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THE POTENTIATING INFLUENCES OF INSULIN ON PANCREOZYMIN-INDUCED HYPERPOLARIZATION AND AMYLASE RELEASE IN THE PANCREATIC ACINAR CELL

BY T. KANNO AND A. SAITO
From the Department of Physiology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, *Japan*

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SUMMARY

1. Insulin (1 u./ml.) potentiated the release of amylase from isolated pancreas of rats perfused with erythrocyte-containing medium and stimulated by 0·5 m-u. pancreozymin (Pz)/ml., whereas the same concentration of insulin failed to potentiate the response evoked by 5 or 200 m-u. Pz/ml.

2. Intracellular measurement of membrane potentials from the acinar cells of the same preparations showed that insulin (1 u./ml.) simultaneously potentiates the hyperpolarization and amylase release in response to 0·5 m-u. Pz/ml.

3. These effects of insulin on the Pz-induced responses were inhibited by ouabain (3×10^{-5} M).

4. The results suggest that insulin, in the concentration studied, has a potentiating action on the activity of an electrogenic Na pump, which maintains the hyperpolarization and amylase release during continuous stimulation with Pz.

5. Insulin also potentiated the Pz-induced amylase release in the rat pancreata *in situ* after vagotomy and ligature of the pyloric region.

INTRODUCTION

More than one hundred years have elapsed since the endocrine pancreas was first described in Paul Langerhans' thesis (1869), and it is now well known that, in birds and mammals, well defined islets of endocrine tissue (the islets of Langerhans) are scattered throughout the entire pancreas. This morphological arrangement of the endocrine and exocrine pancreas is the basis of Henderson's hypothesis (1969) that high concentrations of the islet hormones, insulin and glucagon, might play a significant role in

the secretory response of the exocrine pancreas. Supporting this is the recent observation showing a favourable morphological arrangement; direct connexions between the blood capillaries of the islets and the capillaries of the acinar parenchyma in a wide variety of animal species (see Ando, 1959; Henderson, 1969; Youngs, 1972; Fujita & Watanabe, 1973).

While it has been recently reported that glucagon produces a depression of enzyme release in the stimulated pancreas (Dyck, Rudick, Hoexter & Janowitz, 1969; Dyck, Texter, Lasater & Hightower, 1970; Nakajima & Magee, 1970; DiMagno, Go & Summerskill, 1973; Wizemann, Weppler & Mahrt, 1974), the effect of insulin on the release of pancreatic enzymes has not yet been settled and this is the main purpose of the present experiments. For the present purpose, the pancreas *in situ* is not a suitable preparation, since insulin acts not only on the pancreas *per se* but also on a large variety of other organs and tissues. Danielsson (1974) and Danielsson & Sehlin (1974) have recently incubated pieces of pancreas for *in vitro* studies of the effect of insulin on the secretion from the exocrine pancreas. However the relatively low rates of secretion and the slow responses of the gland to alteration in its environment, limit the usefulness of this preparation (see Kanno, 1974). The isolated rat pancreas, perfused with Krebs–Henseleit solution containing erythrocytes, overcomes these difficulties: the ratio of the maximal rate of amylase release in response to 5 m-u. pancreozymin (Pz)/ml. to the rate of release before stimulation is higher (10–26) using the perfused pancreas than when pieces of pancreas are incubated (Kanno, 1975). And the ratio is still higher (40–50) when the isolated pancreas is perfused with a medium containing 8 vol. % erythrocytes (Kanno, Suga & Yamamoto, 1976). Using this preparation, we have found that insulin augments the hyperpolarizing and secretory effects of a low concentration of Pz. This influence of insulin on the secretory effect of Pz has also been demonstrated in the rat pancreas *in situ:* Pz-induced release of amylase was enhanced and tachyphylaxis of the secreting response to successive stimulation with Pz was inhibited in the presence of insulin.

METHODS

Isolation and perfusion of the pancreas. The experiments were performed on isolated and perfused pancreata or on pancreata *in situ* of Sprague–Dawley strain male rats weighing about 250 g. The rats were fasted for 24 h before the experiments but were allowed water. The isolated and perfused pancreas was prepared as follows. The *flushing preparation* was used except when the rate of flow of pancreatic juice was measured. The *draining preparation* was used when the rate of amylase release and the rate of juice flow were simultaneously measured. Under ether anaesthesia and after cannulation, the vascular system and the common duct were separately perfused. In both preparations, the inlets of the vascular perfusion were the superior

mesenteric artery and the coeliac artery, and the outlet was the protal vein. The rate of vascular flow of both preparations was kept constant at 1 ml./min with the aid of a roller pump. In the flushing preparation, the common duct was flushed at a constant pressure of 5 cm H_2O following the cannulation of both ends. In the draining preparation, the hepatic end of the duct was ligated and the pancreatic juice was collected from the duodenal end following cannulation with a stainless steel tube. The blood supplying stomach, liver and spleen was stopped by tying the arteries. The mesentry with its embedded whole pancreas and the attached duodenum was then removed and mounted on a paraffin block in a Lucite chamber containing 20 ml. modified Krebs–Henseleit solution. Both preparations utilize the whole pancreas and a full description of the procedures has been given in a previous paper (Kanno *et al.* 1976). The temperature of the preparation and perfusing solution was maintained at 37 °C by immersing the apparatus in a water-bath.

Pz (Boots) and bovine insulin (Novo, Copenhagen) were injected through a cannula into the superior mesenteric artery. The concentration of Pz is expressed in Crick, Harpar & Raper (1950) units per millilitre solution, and that of insulin in international units per millilitre solution.

