Influence of Pentachlorophenol on Light Emission from Single Barnacle Muscle Fibers Preloaded with Aequorin

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Experiments show that the resting ouabain-insensitive sodium efflux in giant fibers from the barnacle *Balanus nubilus* is stimulated by external application of pentachlorophenol (PCP). This work has now been extended to include a study of muscle fibers preloaded with the Ca^{2+} indicator aequorin to determine whether PCP is able to increase light emission; and whether its potency depends on the number of chlorine atoms and external pH. The results obtained are as follows: 1) PCP causes a dose-dependent, multiphasic rise in light emission; the threshold concentration in fibers not poisoned with ouabain was in the low micromolar range. 2) The efficacy of PCP is considerably greater than that of less-chlorinated phenols and phenol. 3) The response to PCP is a sigmoidal function of external pH both in unpoisoned and ouabain-poisoned fibers. Reducing external pH potentiates its efficacy. 4) The response to PCP depends on the external Ca^{2+} concentration, and the requirement for Ca^{2+} is usually absolute. *Key words:* aequorin light emission, barnacle muscle fibers, pentachlorophenol. *Environ Health Perspect* 101:622–625(1993)

Interest has been aroused by studies on the pesticide pentachlorophenol (PCP), a known cytotoxicant (*1,2*), that showed PCB can stimulate the sodium efflux in barnacle muscle fibers at a concentration as low as 1 µM and that its efficacy, which exceeds that of less-chlorinated phenols, depends on external pH (pH_e). Additionally, the response of the sodium efflux to PCP is a sigmoidal function of external [Ca^{2+}], the requirement for external Ca^{2+} being absolute (*3*). These findings have raised the question of whether PCP increases internal free Ca^{2+} by promoting the entry of Ca^{2+} from the outside into the myoplasm. This paper presents a study carried out with the photoprotein aequorin in an attempt to address the question of whether stimulation of the sodium efflux is due to a rise in internal free Ca^{2+} resulting from the entry of Ca^{2+} (*4*) and whether the efficacy of PCP depends on the number of chlorine atoms in its aromatic ring, as well as on the pH of the bathing medium (pH_e).

Aequorin, rather than a Ca^{2+} dye substance such as Fura 2 or a Ca^{2+}-selective microelectrode, was chosen for several reasons. First, the aequorin method has been used with considerable success to study internal Ca^{2+} transients in skeletal muscle, most notably in barnacle fibers (*5*). Second, the aequorin signal is known to be relatively free from movement artifacts. Third, aequorin shows high sensitivity to Ca^{2+} and provides not only a sufficiently rapid response time but also a high signal-to-noise ratio. Fourth, aequorin is adequate for detecting internal Ca^{2+} transients over the physiological range of 1×10^{-7} to 5×10^{-6} M and lacks toxicity after injection (*6*).

We obtained specimens of the barnacle *Balanus nubilus* from Puget Sound, Seattle, Washington, and kept them in an aerated aquarium which was maintained at 10°–12°C throughout the study. The methods of dissection and cannulation of single fibers were essentially the same as those described by Bittar (*7*). The artificial sea water (ASW) used had the following composition: NaCl 465 mM; KCl 10 mM; $MgCl_2$ 10 mM; $CaCl_2$ 10 mM, $NaHCO_3$ 10 mM, and pH 7.8. We prepared solutions with varying concentrations of Ca^{2+} by raising or reducing NaCl in osmotically equivalent amounts. Solutions of PCP and other chlorinated phenols and phenol were prepared by dissolving them in dimethyl sulfoxide (DMSO) before their addition to ASW. The final content of DMSO in ASW containing 5×10^{-4} M PCP was 0.5% (v/v). We used the buffers Hepes and Mops in lieu of HCO_3^- in experiments involving ASW at different pH values.

The microinjector used for loading the cannulated fibers with aequorin was of the type described by Bittar and Tallitsch (*8*). A solution of $2–5 \times 10^{-4}$ M aequorin in a volume of 0.4–0.5 µl was injected axially into these fibers with the aid of a micromanipulator and Palmer screw stand. Since the intrafiber fluid volume was on average 40 µl, dilution by the myoplasm of the injected solution was approximately 100-fold. Aequorin was allowed to equilibrate for at least 1 hr. In those fibers poisoned with 10^{-4} M ouabain, external application of this glycoside was carried out some 30 min before the application of pentachlorophenol (PCP) or any of its congeners.

