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Influence of Insulin on Sodium Efflux in Barnacle Muscle Fibers

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Summary. The efflux of radiosodium in single muscle fibers from the barnacle *Balanus nubilus* was irresponsive to internal or external application of insulin. However, this was not the case with fibers isolated from a barnacle specimen pre-exposed overnight to a large dose of insulin. External application of insulin to pre-exposed fibers caused a decrease in the rate of decline of the radiosodium efflux and stopped the decline in the fractional rate constant for Na efflux. Such kinetics were interpreted as indicating that insulin acts either by releasing sequestered Na or abolishing the process of sequestration. Internal application of saline slowed the rate of decline but failed to completely abolish the mechanism of sequestration. Only in the presence of insulin was the fractional loss of Na each second constant. Internal application of insulin caused a prompt step-up in the rate of Na efflux, followed by a reduced efflux rate constant. This meant that injected insulin caused the release of sequestered Na, leading to partial saturation of the efflux. The response of the Na efflux to injected denatured insulin, though resembling that to native insulin was much smaller in size. Internal application of lysozyme produced a transitory step-up in the rate of Na efflux but failed to produce the kinetics observed with native or denatured insulin. Overnight exposure of the barnacle to a dose of denatured insulin failed to render the fiber sensitive to external and internal application of denatured or native insulin *in vitro*. Experiments with ouabain-poisoned fibers showed that external or internal application of native insulin caused stimulation of the remaining Na efflux. They also showed that a 10-fold increase in the concentration of ouabain failed to further reduce the ouabain-insensitive Na efflux. Microinjection of GTP into ouabain-poisoned fibers pre-exposed to insulin resulted in a striking rise in the remaining Na efflux. The magnitude of this effect was considerably greater than that in unexposed fibers. The response which was dose-dependent could be blunted by prior injection of $CaCl_2$. Similarly, the response to $CaCl_2$ injection could be blunted by prior injection of GTP. The evidence brought forward is compatible with the view that insulin acts by abolishing the mechanism of internal Na sequestration and by increasing the activity of the guanylate cyclase system.

Hitherto attempts to study the action of insulin on membrane transport have mostly been made on skeletal muscle bundles rather than on single muscle fibers (*see* Bittar, 1971). Interpretation of such results has proven difficult because the tissue used could not be fresh following loading in a "hot" solution, and because bundles consist of a heterogen-

eous population of fibers separated by intercellular spaces. Nonetheless, the information gained from this work shows that insulin reduces the internal Na concentration (Creese & Northover, 1961) and stimulates the transport of sodium (Kernan, 1962; Moore, 1973). The finding that the hormone stimulates Na transport has been confirmed by carrying out experiments with single *Maia* muscle fibers (Bittar, 1967). These experiments showed quite clearly that it is feasible to render such fibers sensitive to insulin by pre-exposing the crab overnight to a large dose of the hormone. A preparation consisting of a single giant fiber is valuable in more than one way. Firstly, the preparation can be rapidly loaded with radiosodium by microinjection. Secondly, agents such as insulin can be introduced directly into the myoplasm. And thirdly, interpretation of the kinetic results is not as difficult as of those obtained with muscle bundles. Thus, one good reason for employing a crustacean fiber as a preparation is that the argument whether insulin causes the release of internal "bound" or sequestered Na can be put to a direct test. In the present work, therefore, the possibility that insulin releases the fraction of sequestered Na inside the muscle fiber has been explored. A second purpose of this paper is to pursue an enquiry into the theory that insulin acts by increasing *de novo* synthesis of guanylate cyclase or by activating the enzyme system. This is a theory easily tested by microinjecting guanosine triphosphate. As will be amply shown, studies with barnacle fibers may represent an approach that could bring us closer to an understanding of how insulin acts at the membrane level.

Materials and Methods

Single muscle fibers roughly 3–5 cm in length and 1–2 mm in diameter were dissected from the three pairs of depressor muscle bundles of the barnacle *Balanus nubilus* or *B. aquila*. They were then cannulated and mounted on a platform attached to a Palmer screw stand and bathed in artifical sea water. Loading of these fibers with ^{22}Na was done by means of a microinjector, which was similar to that devised by Hodgkin and Keynes (1956) as modified by Caldwell and Walster (1963). The microinjector delivered about 0.1 μl of fluid per cm excursion of the micromanipulator. The $^{22}NaCl$ was supplied by Amersham-Searle Corp (SKS.1). This was dried and then dissolved in distilled water so that volumes of 0.1 μl gave more than 50,000 cpm. Counting of effluent activity in the wash-out samples and the fiber was done as described by Bittar (1966), and Bittar, Caldwell and Lowe (1967). In this type of work, the procedure of radiosodium injection is usually complete within 1 min immediately before $t=0$, and collection of effluent samples is begun at $t=0$. The initial 10- or 15-min phase of the loss of ^{22}Na from the fiber probably represents the period of equilibration. The artificial sea water used as bathing medium had the following composition (mM): NaCl 465, KCl 10, $CaCl_2$ 10, $MgCl_2$ 10,

$NaHCO_3$ 10, at pH 7.8. All experiments were performed at a temperature of 22–24 °C. Insulin supplied by Lilly Research Laboratories, Indianapolis, Indiana as lot #PJ-4609, was recrystallized, trypsin-treated bovine insulin containing $<0.003\%$ glucagon. It had a potency of about 24 units/mg. Also used was porcine sodium insulin, lot #1DG04-94-193 which had a potency of about 26 units/mg. Both lots were free of zinc. Ouabain, lysozyme (grade 1 from egg white) and GTP were obtained from Sigma Chemical Company. Overnight exposure of a barnacle specimen to insulin was carried out by injecting 40 units of protamine zinc insulin (a long-acting preparation) into the *operculum*, then adding 40 units to the artificial sea water (1 liter), and using this sea water as the overnight bathing medium. Estimates of the stimulatory effects caused by insulin were arrived at on the basis of the observed changes in the fractional rate constant for Na efflux and then expressed as a percentage of the immediately preceding rate constant. The fractional rate constant is given by:

$$\text{Rate constant, } k\,(\text{Time}^{-1}) \equiv \frac{\text{Efflux rate}}{\text{Fiber count during collecting period}}.$$