Solution. Perfusion was usually with a modified Krebs–Henseleit solution of the following composition (in mM): NaCl, 131; KCl, 5·6; $CaCl_2$, 2·5; $MgCl_2$, 1·0; $NaHCO_3$ 25; NaH_2PO_4, 1·0; glucose, 5. Dextran T-70 (Pharmacia, Uppsala) was added to this standard solution in a concentration of 5 % (w/v). For the *erythrocyte containing solution*, the dog erythrocytes were washed three times with the standard solution and added to the standard solution to make a final concentration of 8 vol. %. Solutions were equilibrated with 5 % CO_2 in O_2 and had a pH of about 7·2.

Estimation of digestive enzyme and rate of flow of pancreatic juice. Estimation of the rate of flow of pancreatic juice was made as follows. A calibrated tube made of silicon-rubber (about 0·5 mm o.d.) was attached to the free end of the pancreatic duct cannula. Every tenth minute the tube was replaced and the rate of flow of the pancreatic juice down the tube was noted. The sample of juice collected was then diluted with the standard Krebs–Henseleit solution (final volume was 100 ml.) and the amount of amylase assayed by the modified method of Bernfeld (1955) as described previously (Kanno, 1975). One unit of amylase activity in the pancreatic juice or the perfusate was defined as the amount of enzyme which produced 1 mg maltose during a 5 min incubation with the diluted perfusate at 37 °C by the modified method.

Electrical recording. Intracellular recordings were made from the pancreatic acinar cells by manually advancing KCl-filled micro-electrodes under direct visual control. Resting potentials were observed on the screen of a cathode ray oscilloscope (Nihon Koden, ATAC-250, Tokyo) and simultaneously recorded on a direct visual oscillograph (Yokogawa, Type 2915, Tokyo) with a frequency response of 5 kHz. A current pulse of 50 msec duration was passed through the recording electrode in order to polarize the cell membrane. A full description of the procedure has been given in a previous paper (Kanno, 1972).

RESULTS

Experiments on the isolated and perfused preparations

Effect of insulin on Pz-induced amylase output. The isolated pancreas was first perfused for 30 min with the standard Krebs–Henseleit solution to allow amylase output to reach a steady value. Perfusates were collected both from the portal vein and from the common duct at every tenth

minute and were subjected to analysis for amylase. The vascular perfusion was then switched from the solution to the same solution containing 0·5 m-u. Pz/ml. and the perfusion continued for an additional 120 min (Fig. 1*A*). The mean ratio of the maximal amylase output in the presence of Pz to the output before stimulation was about 4.

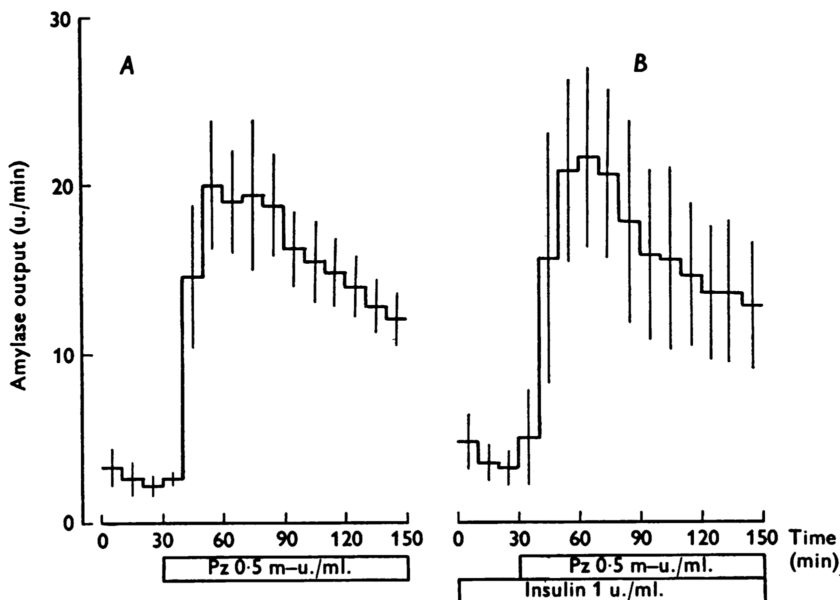

Fig. 1. Amylase output during the continuous perfusion of the flushing preparation of the isolated pancreas with standard Krebs–Henseleit solution and effects of 0·5 m-u. Pz/ml. in the absence (*A*) or presence (*B*) of 1 u. insulin/ml. Each value represents the mean (± S.E. mean) of 10 min measurements from five experiments. The horizontal bars indicate the periods Pz and insulin were present.

The effect of insulin was examined by perfusing the preparation with the same solution to which insulin has been added 30 min before the initiation of continuous stimulation with Pz. The mean ratio of the maximal output in the presence of Pz to the output before stimulation by Pz was also about 4 by perfusion with the solution containing 1 u. insulin/ml. (Fig. 1*B*). Analysis of these results using Student's *t* test showed that the influence of 1 u. insulin/ml. on the secretory effect of 0·5 m-u. Pz/ml. was not significant ($P > 0{\cdot}2$). Amylase output into the portal vein was not influenced by insulin or Pz at the concentrations used and therefore the output was omitted from the figures in the present report.