The method used for recording aequorin light output was a modification of that described by Bittar and Keh (*9*) for monitoring firefly luciferase luminescence. Reading, display, and analysis of the picoammeter data was carried out with the aid of two software programs that were written in our laboratory by Patrick Hamilton. Experience with data processing revealed that analysis is best carried out by collating data based on experiments where basal light emission from the fibers is relatively the same. We estimated increments in light emission on the basis of maximal flash height; that is, by taking the difference between basal light emission and peak emission, expressed in nanoamperes. Significance levels were compared by using Student's *t*-test. A significance level of $p<0.05$ was selected. All experiments were carried out at an environmental temperature of 22°–24°C. All reagents used were analytical grade. Hepes, Mops, and ouabain were purchased from Sigma Chemical Company (St. Louis, Missouri). Pentachlorophenol and other chlorinated phenols and phenol were supplied by Aldrich Chemical Company (Milwaukee, Wisconsin).

In the first series of experiments with unpoisoned and ouabain-poisoned fibers, we investigated the effect on basal light output of external application of PCP at varying concentrations. Initially, it was necessary to determine whether the presence of DMSO (e.g., 0.5% in the bathing medium) influences basal light output from fibers (unpoisoned and ouabain poisoned) preloaded with aequorin. The results obtained in such experiments show that the presence of DMSO is without effect on basal light output. The characteristic signal observed in unpoisoned fibers to external application of PCP (e.g., 5×10^{-4} M within 10 min) is an oscillatory rise in basal light emission, as illustrated in Figure 1a. A peak response to PCP is not immediate in onset. Such an experiment is shown in Figure 1b. Kinetics of this variety are also recorded with ouabain-poisoned fibers, but the onset of a peak response is more rapid and may be followed by a rather large, monophasic or almost monophasic Ca^{2+} transient, as shown in Figure 1c. Notice that the comparison drawn here is between fibers showing fairly similar baseline levels of light output.

It is noteworthy that the time to peak in unpoisoned fibers exposed to PCP shows a correlation with dosage only when the dose of PCP used falls in the high range. For example, time to peak in fibers exposed to 10^{-4} M PCP averages 183 ± 73 sec (n = 5), a value that is not significantly

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different from that obtained with fibers exposed to 2.5 or 5×10^{-4} M PCP, but significantly less than the values of 392 ± 27 sec (n = 6) and 402 ± 52 sec (n = 6) obtained with fibers exposed to 7.5×10^{-4} M and 10^{-3} M PCP, respectively (p <0.02). On the other hand, an analysis of width at half-peak response fails to provide a similar correlation. Another feature that stands out is an increase in the frequency of internal transients in ouabain-poisoned fibers after exposure to PCP and that poisoned fibers are considerably more sensitive to PCP than unpoisoned fibers. For example, fibers poisoned with 10^{-4} M ouabain and exposed to 10^{-3} M PCP 30 min later show an increase in light emission, the magnitude of which averages 224 ± 29% (n = 3), whereas the increase observed in unpoisoned fibers exposed to 10^{-3} M PCP averages 76 ± 17% (n = 3). The difference is significant.

In the second series, we tried to establish the concentration–response curve for the stimulatory effect of PCP on basal light emission using unpoisoned fibers. The time frame of the experiments was 10 min. Two features of the curve shown in Figure 2 are notable: first, light output is increased by PCP at a concentration as low as 10 µM. This and a concentration of 50 µM fall in the toxic range for aquatic organisms (*10*), as well as BF-2 cells derived from blue gill sunfish (*11*). Second, although a 10-fold elevation in PCP concentration fails to elicit a further increase in light output, a sharp inflexion of the curve occurs thereafter with a larger elevation in PCP concentration. Such results are consistent with the hypothesis that two mechanisms are operative: one involving a plasmalemma site which is saturated by PCP in low micromolar concentrations, and the other a myoplasmic site which is not readily saturated by PCP after its passage into the fiber interior.

