"Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function"

Gilon, Patrick ; Henquin, Jean-Claude

ABSTRACT

Acetylcholine (ACh), the major parasympathetic neurotransmitter, is released by intrapancreatic nerve endings during the preabsorptive and absorptive phases of feeding. In beta-cells, ACh binds to muscarinic M(3) receptors and exerts complex effects, which culminate in an increase of glucose (nutrient)-induced insulin secretion. Activation of PLC generates diacylglycerol. Activation of PLA(2) produces arachidonic acid and lysophosphatidylcholine. These phospholipid-derived messengers, particularly diacylglycerol, activate PKC, thereby increasing the efficiency of free cytosolic Ca(2+) concentration ([Ca(2+)](c)) on exocytosis of insulin granules. IP3, also produced by PLC, causes a rapid elevation of [Ca(2+)](c) by mobilizing Ca(2+) from the endoplasmic reticulum; the resulting fall in Ca(2+) in the organelle produces a small capacitative Ca(2+) entry. ACh also depolarizes the plasma membrane of beta-cells by a Na(+-) dependent mechanism. When the plasma membrane is already depolarized by secretagogues such as glucose, this additional depolarization induces a sustained increase in [Ca(2+)](c). Surprisingly, ACh can also inhibit voltage-dependent Ca(2+) channels and stimulate Ca(2+) efflux when [Ca(2+)](c) is elevated. However, under physiological conditions, the net effect of ACh on [Ca(2+)](c) is always positive. The insulinotropic effect of ACh results from two mechanisms: one involves a rise in [Ca(2+)](c) and the other involves a marked, PKC-mediated increase in the efficiency of Ca(2+) on exocytosis. The paper also discusses the mechanisms explaining the glucose depe...

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Mechanisms and Physiological Significance of the Cholinergic Control of Pancreatic β-Cell Function

PATRICK GILON AND JEAN-CLAUDE HENQUIN

Unité d’Endocrinologie et Métabolisme, University of Louvain Faculty of Medicine, B-1200 Brussels, Belgium

Acetylcholine (ACh), the major parasympathetic neurotransmitter, is released by intrapancreatic nerve endings during the preabsorptive and absorptive phases of feeding. In β-cells, ACh binds to muscarinic M3 receptors and exerts complex effects, which culminate in an increase of glucose (nutrient)-induced insulin secretion. Activation of PLC generates diacylglycerol. Activation of PLA2 produces arachidonic acid and lysophosphatidylcholine. These phospholipid-derived messengers, particularly diacylglycerol, activate PKC, thereby increasing the efficiency of free cytosolic Ca2+ concentration ([Ca2+]c) on exocytosis of insulin granules. IP3, also produced by PLC, causes a rapid elevation of [Ca2+]c by mobilizing Ca2+ from the endoplasmic reticulum; the resulting fall in Ca2+ in the organelle produces a small capacitative Ca2+ entry. ACh also depolarizes the plasma membrane of β-cells by a Na+-dependent mechanism. When the plasma membrane is already depolarized by secretagogues such as glucose, this additional depolarization induces a sustained increase in [Ca2+]c. Surprisingly, ACh can also inhibit voltage-dependent Ca2+ channels and stimulate Ca2+ efflux when [Ca2+]c is elevated. However, under physiological conditions, the net effect of ACh on [Ca2+]c is always positive. The insulinotropic effect of ACh results from two mechanisms: one involves a rise in [Ca2+]c, and the other involves a marked, PKC-mediated increase in the efficiency of Ca2+ on exocytosis. The paper also discusses the mechanisms explaining the glucose dependence of the effects of ACh on insulin release. (Endocrine Reviews 22: 565–604, 2001)

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Abbreviations: α, cell, Glucagon-secreting cell; ACh, acetylcholine; ASCI-PLA2, ATP-stimulatable, Ca2+-independent PLA2; β, cell, insulin-secreting cell; [Ca2+]c, free cytosolic Ca2+ concentration; CGRP, calcitonin gene-related peptide; CHO, Chinese hamster ovary; DAG, diacylglycerol; δ, cell, somatostatin-secreting cell; GIP, glucose-dependent insulin-releasing peptide or gastric inhibitory polypeptide; GRP, gastrin-releasing peptide; 5-HT, 5-hydroxytryptamine (or serotonin); 1 IP, inositol 1-phosphate; K+-ATP channel, ATP-sensitive K+ channel; LHA, lateral hypothalamic area; [Na+]c, free cytosolic Na+ concentration; NO, nitric oxide; PA, phosphatidic acid; PACAP, pituitary-adenylate cyclase activating polypeptide; PC, phosphatidylcholine; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP2, phosphatidylglycerol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; PP, pancreatic polypeptide-secreting cell; SP, substance P; SERCA pump, sarco-endoplasmic reticulum Ca2+-ATPase; SOC, store-operated channel; VMH, ventromedial hypothalamic nuclei.
I. Introduction

Despite the alternation of fasting and feeding periods, the concentration of plasma glucose is maintained within a narrow range by a finely tuned balance between insulin, the only hypoglycemic hormone, and glucagon, epinephrine, corticosteroids, and GH, the major hyperglycemic hormones. The secretion of insulin by β-cells of the endocrine pancreas is regulated by glucose and other circulating nutrients. It is also modulated by several hormones and neurotransmitters, among which acetylcholine (ACH) plays a prominent role.

The complex neural control of hormone secretion by the endocrine pancreas has been the subject of other reviews (1–3). It will be addressed only briefly in our contribution, which focuses on the cholinergic control of the β-cell function. After an overview of the in vivo data demonstrating the role of the parasympathetic system in the regulation of glycemia, we analyze and synthesize the in vitro experiments that have elucidated the cellular mechanisms by which ACh influences β-cells. Particular attention is paid to the effects of ACh on phospholipid metabolism, membrane potential, free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)],), and insulin secretion. This article updates and extends other reviews on the subject (4–7).

II. The Innervation of the Endocrine Pancreas

A. General anatomical considerations

The endocrine pancreas is organized in small organs, the pancreatic islets or islets of Langerhans, that are dispersed in the exocrine parenchyma. The islets are composed of a few hundred to several thousands of cells, of which 65–80% are insulin-secreting β-cells. These cells are mainly located in the center of the islet and are surrounded by a mantel of three other cell types, i.e., glucagon-secreting α-cells, somatostatin-secreting δ-cells, and pancreatic polypeptide-secreting cells (PP-cells).

The endocrine pancreas is richly innervated, but the abundance and organization of this innervation are highly variable between species (8). Most of the nerve fibers enter the pancreas along the arteries (9, 10). Unmyelinated nerve fibers are found in the neighborhood of all islet cell types at the periphery and within the islet. At some distance from the islets, glial Schwann cells often form a thin sheet around nerve fibers on their travel toward and within the islet. In the vicinity of islet cells, however, it is not rare to see some nerve fibers lacking this glial protection and coming close to or ending blindly 20–30 nm from the endocrine cells (8, 11–17).

Well differentiated synapses with islet cells have rarely been observed (18–20). Interestingly, the innervation of the islet is very plastic, as suggested by the observation that islets transplanted in the portal vein of diabetic rats became reinnervated by hepatic nerves (21).

The autonomic innervation of the endocrine pancreas has several origins (for review, see Refs. 2 and 3). Classically, the autonomic nervous system uses two interconnected neurons to control effector functions and is divided into two systems, the sympathetic and the parasympathetic nervous systems, according to the location of the preganglionic cell bodies. However, there are indications suggesting that these two systems are not always independent of each other, but display anatomical interactions (22) or share similar neurotransmitters (23–25). The endocrine pancreas also receives other types of nerves, the anatomical origin and the function (motor efferent or sensory afferent) of which are not clearly known. These nerves are of peptidergic and nonpeptidergic nature (2, 3).

B. The parasympathetic innervation

The preganglionic fibers of the parasympathetic limb originate from perikarya located in the dorsal motor nucleus of the vagus (26–33) and possibly also in the nucleus ambiguus (26, 34–37), which are both under the control of the hypothalamus. They are organized in well separated branches traveling within the vagus nerves (cranial nerve X), and through the hepatic, gastric (31, 38), and possibly celiac branches of the vagus (39), they reach intrapancreatic ganglia that are dispersed in the exocrine tissue. These ganglia send unmethylated postganglionic fibers toward the islets (9, 10, 38, 40). Preganglionic vagal fibers release ACh that binds to nicotinic receptors on intraganglionic neurons. Postganglionic vagal fibers release several neurotransmitters: ACh, VIP, gastrin-releasing peptide (GRP), nitric oxide (NO), and pituitary adenylate cyclase-activating polypeptide (PACAP) (3, 27, 41–51). Cholinergic terminals are found in the neighborhood of all islet cell types at the periphery and within the islet (50, 52–56). The importance of the cholinergic innervation of the endocrine pancreas is attested by the presence of a 10-fold higher activity of choline acetyltransferase and acetylcholinesterase (the enzymes involved, respectively, in the synthesis and the degradation of ACh) in the islets than in the surrounding exocrine tissue (57). Cholinergic synapses with endocrine cells have been observed in some species (58, 59).

Understanding the organization of the pancreatic innervation permits correct interpretation of some experiments using different cholinergic antagonists. The stimulation of insulin release occurring upon electrical stimulation of vagal nerves in the dog is abolished by both nicotinic and muscarinic antagonists (60). In the perfused rat pancreas, nicotine produces an increase of insulin secretion that is blocked by atropine (10). These observations can be explained by the presence of nicotinic receptors on pancreatic ganglia and nerves (61–64) and muscarinic receptors on β-cells (see Section X).

The overall effect of a parasympathetic stimulation is an increase of insulin secretion (see Section III). Because postganglionic fibers contain various neurotransmitters in addition to the classic neurotransmitter ACh, it is important to keep in mind that parasympathetic neurotransmission is the sum of various biological effects. VIP and PACAP stimulate insulin secretion by increasing cAMP levels (3). GRP and its amphibian homolog, bombesin (3), are also insulinotropic (3, 42, 65–68). They act on the same family of receptors (69) and exert their action by two mechanisms, directly by stimulating β-cells through the PLC-PKC pathway (3), and indirectly by activating intrapancreatic postganglionic nerves that stimulate insulin secretion (68). NO synthase has been
detected in nerves in several organs wherein NO is considered a neurotransmitter (70, 71), and in pancreatic nitric nerves (45, 48, 49, 67). Various effects of NO on β-cells have been reported (72–75), but it is unclear whether NO is implicated in the parasympathetic modulation of insulin secretion.

