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VALINOMYCIN AND EXCITATION–CONTRACTION COUPLING IN SKELETAL MUSCLE FIBRES OF THE FROG

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SUMMARY

1. Experiments were carried out on intact frog skeletal muscle fibres to study the role of H^+ and K^+ as counter-ions during the release of Ca^{2+} from the sarcoplasmic reticulum (SR). A specific focus was to test whether valinomycin, a potassium ionophore, markedly reduces or abolishes H^+ counter-ion fluxes across the SR membrane in response to electrical stimulation.

2. Single twitch fibres, mounted on an optical bench apparatus and stretch to long sarcomere length (3·6–4·0 μm), were activated by single action potentials (16 °C). Two optical signals related to excitation–contraction coupling were measured: (i) the 'second component' of the intrinsic birefringence signal, which is closely related to the myoplasmic free [Ca^{2+}] transient, and (ii) the transient myoplasmic alkalization (ΔpH) detectable from the pH indicator Phenol Red, a signal thought to reflect the movement of protons from the myoplasm into the SR in partial electrical exchange for released Ca^{2+}.

3. Exposure of a fibre to 5 μM-valinomycin produced a slight, progressive decrease in the amplitude of the birefringence signal, approximately 5–6 % per hour. This result suggests that, if anything, the peak rate at which Ca^{2+} is released from the sarcoplasmic reticulum is slightly decreased by valinomycin.

4. The amplitude of the Phenol Red ΔpH signal, measured after exposure of a fibre to valinomycin for a period of at least 60 min, averaged $0{\cdot}0020 \pm 0{\cdot}0002$ ($\pm$ S.E.M.); this value is slightly smaller than, but not significantly different from ($P > 0{\cdot}05$; two-tailed t test) that measured in fibres not exposed to valinomycin ($0{\cdot}0025 \pm 0{\cdot}0002$). This result does not support the idea that valinomycin, but virtue of increasing the flux of K^+ into the SR, markedly reduces the flux of protons during Ca^{2+} release.

5. Our findings of minimal changes in the birefringence and ΔpH signals are consistent with the idea that, at the time of Ca^{2+} release, the potassium conductance of the SR membrane is large and not substantially increased by the addition of valinomycin to Ringer solution.

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INTRODUCTION

During activation, a frog twitch muscle fibre releases a relatively large total concentration of Ca^{2+} from the sarcoplasmic reticulum (SR) into the myoplasm. This concentration, if referred to the myoplasmic water volume, is estimated to be 0·2–0·3 mM in response to a single action potential (Baylor, Chandler & Marshall, 1983; Cannell & Allen, 1984; Maylie, Irving, Sizto & Chandler, 1987; Baylor & Hollingworth, 1988) and 1–1·5 mM in response to a 1·2 s tetanus (Somlyo, Gonzales-Serratos, Shuman, McClellan & Somlyo, 1981; Cannell & Allen, 1984). These large releases of Ca^{2+} require that there be a concurrent movement of other ions ('counter-ions') across the SR membrane; without substantial counter-ion movement, calculations indicate that, for both twitch and tetanus, there would be an unreasonably large change in SR membrane potential due to the Ca^{2+} movement (Baylor & Oetliker, 1975; Somlyo *et al.* 1981; Baylor, Chandler & Marshall, 1984).

In support of the existence of counter-ion movements, the electron probe measurements of Somlyo *et al.* (1981) detected increases in the concentrations of K^+ and Mg^{2+} within the terminal cisternae of the SR at the end of a 1·2 s tetanus; these increases accounted for 36 and 19%, respectively, of the charge carried by the released Ca^{2+}. (In contrast, the Na^+ and Cl^- movements were not significantly different from zero, each accounting for about 1% of the charge carried by the released Ca^{2+}.) These authors (see also Meissner & Young, 1980) proposed that proton movements from myoplasm to SR, which cannot be detected by the electron probe technique, might account for some or all of the missing charge ('charge deficit') associated with the Ca^{2+} movement.

In a subsequent electron probe study of fibres exposed to the potassium-selective ionophore valinomycin, Kitazawa, Somlyo & Somlyo (1984) reported that, although the Ca^{2+} movement following tetanic stimulation was not altered significantly by valinomycin, K^+ and Mg^{2+} movements into the SR were increased, with the result that the charge deficit was absent. The larger K^+ movement was attributed to an increase in the K^+ permeability of the SR membrane due to valinomycin; the origin of the extra Mg^{2+} movement was unclear.

Because valinomycin appeared to provide a way to perturb the counter-ion flux across the SR, we decided to study the effects of this agent on two optical signals detectable from single fibres stimulated by a single action potential: (i) the transient myoplasmic alkalization (ΔpH) detectable with the pH indicator Phenol Red (Baylor, Chandler & Marshall, 1982*a*; Hollingworth & Baylor, 1990), and (ii) the fibre's intrinsic birefringence signal ('second component' of Baylor & Oetliker, 1975; denoted ΔB). The ΔpH signal is thought to monitor the movement of protons from the myoplasm into the SR in partial electrical exchange for released Ca^{2+} (Pape, Konishi, Hollingworth & Baylor, 1990), whereas the ΔB signal is closely related to the myoplasmic free $[Ca^{2+}]$ transient ($\Delta[Ca^{2+}]$) (Suarez-Kurtz & Parker, 1977; Baylor, Chandler & Marshall, 1982*b*; Kovács, Schümperli & Szücs, 1983; Maylie *et al.* 1987; Baylor & Hollingworth, 1987).

In contrast to the expectation that valinomycin should markedly reduce or abolish counter-ion movements other than those of K^+ and Mg^{2+} (Kitazawa *et al.* 1984), we have found that the amplitude of the Phenol Red ΔpH signal, and hence the H^+

counter-ion flux into the SR, is not altered significantly by valinomycin. Valinomycin also did not markedly alter SR Ca^{2+} release triggered by a single action potential, since the amplitude and time course of the ΔB signal changed only slightly after exposure to valinomycin. Thus the fraction of the counter-ion movement carried by protons also does not appear to have been significantly altered by valinomycin. These findings do not support the conclusion that valinomycin has a significant perturbing effect on counter-ion movements across the SR membrane, at least in fibres stimulated by a single action potential. Possible reasons for the discrepancy between our results and those of Kitazawa *et al.* (1984) are discussed.

