Extracellular Calcium and Acetylcholine-stimulated Insulin Secretion

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SUMMARY

The importance and possible involvement of extracellular calcium in the stimulation of insulin secretion by acetylcholine was examined in the isolated perfused dog pancreas. Acetylcholine (50 μM) failed to stimulate insulin secretion when calcium was omitted from the perfusion medium. In the presence of tetracaine (1 mM) and magnesium (10.16 mM), conditions favoring blockade of Ca^{++} influx into the beta cell, acetylcholine-induced (50 μM) insulin secretion was inhibited. We conclude from these observations that acetylcholine-stimulated insulin secretion is dependent upon extracellular Ca^{++} and an increase in Ca^{++} influx may be involved in this stimulation. DIABETES *23*:494-98, June, 1974.

It is becoming increasingly apparent that the autonomic nervous system may exert modulatory control over insulin secretion. Mediators of alpha-adrenergic activity have been thoroughly documented as inhibitors of insulin release.[1-3] Not only is this blockade suggested to be due to circulating catecholamines,[1,2] but it may also be of a highly specific nature, involving sympathetic nerve fiber innervation of the pancreas.[4] Substantial evidence of cholinergic control also exists. Increased insulin secretion following vagal stimulation has been recorded by numerous investigators,[5-7] and acetylcholine has been found to be a potent insulinotropic agent in vitro whose secretory response is increased following administration of cholinesterase and is inhibited by atropine.[3,8,9]

Acetylcholine is generally believed to function as a cholinergic transmitter by interacting with the plasma membrane of responsive cells to increase the permeability of cations such as sodium and calcium.[10] A number of insulin secretagogues have been found to require extracellular calcium,[11,12] and the movement of calcium into the beta cell has been suggested to be a key event in the secretory mechanism.[13,14] These observations have prompted the present investigation, to determine (1) if acetylcholine-induced secretion is dependent upon extracellular Ca^{++} and (2) if movement of Ca^{++} into the beta cell is of primary importance for this secretion. The isolated perfused dog pancreas was used for this study.

METHODS AND MATERIALS

Pancreases were obtained from mongrel dogs of both sexes, weighing approximately 20 kg., which had no special conditioning. All animals were fasted overnight with free access to water. Each pancreas was prepared as previously described,[15] except we have found by experience that cannulation of the trachea and respiration of the dog (Harvard Apparatus Pump Model 607) expedites removal of the pancreas.

The perfusion procedure was changed from a recirculating system[15] to one that allowed sequential collection of all effluent by an automatic fraction collector. The apparatus consisted of multiple reservoirs maintained at 37° C. by a constant temperature water bath that was continuously gassed with a hydrated mixture of 95 per cent oxygen-5 per cent carbon dioxide. Reservoirs were connected to the main infusion tubing by three way stopcocks aligned in series, thus allowing transfer from reservoir to reservoir without interruption of flow. The flow rate was maintained at 15 ml. per minute with a mean pressure of 75 mm. Hg. Samples were collected at one minute intervals, the volumes were measured, and aliquots were promptly frozen for subsequent immunoreactive insulin determination by the dextran-coated charcoal method of Herbert et al.[16] (125-I-labeled insulin was obtained from New England Nuclear Corp., Boston).

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All perfusion media consisted of Krebs-Ringer bicarbonate buffer containing 4 per cent dextran[17] (Nutritional Biochemicals Co., Cleveland) and 60 mg. per cent glucose. The ionic concentrations of this medium in millimoles per liter were Na^+ 143, K^+ 5.93, Ca^{++} 1.27, $H_2PO_4^-$ 1.19, Mg^{++} 0.6, Cl^- 125.8, SO_4 0.6, and HCO_3^- 24.6. Alterations and additions to this basic medium are described under Results.

Data are reported as the mean ± standard error of the mean (S.E.M.). Statistical significance was established by comparing mean secretion rates of the experimental groups with those of the control group. The Student's two sample *t* test was used for all comparisons.

RESULTS

Each pancreas was perfused for a twenty to thirty minute equilibration period with normal medium. Samples were obtained from -5 to -3 minutes and -3 minutes to time zero to provide a basal rate of secretion. The responsiveness of each pancreas to acetylcholine (50 μM) was determined by administering a five minute acetylcholine pulse at time zero. This pulse caused an immediate (minutes 1 to 2) rise in insulin secretion followed by a delayed return to basal values (figures 1 through 4). This prolonged secretion, which occurred after the acetylcholine pulse, may involve the so-called "off effect" described by Iverson[9] or may represent a residual effect of acetylcholine.

