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FURTHER OBSERVATIONS ON THE BEHAVIOUR OF OUABAIN-INSENSITIVE SODIUM EFFLUX TOWARDS PROCTOLIN IN BARNACLE MUSCLE FIBRES

BY E. EDWARD BITTAR AND JUDE NWOGA*

From the Department of Physiology, University of Wisconsin, Madison, WI 53706, USA

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SUMMARY

1. A further study has been made of the stimulatory action of proctolin on the ouabain-insensitive Na^+ efflux in single muscle fibres from the barnacle, *Balanus nubilus*.

2. (i) Strontium (Sr^{2+}) behaves as a substitute for external Ca^{2+}. In this case, however, the response to proctolin fails to decay. (ii) Injection of Sr^{2+} stimulates the ouabain-insensitive Na^+ efflux. This effect is mimicked by injecting Ca^{2+}.

3. Depolarization of the fibre membrane with 30 and 100 mM-external K^+ augments the response to proctolin.

4. Pre-injection of GTP or Gpp(NH)p (sodium 5′-guanylylimidodiphosphate) prevents the response to proctolin from completely decaying.

5. Pre-injection of guanine nucleotides in conjunction with membrane depolarization stops the response to proctolin from decaying.

6. Measurements of E_m before and during treatment with proctolin indicate a prompt but small and reversible fall in the membrane potential.

7. (i) The aequorin response of fibres pre-treated with ouabain to proctolin is monophasic or multiphasic, and concentration dependent, the minimal effective concentration being in the nanomolar range. (ii) The duration of these signals is usually less than 5 min; this is about half the time it takes for the stimulated Na^+ efflux to reach a maximum. (iii) The aequorin response to proctolin occurs quite often in fibres suspended in nominally Ca^{2+}-free artificial sea water. (iv) Sudden graded elevations in external K^+ following complete decay of the aequorin response to proctolin are rapidly followed by stepwise transitory increments in light emission. (v) The aequorin response to 100 mM-external K^+ is frequently a triplet.

8. (i) Together, these results are in line with the view that the action of proctolin on the ouabain-insensitive Na^+ efflux is the result of a temporary fall in internal pCa and that its point of action is the Ca^{2+} channel, where a putative G protein in the presence of GTP or Gpp(NH)p is able to maintain constancy of the hormonal effect. (ii). They strengthen the argument that the barnacle muscle fibre as a preparation is especially suitable for studies of this kind.

* Permanent address: Department of Physiology, Faculty of Medicine, University of Nigeria, Enugu, Nigeria.

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INTRODUCTION

Relatively little is known so far of the physiological influence of neuropeptides and modulators on ion transport, such as that of sodium. One reasonable but indirect way of approaching this problem is to obtain information by employing the barnacle muscle fibre as an experimental model. Thus, for example, the work of Nwoga & Bittar (1985) suggests that the neuropeptide proctolin stimulates the ouabain-insensitive Na^+ efflux solely by a mechanism which involves activation of Ca^{2+} channels. This is indicated by evidence that the stimulatory response is abolished by Ca^{2+} channel blockers, notably verapamil, Cd^{2+} and Co^{2+}, and that it fails to occur in the nominal absence of external Ca^{2+}. That the modulatory action of proctolin is of physiological significance is shown by the fact that it is effective when it occurs in the nanomolar concentration range. The main aim of the studies described in this communication was to elucidate more fully the nature of the mechanism responsible for the stimulatory response of the ouabain-insensitive Na^+ efflux to proctolin. The evidence brought forward strengthens the view that stimulation of the ouabain-insensitive Na^+ efflux by this pentapeptide (Arg-Tyr-Leu-Pro-Thr) is the result of a temporary decline in internal pCa, and that its receptor site is the Ca^{2+} channel. It is also in line with the view that the response to proctolin is mediated by a Na^+–Ca^{2+} exchanger operating in the reverse mode. Other findings included in this paper indicate that the response to proctolin can be stopped from decaying by pre-injecting GTP or Gpp(NH)p (sodium 5′-guanylylimidodiphosphate) and depolarizing the fibre membrane.

METHODS

Materials

Specimens of the barnacle *Balanus nubilus* were supplied by the Pacific Biomarine Laboratory Inc., Venice, CA, USA and Bio-Marine Enterprises, Seattle, WA, USA. They were kept in a filtered Instant Ocean aquarium containing sea water made from Instant Ocean salts. The temperature of the aquarium water was maintained at 12 °C.

Dissection and cannulation

Single muscle fibres measuring 3–4 cm in length and 1–2 mm in width were isolated by dissection from the depressor muscle bundles and then cannulated in the same way as *Maia* fibres (Caldwell & Walster, 1963). They were kept suspended in a vertical position by means of a 50–80 mg weight attached to the tendon.

Solutions

The experiments were carried out with ASW (artificial sea water) having the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10; pH 7·8.

Solutions of proctolin, GTP and Gpp(NH)p, Ca^{2+} and Sr^{2+} for injection were prepared using a 3 mM-HEPES buffer solution, pH 7·2. Aequorin was prepared by dissolving a sample of the lyophilized protein in a small volume of a 3 mM-HEPES solution (pH 7·5) to give an aequorin concentration of $2{\cdot}5 \times 10^{-4}$ M.

The microinjector

The microinjector used was of the type described by Hodgkin & Keynes (1956) as modified by Bittar & Tallitsch (1975). The volume of fluid usually injected into these fibres was about 0·4 μl. Since the intrafibre fluid volume was on average 40 μl, dilution by the myoplasm of the injected fluid may be taken as 100-fold.

Cannulated fibres were loaded with aequorin with the aid of a 0·5 μl microsyringe (manufactured

by Scientific Glass Engineering PTY Ltd of Ringwood, Australia) onto which a fine capillary tube ($\sim$ 110 μm in width at its proximal end) was mounted and sealed with shellac.

Radioactivity measurements

$^{22}NaCl$ in aqueous solution was supplied by Amersham–Searle Corp., Arlington Heights, IL, USA. The solution was dried down and then redissolved in water so that volumes of 0·4 μl gave $7–9 \times 10^5$ c.p.m. The effluent from the cannulated fibre loaded with radiosodium was collected every 5 min and the residual fibre activity was determined at the end of the experiment. A Beckman auto-γ-counter was used for counting the samples. The data obtained were fed into an Apple II computer programmed to calculate the fractional rate constant for $^{22}Na^+$ efflux (this being efflux rate/fibre count and 1/2 efflux). Stimulation of Na^+ efflux was computed by taking the difference between the maximum rate constant and the value immediately preceding treatment and expressing this as a percentage change. For the case where two stimulatory phases were present, the size of the second stimulation was arrived at by taking the difference between the combined phases and the first phase. The results given in this paper are mean $\pm$ S.E. of the mean. Significance levels were estimated by using Student's unpaired t test.

Measurement of the membrane potential (E_m)

Measurements of the membrane potential of cannulated fibres were made by introducing a Ling–Gerard glass microelectrode axially into the first 5 mm length of the fibre. These electrodes had an external tip diameter of 10–20 μm. The electrodes were filled by boiling in 3 M-KCl under vacuum. The potentials were recorded with a Vibron electrometer (Model 33B-2). In order to monitor the E_m of fibres suspended in ASW containing proctolin, a special chamber with an inlet–outlet system was used. Special precautions were taken to insure that inflow and outflow rates were equal when the system was opened for the addition of proctolin to the bath.