METHODS

The basic experimental procedures have been described (Baylor & Hollingworth, 1990; Hollingworth & Baylor, 1990). Briefly, cold-adapted (4 °C) *Rana temporaria* were killed by decapitation, and intact single twitch fibres were dissected from either the semi-tendinosus or iliofibularis muscles. An isolated fibre was mounted in a transparent chamber on an optical bench apparatus (Baylor & Oetliker, 1977), then stretched to long sarcomere length (3·6–4·0 μm) and lowered onto pedestal supports in order to minimize movement artifacts in the optical records. Fibre activity was initiated by a propagated action potential triggered by a brief shock from a pair of extracellular electrodes positioned 1–2 mm from the site of optical recording. Only fibres that showed all-or-none optical responses were used. In some experiments the residual twitch response was recorded by a tension transducer (Model AE801; SensoNor, Horten, Norway). Valinomycin (1 % ethanol, the solvating agent for valinomycin) had access to the fibres by diffusion from the bath.

Solutions

Two or more of the following four solutions were used in any given experiment:

(i) normal Ringer solution (in mM): 120 NaCl; 2·5 KCl; 1·8 $CaCl_2$; 5 PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)), with pH adjusted to 7·1 by NaOH;

(ii) a 1 % ethanol Ringer solution, composed, by volume, of 1 % ethanol and 99 % normal Ringer solution;

(iii) a valinomycin Ringer solution, identical to the 1 % ethanol Ringer solution but also containing 5 μM-valinomycin (Sigma Chemical Co., St Louis, MO, USA).

(iv) a 'high-Ca^{2+}' Ringer solution, identical to solution (i) except containing 11·8 rather than 1·8 mM-$CaCl_2$. This solution was used at the time of injection of fibres with Phenol Red in order to minimize fibre damage due to the injection procedure (cf. De Mello, 1973; Baylor & Hollingworth, 1988).

The valinomycin solution was made on the day of an experiment by addition of the appropriate weight of valinomycin (MW 1111) to 100 % ethanol to give a 500 μM stock solution. The final valinomycin solution was composed of 1 % by volume of this stock and 99 % normal Ringer solution. The 5 μM final concentration in 1 % ethanol Ringer solution is identical to that used by Kitazawa *et al.* (1984).

Temperature and experimental protocols

Kitazawa *et al.* (1984) used two experimental protocols: (1) a 2 h exposure of bundles of fibres to valinomycin Ringer solution at 4–5 °C, following which electron probe measurements were made; (2) a 1 h exposure of single fibres to valinomycin Ringer solution at 14–15 °C, during which tension measurements were made.

In our experiments, the bath temperature was monitored by thermistor and maintained at 16–16·5 °C. This temperature was desirable for comparison of the pH results of the current study with those of the preceding studies (Baylor & Hollingworth, 1990; Hollingworth & Baylor, 1990). Single fibres at this temperature were exposed to the valinomycin solution for a minimum of 1 h, this time being considered sufficient to permit a comparison of our results with those of Kitazawa

et al. (1984) obtained by protocol (1) above. The latter authors also concluded that, at 14 °C or higher, a 1 h exposure of a single fibre to valinomycin was comparable to that of a 2 h exposure at 4–5 °C for a bundle of fibres.

Phenol Red injections

Since an intracellular Phenol Red concentration of about 0·4 mM or larger is required to adequately resolve the small dye-related absorbance change that normally accompanies fibre activity, fibres were injected with the pH indicator just after, rather than before or during, exposure to valinomycin. This was necessary because (i) indicator concentration is usually less than 0·4 mM by an hour after injection, owing to diffusion of the indicator away from the injection site, and (ii) fibres injected in the valinomycin Ringer solution nearly always lost their all-or-none response because of injection damage. The latter effect is presumed to be due to an adverse effect of either alcohol or valinomycin on the fibre. Based on our previous experience that fibres injected in high-Ca^{2+} Ringer solution tended to survive better than fibres in normal ringer solution, we carried out subsequent injections in the high-Ca^{2+} (valinomycin-free) Ringer solution. Even in high-Ca^{2+} Ringer solution, fibres that had had the 1 h exposure to valinomycin still seemed overly susceptible to damage from the microinjection procedure; in spite of this problem, four fibres were successfully injected with usable quantities of Phenol Red. For the pH measurements in these latter experiments, the fibres were either left in the high-Ca^{2+} Ringer or returned to valinomycin Ringer solution (cf. Fig. 5 and Table 1). As noted in Results of (cf. p. 227), valinomycin is expected to remain membrane bound within the fibre following replacement of the valinomycin Ringer solution with alcohol-free Ringer solution.

Optical measurements

Fibres were illuminated with quasi-monochromatic light from a tungsten–halogen source. Three types of optical signals were measured.

Resting absorbance from Phenol Red. For this purpose, a small spot of light, the diameter of which was less than the fibre diameter, trans-illuminated the fibre near the site of dye injection. The wavelength of the light was selected by interference filter, either from a set of 'narrow-band' (10 nm band pass) or 'wide-band' (30 nm band pass) filters. The central wavelength of the transmission band of each filter was measured in a spectrophotometer and has been used in Results in the description of signal properties according to wavelength. From the light intensity levels transmitted by the fibre, the resting absorbance spectrum of the indicator was determined and thereby the apparent pH of myoplasm (denoted pH_{app}; Baylor & Hollingworth, 1990).

Absorbance changes during fibre activity. Absorbance changes (ΔA) resulting from action potential stimulation were calculated from the equation:

$$\Delta A = -(\Delta I/I)/\log_e 10, \quad (1)$$

where $\Delta I/I$ denotes the fractional change in light intensity transmitted by the fibre. In order to obtain the indicator-related ΔA, $\Delta I/I$ was corrected for the fibre intrinsic change, which was measured at 630 nm (a wavelength where Phenol Red has no absorbance). The correction procedure is illustrated in Results and is described in detail in Hollingworth & Baylor (1990). ΔA was converted to pH units (ΔpH) by the calibration constants given in Hollingworth & Baylor (1990).

Birefringence changes. The birefringence signal was measured with the fibre positioned between crossed polarizers oriented at +45 and −45 deg with respect to the fibre axis. One of two interference filters, with central wavelengths of 700 or 850 nm was used for these measurements. Three components of this signal have been described (Baylor & Oetliker, 1975), and of relevance to this paper is the second component (denoted ΔB), the time course of which is similar to that of $\Delta[Ca^{2+}]$ (Suarez-Kurtz & Parker, 1977; Baylor *et al.* 1982*b*; Maylie *et al.* 1987). Alterations in the amplitude and time course of ΔB have previously been shown to correlate with alterations in the myoplasmic free $[Ca^{2+}]$ transient (Kovács *et al.* 1983; Baylor & Hollingworth, 1987); thus changes in the ΔB signal were used as an indication of changes in $\Delta[Ca^{2+}]$. ΔB is reported as $\Delta I/I$, the fractional change in light intensity measured in response to electrical stimulation.

