (A) the general metabolic blocker dinitrophenol, (B) the Na^+/K^+-ATPase-specific inhibitor ouabain and (C) the P-type ATPase inhibitor vanadate on the intracellular K^+ activity (aK_i) in *Phormia terraenovae* muscles. Values are means ± S.E.M.; values of *N* are given in Table 1. The inhibitors were added at T_0.

P-type ATPase/Na^+-dependent transport inhibitors

Of this group of inhibitors, only ouabain and vanadate caused significant reductions of aK_i (Fig. 2). Ouabain was the more potent in terms both of reducing aK_i (ΔaK_i −61 %) and depolarising E_m (ΔE_m 35 %). The E_K–E_m difference decreased significantly over 60 min, with E_K depolarising to a greater extent than E_m. In addition, R_i also fell significantly. Together, these results suggested that the drop in aK_i occurred largely as a result of inhibition of a Na^+/K^+-ATPase, in accordance with the almost absolute specificity of ouabain for this pump (e.g. Glynn, 1985; Akera, 1981). Similar to the effect of DNP in these muscles (Fig. 3A), the time course for aK_i (Fig. 3B) showed a slight increase in aK_i over the first 10 min before a rapid drop in aK_i during the remainder of the experimental period. Earlier studies have also shown that low concentrations of ouabain initially stimulate the Na^+/K^+ pump (see Bonting, 1970; Lee and Dagostino, 1982).

Vanadate was also highly effective in reducing aK_i (ΔaK_i −51 %, Figs 2, 3C) and depolarising E_m (ΔE_m 33 %). In this case, however, E_K–E_m remained constant throughout the experimental period and R_i was unaffected, suggesting that aK_i decreased through direct inhibition of a K^+ transporter. Since vanadate is an inhibitor of P-type ATPases (Pederson and Carafoli, 1987), this result supports the suggestion that ouabain inhibited a Na^+/K^+ pump in these muscles, the Na^+/K^+-ATPase being an example of a P-type pump. Additional conductance changes may therefore have occurred

in addition to voltage-dependent effects to result in no net change in R_i.

Amiloride had no effect on aK_i (Fig. 2) but did cause depolarisation of E_m (ΔE_m 11 %). In addition, R_i was found to decrease significantly, which implied that there may have been a change in G_{Na} rather than G_K. Whilst one possible action of amiloride on these muscles could be inhibition of a Na^+/H^+ exchanger, a major target of this drug (Kleyman and Cragoe, 1988), it should be noted that Na^+/H^+ exchange is generally found to be inactive in resting cells. Perhaps more likely, therefore, is the possibility that the high concentration of amiloride used may have exerted 'non-specific' effects on a number of Na^+-dependent transport processes, leading to a net increase in conductance (decrease in R_i) and depolarisation of E_m.

When the H^+/K^+-ATPase-specific inhibitor SCH 28080 was applied to *Phormia terraenovae* muscles, no significant change was observed in aK_i (ΔaK_i −9 %, Fig. 2), although E_m hyperpolarised significantly (ΔE_m −8 %) after 60 min. Nonetheless, our results provided no evidence to suggest that SCH 28080 inhibited a K^+ pump in *Phormia terraenovae* muscles.

Anion (Cl^-)-dependent transport inhibitors

Consistent with earlier studies on dipteran muscle which have shown that Cl^- is passively distributed across the membrane with $E_{Cl} \approx E_m$ (Jan and Jan, 1976; Dawson and Djamgoz, 1988), neither bumetanide (1 mmol l^{-1}) nor SITS (1 mmol l^{-1}) was found to exert any significant effects on the parameters recorded in *Phormia terraenovae* muscles (Fig. 2; Table 1). These results clearly demonstrated the complete lack of effect of Cl^--dependent transport inhibitors against aK_i (and therefore the K^+ gradient) and E_m.

Effects of inhibitors on Spodoptera exigua

General metabolic blockers

With the exception of DNP, the general blockers tested in *Spodoptera exigua* all caused significant reduction of aK_i, and they all caused significant depolarisation of E_m (Table 2; Fig. 4). Rotenone was found to be the most potent inhibitor (ΔaK_i −38 %, ΔE_m 28 %). Since E_K–E_m remained constant throughout treatment, aK_i presumably decreased as a result of inhibition of an ATP-dependent K^+ transport mechanism rather than because of an increase in G_K.

KCN also reduced aK_i (ΔaK_i −32 %, Fig. 4) with concurrent depolarisation of E_m (ΔE_m 26 %). During this time, the E_K–E_m difference and R_i exhibited no significant changes. Thus, KCN also apparently reduced aK_i and depolarised E_m by direct inhibition of cell metabolism and hence by inhibiting an ATP-dependent K^+ transport mechanism.

In the presence of azide, the effects on aK_i (ΔaK_i −32 %) and E_m (ΔE_m 16 %) were accompanied by a significant decrease in R_i. If G_K had increased selectively, E_K–E_m might have been expected to decrease as E_m depolarised (E_K being more positive than E_m). However, since the opposite was observed, the main conductance increase must have been due to other ion(s), e.g. G_{Na}. Nonetheless, aK_i apparently decreased as a result of inhibition of a metabolic K^+ pump.