Recording of aequorin light emission

The method used was a modification of that described by Bittar & Keh (1980) for the measurement of firefly luminescence. The light-measuring apparatus, housed in a light-tight black Perspex box, consisted of: (1) a microinjector mounted on a Prior micromanipulator which was driven by an Aminco motor unit under remote control; (2) a platform anchored to a Palmer screw stand on the side of which a cannulated aequorin-loaded fibre could be suspended in a Perspex bathing chamber (covered wherever possible with aluminium foil) that was emptied and filled simultaneously via an outlet–inlet system which was also under outside control. This chamber lay adjacent to (3); an end-window RCA photomultiplier tube (6342) whose dynode chain was connected to a DC power supply unit (Hewlett–Packard 6515A) located outside the box. When applying a voltage of 900 or 1000 V the dark current at 22 $\pm$ 1 °C was $< 0·5$ nA; and (4) the anode current was measured with a Keithley 485 picoammeter and converted to an analogue voltage signal, which was recorded on a Watanabe chart recorder, the response time of which was about 0·3 s for a full-scale deflection (2 V). The digit representation of the picoammeter was simultaneously fed to an IBM Personal Computer through a MetraByte MBC-488 interface card. Reading, display and analysis of the picoammeter data was carried out with the aid of two software programs that were written in this laboratory by Dr Patrick Hamilton.

Freshly dissected fibres of relatively similar dimensions were cannulated and loaded with aequorin by injecting 0·5 μl of a $2·5 \times 10^{-4}$ M solution of the photoprotein axially throughout the length of each fibre. Aequorin was then allowed to equilibrate for at least 1 h. Ouabain (10^{-4} M) was applied externally some 30 min prior to the application of proctolin. All experiments were done at an environmental temperature of 22 $\pm$ 1 °C.

Agents

Ouabain, HEPES, disodium GTP and proctolin were purchased from Sigma Chemical Company, St Louis, MO, USA. Gpp(NH)p (sodium 5′-guanylylimidodiphosphate) was purchased from P.-L. Biochemicals, Inc., Milwaukee, WI, USA. Verapamil HCl was obtained from Knoll Pharmaceutical Company, Whippany, NJ, USA. Aequorin of $> 95\%$ purity was purchased from the Mayo Foundation, Rochester, MN, USA. This was in the lyophilized form from 1 ml of an aqueous solution containing 150 mM-KCl, 5 mM-HEPES buffer and $< 10^{-6}$ M-EDTA, pH 7·5.

RESULTS

The response to proctolin following substitution of external Ca^{2+} with Sr^{2+}

The work of Hagiwara, Fukuda & Eaton (1974) has shown that the selectivity of the Ca^{2+} channel in barnacle muscle fibres follows the sequence $Ba^{2+} > Sr^{2+} \simeq Ca^{2+}$.

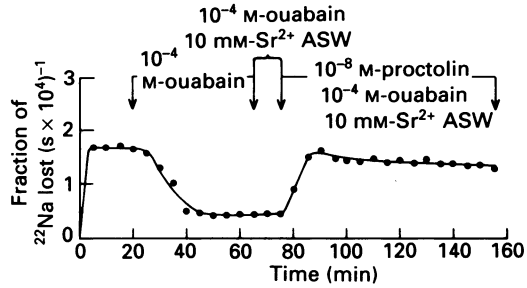

Fig. 1. Sustained stimulation of the ouabain-insensitive Na^+ efflux into 10 mM-Sr^{2+} ASW by external application of 10^{-8} M-proctolin. Notice also the lack of a change in the efflux following the sudden replacement of external Ca^{2+} by Sr^{2+}.

Since the ionic radius of strontium (1·13 Å) is practically the same as that of Ca^{2+} (0·99 Å; Handbook of Chemistry and Physics, 1982), it seemed of special interest to ascertain whether Sr^{2+} is able to fully substitute for Ca^{2+}. As shown in Fig. 1, sudden replacement of external Ca^{2+} by Sr^{2+} fails to modify the ouabain-insensitive Na^+ efflux and that external application of 10^{-8} M-proctolin elicits a prompt and *sustained* stimulatory rise in the efflux, the size of which averages $185 \pm 28\%$ ($n = 5$). This value is not different from $170 \pm 35\%$ stimulation ($n = 5$) observed in companion controls. However, the response in these control fibres was transitory.

Injection of Sr^{2+} and Ca^{2+} in the presence of verapamil

The question next asked was: does the injection of Sr^{2+} mimic the response seen following the injection of Ca^{2+}? To exclude any involvement of the Ca^{2+} channel, the experiments were conducted in the presence of 10^{-4} M-verapamil. As shown in Fig. 2*A* and *B*, the response to the injection of a 0·5 M solution of $SrCl_2$ differs from that obtained with an equimolar concentration of $CaCl_2$ in that the response decays more slowly ($159 \pm 47\%$ stimulation with Sr^{2+} ($n = 4$) *versus* $404 \pm 96\%$ stimulation with Ca^{2+}, $n = 4$, P being $> 0{\cdot}05$). In parallel experiments, 0·5 M-$SrCl_2$ was injected in the absence of verapamil. The stimulatory response obtained averages $338 \pm 42\%$ ($n = 4$), a value which is significantly greater than that obtained in the presence of verapamil (P being $< 0{\cdot}05$). Additionally, in parallel experiments, 0·5 M-$CaCl_2$ was injected in the absence of verapamil. The magnitude of the response averages $379 \pm 79\%$ ($n = 3$), a value which is quite close to that observed in the presence of verapamil (P being $> 0{\cdot}5$).

The response to proctolin when external K^+ is 30 mM

The threshold concentrations of external $K^+(K_o^+)$ at which stimulation of the ouabain-insensitive Na^+ efflux and contraction occur are 20 and 30 mM, respectively (Mason-Sharp & Bittar, 1981). It therefore seemed of interest to see whether the

response to proctolin is augmented in the presence of a raised K_o^+, e.g. 30 mM. The results of these experiments were as follows: (i) simultaneous application of 10^{-8} M-proctolin and elevation of K_o^+ from 10 to 30 mM resulted in a stimulatory response of the order of $569 \pm 26\%$ ($n = 4$). This is to be compared with a stimulatory response

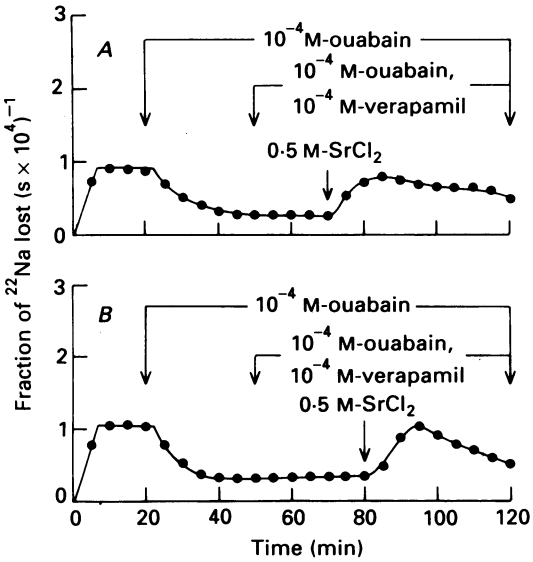

Fig. 2. *A*, stimulation of the ouabain-insensitive Na^+ efflux by injecting 0·5 M-Sr^{2+} in the presence of 10^{-4} M-verapamil in the external medium. Notice that the response decays rather slowly. *B*, stimulation of the ouabain-insensitive Na^+ efflux by injecting 0·5 M-$CaCl_2$ in the presence of 10^{-4} M-verapamil in the external medium.

to proctolin alone of the order of $111 \pm 13\%$, $n = 4$ (P being $< 0{\cdot}001$), and a response to an elevated K_o^+ of the order of $294 \pm 156\%$, $n = 2$ (P being $< 0{\cdot}2$). (ii) In a second group of fibres, the response to proctolin in the presence of 30 mM-K_o^+ and 10^{-4} M-verapamil was of the order of $95 \pm 12\%$, $n = 4$, a value significantly less than $569 \pm 26\%$, P being $< 0{\cdot}001$. Figure 3 illustrates the following : effect of 10^{-8} M-proctolin (panel *A*); effect of 30 mM-K_o^+ (panel *B*); the combined effects of 10^{-8} M-proctolin and 30 mM-K_o^+ (panel *C*), and the effects of 10^{-8} M-proctolin and 30 mM-K_o^+ in the presence of 10^{-4} M-verapamil (applied simultaneously) (panel *D*).