The parasympathetic system also controls the secretion of the other islet hormones. Vagal nerve stimulation increases glucagon (31, 41, 60, 76–78) and PP secretion (41). The effect of vagal stimulation on β-cells is less clear, as it was reported to stimulate (79) or inhibit somatostatin secretion (78, 80). In vitro and in vivo experiments using various cholinergic agents have shown that ACh stimulates glucagon and PP secretion through atropine-sensitive mechanisms (81–84). The effects of cholinergic agonists on in vitro somatostatin secretion are again controversial (2, 80, 82, 85), although this might reflect species differences.

C. The sympathetic innervation

The sympathetic innervation of the pancreas originates from preganglionic perikarya located in the thoracic and upper lumbar segments of the spinal cord (86). The myelinated axons of these cells traverse the ventral roots to form the white communicating rami of the thoracic and lumbar nerves that reach the paravertebral sympathetic chain (87). Preganglionic fibers either communicate with a nest of ganglion cells within the paravertebral sympathetic chain or pass through the sympathetic chain, travel through the splanchnic nerves, and reach the celiac (2, 3, 35, 86, 88) and mesenteric ganglia (86). Ganglia within the paravertebral sympathetic chain, and the celiac and mesenteric ganglia, give off postganglionic fibers that eventually reach the pancreas. The existence of intrapancreatic sympathetic ganglia has also been reported (25, 26, 37). The preganglionic fibers release ACh that acts on nicotinic receptors on intraganglionic neurons, whereas the postganglionic fibers release several neurotransmitters: norepinephrine, galanin, and NPY (3, 51, 52). A rich supply of adrenergic nerves in close proximity of the islet cells has been observed in several mammalian species (53–55, 92).

The net physiological effect of splanchnic nerve stimulation is a lowering of plasma insulin concentration (93–96). This effect is attributed to release of norepinephrine from nerve fibers close to β-cells and to elevation of catecholamine (epinephrine and norepinephrine) plasma levels because of the stimulation of the adrenal medulla. Catecholamines have long been known to inhibit insulin secretion in vitro (1–3, 97) and in vivo (1–3, 98–101). Their action is mediated by α2-adrenoceptors (102), probably of the α2A- and α2C-subtypes (103), which have been identified in β-cells by both pharmacological (104) and molecular approaches (103, 105). Activation of α2-adrenoceptors interferes with the secretory process through several mechanisms that are all prevented by pertussis toxin treatment and are, thus, likely mediated by Gi or G0 (106): an inhibition of adenylate cyclase leading to a lowering of β-cell cAMP, an opening of K+ channels of small conductance leading to partial membrane repolarization and decrease in Ca2+ influx, and a major inhibition of a late step of exocytosis (106). Similar pathways are implicated in the inhibitory action of galanin, which may cooperate with catecholamines to inhibit insulin secretion in response to splanchnic nerve stimulation (3, 106). In contrast, an increase in plasma insulin can be evoked by selective β-adrenergic agonists, particularly of the β2-subtype (2, 95, 107, 108), that activate adenylate cyclase and increase cAMP. However, these usually have little effect on insulin secretion by isolated islets (109). Moreover, the presence of β2-adrenoceptors in β-cells remains controversial (105, 110). It is also important to emphasize that a number of pharmacological studies have been misinterpreted because antagonists of adrenoceptors can influence insulin secretion by acting on other targets, e.g., on ATP-sensitive K+ (K+–ATP) channels (111). The mechanisms by which NPY inhibits insulin release are not clearly known and might involve a decrease in cAMP levels (3).

The sympathetic nervous system exerts profound effects on the secretion of the other islet hormones. Splanchnic nerve stimulation increases glucagon secretion (93–96, 110, 112), and epinephrine stimulates glucagon secretion in vitro and in vivo (84, 100, 114, 115). This effect results from the activation of β-adrenoceptors (101), probably of the β2-subtype (110), although one report implicates α-adrenoceptors (96). It has been shown that pancreatic α-cells express α1A, α1D, and α1F subtypes (103). Splanchnic nerve stimulation decreases somatostatin secretion (80, 96, 116), and norepinephrine inhibits somatostatin release by isolated rat islets (100). The results are less clear for PP secretion. Thus, splanchnic nerve stimulation has been reported to increase (3, 113, 117) or inhibit PP secretion (2, 116). Catecholamines stimulate PP release by isolated islets (118).

Overall, the sympathetic nervous system serves to maintain or increase glycemia in various conditions of stress such as neuroglycopenia, hypovolemia, or physical exercise (3). Its pancreatic action not only involves inhibition of insulin secretion, but also stimulation of glucagon secretion (3, 119).

D. Sensory fibers

Calcitonin gene-related peptide (CGRP) and substance P (SP) are thought to report sensory information in many systems (120). CGRP (51, 121, 122) and SP-immunoreactive (51, 123, 124) nerve fibers have been observed in both the exocrine and endocrine pancreas. Vanilloid receptors, activated by heat, low pH, and various vanilloid agents (such as capsaicin), are localized in sensory fibers and generally report pain information (120). Neonatal treatment of mice with capsaicin destroys the majority of capsaicin-sensitive neurons and has often been used to identify sensory fibers (120). This treatment was followed by a marked reduction of CGRP-immunoreactive fibers in both the endocrine and exocrine pancreas (122) and by a partial reduction in SP-immunoreactive fibers (36, 125).

It is thought that sensory afferents leave the pancreas along the sympathetic fibers within the splanchnic nerves and that the perikarya of the sensory fibers are present in dorsal root ganglia, mainly at the level of the lower thoracic segments of the spinal cord, transmitting noxious information to the central nervous system by synapsing on second-order neurons of the dorsal horn of the spinal cord (2, 86, 126, 127). The existence of such an anatomical route is supported...
by experiments of retrograde labeling (36, 86, 126, 128). It has been suggested that the pancreas is also innervated by sensory afferents that run within the vagus nerve, the perikarya of which are in the nodose ganglion and transmit information to the nucleus tractus solitarius (30, 35, 36, 129, 130).

There is no doubt that sensory nerve fibers report pain information associated with diseases of the exocrine tissue, such as pancreatic cancer and pancreatitis (127, 131), but there are no reports of sensations of pain associated with a destruction of the endocrine pancreas. However, it is possible that sensory fibers play a role in the control of insulin secretion. Thus, neonatal treatment of mice with capsaicin (to destroy these fibers) results in more glucose-stimulated insulin secretion than in nontreated mice, suggesting that sensory fibers exert a direct, tonic inhibition of insulin secretion (132). CGRP may inhibit insulin secretion through a direct action on the islets (121, 133), whereas both inhibitory (134) and stimulatory (124, 135) effects of SP have been reported. Indirect effects of capsaicin-sensitive fibers are also possible. Indeed, it has been reported that removal of endogenous sensory neuropeptides by deafferentation of capsaicin-sensitive sensory nerves improves glucose tolerance by increasing in vivo insulin sensitivity (136, 137).

E. Other types of nerves

Immunocytochemistry has revealed the presence of neurotransmitters other than those described above in pancreatic nerves: cholecystokinin (138), 5-hydroxytryptamine (5-HT or serotonin) (139, 140), and methionine-enkephalin (3, 51). These might also influence insulin secretion: cholecystokinin stimulates insulin release by activating PLC and PLA2 (138), but the effects of 5-HT are controversial, as both inhibition (141, 142) and stimulation (143) of insulin secretion have been reported. Enkephalin also exerts variable effects depending on the concentration used and the species studied (144, 145).

The pancreatic innervation presents other interesting features. The section of extrinsic pancreatic nerves has revealed that many of the intrinsic pancreatic neurons are independent of the integrity of the extrinsic nerves (146), suggesting that the pancreatic innervation might behave as an independent system. This is supported by the observation that intrapancreatic ganglia are interconnected with one another, as are enteric ganglia (37, 140). It has also been suggested that intrapancreatic ganglia are connected with the duodenal myenteric plexus by nerve fibers (50), suggesting the existence of an entero-pancreatic innervation. On the other hand, ganglia from the myenteric plexus of the stomach and duodenum send nerve fibers toward the pancreas (50, 139). Many of these nerves are immunoreactive for 5-HT. Whether these innervations play a physiological role in the regulation of hormone secretion by the endocrine pancreas remains to be investigated.

III. Physiological Role of the Parasympathetic Control of β-Cells

In 1927, Zunz and LaBarre (147), using a cross-perfused canine model, showed that stimulation of the vagus nerve in one dog induced hypoglycemia in the other animal. In 1967, three in vivo studies performed in the dog and the baboon reported that stimulation of the vagus nerve increased plasma insulin, and that this effect involved muscarinic receptors because it was inhibited by atropine (148–150). At the same time, an in vitro study showed that cholinergetic agonists stimulated insulin release from pieces of rat pancreas, and this effect was also antagonized by atropine (99).

The most important characteristic of the influence of ACh on insulin secretion is a tight dependence on the ambient glucose concentration. In vivo, electrical stimulation of the vagus nerve has little effect on the concentration of plasma insulin during hypoglycemia, but increases it more and more efficiently as the concentration of plasma glucose augments (41, 151–155). Similar observations have been made in vitro when the perfused pancreas (81, 156–160) or isolated islets (161–164) were used to study insulin secretion directly (Fig. 1A). This behavior is typical of a potentiating agent. The mechanisms underlying this potentiation will be explained in detail in Section IX.

From here, it is important to bear in mind that the majority of in vitro experiments were conducted with rodent islets, and that the concentration dependence of glucose-induced insulin secretion is different in rodent and human islets. Indeed, the threshold glucose concentration and the half-maximal effective concentration for insulin secretion are, respectively, around 4 and 9 mm for human islets as compared with 7 and 15 mm for mouse islets (165).