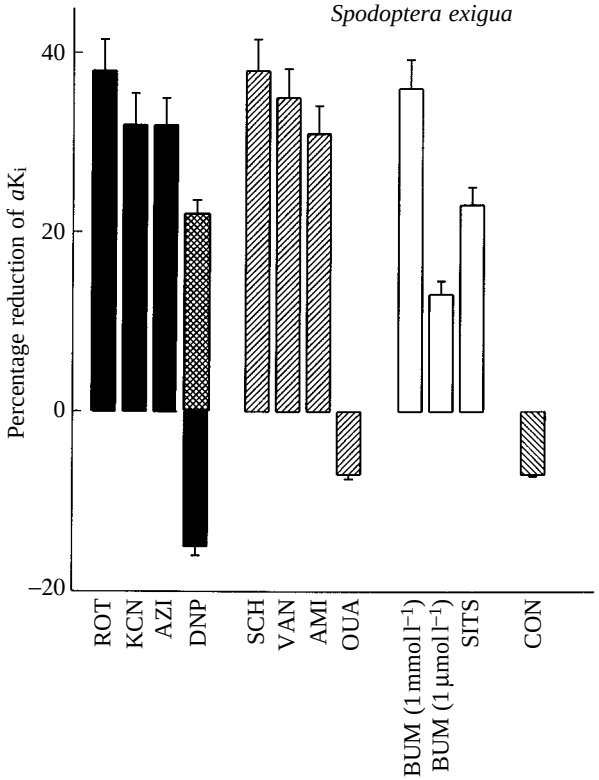

Fig. 4. Percentage reduction of aK_i in *Spodopter*a *exigua* muscle following application of the general metabolic blockers (solid bars) rotenone (ROT), cyanide (KCN), azide (AZI) and dinitrophenol (DNP), and the P-type ATPase/Na^+-dependent transport inhibitors (hatched bars) ouabain (OUA), vanadate (VAN), SCH 28080 (SCH) and amiloride (AMI). The anion (Cl^-)-dependent transport inhibitors used were bumetanide at two concentrations (BUM) and SITS (open bars). With the exception of DNP, all the general metabolic blockers caused significant inhibition of aK_i over 60 min, although only the effects of rotenone and KCN could be attributed directly to inhibition of a K^+ pump. The marked decrease in aK_i observed in the presence of vanadate (35 %) and SCH 28080 (38 %) suggested that a P-type H^+/K^+ ATPase is involved in maintenance of the K^+ gradient in the muscles of this insect. DNP caused an initial increase in aK_i between T_0 and T_{10} min followed by a steady decrease. However, the essential result is unaffected even when comparing the change in aK_i between T_0 and T_{60} (solid bar) *versus* the change between T_{10} and T_{60} (crosshatched bar). Bumetanide (1 mmol l^{-1}) caused a significant reduction of aK_i (36 %, P<0.001). SITS also caused a significant inhibition of 23 % (P<0.05). CON, control. Error bars indicate S.E.M.; values of N are given in Table 2.

DNP, however, appeared to have a very different effect on aK_i from the other general blockers (Fig. 5A). A marked increase in aK_i was observed during the first 10 min, followed by a steady decline over the remaining period. Even after 60 min, aK_i had not regained its initial value (ΔaK_i 15 %). Meanwhile, E_m depolarised steadily throughout DNP application (ΔE_m 21 %). Although E_m depolarised by 2 mV during the first 10 min, contrary to the 5 mV hyperpolarisation

Table 2. *Effects of general metabolic blockers and ion transport inhibitors on* E_m, aK_i, E_K–E_m *and* R_i *in* Spodoptera exigua *muscle*

Treatment	E_m (mV)	aK_i (mmol l^{-1})	E_K–E_m (mV)	Cells/ preparations	R_i (MΩ)	Cells/ preparations
Control						
T_0	–43±0.5	119±2.2	0±0.2	31/5	0.8±0.2	30/4
T_{60}	–43±0.7	127±3.2	–2±0.5	34/5	0.6±0.2	23/4
Dinitrophenol						
T_0	–43±0.7	151±4.1	–6±0.2	24/4	0.4±0.1	22/3
T_{60}	–34±0.9***	174±7.3	–17±0.6	18/4	0.7±0.2	30/3
KCN						
T_0	–43±0.9	130±6.7	–1±0.9	32/4	0.5±0.04	24/3
T_{60}	–34±0.7***	89±2.4***	–1±0.5	18/4	0.4±0.06	23/3
Azide						
T_0	–44±0.8	117±8.3	4±0.8	41/6	0.5±0.1	19/3
T_{60}	–37±0.8***	79±9.6**	9±0.8***	24/6	0.2±0.04***	25/3
Rotenone						
T_0	–40±0.5	146±4.2	–8±0.5	41/6	0.7±0.2	25/4
T_{60}	–29±0.5***	90±2.3***	–7±0.5	31/6	0.2±0.04**	26/4
Vanadate						
T_0	–42±0.7	136±5.0	–4±0.8	51/7	1.1±0.1	51/7
T_{60}	–31±0.5***	89±3.1***	–4±0.7	38/7	0.2±0.03***	52/7
Ouabain						
T_0	–36±0.6	112±3.6	–5±0.6	51/7	1.1±0.2	44/6
T_{60}	–38±1.0	120±5.1	–5±0.6	34/7	0.8±0.2	41/6
Amiloride						
T_0	–42±0.8	130±4.6	–3±0.9	42/6	0.5±0.08	28/4
T_{60}	–35±0.8***	90±2.6***	–1±0.5	43/6	0.3±0.02	28/4
SCH 28080						
T_0	–44±1.0	154±6.9	–5±1.0	37/8	2.4±0.4	25/4
T_{60}	–35±1.1***	96±3.2***	–2±0.7*	38/8	0.6±0.2***	22/4
Control						
T_0	–45±3.5	120±16.0	3±1.5	21/3	0.8±0.2	30/4
T_{60}	–48±2.8	135±16.0	3±1.1	23/3	0.6±0.2	23/4
Bumetanide (1 mmol l^{-1})						
T_0	–46±2.9	159±14.0	–4±1.0	34/5	5.6±0.8	24/3
T_{60}	–35±2.3***	102±4.6***	–4±0.5	34/5	1.1±0.2***	21/3
Bumetanide (1 μmol l^{-1})						
T_0	–46±2.3	164±19.0	–5±2.9	34/5	2.5±0.6	23/3
T_{60}	–48±2.9	143±15.0	1±3.5*	35/5	2.1±0.6	23/3
SITS						
T_0	–44±1.7	151±24.0	–4±2.9	35/5	5.6±1.6	25/4
T_{60}	–43±2.9	117±12.0*	1±2.9*	34/5	2.1±0.6*	21/4