Results are given as means ± S.E.M.

RESULTS

Effect of 1 % *ethanol on the birefringence signal*

One goal of the experiments was to examine the effect of valinomycin on the fibre's intrinsic birefringence signal. However, to distinguish possible effects of valinomycin

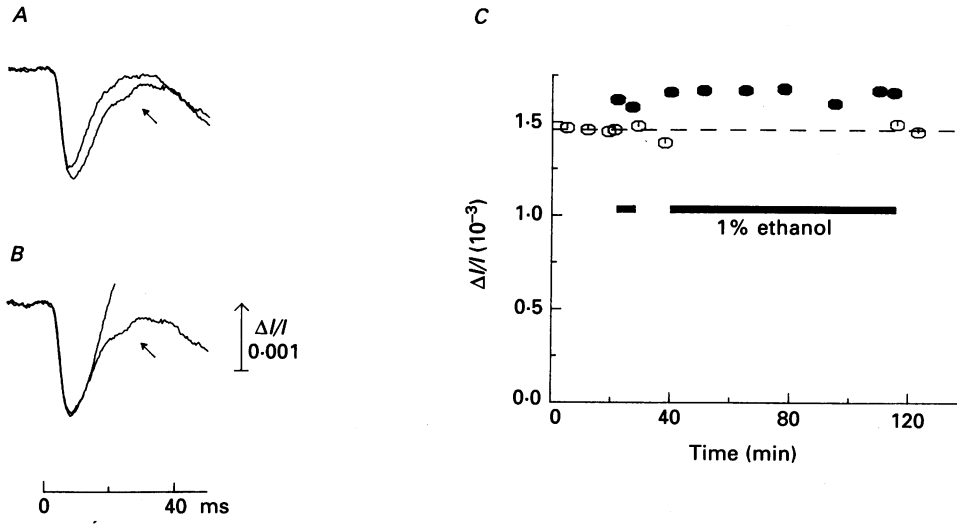

Fig. 1. Effect of 1 % ethanol on the intrinsic birefringence signal. In this fibre, signals were measured over a 2-h period, either in normal Ringer or in ethanol Ringer solution. *A*, two ΔB records superimposed, one taken in normal Ringer near the start of the experiment and the other 1 min later, after bath replacement with 1 % ethanol Ringer (arrowed trace). Zero time marks the moment of action potential stimulation by an external shock. *B*, comparison of the first (arrowed trace) and last ΔB records taken in ethanol Ringer during the experiment (recorded 22 min and 115 min, respectively, after start of the experiment). *C*, plot of the peak amplitude of the ΔB signal (ordinate), measured from records of the type shown in parts *A* and *B*, as a function of time from the start of the experiment (abscissa). Open and filled symbols denote measurements taken in normal and ethanol Ringer solution, respectively. The dashed line represents no change in ΔB amplitude with time. The periods during which the fibre was in the 1 % ethanol Ringer are denoted by black bars. Fibre no. 052190·1; fibre diameter, 63 μm; sarcomere length, 3·8 μm; 16 °C.

from those of the 1 % ethanol solvent, we first examined the effect of 1 % ethanol Ringer solution on the ΔB signal. Figure 1*A* shows two ΔB signals, one measured in normal Ringer and the second (arrowed trace) in ethanol Ringer solution. In this and every other fibre tested (n = 11), 1 % ethanol produced a small (5–20 %) increase in the peak amplitude of the ΔB signal (the early decrease in light intensity that reaches a peak about 10 ms after stimulation) and a slight delay in the time to peak of ΔB. These changes were always observed by the time of the earliest measurement in the ethanol Ringer solution (*ca.* 1 min after start of the Ringer exchange) and in all cases were fully or nearly fully reversed as soon as the fibre was returned to normal Ringer solution. The changes in ΔB probably reflect a slightly larger peak amplitude and

slightly slower falling phase of the myoplasmic free Ca^{2+} transient in the presence of 1% ethanol.

Panels *B* and *C* of Fig. 1 illustrate measurements made to evaluate possible effects of a long-term exposure (1–2 h) of a fibre to 1% ethanol Ringer solution. Figure 1*C* plots the peak amplitude of the ΔB signal as a function of time after the start of one such experiment (○, in normal Ringer; ●, in ethanol Ringer solution). The constant amplitude of the ΔB signal measured while the fibre was in normal Ringer solution (cf. dashed line in Fig. 1*C*) suggests that any effects of either short- or long-term exposures to ethanol are readily reversible. Figure 1*C* also suggests that the augmentation in the peak amplitude of the ΔB signal in ethanol Ringer solution is stable for an extended period. This stability is further illustrated in Fig. 1*B*, which shows the first (arrowed trace) and last ΔB signals recorded from this fibre in the 1% ethanol Ringer solution. Although these records were taken 93 min apart, the ΔB signal was essentially unchanged. (The difference between the late falling phases of the two signals in Fig. 1*B* is probably due to somewhat different movement artifacts associated with the onset of tension (not shown), to which the falling phase of the ΔB signal is sensitive.)

Similar measurements of the ΔB signal during a 1–2 h exposure to ethanol Ringer solution were made in a total of four fibres. To quantitate possible time-dependent effects due to the ethanol exposure, a least squares line was fitted to the ΔB amplitudes measured in the ethanol Ringer solution. For the four experiments, the average slope of the lines, if expressed as a percentage change of ΔB amplitude per hour of exposure to ethanol, was $+1{\cdot}1 \pm 0{\cdot}7\%\ h^{-1}$. This value is not significantly different from zero and confirms that the ΔB signal is stable in 1% ethanol.

Effect of valinomycin on the birefringence signal

The experiment of Fig. 2 illustrates the effect of valinomycin Ringer solution on the ΔB signal. In Fig. 2*A*, a comparison of the superimposed pairs of traces indicates that the peak amplitude of ΔB decreased slightly with time of exposure to valinomycin, while the time to peak of ΔB may have become slightly delayed. In Fig. 2*B*, the peak amplitude of the ΔB signal (○) is plotted as a function of time in valinomycin Ringer solution. The data are well fitted by a straight line with a slight negative slope (see legend of Fig. 2).