The response to proctolin of fibres pre-enriched with GTP and Gpp(NH)p

Bittar & Nwoga (1982*a*) showed that injection of the sodium salt of GTP followed by depolarization of the fibre membrane using 100 mM-K_o^+ leads to a biphasic stimulatory response, i.e. an almost immediate but small and transitory rise in the ouabain-insensitive Na^+ efflux, followed by a large and *sustained* stimulatory response, and that such a response is mimicked by injecting Gpp(NH)p. In view of this, and the fact that G proteins are known to play the role of membrane transducers, it seemed important to test the effect of proctolin on the ouabain-insensitive Na^+ efflux in fibres pre-enriched with GTP or Gpp(NH)p. The main results obtained were as follows: first, the response to 10^{-8} M-proctolin in fibres pre-injected with 0·25 M-GTP stops decaying some 30 min after peaking ($n = 4$), whereas

the response in fibres pre-injected with 0·1 M-GTP is transitory ($n = 3$). Repetition of this type of experiment by injecting the poorly hydrolysable analogue Gpp(NH)p in a concentration of 0·1 M ($n = 4$) and 0·25 M ($n = 4$) prior to external application of 10^{-8} M-proctolin confirmed the finding that it is possible to stop the response to

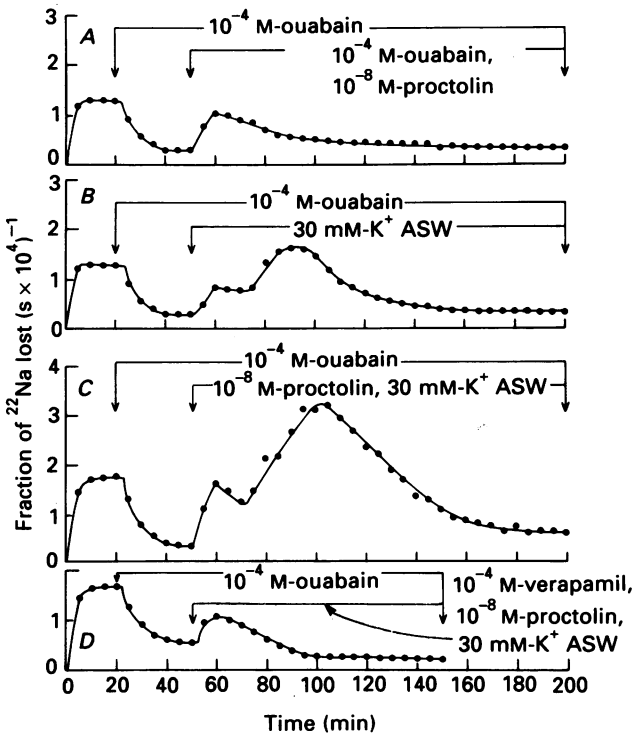

Fig. 3. The response of the ouabain-insensitive Na^+ efflux to *A*, external application of 10^{-8} M-proctolin. *B*, a sudden threefold elevation in external K^+ concentration, and *C*, simultaneous application of 10^{-8} M-proctolin and a threefold elevation in external K^+ concentration. *D*, the small response of the ouabain-insensitive Na^+ efflux to simultaneous application of 10^{-8} M-proctolin, 10^{-4} M-verapamil and a threefold elevation in external K^+ concentration. In experiments of this type, the fibres were isolated from the same barnacle specimen.

proctolin from decaying by pre-injecting a 0·25 M solution of guanine nucleotide. This is illustrated in Fig. 4*A* and *B*, where one sees that proctolin causes a clear-cut step-up in the ouabain-insensitive Na^+ efflux which peaks some 15 min later and then decays but only for a while.

The response to proctolin of fibres pre-enriched with Gpp(NH)p and exposed to 30 and 100 mM-K_o^+

Bittar & Nwoga (1982*a*) and Nwoga (1987) reported that fibres injected with GTP and then exposed to high K_o^+ (e.g. 100 mM) show a delayed but sustained stimulation of the ouabain-insensitive Na^+ efflux. As illustrated in Fig. 5*A* and *B*, this is also true of fibres injected with 0·25 M-Gpp(NH)p and then exposed to 30 or 100 mM-K_o^+. In neither case, however, did a prompt and small step-up following fibre depolarization

occur. Subsequent application externally of 10^{-8} M-proctolin caused a prompt and sustained step-up in the ouabain-insensitive Na^+ efflux (the magnitude of which averages $178 \pm 35\%$ ($n = 4$) and $402 \pm 145\%$ ($n = 4$), respectively, P being $> 0{\cdot}20$).

Measurement of the resting membrane potential before and after proctolin

Measurements were made of the E_m in ouabain-poisoned fibres before and after external application of 10^{-7} M-proctolin. The experimental set-up consisted of a

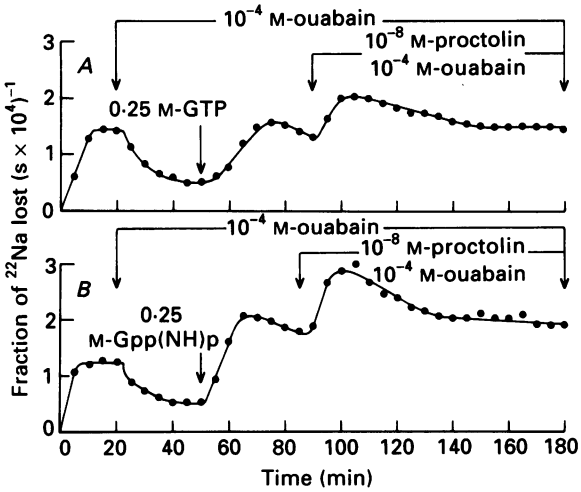

Fig. 4. Persistence of the response to external application of 10^{-8} M-proctolin. *A*, in a fibre pre-injected with 0·25 M-GTP, and *B*, in a fibre pre-injected with 0·25 M-Gpp(NH)p.

bathing chamber with an outlet and inlet, allowing replacement of the medium. The results obtained were as follows: E_m at $t = 0$, $-51 \pm 1{\cdot}5$ mV; at $t = 1$ min following application of proctolin, $-47 \pm 1{\cdot}1$ mV; and at $t = 5$, -49 ± 1 mV ($n = 12$). These measurements were repeated using a more reliable set-up that allowed replacement of the bathing medium without any appreciable agitation of the fibre. The results of these experiments, as summarized in Fig. 6, indicate that the external application of 10^{-7} M-proctolin (upper curve, panel *A*) causes a small reversible fall in the E_m in ~ 1 min but this is not significant enough, whereas the reversible fall (lower curve) also elicited by 10^{-7} M-proctolin is (at $t = 1$ min) significantly less than the original resting E_m. The curve in panel *B* indicates that in this group of fibres 10^{-7} M-proctolin also causes a small fall in the E_m but recovery fails to occur over a 15 min period. Also shown is that a sudden 100-fold increase in proctolin concentration slightly but reversibly reduces the E_m. This particular result is dismissed, since it is most likely related to fibre super-contraction.