A. Difficulties and pitfalls of in vivo studies

Species differences must be considered when interpreting the effects of ACh on insulin secretion in vivo. As already mentioned in Section II, vagal stimulation can release at least five neurotransmitters (ACh, VIP, PACAP, GRP, and NO), the relative contribution of which differs between species. In the dog (49, 60, 76, 149, 166), rat (78), albino mouse (167), and calf (41), vagal stimulation of insulin secretion is mediated mainly or exclusively by muscarinic receptors because it is largely or fully prevented by atropine. This is not the case in the pig (113, 168) and in the cat (10, 169), in which neurotransmitters other than ACh are probably implicated.

A second difficulty is linked to the number of physiological events that are under parasympathetic control. Cholinergetic agonists or antagonists, stimulation of the vagus nerve, and vagotomy induce multiple effects that can indirectly interfere with insulin secretion. Cholinergetic agents influence the secretion of the other islet hormones (see Section II), but it is difficult to establish to what extent the observed changes in insulin secretion are influenced by paracrine or endocrine interactions. Such interactions depend on the organization of the microvascularization and the direction of blood flow, which are still a matter of debate (170–172). In addition, clear evidence for intraislet paracrine influences has not yet been reported (172). Importantly, the effects of ACh on insulin secretion are observed at glucose concentrations that are substimulating or stimulating for insulin release but inhibitory for glucagon release.

Several intestinal hormones, particularly glucose-dependent insulin-releasing peptide (GIP) and glucagon-like peptide-1, potently increase insulin secretion (173–175). GIP- and
glucagon-like peptide-1-secreting cells possess muscarinic receptors (176, 177), and both the stimulation of the vagus nerve and cholinergic agonists stimulate their release (178).

ACh also stimulates gastric emptying (179), which may affect the rate of glucose absorption, change in glycemia, and hence, insulin secretion (180, 181). It has also been reported that the increase in islet blood flow produced by a rise in blood glucose is mediated by the central nervous system, which senses the changes in glycemia and sends signals to islet vessels through the vagus nerves (182).

**B. Physiological situations**

1. Role of the vagus nerve on glucose tolerance. In lean animals or humans, basal insulin secretion is not affected or is only slightly decreased by vagotomy or atropinization (9, 149, 183–196), which indicates that there is no significant tonic stimulation of the β-cells by the parasympathetic system in the fasting state. In contrast, it is generally agreed that the vagus nerves participate in the control of insulinemia during the periods of feeding. The difficulty is to assess their contribution to the overall insulin-secretory response. Thus, depending on the study, the tolerance to a glucose load is unaffected or impaired by atropine or vagotomy, whereas the associated insulin response is larger, similar, or smaller. The results become more consistent when the insulinogenic index (Δ insulinemia/Δ glycemia) is calculated, and the mode of administration of glucose (oral or iv) is taken into account (197–199). Thus, when glucose is administered iv, the insulinogenic index is not affected or is hardly modified by atropine or vagotomy (184, 197, 199, 200, 202). In addition, the rise in plasma insulin is delayed, which also contributes to the glucose intolerance of vagotomized or atropine-treated rats (1, 199, 203). These results suggest that ACh potentiates the insulin response to glucose after a glucose load, a conclusion that is supported by experiments using animal models without parasympathetic innervation of the β-cells. After destruction of their β-cells by alloxan or streptozotocin, rats were transplanted with isolated islets. The vagus nerves of the receivers were intact, but the transplanted β-cells were presumably denervated at the time of test (203–205). Meal ingestion induced a glucose increase that was larger in transplanted than in normal control rats, and that was associated with a delayed insulin response (40, 206, 207). This confirms that a direct parasympathetic innervation of β-cells improves glucose tolerance.

2. The vagus nerves transmit signals of several origins. When evaluating the physiological role of the muscarinic control of β-cells, it is important to bear in mind that the vagus nerves are the parasympathetic effectors of signals that are all integrated in the brain but come from at least four sources: cephalic sensory organs including those of the oral cavity and the visual and olfactory systems, the gut, the liver, and the brain itself (208). The sequential activation of all these inputs will affect insulin secretion in a time-dependent manner upon meal ingestion.

a. Cephalic sensory organs and the gastrointestinal tract. The preabsorptive insulin phase corresponds to the earliest plasma insulin rise during the first minutes of food ingestion. It does not depend on nutrient assimilation, as it occurs before the glycemia has increased (1, 200, 206, 207, 209–216) and is sometimes associated with a transient hypoglycemia (28, 203, 217). The amplitude of the preabsorptive insulin phase is highly variable from one study to another, but it is consistently much smaller than the postabsorptive insulin phase occurring when glycemia starts increasing. It corresponds to a rise of approximately 20% (28, 216, 217) or more.
channels similar to those of \( /H9252 \) (233, 234). Glucoresponsive neurons equipped with \( K \)-rectify by a reflex involving gut glucoreceptors augmenting by a vagally induced release of incretins (203), or more di- atropine implicates the vagus nerve (176, 217, 232), but it linemia peak (203). Abolition of this phase by vagotomy and is sometimes reflected by a single preabsorptive insu- lation. Insulin is indeed very rapidly degraded by the liver peripheral or heart blood and not directly in the portal circu- lation. Insulin is indeed very rapidly degraded by the liver (50% during the first passage of blood), and the amplitude of the changes in insulinemia is much smaller in peripheral than in portal blood (192, 218). The preabsorptive insulin response involves both cholinergic and noncholinergic mechanisms (3, 216). It can be subdivided into the cephalic phase and the enteric phase.

The cephalic phase does not even require ingestion of nutrients, as it can occur in response to oral saccharin or water intake in animals (1, 204), but not in humans (219). Its mechanisms involve stimulation of oropharyngeal receptors (210, 220, 221) and probably also conditioned visual and olfactory reflexes, because an early peak of insulin secretion can be observed in animals that simply see or smell food (28).

A cephalic phase exists in humans (Refs. 28 and 222–225, but see Ref. 226), but is less easily conditioned than in animals (227, 228). Because no cephalic phase occurs after vagotomy, nor is this phase observed in diabetic animals transplanted with denervated islets, it is ascribed to a direct stimulation of \( \beta \)-cells by both cholinergic and noncholinergic fibers of the vagus nerves (3, 203–206). The sympathetic nervous system might also contribute to the cephalic phase in the dog by activating \( \beta_2 \)-adrenoceptors (229).

The enteric phase has been much less extensively studied because of the difficulty in separating it from the preceding cephalic phase and the following postabsorptive phase, which rapidly causes an increase in glycemia (28, 203, 217, 230, 231). This phase has been observed after direct infusion of a meal into the stomach or the duodenum (176, 203, 232) and is sometimes reflected by a single preabsorptive insulinemia peak (203). Abolition of this phase by vagotomy and atropine implicates the vagus nerve (176, 217, 232), but it remains unclear whether the response is mediated indirectly by a vagally induced release of incretins (203), or more directly by a reflex involving gut glucoreceptors augmenting efferent activity of the pancreatic branch of the vagus nerve (233, 234). Glucoresponsive neurons equipped with \( K^+ \)-ATP channels similar to those of \( \beta \)-cells have recently been identified in the myenteric plexus of the guinea pig ileum (235).

The role of the preabsorptive phases of insulin secretion was initially addressed by comparing the insulin and glucose responses to oral vs. iv glucose administrations (203, 236). However, it was later found that this type of comparison might be misleading because a lesser glucose tolerance after iv administration of the sugar could result from the lack of incretin effect rather than the lack of preabsorptive insulin secretion. The importance of the cephalic phase for glucose homeostasis was established by experiments showing that plasma glucose and insulin concentrations increased more after direct administration into the stomach than after oral intake of the same amount of nutrients (237, 238). These results were confirmed in a more recent study that compared the insulin and glucose responses to gastric glucose admin- istration in humans allowed or not allowed to taste food (239). Prevention of cephalic phase during food intake di- minished the glucose tolerance without changing insulin secretion during the 3-h period after the beginning of food intake. This glucose intolerance was attributed to differences in the kinetics of the changes in insulinemia, and possibly also to a larger glucagon secretion over the same period of time (239).

That the cephalic phase exerts a beneficial, long-lasting effect has also been elegantly demonstrated after oral glu- cose administration to insulin-deficient rats transplanted with denervated islets. The missing cephalic phase in these rats was mimicked by a small premeal iv injection of insulin. This restored early insulin peak did not affect subsequent plasma insulin levels during the period of glucose intake, but attenuated (without normalizing) the rise in plasma glucose levels (40).

With the exception of two reports (196, 240), all the above-described studies suggest that the timing of insulin secretion is important for optimal glucose homeostasis. By promoting anticipatory use of glucose by the liver, muscles, and adipose tissue, and by inhibiting glucose production by the liver, the preabsorptive insulin phases restrain the changes in glycemia and insulinemia within a narrow range. This may serve as a protection against overworking of the \( \beta \)-cells.

b. Liver. Like \( \beta \)-cells, the liver receives efferent vagal stim- uli in response to an oral stimulus (220, 241). Neurophysiological studies have also revealed that portal glucose injection increases efferent vagus activity innervating the pancreas, and it has been suggested that hepatic glucosensitive mechanisms may affect pancreatic function by involving hepatic vagus afferents and pancreatic vagus efferents (208). These results are supported by some physiological experiments demonstrating that a rise of the glucose concentration in the portal circulation to the liver induces an increase of insulin secretion that is prevented by vagotomy of the hepatic branch (242) and is mimicked by hepatic vagal stimulation (243). However, other studies have reported that a rise in insulinemia is mimicked by a section of the hepatic branches of the vagus nerve, whereas a drop in insulinemia is induced by electrical stimulation of this branch (241, 244).

Therefore, it has been suggested that afferent fibers exert a tonic inhibition in brainstem centers of an efferent vagal branch innervating the pancreas (241, 244). This implies that the afferent hepatic nerve activity is inversely related to the portal glucose level, which is confirmed by neurophysiological data (245–247). It is difficult to establish how glucose homeostasis is influenced by these vagally mediated messages from the liver to the endocrine pancreas.

c. Brain. Several studies suggest that an increase in the glucose concentration in the brain can increase vagal tone. Indeed, injection of glucose in the carotid artery of rats, in an amount insufficient to modify systemic plasma glucose concentra- tion, induced a rapid increase in insulin secretion that was abolished by vagotomy (182). This effect likely involves glucoresponsive neurons in the hypothalamus and the nucl- ear tractus solitarius (220, 248).

