tainer tubes. Hematocrits and ESR values were determined by procedures developed at Memorial Sloan-Kettering Cancer Center (*9*). The cancer detection clinic did not accept patients presenting any specific or general health complaints. Such individuals were referred to the adjoining Memorial Center outpatient clinic for comprehensive examination and further appropriate medical action. Blood samples obtained from patients with breast lesions were tested daily for ESR and other parameters, along with other blood samples taken from all patients examined in the medical center. Thus, identification of the patients and matching of their diagnosis and ESR occurred days or weeks later, following hospitalization and biopsy or surgery. The breast cases were later segregated from the other patients, and their specific diagnoses were tabulated and correlated with the "blind" ESR findings. All diagnoses were established through standard pathological procedures.

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Microtubule Assembly and the Intracellular Transport of Secretory Granules in Pancreatic Islets

Abstract. *Polymerized and depolymerized tubulin were measured in pancreatic islets under various physiological and pharmacological conditions. Variations in insulin release from islets of fed or fasted rats were accompanied by concomitant changes in tubulin polymerization. Glucose induced the formation of microtubules in vitro independent of extracellular calcium. Total and polymerized tubulin content were decreased by fasting and restored by glucose feeding.*

Microtubules have been implicated in various secretory processes occurring via exocytosis (*1, 2*), but the mechanism of their involvement remains obscure. Most of these reports are based on the observation that agents which disrupt the microtubules, such as colchicine and vinblastine, inhibit the specific secretory activity under study. On the basis of the observation that colchicine inhibits the second phase of glucose-induced insulin release, Lacy proposed that the vectorial transport of insulin granules to the cell membrane occurs along a microtubular-microfilamentous network (*1, 3*). In analogy to other microtubule-dependent movements (*4*), it seemed possible that the second and sustained phase of insulin secretion may be regulated, at least in part, by the degree of tubulin polymerization. To test this possibility, a method has been developed in our laboratory to measure the cellular pools of polymerized (that is, microtubules) and depolymerized tubulin (*5*). The sensitivity of this technique permits measurement of as little as 40 ng of tubulin, an amount present in about ten isolated rat islets.

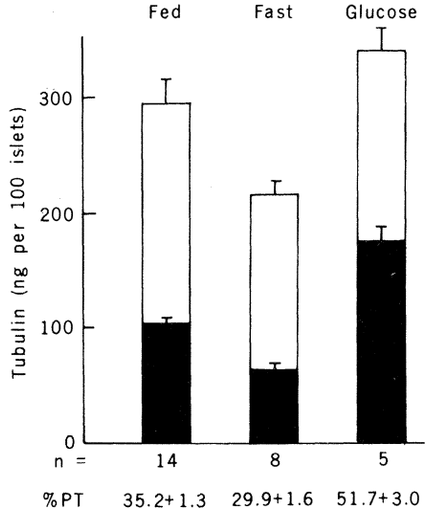

Fig. 1. Total tubulin (open columns) and polymerized tubulin (closed columns), measured in islets from fed, 72-hour fasted and dextrose fed rats. The degree of tubulin polymerization (percent PT) represents polymerized tubulin (SN-2)/total tubulin (SN-1 + SN-2). All values represent means ± the standard error of the mean; the significance of differences was measured by Student's *t*-test.

After isolation (*6*) or incubation under varying experimental conditions, 200 rat islets were homogenized in 125 μl of a microtubule-stabilizing solution (MTS) (*7*) and centrifuged at 8500*g* for 10 minutes at room temperature. Depolymerized tubulin was assayed in duplicate 25- μl portions of the supernatant fraction (SN-1) by a modification of a previously described colchicine binding assay (*8*) with 0.12 nmole of colchicine containing 5 nc of ^{3}H-labeled colchicine and incubating for 150 minutes at 37°C. The pellet (PP-1), containing the precipitated microtubules, was resuspended in 75 μl of ice-cold microtubule depolymerizing solution (TS) (*7*), and centrifuged at 8500*g* for 10 minutes at 4°C; and the tubulin activity was assayed in duplicate in the resultant supernatant fraction (SN-2). Colchicine binding activity was found exclusively in SN-1 and SN-2 and coeluted with ^{125}I-labeled tubulin (*9*) on a Bio-Gel A-5m column. No detectable colchicine binding activity was observed in the second precipitate (PP-2) when resuspended in 75 μl of TS. Furthermore, when sections of PP-1 and PP-2 were examined by electron microscopy, microtubules were readily apparent in PP-1, and disappeared completely after exposure to the TS solution. When islets were exposed to 4°C for 20 minutes prior to homogenization in MTS, colchicine binding activity of SN-1 was significantly increased and that of SN-2 was decreased, an indication of the lability of islet microtubules at low temperature comparable to that reported in other systems (*10*).