In the third series of experiments, the response to PCP as a function of pH_e was examined. Since PCP is a weak acid with a pK_a of 4.8 (*12*), experiments were done to ascertain whether the efficacy of PCP depends in part on the pH of the suspending medium. These experiments involved the use of two different inert buffers, Hepes (pK_a 7.5) and Mops (pK_a 7.2). The results obtained with 10 mM Hepes ASW containing 5×10^{-4} M PCP show that light emission is a sigmoidal function of the medium pH. Light emission increases as pH_e is reduced. Similar results were obtained using unpoisoned and ouabain-poisoned fibers suspended in 10 mM Mops-ASW containing 5×10^{-4} M PCP. Summarized in Figure 3 are the results obtained with Mops as the buffer over a pH_e range of 8–6. Notice that pH_e 8 (10 nM)

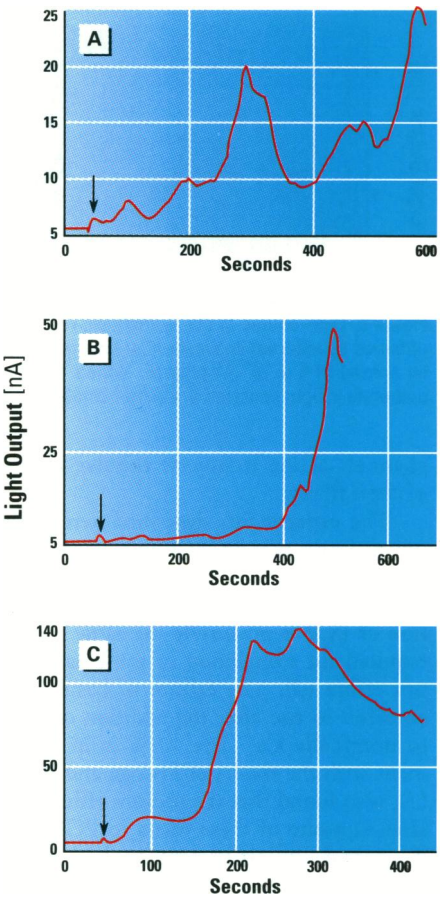

Figure 1. (A) Time-course of aequorin signals recorded from an unpoisoned fiber exposed to 5×10^{-4} M pentachlorophenol (PCP) at time indicated by arrow. Light output is expressed in nanoamperes. (B) A lag period precedes the onset of a marked outburst of light emission from aequorin. The PCP concentration was 5×10^{-4} M. (C) Time-course of a somewhat monophasic aequorin signal recorded from a fiber treated with 10^{-4} M ouabain 30 min before exposure to 5×10^{-4} M pentachlorophenol at time indicated by arrow.

is fairly close to the threshold value for ouabain-poisoned fibers. Also notice that the midpoint of the slope is about 100 nM H^+. In a typical experiment, an unpoisoned fiber suspended in 10 mM Mops-ASW at pH 7 (i.e., 100 nM) shows a multiphasic rise in light emission when exposed to 5×10^{-4} M PCP, as illustrated in Figure 4a. The rise is from approximately 15 nA to less than 100 nA over less than 10 min. Multiphasic behavior is also seen in ouabain-poisoned fibers suspended in 10 mM Mops containing 5×10^{-4} M PCP at pH 6 (1000 nM), but the peak response is larger, as illustrated in Figure 4b. Notice that the basal level of light output before the addition of PCP is practically the same as that of the fiber in the preceding experiment.

Next, we studied the response to PCP as a function of chlorine atoms. The potency of chlorinated phenols is known

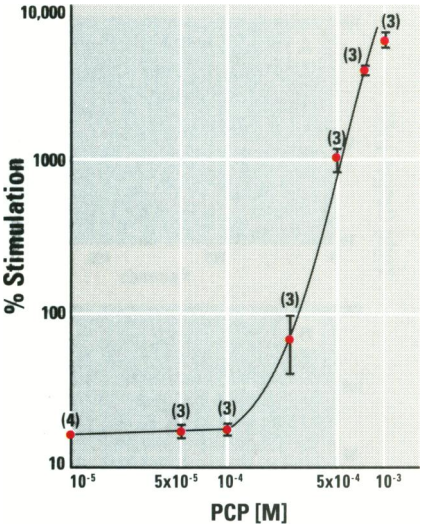

Figure 2. Concentration–response curve for the stimulatory effect of pentachlorophenol on light emission from aequorin in unpoisoned fibers. Vertical bars indicate ±1 SEM; the number of measurements carried out is in parentheses. All fibers used were isolated from the same barnacle specimen (log–log plot).