The effect of insulin was further examined by perfusing the preparation

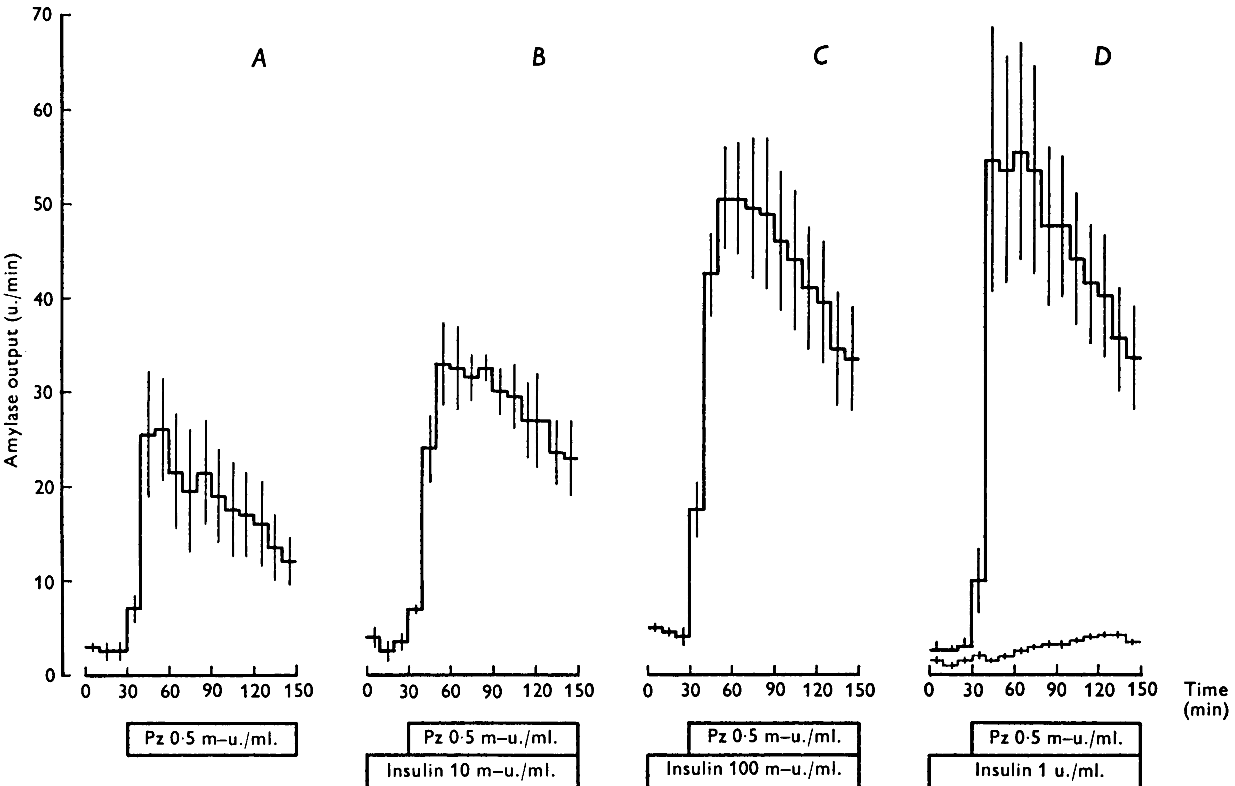

Fig. 2. Amylase output during the continuous perfusion of the flushing preparation of the isolated pancreas with Krebs–Henseleit solution containing erythrocytes and effects of 0·5 m-u. Pz/ml. in the absence (*A*), or presence of 10 m-u. insulin/ml. (*B*), 100 m-u. insulin/ml. (*C*), or 1 u. insulin/ml. (*D*). Thin line in *D* represents amylase output during continuous perfusion with insulin alone (1 u./ml.) in the absence of Pz. Each value represents the mean (± S.E. of mean) of 10 min measurement from five experiments. Symbols as in Fig. 1.

with the Krebs–Henseleit solution containing dog erythrocytes (8 vol. %). In the absence of insulin, the mean ratio of the maximal anylase output in the presence of Pz to the output before stimulation was about 10 (Fig. 2*A*). The mean ratio rose to about 11 by perfusion with the solution containing 10 m-u. insulin/ml., to about 15 by perfusion with solution containing 100 m-u. insulin/ml. and to about 18 by perfusion with the

