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A STUDY OF THE OUABAIN-INSENSITIVE SODIUM EFFLUX IN BARNACLE MUSCLE FIBRES USING PHORBOL DIBUTYRATE AS A PROBE

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SUMMARY

1. The resting ouabain-insensitive Na^+ efflux in muscle fibres isolated from the barnacle, *Balanus nubilus*, is stimulated by external or internal application of phorbol 12,13-dibutyrate (PD). The response occurs fairly promptly and may not decay at all, or more commonly, decay rather slowly. The magnitude of the response to external or internal application of PD is dose-dependent, the minimum effective concentration being about 10^{-8} M.

2. The response to PD fails to occur in the nominal absence of external Ca^{2+}. Sudden removal of external Ca subsequent to peak stimulation by PD leads to almost complete reversal of the response. The response to PD of fibres suspended in Li^+-ASW (artificial sea water) is similar in magnitude to that of fibres suspended in Na^+-ASW. However, it differs in that it is of a sustained nature.

3. Calcium channel blockers, e.g. verapamil, completely prevent the response to PD from occurring. Both Cd^{2+} and Co^{2+} are less effective than verapamil.

4. Pre- but not post-injection of EGTA reduces the response to PD. Pre- or post-injection of Mg^{2+} reduces the response considerably.

5. Fibres pre-injected with GTP show a reduced response to PD. Fibres pre-injected with PD show a reduced response to GTP. Pre-injection of protein kinase inhibitor is without effect on the response to PD.

6. Furosemide, piretanide and bumetanide are without effect on the response to PD.

7. DIDS (4,4′-diisothiocyanostilbene-2,2-disulphonic acid) is a potent inhibitor of the response to PD but not amiloride. Pyridoxal 5-phosphate and benzolamide are also powerful inhibitors. Pyridoxal 5-phosphate in combination with benzolamide fails to completely abolish or reverse the response to PD.

8. Luminescence from aequorin is promptly increased by PD in a dose-dependent manner, the minimal effective concentration being in the nanomolar range. The signal is monophasic or multiphasic in shape, and is often less than 5 min in duration. Not infrequently, however, the aequorin response fails to completely decay and the new level of resting glow remains above the original baseline level.

9. Collectively, these observations accord with a tentative general hypothesis stating that the stimulatory response of the ouabain-insensitive Na^+ efflux to PD is triggered by two mechanisms. One involves a rise in myoplasmic free [Ca^{2+}] resulting

from the entry of external Ca^{2+} via opened Ca^{2+} channels which is followed by the operation of the Na^+–Ca^{2+} exchanger in the reverse mode. The other involves stimulation of the Na^+/HCO_3^-–Cl^-/H^+ exchanger, presumably as the result of phosphorylation and/or an internal acidosis brought about by a sufficient rise in myoplasmic free [Ca^{2+}].

INTRODUCTION

Studies of the Na^+ efflux in barnacle muscle fibres suspended in 10 mM-Mg^{2+}-artificial sea water (ASW) show that it can be partitioned operationally into several distinct phases (see Bittar, 1983): (1) a phase reflecting the properties of the Na^+–K^+-ATPase, a system which is specifically inhibited by the cardiac glycoside, ouabain; (2) rather minor phase involving Na^+–Na^+ exchange diffusion which is identifiable by employing Li^+ as a substitute for external Na^+; (3) a phase which is modulated by external pH and HCO_3^-, as well as the P_{CO_2} (this phase is completely abolished by the carbonic anhydrase inhibitor, benzolamide); (4) a phase which is ouabain-insensitive and largely abolished by reducing the environmental temperature to 0 °C. Furthermore, by employing ouabain in a maximally effective concentration, viz. 10^{-4} M, to inactivate the membrane Na^+–K^+-ATPase system, it has been possible to specify those components of the ouabain-insensitive Na^+ efflux that are mediated by cyclic AMP-dependent protein kinase, and *putative* Ca^{2+}–calmodulin-dependent protein kinase. More recently, attempts have been made to address the question of whether Ca^{2+}–phospholipid-dependent protein kinase C plays a role in regulating the ouabain-insensitive Na^+ efflux. This possibility has been explored in particular detail by using the tumour-promoting phorbol ester, phorbol 12,13-dibutyrate (PD), which is a specific activator of Ca^{2+}–phospholipid-dependent protein kinase C (Castagna, Takei, Kaibuchi, Sano, Kikkawa & Nishizuka, 1982), and known to be more potent than diglycerides as a protein kinase C activator, and not to be as readily metabolized. The following communication describes this work which demonstrates that PD at nanomolar concentrations stimulates the ouabain-insensitive Na^+ efflux from these large muscle fibres, and increases phasically the myoplasmic free Ca^{2+} concentration by stimulating Ca^{2+} influx through verapamil-sensitive surface membrane Ca^{2+} channels. The requirement for external Ca^{2+} is found to be absolute. The study also shows that DIDS, pyridoxal 5-phosphate and benzolamide in addition to internally applied Mg^{2+} are potent inhibitors of the response to PD. Fitting the data together, the results support the view that the activation of Ca^{2+}–phospholipid-dependent protein kinase C is also associated with the stimulation of the Na^+–Ca^{2+} and Na^+/HCO_3^-–Cl^-/H^+ exchangers.

A preliminary report of this work has appeared elsewhere (Bittar & Nwoga, 1989*a*).

METHODS

The species of barnacles, the methods of dissection, cannulation, microinjection and counting of $^{22}Na^+$ activity in the effluent and in the fibre, as well as the measurement of the membrane potential, were essentially the same as those described previously (Bittar & Nwoga, 1989*b*). Solutions with varying concentrations of Ca^{2+} were prepared by raising or reducing NaCl in osmotically equivalent amounts. This was also the case when preparing 50 mM-Mg^{2+}-ASW. In those experiments where the medium was Li$^+$-ASW, its composition was as follows (mM): LiCl, 475; CaCl$_2$, 10; MgCl$_2$, 10; KHCO$_3$, 10 and pH 7·8. To prepare solutions of furosemide, piretanide and bumetanide in millimolar concentrations, methanol was used as the solvent and its final concentration in ASW was 1%. Changes in myoplasmic free Ca^{2+} were monitored with the Ca^{2+}-sensitive photoprotein, aequorin, as described by Ashley & Ridgway (1970) in this preparation and by Bittar & Nwoga (1989*b*). All experiments were performed at room temperature (between 22 and 24 °C).

The results presented in this paper are means ± S.E. of mean and significance levels were computed by using Student's *t* test. Estimates of the size of the observed effects on the ouabain-insensitive Na$^+$ efflux were computed on the basis of the rate constants for ^{22}Na$^+$ efflux. In the situation where two stimulatory phases were present, the size of the second response was computed by taking the difference between the two combined phases and the first phase. A similar method of computation was applied in the situation where two inhibitory responses were present.