Data are presented as means ± S.E.M.
The results of Student *t*-tests between values at T_0 and T_{60} are given as follows; significant at *P<0.05, **P<0.01, ***P<0.001.

of E_m predicted for this increase in aK_i, the difference between the real and predicted change was not significant. It is possible, therefore, that the increase in aK_i at T_{10} was real and not due to an artefact. The effects on E_m and E_K from T_{10} onwards were reminiscent of pump inhibition, i.e. E_K and E_m changing in the same direction. It is possible, therefore, that DNP caused initial stimulation of a K^+ pump, followed by inhibition. As outlined below, such an effect could be due to the action of DNP as a protonophore.

P-type ATPase/Na^+-dependent transport inhibitors

Treatment with three of the four inhibitors caused significant reductions of aK_i with concurrent depolarisation of E_m (Fig. 4). However, in this case the exception was ouabain,

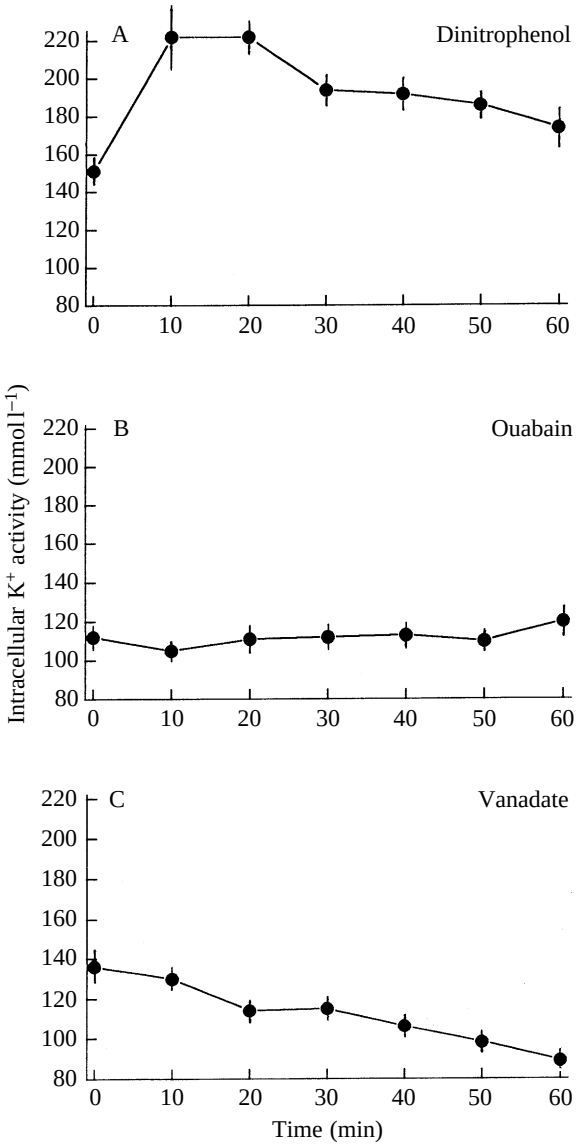

Fig. 5. Time courses showing the effects of (A) the general metabolic blocker dinitrophenol, (B) the Na^+/K^+-ATPase-specific inhibitor ouabain and (C) the P-type ATPase inhibitor vanadate on the intracellular K^+ activity (aK_i) in *Spodoptera exigua* muscles. Values are means ± S.E.M.; values of *N* are given in Table 2. Inhibitors were added at time T_0.

which had no significant effect on either aK_i or E_m in these muscles. The E_K–E_m difference and R_i also remained constant. Thus, ouabain was found to be totally ineffective as an inhibitor of K^+ transport in this insect (Fig. 5B).

Vanadate, in contrast, caused a highly significant drop in aK_i (ΔaK_i −35 %) and depolarisation of E_m (ΔE_m 26 %) (Figs 4, 5C). As in *Phormia terraenovae* (Table 1), the time courses for both aK_i and E_m were very similar, and the E_K–E_m difference consequently maintained a steady level throughout the experimental period. Hence, the decrease in aK_i was presumably due to inhibition of a K^+ pump. R_i, however, fell significantly, suggesting that vanadate may also have affected membrane conductance (possibly voltage-dependent).

Similarly, the application of the H^+/K^+-ATPase-specific inhibitor SCH 28080 also caused a significant decrease in aK_i (ΔaK_i −38 %) and depolarisation of E_m (ΔE_m 21 %). These effects were comparable to those of ouabain on aK_i in *Phormia terraenovae* muscle. R_i and the E_K–E_m difference also decreased significantly, suggesting that there had been a net increase in membrane conductance. However, since the change in E_K–E_m was only very slight (3 mV), it seems most likely that the majority of the fall in aK_i was due to inhibition of a K^+ pump. The decrease in R_i might then result from a voltage-dependent increase in conductance involving G_K and possibly some other ion(s) (Salkoff and Wyman, 1983; Miller and Usherwood, 1990; Ramaswami and Tanouye, 1989).

Amiloride-treatment of *Spodoptera exigua* muscles also had a pronounced effect on both aK_i (ΔaK_i −31 %, Fig. 4) and E_m (ΔE_m 17 %). E_K–E_m remained constant throughout the experimental period and there was no change in R_i. Thus, amiloride was also presumed to reduce aK_i by inhibition of active K^+ transport.