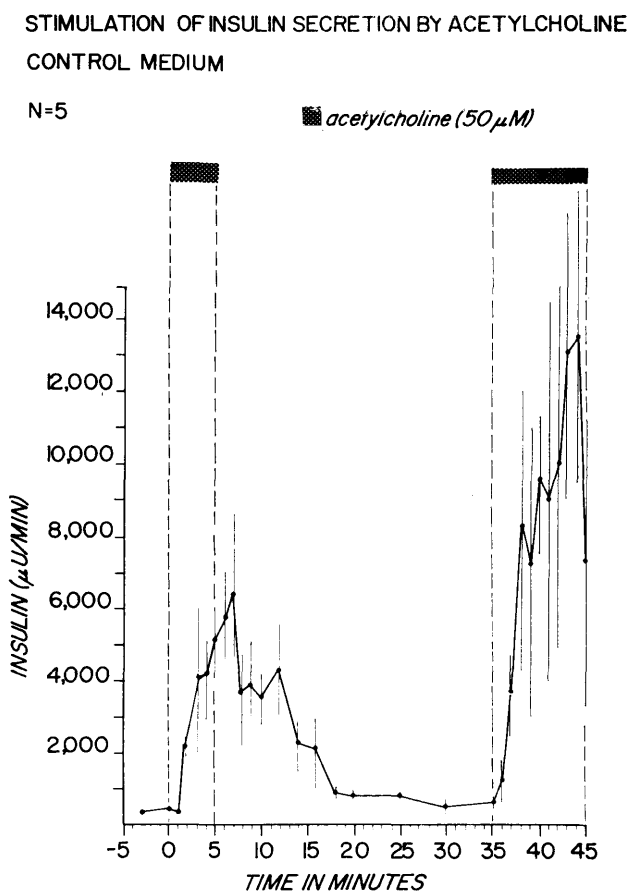

FIG. 1. Control experiments. Each value represents the mean ± S.E.M. of five perfusions.

Stimulation of Insulin Secretion by Acetylcholine

After the first pulse of acetylcholine (50 μM), control medium was perfused for thirty minutes, followed by a second perfusion (minutes 35 to 45) of 50 μM acetylcholine (figure 1). During the second acetylcholine exposure, secretion increased immediately and continued to be high throughout the pulse. It should be noted that the mean secretion rate from minutes 35 to 40 was significantly greater than the rate of secretion from minutes 0 to 5 ($p<.05$). This phenomenon of "hypersensitivity to further stimulation" has also been reported when glucose was used as the stimulus.[18]

Effect of Acetylcholine on Insulin Secretion in a Ca^{++} Free Medium

In this series the initial five minute acetylcholine pulse was followed by a Ca^{++} free infusion for twenty minutes (figure 2) and an acetylcholine (50 μM) infusion during the last five minutes (minutes 35 to 40). From minutes 40 to 45 the perfusion medium was changed to a normal medium containing Ca^{++} plus 50 μM acetylcholine. There was no stimulation of insulin secretion ($p<.01$) in the absence of extracellular Ca^{++} (minutes 35 to 40), but the reintroduction of Ca^{++} from minutes 40 to 45 allowed acetylcholine to elicit a prompt release of insulin.

Effect of Tetracaine on Acetylcholine-induced Insulin Secretion

Tetracaine (1 mM) was infused for twenty-five minutes following the usual equilibration and five minute acetylcholine pulse (figure 3). Acetylcholine failed to stimulate insulin release in the presence of tetracaine (minutes 35 to 40, $p<.01$), but release could be elicited from minutes 40 to 45 when supernormal amounts of Ca^{++} (5.08 mM) were added to the perfusion medium.