The monitoring of light from aequorin before and after proctolin

To marshal evidence that the mechanism underlying stimulation of the ouabain-insensitive Na^+ efflux by proctolin is mediated by a transitory fall in myoplasmic pCa, and that this effect is dose-dependent, the time-course of luminescence from

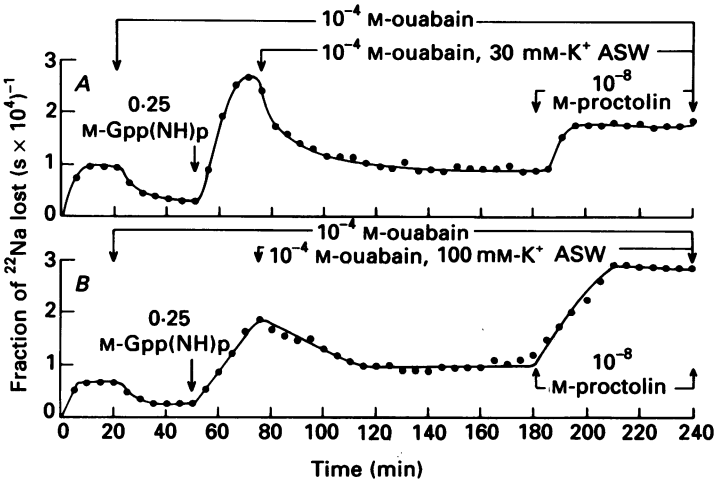

Fig. 5. Persistence of the response to external application of 10^{-8} M-proctolin. *A*, in a fibre pre-injected with 0·25 M-Gpp(NH)p and then depolarized by raising K_o^+ from 10 to 30 mM, and *B*, in a fibre pre-injected with 0·25 M-Gpp(NH)p and then depolarized by a sudden tenfold elevation in K_o^+. Notice that the response to guanine nucleotides fails to completely decay in the presence of an elevated K_o^+. As will be recalled, the rule is that the response to high K_o^+ disappears both rapidly and completely (Mason-Sharp & Bittar, 1981).

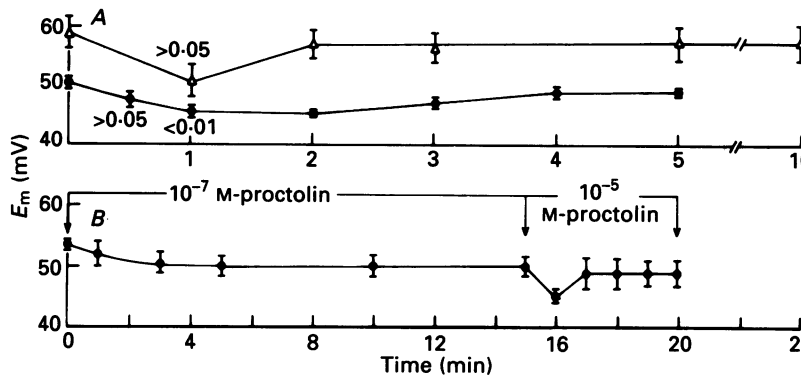

Fig. 6. Time course of the resting membrane potential of fibres treated with 10^{-4} M-ouabain some 30 min prior to treatment with proctolin. *A*, 10^{-7} M-proctolin (●, $n = 7$) and 10^{-5} M-proctolin (△, $n = 3$) applied at $t = 0$. *B*, 10^{-7} M-proctolin, followed at $t = 15$ min by 10^{-5} M-proctolin ($n = 7$). Vertical bars indicate ±1 S.E.M.

aequorin was investigated before and after external application of proctolin. Fibres having relatively similar dimensions were selected and isolated from the same barnacle specimen, and subsequently loaded with aequorin by injecting a $2{\cdot}5 \times 10^{-4}$ M solution of the photoprotein at least 1 h prior to monitoring the resting level of light emission. Furthermore, treatment of these fibres with 10^{-4} M-ouabain was begun some 30 min following loading with aequorin. The main results obtained in

these experiments were as follows: an increase in luminescence from aequorin occurs almost immediately or within 1 min of adding proctolin to the bathing medium. As illustrated in the chart tracing shown in Fig. 7, external application of proctolin in a concentration as low as 10^{-9} M at $t = 0$ is promptly followed by a series of repetitive

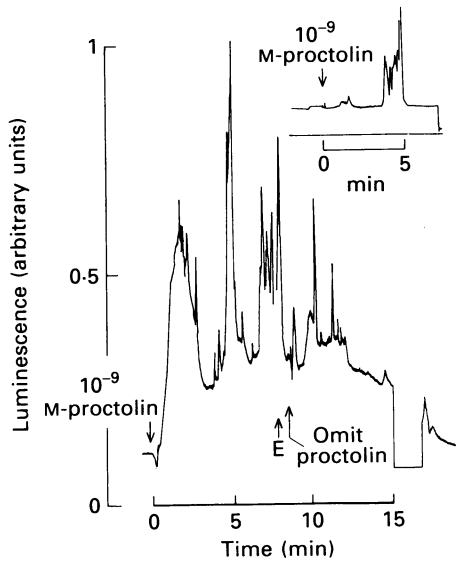

Fig. 7. Chart recording of aequorin signals from a ouabain-poisoned fibre treated externally with 10^{-9} M-proctolin at $t = 0$ (first arrow). At the second arrow, *E*, the bathing chamber was emptied and ASW without proctolin was added at the third arrow. The DC power supply unit was turned off at $t = 15$ and then on again. Inset: aequorin signals from a companion fibre treated with 10^{-9} M-proctolin at $t = 0$. The settings of the Watanabe chart recorder were the same as in the preceding experiment. Temperature 23 ± 1 °C.

Ca^{2+} transients. At arrow E, the bathing chamber was emptied and refilled with ASW only. As can be seen, the multiphasic response persists for a few more minutes. At $t = 15$ min, the DC power supply unit was turned off and then on, thereby indicating glow intensity as it approaches the original resting level. This mode of behaviour was also seen in a companion fibre. However, in two other fibres isolated from the same bundle, the aequorin response was not only slower in onset but also the size and frequency of the spikes were considerably less, as indicated in the inset. Notice the brief and small transient preceding the doublet, and that light emission returns to its original baseline level at $t = 5$ min. Also notice that the peak of the aequorin response is about one-fourth that recorded in the preceding experiment. This degree of variability among fibres obtained from the same bundle is not a rare happening. It is commonly found among barnacle specimens. Another difficulty encountered in this type of work is batch-to-batch variation. Similar considerations apply to the case where the effect of proctolin on the ouabain-insensitive Na^+ efflux is being tested.

A second salient feature of the aequorin response to proctolin is that the shape of the signal is either monophasic or multiphasic, and rarely biphasic. The monophasic signal can be extremely brief in duration, as illustrated in Fig. 8*A*, or multiphasic and a few minutes in duration (Fig. 8*B*). For the case where 10^{-7} M-proctolin (an intermediate concentration) is applied, the duration of the aequorin response (in

fibres derived from California specimens) averages $3{\cdot}2 \pm 0{\cdot}5$ min ($n = 8$). This is a time frame that falls in the range of the time taken by the ouabain-insensitive Na^+ efflux to reach a maximum in response to 10^{-7} M-proctolin, viz. $10{\cdot}5 \pm 0{\cdot}9$ min ($n = 10$).

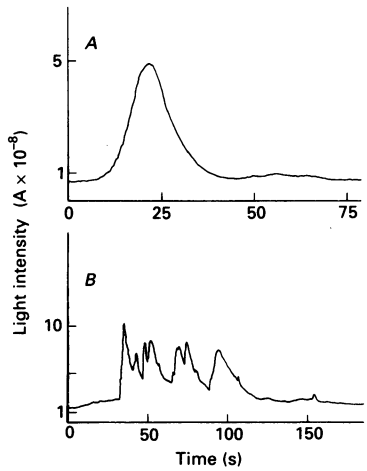

Fig. 8. *A*, computer print-out showing the time course of a monophasic aequorin signal from a Californian fibre exposed to 10^{-7} M-proctolin at $t = 0$. *B*, computer print-out showing the time course of a multiphasic aequorin response of a Californian fibre to 10^{-7} M-proctolin applied externally at $t = 0$. Notice that in both instances the signals decline to a level which is slightly above the original resting level.