All reagents used were of analytical grade. Phorbol 12,13-dibutyrate (PD), 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), 4α-phorbol 12,13-didecanoate, ouabain, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) and ethyleneglycol-bis(β-aminoethylether)*N*,*N*′-tetraacetic acid (EGTA) were purchased from Sigma Chemical Company, St Louis, MO, USA. 4α-Phorbol 12,13-dibutyrate was purchased from LC Services Corporation, Woburn, MA, USA. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) was purchased from K and K Laboratories, Inc., Plainview, NY, USA. Verapamil hydrochloride was obtained from Knoll Pharmaceutical Company, Whippany, NJ, USA. Dimethylsulphoxide (DMSO) was purchased from Fisher Scientific Co., Fair Lawn, NJ, USA. Benzolamide was a gift from Dr T. Maren of the Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL, USA. Amiloride hydrochloride was obtained from the Merck Institute for Therapeutic Research, West Point, PA, USA. Both furosemide and piretamide were supplied by Hoechst–Roussel Pharmaceuticals Inc., Somerville, NJ, USA, and bumetanide by Dr P. W. Feit, Leo Pharmaceutical Products, Ballerup, Denmark. Aequorin (> 95% purity) was purchased from the Mayo Foundation, Rochester, MN, USA. Pure protein kinase inhibitor (PKI) was obtained as a gift from Dr E. Fischer of the Department of Biochemistry, University of Washington, Seattle, WA, USA.

RESULTS

For microinjection of phorbol ester 1% DMSO was used as the vehicle. Control experiments involving the injection of 1% DMSO into unpoisoned and ouabain-poisoned fibres twice in succession showed that this had little or no effect on the Na$^+$ efflux. Initially, it was necessary to determine the efficacy of several phorbol esters, notably 12-*O*-tetradacanoyl-phorbol-13-acetate (TPA) and phorbol 12,13-dibutyrate (PD). PD was found to be considerably more effective than TPA. The inactive isomers, 4α-pborbol 12,13-didecanoate and 4α-phorbol 12,13-dibutyrate, were completely ineffective. For example, injection of the latter isomer in a concentration of 10^{-3} M into five fibres pre-treated with 10^{-4} M-ouabain was ineffective, whereas injection of 10^{-3} M-PD caused stimulation of the ouabain-insensitive Na$^+$ efflux in the order of 454 ± 89% ($n = 6$).

Effect of phorbol 12,13-dibutyrate

External application of PD leads to stimulation of the ouabain-insensitive Na$^+$ efflux. This response either fails to decay, or more commonly decays rather slowly, as illustrated in Fig. 1*A*. In this instance, the response to 10^{-5} M-PD reaches a peak very slowly. Alternatively, the response may decay rapidly, but only for a while, as illustrated in Fig. 1*B*, or it may decay quite rapidly. As a general rule, ouabain-poisoned fibres injected with 10^{-2} or 10^{-3} M-PD always respond. This occurs promptly or within about 10 min of injection. A rapid onset of the response correlates with the phasic aequorin signal described on p. 277. The magnitude of the response in fibres isolated from the same barnacle, and injected with 10^{-2} M-PD, averages $617 \pm 82\%$ ($n = 4$), while that resulting from external application of 10^{-5} M-PD averages $579 \pm 128\%$ ($n = 4$). Another basic feature is that these fibres contract promptly on injection of 10^{-2} M-PD, or do so gradually within 10 min of external treatment with 10^{-5} M-PD.

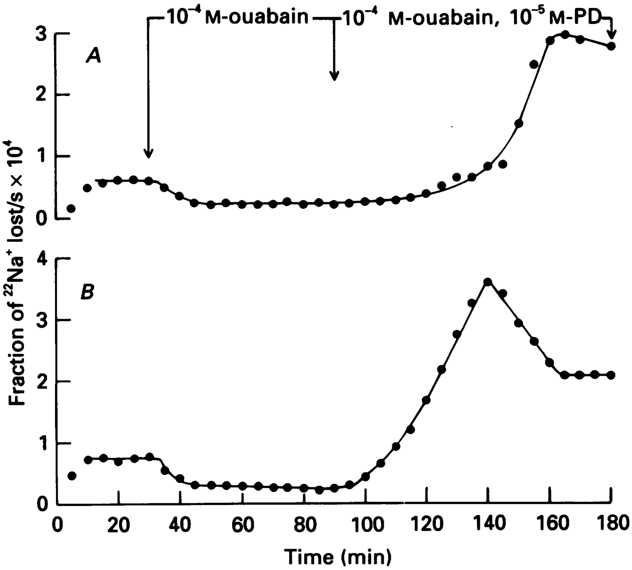

Fig. 1. Kinetics of the stimulatory response of the ouabain-insensitive Na^+ efflux to external application of 10^{-5} M-PD.

Concentration–response curves

Summarized in Fig. 2*A* and *B* are the results obtained by internal and external application of PD in varying concentrations. In the former case, fibres poisoned with 10^{-4} M-ouabain are shown to be sensitive to as little as 10^{-8} M-PD, assuming 100-fold dilution by the myoplasm. In the latter case, the minimum effective concentration of PD is also about 10^{-8} M.

The response as a function of external Ca^{2+}

The requirement for external Ca^{2+} is absolute. Injection or external application of PD in the nominal absence of external Ca^{2+} is ineffective. When, however, external Ca^{2+} is restored in the presence of 10^{-5} M-PD, a sharp step-up in the ouabain-insensitive Na^+ efflux averaging $864 \pm 99\%$ (as compared with $957 \pm 92\%$ in controls, $n = 5$) is observed. Identical results were obtained when 10^{-2} M-PD was injected (i.e. $755 \pm 275\%$ stimulation upon restoring external Ca^{2+}, $n = 5$ *vs.* $542 \pm 74\%$ in controls, $n = 5$, $P > 0{\cdot}4$). In a third series, external Ca^{2+} was suddenly omitted following the onset of peak stimulation by the injection of 10^{-2} M-PD. Characteristically, a prompt and appreciable decline occurs which, as illustrated

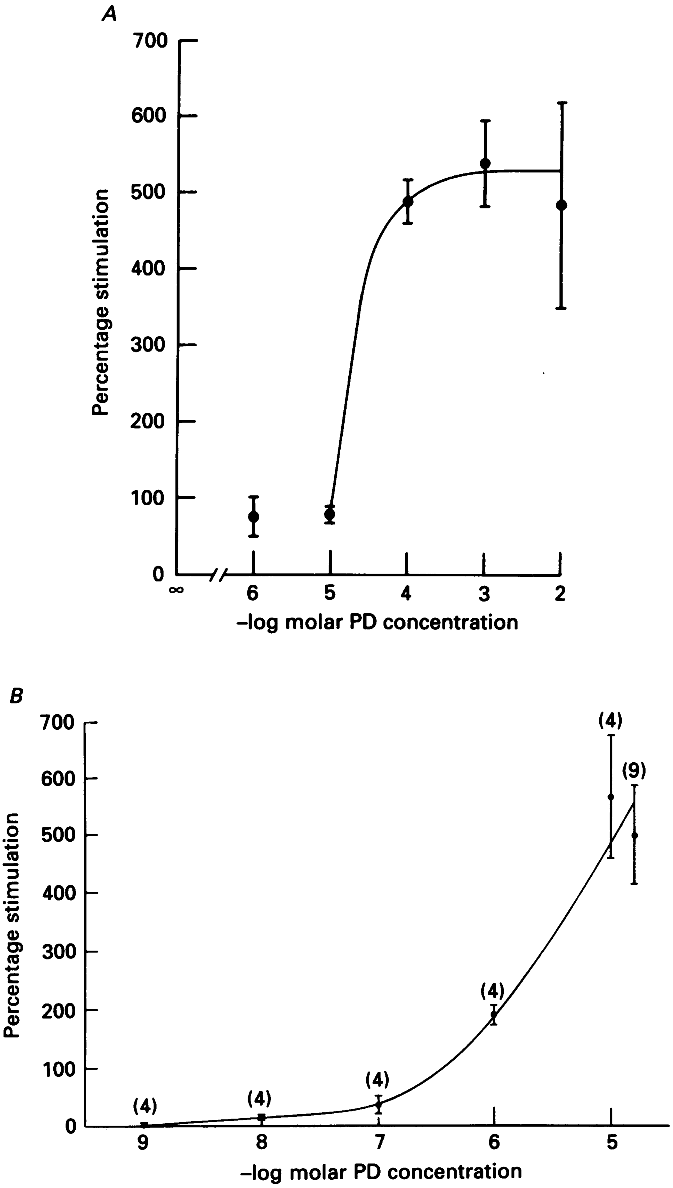

Fig. 2. *A*, concentration–response curve for the stimulatory effect of injected PD on the ouabain-insensitive Na^+ efflux. Each plotted point is the mean value of three measurements. Vertical bars indicate ±S.E.M. The fibres used were isolated from one barnacle specimen. *B*, concentration–response curve for the stimulatory effect of external application of PD on the ouabain-insensitive Na^+ efflux. The number of measurements made is given in parentheses. Each point and its bar represent average and S.E.M. The fibres used were isolated from two barnacle specimens of the same batch.