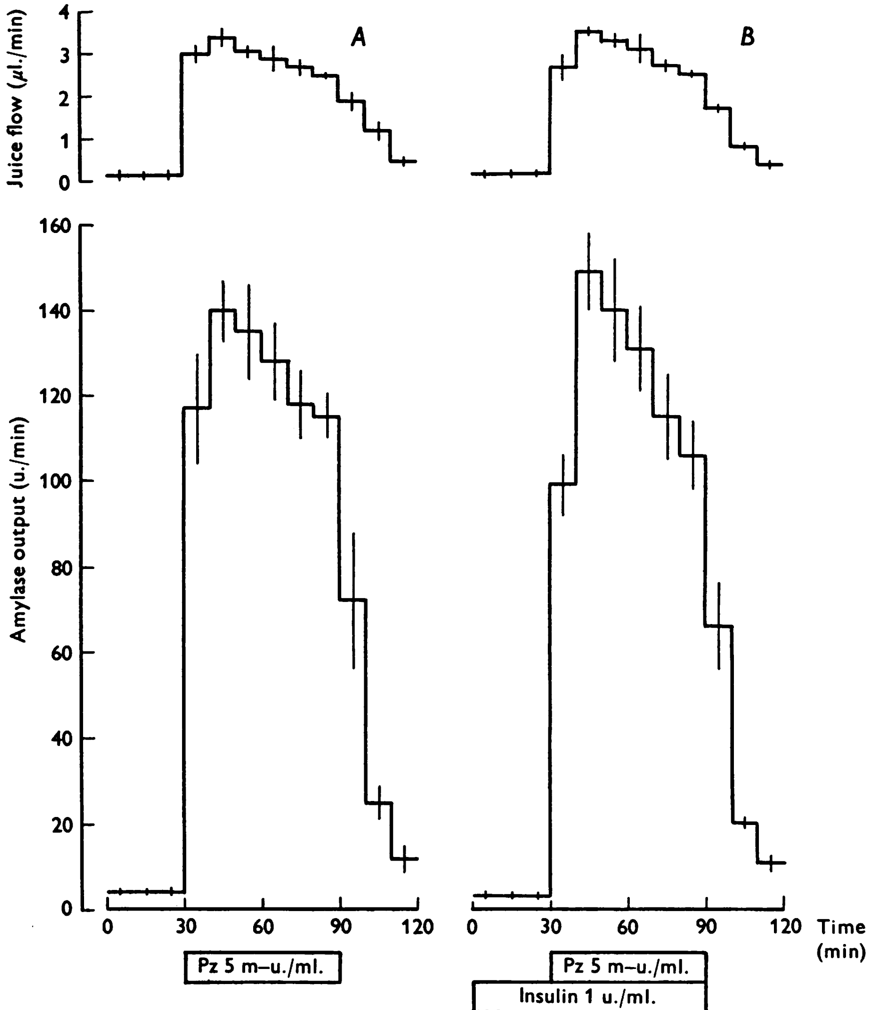

Fig. 3. Amylase output during the continuous perfusion of the *draining preparation* of the isolated pancreas with Krebs–Henseleit solution containing erythrocytes and the effect of 5 m-u. Pz/ml. in the absence (*A*) or presence of (*B*) 1 u. insulin/ml. Each value represents the mean of five experiments. Symbols as in Fig. 1.

solution containing 1 u. insulin/ml. (Fig. 2*B*, *C* and *D*). Analysis of these results using Student's *t* test showed that the influences of 100 m-u. and 1 u. insulin/ml. on the secretory effect of 0·5 m-u. Pz/ml. were significant ($P < 0.05$) but the influence of 10 m-u. insulin/ml. was not significant ($P > 0.2$). In the absence of Pz, 1 u. insulin/ml. produced a slight and gradual increase in amylase output into the common duct (Fig. 2*D*).

When higher concentrations of Pz were used to stimulate the preparation, insulin did not augment the response. Fig. 3 shows the time courses of amylase output and pancreatic juice flow before and after stimulation with 5 m-u. Pz/ml. in the absence or presence of 1 u. insulin/ml. Insulin was likewise without effect when preparations were stimulated with 200 m-u. Pz/ml.

Influence of insulin on the hyperpolarizing effect of Pz. Previous papers have shown that perfusion with Krebs–Henseleit solution containing 200 or 5 m-u. Pz/ml. produced a hyperpolarization of the acinar cells and increased the amylase output from the isolated rat pancreas (Kanno, 1972, 1974, 1975). These effects of Pz were enhanced when the pancreas was perfused with an oxygenated Krebs–Henseleit solution containing dog erythrocytes (Kanno *et al.* 1976). The present experiments confirmed these effects during perfusion with the solution containing 0·5 m-u. Pz/ml. and 8 vol. % dog erythrocytes. Fig. 4 shows a typical recording of intracellular membrane potential and effective membrane resistance from an acinar cell of the perfused pancreas. Perfusion with the standard solution containing 0·5 m-u. Pz/ml. and erythrocytes (8 %) resulted in a gradual hyperpolarization from −35 mV to the maximum value of −40 mV 10 min after the initiation of stimulation, and this change coincided with a gradual increase in amylase output.

When 1 u. insulin/ml. was added to the perfusing erythrocyte-containing solution, the hyperpolarization and amylase release produced by 0·5 m-u. Pz/ml. were potentiated: the membrane potential increased from −35 to a maximum value of −50 mV 1 min after the initiation of stimulation and this change coincided with a higher amylase output (Fig. 5).

To correlate the secretory response with the electrical phenomena, intracellular membrane potential and effective membrane resistance have been recorded from as many cells as possible during the course of perfusing the preparation instead of recording continuously from a single cell. This was done because amylase output is a summation of the output from every cell in the pancreas and the secretory response seems to be more closely correlated to the mean of electrical properties of acinar cells in the preparation. Fig. 6*A* shows the means (± S.E.) of the electrical measurements, the flow of pancreatic juice and the amylase output from five experiments on different preparations before and after stimulation with 0·5 m-u. Pz/ml.

