here were found to be of the monohydrate type. We conclude that X-ray microarea diffractometry, as demonstrated here, is indeed a valuable method for examining the structure of biominerals in situ on a molecular level, and it can be expected to give a considerable impetus to structural analysis in the fields of biology and medicine.

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Microinjection of synthetic protein kinase inhibitor into single barnacle muscle fibers before and after cyclic AMP

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Summary. Single muscle fibers from the barnacle *Balanus nubilus* have been used as a preparation to see if a synthetic 20-residue PKI (5–24)-peptide is able to block or reverse the stimulatory response of the ouabain-insensitive Na efflux to injected cAMP. The results obtained show that this peptide behaves as a powerful inhibitor of the cAMP-mediated response and is also able to partially reverse the sustained stimulation of the Na efflux observed in ouabain-poisoned fibers following the injection of subunit A of cholera toxin.
Key words. Synthetic PKI; cAMP; sodium efflux; barnacle muscle fibers.

Earlier work involving single muscle fibers from the barnacle, *Balanus nubilus* has shown that the Na efflux is stimulated by the microinjection of cyclic AMP[3] in a concentration as low as 10^{-6} M[4] and that pure protein kinase inhibitor (PKI) has the ability following its injection to interrupt or reverse the effect of cAMP[5], as well as that of pure catalytic subunit of cAMP-dependent protein kinase[6]. These observations are in keeping with the widely held view that PKI specifically inhibits the free catalytic subunit of cAMP-protein kinase, which is formed as the result of the dissociation of the holoenzyme by cAMP[7,8]. More recently, Scott and coworkers[9–11] have succeeded in defining the inhibitory domain of PKI and reported it to be represented by a 20-peptide residue. They also showed that potent inhibitory analogs contain at least 20–24 residues and that the 20-residue PKI (5–24)-peptide exhibits a Ki of ~ 9 nM. That is to say, it is as potent as native PKI. The purpose of this communication is to report that this particular peptide when microinjected into single barnacle muscle fibers behaves as a powerful inhibitor of the stimulatory response of the ouabain-insensitive Na efflux to injected cAMP.

Specimens of the barnacle *Balanus nubilus* were obtained from the Pacific Biomarine Laboratory, Inc. in Venice, CA, and kept in a 150-gallon Instant Ocean aquarium containing artificial seawater. The temperature of the aquarium water was maintained at about 12 °C throughout. Single fibers measuring 3–4 cm in length and 1–2 mm in diameter were isolated by dissection from the depressor muscle bundles and then cannulated. A 50–80-mg weight was attached to the tendon of the cannulated fiber, thereby keeping it in a vertical position while suspended in artificial seawater (ASW). The experiments were carried out with ASW having the following composition (mM): NaCl, 465; KCl, 10; $CaCl_2$, 10; $MgCl_2$, 10; $NaHCO_3$, 10 and pH 7.8. The microinjector used was of the type described by Bittar and Tallitsch[12]. The volume of test fluid injected into a fiber was about 0.4 µl. This is diluted by the myoplasm by a factor of roughly 100. $^{22}NaCl$ in aqueous solution was obtained from Amersham-Searle Corp., Arlington Heights, IL. The solution was dried down and then redissolved in distilled water, so that volumes of around 0.4 µl gave at least 750,000 counts per min. The effluent from the cannulated fiber loaded with radiosodium was collected every 5 min and the residual fiber activity was determined at the end of the experiment. A Beckman au-

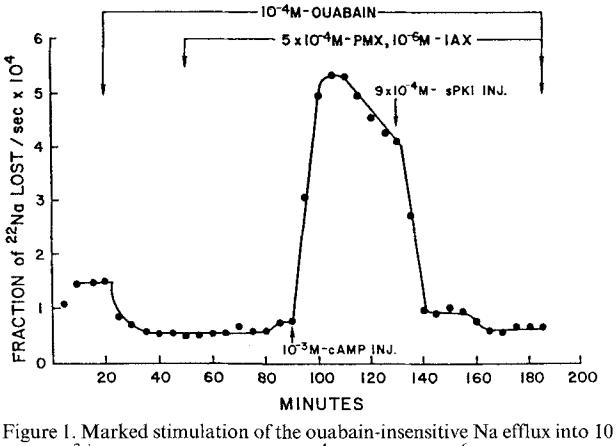

Figure 1. Marked stimulation of the ouabain-insensitive Na efflux into 10 mM-Mg^{2+}-ASW containing 5×10^{-4} M PMX & 10^{-6} M IAX by injecting 10^{-3} M cAMP and complete reversal of this response by injecting 9×10^{-4} M sPKI (rate constant for ^{22}Na efflux plot).