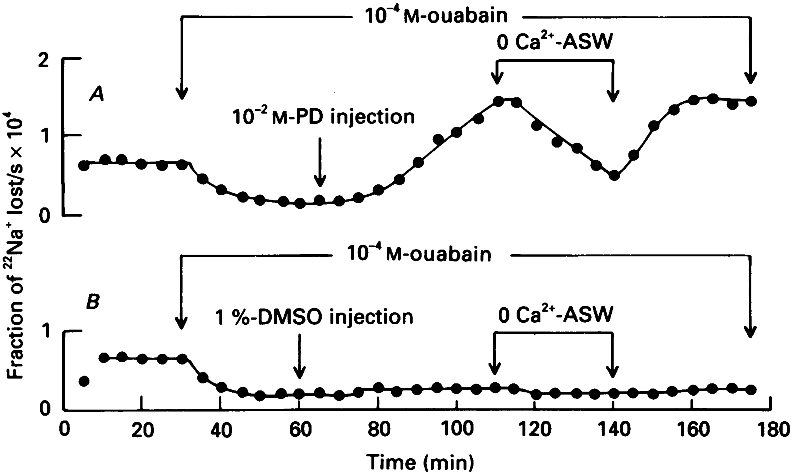

Fig. 3. *A*, the effect of sudden omission of external Ca^{2+} 50 min following the onset of peak stimulation by injecting 10^{-2} M-PD and its complete reversal by restoring external Ca^{2+} at $t = 140$ min. *B*, a companion control fibre.

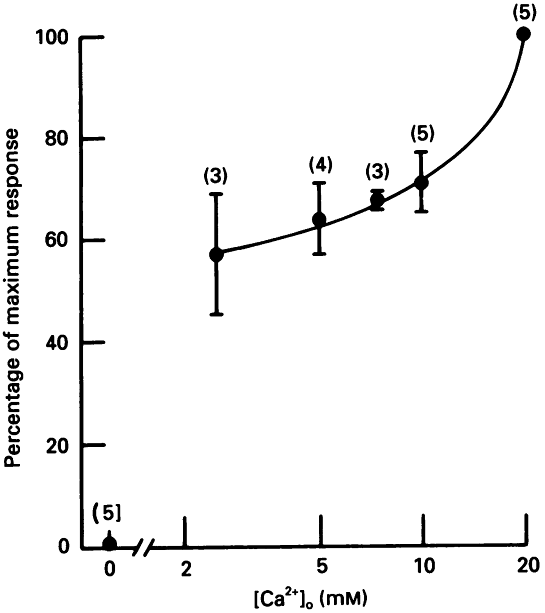

Fig. 4. The dependence on external Ca^{2+} of the response of the ouabain-insensitive Na^+ efflux to external application of 10^{-5} M-PD. The number of measurements made is indicated in parentheses. Each point and its bar represent average and S.E.M. The fibres used were isolated from the same barnacle specimen.

in Fig. 3, is reversed by restoring external Ca^{2+} ($n = 5$). The same is true of external application of 10^{-5} M-PD ($n = 5$). Shown in Fig. 4 is the external Ca^{2+} concentration dependence curve determined in the presence of 10^{-5} M-PD externally. Collectively, these results provide conclusive evidence that the response to PD is dependent on the external Ca^{2+} concentration, and that it does not occur in the nominal absence of external Ca^{2+}.

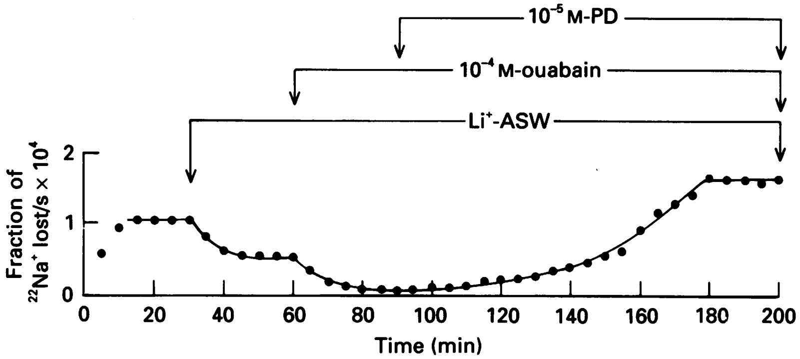

Fig. 5. The response of the ouabain-insensitive Na^+ efflux into Li^+-ASW following external application of 10^{-5} M-PD. Notice that the response is preceded by a 10 min latent period and that it develops slowly and is of a sustained nature.

The response in the presence of external Li^+

One way of elucidating the mechanism underlying the response to PD is to find out whether it depends on the presence of external Na^+ and the Na^+ gradient across the fibre membrane. Thus, Li^+ was used as a substitute for external Na^+. It is apparent from Fig. 5 that the ouabain-insensitive Na^+ efflux into 475 mM-Li^+-ASW rises steadily following external application of 10^{-5} M-PD and about 90 min later the response is of a sustained nature. This averages 989 ± 170 % in size ($n = 5$), which is not different from 965 ± 88 % obtained in control fibres ($n = 5$). These results support the concept that Na^+–Na^+ exchange is not involved in the response to PD and that reversal of the Na^+ gradient further promotes the operation of the Na^+–Ca^{2+} exchanger in the reverse mode. Though such data do not rule in or out a role for Na^+–Li^+ exchange (Allen & Hinke, 1971), Na^+–Na^+ exchange is thought to play a minor role in barnacle muscle fibres (Bittar, 1983). One of the best pieces of evidence in support of this view is that showing a fall in intracellular Na^+ when external Na^+ is omitted (Menard & Hinke, 1981).

Since the validity of the interpretation that PD stimulates Na^+–Ca^{2+} exchange in the reverse mode partly depends on demonstrating that a larger response is seen when the external Ca^{2+} concentration is raised, the above type of experiment was repeated using 20 mM-Ca^{2+}–Li^+-ASW. These experiments yielded the following results: the response to the injection of 10^{-3} M-PD into four test fibres averages 426 ± 117 %, as compared with 108 ± 36 % in four companion control fibres. The difference is significant ($P < 0.05$).