Results

Effect on Na Efflux of External Insulin

In the first group of 20 experiments, barnacle fibers were treated internally with a suspension of 100 U/ml insulin (pH = 7.0) or externally (1 U/ml). In eight of these experiments, microinjection was followed by external application of insulin to see if the fibers would be made responsive to the hormone. None of the 20 fibers tested showed any sensitivity to insulin. This result differs from that obtained by Bittar (1967) who reported that the Na efflux in *Maia* fibers is not infrequently promptly stimulated by external application of 1 U/ml insulin. This being so, the purpose of the next group of experiments was to find out whether these fibers could be rendered sensitive by pre-exposing the barnacle *in vivo* to a large dose of insulin. The results obtained with fibers pre-exposed overnight to a dose of insulin showed that sensitivity to external or internal application of the hormone was acquired. As illustrated in Fig. 1, external application of 1 U/ml insulin led to a prompt change in slope of the efflux plot. Inspection of the fractional rate constant plot indicates that the fraction of Na lost each second was declining before application of insulin, and that the hormone stopped the decline ($n = 5$). Kinetics of this type can be explained by assuming that (*i*) insulin abolished the mechanism of Na sequestration. Broadly, by sequestration is meant exclusion of a fraction of internal Na from exchange or its less ready exchange with the internal free fraction or external Na. (*ii*)

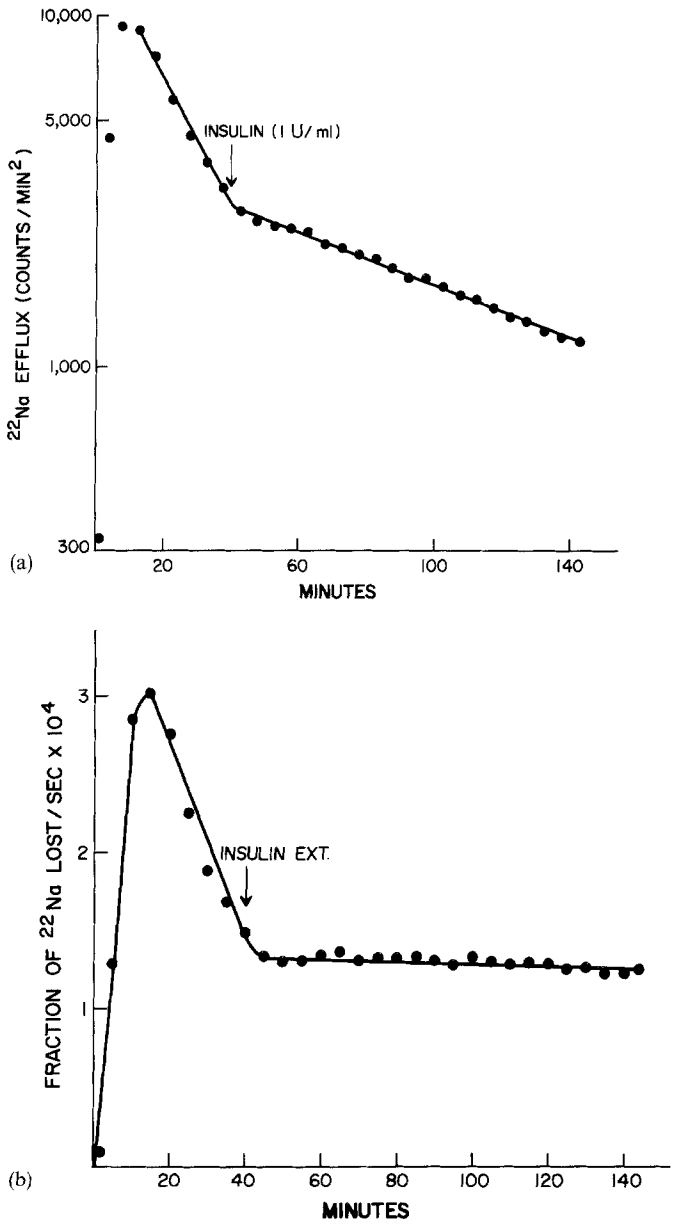

Fig. 1. Effect of external application of 1 U/ml insulin on Na efflux from a pre-exposed fiber. (*a*) Efflux plot. (*b*) Rate constant plot

Insulin causes the release of sequestered Na as well as abolishes sequestration. And (*iii*) insulin causes the release of sequestered Na, and the rise in internal Na leads in turn to abolition of the sequestration mechanism. An alternative explanation is that the Na pump of pre-exposed

fibers is running down and that insulin reduces the Na influx so that influx and efflux become equal. However, the kinetic results, namely, a reduced rate of decline of the Na efflux and a declining fractional rate constant becoming a constant in the presence of insulin are identical to those seen with aldosterone (Bittar & Tallitsch, 1975*a*) and hence suggest that the former explanation is the more plausible.

It should be recalled here that barnacle fibers fall into two groups: in one group, little or no internal Na is sequestered and in the other a varying amount of Na is sequestered (Bittar, Chen, Danielson, Hartmann & Tong, 1972). Since almost all fibers pre-exposed overnight to insulin showed falling rate constants for Na efflux prior to re-application of insulin *in vitro,* it was deduced that they belong to the second group and that overnight pre-exposure to the hormone *in vivo* somehow leads to activation of the mechanism of sequestration in fibers freshly cannulated and injected with radiosodium in the absence of external insulin. The extent of sequestration before and after re-application of insulin was determined by the slope analysis method of Dick and Lea (1967). As pointed out by these workers, the equation only allows a very rough estimate to be made of the sequestered Na fraction. Furthermore, it could be argued that the method cannot be applied here since loading by microinjection fails to allow enough time for the labelling of the sequestered Na fraction or that the observed change in slope of the efflux plot caused by external insulin does not involve a change in size of the two internal compartments, but does involve two simultaneous effects on k_{23} and k_{21} (membrane rate constant), e.g.