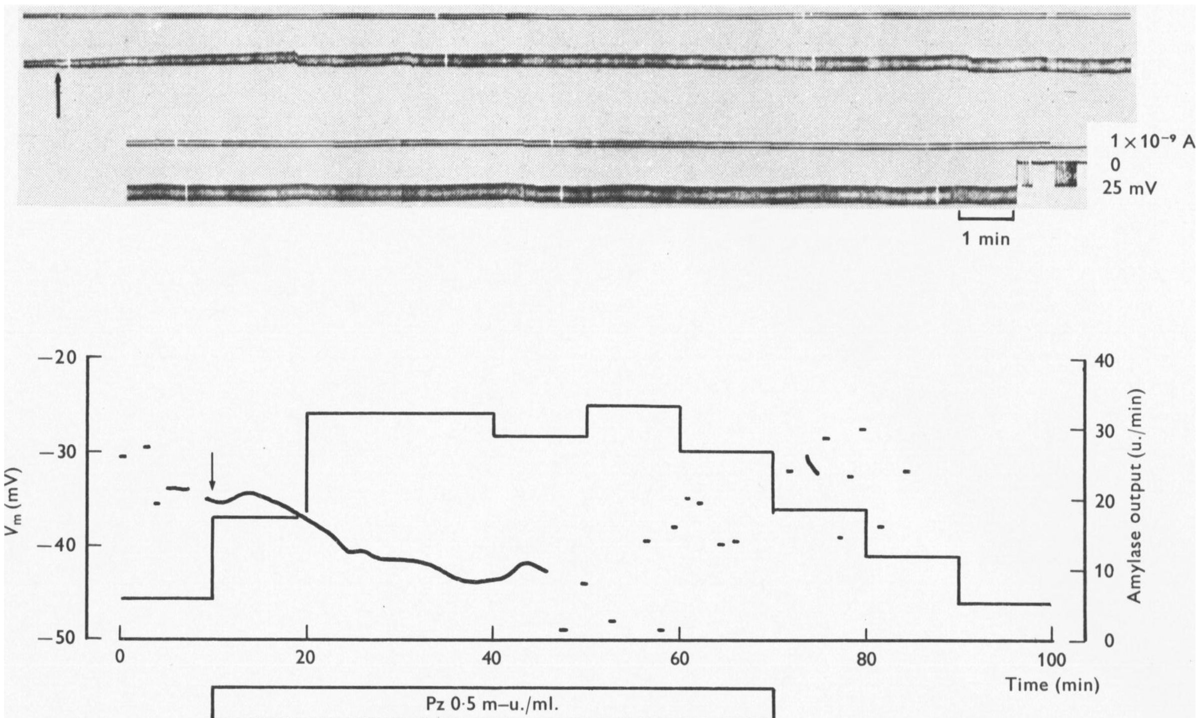

Fig. 4. An example of a long recording of membrane potential with superimposed displacements resulting from passage of current pulses (50 msec duration) through the recording electrode. The recording was traced in the Figure below, and the potentials before and after the long recording are also traced by thick lines. The time course of amylase output of the flushing preparation is shown by the stepped line. The horizontal bar indicates the period Pz (0·5 m-u./ml.) was present. The arrows correspond to the time when Pz reached to the preparation.

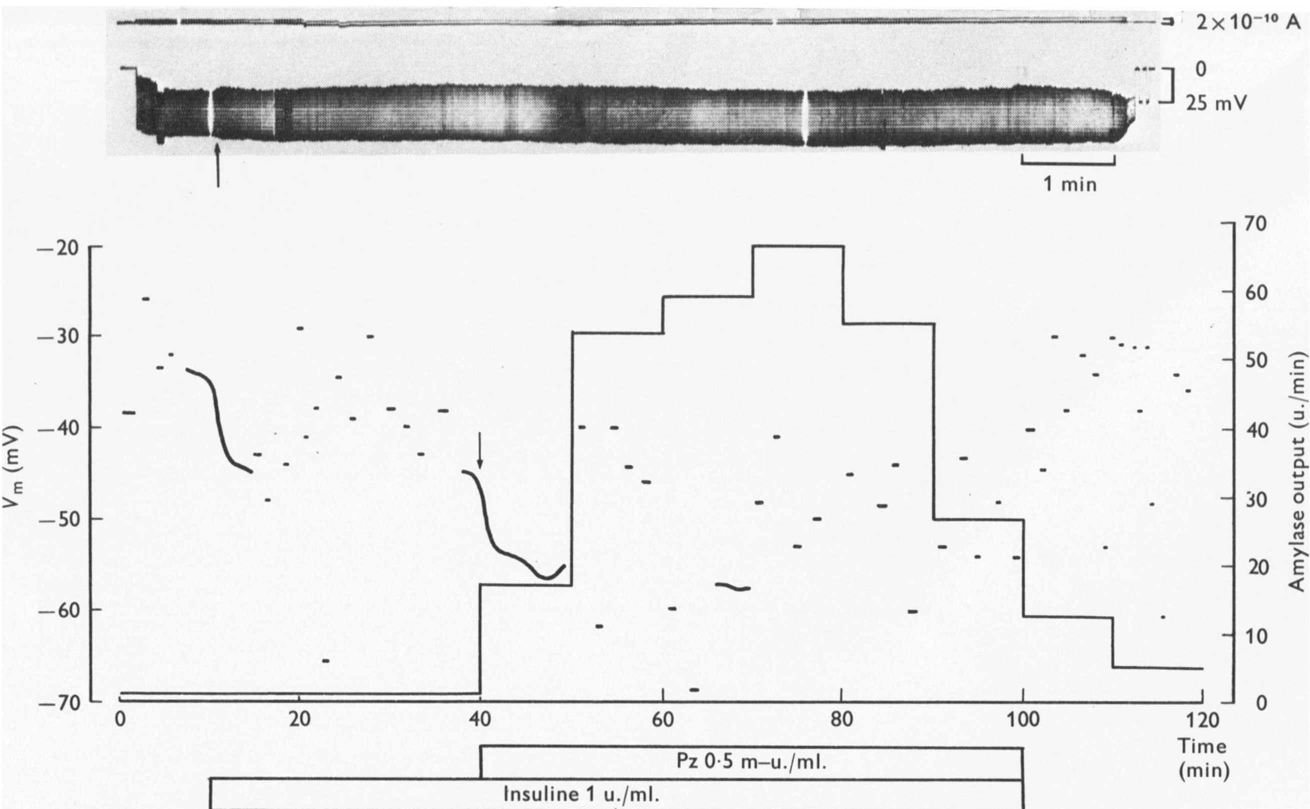

Fig. 5. An example of a long recording of membrane potential with superimposed displacements resulting from passage of current pulses (50 msec duration) through the recording electrode. The recording was traced in the Figure below, and the potentials before and after the long recording are also traced. The time course of amylase output of the flushing preparation is shown by the stepped line. The horizontal bars indicate the period Pz (0·5 m-u./ml.) and the period insulin (1 u./ml.) were present respectively. The arrows correspond to the time when Pz reached to the preparation.