3. Is there a long-lasting vagal stimulus during the absorptive phase? Whereas it is clear that the parasympathetic system contributes to the preabsorptive insulin response, it is less obvious whether a parasympathetic stimulus of the endo- crine pancreas persists during the meal. Because ACh is
quickly degraded by cholinesterases in plasma, it is impossible to reliably measure the pancreatic ACh spillover as an index of parasympathetic neural activity of the pancreas (229). Measurements of plasma PP provide an alternative approach to evaluate the parasympathetic activity. Indeed, PP secretion is predominantly under vagal control because its secretion in vivo is nearly completely prevented by atropine or vagotomy (249–251). Meal ingestion induces a biphasic PP secretion characterized by a rapid first phase followed by a second sustained response. Both phases are prevented by atropine (229). The first phase has sometimes been correlated to the preabsorptive insulin response (250, 252). The presence of the second sustained phase supports the existence of a long-lasting cholinergic stimulus. Because cholinergic nerve fibers innervating PP and β-cells likely have a common origin, it is highly plausible that β-cells are also under the influence of a long-lasting vagal stimulus during meals. This suggestion is corroborated by the observation that atropine markedly suppressed insulin response to a meal (251). However, recent data obtained with mice lacking the M3 muscarinic receptor (the main muscarinic receptor on β-cells, see Section X) do not clarify the issue. These mice do not show any signs of impaired glucose intolerance after oral or ip glucose administration (253), but it is unclear to which extent other factors that were observed in these mice, such as increased insulin sensitivity, hypoleptinemia, and hypophagia, contributed to glucose tolerance.

C. Pathophysiological situations: hyperinsulinemia, obesity, and insulin resistance

The net effect of the central nervous system on insulin secretion is the result of a balance between the influence of the inhibitory sympathetic system and the stimulatory parasympathetic system. The metabolic consequences of a dysregulation of this subtle balance have been reviewed recently (254). Only the troubles associated with an anomaly of the parasympathetic system will be briefly mentioned here.

Two areas in the brain play a major role in the control of the efferent autonomic pathways, the ventromedial hypothalamic nuclei (VMH, also called the satiety center) and the lateral hypothalamic area (LHA, also called the feeding center) (241, 254). The VMH increases the activity of the sympathetic nervous system and decreases that of the parasympathetic nervous system, whereas opposite effects are produced by the LHA. The VMH and the LHA reciprocally inhibit each other.

Several animal models of hyperinsulinemia are characterized by a dysregulation of the sympathetic and parasympathetic pathways. A lesion of the VMH in the rat causes an exaggerated insulin response to an iv or intragastric glucose load. This hyperinsulinemia occurs rapidly, 10 min after the lesion (255), and is abolished by atropine or vagotomy (256–259). In animal models of hyperinsulinemia associated with a defect in leptin signaling, such as the ob/ob mice (abnormal leptin) or fa/fa (Zucker) rats (abnormal leptin receptors), the earliest detectable alteration of insulin secretion is a hyperresponsiveness to glucose that occurs before the animals become hyperphagic (193, 260–265). It is mediated by the vagus nerve, as it is abolished by atropine (194, 255, 260, 266) or vagotomy (193, 266). NPY is a potent physiological stimulator of feeding that is present at abnormally high levels in the hypothalamus of fa/fa rats and ob/ob mice. Rats that undergo a chronic intracerebroventricular infusion of NPY display basal and glucose-induced hyperinsulinemia that is prevented by vagotomy (267, 268). A common feature of all these animal models is a hyperinsulinemia that results from an excessive vagal cholinergic tone and an attenuation of the inhibitory sympathetic tone (254). The chronic influence of hyperglycemia on the autonomic system may also aggravate the syndrome (269).

An increased sensitivity of β-cells to ACh might also contribute to hyperinsulinemia. This has been reported in ob/ob mice (262) and in genetically normal mice subjected to a high-fat diet (46, 270, 271). The hyperinsulinemia brought about by an enhanced cholinergic over sympathetic tone and/or an exaggerated sensitivity of β-cells to ACh might be a compensatory mechanism for insulin resistance.

This fairly clear picture obtained in experimental animals cannot readily be extrapolated to human subjects. Although indirect evidence suggests that insulin secretion is more sensitive to cholinergic stimulation in insulin-resistant obese subjects than in lean subjects (272), it is widely agreed that atropine does not correct the hyperinsulinemia of obese subjects (195, 196). Moreover, the preabsorptive phase of insulin secretion was found to be enhanced (273), normal (224, 274, 275), or absent in obese subjects (276). In type 2 diabetes, the initial rise in insulin levels after a meal is often delayed or deficient (277, 278), but it is unknown whether an impaired preabsorptive vagal stimulus contributes to this defect.

IV. General Characteristics of Acetylcholine (ACh)

Effects on Insulin Secretion in Vitro

The glucose dependence of the effects of ACh on insulin release has already been emphasized (see Section III and Refs. 162 and 163) and is illustrated in Fig. 1A. At the concentration of 1 μM, ACh does not affect basal insulin secretion (3 mM glucose), but causes a rapid, marked, and sustained potentiation of insulin secretion induced by 15 mM glucose (the half-maximally effective concentration in this model). The effect of ACh starts to appear between 5 and 7 mM glucose, i.e., around the threshold concentration of the sugar (not illustrated), and persists in the presence of a maximally effective concentration of glucose (30 mM). ACh also increases insulin secretion in the presence of nutrients other than glucose, e.g., leucine (279). However, nutrients can be replaced by tolbutamide to unmask the insulinotropic effect of ACh. As shown in the lower panel of Fig. 1A, the addition of 100 μM tolbutamide to a medium containing 3 mM glucose evokes a small increase in insulin secretion (mediated by K+ ATP channel closure and membrane depolarization), and the subsequent addition of 1 μM ACh slightly potentiates insulin secretion. The larger efficacy of ACh in the presence of high glucose than in the presence of low glucose plus tolbutamide can largely be ascribed to the amplification of insulin secretion (increase in Ca2+ efficiency in exocytosis) that the sugar produces (280). This glucose dependence persists when ACh is used at high concentrations (e.g., 100 μM), which, however,
also induce a small sustained elevation of basal insulin secretion (not shown).

The pattern of ACh-induced insulin secretion critically depends on whether Ca\(^{2+}\) influx can occur (281–288). In the presence of a control medium containing extracellular Ca\(^{2+}\), the stimulation of secretion is sustained. When Ca\(^{2+}\) is omitted from the medium, only high ACh concentrations (≥10 μM) trigger a rapid, transient peak of secretion that also requires the presence of a high concentration of glucose (or other nutrients) (Refs. 162 and 289–291 and Fig. 1B). Ca\(^{2+}\) influx can also be prevented by opening K\(^{+}\)-ATP channels with diazoxide and holding the membrane at the resting potential. Under these conditions, the effect of ACh is similar to that produced in the absence of extracellular Ca\(^{2+}\) (289) (Fig. 1B, lower panel). Blockade of voltage-operated Ca\(^{2+}\) channels similarly affects the action of ACh on secretion (not shown) (287).

The mechanisms underlying these glucose, membrane potential, and Ca\(^{2+}\) dependencies of ACh-induced insulin secretion will be explained in the following paragraphs.

V. Effects of ACh on β-Cell Phospholipases

A. Activation of PLC

1. Type of PLC and mechanisms of activation. PLC enzymes hydrolyze the phosphodiester bond on the third (sn-3) position of phosphoglyceride molecules to release diacylglycerol (DAG) and a phosphorylated polar head group (Figs. 2 and 3). There exist three main groups of PLC (PLC-β, PLC-γ, and PLC-δ), each containing several subtypes (292–294). PLC-β are activated by heterotrimeric G proteins, whereas PLC-γ are activated by tyrosine kinases. The mechanisms of activation of PLC-δ are unknown (294). All three types hydrolyze phosphatidylinositol (PI), PI 4-phosphate (PIP), and PI 4,5-bisphosphate (PIP\(_2\)) in a Ca\(^{2+}\)-dependent manner to produce DAG and inositol 1-phosphate (1 IP), inositol 1,4-bisphosphate, and IP3, respectively. At low [Ca\(^{2+}\)]\(_{i}\), PIP\(_2\) is the preferred substrate (293). In some tissues, certain PLC isoforms can hydrolyze plasmeylcholine, phosphatidylethanolamine, or phosphatidylcholine (PC) (295–298).

In pancreatic cells, PLC is both cytosolic and membrane associated (299–301) and specifically hydrolyzes phosphoinositides (300, 302). Stimulation of normal β-cells (5, 286, 303, 309) and insulin-secreting tumor cells (310–313) with cholinergic agonists has long been shown to cause DAG and IP3 accumulation. By analogy with other tissues (314, 315), it is assumed that this results from activation of a PLC-β isozyme. However, it is not known which of the three PLC-β isoforms (β1, β2, or β3) identified in β-cells (316–320) is coupled to the muscarinic receptor. Activation of PLC by cholinergic agonists involves a G protein (310, 312, 322). One single study, performed with rat islets, reported that the G protein activated by carbchol is pertussis toxin sensitive and suggested that it corresponds to a G\(_{\alpha}\) protein (323). All other studies, performed with RINm5F cells (324, 325), rat islets (321, 326, 327), and β-TC3 cells (328), found the G protein coupled to the muscarinic receptor to be pertussis and cholera toxin insensitive. It is thought to belong to the G\(_{\alpha}\) subfamily (328), like the G protein that couples muscarinic receptors to PLC-β in other tissues. Activation of PLC-β has been reported to be directly mediated by the α-subunit of the G\(_{\alpha}\) protein (329). The G\(_{\alpha}\) subfamily contains several members (G\(_{\alpha5}^\alpha\text{G}_{\alpha13}\), G\(_{\alpha14}\), G\(_{\alpha15}\), and G\(_{\alpha16}\)) (330, 331) that seem to specifically interact with the different PLC-β subtypes (292). The nature of the G\(_{\alpha}\)-subunit involved in the coupling of the muscarinic receptors to PLC-β in β-cells is unknown.