Anion (Cl^-)-dependent transport inhibitors

In lepidopteran muscles, unlike dipteran muscles, there is evidence that Cl^- makes a significant contribution to E_m, there being a strong inward Cl^- gradient associated with these cells (Dawson *et al.* 1989). Thus, a Cl^--dependent mechanism utilising Cl^- influx could possibly be involved in maintenance of aK_i in *Spodoptera exigua* muscles. Of the anion-dependent transport inhibitor 'treatments' tested on these muscles, two (1 mmol l^{-1} bumetanide and SITS), caused significant changes in aK_i after 60 min.

Treatment of *Spodoptera exigua* muscles with a low concentration of bumetanide (1 μmol l^{-1}) was ineffective in reducing aK_i (ΔaK_i −13 %; Fig. 4). Neither R_i nor E_m (ΔE_m 4 %) changed, although the absolute difference E_K–E_m did change significantly, suggesting that there may have been some effect on membrane conductance.

In contrast, the application of 1 mmol l^{-1} bumetanide caused a substantial decrease in aK_i (ΔaK_i −36 %; Fig. 4) and depolarisation of E_m (ΔE_m 24 %). Although R_i decreased significantly, suggesting that membrane conductance had increased, E_K–E_m did not change after 60 min. This latter result implied that no significant change had occurred in G_K. Presumably, therefore, 1 mmol l^{-1} bumetanide exerted its effects on the K^+ gradient *via* inhibition of a Cl^--dependent K^+ transporter. The decrease in R_i might then be explained as follows: (1) an increase in some voltage-dependent conductance resulting from depolarisation of E_m; and (2) an increase in G_{Cl} could also have been involved, although there would also need to be an increase in conductance of some other ion(s), e.g. Na^+, H^+, in order for E_m to depolarise.

Treatment with 1 mmol l^{-1} SITS also caused a significant decrease in aK_i (ΔaK_i −23 %). Meanwhile, R_i decreased significantly, as did the absolute difference E_K–E_m. Both results would suggest that membrane conductance, possibly involving G_K, had increased, although there was no significant change in E_m (ΔE_m 2 %).

Discussion

The maintenance of transmembrane ion gradients can be achieved by means of two broad classes of mechanisms as follows. (1) Primary active transport, in which a primary energy source, such as energy derived from hydrolysis of adenosine triphosphate (ATP), is used to move ions against their electrochemical gradients. These mechanisms are generally referred to as metabolic ion pumps, e.g. the Na^+/K^+-ATPase. (2) Secondary active transport, in which the movement of one ion against its electrochemical gradient is coupled to the movement of another ion down its electrochemical gradient, such that the total free energy is negative (e.g. Na^+/Ca^{2+} exchanger, $Na^+/K^+/Cl^-$ cotransporter). Either type of mechanism can be 'electrogenic' (i.e. transfer a net quantity of electrical charge) and may directly generate a portion of the resting membrane potential E_m in a given cell.

In the present study, we have investigated the mechanisms involved in maintenance of the K^+ activity gradient in the body-wall muscles of two insects with very different transmembrane ionic gradients. Using a comparative approach, we have elucidated the principal active transport mechanisms involved in maintenance of the K^+ activity gradient in the muscles of both insects.

Maintenance of the K^+ activity gradient in Phormia terraenovae

Relatively few data are available for the role of metabolic ion pumps in dipteran muscle. However, preliminary studies in the body-wall muscles of larval and prepupal *Calliphora erythrocephala* (Djamgoz, 1986) showed that application of either DNP or ouabain (1 mmol l^{-1}) caused a gradual depolarisation (by 20–60 %) of E_m over a period of up to 3 h. In the same study, cooling the preparation from 18 to 5 °C caused E_m to depolarise by 10 mV in 1 min. Henon and Ikeda (1981) obtained essentially the same result in *Drosophila melanogaster* flight muscles, where addition of 1 mmol l^{-1} ouabain caused a peak depolarisation of 19 mV after 10–15 min. Studies in dictyopteran and orthopteran muscle, which have similar transmembrane ion gradients, have also shown that application of metabolic blockers, including ouabain, causes gradual depolarisation of E_m over 1–3 h (Wareham *et al.* 1974; Leech, 1986; Djamgoz, 1986). Thus, although no data exist for the effects of metabolic blockers on aK_i, these preliminary studies suggested that a DNP- and ouabain-sensitive K^+ pump, presumably a Na^+/K^+-ATPase, could be present in dipteran muscle.

In *Phormia terraenovae* muscle, the application of the general metabolic blockers DNP, KCN and rotenone caused a significant reduction of aK_i and a depolarisation of E_m (Table 1), which could be attributed to inhibition of an ATP-dependent mechanism. Of these drugs, DNP was by far the most potent, causing a 68 % reduction in aK_i over 60 min, suggesting the presence of a strongly ATP-dependent K^+ pump in these muscles. Similar effects, which could also be attributed to inhibition of a K^+ pump, were observed following application of the P-type ATPase inhibitor vanadate (ΔaK_i −51 %) and the Na^+/K^+-ATPase-specific inhibitor ouabain (ΔaK_i −61 %). In agreement with earlier studies, the sensitivity of aK_i to these specific inhibitors suggested that a Na^+/K^+-ATPase is responsible for the bulk of ATP-dependent K^+ transport in these muscles. Furthermore, this conclusion is consistent with studies of resting membrane electrogenesis in dipteran muscles, which have shown that E_m can be fully accounted for by the partial permeabilities of K^+ and Na^+, the K^+ and Na^+ gradients presumably being maintained by the Na^+/K^+-ATPase. In addition, the characterisation and expression of a ouabain-sensitive α-subunit from *Drosophila melanogaster* flight and tubular muscles (Lebovitz *et al.* 1989) has provided direct evidence for the presence of a Na^+/K^+-ATPase in dipteran muscle.