Similar experiments, on other different preparations, were done in the presence of 1 u. insulin/ml. (Fig. 6*B*). It can be seen that Pz (0·5 m-u./ml.) hyperpolarized the cells, increased the effective membrane resistance, the amylase output into the duct and the flow of pancreatic juice. Analysis

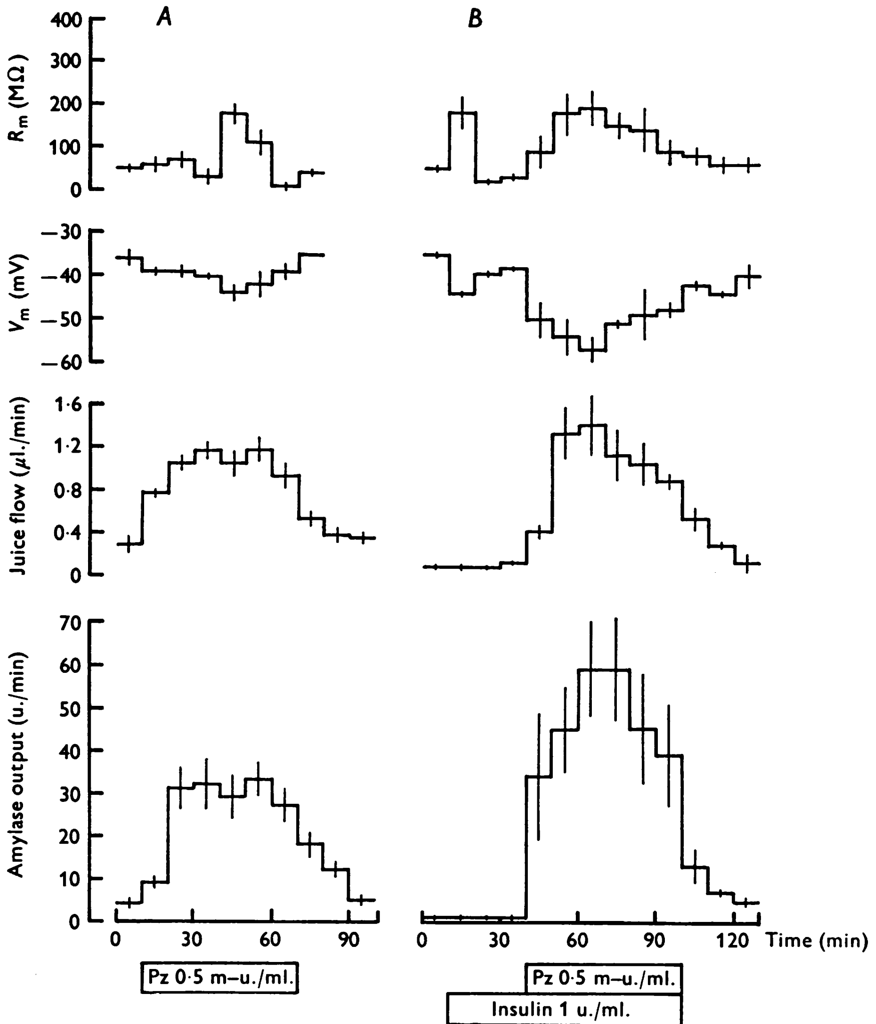

Fig. 6. Time courses of the changes in effective membrane resistance (R_m), membrane potential (V_m), juice flow and amylase output during the continuous perfusion of the draining preparation of the isolated pancreas with Krebs–Henseleit solution and the effects of 0·5 m-u. Pz/ml. in the absence (*A*) or presence (*B*) of 1 u. insulin/ml. Each value represents the mean of five experiments. Symbols as in Fig. 1.

of the results using Student's t test showed that each of the maximum values was significantly higher than the corresponding control value taken before stimulation ($P < 0.05$). Administration of insulin (1 u./ml.) potentiated these effects of Pz on membrane potential, effective membrane resistance and amylase output but has little influence on the flow of

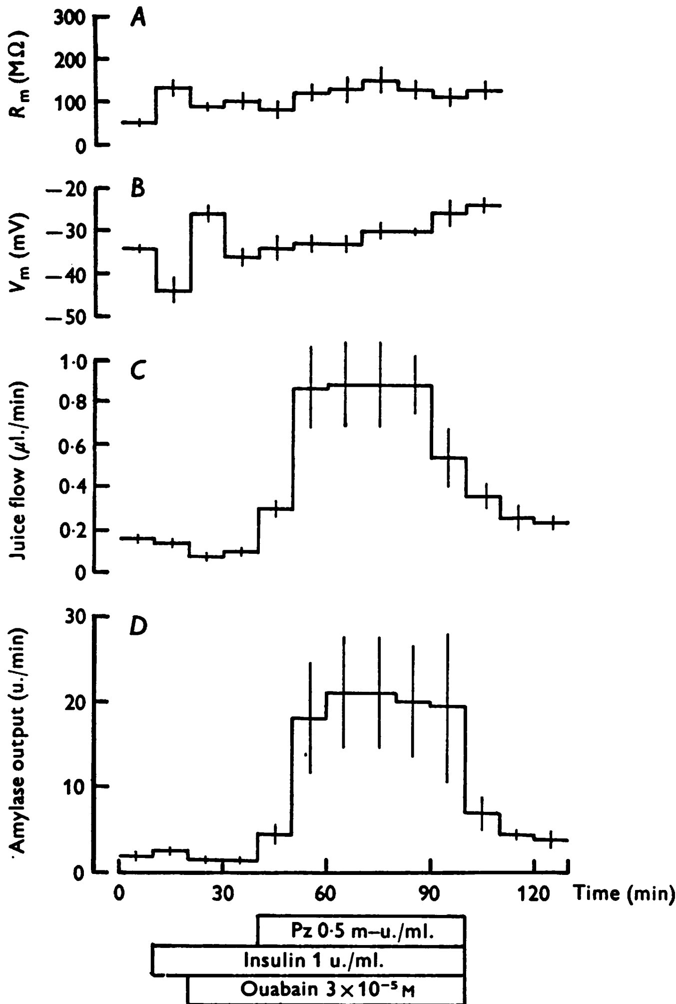

Fig. 7. Time courses of the changes in effective membrane resistance (R_m), membrane potential (V_m), juice flow and amylase output during the continuous perfusion of the draining preparation of the isolated pancreas with Krebs–Henseleit solution and the effect of 0·5 m-u. Pz/ml. in the presence of insulin (1 u./ml.) and ouabain (3×10^{-5} M). Each value represents the mean of five experiments.