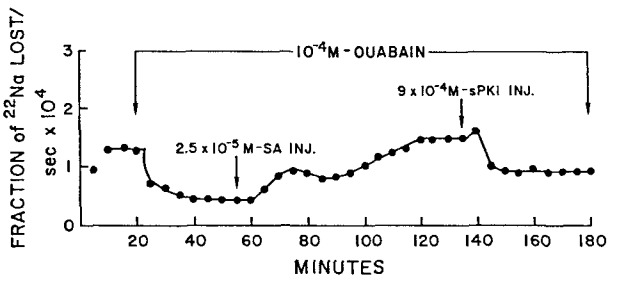

Figure 2. Sustained stimulation of the ouabain-insensitive Na efflux by injecting 2.5×10^{-5} M subunit A of cholera toxin, followed by partial reversal of this response by injecting 9×10^{-4}M sPKI. Note two additional features: one is the characteristic latency period preceding the onset of the effect of SA, and the other is the long delay in the effect reaching a peak. In experiments of this type where microinjection of a substance is involved, dilution of the substance by the myoplasm may be assumed to be 100-fold.

togamma counter was used for counting the samples. The data were fed into an Apple II computer programmed to compute the fractional rate constant for ^{22}Na efflux (this being efflux rate/fiber count + 1/2 efflux). Stimulation of Na efflux was estimated by taking the difference between the maximum rate constant and the rate constant immediately before the onset of stimulation. Inhibition was estimated by taking the difference between the rate constant at maximal inhibition or that found by extrapolating the last few points of the inhibitory phase back to the time of application of the agent, and the rate constant before the onset of inhibition. The results given in this paper are means ± SEM and significance levels were determined by using Student's unpaired t-test. All experiments were carried out at room temperature, 23 ± 1 °C. Ouabain, HEPES and cyclic AMP were supplied by Sigma Chemical Company, St. Louis, MO. Subunit A of cholera toxin was obtained from Schwartz/Mann, Orangeburg, NJ. Synthetic PKI was a gift from Prof. E.H. Fischer of the University of Washington, Seattle, WA. PMX [1-propyl-3-methyl-7-(5-hydroxyhexyl)-xanthine] was a gift from Dr V. Stefanovich of Hoechst Aktiengesellschaft, Wiesbaden, FRG. IAX(1-isoamyl-3-isobutylxanthine) was a gift from Dr Jack N. Wells of the Department of Pharmacology, Vanderbilt University, Nashville, TN. Test solutions of cAMP and sPKI for injection were prepared by using 3 mM-HEPES, pH 7.2.

In the first group of experiments, fibers pretreated with 10^{-4}M ouabain were injected with 9×10^{-4}M-sPKI and 40 min later injected with 10^{-3}M cAMP. Companion controls were injected with 3 mM-HEPES in lieu of sPKI in 3-mM HEPES. The results obtained show that the stimulatory response in test fibers was in the order of 170 ± 18%, n = 7, as compared to 512 ± 30%, n = 7 in controls ($p < 0.001$). This finding is essentially the same as that obtained with pure PKI[5]. Failure of sPKI to completely abolish the response to cAMP could be due to the action of Ca^{2+}-dependent proteases.

In the second group of experiments, ouabain-poisoned fibers were suspended in a bathing medium containing 5×10^{-4}M PMX and 10^{-6} M IAX prior to the injection of 10^{-3} M cAMP. The reason why this was done is that in the absence of these PDE inhibitors the response to cAMP decays rather rapidly[13]. As illustrated in figure 1, decay of the response to cAMP is rapid despite the presence of both PDE inhibitors. This is the exception rather than the rule, and is possible if in some fiber batches the phosphatase system is more brisk than it is in other batches. It can also be seen that injection of sPKI following the onset of peak stimulation by cAMP leads to a rapid and complete reversal of the response (92 ± 2% step-down, n = 4). Companion controls show decay of the response to injected cAMP and that the Na efflux returns to the original baseline level at about t = 170 min (n = 3).

To further confirm this result, experiments were done in which subunit A of cholera toxin was injected, followed by sPKI. Justification for this choice of an agonist is that subunit A is known to directly activate membrane adenylate cyclase[14, 15] and to cause sustained stimulation of the ouabain-insensitive Na efflux following its injection into barnacle fibers[16]. Shown in figure 2 is that the injection of 2.5×10^{-5}M SA induces sustained stimulation of the ouabain-insensitive Na efflux following a characteristic short latency period, and that this is partially reversed by the injection of sPKI (the step-down being 56 ± 5% of the response, n = 3). These experiments also reveal that the residual stimulatory response at t = 180 min is virtually the same as that at t = 145 min. A similar effect on the response to injected cholera toxin is seen in fibers injected with pure PKI[13].

Taken together, the present results support the conclusion that the 20-residue PKI (5–24)-peptide is able to mimic the action of native PKI. Whether the synthetic 20-residue peptide reported by Cheng et al.[17, 18] is equipotent remains to be seen.

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