The total tubulin content of islets obtained from fed rats averaged 295.3 ± 19.5 ng of tubulin per 100 islets of which 35.2 ± 1.3 percent (104.16 ± 3.85 ng per 100 islets) was in the polymerized form (Fig. 1). In rats fasted for 72 hours, total tubulin decreased about 27 percent (217 ± 13 ng per 100 islets; $P < .02$), and polymerized tubulin decreased to an even greater extent (64.55 ± 3.74 ng per 100 islets; $P < .01$). When rats were maintained exclusively on a solution of 30 percent dextrose drinking water in 0.2 percent saline, total tubulin increased slightly but not significantly, whereas polymerized tubulin increased approximately 70 percent (175.82 ± 11.39 ng per 100 islets; $P < .001$). These changes in microtubule content parallel comparable alterations in the insulin secretory response to glucose observed either in vivo or with isolated rat islets under similar conditions (*11, 12*).

To determine the role of glucose in modulating the polymerization of tubulin, islets

from fed and fasted rats were incubated for 2 hours in the presence and absence of glucose (300 mg/100 ml). Under these conditions, no detectable change in the total tubulin content was noted during the course of incubation. As shown in Fig. 2, this amount of glucose increased polymerized tubulin (the change was 46.59 ± 3.92 ng per 100 islets; $P < .01$) and stimulated insulin release (the change was 152.02 ± 6.85 microunits per islet per 120 minutes). This glucose effect on tubulin polymerization was not dependent upon extracellular calcium, and was not influenced by cycloheximide at a concentration (5 μg/ml) sufficient to inhibit protein synthesis greater than 95 percent. The addition of theophylline had no detectable effect on polymerized tubulin content, but resulted in a further increase in insulin release (about 23 percent; $P < .01$). In fasted rats, glucose at 300 mg/100 ml elicited an impaired insulin response (the change was 65.3 ± 7.0 microunits per islet per 120 minutes) and was not associated with any detectable change in polymerized tubulin. This finding and similar observations by others (*11–13*) that glucose-induced insulin release during prolonged incubation (90 to 120 minutes) is severely depressed by fasting, is consistent with the concept that the second phase of insulin secretion is a microtubule-dependent process. This proposal is further supported by the finding that theophylline normalized glucose-induced insulin release (Fig. 2) [as has been found previously in vivo (*11*) and in vitro (*13*)] and increased polymerized tubulin about 47 percent in fasted islets (the change was 43.78 ± 3.92 ng per 100 islets; $P < .01$). The low content of polymerized tubulin in fasted islets might be attributed to the reported decrease in concentrations of cyclic adenosine monophosphate (*14*), adenylate cyclase, and cyclic AMP-dependent protein kinase (*15*) under these conditions.

Insulin release during prolonged glucose stimulation depends on the synthetic activity of the beta cell (*16*) and on the rates of intracellular migration of insulin granules to the cell periphery and their fusion with the plasma membrane (*17*). The inhibition of glucose-induced insulin release by colchicine has been attributed to an impaired transport of beta granules to the cell membrane, since disruption of microtubules does not affect glucose-induced insulin synthesis (*18*) or inhibit fusion of the beta granule with the plasma membrane after stimulation with tolbutamide (*3*). To define more explicitly the role of polymerized tubulin in the intracellular transport of granules, the release of newly synthesized insulin (labeled with [^{3}H]leucine) was compared to that of total insulin (determined by immunoassay) under conditions where the degree of tubulin polymerization was varied. If microtubules are involved in the intracellular transport of insulin (*1*) and there is a temporal sequence favoring the release of peripheral and previously synthesized hormone (*19*), it would be anticipated that the release of newly synthesized hormone, to be transported from the endoplasmic reticulum to the plasma membrane, would be more affected by microtubule disruption than would total immunoassayable hormone secretion. To further accentuate this differential effect, a concentration of colchicine (0.3 m*M*) was selected which produced a significant but minimal inhibition of glucose-induced insulin release as measured by immunoassay. A decrease in polymerized tubulin, as seen in fasted or colchicine-treated islets (Table 1), is associated with a greater decrease in ^{3}H-labeled (pro)insulin release than in total (by immunoassay) insulin secretion and supports the view that microtubules are involved in hormone transport.