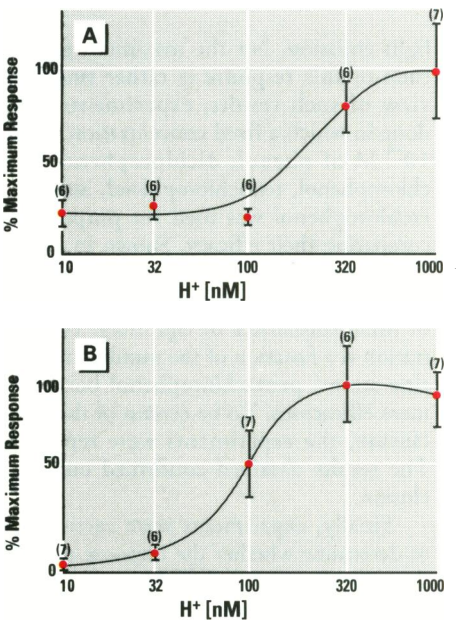

Figure 3. (A) The dependency on pH_e of the magnitude of the increase in light emission in unpoisoned fibers and (B) ouabain-poisoned fibers in response to external application of 5×10^{-4} M pentachlorophenol in 10 mM Mops-ASW. Each plotted point in panel A is the mean of six measurements, except that at pH 6. Vertical bars indicate ±1 SEM. The fibers used were isolated from three barnacle specimens taken from the same batch (semilog plot).

to increase with chlorination of the parent phenol (*3,10,13*). Figure 5 shows a representative experiment carried out with phenol. External application of 5×10^{-4} M phenol in ASW containing 10 mM Hepes at pH 7.4 produces a multiphasic rise in

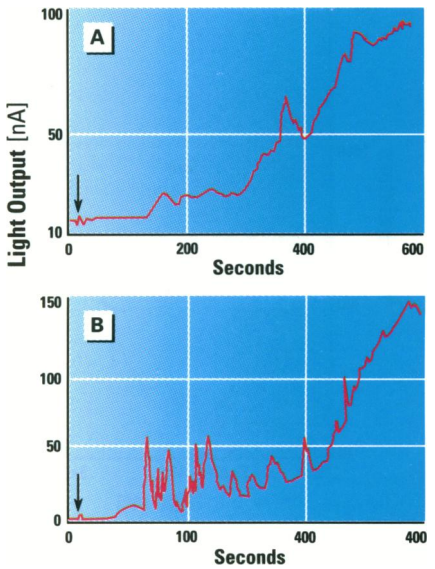

Figure 4. Time-course of a multiphasic aequorin response of an ouabain-poisoned fiber suspended in 10 mM Mops-ASW at (A) pH 7 and (B) pH 6 to external application of 5×10^{-4} M pentachlorophenol (PCP) at time indicated by arrow. Notice the brief lag period preceding the onset of a rise in basal light emission in panel A.

light emission, but the maximum magnitude of this response is rather small. In view of such results, experiments were done in which a fixed concentration of 5×10^{-4} M of phenol, dichlorophenol, trichlorophenol, tetrachlorophenol, and pentachlorophenol was used for purposes of comparing their efficacy. Shown in Figure 6 is clear-cut evidence that an increase in light output in response to the application of these substances in equimolar concentration is a function of the number of chlorine atoms, pentachlorophenol being the most efficacious. To be certain of this conclusion, the experiments were repeated. The results obtained confirmed our conclusion.