Phospholipid hydrolysis and inositol production is larger in β-cells maintained in a Ca\(^{2+}\)-containing medium than in a Ca\(^{2+}\)-free medium (5, 162, 290, 303, 332), and are markedly reduced in insulin-secreting cells loaded with the Ca\(^{2+}\)-chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetracetic acid (BAPTA) (333). Two hypotheses have been put forward to explain this Ca\(^{2+}\) dependence. Because PLC is strictly Ca\(^{2+}\)-dependent (293), the enhanced hydrolysis of phosphoinositides observed in cells bathed in a Ca\(^{2+}\)-containing medium has sometimes been attributed to a direct activation of PLC by Ca\(^{2+}\). (300, 305, 307, 322, 332–338), independently from calmodulin.

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![Fig. 2. Influence of ACh on phosphoinositol metabolism in pancreatic β-cells. See text (Section V.A.2) for explanations.](image-url)
The effect of high $K^+$ on IP3 levels is species dependent and larger in the rat than in the mouse (340), perhaps because of the expression of different PLC isoforms (317, 320, 341, 342). Second, even in the rat, phosphoinositide breakdown is much larger in response to carbachol than to high $K^+$ (300, 337). This proposal was supported by the observations that high $K^+$, which induces a large rise in $[Ca^{2+}]_c$, increased IP3 or total IP levels (332, 339, 340) and accelerated the efflux of radioactivity from rat islets prelabeled with $[^3H]$inositol (335).

However, these results must be interpreted with caution. First, the effect of high $K^+$ on IP3 levels is species dependent and larger in the rat than in the mouse (340), perhaps because of the expression of different PLC isoforms (317, 320, 341, 342). Second, even in the rat, phosphoinositide breakdown is much larger in response to carbachol than to high $K^+$ (316, 332, 335, 339). Therefore, a second hypothesis suggests that the potentialization by $Ca^{2+}$ of ACh-induced IP3 production results from a synergistic effect between $Ca^{2+}$ and muscarinic activation (309, 316). As G proteins have been reported to enhance the $Ca^{2+}$ sensitivity of PLC (343–346), the $Ca^{2+}$ requirement would be reduced to the resting $[Ca^{2+}]_c$ levels. This would also explain how muscarinic agonists can elicit a significant phosphoinositide hydrolysis in cells bathed in a $Ca^{2+}$-free medium. Finally, three studies have proposed that PLC activity can also be controlled by the membrane potential, independently from a change in $[Ca^{2+}]_c$. Depolarization in a medium supplemented with methoxyverapamil or in a $Ca^{2+}$-free medium was reported to increase PLC activity in ob/ob islets (347) or in insulin-secreting β-TC3 cells loaded with 1,2-bis(o-aminophenoxy)ethane-$N,N,N',N''$-tetraacetic acid (BAPTA) (338). The opposite effect, a decrease in PLC activity, was measured in rat islets (339). Thus, the question remains unanswered.

2. Phosphoinositid and phosphoinositide metabolism. Experiments with RINm5F cells or rat islets have shown that IP3 is very rapidly transformed into inositol 1,3,4,5-tetrakisphosphate (307, 332, 348) by a kinase activated by $Ca^{2+}$-calmodulin (305, 349, 350), and into inositol 1,4-bisphosphate by a phosphatase (305, 348, 351, 352) (Fig. 2). Inositol 1,3,4,5-tetrakisphosphate can then be degraded into inositol 1,3,4-trisphosphate. All inositol phosphate isomers can be further metabolized through complex pathways (350, 353). In several studies in which the different isomers of inositol phosphate were not separated (5, 290, 311, 313), cholinergic agonists caused a monotonic increase of inositol trisphosphate levels to a plateau that was reached within approximately 1 min and thereafter maintained with only a minor decline. When the two major isomers of inositol trisphosphate, i.e., IP3 and inositol 1,3,4-trisphosphate, were separated, very different time courses of accumulation emerged (305, 311, 352). Indeed, IP3 accumulation consisted in a burst, reaching a peak within the first 5 sec of stimulation, followed by a decrease and then a lower sustained phase. By contrast, inositol 1,3,4-trisphosphate levels increased slowly, reached a maximum after approximately 30 sec of stimulation, and plateaued at that level thereafter. The biphasic increase in IP3 probably involves both a negative feedback effect of PKC on PLC activity (see Section V.A.3) and a rapid degradation of IP3 (352, 354). Indeed, the two-step conversion of IP3 into inositol 1,3,4-trisphosphate initiated by the $Ca^{2+}$-calmodulin-sensitive IP3 kinase was markedly attenuated if the rise in $[Ca^{2+}]_c$ that IP3 produces (see Section VIII.A.1) was abolished (305). This suggests that the rise in $[Ca^{2+}]_c$ contributes to the rapid degradation of IP3 and, therefore, also contributes to the transient nature of its accumulation (305, 352).

Whereas it is well established that muscarinic stimulation of insulin secretion increases with the glucose concentration, it remains unclear whether glucose, per se, potentiates ACh-induced IP3 accumulation. The larger effect that carbachol produces in the presence of high glucose (164, 304, 316, 355) might well result from an additional $Ca^{2+}$-dependent activation of PLC due to a greater increase in $[Ca^{2+}]_c$ (see Section VIII.A.3). This interpretation is supported by the observation that glucose failed to enhance carbachol-induced accumulation of inositol trisphosphate in rat islets incubated in a $Ca^{2+}$-free medium (339). Glucose itself and various intermediates of its metabolism were without effect on PLC activity in a cytosolic fraction of mouse islet homogenate.
(300). However, other reports have suggested that glucose metabolism might interact with phosphoinositid metabolism. Thus, glucose metabolites inhibited IP3 degradation (356, 357) and increased IP3 production (358) in rat islet and RINm5F cell homogenates, and directly stimulated Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores of HIT-T15 cell homogenates (359). Moreover, glucose has been reported to stimulate the de novo synthesis of DAG, inositol phosphate, phosphoinositides, or polyphosphoinositides (4, 302, 305, 311, 358, 360–366). More experiments must be performed to evaluate the possible direct effects of glucose on ACh-induced IP3 accumulation.

In many tissues, PLC mainly hydrolyzes PIP$_2$. In pancreatic $\beta$-cells, cholinergic agonists also stimulate PI hydrolysis as shown by the rapid accumulation of 1 IP independently from the other phosphoinositol intermediates in rat islets challenged with carbachol (348) (Fig. 2). In agreement with this observation, PI was found to be a better substrate than PIP$_2$ for PLC from the cytosolic fraction of mouse islet homogenates (300). Surprisingly, carbachol-induced accumulation of 1 IP in rat islets is stimulated by hyperpolarization and is inhibited by depolarization of the plasma membrane (339). The underlying mechanisms are not known. The predominance of PI hydrolysis over that of PIP$_2$ during prolonged stimulation (348) implies that DAG can be formed independently of IP3 formation. However, the potential importance of this pathway for the stimulation of insulin release has yet to be established.

At the same time cholinergic agonists hydrolyze phospholipids, they also accelerate PI turnover. DAG can be re-synthesized back to PI by the following steps (Fig. 2): 1) diacylglycerol kinase converts DAG into phosphatidic acid (PA) at the expense of an ATP; and 2) PA then reacts with CTP to form CMP-phosphatidate (CDP-DAG), which in turn reacts with inositol to form PI (346, 367–369). The enzymes involved in this cycle are present in rat islets (370, 371). Because the cycle requires phosphorylated nucleotides, its turnover was estimated by labeling islets with $^{32}$PO$_4$–. It was found that carbachol stimulates the labeling of PA and decreases that of PIP and PIP$_2$, which suggests that cholinergic agonists accelerate PI turnover after PLC activation in rat islets (5, 312, 306, 372, 373). This effect was strongly inhibited in a Ca$^{2+}$-free medium, which might result from the Ca$^{2+}$ dependence of PLC (334, 372). Concomitantly, there is an enhanced flux from PI $\rightarrow$ PIP $\rightarrow$ PIP$_2$ which is necessary for resynthesis of PIP$_2$ (322). This increased flux might result from a direct stimulation of the activity of PI 4-kinase, the enzyme responsible for the synthesis of PIP from PI (322). Because PIP kinase is inhibited by its product, PIP$_2$, hydrolysis of PIP$_2$ by PLC could also relieve this inhibition, therefore stimulating the flux for PIP$_2$ synthesis. This latter mechanism has been demonstrated in other cell types (374), but not in $\beta$-cells. Cholinergic agonists not only accelerate PI turnover, they also stimulate de novo synthesis of phospholipids, as deduced from the enhanced incorporation of $[^3H]$glycerol into DAG (375), PA, and PI in rat islets (360). Again, this de novo synthesis pathway is very much Ca$^{2+}$ dependent (360).

Of all inositol species formed upon ACh stimulation, IP3 is the physiologically more important isomer. Its effects will be described later (see Section VI.A.1).

### 3. Diacylglycerol and PKC

DAG is liposoluble and remains in the plasma membrane. It causes the translocation of its target, PKC, from the cytosol to the membrane. This translocation also requires Ca$^{2+}$ and an acidic phospholipid, such as phosphatidylserine (376, 377). Metabolism of DAG, either by DAG lipase-catalyzed deacylation (which yields arachidonic acid) or by DAG kinase-catalyzed phosphorylation (which yields PA), terminates its action on PKC (299, 378) (Fig. 3).

Cholinergic agonists produce two major species of DAG that are enriched in either arachidonate (a polyunsaturated fatty acid) or palmitate (a saturated fatty acid), and accumulate with different time courses in $\beta$-cells (Fig. 3). The concentration of arachidonate-enriched DAG increases quickly during the first seconds of stimulation, before declining and remaining at a lower sustained level. This type of DAG probably originates from PIP$_2$ that mostly contains arachidonate at the sn-2 position of the phospholipid (302, 379). By contrast, the palmitate-enriched DAG accumulates monotonically during the first minutes of stimulation (302, 375). This species resembles that produced upon glucose stimulation (365, 375), but its source upon ACh stimulation is unknown. It might originate from PLD activation (see Section V.C) and hydrolysis of PC, a phospholipid enriched in palmitate in islets, from a synergistic effect with glucose on de novo DAG synthesis, or from other undefined pathways (302, 380). A biphasic increase in polyunsaturated DAG and a delayed accumulation of saturated DAG have been documented in many other cell types (381). The differential time course of accumulation of the two DAG species might have an impact on PKC activation. Indeed, polyunsaturated DAG is a much more potent PKC activator than saturated DAG in HIT cells (365, 382) and other cell types (379).