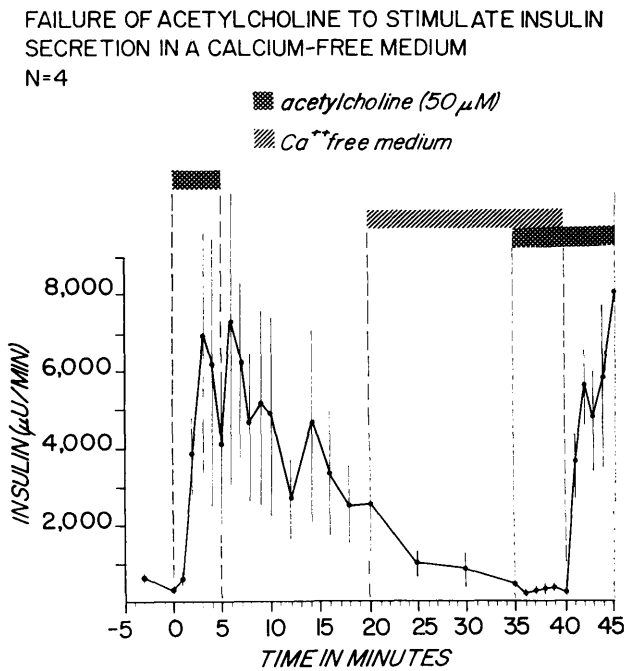

FIG. 2. The failure of acetylcholine to stimulate insulin secretion through a calcium-free medium was followed by a restoration of secretion when calcium was reintroduced. Each value represents the mean ± S.E.M. of four perfusions.

Effect of a High Extracellular Mg^{++} Concentration on Acetylcholine-induced Insulin Secretion

The ability of acetylcholine to stimulate insulin secretion in the presence of an increased level of Mg^{++} was tested by raising the concentration of this ion to 10.16 mM (figure 4). Following the control pulse of acetylcholine, the pancreases were perfused with high Mg^{++} for fifteen minutes. After this period, acetylcholine was added to the high Mg^{++} medium for five minutes (minutes 35 to 40), and insulin secretion was inhibited ($p<.05$). The inhibition imposed by the high level of Mg^{++} was reversed by returning to a medium containing the normal Mg^{++} concentration. This reversal of inhibition was noted as a high rate of secretion which occurred from minutes 40 to 45. It should be pointed out that the high peak of secretion resulting from the five minute pulse (from minutes 0 to 5) of acetylcholine was due primarily to one hypersecreting pancreas.

DISCUSSION

Several investigators have shown that insulin secretion can be elicited by stimulation of the vagus nerve[4-7] and can be blocked by prior administration of atropine.[4,6] Furthermore, since acetylcholine is a potent insulin secretagogue in vitro,[3,8,9] this strongly suggests that cholinergically mediated insulin secretion is a result of a direct action of acetylcholine on the pancreatic beta cell. The mechanism whereby acetylcholine elicits insulin secretion is not known. An increased calcium influx is known to be associated with the action of acetylcholine at other tissues,[10] while extracellular calcium has been found to be required for the insulinotropic action of various stimuli.[11-14] These two facts point to a possible role of extracellular calcium in the mechanism of acetylcholine-induced insulin secretion.

From the data in figure 2 it can be seen that the ability of acetylcholine to stimulate insulin secretion was completely lost when extracellular Ca^{++} was removed from the perfusion medium. However, when Ca^{++} was reintroduced, there was a sharp rise in secretion, similar to that seen under control conditions. This observation indicates that the failure of acetylcholine to release insulin was due to a lack of Ca^{++} and not to decreased tissue viability. It would appear, therefore, that acetylcholine shares in com-

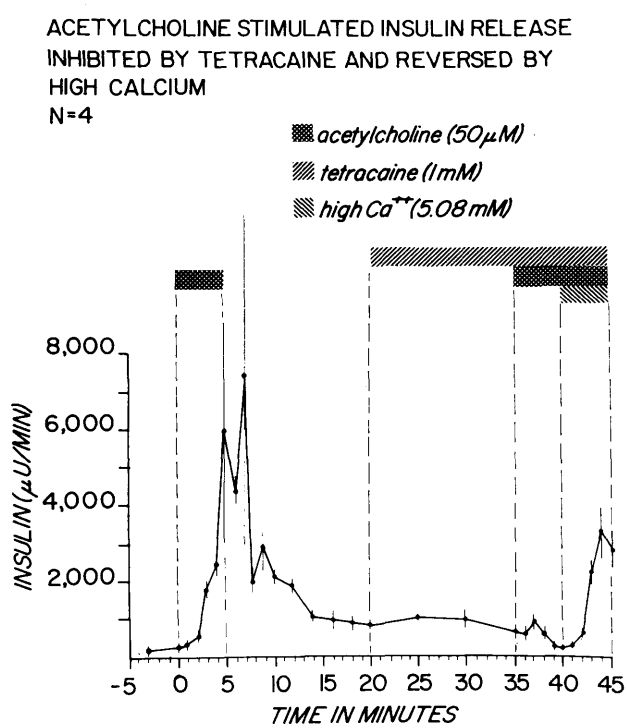

FIG. 3. The inhibition of acetylcholine-induced insulin secretion by tetracaine was reversed with high calcium levels. Each value represents the mean ± S.E.M. of four perfusions.