Consistent with the lack of a Cl^- gradient in dipteran muscle, no evidence for the involvement of a Cl^--dependent K^+ transport mechanism in K^+ maintenance was obtained using bumetanide and SITS. We conclude, therefore, that a vanadate- and ouabain-sensitive Na^+/K^+-ATPase is the principal mechanism involved in maintenance of the K^+ gradient in *Phormia terraenovae* muscle.

Maintenance of the K^+ activity gradient in Spodoptera exigua

In contrast to the situation in Diptera, previous studies have provided some evidence to suggest that a metabolic mechanism, possibly an ion pump, may contribute directly to E_m in lepidopteran muscle. As mentioned previously, Dawson *et al.* (1989) found that resting membrane electrogenesis in *Chilo partellus* skeletal muscle could not be fully accounted for by the partial permeabilities of K^+, Na^+ and Cl^-, and some 15 % of E_m remained unaccounted for. In other studies, it has also been shown that application of DNP to lepidopteran skeletal muscle induces a depolarisation of E_m, albeit apparently without a change in intracellular $[K^+]$ ($[K^+]_i$) (Rheuben, 1972; Piek *et al.* 1973). Unlike dipteran muscle, for Lepidoptera, Rheuben (1972) noted that ouabain was ineffective on E_m of the subalar muscles of adult *Antherea polyphemus*, while Dawson *et al.* (1989) also found that ouabain applied for up to 5 min had no effect on the E_m of *Chilo partellus* muscle and that E_m was constant throughout the $[K^+]_o$ series applied (1–42 mmol l^{-1}). Thus, in contrast to previous suggestions by Akera (1971), Akera *et al.* (1974) and Baker and Willis (1970), the apparent insensitivity of lepidopteran muscle to ouabain may not be due to the relatively high concentrations of external K^+ present in the haemolymph (23–54 mmol l^{-1}; Djamgoz, 1986). A ouabain-insensitive metabolic pump could therefore be involved in maintenance of E_m in Lepidoptera.

General metabolic blockers

In comparison with *Phormia terraenovae*, the application of the general metabolic blockers rotenone, KCN and azide to *Spodoptera exigua* muscles caused considerably less reduction of aK_i (ΔaK_i −32 % to −38 %) which could be attributed directly to inhibition of a metabolic K^+ pump. Importantly,

DNP, the most potent inhibitor in *P. terraenovae*, was largely ineffective in *S. exigua*. This effect could be due to the action of DNP as a protonophore. Intracellular acidification following DNP application has been reported in a number of cells, including the Malpighian tubules of the ant *Formica polyctena* (Dijkstra *et al.* 1994; Leyssens *et al.* 1993; McLaughlin and Dilger, 1980). Briefly, neutral DNP molecules entering a cell return to the bath side as DNP and DNP^-, leaving a proton inside the cell. Since the cell has a limited volume, the uptake of protons can alter intracellular pH, depending on the buffering capacity of the cell (McLaughlin and Dilger, 1980). DNP could therefore have caused an increase in intracellular H^+ activity (aH_i), which would necessitate the active extrusion of protons in order to maintain the pH balance of the cell. We have suggested that an H^+/K^+-ATPase is active in these muscles (see below) and an initial increase in $[H^+]_i$ might stimulate this pump to remove H^+ from the cell and, in so doing, cause aK_i to increase.

Although we have shown previously that DNP 'interferes' with K^+ activity recordings, this is unlikely to affect intracellular measurements (Fitzgerald and Djamgoz, 1995). Nevertheless, DNP-induced poisoning of the cells could initially generate an unknown 'intracellular interferent'. Despite this possibility, however, the essential result is unaffected even when comparing the change in aK_i between T_{10} and T_{60} min (see Fig. 4), i.e. DNP has little overall effect on aK_i in *S. exigua* compared with *P. terraenovae* (see Fig. 3). Nonetheless, our results would suggest that a rotenone-, KCN- and azide-sensitive ATP-dependent K^+ pump could contribute up to 38 % of K^+ transport in *S. exigua* muscles.

P-type ATPase/Na^+-dependent transport inhibitors

Unlike the situation in *Phormia terraenovae*, ouabain was found to be totally ineffective as an inhibitor of K^+ transport in *Spodoptera exigua*. However, the vanadate-sensitivity of aK_i did suggest the presence of a P-type ATPase in the muscle cells of this insect (Macara, 1980; Pederson and Carafoli, 1987). Of the P-type ATPases, only two, namely the Na^+/K^+-ATPase and the H^+/K^+-ATPase, are involved in K^+ transport (Pederson and Carafoli, 1987; Sachs and Munson, 1991). Whilst some Na^+/K^+-ATPase isoforms are often referred to as being 'ouabain-insensitive' or 'ouabain-resistant', they are generally inhibited by 'high' concentrations (1 mmol l^{-1}) of ouabain (Sweadner, 1989; Fambrough, 1988; Proverbio *et al.* 1991; Canessa *et al.* 1992). Ouabain-insensitivity, therefore, suggested the possible involvement of an H^+/K^+ pump in maintenance of aK_i in *S. exigua* muscle. This was supported by the application of the H^+/K^+-ATPase-specific inhibitor SCH 28080, which also caused a significant decrease in aK_i. SCH 28080 is well established as a specific inhibitor of the gastric H^+/K^+-ATPase and has been shown to act primarily by competitive binding to the K^+ binding site on the extracellular surface of the pump (Wallmark *et al.* 1987; Scott *et al.* 1987; Keeling *et al.* 1988). Hence, a SCH-28080-sensitive H^+/K^+ pump similar to the mammalian enzyme is likely to be involved in maintenance of aK_i in *S. exigua* muscle.