Next, the concentration–response relation for the stimulating effect of proctolin on light emission from aequorin in fibres isolated from California *B. nubilus* specimens was determined. As summarized in Fig. 9*A*, the minimal effective concentration of proctolin in this series of experiments lies in the region of 10^{-8} M. In confirmation of dose dependence and the fact that the threshold concentration of proctolin tends to vary among barnacle specimens is the example given in Fig. 9*B*. This indicates a threshold level of about 10^{-7} M. Notice that the basal levels of resting glow are not practically the same, a situation which is likely to be due to non-uniform distribution of the injected aequorin and variability in the degree of Ca^{2+} leakage, for example, from the sarcoplasmic reticulum (SR). Notice also that the signal is monophasic, whereas that obtained with 10^{-5} M-proctolin is multiphasic.

Experience amply indicates the existence of several basic differences between fibres isolated from *B. nubilus* specimens collected from Californian waters (Monterey Bay) and those from Washington waters (Puget Sound). First, the resting glow in Washington fibres is usually considerably less than that found in Californian fibres ($5{\cdot}5 \pm 0{\cdot}9$ A $\times 10^{-10}$, $n = 12$ *vs.* $49{\cdot}6 \pm 6{\cdot}3$ A $\times 10^{-10}$, $n = 12$). Secondly, the signal induced by proctolin in Washington fibres is usually monophasic or biphasic, rather than multiphasic. Thirdly, the new level of resting glow following complete disappearance of the aequorin response to proctolin is often below the original baseline level. These features are illustrated by the experiment given in Fig. 10. Note that proctolin in a concentration of 10^{-6} M was applied at $t = 0$. Other parameters, including the latent period, $t_{\frac{1}{2}}$, for the response to reach peak height, and the duration

of the signal, however, are not found to be very different from those of monophasic signals occurring in Californian fibres.

An important question raised by the observation that the new level of resting glow in Washington fibres is often lower than the original level of resting glow is whether

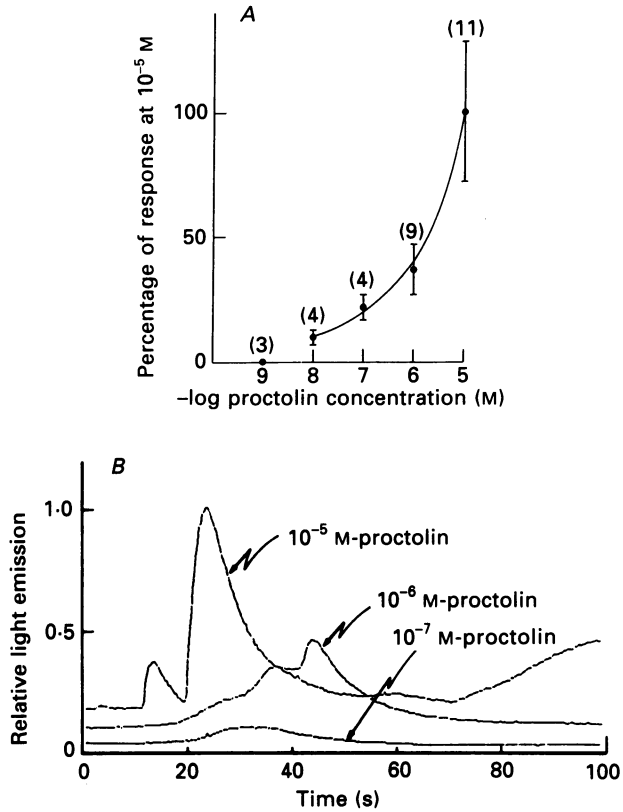

Fig. 9. *A*, concentration–response curve for the effect of proctolin on light emission from aequorin. The Californian fibres used were of roughly similar dimensions and were injected with $2{\cdot}5 \times 10^{-4}$ M-aequorin in a volume of 0·5 μl. Vertical bars indicate ± 1 S.E.M. and the number of measurements carried out is given in parentheses. *B*, aequorin signals from three companion Californian fibres treated with 10^{-7}, 10^{-6} and 10^{-5} M-proctolin. Notice the multiphasic response of the fibre treated with 10^{-5} M-proctolin.

the undischarged aequorin remaining after the complete disappearance of the response to proctolin (for example, 10^{-6} M) is plentiful. This was addressed by suddenly raising external K^+ tenfold at the end of most of the experiments carried out with Washington fibres, as well as by raising external K^+ step-wise in certain experiments. Shown in the middle and lower panels of Fig. 10 are the remaining parts of the experiment appearing in the upper panel. Notice that a sudden elevation from 10 to 20 mM (at the arrow, middle panel) is almost immediately followed by one or two motion artifacts, and then a gradual rise in light output. At the next arrow, external K^+ was raised to 30 mM. This is again followed by a 'dip' (motion artifact), and then a step-up in light output which upon reaching a peak begins to decay rather slowly. Notice that the time bases appearing in the three panels are not the same. As

shown in the lower panel, the characteristic response to suddenly raising external K^+ to 100 mM is a triplet, the peak of which exceeds 60 nA. At $t = 30$ min (not shown) resting glow measures ~ 0·8 nA. Essentially similar kinetics were observed in the five other companion test fibres. Collectively, these results are significant, not only

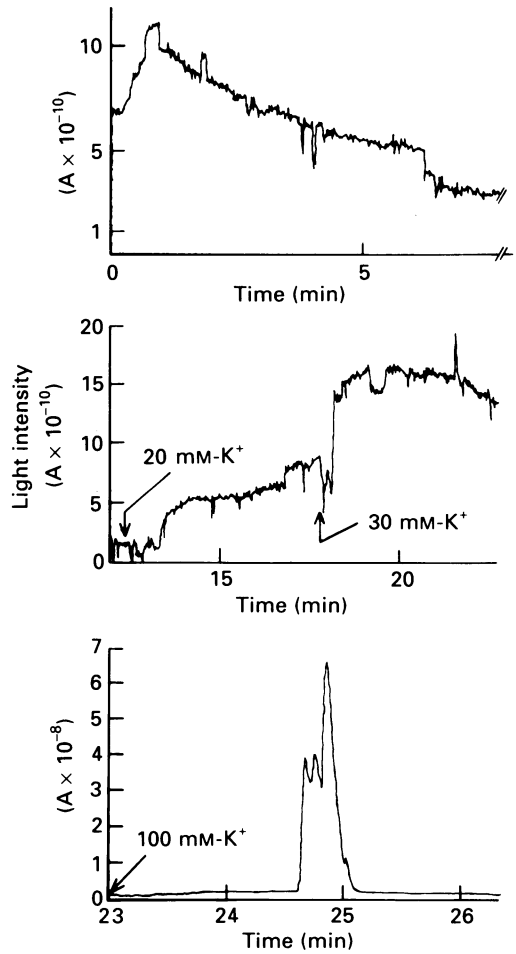

Fig. 10. Upper panel: computer print-out of the monophasic aequorin signal (expressed in A) from a Washington fibre treated with 10^{-6} M-proctolin at $t = 0$. This representative experiment was continued. Mid-panel: at the first arrow, external K^+ was suddenly doubled and at the second arrow, it was raised to 30 mM. Lower panel: at the arrow external K^+ was raised from 30 to 100 mM. Note that the time bases of the three portions of this experiment are not the same.

because they indicate that the undischarged aequorin prior to raising external K^+ is substantial, but also because they provide evidence supporting the view that the threshold concentration at which external K^+ stimulates the ouabain-insensitive Na^+ efflux via a fall in myoplasmic pCa (see Fig. 3) lies in the region of 20 mM (Mason-Sharp & Bittar, 1981). Moreover, the experiments involving 100 mM-K^+ are particularly instructive in that the simplest interpretation of the triplet found in the

rising phase of the aequorin signal is that the first phase is ascribable to Ca^{2+} entry following T-tubule membrane depolarization, while the other two phases are ascribable to SR Ca^{2+} release. In the latter case, evidence is available that Ca^{2+} release by skeletal muscle SR is biphasic (Ikemoto, Antoniu & Kim, 1984).