Similar results were seen in a total of fourteen experiments. The average rate at which the amplitude of the ΔB signal changed with exposure time to valinomycin was $-5{\cdot}5 \pm 0{\cdot}9\%\ h^{-1}$. While small, this change is significantly different ($P < 0{\cdot}01$, two-tailed t test) from the $+1{\cdot}1 \pm 0{\cdot}7\%$ change h^{-1} observed for fibres in ethanol Ringer solution alone (see preceding section). The small time-dependent decrease in the amplitude of the ΔB signal suggests that, if anything, valinomycin slightly decreased the amplitude and rate of rise of the Ca^{2+} transient (cf. Methods) and hence, if anything, slightly decreased the peak rate of SR calcium release.

Effect of valinomycin on fibre electrical properties

Several measurements were made to evaluate possible effects on fibre electrical properties of a long-term exposure to valinomycin. One parameter examined was the threshold voltage of the stimulating shock required to generate an action potential.

In five fibres, the threshold voltage was noted prior to exposure of the fibre to valinomycin Ringer and at various times during the subsequent exposure to valinomycin. On average, the threshold voltage changed by less than 2% (range, −18 to +17%) between the first and last measurements of the threshold voltage

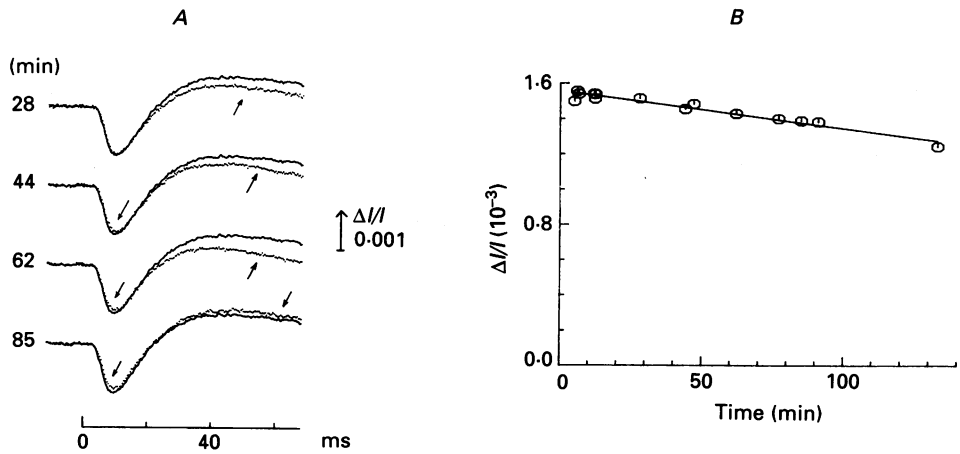

Fig. 2, Changes in the ΔB signal with time of exposure to valinomycin Ringer. *A*, four pairs of traces are shown: in each pair, the trace marked with arrows is the ΔB signal measured at the indicated time after change of the fibre to valinomycin Ringer; the other trace is the ΔB signal measured 6 min after the change to valinomycin Ringer. *B*, plot of the peak amplitude of the ΔB signal (○) as a function of time after the fibre was placed in valinomycin Ringer. Also shown is the least squares line fitted to the data. The slope of the line is $-8{\cdot}2\%\ h^{-1}$ if expressed relative to the amplitude of the y-intercept of the line (time = 0 min). The amplitude of the ΔB signal measured in normal Ringer just before the change to valinomycin Ringer was $-1{\cdot}3 \times 10^{-3}$ ($\Delta I/I$). Fibre no. 111585.1; sarcomere length, 4·0 μm; 16 °C.

(times at final evaluation of threshold voltages ranged from 45 to 151 min after the change to valinomycin Ringer solution). A second property examined was the propagation velocity (Θ) of the action potential, estimated from the temporal shift between the rising phases of the ΔB signal measured at two locations along the fibre separated by about 2 mm (cf. Oetliker & Schümperli, 1982). In five fibres, the average value of Θ measured shortly (< 10 min) after exposure to valinomycin Ringer solution was $2{\cdot}2 \pm 0{\cdot}5\ m\ s^{-1}$, whereas after an extended exposure to valinomycin (64 to 122 min) the average value of Θ was $2{\cdot}0 \pm 0{\cdot}5\ m\ s^{-1}$. The average difference in Θ, $-0{\cdot}2 \pm 0{\cdot}1\ m\ s^{-1}$, is not significantly different from zero ($P > 0{\cdot}05$, two-tailed t test). The resting membrane potential, V_m, was also measured in three fibres after impalement with a conventional intracellular microelectrode filled with 3 M-KCl. The values of V_m observed (−91, −81 and −76 mV; times of measurements during exposure to valinomycin, 90, 100 and 148 min, respectively) are within or close to the range expected for normal fibres (−80 to −90 mV). These results indicate that, for the parameters measured, a 1–2 h exposure to valinomycin Ringer solution produced little or no change in fibre electrical properties. This conclusion is consistent with that of Hinkel and Van der Kloot (1973), who found that

valinomycin at concentrations below $1{\cdot}25 \times 10^{-5}$ g ml^{-1} (11 μM) had no detectable effect on the electrical resistance of the exterior membranes (surface plus T-system) of frog skeletal muscle fibres.

pH measurements with Phenol Red

Previous work carried out on frog muscle with the electron probe technique (cf. Introduction) indicated that an apparent 'charge deficit' is normally associated with