$$Na_3 + Na_2^* \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} Na_3^* + Na_2$$

at equilibrium. From the results given in Table 1 it will be noticed that insulin caused a marked reduction in the size of the fraction of sequestered Na. This approach, however, gave no clue as to whether insulin acts directly by abolishing the mechanism of sequestration or by releasing sequestered Na and then, abolishing the mechanism of sequestration as the result of the rise in myoplasmic free Na concentration. One way of distinguishing between these two possibilities is to inject pre-exposed fibers with graded saline and then apply external insulin. Hence experiments with 0.1 and 1 M solutions of NaCl were done. As illustrated in Fig. 2*a* and *b*, the rate of decline of the Na efflux from a pre-exposed fiber injected with 0.1 M saline was reduced but the fractional rate constant continued to decline though less steeply. The decline stopped only

Table 1. Estimates of the sequestered Na fraction before and after insulin application

Exp. No.	$d/dt \ln [Na^*]_i$ ($\times 10^{-3}$ min^{-1})	$d/dt (\ln d[Na]_i/dt)$ ($\times 10^{-3}$ min^{-1})	% Sequestered
(a) Before Insulin			
25	12.6	24.0	47.5
26	12.2	23.1	47.2
27	12.6	23.0	45.2
28	7.5	23.0	67.4
29	4.8	27.7	82.7
30	9.1	24.8	63.3
			Mean $\pm$ SE 58.9 $\pm$ 6.0
(b) After Insulin			
25	7.1	7.4	4.1
26	5.5	5.9	6.8
27	8.2	7.2	0.0
28	4.4	6.7	34.3
29	3.8	6.1	37.7
30	4.7	9.0	47.8
			Mean $\pm$ SE 21.8 $\pm$ 8.37
			$p < 0.01$

after external application of 1 U/ml insulin. This is taken to mean that insulin acts by abolishing or reducing the extent of sequestration of Na. Fig. 3*a* shows that injection of 1 M NaCl caused a step-down in the Na efflux but no alteration of the efflux rate constant. These kinetic changes are represented in Fig. 3*b* as a step-down and persistence in the decline of the rate constant. Only in the presence of 1 U/ml insulin was the decline abolished. The observed fall in Na efflux following the introduction of 1 M inactive sodium is in line with the view that the efflux in barnacle fibers saturates when the internal free Na concentration is high (Brinley, 1968). The same is true of *Maia* fibers (Bittar *et al.*, 1967). However, as pointed out by Brinley (1968), the ouabain-sensitive Na efflux tends to slow down when $[Na]_i$ is high, presumably because of damage or partial inactivation of the transport system. This could be the case here.

The idea that insulin abolishes the mechanism of sequestration is confirmed by the results summarized in Table 2. By applying the slope ratio method it is clear that the effect of insulin is genuine. For the case where 1 M saline was injected, the slope change caused by insulin

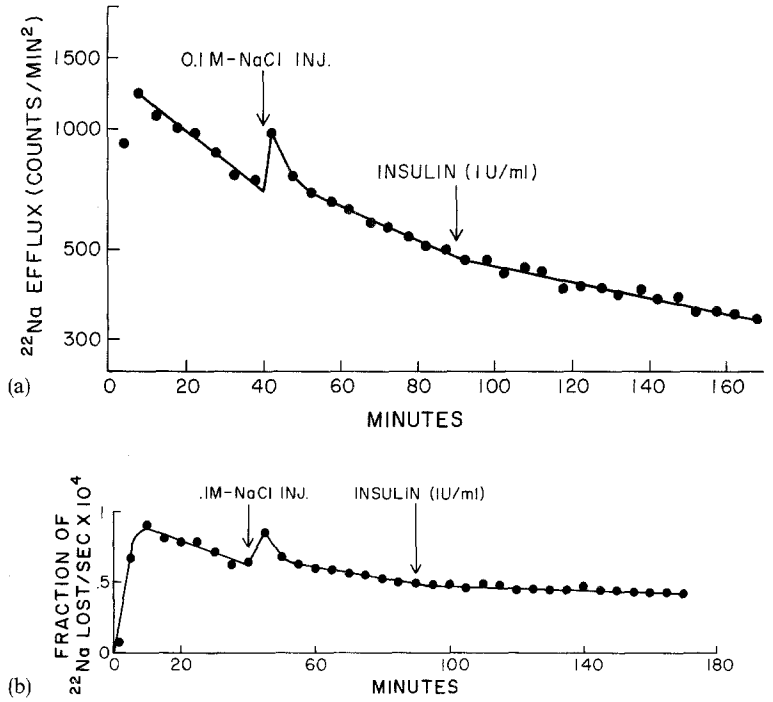

Fig. 2. Effect on Na efflux of internal application of 0.1 M NaCl, followed by external application of 1 U/ml insulin. (*a*) Efflux plot. (*b*) Rate constant plot

in each experiment argues against the idea that the extra sodium added damages the membrane.

Effect on the Na Efflux of Internal Insulin

The next step was to see what happens when insulin is injected. Fig. 4 shows that internal application of a suspension of 100 U/ml insulin caused a prompt rise in the Na efflux followed by a reduced rate of decline. A glance at the rate constant plot confirms that insulin stopped the decline in the fractional rate constant. Thus, the effect of insulin was to mobilize the fraction of sequestered Na, leading to partial saturation of the efflux. As for the step-up in the rate of Na efflux, this is a feature which was not present in the preceding experiments. Estimates of its magnitude gave an average value of $23.2 \pm 6.2\%$ (SEM) ($n=11$). Since it might be argued that these changes in the Na efflux produced by insulin are nonspecific, experiments were carried out with denatured