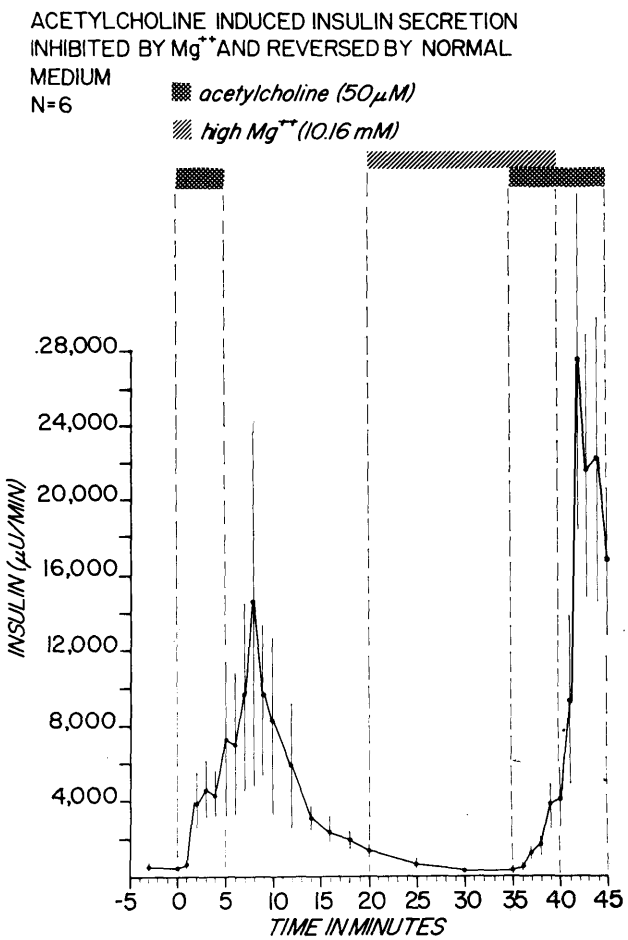

FIG. 4. Insulin secretion induced by acetylcholine was inhibited when high magnesium levels were present and reversed by the return to a normal medium. Each value represents the mean ± S.E.M. of six perfusions.

mon with many other insulinotropic agents an absolute requirement for extracellular Ca^{++}.

Having established the stimulative action of acetylcholine to be calcium dependent, we decided to investigate the possibility that an influx of Ca^{++} might be involved in this process. To answer this question we tested the insulinotropic action of acetylcholine in the presence of the local anesthetic tetracaine and at high extracellular Mg^{++} levels, conditions which are known to interfere with Ca^{++} entry into cells.[19-21]

Tetracaine proved to be a potent inhibitor of acetylcholine-induced insulin secretion (figure 3, minutes 35 to 40). This compound has been reported to inhibit competitively Ca^{++} entry in other cells.[19] If tetracaine was inhibiting insulin secretion by a similar action on the beta cell, then a rise in extracellular Ca^{++} concentration would antagonize this inhibition. This, indeed, was the finding, as illustrated, from minutes 40 to 45, viz. by raising the Ca^{++} concentration to 5.08 mM, the inhibition imposed by tetracaine was partially reversed.

Excess Mg^{++} (10.17 mM), while not completely abolishing insulin secretion, did reduce significantly ($p<.05$) the stimulative action of acetylcholine (figure 4, minutes 35 to 40). The high rate of secretion which occurred from minutes 40 to 45 suggests that reducing the extracellular Mg^{++} concentration to normal could reverse this inhibition. However, the possibility that this high rate of secretion would have occurred had the elevated Mg^{++} level been maintained cannot be excluded.

Based upon the tetracaine and high magnesium experiments, it would appear that whenever Ca^{++} movement into the cell is inhibited, insulin secretion is also inhibited. This observation, together with the finding that acetylcholine is without effect in a Ca^{++} deficient media, led us to conclude that acetylcholine-stimulated insulin secretion is Ca^{++} dependent and an increase in Ca^{++} influx may be a necessary requirement for this stimulation.

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