Response in the presence of 50 mM-Mg^{2+}

The standard ASW used in this laboratory contains 10 mM-Mg^{2+} which is roughly one-fifth the concentration of Mg^{2+} in Pacific ocean waters. To determine whether the effect of PD is influenced by a raised external Mg^{2+} concentration, and whether there is competition between external Ca^{2+} and Mg^{2+}, ouabain-poisoned fibres were suspended in 50 mM-Mg^{2+}-ASW and then exposed to 10^{-5} M-PD. The magnitude of the observed response averages 1050 ± 171 % ($n = 4$), a value not significantly different from the 871 ± 242 % observed in controls ($n = 4$, $P > 0{\cdot}3$). In a second series, fibres pre-treated with 10^{-4} M-ouabain were injected with 10^{-3} M-PD and after the onset of peak stimulation they were suspended in 50 mM-Mg^{2+}-ASW. This had no effect ($n = 4$). In a parallel series of experiments, fibres suspended in 50 mM-Mg^{2+}-ASW were injected with 10^{-3} M-PD. The response obtained averages 382 ± 99 % ($n = 3$), a value practically the same as that found in controls, viz. 314 ± 54 % ($n = 4$).

The effect of Ca^{2+} channel blockers

Verapamil completely abolishes stimulation of the ouabain-insensitive Na^+ efflux by 100 mM-K^+ (Mason-Sharp & Bittar, 1981). This is also the case when 10^{-4} M-verapamil is applied externally prior to 10^{-5} M-PD ($n = 5$). Repetition of this type of experiment led to similar results (20 ± 13 % stimulation, $n = 3$ *vs.* 616 ± 71 % stimulation in controls, $n = 5$, $P < 0{\cdot}001$). Verapamil is also found to completely abolish the response to 10^{-2} M-PD injection ($n = 3$). Collectively, such results give support to the view that the mechanism by which PD stimulates the ouabain-insensitive Na^+ efflux involves activation of Ca^{2+} channels. However, they do not exclude Ca^{2+} release channels of the sarcoplasmic reticulum since these channels are also vulnerable to blockage by verapamil (Valdivia & Coronado, 1989).

Cadmium, a known Ca^{2+} channel blocker (e.g. Stefani & Chiarandini, 1982), is able, when applied in a concentration of 2 mM, to completely block the stimulatory response of the ouabain-insensitive Na^+ efflux to proctolin (Nwoga & Bittar, 1985). The results obtained with 2 mM-Cd^{2+} were as follows: 328 ± 76 %, $n = 4$ *vs.* 616 ± 71 % in controls, $n = 5$ ($P < 0{\cdot}05$), and on repetition: 111 ± 41 %, $n = 4$ *vs.* 213 ± 6 % in controls, $n = 4$ ($P < 0{\cdot}05$).

Cobalt is also a known Ca^{2+} channel blocker (Stefani & Chiarandini, 1982), which when applied in a concentration of 10 mM completely abolishes the response of barnacle fibres to proctolin (Nwoga & Bittar, 1985). The results obtained here show that Co^{2+} fails to completely abolish the response to 10^{-5} M-PD (119 ± 9 %, stimulation, $n = 3$ *vs.* 213 ± 6 % in controls, $P < 0{\cdot}05$); and upon repetition: 327 ± 19 %, $n = 4$ *vs.* 616 ± 71 % in controls, $n = 5$ ($P < 0{\cdot}01$).

Effect of pre-injecting EGTA

To further substantiate the view that the response of the ouabain-insensitive Na^+ efflux depends on myoplasmic free Ca^{2+} concentration, 250 mM-EGTA at pH 7·2 was injected along the entire length of ouabain-poisoned fibres 30 min before external application of 10^{-5} M-PD. The magnitude of the observed response averages 399 ± 40 %, $n = 4$ *vs.* 736 ± 18 % in controls, $n = 4$ ($P < 0{\cdot}01$). However, the injection of 250 mM-EGTA 40 min following the onset of peak stimulation by 10^{-5} M-PD was without effect ($n = 4$). This is an intriguing finding because it suggests two possibilities. First, that an increase in free $[Ca^{2+}]_i$ is an early event; and second, that stimulation by PD of the ouabain-insensitive Na^+ has a substantial component which is independent of $[Ca^{2+}]_i$ changes.

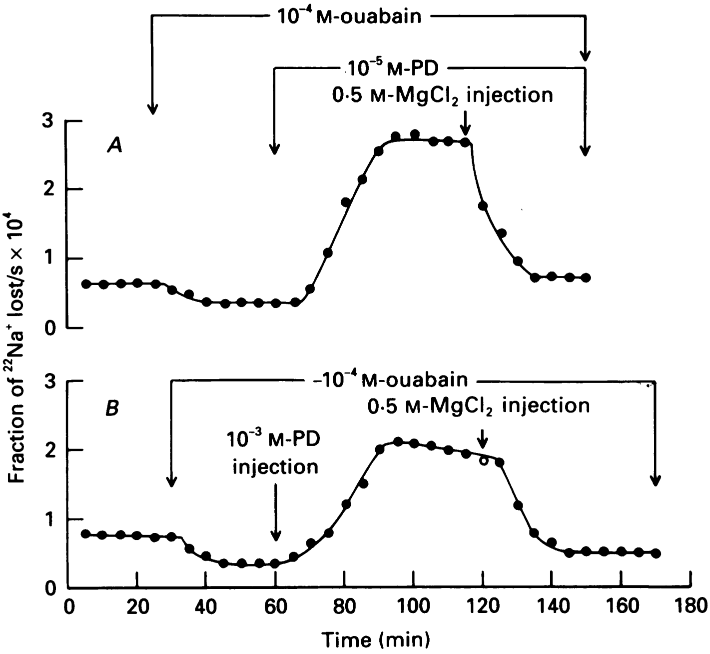

Fig. 6. Almost complete reversal by injecting 0·5 M-$MgCl_2$ of the response to: *A*, external application of 10^{-5} M-PD; and *B*, internal application of 10^{-3} M-PD.

Reversal of the response by injecting $MgCl_2$

There is ample evidence that Ca^{2+} release by the sarcoplasmic reticulum, e.g. in rabbit skeletal muscle, is mediated by Ca^{2+} channels that can be blocked by Mg^{2+}, e.g. in SR vesicles, as well as SR Ca^{2+} channels in lipid bilayers (Smith, Coronado & Meissner, 1985), and that these release channels are controlled by the T-tubule membrane Ca^{2+} channels, which behave as voltage-sensors (Rios & Brum, 1987). The results of experiments indicate that the responses to 10^{-5} M-PD are significantly reduced by pre-injecting 0·5 M-$MgCl_2$ (in a 3 mM-HEPES solution, pH 7·2): 881 ± 53 %, $n = 4$ *vs.* 1408 ± 126 % in controls, $n = 8$, $P < 0·02$. They also indicate a 74 ± 4 % reversal of the response to 10^{-5} M-PD by injecting 0·5 M-$MgCl_2$ 25 min after peak stimulation, as shown in Fig. 6*A* ($n = 3$). Repetition indicates 75 ± 4 % reversal ($n = 5$). The same is true when 0·5 M-$MgCl_2$ is injected 1 h after injecting 10^{-3} M-PD, as shown in Fig. 6*B* (84 ± 4 % reversal, $n = 4$). Taken together, these results not only parallel those showing that pre-injection of 0·5 M-$MgCl_2$ practically abolishes the response of the ouabain-insensitive Na^+ efflux to 100 mM-K^+ (Bittar & Nwoga, 1982*b*) but also raise the possibility that the release channel of the SR may be regulated by myoplasmic pMg, as well as by protein kinase C.