Table 1. Groups of 50 islets were incubated for 30 minutes in 500 μl of bicarbonate-buffered Krebs-Ringer medium, containing 50 μc of L-[^{3}H]leucine. After the islets were washed, incubation was continued for 90 minutes under the same experimental conditions in the presence of 1 m*M* unlabeled leucine. Total ^{3}H-labeled (pro)insulin synthesis and the release of labeled hormone during the 90-minute incubation were determined as described (*20*). ^{3}H-labeled (pro)insulin release is expressed as the percentage of total ^{3}H-labeled (pro)insulin synthesis. Numbers in parentheses indicate the number of tests.

Experimental conditions			Hormone release	
Source of islets	Glucose (mg/100 ml)	Colchicine (m*M*)	[^{3}H](pro)insulin (percent)	Immunoreactive insulin (microunit/islet)
Fed rats	0	0		21 ± 3 (5)
	300	0	8.75 ± 0.85 (8)	141 ± 13 (8)
	300	0.3	3.53 ± 0.80* (5)	109 ± 14† (5)
Fasted rats	300	0	2.92 ± 0.58* (3)	91 ± 9* (3)

*$P < .01$; †$P < .025$.

These studies demonstrate that tubulin exists in a dynamic equilibrium between polymerized and depolymerized forms in pancreatic islets. Known insulin secretagogues, such as glucose and theophylline, alter this equilibrium significantly in favor of microtubule formation. Both total tubulin and the degree of tubulin polymerization are significantly decreased in islets of rats fasted for 72 hours, and this effect is completely reversed by dextrose feeding.

These observations suggest that modulation of the degree of tubulin polymerization in pancreatic islets represents another mechanism by which various physiological factors and pharmacological agents may regulate insulin secretion.

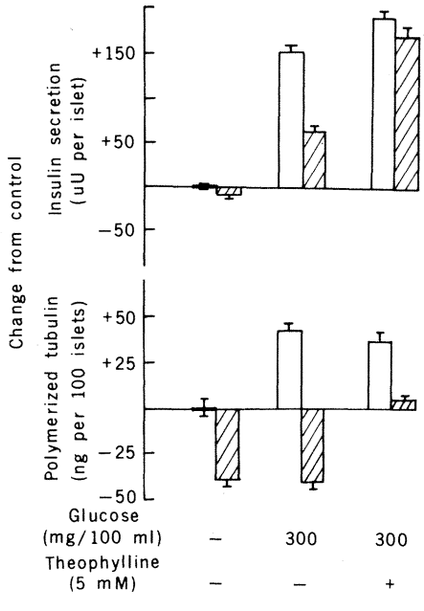

Fig. 2. Islets from fed (open columns) and fasted (hatched columns) rats were incubated for 120 minutes at 37°C in bicarbonate-buffered Krebs-Ringer medium (*p*H 7.40). The total insulin release and polymerized tubulin are expressed as the change from the controls. Control values were obtained from islets isolated from fed rats and incubated for 120 minutes in the absence of glucose. All values represent means ± the standard error of the mean in four to ten experiments.

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Extracellular Potassium Accumulation and Inward-Going Potassium Rectification in Voltage Clamped Ventricular Muscle

Abstract. *Measurements of afterpotential, action potential duration, and output of a potassium-sensitive microelectrode indicate that the application of long clamp pulses (1 to 8 seconds) to frog ventricular muscle is accompanied by a change in the extracellular potassium concentration. The plot of the magnitude of the potassium accumulation against the clamped membrane potential yields an N-shaped relation similar to the "steady state" current-voltage relation. The accumulation studies confirm a strong inward-going (anomalous) potassium rectification.*

It is generally accepted that K efflux plays an important role in the repolarization of the cardiac action potential. Attempts to measure time-dependent changes in the radioactive K efflux during a single action potential have been made by using rapid perfusion. The results, however, indicated that diffusion across an unstirred extracellular space delays the efflux to the perfusing solution up to several seconds (*1*). The presence of a diffusion barrier may lead to poor time resolution of flux measurements, false low estimates of unidirectional transmembrane fluxes, and accumulation of K in the extracellular space.