The PKC family comprises a number of phosphatidylinerine-binding isoforms that can be classified in four groups. The conventional isoforms, or cPKC, are activated by Ca$^{2+}$ and DAG or phorbol esters; they include $\alpha$, $\beta$, $\beta\prime$, and $\gamma$, of which $\beta$ and $\beta\prime$ refer to the two gene products resulting from alternative splicing of the same PKC$\beta$ gene. The novel isoforms, or nPKC ($\delta$, $\epsilon$, $\eta$, and $\theta$), are unresponsive to Ca$^{2+}$ but are activated by DAG alone or phorbol esters. The atypical isoforms, or aPKC ($\zeta$, and $\iota$; PKCl$\alpha$ is the mouse homolog of human PKC$\alpha$), are Ca$^{2+}$ independent and do not bind DAG or phorbol esters. The PKC$\mu$ isoform is also Ca$^{2+}$ independent and is activated by phorbol esters, but has a structure different from the other isoforms (298, 381, 383–386). The nature of the PKC isoforms present in insulin-secreting cells remains controversial (reviewed in Ref. 376). In pancreatic islets, the isoform $\alpha$ predominates, but one or several of the isoforms $\beta$, $\delta$, $\epsilon$, $\zeta$, $\iota$, $\nu$, and $\mu$ (382, 387, 392, 394, 397–400).

Several studies demonstrate that cholinergic agonists induce the translocation of PKC to membranes (163, 312, 365, 382, 401), but they do not establish which isoforms are translocated. Because cholinergic agonists induce DAG accumu-
agonists differ from those of normal insulin-secreting cell lines whose responses to cholinergic stimulation of PLC from the ACh receptor (411), either by a PKC-mediated negative feedback might result from the uncoupling of PLC from the ACh receptor (411), or by phospholipase c (413), by a modification of the G protein that binds actin and Ca^{2+}-calmodulin and that has been implicated in cell movement and vesicle transport (405, 406). This substrate is phosphorylated in response to carbachol (407, 408). Other PKC substrates might be the G proteins that are associated with the α2-adrenoceptor and uncouple from the receptor after phosphorylation. This mechanism might explain how phorbol esters and carbachol reduce the ability of adrenoceptors to inhibit glucose-induced insulin secretion from rat islets (409, 410). In view of the importance of the PKC-dependent pathway in the stimulation of insulin secretion by ACh (see Section IX.B.1), identification of the targets of PKC in β-cells is an important question.

It has been suggested that PKC activation also exerts a negative feedback control on the signal transduction linked to PLC and activated by ACh. Indeed, stimulation of PKC inhibits the production of inositol phosphates induced by cholinergic agonists (290, 312, 322, 352, 393). This effect occurs within 10 min (perhaps within even less time) of stimulation with phorbol esters (352). It likely contributes to the biphase time course of accumulation of IP3 and arachidonic activation of PLC (293, 433, 434, 436). The other fraction of arachidonic acid could derive from DAG lipase-catalyzed deacylation of DAG formed after 433, 434). The other fraction of arachidonic acid could derive from DAG lipase-catalyzed deacylation of DAG formed after PLC activation (299, 434). Because PLC can be activated by ACh in Ca^{2+}-dependent and Ca^{2+}-independent ways, this pathway could explain how some arachidonic acid accumulates even in the absence of Ca^{2+}. Carbachol has also been suggested to activate ASCI-PLA2, but this proposal was based on the use of haloenol lactone suicide substrate (HELSS), an inhibitor of ASCI-PLA2 (438) that has since been shown to exert nonspecific effects in β-cells (435).

The transduction mechanisms leading to activation of PLA2 in β-cells are not known. It is likely that the increase in

PLA2 enzymes hydrolyze the sn-2 ester linkages in phosphoglyceride molecules to release a lysophospholipid and a free acid, such as arachidonate (Fig. 3). They can hydrolyze various substrates, such as PC, phosphatidylethanolamine, phosphatidylserine, PI, PA, and plasmalogens (420).

There exist several types of mammalian PLA2 (421). Types I, II, V, and VII are associated with membranes and, because they are secreted, are referred to as secretory PLA2 (sPLA2). Types I, II, and V are stimulated by millimolar Ca^{2+} concentrations, whereas type VII is Ca^{2+} independent (421). Types IV, VI, and VIII are cytosolic. Type IV is Ca^{2+} dependent, requiring micromolar Ca^{2+} concentrations to be translocated to the membrane, whereas types VI and VIII are Ca^{2+} independent (421). Types IV and VI PLA2 display a specificity for phospholipids with arachidonic acid esterified to the second carbon of the glycerol backbone (422, 423), whereas types I and II show little specificity for the hydrolyzed fatty acid chain (293, 423).

The presence of sPLA2 in insulin-secreting cells is well documented, but controversies persist concerning the type of sPLA2 that is expressed (424–426). sPLA2 might be associated with insulin-secretory granules (427). Pancreatic islets and insulin-secreting cell lines also contain type IV PLA2 and type VI cytosolic, ATP-stimulatable Ca^{2+}-independent PLA2 (ASCI-PLA2) (422, 425, 426, 428–432). Several studies suggest that ACh activates PLA2 in islets (Fig. 3). Thus, cholinergic agonists stimulate efflux of radioactive material from rat or mouse islets prelabeled with radioactive arachidonic acid (5, 433–435), and arachidonate represents the major metabolite present in the effluent fractions (433). This effect is largely Ca^{2+} dependent, as the stimulated efflux of radioactive arachidonic acid was markedly reduced by verapamil or removal of external Ca^{2+} (433, 434, 436). The persistence of a small stimulation of the efflux in a Ca^{2+}-free medium is compatible with the activation of a Ca^{2+}-independent PLA2 (433, 434, 436). Similar results were obtained by studying carbachol-induced production of PGE_{2,} an eicosanoid derived from arachidonic acid (327). Muscarinic activation of PLA2 is also supported by the demonstration that both lysophosphatidylcholine (434) and arachidonic acid (375, 434, 437) accumulate in rat islets upon stimulation with carbachol. However, because the accumulation of arachidonic acid was larger than that of lysophosphatidylcholine and was approximately 65% inhibited by RG80267, an inhibitor of DAG lipase, it is likely that only a fraction of arachidonic acid accumulation results from PLA2 activation (434). The other fraction of arachidonic acid could derive from DAG lipase-catalyzed deacylation of DAG formed after PLC activation (299, 434). Because PLC can be activated by ACh in Ca^{2+}-dependent and Ca^{2+}-independent ways, this pathway could explain how some arachidonic acid accumulates even in the absence of Ca^{2+}. Carbachol has also been suggested to activate ASCI-PLA2, but this proposal was based on the use of haloenol lactone suicide substrate (HELSS), an inhibitor of ASCI-PLA2 (438) that has since been shown to exert nonspecific effects in β-cells (435).

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[Ca\textsuperscript{2+}]\textsubscript{i} produced by cholinergic agonists activates the cytosolic Ca\textsuperscript{2+}-dependent PLA\textsubscript{2} (439). Indeed, high K\textsuperscript{+} and the Ca\textsuperscript{2+} ionophore A23187 also increased arachidonic acid accumulation within rat islets and also increased PGE\textsubscript{2} release from rat islets (440). Other mechanisms might also activate PLA\textsubscript{2}, including emptying of intracellular Ca\textsuperscript{2+} stores (441), G protein regulation (420), PKC (297, 298), and MAPK (442).

The two primary products formed upon PLA\textsubscript{2} activation are arachidonic acid and lysophosphatidylcholine (Fig. 3). Arachidonic acid has been reported to exert various effects in β-cells (380). These include Ca\textsuperscript{2+} mobilization from the endoplasmic reticulum (Refs. 443–448, but see Refs. 324 and 448), facilitation of voltage-dependent Ca\textsuperscript{2+} entry (380, 449), increase in [Ca\textsuperscript{2+}]\textsubscript{i} through voltage-independent Ca\textsuperscript{2+} channels (449), activation of K\textsuperscript{+}-ATP channels (450), and stimulation of PKC (446, 451). Among all these effects, the last one deserves particular attention because it also exists in other cell types and consists of a direct activation of PKC or a potentiation of the PKC stimulation by DAG (297, 298, 380, 452, 453). Arachidonic acid is also the precursor of cyclooxygenase (PGs, prostacyclins, and thromboxane) and lipoxygenase products (hydroperoxyeicosatetraenoic acids, hydroxyeicosatetraenoic acids, and leukotrienes) (297) that seem to exert various, although not major, modulatory effects on insulin secretion (380, 454, 455). Lysophosphatidylcholine also activates PKC in the presence of DAG in various cell types (381). In β-cells and insulin-secreting cell lines, it stimulates 45Ca\textsuperscript{2+} efflux and insulin secretion (456–459).

C. Activation of PLD

PLD catalyzes the hydrolysis of the terminal diester bond of the membrane glycerophospholipids, resulting in the formation of PA and a free polar head group (Fig. 3). Different PLD isoforms hydrolyze various substrates, such as PC, PI, phosphatidylserine, or phosphatidylethanolamine (460, 461). PC is the most abundant phospholipid in pancreatic islets (462–464), and its hydrolysis by PLD yields PA and choline. PLD can be activated by numerous pathways, including tyrosine kinases, PKC, or small G proteins, and seems to require various cofactors, such as fatty acids, or Ca\textsuperscript{2+}, for its activation (461, 465, 466).

The studies of PLD activation in islets have yielded conflicting results. Carbachol has been reported to increase the production of [\textsuperscript{3}H]choline in mouse islets prelabeled with [methyl\textsuperscript{3}H]choline (467), which suggests that PLD was activated. Because this effect was mimicked by sodium fluoride, which activates PLC, and by a phorbol ester, it might result from PKC activation (467, 468). Activation of PLD by PKC has been documented in other cell types (293, 460, 461, 466). However, carbachol did not affect [\textsuperscript{3}H]choline production in rat islets prelabeled with [\textsuperscript{3}H]choline (302). Therefore, the carbachol-induced accumulation of PA in rat islets (437) was not ascribed to PLD activation, but to phosphorylation by DAG kinase of DAG derived from PLC activation (318).