Response of fibres pre-enriched with $GTPNa_2$

The next step was to test the validity of the idea that a reciprocal relationship exists between the Ca^{2+}–phospholipid-dependent protein kinase C and the membrane adenylate cyclase systems (Anderson, Estival, Taptovaara & Gopalakrishna, 1985), or that the relationship is bi-directional as for example in platelet membranes (Katada, Gilman, Watanabe, Bauer & Jakobs, 1985). Since the injection of guanine nucleotide into barnacle fibres is associated with an appreciable rise in internal cyclic AMP (Baker & Carruthers, 1983), and stimulation of the ouabain-insensitive Na^+ efflux (Bittar & Nwoga, 1982*a*), it seemed worthwhile to inject 0·5 M-$GTPNa_2$ before and after the external application of 10^{-5} M-PD. The results were as follows: the response to PD of fibres injected with $GTPNa_2$ beforehand averages 514 ± 127% ($n = 4$), as compared with 1279 ± 290% in controls ($n = 4$, $P > 0{\cdot}05$). Interestingly, the stimulatory response of the test fibres failed to decay. Additional experiments were carried out but this time ouabain-poisoned fibres were injected with 10^{-2} M-PD before and after injecting 0·5 M-$GTPNa_2$. The results obtained show: (i) the response to GTP after PD averages 132 ± 24% ($n = 4$), as compared with a response to GTP before PD which averages 285 ± 38% ($n = 8$, $P < 0{\cdot}05$), and (ii) the response to PD after GTP averages 61 ± 41% ($n = 4$), as compared with a response to PD before GTP which averages 643 ± 76% ($n = 8$, $P < 0{\cdot}01$).

Lack of effect of protein kinase inhibitor

Fibres pre-treated with 10^{-4} M-ouabain were injected with 5×10^{-5} M-PKI, and then exposed to 10^{-5} M-PD, while companion controls were pre-injected with a 3 mM-HEPES solution. The results show no significant difference between the response of test (355 ± 91%, $n = 4$) and control fibres (546 ± 135% $n = 4$, $P > 0{\cdot}3$).

Lack of effect of amiloride

The activation of protein kinase C by phorbol esters often leads to stimulation of Na^+–H^+ exchange in a wide variety of vertebrate cells (Grinstein & Rothstein, 1986), and this exchange mechanism is competitively inhibited by the pyrazine diuretic, amiloride, and several of its analogues. However, the results of experiments show that the response to 10^{-5} M-PD in the presence of 10^{-3} M-amiloride is not significantly different from that in the absence of amiloride (900 ± 161%, $n = 8$ *vs.* 1210 ± 97%, $n = 10$) and that 10^{-3} M-amiloride is also ineffective when it is applied after peak stimulation by PD ($n = 10$).

Inhibition by DIDS

DIDS (4,4′-diisothiocyanostilbene-2,2-disulphonic acid) is an inhibitor of the anion exchange process, e.g. in red blood cells (Cabantchik & Rothstein, 1974), as well as an inhibitor of acid extrusion in barnacle muscle fibres (Boron, 1977). Furthermore, DIDS or SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid) are able to inhibit Cl^- efflux in barnacle muscle fibres (Ashley, Ellory, Lea & Ramos, 1978; Russell & Brodwick, 1979; Bittar, Schultz & Tesar, 1980). The results of experiments show that the response of the ouabain-insensitive Na^+ efflux to external application of 10^{-5} M-PD is drastically reduced when 10^{-4} M-DIDS is applied beforehand ($337 \pm 37\%$, $n = 7$ *vs.* $829 \pm 90\%$ in controls, $n = 16$, $P < 0{\cdot}01$). A representative experiment is shown in Fig. 7*A*. Notice that DIDS does not disturb the ouabain-insensitive Na^+ efflux and that the onset of the PD effect is not prompt. Such a result suggests the possibility that the Na^+/HCO_3^-–Cl^-/H^+ exchanger plays a primary role in the response of the Na^+ efflux to PD.

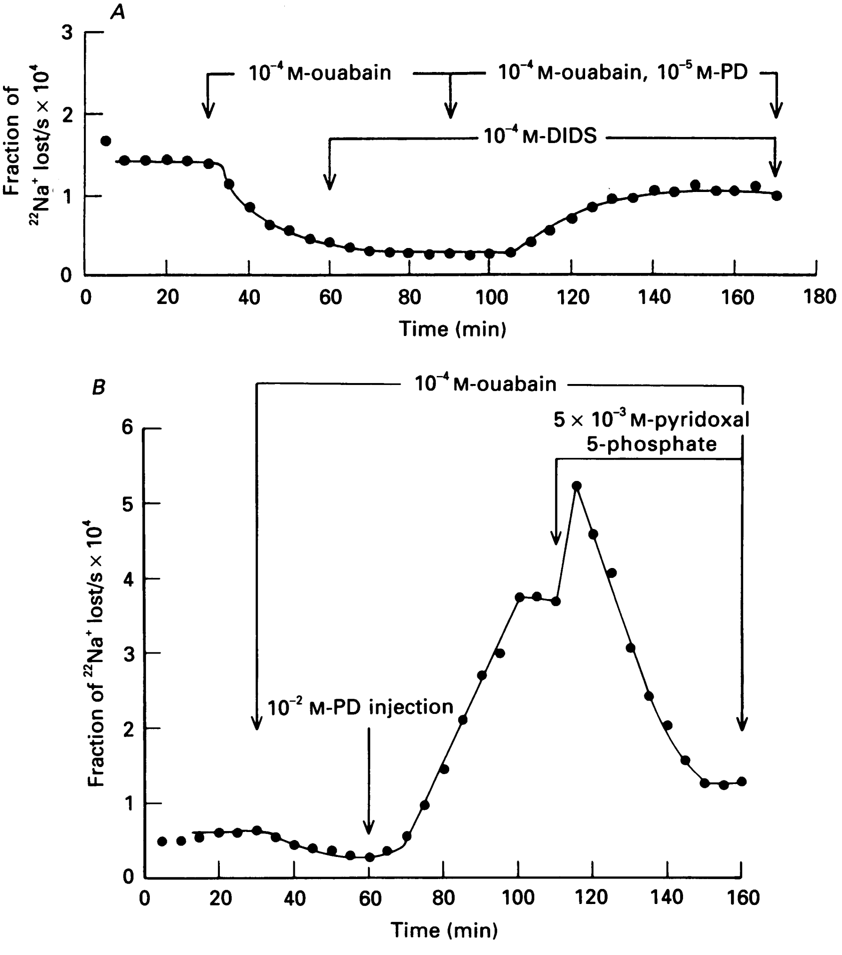

Fig. 7. *A*, the suppressing effect of external application of 10^{-4} M-DIDS on the response of the ouabain-insensitive Na^+ efflux to external application of 10^{-5} M-PD. *B*, biphasic effect of external application of 5×10^{-3} M-pyridoxal 5-phosphate on the response to the injection of 10^{-2} M-PD.

Inhibition by pyridoxal 5-phosphate

Boron (1977) produced compelling evidence that the amino-reactive agent, pyridoxal 5-phosphate (Cabantchik, Balshin, Breuer & Rothstein, 1975), is a potent inhibitor of the acid-extrusion system in barnacle muscle fibres. Experiments were therefore performed in which 5×10^{-3} M-pyridoxal 5-phosphate was applied before and after the injection of 10^{-2} M-PD. The results obtained show: (i) a stimulatory response to PD averaging $137 \pm 19\%$, $n = 5$ *vs.* $1124 \pm 82\%$ in controls, $n = 5$ ($P <$ $0{\cdot}001$) and (ii) a $58 \pm 3\%$ reversal of the response ($n = 5$). As illustrated in Fig. 7, the effect of pyridoxal 5-phosphate is biphasic: a transitory stimulation is followed by a sustained plateau phase. It is noteworthy that pyridoxal 5-phosphate is without effect on the resting ouabain-insensitive Na^+ efflux ($n = 5$).