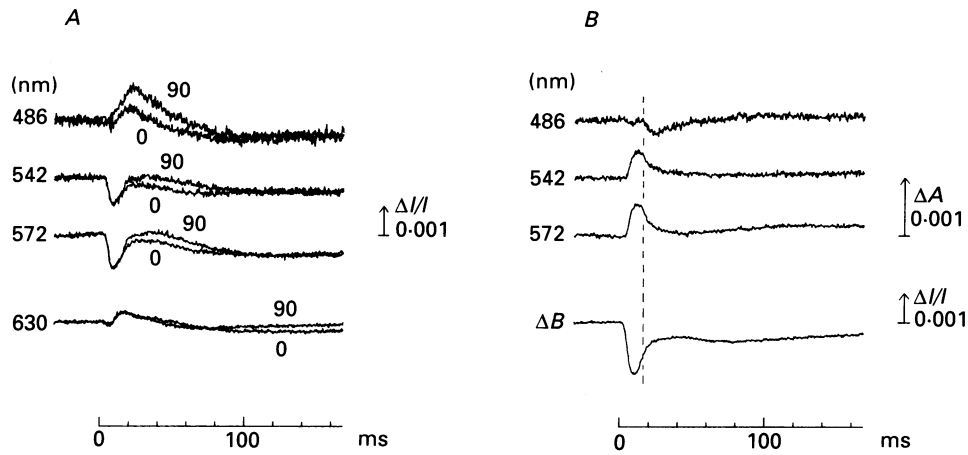

Fig. 3. Absorbance records from a fibre injected with Phenol Red following a 1 h exposure to valinomycin Ringer. The central wavelength of the wide-band interference filters is indicated in nm to the left of the traces. The lowermost trace of panel *B* is the intrinsic birefringence signal taken at the time of the absorbance records. *A*, at each wavelength, two traces have been superimposed, representing the raw transmission changes recorded with two forms of polarized light (0 or 90 deg, as indicated). *B*, Phenol Red-related isotropic absorbance changes from panel *A*, obtained as the 1:2 weighted average of the 0 and 90 deg records at wavelength λ after subtraction of the fibre intrinsic change, estimated from the 630 nm record after a scaling by the factor $(630/\lambda)^{1{\cdot}6}$ (Hollingworth & Baylor, 1990). The calibration bar in ΔA units applies to the top three records; the lower calibration bar applies to the ΔB signal. The dashed vertical line indicates the time after which contamination of the records by a movement artifact was obviously significant (cf. the 486 nm trace, which, for a pH change, should be flat). At the time of the measurements (104 min after exposure of the fibre to valinomycin), the estimated values of Phenol Red concentration, resting pH and ΔpH for this fibre region were, respectively, 1·71 mM, 7·06 and 0·0022. Fibre no. 070188·1; fibre diameter, 120 μm; spot size, 73 μm; sarcomere length, 3·8 μm; 16·2 °C.

SR Ca^{2+} release (Somlyo *et al.* 1981) and that this deficit is abolished in fibres exposed to valinomycin (Kitazawa *et al.* 1984). We therefore wished to determine whether valinomycin altered the myoplasmic alkalization (ΔpH) normally detectable during a single twitch in fibres injected with the pH indicator Phenol Red. Since it is likely that this ΔpH signal reflects a movement of H^+ into the SR as a counter-ion for Ca^{2+} release (Pape *et al.* 1990), valinomycin would be expected to greatly reduce or eliminate the Phenol Red signal (Note: under physiological conditions, any increase in proton conductance due to valinomycin is expected to be negligible in comparison

with the increase expected for the K^+ conductance: cf. Andreoli, Fieffenberg & Tosteson, 1967).

To examine valinomycin's effect on ΔpH, fibres were exposed to valinomycin Ringer solution at 16 °C for a minimum of 1 h. Following this exposure, a high-Ca^{2+} Ringer solution was introduced in preparation for microinjection of the fibre with Phenol Red and measurement of ΔpH (cf. Methods). Since, in the absence of a solvating agent (e.g. 1% ethanol), valinomycin is quite insoluble in aqueous solutions, it is likely that the valinomycin that entered a fibre during the initial exposure remained in the fibre after the change to the high-Ca^{2+} Ringer solution (cf. Stark & Benz, 1971, who, in bilayer experiments, detected no reduction in valinomycin-induced conductance even after exposure of the bilayer to a bulk aequeous phase without valinomycin for periods exceeding 1 h). Moreover, in one experiment (see Fig. 5, described in detail below) a fibre was injected with a sufficiently large quantity of Phenol Red that pH changes could be measured for an extended period in the high-Ca^{2+} Ringer solution used for the injection as well as for a further 40 min period after re-introduction of valinomycin Ringer solution. No significant difference was found in the amplitudes of the ΔpH signals measured before and after the re-introduction of valinomycin Ringer solution.

Figures 3–5 show results obtained from one of four fibres successfully studied with these protocols. Figure 3*A* shows raw transmission changes ($\Delta I/I$) measured in response to action potential stimulation, whereas Fig. 3*B* estimates the Phenol Red-related absorbance changes (denoted ΔA). Through time to peak, the time course of ΔA, which is best resolved in the 572 nm trace, is closely similar to that observed in normal fibres (Hollingworth & Baylor, 1990). At later times in the transient, beginning about the time indicated by the dashed vertical line in Fig. 3*B*, the ΔA signals are contaminated by a movement artifact related to the development of fibre tension (not shown). Such movement artifacts are also often seen in fibres not exposed to valinomycin (Hollingworth & Baylor, 1990; Pape *et al.* 1990); when present, the movement artifact renders unreliable a determination of the exact time course of ΔpH at peak and into the falling phase of the transient. For the experiment of Fig. 3, the apparent peak amplitude of ΔA, which may be slightly contaminated by the movement artifact, corresponds to a ΔpH of 0·0022. This value is not significantly different from the average peak value of $0{\cdot}0025 \pm 0{\cdot}0002$ observed in fibres not exposed to valinomycin Ringer solution (Hollingworth & Baylor, 1990).

Figure 4*A* (●) plots the resting absorbance spectrum of Phenol Red measured in this fibre. The curve in Fig. 4*A* shows a fit of a Phenol Red *in vitro* spectrum to the muscle data, after least squares adjustment for the level of pH. The indicated value of resting pH (denoted pH_{app}) was 6·97; this value is slightly smaller than the average value of pH_{app} (7·17; range 6·8–7·5) determined by the identical method in eight fibres not exposed to valinomycin (Baylor & Hollingworth, 1990). Figure 4*B* shows an analogous plot for the peak value of ΔA measured during fibre activity. These data are well described by a ΔpH spectrum (curve), as similarly found for fibres injected with Phenol Red but not exposed to valinomycin (Hollingworth & Baylor, 1990). The good fits in Fig. 4 indicate that the resting and active pH signals from Phenol Red are not modified in some unexpected way by valinomycin.