In various cell types, PLD activation is implicated in the regulation of vesicular trafficking, and its main product, PA, is involved in secretion, mitogenesis, and inflammation (461). PA has been reported to directly activate PKC (469). However, early suggestions that PA can act as a second messenger to stimulate insulin release (470, 471) still await confirmation. The action of PA is terminated by its conversion into lyso-PA by a PLA\textsubscript{2} or DAG by PA-phosphohydrolase (472). The resulting DAG accumulation might theoretically activate PKC. However, this mechanism is probably of minor importance because DAGs derived from PLD contain saturated or mono-unsaturated fatty acids at the sn-2 position and are poor activators of PKC (379).

VI. Effects of ACh on the Membrane Potential of β-Cells

To understand how ACh influences the membrane potential of β-cells, it is important to bear in mind the mechanisms by which glucose regulates this membrane potential. Glucose enters the β-cell by a facilitated transport system belonging to the GLUT family (473–475), and its metabolism leads to a rapid increase in the ATP/ADP ratio (476), which closes K\textsuperscript{+}-ATP channels in the plasma membrane. In the absence of glucose or at a nonstimulating glucose concentration, the ATP/ADP ratio is low. Enough K\textsuperscript{+}-ATP channels are open to confer a low electrical resistance to the plasma membrane and to keep it at the resting potential, close to the equilibrium potential of K\textsuperscript{+}. In the presence of a stimulating glucose concentration, the ATP/ADP ratio is high, and K\textsuperscript{+}-ATP channels are largely closed, which increases the resistance of the membrane. The decrease of the K\textsuperscript{+} permeability allows a yet unidentified current to depolarize the plasma membrane. When the threshold potential for activation of voltage-dependent Ca\textsuperscript{2+} channels is reached, an oscillating electrical activity starts (477, 478). Each oscillation of the membrane potential is characterized by a sustained depolarizing phase, commonly called slow wave, on top of which Ca\textsuperscript{2+} spikes occur. The effect of glucose on the membrane potential can be mimicked by other nutrients, e.g., leucine, that are metabolized by the β-cell. It can also be reproduced by a pharmacological agent, such as tolbutamide, that directly closes K\textsuperscript{+}-ATP channels (479). In contrast, the effect of glucose on the membrane potential can be antagonized by diazoxide, which directly opens K\textsuperscript{+}-ATP channels even when the ATP/ADP ratio has been increased by glucose.

A. Dependence on the electrical resistance of the plasma membrane

The effects of ACh on the membrane potential depend on the glucose concentration (Fig. 4, A–C). In the presence of a low glucose concentration (<5 mM), when the membrane potential is high (resting potential), cholinergic agonists (1–100 μM) produce only a small and sustained depolarization and do not induce electrical activity (279, 480–483) (Fig. 4A). In contrast, when the membrane has already been partially depolarized by a stimulatory concentration of glucose, the depolarizing effect of ACh is larger and is accompanied by an increase of the electrical activity (Fig. 4B). However, if glucose-induced depolarization is reversed by diazoxide, the effect of ACh on the membrane potential is again similar to that produced in low glucose (Fig. 4C). This difference is not due to the absolute level of the membrane potential before addition of ACh. Thus, the effect of ACh remains small when
the membrane is depolarized by high K⁺ or arginine in the presence of diazoxide, and is large when the membrane is depolarized by tolbutamide in low glucose (279). These results indicate that the depolarizing action of ACh critically depends on the resistance of the plasma membrane. When K⁺-ATP channels are open, either because the glucose concentration is low or because of the presence of diazoxide, the plasma membrane has a low resistance, and ACh produces only a minor depolarization. The depolarizing action of ACh is much larger when the plasma membrane has a high resistance because of the closure of K⁺-ATP channels by glucose or tolbutamide. In the presence of a stimulating glucose concentration, cholinergic agonists accelerate the slow waves of membrane potential or produce a sustained depolarization with continuous electrical activity (161, 279, 482, 484–487) (Fig. 4B). This depolarizing effect is already manifest at low concentrations of ACh (~0.1 μM) or cholinergic agonists (161, 279, 482). One report has described a peculiar inhibitory effect of muscarinic agonists on glucose-induced electrical activity in β-cells (488).

### B. Mechanisms of the depolarization

Several ionic mechanisms may depolarize the plasma membrane: a decrease of K⁺ permeability, an increase of Na⁺, Ca²⁺, or Cl⁻ permeability, or an inhibition of the electrogenic Na⁺ pump.

Before K⁺-ATP channels were identified in β-cells and were shown to be the target of glucose metabolism, measurements of ⁸⁶Rb⁺ efflux (a tracer of K⁺ efflux) from mouse islets indicated that ACh depolarizes the β-cell membrane by a mechanism other than a decrease in K⁺ conductance (285). Thus, under no experimental condition did ACh decrease ⁸⁶Rb⁺ efflux as do glucose and tolbutamide. Moreover, the effects of ACh on the electrical activity were very different from those induced by glucose through closure of K⁺-ATP channels. Indeed, a rise in the glucose concentration increased the duration of the plateau phase without affecting the frequency of slow waves, whereas low concentrations of ACh increased the frequency of slow waves of the membrane potential without affecting the duration of the plateau phase (279, 484). All available data, except those of one study (486), speak against an effect of ACh on K⁺-ATP channels. However, no direct test with the patch-clamp technique has been reported. In view of the recent suggestion that PIP₂ might negatively modulate K⁺-ATP channels, and that its hydrolysis by PLC-linked agonists might decrease K⁺-ATP channel

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**Fig. 4.** Effects of ACh on the membrane potential (A–C) and voltage-dependent Ca²⁺ current (D) of mouse pancreatic β-cells. **A**, The membrane potential of a single cell within an islet was recorded with high resistance microelectrode. A, Sodium dependence of the effect of 20 μM ACh on the membrane potential of β-cells perfused with a medium containing 3 mM glucose (G) and 2.5 mM Ca²⁺. ACh was added when indicated to a medium containing 135 mM Na⁺ (Na 135 mM) or to a medium in which Na⁺ has been replaced by N-methyl-D-glucamine (Na 0). [Redrawn from J. C. Henquin et al.: Endocrinology 122:2134–2142, 1988 (480) © The Endocrine Society.] B, Effects of two concentrations of ACh (1 and 100 μM) on the membrane potential of a β-cell perfused with a medium containing 15 mM glucose throughout. The two recordings are shown without interruption. [Redrawn from P. Gilon et al.: Biochem J 311:259–267, 1995 (545). © the Biochemical Society.] C, Effect of 1 μM ACh on the membrane potential of a β-cell perfused with a medium containing 10 mM glucose throughout. Diazoxide (100 μM) was added when indicated. By reducing the resistance of the plasma membrane, it decreases the depolarizing action of ACh. [Redrawn from M. P. Hermans et al.: Endocrinology 120:1765–1773, 1987 (279). © The Endocrine Society.] D, Inhibition of voltage-dependent Ca²⁺ current in an isolated β-cell. The current, recorded in the whole-cell mode of the patch-clamp technique, was elicited by a depolarization from −80 to +10 mV every 10 s. The upper trace shows control current and current after the application of ACh. The lower trace represents the time course of the peak Ca²⁺ current. An upward deflection corresponds to a decrease of its amplitude. The inhibitory effect of ACh depends on the concentration used and is reversible. [Redrawn from P. Gilon et al.: J Physiol 499: 65–76, 1997 (630).]
activity (489, 490), it would be interesting to evaluate whether ACh indirectly influences K+-ATP channels in β-cells.

It has been suggested that ACh inhibits Cl− channels in outside-out patches of β-cell membrane (491). However, ACh was found not to affect 36Cl− efflux from normal mouse islets (492) and 39Cl− retention by ob/ob mouse islets (493). Moreover, the depolarization produced by ACh was unaffected in a Cl−-free medium (492). Overall, these observations indicate that Cl− plays no major role in the effect of ACh on the β-cell membrane potential.

The currently accepted hypothesis is that ACh depolarizes the β-cell membrane by increasing its permeability to Na+. (480, 493). The cornerstone of this proposal is the abolition of the depolarization by omission of extracellular Na+ (Fig. 4A) (480) and the activation of a small Na+-dependent inward current by ACh (494). The hypothesis is also supported by the observations that ACh increases total Na+ content (495), 22Na+ uptake (480, 493), and free cytosolic Na+ concentration ([Na+]i) (496) in islet cells. The mechanisms by which ACh activates a Na+ current are not known. Activation of voltage-dependent Na+ channels has been ruled out for two reasons: 1) these channels are already completely inactivated at the resting potential in mouse β-cells (497) or at the plateau potential in the rat (498); and 2) tetrodotoxin, a blocker of voltage-dependent Na+ channels, does not prevent the depolarization, the 22Na+ uptake, the [Na+]i increase, or the inward current produced by ACh (480, 494, 496). Nicotinic receptors are nonselective cation channels (416, 499). However, these channels are not present in pancreatic β-cells. All effects of ACh on membrane potential, Na+ current, and [Na+]i measurements are completely prevented by atropine, whereas they are unaffected by tubocurarine or hexamethonium, two nicotinic antagonists, and are not mimicked by nicotine (480, 484, 486, 493, 494, 496).

In cardiac Purkinje cells, ACh was found to increase [Na+]i, by blockade of the Na+ pump (500). This is not the case in β-cells, because ACh- and ouabain-induced [Na+]i increases were additive (495, 496).

In various cell types, emptying of intracellular Ca2+ pools activates different conductances (Ca2+–, Na+, or K+) (501–505) carried by a family of channels called store-operated channels (SOCs) (503–505). The tumoral insulin-secreting MIN6 cells express the transient receptor potential 1 gene (506), whose human homolog encodes a nonselective channel permeable to Na+ and Ca2+ and is activated by Ca2+ store depletion (507). In platelets and lymphocytes (508, 509), intracellular Ca2+ store depletion by thapsigargin and cyclopiazonic acid, two inhibitors of the sarcoplasmic reticulum Ca2+-ATPase (SERCA) pump, activates Na+ influx. It has been hypothesized that ACh, which also depletes intracellular Ca2+ stores (see Section VIII.A.1), activates Na+ influx by a similar mechanism (487). However, this pathway accounts for only a small fraction of the influx of Na+ elicited by ACh in β-cells, because thapsigargin or cyclopiazonic acid, which empty intracellular Ca2+ pools much more efficiently than does ACh, did not mimic or abolish the rise in [Na+]i produced by ACh (510). Likewise, thapsigargin did not prevent ACh from activating an inward Na+ current (494).