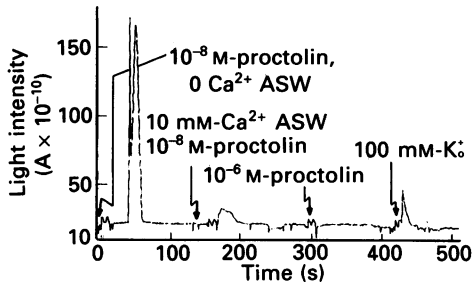

Fig. 11. Computer print-out showing the aequorin response of a Washington fibre suspended in nominally Ca^{2+}-free ASW and exposed to 10^{-8} M-proctolin at $t = 0$ (first arrow). At the second arrow, Ca^{2+} was restored to the bathing medium. At the third arrow, the proctolin concentration was increased 100-fold. At the fourth arrow, external K^+ was raised from 10 to 100 mM.

It remained to be asked whether an external Ca^{2+} requirement for an aequorin response to proctolin is absolute. As will be recalled, it is absolute in the case of the response of the Na^+ efflux to proctolin (Nwoga & Bittar, 1985). Experiments were therefore performed in which Washington fibres pre-loaded with aequorin and pre-treated with 10^{-4} M-ouabain were suspended in nominally Ca^{2+}-free ASW for 10–15 min prior to external application of 10^{-8} M-proctolin. Figure 11 shows that the aequorin response, which is a doublet, is quite striking and sharp (and averages almost 60-fold with a range of 8- to 82-fold, $n = 8$). At the second arrow, the replacement of Ca^{2+}-free ASW by 10 mM-Ca^{2+} ASW leads to a small rise in light emission, a result which was also seen in three other companion fibres. However, in two of the remaining four fibres, the results were very different: in one the response to proctolin in 10 mM-Ca^{2+} ASW was slightly greater than the response in Ca^{2+}-free ASW. In the other fibre, a response to proctolin failed to occur in the absence of external Ca^{2+}, while in the presence of Ca^{2+}, the rise in light emission was practically 80-fold. Also shown at the third arrow in Fig. 11 is that 10^{-6} M-proctolin is without effect, and that at the fourth arrow, a sudden tenfold increase in external K^+ is quickly followed by a rise in light emission which is not large and very brief in duration. Notice that the final level of resting glow is virtually the same as that prior to depolarization of the fibre membrane. A sensitivity this low to high K_o^+ was also found in seven companion fibres, as well as in fibres whose ouabain-insensitive Na^+ efflux was being monitored at this particular time. Again, this confirms the existence of wide variation in sensitivity to K^+ among barnacle specimens.

DISCUSSION

One of the steps taken towards explaining the mechanism by which proctolin stimulates the ouabain-insensitive Na^+ efflux was to monitor myoplasmic pCa with

the aid of aequorin. The results of these experiments clearly indicate that proctolin promptly brings about a transitory fall in myoplasmic pCa. Evidence of its physiological significance is partly provided by two observations: one is that a fall in internal pCa is found to occur when the concentration of proctolin is in the nanomolar range, and the other that proctolin in this concentration range is able to stimulate the ouabain-insensitive Na^+ efflux. Moreover, the multiphasic shape of the aequorin signal is reminiscent of the repetitive Ca^{2+} transients seen in a variety of cells, including single hepatocytes treated with Ca^{2+}-mobilizing hormones (Woods, Cuthbertson & Cobbold, 1987).

Aequorin was chosen as the internal Ca^{2+} indicator because of its manifold advantages. Chief among these are its sensitivity to free Ca^{2+}, especially in the full range of the internal pCa occurring in living cells, as well as its relative specificity for Ca^{2+} (Blinks, Wier, Bess & Prendergast, 1982). Another is its response time of 5–10 ms, and the fact that it is inert and does not interfere with cell function (Blinks *et al.* 1982). However, such an approach is not without certain assumptions. For example, it is assumed that the fibres are homogeneous and that both aequorin and free Ca^{2+} in the myoplasm are uniformly distributed. Since the relation between light intensity and Ca^{2+} is sigmoidal rather than linear (the rate of the aequorin reaction being proportional to $[Ca^{2+}]^{2.5}$ – Allen, Blinks & Prendergast, 1977), it is not easy to interpret both monophasic and multiphasic signals quantitatively. More to the point, in a preparation as large as the barnacle muscle fibre, one would expect the internal pCa changes elicited by proctolin to be largely confined to the periphery. If this is true, then the total aequorin signal would be dominated by the light coming from those peripheral regions where the Ca^{2+} is greatest, for example, just under the T-tubule surface membrane. This is also the case with other large preparations, including the squid axon (Baker, Hodgkin & Ridgway, 1971), and frog muscle (Cannell & Allen, 1984). Other factors, such as Mg^{2+}, which is known to interfere with the action of Ca^{2+} on aequorin in addition to reducing the level of Ca^{2+}-independent luminescence (Blinks *et al.* 1982), are unlikely to be major when the changes in internal free Mg^{2+} are not in the millimolar range. This partly follows from the observation that the internal free Mg^{2+} in barnacle fibres lies in the 4–5 mmol (kg wet weight) range (Ashley & Ellory, 1972). Further, any transitory rise in internal pMg would primarily depend on the magnitude of the Ca^{2+} released by the SR, and the extent to which Mg^{2+} uptake from the myoplasm is coupled to such release (Yoshioka & Somlyo, 1984). One is thus left with the tentative conclusion that the aequorin response to proctolin primarily reflects an 'overall' pCa change in the periphery of the myoplasm rather than of a mean pCa change across the myoplasmic compartment.

In addition, the aequorin experiments have yielded useful information about the time course of the Ca^{2+} transients caused by proctolin. At least two points are notable: first, resting luminescence increases in most instances within 1 min of external application of proctolin. Secondly, the duration of these Ca^{2+} transients is usually 3–5 min. This is a time frame that corresponds quite closely with the onset of the stimulatory response of the ouabain-insensitive Na^+ efflux to proctolin, as well as the time it usually takes to reach a maximum, viz. 10 min. An onset this rapid agrees with the observation that fibres treated with proctolin, for example,

concentrations of 10^{-7} M or more, undergo a contracture almost immediately. This is in keeping with the general property of proctolin as a contractile principle. Thus, for example, it causes the contracture of *Limulus* heart muscle (Watson & Hoshi, 1985), cockroach hindgut (Brown, 1975), and lobster opener muscle (Schwartz, Harris-Warrick, Glusman & Kravitz, 1980). In the case of lobster muscle, proctolin in concentrations as low as 10^{-10} M causes an increase in tension while barely reducing the E_m and even with 10^{-7} M, the fall in E_m is less than 1 mV (Schwartz *et al.* 1980). Importantly, then, contracture is interpreted as strengthening the conclusion that proctolin reduces myoplasmic pCa. This may occur through the entry of external Ca^{2+} or the redistribution of internal Ca^{2+} (e.g. via an inositol trisphosphate (IP_3) mechanism) or through both mechanisms. Calcium entry *per se* is far from proven by the observation of an aequorin response to proctolin in fibres suspended in Ca^{2+}-free ASW. Several reasons as to why this is so can be given. First, those channels lying deep in the cleft T-tubule systems of fibres immersed in nominally Ca^{2+}-free ASW probably still contain some Ca^{2+}, more particularly because of the occurrence of Ca^{2+} efflux. EGTA was not added to the bathing medium since experience shows that the chelator aggravates the labile condition these fibres develop when immersed too long in Ca^{2+}-free ASW. Secondly, Ca^{2+}-free solutions invariably contain 10^{-6} M–Ca^{2+} or slightly less. Thirdly, some fibres, as found, respond to proctolin only if Ca^{2+} is restored, while other fibres respond in the absence of external Ca^{2+}, and respond, once more, when Ca^{2+} is restored.