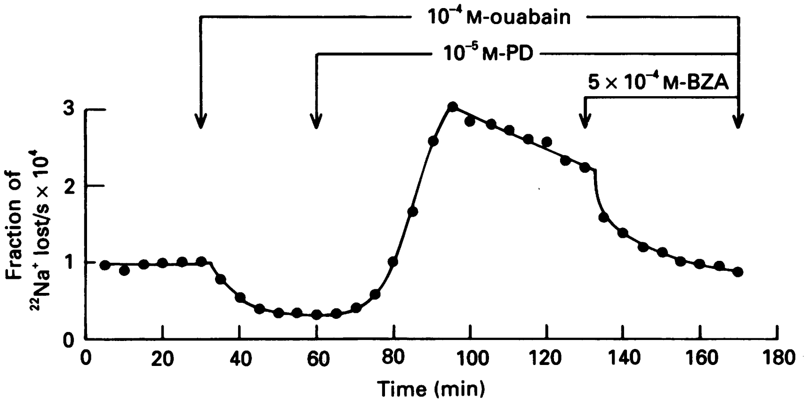

Fig. 8. Partial reversal by external application of 5×10^{-4} M-BZA following peak stimulation by external application of 10^{-5} M-PD.

Inhibition by benzolamide

Benzolamide (BZA), a powerful inhibitor of carbonic anhydrase (Maren, 1977), has the ability of reversing the stimulatory response of the ouabain-insensitive Na^+ efflux, and the Cl^- efflux to external acidification (Schultz & Bittar, 1978; Bittar *et al.* 1980; Chambers & Bittar, 1981). The results obtained were as follows: (i) $491 \pm 53\%$ stimulation is caused by 10^{-5} M-PD in the presence of 5×10^{-4} M-BZA, $n = 8$ *vs.* $829 \pm 90\%$ in controls, $n = 16$ ($P < 0{\cdot}02$). It is also noteworthy that BZA does not alter the resting ouabain-insensitive Na^+ efflux ($n = 8$). (ii) There is $44 \pm 5\%$ reversal by 5 ± 10^{-4} M-BZA when it is applied externally, after the onset of the peak response to PD, as shown in Fig. 8 ($n = 4$). (iii) In view of these results, a concentration–response curve for the inhibitory effect of BZA on the response to the injection of 5×10^{-3} M-PD was determined. This is presented in Fig. 9 where it can be seen that 10^{-3} M-BZA reduces the response to PD by almost 70%. It will be recalled that half this concentration of BZA is enough to practically abolish the response to external acidification (Schultz & Bittar, 1978). (iv) External application of 5×10^{-4} M-BZA following the onset of peak stimulation by 10^{-5} M-PD causes a $44 \pm 5\%$ reversal of the response ($n = 4$).

The present additional data indicating that the response to PD is drastically reduced not only by pyridoxal phosphate but also by BZA raised the question as to whether a larger effect would be obtained if the two were used together. The results of experiments were as follows: (i) The response to 10^{-2} M-PD injection in the presence of 5×10^{-3} M-pyridoxal 5-phosphate and 5×10^{-4} M-BZA averages $363 \pm 77\%$, $n = 5$ *vs.* $720 \pm 38\%$ in companion controls ($P < 0{\cdot}01$). (ii) External application of 5×10^{-3} M-pyridoxal 5-phosphate and 5×10^{-4} M-BZA following peak stimulation by injecting 10^{-2} M-PD leads to $69 \pm 2\%$ reversal of the response but the difference between the size of this residual response and the response to PD in the presence of both inhibitors is not significant (218 ± 11% *vs.* 363 ± 77%, $P > 0{\cdot}05$).

As shown earlier, the ouabain-insensitive Na^+ efflux into 475 mM-Li^+-ASW is stimulated by PD and this response is of a sustained nature. Hence the question asked was: Would BZA be able to reverse this response? The results of experiments show that 10^{-3} M-BZA promptly curtails the response to the injection of 10^{-2} M-PD by 76 ± 7% ($n = 5$), and the residual ouabain-insensitive Na^+ efflux is of a sustained nature.

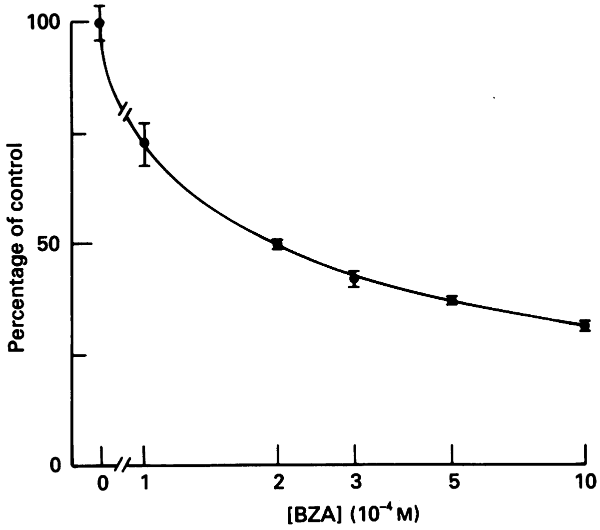

Fig. 9. Concentration–response curve for the inhibiting action of BZA on the response of the ouabain-insensitive Na^+ efflux to the injection of 5×10^{-3} M-PD. Each plotted point is the mean of three determinations. Vertical bars indicate ±S.E.M. The fibres used were isolated from the same barnacle specimen.

Lack of effect of furosemide, piretanide and bumetanide

It is generally recognized that a wide variety of cells, including non-epithelial cells, e.g. cultured chick heart cells (Frelin, Chassande & Lazdunski, 1986) and squid axon (Russell, 1983), possess an electroneutral Na^+–K–Cl^- co-transport system, which is inhibited by the loop diuretics furosemide, piretanide, bumetanide and benzmetanide, and unaffected by amiloride, SITS or DIDS (Geck & Heinz, 1986; O'Grady, Palfrey & Field, 1987). Initially, experiments were performed using 5×10^{-5} M-furosemide and 5×10^{-5} M-piretanide. Because the results obtained were negative, the study was repeated but this time 10^{-4} M-bumetanide was included and the concentration of both furosemide and piretanide was increased to 10^{-3} M. Again, the results were negative and 1 % methanol was also without effect (control).

Measurement of the membrane potential before and after phorbol 12,13-dibutyrate

A rather large number of measurements were made of the membrane potential in ouabain-poisoned fibres in the absence and presence of 10^{-6} and 10^{-5} M-PD. However, no significant changes were recorded.