Interestingly, amiloride also caused a marked reduction in aK_i which was also apparently due to inhibition of active K^+ transport. Since amiloride is primarily a Na^+ transport inhibitor (Benos, 1982; Kleyman and Cragoe, 1988), it is possible that Na^+ and K^+ transport are linked in *S. exigua* muscles. As previously suggested, however, the involvement of a Na^+/K^+-ATPase in *S. exigua* would be unlikely. Although amiloride-sensitive but ouabain-resistant K^+ pumping has been induced in some mammalian cell lines transfected with a ouabain-resistance gene, oua^R (English *et al.* 1985; Epstein and Lechene, 1988; Schulz and Cantley, 1988), there have been no reports of similar effects in non-transfected cells. Alternatively, amiloride may have acted indirectly on the K^+ gradient through inhibition of Na^+/H^+ exchange, thus causing a change in intracellular pH (pH_i). This may, in turn, have affected a pH_i-dependent mechanism such as the H^+/K^+-ATPase. Notably, English and Cantley (1984) found that $[Na^+]_i$ in a cell line from *Manduca sexta* was vanadate-sensitive and ouabain-insensitive. It was suggested by English and Cantley (1984) that a Na^+/H^+ exchanger may be present in these cells. This hypothesis, however, is also questionable, assuming a weak Na^+ gradient to be present in *S. exigua* muscle cells as found in the muscle cells of other Lepidoptera (Djamgoz and Dawson, 1989). Although it has been suggested that the amiloride-sensitive Na^+/H^+ exchanger can act in 'reverse', apparently accepting K^+ as a substitute for Na^+ (Cala, 1986), there is not an overwhelming body of evidence to support such a mechanism. Finally, amiloride may directly inhibit other K^+ transport mechanisms (e.g. H^+/K^+-ATPase, K^+/Cl^- exchange) also found in these muscles (see below). Considering the high concentration of this drug applied, such non-specific effects may well have occurred (Kleyman and Cragoe, 1988).

The vanadate- (ΔaK_i −35 %) and SCH-28080-sensitivity (ΔaK_i −38 %) of aK_i therefore strongly suggests that a K^+ pump, not unlike the mammalian gastric H^+/K^+-ATPase (e.g. Ganser and Forte, 1973; Berglindh *et al.* 1980; Sachs *et al.* 1976; Rabon and Reuben, 1990), could be involved in maintenance of aK_i and hence the K^+ gradient in *S. exigua* muscle. Support for this conclusion is given by English and Cantley (1984), who also provided some evidence to suggest the presence of a ouabain-insensitive but vanadate-sensitive mechanism, presumed to be an H^+/K^+-ATPase, in a lepidopteran embryonic cell line (CHE) from *Manduca sexta*. Interestingly, Woodruff *et al.* (1992) have shown that an H^+ gradient can make a direct contribution to the E_m in ovarian follicles of the moth *Hyalophora cecropia*, which like lepidopteran muscles are also bathed directly in the haemolymph. In this case, the fractional contribution of H^+ to E_m was found to be as much as 24 %. It is important to note that active K^+ transport is also well documented in insect, particularly lepidopteran, epithelia (e.g. Harvey and Nedergaard, 1964; Harvey *et al.* 1983; Harvey, 1992; Dow, 1984). A V-type H^+-ATPase coupled to H^+/K^+ exchange is believed to be responsible for this mechanism of epithelial K^+ transport (Wieczorek *et al.* 1991; Wieczorek, 1992; Moffett and Koch, 1992; Dow, 1992; Lepier *et al.* 1994). Although the

V-type-ATPase-specific inhibitor bafilomycin A_1 has not been used in the present study, the fact that this K^+ transport mechanism has only ever been reported in insect epithelia would suggest that a similar mechanism is unlikely to operate in *S. exigua* muscle. Indeed, it was recently reported that the active K^+ secretion in Malpighian tubules of *F. polyctena* was unaffected by application of SCH 28080 (Leyssens *et al.* 1994). Furthermore, the observation that SCH 28080 reduced aK_i by exactly the same percentage as rotenone (38 %) would imply that an H^+/K^+ pump is the principal ATP-dependent mechanism involved in K^+ transport in this insect.

Assuming, then, that an H^+/K^+-ATPase is present, it would follow that the membrane potential (E_m) in *Spodoptera exigua* muscle must be pH-dependent. In several studies on insect muscle, changes in extracellular pH (pH_e) and other external influences which may be expected to influence pH_i have been found to affect the E_m of lepidopteran muscle (see Djamgoz, 1987, and references therein). Rheuben (1972) showed that the E_m in *Antherea polyphemus* was highly pH-sensitive, E_m hyperpolarising and R_i decreasing when pH_e was raised and the opposite occurring when pH_e was lowered. Such effects can be explained by changes in chloride conductance (G_{Cl}) which, in insects, exhibits a marked pH-dependence (see Djamgoz, 1987). However, pH effects on other mechanisms not previously investigated, such as H^+ conductance (G_H) or inhibition of the H^+/K^+-ATPase, may also affect E_m in Lepidoptera.

In its normal mode of operation, the gastric H^+/K^+-ATPase is electroneutral (Rabon *et al.* 1982). A similar H^+/K^+ pump in Lepidoptera might not, therefore, be the electrogenic mechanism proposed by Dawson *et al.* (1989) to be directly responsible for maintaining a part of the E_m in *Chilo partellus* muscle. Nevertheless, it is possible that the H^+ gradient, as maintained by the H^+/K^+ pump, could be the remaining ionic component needed to account fully for E_m, according to the model of Dawson *et al.* (1989). Further experiments to monitor the effects of pH_e on E_m and aK_i, however, are required before confirmation of the role of an H^+ gradient in maintenance of E_m. Nonetheless, it seems likely that an H^+/K^+ pump is responsible for ATP-dependent K^+ transport in *Spodoptera exigua*.