The provisional conclusion that the external medium is the origin of trigger Ca^{2+} is supported by the experiments carried out with verapamil, Cd^{2+} and Co^{2+} (Nwoga & Bittar, 1985), high K^+ and guanine nucleotides (Mason-Sharp & Bittar, 1981; Bittar & Nwoga, 1982*a*, *b*). Such studies are obviously relevant to an understanding of so-called Ca^{2+}-induced Ca^{2+} release. This mechanism can best be envisaged if allosteric coupling does exist between the Ca^{2+} channels of the transverse tubule membrane, and the Ca^{2+} release channel of the sarcoplasmic reticulum (Campbell, Leung & Sharp, 1988; Fill & Coronado, 1988). Thus membrane depolarization augments the response to proctolin because voltage-gated Ca^{2+} channels (or the voltage sensor) and proctolin receptor-operated Ca^{2+} channels are the same. Alternatively, these channels may be of two distinct types: voltage-gated and receptor-operated, but closely interacting. It may also be plausibly maintained that proctolin acts specifically through a second messenger-operated channel such as that whose messenger is inositol 1,4,5-triphosphate (IP_3). This would account for an aequorin response in some fibres suspended in nominally Ca^{2+}-free ASW, but evidence that proctolin raises internal IP_3 is still lacking. More importantly, at this stage, one has to consider whether IP_3 is able to release Ca^{2+} from the SR of barnacle fibres (Vergara, Tsien & Delay, 1985; Rojas, Nasser-Gentina, Luxoro, Pollard & Carrasco, 1987; Campagnon, Lagos & Vergara, 1989) or whether it is, in fact, ineffective (Lea, Griffiths, Tregear & Ashley, 1986). It could well be that its effectiveness depends on the T-tubule membrane potential, for example, in peeled rabbit skeletal muscle (Donaldson, Goldberg, Walseth & Huetteman, 1988).

It is too early to do more than speculate about the mechanism by which excess internal free Ca^{2+} leads to stimulation of the Na^+ efflux. One reasonable hypothesis is that Ca^{2+} activates the putative Ca^{2+}–calmodulin-dependent protein kinase branch

of the Ca^{2+} messenger system (e.g. Kennedy, Bennett, Erondu & Miller, 1987; Schulman & Lou, 1989), thereby leading to phosphorylation of the sarcolemmal Na^+–Ca^{2+} exchanger (Caroni, Soldati & Carafoli, 1984). This would explain not only the transient nature of the response, but also the fact that it outlasts the aequorin response. For this is a kinase that is rendered independent of both Ca^{2+} and calmodulin upon autophosphorylation (Kennedy *et al.* 1987; Schulman & Lou, 1989). Thus, if this is the case, then the duration of the response of the Na^+ efflux to proctolin would be expected to be governed by the relative rates of two processes, autophosphorylation and dephosphorylation by phosphatase, e.g. calcineurin. This Ca^{2+}–calmodulin-dependent enzyme (Stewart, Ingebretsen, Manalan, Klee & Cohen, 1982) is known to dephosphorylate the Na^+–Ca^{2+} exchanger, for example, in heart muscle (Caroni *et al.* 1984; Carafoli & Longoni, 1987), as well as the α_1 subunit of the Ca^{2+} channel protein in skeletal muscle T-tubule membrane (O'Callahan & Hosey, 1988). A dephosphorylation reaction would account for the transitory nature of the response of the Na^+ efflux to proctolin. Whether this presages desensitization it is impossible to say, since proctolin at a higher concentration was not subsequently applied. However, what is of special interest is that pre-injection of GTP or Gpp(NH)p prolongs the response to proctolin. Remarkably this closely resembles the response to serotonin of fibres pre-injected with guanine nucleotides (Bittar & Chambers, 1985).

Having adopted the viewpoint that phosphorylation is the mechanism underlying stimulation of the Na^+–Ca^{2+} exchanger, one now begins to see in outline that the various conditions necessary for the operation of the exchanger in the reverse mode, for example, as in the squid axon (Baker, 1986; DiPolo & Beaugé, 1987) are present here, that is, a reduced Na^+ electrochemical gradient resulting from inactivation of the membrane Na^+–K^+-ATPase by ouabain, ASW containing 10 mM–Ca^{2+} and a decline in internal pCa almost immediately following the application of proctolin. However, this Ca^{2+} transient is very brief and it disappears presumably because of SR buffering and active Ca^{2+} extrusion by the sarcolemmal Ca^{2+}–Mg^{2+}-ATPase. The idea of SR buffering gains further credence from the finding of a reduced resting glow following the full decay of the aequorin signal elicited by proctolin. This change in baseline may reflect an overshoot of the SR pump. Assuming then that the original Ca^{2+} electrochemical gradient is restored, the question arising is not so much whether $\Delta\tilde{\mu}_{Ca} > 3\Delta\tilde{\mu}_{Na}$ (Rasgado-Flores & Blaustein, 1987) but rather whether the key event leading to stimulation of the ouabain-insensitive Na^+ efflux is phosphorylation of the exchanger. As yet, information concerning this question is unavailable.

A vexing but key question now to be asked is: does proctolin depolarize the T-tubule membrane? Although the measurements of E_m carried out leave this question in abeyance, it has been addressed in a different way, though indirectly. On the view that Ca^{2+} channel behaviour is partly governed by G protein (e.g. Brown & Birnhaumer, 1988), GTP or Gpp(NH)p was injected before the application of proctolin. Justification for adopting this approach is to be found in the work of Bittar & Nwoga (1982*b*) who demonstrated that the stimulatory response of the ouabain-insensitive Na^+ efflux to the injection of guanine nucleotides is partly abolished by pre-applying verapamil externally, or pre-injecting protein kinase inhibitor. As found here, the response to proctolin of fibres pre-injected with Gpp(NH)p does not

decay. This is a matter of some significance not only because it is new but also because the same is also true of the response to proctolin in fibres pre-injected with Gpp(NH)p and depolarized with high K_o^+. What, then, is the fundamental difference between these two observations? A clue, it would appear, comes from the finding that verapamil completely abolishes the sustained response of the ouabain-insensitive Na^+ efflux in fibres pre-injected with GTP and depolarized with high K_o^+ (Bittar & Nwoga, 1982*a*). That is to say, the sustained response is wholly attributable to activation of voltage-gated Ca^{2+} channels, a condition which would be expected to exist if G_s and G_{CaCh} are one and the same (see Mattera, Graziano, Yatani, Zhou, Graf, Codina, Birnbaumer, Gilman & Brown, 1989). This line of reasoning is supported by indirect evidence that these fibres possess G_s protein: for example, the two universal activators of G_s protein, F^- and cholera toxin, when injected, cause sustained stimulation of the ouabain-insensitive Na^+ efflux (Bittar & Nwoga, 1982*a*, *b*). Whether this G protein plays a role in the mechanism of proctolin action is a subject that awaits investigation.

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REFERENCES

ALLEN, D. G., BLINKS, J. R. & PRENDERGAST, F. G. (1977). Aequorin luminescence: relation of light emission to calcium concentration – a calcium-independent component. *Science* **196**, 996–998.

ASHLEY, C. C. & ELLORY, J. C. (1972). The efflux of magnesium from single crustacea muscle fibres. *Journal of Physiology* **226**, 653–674.

BAKER, P. F. (1986). Exchange of sodium for calcium across the plasma membrane. *Journal of Cardiovascular Pharmacology* **8**, suppl. 8, S25–32.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *Journal of Physiology* **218**, 709–755.

BITTAR, E. E. & CHAMBERS, G. (1985). The modulatory action of 5-hydroxytryptamine on sodium efflux: the barnacle muscle fibre as a model system. *Comparative Biochemistry and Physiology* **80C**, 421–429.

BITTAR, E. E. & KEH, T. (1980). An investigation of myoplasmic magnesium adenosine triphosphate in single barnacle muscle fibres with the firefly method. *Journal of Physiology* **302**, 73–88.

BITTAR, E. E. & NWOGA, J. (1982*a*). Some further observations on the stimulation by high external potassium of the sodium efflux in barnacle muscle fibers. *Pflügers Archiv* **395**, 318–325.