Finally, experiments were carried out to determine whether the response to PCP depends on external Ca^{2+}. Using the recent results that the stimulatory response of the sodium efflux to PCP is a sigmoidal function of external Ca^{2+} and that the requirement for external Ca^{2+} is absolute (*3*), studies with ASW containing Ca^{2+} at varying concentrations were undertaken. Figure 7 illustrates that the magnitude of the observed rise in light emission after the application of 5×10^{-4} M PCP depends on the external Ca^{2+} concentration and that PCP is ineffective in the nominal absence of Ca^{2+} in the bathing medium. Other experiments, however, indicate that, on occasion, a small increase in basal light emission is observed with 5×10^{-4} M PCP. This is accounted for by assuming that some Ca^{2+} is retained in the fluid of the

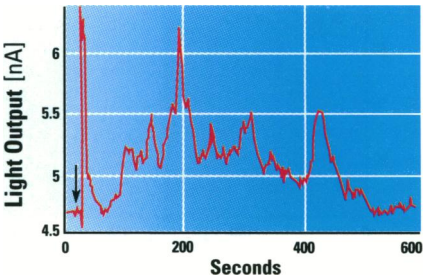

Figure 5. Time-course of the multiphasic response obtained by external application (at time indicated by arrow) of 5×10^{-4} M phenol. Notice that the outbursts in light emission are rather small.

channels of the transverse tubular system of these fibers.

The experiments described here show quite clearly that PCP is able to increase light emission from fibers preloaded with aequorin and that the threshold concentration of PCP is practically the same as that required for stimulation of the basal sodium efflux (*3*). The failure of basal light emission to rise after the addition of PCP to nominally Ca^{2+}-free ASW is in agreement with the results of Nwoga and Bittar (*3*), who found that the sodium efflux rises after exposure of these fibers to PCP only if external Ca^{2+} is present in the bathing medium. Although the provisional conclusion which emerges is that "trigger" Ca^{2+} derives from the bathing medium, the possibility still remains that a fraction of the Ca^{2+} comes from internal storage sites, such as the sarcoplasmic reticulum. This possibility is not unlikely considering evidence that ryanodine and TMB, both of which act as blockers of the sarcoplasmic reticulum Ca^{2+} release channel, are able to reduce the response of the sodium efflux to PCP (*3*). It could be that the increase in internal free Ca^{2+} caused by PCP is partly the result of IP_3-promoted release of Ca^{2+} from the sarcoplasmic reticulum, but there is evidence that the ouabain-insensitive sodium efflux is unresponsive to the injection of IP_3 (*14*). This is also true of thapsigargin (Bittar EE, unpublished data). Another possibility is that PCP, a known uncoupler (*15,16*), might cause the release of Ca^{2+} from mitochondria, as well as a reduction in internal ATP-Mg. However, neither possibility seems likely, partly because the classical uncoupler 2,4-dinitrophenol is without effect on the sodium efflux (Bittar EE, unpublished data), and partly because phosphagen occurs in abundance in these fibers [e.g., ^{31}P-NMR reveals an ArP content of 24 mmol/kg fiber water (*17*)].

There is circumstantial evidence favoring the view that the Ca^{2+} channel (L-type in barnacle fibers) is a primary point of action of PCP and that external Ca^{2+}

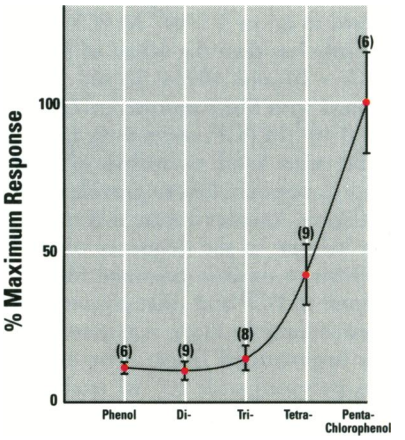

Figure 6. Comparison of the potency of phenol and four chlorinated derivatives. The ouabain-poisoned fibers were suspended in 10 mM Hepes-ASW at pH 7.4. The concentrations used were equimolar (5×10^{-4} M). Vertical bars indicate ±1 SEM; the number of individual experiments carried out is in parentheses. The fibers used in these experimental sessions were isolated from three barnacle specimens taken from the same batch.