Anion (Cl^-)-dependent transport inhibitors

In lepidopteran muscles, unlike dipteran muscles, there is evidence that Cl^- makes a significant contribution to E_m, there being a strong inward Cl^- gradient associated with these cells (Dawson *et al.* 1989). Thus, a Cl^--dependent mechanism utilising Cl^- influx could be involved in maintenance of aK_i in *Spodoptera exigua* muscles. In vertebrates, high concentrations of bumetanide (1 mmol l^{-1}) have been proposed to inhibit K^+/Cl^- cotransport whilst at low concentrations (1 μmol l^{-1}), $Na^+/K^+/Cl^-$ cotransport is inhibited (Ellory *et al.* 1982; Lauf, 1984, 1985; Bernhardt *et al.* 1988). Whilst 1 μmol l^{-1} bumetanide was found to be ineffective on K^+ transport in *Spodoptera exigua* muscle, 1 mmol l^{-1} bumetanide caused a significant reduction of aK_i (36 %) which could be attributed to active K^+ transport. Although in the past there has been some conjecture as to whether the $Na^+/K^+/Cl^-$ and K^+/Cl^- cotransporters are separate mechanisms (e.g. Lauf, 1985; Garay *et al.* 1986; Hall *et al.* 1989; Geck and Heinz, 1986), the observation that the low (1 μmol l^{-1}) concentration of bumetanide was ineffective would suggest that the mechanism could be similar to the K^+/Cl^- cotransporter found in mammals (e.g. Bernhardt *et al.* 1988). In turn, this could agree further with the lack of a substantial inward Na^+ gradient, as found in the muscles of most other Lepidoptera (Dawson *et al.* 1989). By analogy with the well-documented effects of bumetanide in mammals combined with the available data for its effects in insects (e.g. Schwiening and Thomas, 1992; Leyssens *et al.* 1994), the results of the present study and the prevailing ionic gradients in lepidopteran muscle support the conclusion that a K^+/Cl^- cotransport mechanism with little or no involvement of Na^+ is possible. All the anion-dependent cotransporters so far studied have been found to be electroneutral (e.g. Bernhardt *et al.* 1988; Shetlar *et al.* 1990). Hence, the proposed Cl^--dependent K^+ cotransporter could be involved in maintenance of E_m through regulation of the transmembrane K^+ and Cl^- gradients in these muscles.

Finally, treatment with 1 mmol l^{-1} SITS also caused a significant reduction in aK_i (23 %) in *S. exigua* muscle which could be explained by the following. (1) Any direct effect of SITS on the proposed bumetanide-sensitive Cl^--dependent K^+ cotransporter would seem unlikely since, at least in mammals, the disulphonic stilbenes are ineffective against $Na^+/K^+/Cl^-$ or K^+/Cl^- cotransporters (Haas *et al.* 1982; Hoffmann, 1982; Knauf and Rothstein, 1971). (2) The disulphonic stilbenes SITS and DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) have also been found to block Cl^- channels in some epithelia (e.g. Hanrahan *et al.* 1985). Any effect of SITS on G_{Cl}, however, also seems unlikely to have occurred since there was no significant change in E_m. A decrease in G_{Cl} would be expected to cause some depolarisation of E_m. (3) Since, at least in vertebrates, none of the anion exchange/cotransport mechanisms which are inhibited by the disulphonic stilbenes transports K^+, SITS is most likely to have an indirect effect on aK_i. Involvement of either the Na^+/HCO_3^- cotransporter or Na^+-dependent Cl^-/HCO_3^- exchanger seems unlikely to have occurred, bearing in mind the lack of an inward Na^+ gradient in these muscles (Dawson *et al.* 1989). Alternatively, it is possible that SITS may have inhibited a Cl^-/HCO_3^- exchanger and, by disrupting the Cl^- gradient in this way, may indirectly have inhibited the proposed bumetanide-sensitive K^+/Cl^- cotransporter, resulting in a reduction of aK_i. This mechanism is electroneutral (Thomas, 1984), and indeed no significant change in E_m was observed after application of SITS for 60 min. Under certain conditions, e.g. reduced pH_i, Cl^-/HCO_3^- exchange in vertebrates can be found operating in reverse, with Cl^- efflux coupled to HCO_3^- influx (Jennings, 1992). In *S. exigua* muscle, then, either the HCO_3^- gradient would need to be steeper than the Cl^- gradient or, as has been suggested by Thomas (1984), some energy must be provided to extrude Cl^- against its electrochemical gradient. Nonetheless, inhibition by SITS of such a Cl^--extruding

mechanism would increase the intracellular Cl^- activity, aCl_i, and therefore reduce Cl^- influx which, in turn, would reduce aK_i, assuming that K^+ and Cl^- are indeed coupled.

Comparison of the mechanisms involved in maintenance of the K^+ activity gradient in Phormia terraenovae *and* Spodoptera exigua *muscles*

A marked difference in the effects of the general metabolic blockers was observed in the two insects. DNP was far more potent in *P. terraenovae* than in *S. exigua*, and even rotenone, the most potent inhibitor in *S. exigua*, caused only some 50 % of the effect of DNP in *P. terraenovae*. This difference in potency of the general blockers, in particular DNP, between the two insects would suggest a greater dependence on ATP for maintenance of the K^+ activity gradient in *P. terraenovae* compared with *S. exigua*.