BITTAR, E. E. & NWOGA, J. (1982*b*). Stimulation by injected guanosine triphosphate of the sodium efflux in barnacle muscle fibres. *Journal of Physiology* **322**, 389–397.

BITTAR, E. E. & TALLITSCH, R. B. (1975). Stimulation by aldosterone of the sodium efflux in barnacle muscle fibres. *Journal of Physiology* **250**, 331–346.

BLINKS, J. R., WIER, W. G., HESS, P. & PRENDERGAST, F. G. (1982). Measurement of Ca^{2+} concentrations in living cells. *Progress in Physics and Molecular Biology* **40**, 1–114.

BROWN, A. M. & BIRNBAUMER, L. (1988). Direct G protein gating of ion channels. *American Journal of Physiology* **254**, H401–410.

BROWN, B. E. (1975). Proctolin: a peptide transmitter candidate in insects. *Life Sciences* **17**, 1241–1252.

CALDWELL, P. C. & WALSTER, G. E. (1963). Studies of the microinjection of various substances into crab muscle fibres. *Journal of Physiology* **169**, 353–372.

CAMPBELL, K. P., LEUNG, A. T. & SHARP, A. H. (1988). The biochemistry and molecular biology of the dihydropyridine-sensitive calcium channel. *Trends in Neurosciences* **11**(10), 425–430.

CANNELL, M. B. & ALLEN, D. G. (1984). Model of calcium movements during activation in the sarcomere of frog skeletal muscle. *Biophysical Journal* **45**, 913–925.

CARAFOLI, E. & LONGONI, S. (1987). The plasma membrane in the control of the signaling function of calcium. In *Cell Calcium and the Control of Membrane Transport*, ed. MANDELL, L. J. & EATON, D. C., pp. 21–29. New York, NY, USA: Rockefeller University Press.

CARONI, P., SOLDATI, L. & CARAFOLI, E. (1984). The Na^+/Ca^{2+} exchanger of heart sarcolemma is regulated by a phosphorylation–dephosphorylation process. In *Electrogenic Transport: Fundamental Principles and Physiological Implications*, ed. BLAUSTEIN, M. P. & LIEBERMAN, M., pp. 149–160. New York, NY, USA: Raven Press.

COMPAGNON, D., LAGOS, N. & VERGARA, J. (1989). Metabolic support for a chemical mechanism of E–C coupling in barnacle muscle fibers. *Biophysical Journal* **55**, 87a.

DIPOLO, R. & BEAUGÉ, L. (1987). Characterization of the reverse Na/Ca exchange in squid axons and its modulation by Ca_i and ATP. *Journal of General Physiology* **90**, 505–525.

DONALDSON, S. K., GOLDBERG, N. D., WALSETH, T. F. & HUETTEMAN, D. A. (1988). Voltage dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in peeled skeletal muscle fibers. *Proceedings of the National Academy of Sciences of the USA* **85**, 5749–5753.

FILL, M. & CORONADO, R. (1988). Ryanodine receptor channel of sarcoplasmic reticulum. *Trends in Neurosciences* **11** (10), 453–457.

HAGIWARA, S., FUKUDA, J. & EATON, D. C. (1974). Membrane currents carried by Ca, Sr, and Ba in barnacle muscle fiber during voltage clamp. *Journal of General Physiology* **63**, 564–578.

HANDBOOK OF CHEMISTRY AND PHYSICS (1982). 62nd edn, ed. WEAST, R. C. & ASTLE, M. J. Boca Raton, FL, USA: CRC Press.

HODGKIN, A. L. & KEYNES, R. D. (1956). Experiments on the injection of substances into squid axons by means of a microsyringe. *Journal of Physiology* **131**, 592–616.

IKEMOTO, N., ANTONIU, B. & KIM, D. H. (1984). Rapid calcium release from the isolated sarcoplasmic reticulum is triggered via the attached transverse tubular system. *Journal of Biological Chemistry* **259**, 13151.

KENNEDY, M. B., BENNETT, M. K., ERONDU, N. E. & MILLER, S. G. (1987). Calcium/calmodulin dependent protein kinases. *Calcium and Cell Function* **7**, 61–107.

LEA, T. J., GRIFFITHS, P. J., TREGEAR, R. T. & ASHLEY, C. C. (1986). An examination of the ability of inositol 1,4,5-trisphosphate to induce calcium release and tension development in skinned skeletal muscle fibres of frog and crustacea. *FEBS Letters* **207**, 153–161.

MASON-SHARP, D. & BITTAR, E. E. (1981). Stimulation of high external potassium of the sodium efflux in barnacle muscle fibers. *Journal of Membrane Biology* **58**, 213–226.

MATTERA, R., GRAZIANO, M. P., YATANI, A., ZHOU, Z., GRAF, R., CODINA, J., BIRNBAUMER, L., GILMAN, A. G. & BROWN, A. M. (1989). Splice variants of the α subunit of the G protein G_s activate both adenyl cyclase and calcium channels. *Science* **243**, 804–807.

NWOGA, J. (1987). Concerning stimulation by high external potassium and calcium injection of the ouabain-insensitive sodium efflux in barnacle muscle fibres. *Comparative Biochemistry and Physiology* **86A**, 733–737.

NWOGA, J. & BITTAR, E. E. (1985). Stimulation by proctolin of the ouabain-insensitive sodium efflux in single barnacle muscle fibers. *Comparative Biochemistry and Physiology* **81C**, 345–350.

O'CALLAHAN, C. M. & HOSEY, M. M. (1988). Multiple phosphorylation sites in the 165-kilodalton peptide associated with dihydropyridine-sensitive calcium channels. *Biochemistry* **27**, 6071–6077.

RASGADO-FLORES, H. & BLAUSTEIN, M. P. (1987). Na/Ca exchange in barnacle muscle cells has a stoichiometry of 3 Na^+/1 Ca^{2+}. *American Journal of Physiology* **252**, C499–504.

ROJAS, E., NASSAR-GENTINA, V., LUXORO, M., POLLARD, M. E. & CARRASCO, M. A. (1987). Inositol 1,4,5,-trisphosphate-induced Ca^{2+} release from the sarcoplasmic reticulum and contraction in crustacean muscle. *Canadian Journal of Physiology and Pharmacology* **65**, 672–680.

SCHULMAN, H. & LOU, L. L. (1989). Multifunctional Ca^{2+}/calmodulin-dependent protein kinase: domain structure and regulation. *Trends in Biochemical Sciences* **14**, 62–67.

SCHWARTZ, T. L., HARRIS-WARRICK, R. M., GLUSMAN, S. & KRAVITZ, E. A. (1980). A peptide action in a lobster neuromuscular preparation. *Journal of Neurobiology* **11**, 623–628.

STEWART, A. A., INGEBRETSEN, T. S., MANALAN, A., KLEE, C. B. & COHEN, P. (1982). Discovery of a Ca^{2+} and calmodulin-dependent protein phosphatase. Probable identity with calcineurin (CaM BP80). *FEBS Letters* **137**, 80–84.

VERGARA, J., TSIEN, R. Y. & DELAY, M. (1985). Inositol 1,4,5-trisphosphate: a possible chemical

link in excitation–contraction coupling in muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 6352–6356.

WATSON, W. H. & HOSHI, T. (1985). Proctolin induces rhythmic contractions and spikes in *Limulus* heart muscle. *American Journal of Physiology* **249**, R490–495.

WOODS, N. M., CUTHBERTSON, K. S. R. & COBBOLD, P. H. (1987). Agonist-induced oscillations in cytoplasmic free calcium concentration in single rat hepatocytes. *Cell Calcium* **8**, 79–100.

YOSHIOKA, T. & SOMLYO, A. P. (1984). The calcium and magnesium contents and volume of the terminal cisternae in caffeine-treated skeletal muscle. *Journal of Cell Biology* **99**, 558–568.