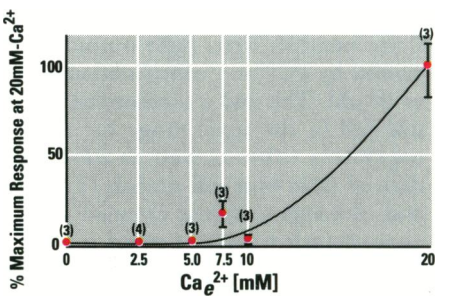

Figure 7. The dependency of light emission from aequorin in ouabain-poisoned fibers treated with 10^{-3} M pentachlorophenol externally on the external Ca^{2+} concentration. Vertical bars indicate ±1 SEM; the number of measurements recorded is in parentheses. The fibers used were isolated from the same barnacle specimen.

reaches the myoplasm via this pathway (*3*). However, this cannot be the complete picture: for example, to account more fully for the Ca^{2+} transients recorded with aequorin, one has to explain the part played by the Na^+–Ca^{2+} exchanger in maintaining cellular Ca^{2+} homeostasis in the face of an increased Ca^{2+} influx and a reduced sodium gradient in ouabain-poisoned fibers. This is precisely the rationale for using 10^{-4} M ouabain; that is, to maximally inhibit the membrane Na^+–K^+-ATPase system, thereby raising the internal sodium concentration in these fibers (*18*). Thus, for example, under conditions where $\Delta\tilde{\mu}_{Ca} > 3\Delta\tilde{\mu}_{Na}$, the energy provided by the Ca^{2+} electrochemical gradient wouldbe expected to drive the sodium efflux (*19*). In other words, the marked outbursts of Ca^{2+} transients observed, particularly in ouabain-poisoned fibers, is

largely related to the operation of the Na^+–Ca^{2+} exchanger in the reverse mode. Because ATP behaves as a positive effector of this exchanger in barnacle fibers (*20*), we suggest that PCP stimulates reverse Na^+–Ca^{2+} exchange as the result of transitorily raising the internal ATP level. This is likely to be the case if PCP acts as a competitive inhibitor of certain ATP-dependent processes. Evidence supporting this possibility comes from recent studies carried out with the firefly luciferase reaction *in vitro* (Xiang Z, Bittar EE, unpublished data). PCP is found to increase the K_m for ATP but not affect V_{max}. Because a specific and powerful inhibitor of the Na^+–Ca^{2+} exchanger is not yet available (*21*), this rather interesting problem cannot be addressed in a direct manner at this time.

The pH-reaction profile is a sigmoid curve with a mid-point of about pH 7. This is compatible with a mechanism involving ionization of a group located in an active site, e.g., the Ca^{2+}-channel protein that facilitates the passage of external calcium into the myoplasm after interaction of PCP at this active site. An additional explanation of the observed dependency of PCP potency on pH_e is to invoke the law of nonionic diffusion (*22,23*). In other words, protonation of PCP renders it more lipophilic, thus leading to greater entry of the neutral species (e.g., into the plasmalemma compartment per se). This could bring about changes in lipid bilayer organization. Thus, to understand more fully the mechanism underlying the action of PCP, information is required not only about the role of the Ca^{2+} channel but also about whether PCP affects membrane fluidity. Until now, the few studies carried out with synthetic lipid bilayer membranes and biological membranes indicated that PCP in the micromolar range alters the physical properties of these systems (*24–26*). Another approach is to use an equilibrium model: this shows that at pH values less than pH 7 the distribution of the weak acid, e.g., between octanol and water, is dominated by the neutral species and does not depend on ionic strength (*27*). Such studies remain meager but are in keeping with the classical studies of Goodnight, who was the first to demonstrate that PCP can be toxic and that lowering pH potentiates this toxicity (*28*).

Finally, this study draws attention to the possibility that the cytotoxic action of PCP may be chiefly due to a raised cytosolic free Ca^{2+} concentration (*29*). Although human deaths have taken place after acute industrial, accidental, or suicidal exposure to PCP, the minimal lethal blood concentration remains unknown (*2*). Whole-body exposure to PCP and urinary levels in the general population are very low [0.001–0.17 mg/person/day and 1–10 µg/l, respectively (30)]. Aquatic species show extreme susceptiblity to PCP toxicity. This is borne out by evidence of an LC_{50} value for PCP of about 1 µM (*31,32*), which is a concentration close to that found to cause a rise in internal free Ca^{2+} in barnacle fibers.

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