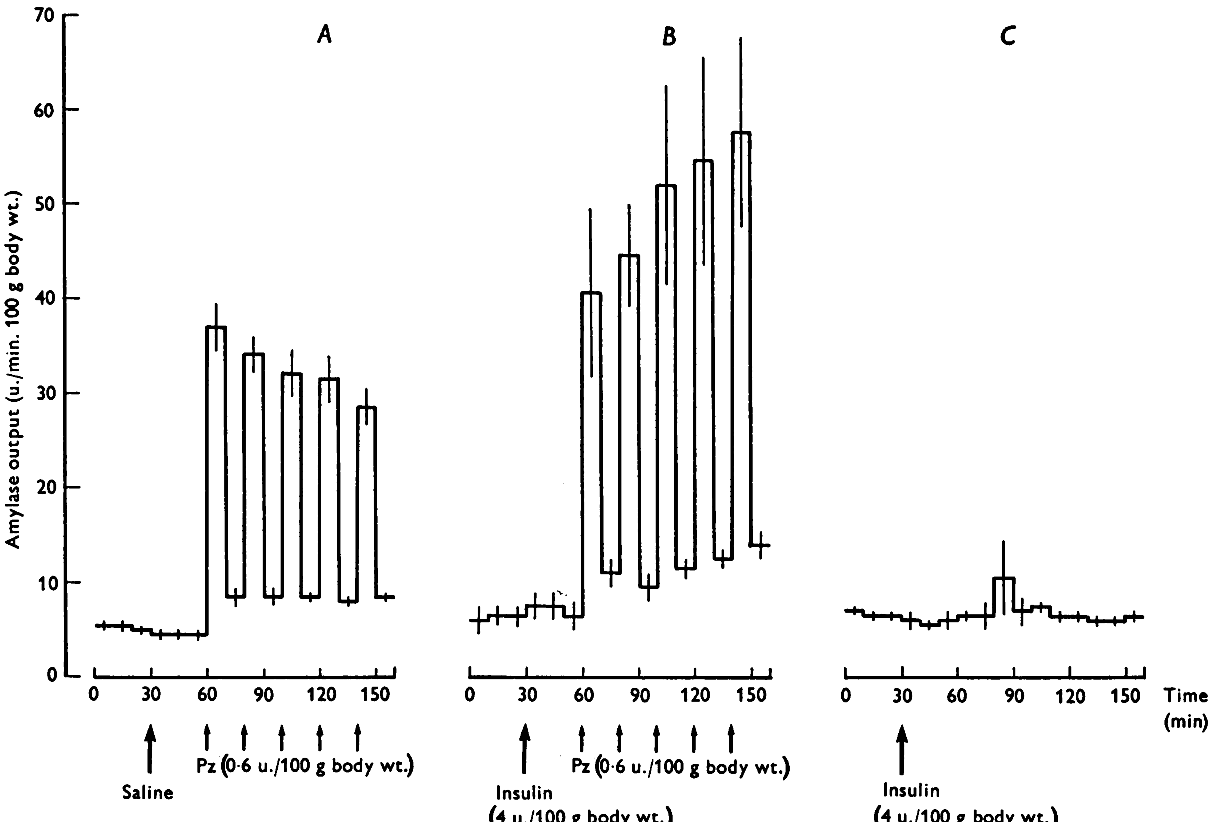

Fig. 8. Amylase output of the rat pancreas *in situ* during the course of intermittent stimulations with Pz (0·6 u./100 g body wt.) after injection of saline (*A*) or insulin (4 u./100 g body wt.) (*B*). Amylase output after injection of insulin (4 u./100 g. body wt.) without Pz stimulation is shown in *C*. Each value represents the mean of four experiments.

pancreatic juice. Analysis of the results using Student's t test showed that the effects of Pz on membrane potential, effective membrane resistance and amylase output in the presence of insulin were significantly higher than the corresponding values in the absence of insulin ($P < 0.001$, 0·01 and 0·05 respectively).

If these potentiating influences of insulin are due to an increase in the activity of an electrogenic Na pump, simultaneous administration of ouabain may be expected to inhibit these effects. Fig. 7 illustrates the inhibitory action of ouabain on the potentiating influences of insulin. When Pz was added to the perfusing solution which contained ouabain (3×10^{-5} M), insulin (1 u./ml.) and erythrocytes (8 %), it failed to produce hyperpolarization, and increased amylase output and juice flow to amounts lower than the corresponding value (Fig. 6*B*) that obtained in the absence of ouabain.

Experiments on the pancreas in situ

After vagotomy and ligaturing the pyloric region in the anaesthetized rats, amylase output into the common duct was estimated during the course of administration of 0·6 u. Pz/100 g body wt. injected regularly at 20 min intervals into the femoral vein. The amylase output into the common duct initially increased about 7 times above the control level and this was followed by a gradual diminution in the subsequent responses. Fig. 8*A* shows the mean (± S.E.) of four experiments. The amylase output in response to the fourth stimulus was about 60 % of the initial response.

Administration of insulin before Pz potentiates the secretory effects of Pz. Fig. 8*B* shows the mean (± S.E.) of four experiments, in which 4 u. insulin/100 g. body wt. has been injected into the femoral vein 30 min before stimulation with 0·6 u. Pz/100 g body wt. The mean initial amylase output was about 8 times greater than the control output and the response was further increased in the four subsequent responses. Analysis of the results, depicted in Fig. 8*A* and *B*, using Student's t test showed that the value of the initial response in Fig. 8*A* was not significantly larger than that in Fig. 8*B* ($P > 0.05$), however, the value of the fourth response in Fig. 8*B* was significantly larger than that in Fig. 8*A* ($P < 0.05$). Insulin, at the dose used, had no effect on the pancreas *in situ* in the absence of Pz (Fig. 8*C*).