Figure 5 plots, as a function of time, the peak amplitudes of the ΔB signals (×) and

ΔpH signals (●) measured from all runs taken during this experiment. As indicated by the horizontal lines at the bottom of Fig. 5, this fibre was initially exposed to valinomycin Ringer solution for a 60 min period, followed by a 53 min period in the high-Ca^{2+} Ringer solution (dashed line), followed by a re-exposure to valinomycin

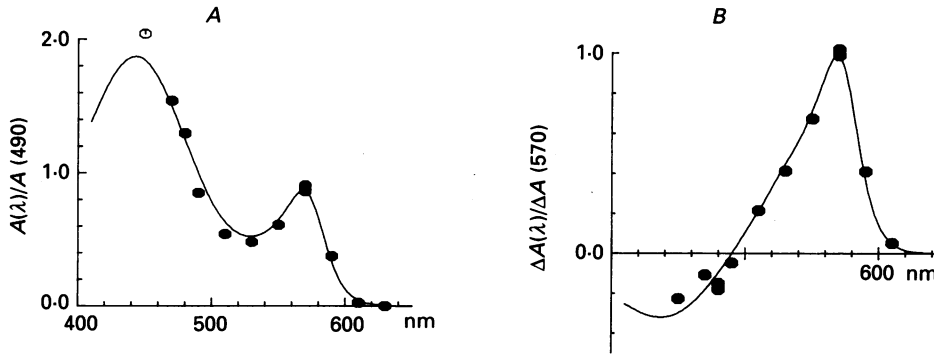

Fig. 4. Wavelength dependence of the Phenol Red resting (part *A*) and active (part *B*) absorbance signals measured from a single fibre after a 1 h exposure to valinomycin. *A*, the circles plot the value of indicator-related absorbance (ordinate) as a function of the central wavelength (abscissa) of the 10 nm interference filters used for the measurements. For normalization, absorbance at 490 nm, the presumed isosbestic wavelength for Phenol Red in myoplasm, was used. The curve is the least squares fit of the muscle data (excluding the 450 nm point) by *in vitro* calibration spectra for pH; the *in vitro* spectra were red-shifted by 10 nm prior to calculation of the best fit (cf. Baylor & Hollingworth, 1990). A myoplasmic pH of 6·97 was estimated from the fit. *B*, ●: wavelength dependence of the peak of the Phenol Red absorbance change detected in response to action potential stimulation. Peak ΔA was estimated from records of the type shown in Fig. 3*B* and amplitudes have been normalized by that observed at 570 nm. The curve is an *in vitro* calibration curve for ΔpH (shifted by 10 nm), with its amplitude set by a least squares fit to the muscle data. The amplitude of ΔA corresponds to a peak ΔpH of +0·0024. Same fibre as Fig. 3; average indicator concentration at the site of optical recording, 1·55 mM; 49 min after completion of the initial 60 min exposure to valinomycin. See also Table 1.

Ringer solution for the remainder of the experiment (second continuous line in Fig. 5). The increase in the absolute amplitude of the ΔB signal after the initial change from normal Ringer to the valinomycin Ringer solution (time = 0 min) is attributable to the effect of 1 % ethanol (cf. Fig. 1); conversely, the decrease in the ΔB signal following the change back into the high-Ca^{2+} Ringer solution (time = 60 min) is attributable to removal of ethanol. In response to the first injection of this fibre with Phenol Red (injection time indicated by the first vertical arrow in Fig. 5), the ΔB amplitude was unchanged, indicating that the fibre condition was not altered by the injection. After two measurements of pH_{app} and ΔpH (cf. Table 1 and Fig. 5), the fibre was injected a second time with Phenol Red; a second injection was desirable because the amount of Phenol Red injected initially was relatively small and the dye concentration was decreasing with time due to indicator diffusion away from the first injection site. The decrease in the ΔB amplitude following the second injection of Phenol Red (injection time indicated by the second vertical arrow) may reflect some damage from the second injection; however, the fibre appeared to recover from this damage, since the ΔB amplitude subsequently returned to that

observed at earlier times. Within experimental error, the measured values of ΔpH (●) were constant, both after the first exposure and during the second exposure to valinomycin.

In three other experiments, Phenol Red was injected and ΔpH was measured after return of a fibre to high-Ca^{2+} Ringer solution following the initial 60 min exposure to

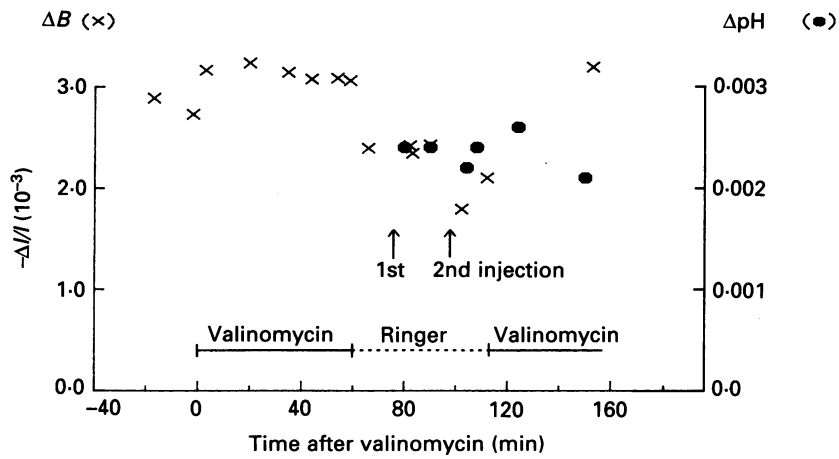

Fig. 5. Plot of the peak amplitudes of the ΔB signals (×; left-hand ordinate) and ΔpH signals (●; right-hand ordinate) as a function of time after initial exposure of a fibre to valinomycin Ringer. After the first exposure of the fibre to valinomycin, the bathing solution was exchanged for high-Ca^{2+} Ringer (dashed time period indicated on the plot); while in the latter solution, the fibre was subjected to two injections with Phenol Red (injection times indicated by the arrows). At 113 min, the bath was again exchanged for valinomycin Ringer and two additional pH measurements were made. At 'negative' times (see abscissa) the fibre was in normal Ringer solution. Same fibre as Figs 3 and 4. See Table 1 for a summary of all pH measurements from this fibre. (Note: the absence of a significant change in amplitude of the ΔpH signal following the re-introduction of valinomycin Ringer suggests that 1 % ethanol itself has at most a minor effect on the ΔpH signal; however, this conclusion was not tested in independent experiments.)