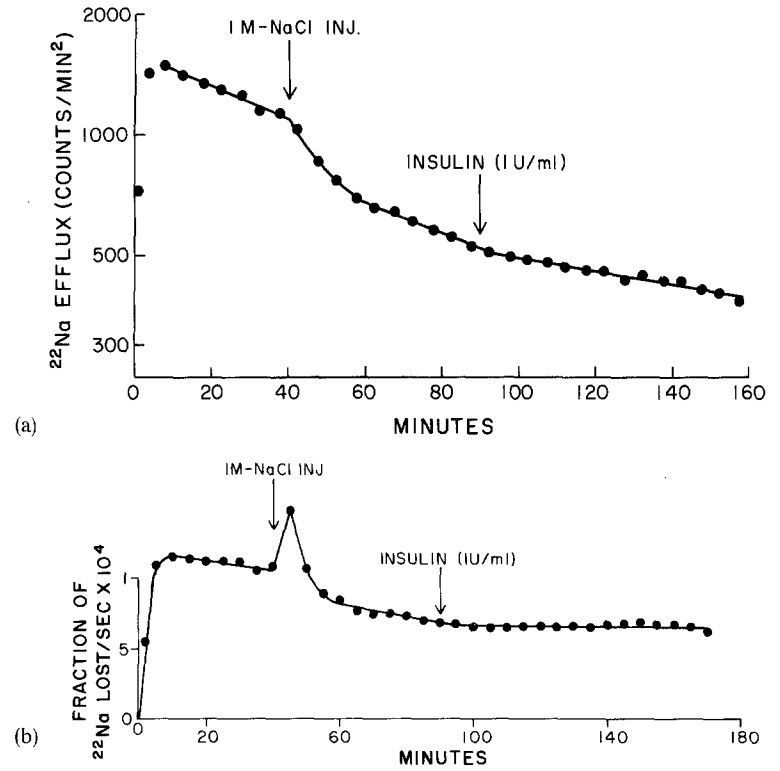

Fig. 3. Effect on Na efflux of internal application of 1 M NaCl, followed by external application of 1 U/ml insulin. (*a*) Efflux plot. (*b*) Rate constant plot

Table 2. Estimates of the sequestered Na fraction before and after injecting NaCl, and before and after applying external insulin

Exp. No.	Before NaCl (% sequestered)	After NaCl (% sequestered)	After insulin (% sequestered)
(a) Experiments with 0.1 M saline			
6	72.3	60.4	36.4
1	31.7	36.6	21.7
2	63.3	60.3	14.6
5	42.4	30.5	18.2
Mean ± SE	52.43 ± 9.32	46.95 ± 7.83	22.73 ± 4.78 $p < 0.05$
(b) Experiments with 1 M saline			
7	38.4	44.2	0
6	66.7	54.3	0
4	45.2	29.0	0
8	37.9	33.3	0
Mean ± SE	47.05 ± 6.76	40.20 ± 5.69	0

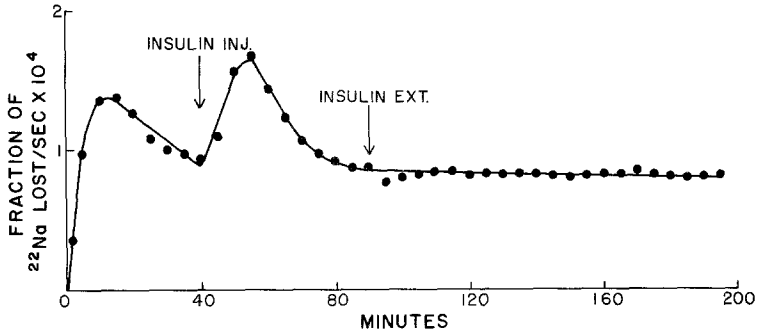

Fig. 4. Effect on Na efflux of internal application of 100 U/ml insulin. Also shown is that external application of 1 U/ml insulin following the development of a new steady state is ineffective

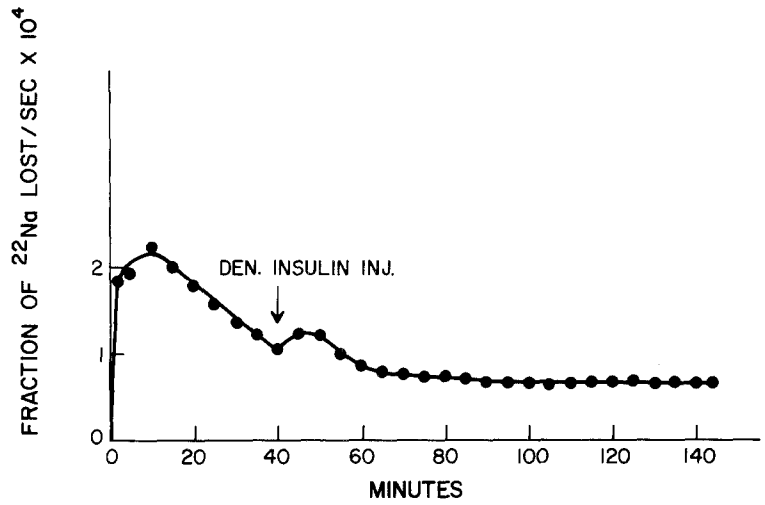

Fig. 5. Effect on Na efflux of internal application of 100 U/ml denatured insulin

insulin. Denaturation of the insulin used was accomplished by boiling for 1 hr. As shown in Fig. 5, internal application of 100 U/ml denatured insulin caused a prompt rise in the Na efflux, followed by a reduced rate of decline. Although these kinetics resembled those obtained with native insulin, the magnitude of the step-up in the rate of Na efflux was considerably smaller, being $10.5 \pm 1.8\%$ ($n=11$). In these circumstances, the effect of external application of 1 U/ml denatured insulin was tried. The two experiments done showed that denatured insulin was without effect.

In view of these results, the problem was to decide whether other proteins may modify the Na efflux in the same way as insulin and

whether overnight exposure of a barnacle to denatured insulin would render its fibers sensitive *in vitro* to external or internal application of native and denatured insulin. For this reason experiments were first done with lysozyme, a protein with a molecular weight of 13,900 daltons and of known structure.

Internal application of a solution of 6×10^{-4} M lysozyme failed to stop the decline in the rate constant for Na efflux. However, the same was not true following internal application of a suspension of 6×10^{-4} M native insulin. The results obtained with the slope ratio method given in Table 3 bear out the view that insulin but not lysozyme reduces the size of the fraction of sequestered Na.