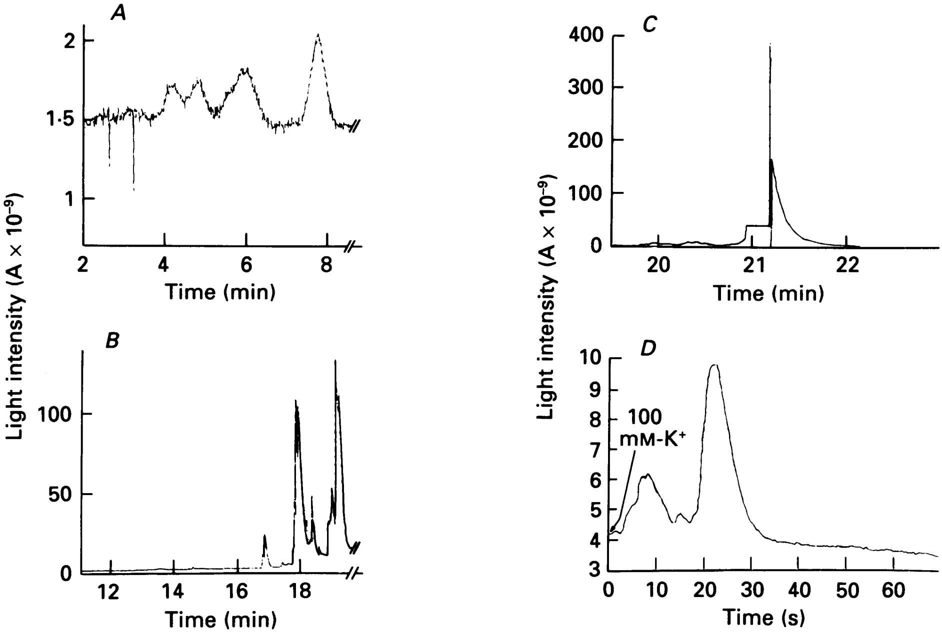

Fig. 10. Computer printouts of aequorin signals obtained following external application of 10^{-5} M-PD. *A*, a slight rise in light intensity after $t = 2$ min is followed at about $t =$ 4 min by a burst of repetitive Ca^{2+} transients. *B*, this is repeated shortly after $t = 16$ min. *C*, this is repeated after $t = 20$ min (off-scale). *D*, $[K^+]_0$ was raised to 100 mM at arrow. Note that the time bases are not the same. Ordinate light intensity expressed in A × 10^{-9}.

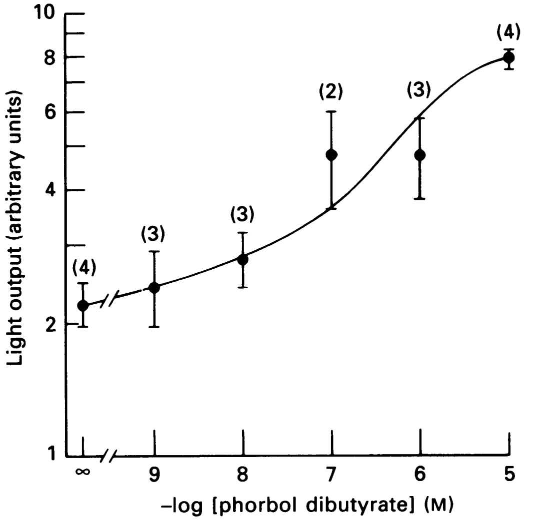

Fig. 11. Concentration–response curve for the stimulating effect of external application of PD on luminescence from aequorin. The number of determinations carried out is given in parentheses. Vertical bars indicate ±S.E.M. The fibres used were isolated from two barnacle specimens.

Aequorin luminescence before and after phorbol 12,13-dibutyrate

The response of ouabain-poisoned fibres pre-loaded with aequorin to PD is monophasic or multiphasic. This is true of fibres isolated from *Balanus nubilus* specimens collected in Monterey Bay (California) and Puget Sound (Washington) waters. The duration of a multiphasic response to external application of 10^{-5} M-PD is usually rather brief (e.g. $3{\cdot}6 \pm 0{\cdot}5$ min, $n = 6$). In the example given in Fig. 10 of a Washington fibre, 10^{-5} M-PD was applied at $t = 0$ (not shown). A slight rise in light intensity after $t = 2$ min precedes the occurrence of repetitive spikes of $[Ca^{2+}]_i$ (*A*). This is repeated twice: after $t = 16$ min (*B*) and $t = 20$ (*C*). In the latter case, the signal was off-scale, and at $t = 0$ s (*D*), $[K^+]_o$ was suddenly raised tenfold. The shape of the aequorin response is a triplet, as reported recently (Bittar & Nwoga, 1989*b*). Notice that the signal disappears quite rapidly, following which there is a decline in light emission to a level lower than the original baseline.

Next, the concentration–response relation for the effect of PD on resting luminescence was determined. In Fig. 11 it is shown that the minimal effective concentration of PD falls in the nanomolar range. This is in agreement with the $^{22}Na^+$ efflux data which indicate that the threshold concentration is about 10^{-8} M.

The finding that pre- or post-injection of $MgCl_2$ almost totally eliminates the response of the ouabain-insensitive Na^+ efflux to PD raised the possibility that Mg^{2+} might also reduce the aequorin response to PD. This seemed reasonable, particularly since Mg^{2+} is known to compete with Ca^{2+} for the active sites of the photoprotein (Blinks, Wier, Hess & Prendergast, 1982). Experiments therefore were performed in which aequorin-loaded fibres, pre-treated with ouabain, were injected with 0·5 M-$MgCl_2$ about 15 min before external application of 10^{-5} M-PD. Companion control fibres were injected with a 3 mM-HEPES solution in lieu of $MgCl_2$. The results show an aequorin response to PD, the magnitude of which averages $103 \pm 19\%$ ($n = 5$), as compared with $779 \pm 273\%$ in companion controls ($n = 5$). The difference is significant ($P < 0{\cdot}05$).

DISCUSSION

The picture which emerges is that phorbol dibutyrate is able to stimulate the ouabain-insensitive Na^+ efflux as a result of activation of Ca^{2+}–phospholipid-dependent protein kinase C, and that this response is initiated following the entry of external Ca^{2+} through Ca^{2+} channels. This is supported by several lines of evidence. First, barnacle muscle fibres possess Ca^{2+}–phospholipid-dependent protein kinase C which is activated by TPA (Bittar & Girard, 1987), a finding which is in accordance with expectation, since the presence of this enzyme in the animal kingdom is ubiquitous (Kuo, Andersson, Wise, Mackerlova, Salomonsson, Brackett, Katoh, Shoji & Wrenn, 1980). Second, the specific receptor in cells for phorbol dibutyrate is protein kinase C (Castagna *et al.* 1982). Third, the requirement for external Ca^{2+} is absolute. Fourth, the response fails to occur in the presence of verapamil. The failure of Cd^{2+} to block completely the response to PD is a surprising finding which seems to emphasize the possibility that the efficacy of Cd^{2+} may depend on the phosphorylation state of the Ca^{2+} channel protein and/or that Cd^{2+} may reach the myoplasm via the Ca^{2+} channel (e.g. in cell line GH_4C; Hinkle, Kinsella & Osterhoundt, 1987) and then inhibit Ca^{2+}–phospholipid protein kinase C (e.g. in rat vascular smooth muscle; Mazzei, Girard & Kuo, 1984). Alternatively, Cd^{2+}, unlike verapamil, may be unable to block the SR Ca^{2+} release channel. Fifth, the response is drastically reduced or reversed by injecting Mg^{2+}. Sixth, pre-injection of EGTA reduces the size of the response to PD. And seventh, luminescence from aequorin is promptly increased by PD.