valinomycin. Table 1 summarizes the main findings from the four experiments. The average values observed for the peak amplitude, time to half-peak and time to peak of ΔpH were $0{\cdot}0020 \pm 0{\cdot}0002$, $6{\cdot}7 \pm 0{\cdot}4$ ms and $14{\cdot}4 \pm 0{\cdot}3$ ms, which may be compared with the values $0{\cdot}0025 \pm 0{\cdot}0002$, $8{\cdot}6 \pm 0{\cdot}3$ ms and $17{\cdot}0 \pm 1{\cdot}0$ ms, respectively, recorded from fibres not exposed to valinomycin (Hollingworth & Baylor, 1990). Although the differences in times to half-peak and to peak (but not peak amplitude) are statistically significant ($P < 0{\cdot}05$, two-tailed t test), these differences are not large and are reasonably attributed to the influence of movement artifacts, which were somewhat greater in the experiments of this paper compared with the earlier study (cf. Fig. 3*B* *vs.* e.g. Fig. 2 of Hollingworth & Baylor, 1990). When present, the movement artifact appears to have had the general effect of shortening the 572 nm signal (cf. Fig. 3*B*), thus tending to reduce the peak amplitude and to abbreviate the time course of the transient. Therefore the principal conclusion of a comparison between the results of this paper and those of the earlier study is that valinomycin had little if any effect on the Phenol Red ΔpH signal.

TABLE 1. Characterization of myoplasmic pH transients measured from fibres after exposure to valinomycin Ringer solution

					ΔpH_{app}		
Fibre no.	Time of the measurements (min)	N	Phenol Red concentration (mM)	pH_{app}	Peak amplitude	Time to half-peak (ms)	Time to peak (ms)
070188·1	20–83 [11–39]	6	0·52–1·71	6·97–7·17	0·0024	5·8	13·7
071188·1	24	1	0·40	7·02	0·0020	6·0	14·0
071789·1	40–57 [4–21]	2	0·86–1·33	6·97–6·98	0·0021	6·9	14·7
071889·1	26	1	0·99	7·02	0·0016	7·6	15·2
Mean ± S.E.M. ($n = 4$)					0·0020 ± 0·0002	6·7 ± 0·4	14·4 ± 0·3

All fibres were exposed to valinomycin Ringer for a minimum of 60 min, then returned to high-Ca^{2+} Ringer and injected with Phenol Red. The second column gives the time of the measurements recorded in the remaining columns relative to the time that the fibre was returned to high-Ca^{2+} Ringer (the numbers in brackets give time relative to return of the fibre to valinomycin Ringer for those fibres in which pH was measured after re-exposure to valinomycin Ringer; see Fig. 5). N, number of measurements. The measurements of ΔpH_{app} were all made in response to a single action potential.

DISCUSSION

Valinomycin had minimal effects on the signals studied in our experiments. Firstly, the intrinsic birefringence signal revealed a slight decrease in amplitude with time of exposure to valinomycin, suggesting that the peak rate of SR Ca^{2+} release may have been reduced slightly following exposure to valinomycin. This effect is opposite to that expected if: (i) in the absence of valinomycin, a significant SR membrane potential change (ΔV_{SR}) developed at the time of Ca^{2+} release that limited further release, and (ii) in the presence of valinomycin, ΔV_{SR} was reduced by virtue of an increase in the K^+ permeability of the SR membrane. Secondly, following exposure of fibres to valinomycn, the amplitude of the myoplasmic alkalization (ΔpH) detected with Phenol Red was not significantly different from that previously reported in fibres not exposed to valinomycin (Hollingworth & Baylor, 1990). Thus, our results do not support the conclusion of Kitazawa *et al.* (1984) that valinomycin markedly reduces or abolishes the movements of counter-ions other than K^+ and Mg^{2+} (see Introduction).

In considering possible explanations for the apparent inconsistency between the findings of Kitazawa *et al.* (1984) and our own, it is relevant to consider possible methodological errors as well as differences in the experimental protocols.

Indicator dye measurements. In recent years a number of methodological problems have been identified in the use of indicator dyes under intracellular conditions (for some examples, see Baylor & Hollingworth, 1990). The major source of the problems appears to be related to the binding of indicator molecules to intracellular constituents, such as soluble and structural proteins. As a result of the binding, indicator properties are altered, raising the possibility of artifacts in the

measurements and/or errors in the interpretations. Although we cannot rule out this possibility with certainty, the detailed methodological evaluations carried out in the case of Phenol Red (Baylor & Hollingworth, 1990; Hollingworth & Baylor, 1990; Pape *et al.* 1990) support the conclusion that the ΔpH_{app} signal from this indicator primarily reflects a genuine myoplasmic alkalization associated with an H^+ counter-ion movement into the SR.

Electron probe measurements. An alternative possibility is that some aspect of the measurements reported by Kitazawa *et al.* (1984) is not correct. One puzzling finding of that study is that valinomycin significantly increased the relative contribution of Mg^{2+} to the total counter-ion movement, whereas the expected result, if valinomycin simply increased the K^+ permeability of the SR membrane, is a decrease in the movement of other ions, including Mg^{2+}, relative to the K^+ movement. The unexplained increase in the Mg^{2+} movement raises concerns about the underlying significance of the changes detected. More generally, with the electron probe technique, statistical uncertainties arise in comparisons between resting and tetanized conditions, as well as between control and valinomycin-treated conditions, because of the need to use different frogs for each measurement condition. The consequent sample variations for the ions of interest are considerable (cf. Tables 4 and 5 of Kitazawa *et al.* 1984), thus also raising concerns about the reliability of this technique in quantitating changes in the counter-ion movements.

Differences in stimulation protocols. A third explanation for the apparent contradiction between our results and those of Kitazawa *et al.* (1984) is related to the different types of stimulation employed. Our fibres were activated by single action potentials, whereas Kitazawa *et al.* (1984) utilized a 1·2 s tetanus. One likely consequence of the latter protocol is that the measured ionic movements reflect processes in addition to Ca^{2+} release and the associated ion fluxes that result from changes in electro-chemical driving forces across the SR membrane. In particular, the SR Ca^{2+} pump, which presumably turns over at a near maximal rate during most of a 1·2 s tetanus, probably makes a substantial contribution to the net ionic distribution across the SR membrane at the end of the tetanus. In contrast, by 20 ms after a single action potential, the latest time period considered for the measurements of our study, the turnover of the Ca^{2+} pump is estimated to be negligible (cf. Pape *et al.* 1990). Although we have no specific hypothesis to explain how differences in pump-related ionic movements might reconcile the results of Kitazawa *et al.* with our own, it should be borne in mind that the stimulation protocols used in the two studies did have substantial differences. Thus, it is possible that the measurements both of this study and of Kitazawa *et al.* (1984) are correct and that some fundamental difference exists between the effects of valinomycin on counter-ion movements during a twitch and long tetanus. (Note: any increase in g_K due to the presence of valinomycin should not, in terms of the K^+ movements that occur during either twitch or tetanus, be limited by the activation kinetics of the ionophore, since in bilayer measurements a step change in driving force activates the valinomycin conductance with a time constant of tens of microseconds (cf. Stark, Ketterer, Benz and Laüger, 1971).)