In order to rule out the second possibility, fibers isolated from a barnacle specimen exposed overnight to denatured insulin (the procedure was to inject 40 units of denatured insulin into the *operculum* and add 40 units to 1 liter ASW) were used. It was found in the eight fibers

Table 3. Estimates of the sequestered Na fraction before and after lyosozyme or insulin application

Exp. No.	$d/dt \ln [Na^*]_i$ ($\times 10^{-3}$ min^{-1})	$d/dt(\ln d[Na]_i/dt)$ ($\times 10^{-3}$ min^{-1})	% Sequestered
(a) Before Lysozyme			
1	6.5	13.1	50.4
2	6.0	8.8	31.8
5	5.6	8.5	34.1
6	5.9	15.4	61.7
			Mean 44.5 ± 7.1
(b) After Lysozyme			
1	6.5	13.1	50.4
2	6.0	9.1	34.1
5	5.6	8.6	34.9
6	5.9	16.1	63.4
			Mean 45.7 ± 6.9
(c) After Insulin			
1	5.5	6.7	17.9
2	4.5	5.3	15.1
5	4.7	6.2	24.2
6	4.9	7.2	31.9
			Mean 22.3 ± 3.73
			$p < 0.05$

studied that neither denatured nor native insulin, whether applied internally or externally, exerted any effect on the Na efflux. Another significant feature of these experiments was the absence of a declining rate constant for Na efflux. It is clear, therefore, that the development of sensitivity overnight to insulin is only possible if native insulin is employed.

Response of the Ouabain-Insensitive Na Efflux to Insulin

It now remained to establish whether membrane stimulation caused by injected insulin is the result of increased activity of the membrane (Na^+-K^+)-ATPase system or the ouabain-insensitive Na efflux and whether the ouabain-insensitive Na efflux is responsive to external application of insulin. Fig. 6 shows that external application of 10^{-4} M ouabain caused a large fall in the Na efflux and that internal application of 100 U/ml insulin caused a prompt and fairly appreciable rise in Na loss. The magnitude of the stimulation was in the order of $57.7 \pm 5.3\%$ ($n=6$). This is to be compared with a value of $36.2 \pm 9\%$ ($n=3$) obtained with controls where ouabain-poisoned fibers were injected with 100 U/ml denatured insulin. To exclude the possibility that other proteins, e.g. lysozyme, are able to exert a similar effect on the ouabain-insensitive Na efflux and that the concentration of ouabain used here was insufficient to fully inactivate the membrane (Na^+-K^+)-ATPase system, three experiments were done in which 10^{-3} M ouabain was applied followed by internal application of lysozyme and then insulin. 10^{-3} M ouabain failed to reduce any further the remaining Na efflux, and lysozyme but not insulin failed to stop the decline in the fractional rate constant. Both lysozyme and insulin caused a step-up in the rate of Na efflux. With

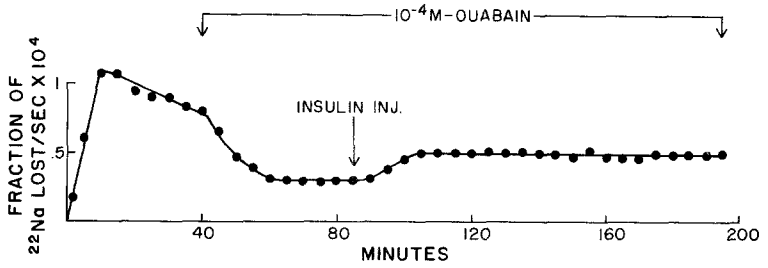

Fig. 6. Effects on Na efflux of external application of 10^{-4} M ouabain, followed by internal application of 100 U/ml insulin

lysozyme the rise was in the order of $51.0 \pm 13.8\%$ and with insulin $54.8 \pm 19.2\%$ ($n=3$). Since similar findings have been reported with the microinjection of troponin and myosin (Bittar, Tong & Greaser, 1973), one cannot avoid the conclusion that the transitory step-up in efflux caused by injected insulin is nonspecific and very likely to be due to chelation of internal free Ca^{2+}.

The effect of external application of insulin on the ouabain-insensitive Na efflux was next examined. External application of 1 U/ml insulin, as shown in Fig. 7*a*, to a fiber treated with 10^{-4} M ouabain, caused a delayed rise in the remaining Na efflux. This effect was in the order of $158.3 \pm 58.6\%$ ($n=4$). A similar effect was found with injected insulin (in the presence of external insulin), as shown in Fig. 7*b*. The magnitude of this effect averaged $94.7 \pm 16.7\%$ ($n=3$). It is clear, therefore, that the sites of action of external and internal insulin are not the same. The finding that external insulin stimulates the ouabain-insensitive Na efflux is in accord with the concept that the initial step underlying insulin action involves interaction of the hormone with a receptor lying on the external side of the cell membrane. On the basis of this result, an attempt was made to establish the minimal concentration of external insulin required to stimulate the ouabain-insensitive Na efflux. Shown

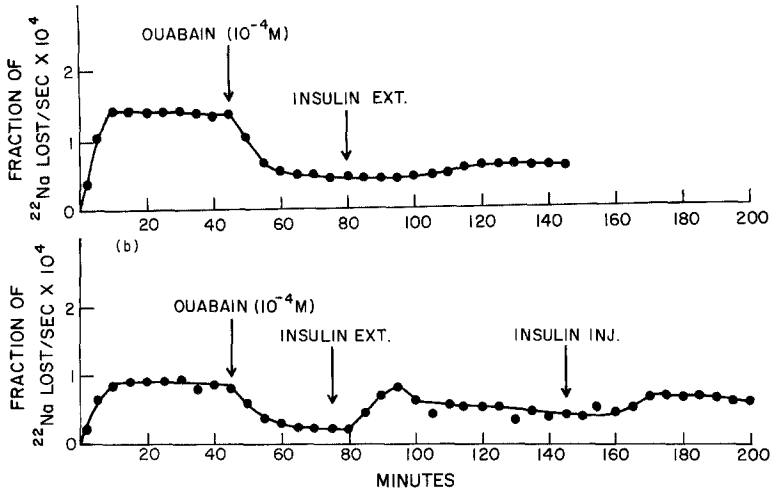

Fig. 7. (a) Effect on the ouabain-insensitive Na efflux of external application of 1 U/ml insulin. (*b*) Effects on the ouabain-insensitive Na efflux of external application of 1 U/ml insulin followed by internal application of 100 U/ml insulin. First arrow indicates time at which 10^{-4} M ouabain was applied externally. Ouabain was not omitted following treatment with insulin