DISCUSSION

Effects of insulin on pancreatic enzyme release. In the present experiments, the hormone *per se* produced a transient hyperpolarization associated with little increase in amylase release. Robberecht & Christophe (1971) showed that the amylase release was reduced when the islet cells in pieces

of rat pancreas were stimulated with 10 mM glucose. Case & Clausen (1973) have also shown that insulin had little effect on the rate of Ca and amylase release in the isolated uncinate pancreas of baby rats.

The principal conclusion permitted by our experiments is that insulin potentiates the Pz-induced hyperpolarization and amylase release in the isolated and perfused rat pancreas. The potentiation was detected when 1 u. insulin/ml. was added to the perfusing solution containing erythrocytes and a low concentration of Pz (0·5 m-u./ml.). Each of these experimental conditions is critical for detecting the potentiating effect: insulin in the same concentration had no effect when the pancreas was perfused with a solution containing no erythrocytes, or when the pancreas was stimulated with higher concentrations of Pz (5 m-u./ml.). In 1938, Sergeyeva observed that continuous stimulation of the vagus nerves of the cat caused almost entire depletion of zymogen granules of the acinar cells with the exception of those forming 'halos' around the islets of Langerhans. On this and other observations, she suggested that the stimulation also caused the release of insulin, which would inhibit the discharge of zymogen granules from the near-by acinar cells. The strength of stimulation she used may probably exceed a threshold strength to produce the potentiating influence of insulin. Danielsson (1974) found that, in batch-incubated and perfused pieces of mouse pancreas, 100 μg (approximately 2·4 u.) insulin/ml. reduced the amylase output produced by 100 m-u. Pz/ml. It may be possible that the preparation used by Danielsson (1974) was not under the best metabolic conditions and that the concentration of Pz used was too high to demonstrate the potentiating influence of insulin. In fact, in the present experiments, the potentiating influence of insulin could not be detected in the isolated rat pancreas perfused with an oxygenated Krebs–Henseleit solution containing no erythrocytes. Wizemann *et al.* (1974) showed in the isolated and perfused cat pancreas that insulin (4–40 u.) did not produce any effect on amylase output induced by Pz (0·5 u.). Although the concentration of Pz he used was not mentioned, the ineffectiveness of insulin may be also due to a higher concentration of Pz as well as no erythrocyte in the perfusing medium.

Insulin and electrogenic Na pump. Our results raise the question whether there is a common basis for these effects of insulin potentiating Pz-induced hyperpolarization and amylase release. Kanno (1975) has postulated that the carrier-mediated Ca influx may have a direct correlation with the Na gradient between inside and outside of the acinar cell and the Ca influx may be the essential event to initiate the exocytosis of zymogen granules. The Na gradient may be maintained by a Na pump, which, at least in part, seems to be electrogenic. The action of insulin to potentiate the electrogenic Na pump may thus be a most probable common

basis for the effects of insulin potentiating the Pz-induced hyperpolarization and amylase release.

One of the most familiar effects of insulin, on a variety of cells, is to hyperpolarize the cells and this is associated with a new distribution of intracellular ions: there is ample evidence that insulin hyperpolarizes frog skeletal muscle, fat cells in rat adipose tissue, rabbit ciliary epithelium, frog gastric mucosa, toad bladder and colon mucosa and there is also evidence that insulin causes a net uptake of K by skeletal muscle, adipose tissue and liver (see Zierler, 1972). One possible explanation for the effect is that insulin increases the activity of an electrogenic Na pump, although some investigators do not agree with this explanation (see Zierler, 1972; Moore, 1973).

Concentration of insulin around the acinar cells. The present experiments have shown that insulin produced its influences only when the concentration of insulin was in large excess over the normal plasma level. Although the insulin concentrations to which the pancreatic acinar cells are normally exposed *in situ* are unknown but there are reasons for believing that the concentrations may far exceed the plasma level. Kanazawa, Kuzuya & Ide (1968) reported a maximum plasma concentration of insulin in the superior pancreatico-duodenal vein of about 10 m-u./ml. after the injection of glucose (1 g/kg) into the anaesthetized dog. Furthermore, the presence of direct capillary connexions between the islets and the surrounding acinar parenchyma may provide a much higher concentration of insulin in the extracellular environment to which the acinar cells are normally exposed. The direct capillary connexions have been demonstrated in pancreata of a wide variety of animal species (see Henderson, 1969; Youngs, 1972; Fujita & Watanabe, 1973). The connexions may be responsible to give a halo appearance: the cells of the peri-insular acini have larger nuclei and zymogen (see Kramer & Tan, 1968).

The present experiments showed that 100 m-u. bovine insulin/ml. caused a significant potentiation of the Pz-induced amylase output. Recent experiments showed that the influences of 20 m-u. rat insulin/ml. exceeded that of 100 m.-u. bovine insulin/ml. (T. Kanno and A. Saito, unpublished). This concentration of insulin, 20 m-u./ml., seems to be in a physiological range of plasma level to which the acinar cells are exposed *in situ*.

Effects of insulin in situ. The present *in situ* pancreatic studies were performed after vagotomy and ligature of the pyloric region and these effects of insulin were similar to those effects observed in our previous experiments (Kanno, Ueda & Saito, 1976), in which the vagus nerve was intact and the pyloric region not ligatured. Thus the effects of insulin may be mainly on the exocrine pancreas *per se* and this view agrees

with the preceding results, that insulin has direct actions on Pz-induced hyperpolarization of the pancreatic acinar cell and amylase release in the isolated and perfused preparation of the rat pancreas.

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