In the experiments reported here, we have attempted to measure changes in the extracellular K concentration and relate them to the net membrane currents measured during a voltage clamp step. To estimate these changes, we have taken advantage of the specific effect of K on the resting potential (*2*) and on the duration of the action potential (*3*) of frog ventricular muscle. In some experiments the K activity was directly measured with a K-sensitive microelectrode (*4*) inserted into the preparation.

Strips of frog ventricular muscle (0.3 to 0.5 mm in diameter) were mounted in a single sucrose gap voltage clamp setup (*5*). Isometric tension, transmembrane potential, and membrane current were monitored simultaneously from the segment of muscle (length $\simeq$ 0.5 mm) bathed in Ringer solution (116 m*M* NaCl, 3 m*M* KCl, 2 m*M* $NaHCO_3$, and 0.2 m*M* $CaCl_2$). The other end of the muscle was depolarized with 120 m*M* KCl solutions. All experiments were performed at room temperature (20° to 24°C). In these experiments no corrections were made for "leakage current" across the sucrose gap or for potential drop across the series resistance. The preparation was considered to be satisfactory only if the current trace was smooth, without any indication of an inverted action potential, and the tension trace was free of transient contractile responses and well maintained during the clamp pulse.

Figure 1, A to D, shows voltage clamp steps to various potentials and the accompanying contractions and membrane currents superimposed on the normal action potential and contraction. The amplitude and time course of the afterpotential are monitored after releasing the clamp by opening the feedback loop. Five superimposed clamp steps are shown in each panel. In Fig. 1E the afterpotentials from A to D are plotted as functions of the clamp duration. In Fig. 1A, where the membrane is hyperpolarized from rest (from –85 to –106 mv), a hyperpolarizing afterpotential develops rapidly while the inward current decreases. In Fig. 1B the membrane is depolarized to –43 mv; the outward current is fairly constant, and the afterpotentials seem to approach a constant value. Clamp pulses of 2 seconds or less, which are accompanied by fairly con-

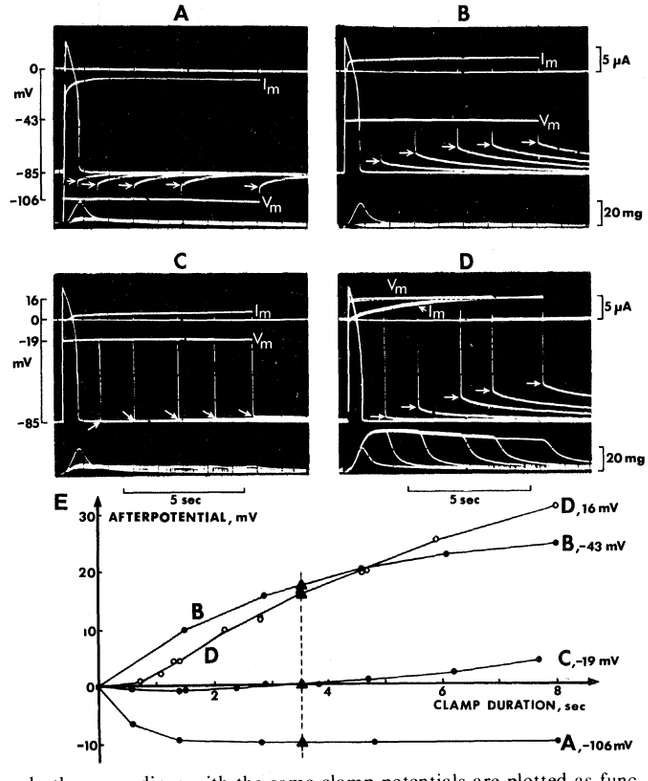

Fig. 1. Afterpotential as a function of clamp duration for four different clamp potentials: (A) –106 mv, (B) –43 mv, (C) –19 mv, and (D) +16 mv). In each panel five clamp pulses and the associated membrane currents and contractions are superimposed on a normal action potential and contraction. The continuous horizontal line near the top of each panel is the zero current trace. The current trace is labeled I_m and the membrane potential during the clamp is labeled V_m. The afterpotentials are measured shortly after release of the clamps as the difference between the resting potential and the potentials at the sharp bends indicated with arrows. Bottom traces are contraction recordings. (E) Afterpotentials from the recordings in (A) to (D) and other recordings with the same clamp potentials are plotted as functions of clamp duration. The dashed line indicating a clamp duration of 3.4 seconds intersects the curves in points marked with triangles and corresponding to values plotted in Fig. 2.