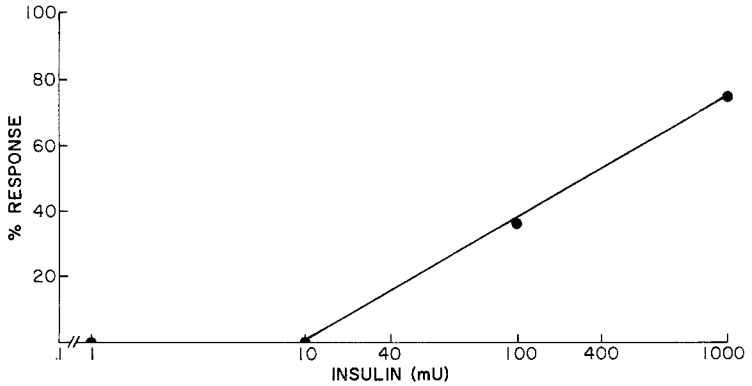

Fig. 8. Dose-response curve for the effect of external insulin on the Na efflux following inactivation of the membrane ($Na^+ - K^+$)-ATPase with 10^{-4} M ouabain. Each point represents the mean of data obtained from four experiments. The fibers used were isolated from the same barnacle specimen

in Fig. 8 is the dose-response curve. The main point of interest is that if a concentration of insulin in the region of 0.01–0.1 U/ml is the minimal effective concentration, then this is not very different from that reported by Moore (1973) or Grinstein and Erlij (1974) for unpoisoned frog muscle.

Response of the Ouabain-Insensitive Na Efflux to Internal Application of Guanosine Triphosphate

Illiano, Tell, Siegel and Cuatrecasas (1973) produced evidence that insulin causes a rise in cGMP concentration in isolated fat cells and liver cells. This observation raised the possibility that overnight exposure of the barnacle to insulin (0.04 U/ml) results in increased guanylate cyclase system activity. A direct way of obtaining information regarding this possibility is to microinject graded amounts of GTP and find out whether the response of the ouabain-insensitive Na efflux to GTP in pre-exposed fibers differs from that of unexposed fibers. Microinjection of 0.5 M GTP was found to cause a marked rise in the ouabain-insensitive Na efflux in pre-exposed fibers. The magnitude of this effect was in the order of $606.7 \pm 20.6\%$ ($n=6$). In conspicuous contrast, unexposed fibers pretreated with 10^{-4} M ouabain responded to internal application of 0.5 M GTP by showing $236.4 \pm 20.6\%$ stimulation ($n=6$) ($p<0.02$).

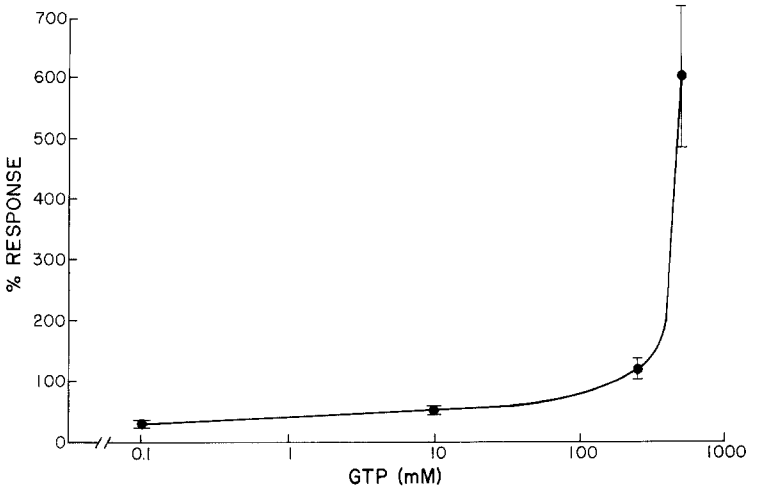

Fig. 9. Dose-response curve for the effect of internal application of GTP on the Na efflux following inactivation of the membrane ($Na^+ - K^+$)-ATPase with 10^{-4} M ouabain. Each point represents the mean of data obtained with GTP from the following number of experiments: 10^{-4} M, $n=5$; 10^{-2} M, $n=4$; 2.5×10^{-1} M, $n=4$; and 5×10^{-1} M, $n=6$

Given in Fig. 9 is the dose-response curve, which shows quite clearly that micromolar amounts or less of GTP when added to the myoplasm (i.e. assuming 100-fold dilution) can cause a rise in the ouabain-insensitive Na efflux. This is found to be of special significance in the light of the fact that the concentration of GTP in cells is reported to be in the region of 0.5 mM.

The next question to be asked was whether the response of the ouabain-insensitive Na efflux to external insulin is altered in fibers enriched with GTP. The answer to this question is as follows: (*i*) fibers pretreated with 10^{-4} M ouabain and injected with 0.1 M GTP showed $36.1 \pm 10.9\%$ stimulation by 1 U/ml insulin ($n=4$); controls showed $33.1 \pm 7.7\%$ stimulation ($n=3$). (*ii*) Fibers injected with 0.25 M GTP showed $29.1 \pm 12.8\%$ stimulation by insulin ($n=2$); controls showed $58.7 \pm 0.6\%$ stimulation by insulin ($n=2$). And (*iii*) fibers injected with 0.5 M GTP showed $5.5 \pm 5.5\%$ stimulation by insulin ($n=2$); controls showed $69.2 \pm 36.1\%$ stimulation by insulin ($n=2$). Though the differences here were not statistically significant, the trend is clearly towards reduced sensitivity to insulin as the myoplasmic GTP concentration is gradually raised. This was to be expected if phosphorylation of the fiber membrane prior to re-application of insulin leads to reduced accessibility of the insulin-receptor as the result of conformational changes in the membrane.