Hitherto relatively little is known about the steps intervening between protein kinase C activation and physiological responses. However, evidence is already available that this enzyme participates in the phosphorylation of the Ca^{2+} channel protein lying in the T-tubule membrane of skeletal muscle, e.g. rabbit muscle (O'Callahan, Ptasienski & Hosey, 1988). Other experimental models, notably *Aplysia* neurones (Strong, Fox, Tsien & Kaczmarek, 1987), have yielded evidence which indicates that phorbol esters increase the inward Ca^{2+} current. If this is true, and if protein kinase C activation by PD leads to activation of Ca^{2+} channels in barnacle fibres, then the simplest and preferred explanation of the rise in Na^+ efflux is that it is the result of activation by trigger Ca^{2+} of not only Ca^{2+}–calmodulin-dependent protein kinase, which leads to phosphorylation of Ca^{2+} channel protein (e.g. rabbit skeletal muscle; O'Callahan & Hosey, 1988), and phosphorylation of the Na^+–Ca^{2+} exchanger (e.g. in heart muscle sarcolemma; Caroni & Carafoli, 1983), but also enhanced binding of protein kinase C to the inner side of the plasmalemma (Anderson, Estival, Taptovaara & Gopalakrishna, 1985). Ca^{2+} is also known to enhance the affinity of PD to protein kinase C, but once the enzyme intercalates into the membrane no further increase in activity can be elicited by adding Ca^{2+}, or phorbol ester (Bazzi & Nelsestuen, 1988).

It is clear from earlier studies that protein kinase inhibitor (PKI) stops injected cyclic AMP from stimulating the ouabain-insensitive Na^+ efflux (Bittar, Demaille, Fischer & Schultz, 1979). Hence the inability of injected PKI to reduce the size of the response to PD may be taken as an indication that the response is not the result of newly formed cycle AMP. Moreover, such a result is what one might expect if there is a reciprocal relationship between the Ca^{2+}–phospholipid-dependent protein kinase C and the membrane adenylate cyclase systems. Alternatively, the lack of effect with PKI may be attributed to proteolysis of the inhibitor by Ca^{2+}-dependent proteases.

The observation that DIDS and pyridoxal 5-phosphate are potent inhibitors of the response of the ouabain-insensitive Na^+ efflux to PD, whilst furosemide, piretanide and bumetanide are not, is a matter of special interest for a number of reasons. First, it eliminates the involvement of Na^+–K–Cl^- co-transport and suggests that the Na^+/HCO_3^-–Cl^-/H^+ exchanger plays a major role in mediating the observed rise in Na^+ efflux. Although no direct evidence is available as yet to link an increase in both Na^+ influx and H^+ efflux, the hypothesis adopted here is that the rise in Na^+ efflux is due to increased Na^+–Ca^{2+} exchange in the reverse mode resulting from a reduction in the Na^+ gradient and myoplasmic pCa. The influence of pH_i is probably not negligible, judging by the work of Philipson, Bersohn & Nishimoto (1982) who used membrane vesicles from cardiac muscle sarcolemmae and found the stimulatory response of the exchanger to pH_i to be a sigmoidal function of pH_i. Substantiation of the view that barnacle fibres possess an Na^+/HCO_3^-–Cl^-/H^+ exchanger in lieu of an Na^+–H^+ exchanger is provided by the work of Boron (1985). Consistent with this view is the demonstration that an Na^+–H^+ exchanger can be expressed in barnacle fibres by injecting poly $(A)^+$ RNA into them (Knakal, Summers, Cragoe & Boron, 1985). Second, it is now widely recognized that activation of protein kinase C is followed by stimulation or inhibition of Na^+–H^+ exchange in practically all mammalian tissues examined so far (Grinstein & Rothstein, 1986). This raises the intriguing possibility that a phosphorylation process might be responsible for the activation of the Na^+/HCO_3^-–Cl^-/H^+ exchanger. Third, a striking feature of the present results is the ability of BZA to drastically reduce the response to PD or reverse it. Such a finding lends credence to the view that the Na^+/HCO_3^-–Cl^-/H^+ exchanger and the carbonic anhydrase system are closely connected. As will be recalled skeletal muscle contains carbonic anhydrase not only in the myoplasm but also in the sarcolemma and SR (Gros & Dodgson, 1988), and barnacle muscle is no exception (T. H. Maren, private communication; H. Deutsch & E. E. Bittar, unpublished). The question then arising is: What triggers carbonic anhydrase activity? Two plausible explanations come to mind. One lies in the finding by various workers of a fall in internal pH following a sufficient rise in cytosolic free Ca^{2+}. For example, this is the case in mammalian heart muscle in which the influence of Ca^{2+} on pH_i seems independent of the Na^+–H^+ exchanger (Vaughan-Jones, 1988). Moreover, an acid load *per se* is known to activate the Na^+–H^+ and Na^+/HCO_3^-–Cl^-/H^+ exchangers (Boron, 1977; Aronson, Nee & Sutton, 1982). The other explanation is that one of the phosphorylation steps mediated by protein kinase C is phosphorylation of carbonic anhydrase. As yet, supporting evidence for such a mechanism is unavailable. Fourth, the inhibiting effect of injected Mg^{2+} may not be limited to the Ca^{2+} release channel of the SR. It may include inhibition of the Na^+/HCO_3^-–Cl^-/H^+ exchanger in the light of evidence that a fall in internal pMg in barnacle fibres inhibits the SITS- or DIDS-sensitive Cl^- effluxes and net acid extrusion (Russell & Brodwick, 1988). And fifth, it is notable that the magnitude of the response of fibres suspended in Li^+-ASW to the injection of PD is virtually the same as that of fibres suspended in Na^+-ASW. This suggests that under conditions of protein kinase C activity, the Na^+–Ca^{2+} exchanger stops discriminating between Na^+ and Li^+. Alternatively, if the sequence of discrimination $Na^+ \gg Li^+$ persists, one would then have to suppose that the rise in myoplasmic free $[Ca^{2+}]$ in fibres immersed in Li^+-ASW exceeds that in fibres immersed in Na^+-ASW. This can be inferred on simple grounds of reversal of the Na^+ gradient, as well as Na^+–Li^+ exchange (Bittar, Chambers & Brown, 1983). A persistently raised myoplasmic free Ca^{2+} concentration may well account for the sustained nature of the response of these fibres to PD. Expressed in a different way, Ca^{2+} cycling and protein kinase C activity are essential features of a sustained response (Alkon & Rasmussen, 1988). This, however, leaves unanswered the question of why BZA is able to reduce the response to injected PD of the ouabain-insensitive Na^+ efflux into Li^+-ASW. Reversal of the response by BZA may be due to an intracellular acidosis if the correct view is that a marked increase in myoplasmic free $[Ca^{2+}]$ leads to a fall in pH_i and hence activation of carbonic anhydrase.

In summary, therefore, it can be said that the use of the barnacle muscle fibre as a preparation has led to evidence supporting the view that stimulation of the ouabain-insensitive Na^+ efflux by PD is due to the activation of the Ca^{2+}–Na^+ and Na^+/HCO_3^-–Cl^-/H^+ exchangers, and that this is preceded by a rise in myoplasmic free [Ca^{2+}] resulting from the entry of trigger Ca^{2+} via verapamil-sensitive Ca^{2+} channels. In the absence of direct evidence, it is tacitly assumed that phosphorylation reactions mediated by protein kinase C in addition to Ca^{2+}–calmodulin-dependent protein kinase are partly responsible for the activation process. More data about the exchanger which exchanges internal H^+ and Cl^- for external Na^+ and HCO_3^-, and is inhibited by DIDS and pyridoxal phosphate but not by amiloride, have been obtained with BZA. Its physiological significance rests on the assumption that BZA is a specific inhibitor of the carbonic anhydrase system. Whether there is a close connection between the operation of this system and protein kinase C activity is not yet known.

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