Vanadate-sensitive, i.e. P-type, ATPases were found to be involved in maintenance of the K^+ gradients in both insects. In *P. terraenovae*, there appears to be a ouabain-sensitive Na^+/K^+ pump. This is consistent with the prevailing ion gradients and agrees with previous physiological and molecular studies of dipteran muscle (Djamgoz and Dawson, 1988; Henon and Ikeda, 1981; Djamgoz, 1986; Lebovitz *et al.* 1989). Furthermore, this mechanism is likely to account for the bulk of ATP-dependent K^+ transport in this insect, since the aK_i values for ATP-dependence generally, and Na^+/K^+-ATPase specifically, were very similar (68 % and 61 %, respectively). Since the Cl^--dependent transport inhibitors bumetanide and SITS were totally ineffective, in agreement with Cl^- being in passive equilibrium across dipteran muscle membranes, the Na^+/K^+-ATPase would appear to be the principal mechanism maintaining the K^+ gradient in this insect (Fig. 6A).

In contrast, a ouabain-insensitive but vanadate- and SCH-28080-sensitive pump, probably an H^+/K^+-ATPase, appears to be present in *S. exigua*. This is consistent with there being a strong K^+ gradient but a very weak Na^+ gradient in lepidopteran muscle (Djamgoz and Dawson, 1989), and would further agree with preliminary studies in other lepidopteran tissues which suggested that the H^+ gradient may be involved in K^+ transport (English and Cantley, 1984; Woodruff *et al.* 1992). This mechanism could account for approximately 40 % of the total K^+ transport in this insect, the aK_i values for both ATP-dependence generally, and H^+/K^+-ATPase specifically, being the same. However, consistent with the presence of a strong inward Cl^- gradient, a bumetanide-sensitive K^+/Cl^- cotransporter appeared to be responsible for a further 36 % of K^+ transport. In turn, a SITS-sensitive exchanger, possibly a Cl^-/HCO_3^- exchanger, could also affect K^+ transport indirectly, perhaps by regulating the inward Cl^- gradient and hence K^+/Cl^- cotransport. Together, it would be possible

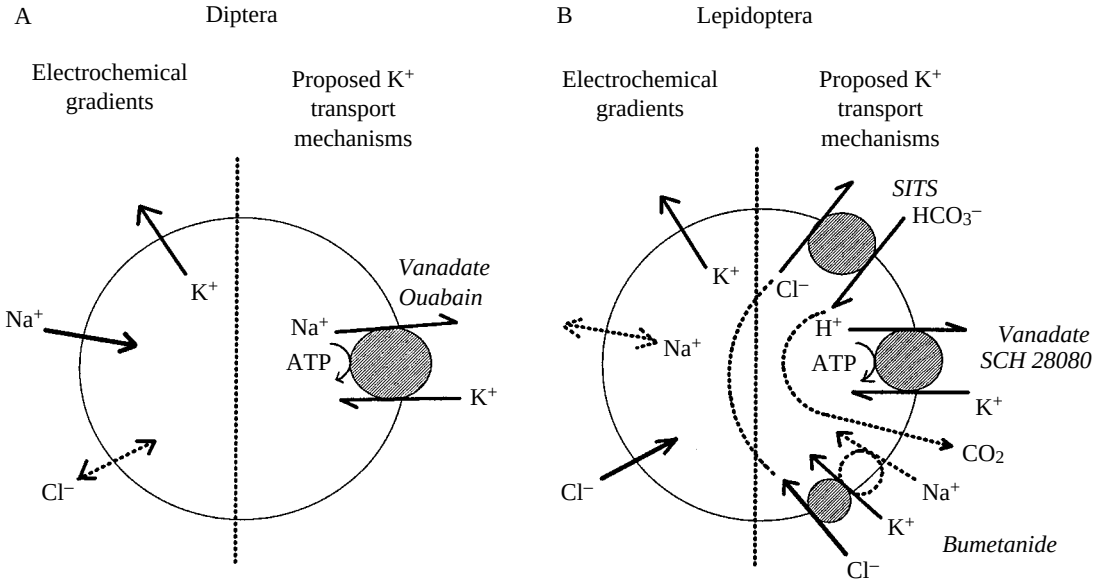

Fig. 6. (A) Proposed scheme for maintenance of the K^+ gradient in *Phormia terraenovae* muscle. In dipteran muscles, strong inward Na^+ and outward K^+ gradients prevail, whilst Cl^- is in passive equilibrium as shown on the left (the relative strengths of these gradients are indicated by the thickness of the lines, i.e. the thicker the line, the steeper the gradient). In accordance with the predominant gradients, a ouabain- and vanadate-sensitive Na^+/K^+-ATPase is found to account for the bulk of the ATP-dependent transport (ΔaK_i –61 %) in this insect (shown on the right). (B) Proposed scheme for maintenance of the K^+ gradient in *Spodoptera exigua* muscle. In lepidopteran muscle, outward K^+ and inward Cl^- gradients prevail, whilst Na^+ forms a very weak, almost non-existent gradient (shown on the left). On the right, the proposed K^+ transport mechanisms are illustrated. A vanadate- and SCH-28080-sensitive H^+/K^+-ATPase was found to account for the bulk of ATP-dependent K^+ transport. Consistent with there being a strong inward Cl^- gradient, a bumetanide-sensitive K^+/Cl^- cotransporter was also found to be involved in K^+ transport. The possibility, however, that some Na^+ influx is also associated with this cotransport cannot be ruled out (indicated by the dotted line). Together, these two mechanisms could account for a large part (approximately 70 %) of K^+ transport in this insect. An additional SITS-sensitive Cl^-/HCO_3^- exchanger could indirectly affect K^+ transport through regulation of the Cl^- gradient and hence K^+/Cl^- cotransport. It is possible that these different mechanisms operate in synchrony, as indicated by the dotted routes.

for these mechanisms to operate in concert as shown in Fig. 6B. Accordingly, active extrusion of Cl^- from the cell by Cl^-/HCO_3^- exchange would maintain the inward Cl^- gradient and, hence, K^+ transport *via* K^+/Cl^- cotransport; incoming HCO_3^- could be removed from the cell as CO_2 and H_2O.

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