Response of the Ouabain-Insensitive Na Efflux to External Application of $CaCl_2$ Before and After Fiber Enrichment with GTP

Guanylate cyclase activity is reported to be present in almost all tissues hitherto examined, including those of arthropods (Kuo, Wyatt & Greengard, 1971). The enzyme, unlike the adenyl cyclase system, is loosely bound to the cell membrane. Ca^{2+} is known to act as a regulator of its activity; so does Mn^{2+}, which is a more potent regulator (Chrisman, Garbers, Parks & Hardman, 1975; Garbers, Dyer & Hardman, 1975). Calcium, however, as shown by these workers, can act as an inhibitor when the GTP concentration is high. In order to strengthen the argument that insulin induces *de novo* synthesis of guanylate cyclase or activates the system, experiments were done by microinjecting $CaCl_2$ into pre-exposed fibers. The experiments fall into four groups. In the first, 1 M $CaCl_2$ was injected following inactivation of the membrane $(Na^+ - K^+)$-ATPase with 10^{-4} M ouabain. The results showed $303.3 \pm 100.7\%$ stimulation ($n=4$). Controls showed $208.2 \pm 27.5\%$ stimulation ($n=6$) ($p>0.3$). The lack of a difference is in line with what one might expect if preexposed fibers have a higher GTP concentration than unexposed fibers.

In the second group of experiments, 1 M $CaCl_2$ was injected following enrichment of ouabain-poisoned fibers with 0.5 M GTP. Shown in Fig. 10*a* is the result of such an experiment. It is at once clear that the size of the response to injected $CaCl_2$ is small. Estimates of the stimulation gave an average figure of $18.4 \pm 8.8\%$ ($n=4$). A similar pattern was seen with unexposed fibers, the size of the stimulation caused by $CaCl_2$ being $62.03 \pm 16.5\%$ ($n=4$) ($p<0.1$). Not only is this result compatible with the see-saw theory of control of guanylate cyclase activity by GTP and Ca^{2+}, but also with the view that GTP enriched fibers, whether pre-exposed to insulin or not, show a slight stimulatory response following a fall in myoplasmic pCa because of activation by Ca^{2+} of the phosphorylase kinase system (Bittar *et al.*, 1972). The observation that fibers enriched with 0.5 M GTP start contracting some 15–20 min after injection is important for the light it sheds on the problem whether the cGMP formed causes a fall in myoplasmic pCa and hence activation or suppression of guanylate cyclase activity. This possibility which remains unexplored may partly explain why the stimulation caused by injected GTP is so great and somewhat sustained. To confirm the see-saw relationship, the third group of experiments was carried out by microinjecting GTP following $CaCl_2$. Fig. 10*b* shows that an injection of 0.5 M GTP

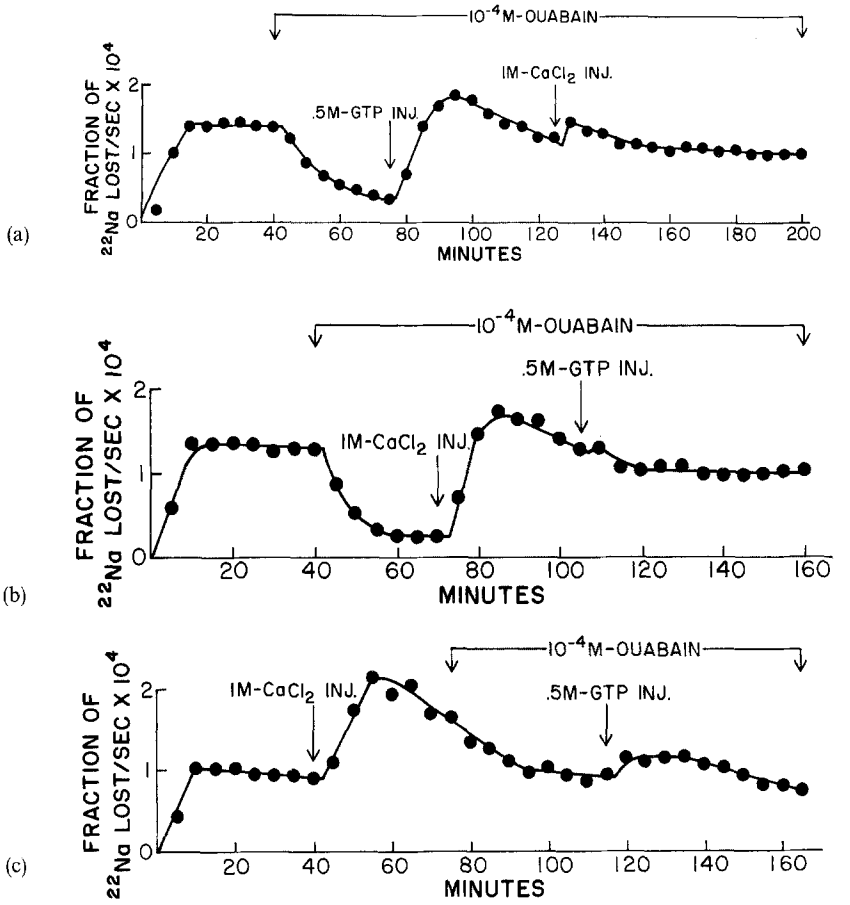

Fig. 10. (*a*) Effects on the Na efflux of external application of 10^{-4} M ouabain, followed by internal application of 0.5 M GTP and 1 M $CaCl_2$. (*b*) Effects on the Na efflux of external application of 10^{-4} M ouabain, followed by internal application of 1 M $CaCl_2$ and 0.5 M GTP. (*c*) Lack of effect of 10^{-4} M ouabain on the Na efflux following internal application of 1 M $CaCl_2$. Also shown is the effect of internal application of 0.5 M GTP following addition of ouabain to the external medium

had only a small stimulatory effect on the ouabain-insensitive efflux. Estimates gave an average figure of $29.0 \pm 11.4\%$ ($n=4$) (controls: $12.1 \pm 8.5\%$, $n=4$).