Would valinomycin be expected to significantly increase the resting K^+ conductance of the SR membrane?

Our findings concerning ΔpH and ΔB would be expected if the potassium conductance (g_K) of native SR membrane is large and not substantially changed by valinomycin. In this regard, it is of interest to estimate the possible increase in g_K that may arise due to the presence of 5 μM-valinomycin in Ringer solution. An upper limit for this increase may be calculated from the measurements of Andreoli *et al.* (1967), who measured the conductance increase of black lipid membranes made from sheep red cells and exposed to variable concentrations of valinomycin. At the largest concentration tested, 0·5 μM, the increased in g_K was 1 mS cm^{-2} (measured in 0·1 M-KCl). Thus, if the increase in g_K remains linear with larger valinomycin concentrations, an increase of about 10 mS cm^{-2} is expected at 5 μM-valinomycin. The actual value applicable to the SR membrane might, however, be considerably smaller than 10 mS cm^{-2} since a large fraction of the SR membrane area is non-lipidic, being occupied by the SR Ca^{2+} pump molecules, which are present at a density of ~ 20000–30000 μm^{-2} (Scales & Inesi, 1976; Franzini-Armstrong, Ferguson, Castellani & Kenney, 1986).

An increase in g_K of 10 mS cm^{-2} (or smaller) may or may not represent a substantial change in the resting value of g_K of the SR membrane, for which the literature contains a wide range of estimates (cf. Meissner, 1983). If g_K is as low as 0·03–0·1 mS cm^{-2} (Bezanilla & Horowicz, 1975; Vergara, Bezanilla and Salzberg, 1978; Best & Abramcheck, 1985) clearly a valinomycin-related increase of up to 10 mS cm^{-2} would represent a major change. On the other hand, such a change would be relatively minor compared with the estimate of 60–100 mS cm^{-2} for g_K obtained from the work of Labarca & Miller (1981) and Garcia & Miller (1984).

Likely contribution to g_K from the Ca^{2+} release pathway itself

In assessing the magnitude of the SR g_K during excitation–contraction coupling, one should bear in mind that as Ca^{2+} is released, the release pathway itself may also allow K^+ (and other ions) to transiently move across the SR membrane under an electro-chemical driving force (cf. Baylor *et al.* 1984). Interestingly, bilayer studies of the ryanodine receptor protein (the presumed SR Ca^{2+} channel for excitation–contraction coupling; see, e.g. Lai, Erickson, Rousseau, Liu & Meissner, 1988) reveal a substantial monovalent cation conductance. In fact, in the presence of 0·1 M-KCl and physiological Ca^{2+} levels, the channel is reported to be about six times more conductive to K^+ than to Ca^{2+} (Smith, Imagawa, Ma, Fill, Campbell & Coronado, 1988).

An estimate of the contribution of the open Ca^{2+} release channels to g_K may be made as follows. In frog muscle, the density of foot proteins (presumed to be the ryanodine receptors and Ca^{2+} release channels; Lai *et al.* 1988) is about 800×10^8 cm^{-2} of junctional transverse tubular membrane (Franzini-Armstrong, 1975, or about 500×10^8 cm^{-2} of total tubular membrane (cf. Mobley & Eisenberg, 1975). If the ratio of SR membrane to total tubular membrane is taken as 15:1 (the average of the values reported by Peachey (1965) and Mobley & Eisenberg (1975)), then, referred to the SR membrane, the density of Ca^{2+} release channels is 33×10^8 cm^{-2}. If g_K for a

single open ryanodine receptor is taken as 200 pS (Smith *et al.* 1988), the total g_K potentially available from all the Ca^{2+} release channels is then about 600 mS cm^{-2} of SR membrane. However, it is not likely that all channels open with each release event. The number of channels that open during a single twitch may be estimated as follows. As reported by Maylie *et al.* (1987) and Baylor & Hollingworth (1988), the peak rate at which Ca^{2+} enters the myoplasm from the SR is likely to lie in the range 100–150 μM ms^{-1} at 16–18 °C (i.e. 0·1–0·15 mmol of Ca^{2+} cm^{-3} of myoplasm s^{-1}). If the quantity of SR membrane area is taken as 5×10^4 cm^2 cm^{-3} of myoplasm (the average of the values reported by Peachey (1965) and Mobley & Eisenberg (1975); see Baylor *et al.* (1983) for conversion of units to myoplasmic volume), the peak Ca^{2+} current density is 0·4–0·6 mA cm^{-2}. On the other hand, the data of Smith *et al.* (1988) suggest that an open release channel at physiological K^+ and 5 mM-'trans' Ca^{2+} (corresponding to SR luminal Ca^{2+}) passes about 1 pA of Ca^{2+} current if SR membrane potential is near 0 mV. This current would presumably be somewhat smaller, perhaps 0·2–0·4 pA if trans Ca^{2+} were 1–2 mM, a range probably more appropriate to the free $[Ca^{2+}]$ in the SR under physiological conditions (cf. Hasselbach & Oetliker, 1983). Hence, the number of open Ca^{2+} channels required to give a current density of 0·4–0·6 mA cm^{-2} is estimated to be $10–30 \times 10^8$ cm^{-2} of SR membrane, representing 30–90 % of the available channel density. Thus, at the peak of the release event, g_K from the open release channels is estimated to be 200–600 mS cm^{-2}, which is much larger than the expected increase in resting g_K due to valinomycin as calculated in the preceding section. Thus, irrespective of the value of g_K that applies to the SR in the resting state, this calculation suggests that it is unlikely that 5 μM-valinomycin would substantially increase the g_K of the SR membrane that is operational at the time of SR Ca^{2+} release. Our findings of minimal changes in ΔB and ΔpH after exposure of fibres to valinomycin are also consistent with the idea that, during activation, g_K of the SR is sufficiently large that it cannot be substantially enhanced by valinomycin.

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