The fourth group of experiments was designed in such a way as to make it possible to test the idea that the stimulated Na efflux following a fall in myoplasmic *p*Ca is refractory to 10^{-4} M ouabain in the same way as a phosphorylated membrane is found to be following stimulation of the Na efflux by injecting cAMP (Bittar, Chambers & Schultz, 1976). Thus, when 1 M $CaCl_2$ was injected, followed by 10^{-4} M ouabain (ext)

and 0.5 M GTP (int), the results were as follows: $86.0 \pm 26.0\%$ stimulation by $CaCl_2$ and $44.6 \pm 23.3\%$ stimulation by GTP ($n=4$). Ouabain as shown in Fig. 10*c* was without any effect. Controls (i.e. unexposed fibers) showed $43.3 \pm 0.2\%$ stimulation by $CaCl_2$ but no effect whatsoever following external application of ouabain or injection of GTP ($n=2$).

Discussion

The results described in this paper show that it is possible to render barnacle fibers sensitive to insulin by simply pre-exposing the barnacle *in vivo* long enough to a large dose of the hormone. The mechanism underlying the development of insulin sensitivity is not yet known but it could be the same as that observed in *Maia* fibers treated with insulin (Bittar, 1967) and barnacle fibers treated with aldosterone (Bittar & Tallitsch, 1975*a*), namely a mechanism that is abolished by actinomycin D. However, barnacle fibers do not seem to exhibit the same pattern of sensitivity to insulin as *Maia* fibers. For example, injected insulin stimulates the Na efflux in pre-exposed barnacle fibers, whereas it inhibits the efflux in *Maia* fibers. Moreover, external application of insulin to barnacle fibers following internal application is without effect. This is not the case in *Maia* fibers since external insulin causes stimulation following suppression of the efflux by internal application of the hormone. The simplest way of explaining the results obtained with barnacle fibers is that overnight exposure of the barnacle specimen leads to induction or activation of a receptor system to which external or internal insulin is accessible. It may be that the induced receptor system lies in membranes that belong to the same compartment that sequesters Na, e.g. the sarcoplasmic reticulum (SR), where protein synthesis supposedly takes place and where the bulk of the internal Na in skeletal muscle has been reported to be located (Rogus & Zierler, 1973). Failure of injected saline to stop the decline in the fractional rate constant for Na efflux suggests that the rise in myoplasmic free Na concentration resulting from the release of sequestered Na does not bring about complete abolition of the mechanism of sequestration. Only in the presence of insulin is total abolition seen. This result differs from that obtained with aldosterone (Bittar & Tallitsch, 1975*b*). It will be recalled that injected saline promptly stopped the decline in the rate constant in fibers pre-exposed overnight to aldosterone. The underlying reason for this difference is unclear. Why, on the other hand, denatured insulin is able to mimic

the effect of native insulin only when it is injected is a matter for surprise. It could be that denatured insulin still carries a net negative charge or that boiling fails to disrupt the binding site of the insulin molecule.

The experiments with ouabain yielded data which give support to the view that the primary site of insulin action is not the membrane ($Na^+ - K^+$)-ATPase system (Rogus, Price & Zierler, 1969; Clausen, 1975). However, other workers have found that insulin stimulates this system *in situ* (Moore, 1973; Grinstein & Erlij, 1974) or in the isolated form (Brodal, Jebens, Öy & Iversen, 1974). In a more recent paper, Gavryck, Moore and Thompson (1975) point out that a reduction in ATP concentration from 2 to 0.5 mM is enough to double the stimulatory action of insulin on ($Na^+ - K^+$)-ATPase isolated from frog muscle. This is an observation which could account for the failure of insulin in the present work to stimulate the ouabain-sensitive Na efflux if it were only assumed that pre-exposed fibers have a high myoplasmic ATP content. An alternative explanation is that pre-exposed fibers have a high internal Na concentration particularly following re-application of insulin *in vitro* and hence the membrane ($Na^+ - K^+$)-ATPase system is already maximally activated. This interpretation agrees with that of Gavryck and co-workers who reported not only failure of ouabain to abolish the insulin effect when the Na concentration was raised but also failure of insulin to stimulate the enzyme when Na was high.

Other actions have been ascribed to insulin, e.g. inhibition of the membrane adenylate cyclase system (Walaas, Walaas & Grønnerød, 1972) and stimulation of the phosphodiesterase system (Woo & Manery, 1973). The experiments, however, carried out with GTP lead to the conclusion that the problem of how insulin acts is closely connected with the guanylate cyclase system. Granted that both lines of argument, that of the observed effects of GTP and Ca^{2+}, are not quite conclusive, it at least seems now more certain that guanylate cyclase rather than adenylate cyclase or phosphodiesterase is the control-point affected by insulin. Thus, a plausible explanation for the lack of an insulin effect on the ouabain-sensitive Na efflux may lie in the Yin-Yang theory advanced by Goldberg and co-workers (1975). This theory, in its simplest form, states that cAMP and cGMP exert dualistic actions on cell function. The objection may immediately be raised that this theory is inapplicable since both cyclic nucleotides stimulate the ouabain-insensitive Na efflux in barnacle fibers (Bittar, Hift, Huddart & Tong, 1974). This objection can be met on the grounds that if insulin increases guanylate cyclase activity, then the concentration of myoplasmic GTP would tend to fall

off, and as a result, there would be reduced adenylate cyclase activity. This is a likely explanation particularly since it is known from the work of Rodbell, Birnbaumer, Pohl and Krans (1971) that GTP is a necessary effector of the adenylate cyclase system (in isolated fat cells). Whether insulin also increases the amount of cGMP-dependent protein kinase and protein modulator is not yet known, but this possibility cannot be overlooked in view of the work of Donnelly, Kuo, Reyes, Liu and Greengard (1973) showing that both proteins are found in a wide variety of tissues, e.g. lobster tail muscle.

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