It is clear that K+ is the major counterion for the increased Na+ influx in β-cells. Indeed, ACh induces a sustained stimulation of 86Rb+ efflux from mouse islets, which is abolished in a Na+-free medium (162, 285, 480). This acceleration of K+ efflux is a very sensitive response to ACh, similar to that of the membrane potential, as it is almost maximally stimulated by 1 μM ACh. Its resistance to omission of extracellular Cl− and to furosemide rules out the intervention of the Na+K+2Cl− cotransport system (492). It remains unclear whether the channel activated by ACh is highly selective for Na+ or is nonselective, carrying both K+ efflux and Na+ influx.

Activation of a Na+ conductance by muscarinic receptors is not classical, but it has also been reported in other systems. M2 receptors induce a tetrodotoxin- and pertussis toxin-resistant Na+ current in ventricular myocytes (511–513). Muscarinic stimulation activates a nonselective cationic conductance in guinea pig gastric and ileal smooth muscle cells (514–517), rabbit jejunal longitudinal cells (518), canine pyloric circular muscle cells (519), and chromaffin cells (520). Recently, an inward monovalent cation current activated by carbachol has been reported in Chinese hamster ovary (CHO) cells expressing the M3 receptor (521).

It is important to emphasize here that although the SOC current is not responsible for the influx of Na+ triggered by ACh, it can depolarize the plasma membrane by stimulating Ca2+ influx (capacitative Ca2+ entry; see Section VIII.A.2). Indeed, thapsigargin has been reported to stimulate Ca2+ influx and to depolarize β-cells (522, 523). This mechanism is activated by high concentrations of ACh (100 μM), but contributes much less to the depolarization of the plasma membrane than does the stimulation of Na+ influx that is already operative at low concentrations of the neurotransmitter (~1 μM).

C. Paradoxical hyperpolarization by ACh

Several (482, 483, 486, 487, 524, 525), but not all (480, 484), studies have reported that high concentrations of cholinergic agonists (~10 μM) produce an early transient hyperpolarization of β-cells when islets are perifused with a stimulating concentration of glucose. Because this hyperpolarization is blocked by charybotoxin, it might result from the transient opening of large conductance maxi K+ (KCa) channels activated during the large [Ca2+]i increase resulting from Ca2+ mobilization (482). Activation of a K+ current synchronized with Ca2+ release from intracellular Ca2+ stores is well documented in pancreatic acinar cells (526) and β-cells (527–530).

The rise in [Na+]i, brought about by ACh activates the sodium pump, which is electrogenic and produces a repolarizing current. However, the impact of this current only becomes evident when the depolarizing current produced by ACh stops. It is responsible for the marked and transient repolarization of the β-cell membrane upon washing of ACh (161, 480, 486, 531). It is also possible that this pump current is involved in the acceleration of the slow waves by ACh (480, 484).

VII. Other Effects of ACh in Islet Cells

Many other effects of ACh in β-cells have been reported, but they have remained controversial. Only those that were
believed to be important for the control of insulin secretion will be mentioned briefly.

A. Effects on glucose metabolism

ACh has been reported to slightly increase glucose utilization (532) and nicotinamide adenine dinucleotide (reduced form) (NADH) content in rat islets (533, 534). This effect might result from the \([\text{Ca}^{2+}]_c\) increase produced by ACh. However, other studies found glucose oxidation by mouse islets (493) and reduced nicotinamide-adenine dinucleotide (phosphate) [NAD(P)H] fluorescence (535) and glucose utilization (341) in rat islets to be unaffected by ACh. We have already emphasized that the effects of ACh on ionic fluxes and \(\beta\)-cell membrane potential differ from those induced by an increase in nutrient concentration.

B. Effects on cyclic nucleotides

ACh induced a small, rapid (284), and transient (534) increase in cAMP levels in rat islets incubated in low glucose, probably via the activation of \(\text{Ca}^{2+}\)-calmodulin-sensitive adenylyl cyclase (536, 537). However, in the presence of stimulating glucose concentrations, ACh did not affect islet cAMP levels (161, 284, 286). In contrast to the situation in the exocrine pancreas (538) and various other cell types (539), cholinergic agonists do not increase cyclic GMP (161) and NO production (540) in islets.

C. Effects on cytoplasmic pH

It has been suggested that alkalization of \(\beta\)-cells increases insulin release under certain conditions (541). ACh slightly increases intracellular pH in mouse \(\beta\)-cells and probably does so through the activation, by PKC, of the Na\(^+\)/H\(^+\) exchanger, because the effect was observed in a HEPES-buffered, bicarbonate-free medium (542, 543).

VIII. ACh Controls Free Cytosolic \(\text{Ca}^{2+}\) Concentration \((\text{[Ca}^{2+}]_c\)) in \(\beta\)-Cells

The rise of \([\text{Ca}^{2+}]_c\) in \(\beta\)-cells serves as a triggering signal for exocytosis of insulin granules. The complex effects of ACh on this triggering signal were first deciphered by \(^{45}\text{Ca}^{2+}\) efflux measurements. The conclusions of these experiments were later confirmed by more direct approaches using fluorescent probes to measure \([\text{Ca}^{2+}]_c\) directly inside the cells (Fig. 5). ACh has only small effects on \(\beta\)-cell \([\text{Ca}^{2+}]_c\), in the presence of low, nonstimulatory glucose concentrations (Fig. 5A), but causes a sustained \([\text{Ca}^{2+}]_c\) rise in the presence of high glucose (Fig. 5, B and C) (544–546). This sustained response, however, requires the presence of extracellular \(\text{Ca}^{2+}\) and the possibility for \(\text{Ca}^{2+}\) to enter \(\beta\)-cells through voltage-operated \(\text{Ca}^{2+}\) channels (Fig. 5A). At high concentrations, ACh also unexpectedly lowers \([\text{Ca}^{2+}]_c\) in \(\beta\)-cells (Fig. 5C) (545). The following paragraphs describe the mechanisms by which ACh produces these changes.

A. Mechanisms by which ACh increases \([\text{Ca}^{2+}]_c\)

1. Mobilization of \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) stores (Figs. 5A and 6, A and B). Mobilization of \(\text{Ca}^{2+}\) from intracellular \(\text{Ca}^{2+}\) stores can be studied by monitoring \(^{45}\text{Ca}^{2+}\) efflux from or into \([\text{Ca}^{2+}]_c\) in islets perfused with a \(\text{Ca}^{2+}\)-free medium, i.e., when no \(\text{Ca}^{2+}\) influx can occur. Under these conditions, cholinergic agonists increase the \(^{45}\text{Ca}^{2+}\) efflux rate (162, 279, 285–287, 291, 304, 483, 547, 548) and \([\text{Ca}^{2+}]_c\) (545, 546, 549, 550). The mechanisms underlying this \([\text{Ca}^{2+}]_c\) rise have been extensively studied with subcellular fractions or permeabilized insulin-secreting cells (324, 351, 354, 357, 443, 547, 551–561). They involve rapid production of IP3, catalyzed by PLC (see Section V.A.1), and its binding to specific IP3 receptors located on intracellular \(\text{Ca}^{2+}\) stores. The concentration of IP3 accumulated in response to maximal concentrations of carbacol has been estimated in experiments performed with RINm5F cells in which phosphoinositides were labeled to isotopic equilibrium with \(^{[3]H}\)inositol (311). An increase of IP3 of 1.5 \(\mu M\) was calculated, which is close to the reported half-maximal concentration (0.5–3 \(\mu M\)) that releases \(\text{Ca}^{2+}\) from the endoplasmic reticulum in permeabilized insulin-secreting cells (351, 552, 562). Accumulation of IP3 was very fast, in keeping with the rapidity of \(\text{Ca}^{2+}\) mobilization by ACh (311, 333). This \(\text{Ca}^{2+}\) mobilization is not produced by physiological phosphoinositols other than IP3 (367, 447, 552, 554, 563) and is prevented by injecting \(\beta\)-cells with heparin, an antagonist of IP3 receptors (523, 557, 564, 565).

The response to ACh is different in whole islets and in single cells. In whole islets, ACh induces a concentration-dependent transient peak of \([\text{Ca}^{2+}]_c\), followed by a small sustained elevation (545) (Figs. 5A and 6A). A similar biphasic pattern was reported for \(^{45}\text{Ca}^{2+}\) efflux (162, 279, 285, 287, 547, 548). This contrasts with the two types of responses occurring in single cells: a rapid single \([\text{Ca}^{2+}]_c\), transient (510, 546, 550, 566) or a series of \([\text{Ca}^{2+}]_c\) oscillations (510, 566, 567) (Fig. 6B). Similar oscillations can be produced by infusing \(\beta\)-cells with guanosine 5’-[\(\gamma\)-thio]triphosphate (527, 529, 568, 569). The reason why islets do not display \([\text{Ca}^{2+}]_c\) oscillations in response to ACh in a \(\text{Ca}^{2+}\)-free medium is attributed to the fact that the recorded \(\text{Ca}^{2+}\) signal is the average of the \([\text{Ca}^{2+}]_c\) responses of all \(\beta\)-cells within the islet. Contrary to glucose-induced \([\text{Ca}^{2+}]_c\) oscillations that result from periodic depolarizations of the plasma membrane and are coupled between all \(\beta\)-cells of the islet through gap junctions (289, 570, 571), IP3-induced \([\text{Ca}^{2+}]_c\) oscillations are not synchronized between electrically coupled \(\beta\)-cells (572).

The amplitude of the transient peak of \([\text{Ca}^{2+}]_c\), or \(^{45}\text{Ca}^{2+}\) efflux triggered by ACh largely depends on the glucose concentration present before and during ACh stimulation (162, 547, 548, 556, 573–575). It is much smaller at a low glucose concentration than at a high glucose concentration (Fig. 5A). This difference is attributed to the filling of intracellular \(\text{Ca}^{2+}\) stores by glucose (562, 565, 574). Other mechanisms, such as an enhanced production or a decreased degradation of IP3 in the presence of glucose (see Section V.A.1.b), might also be involved.

Three isoforms of the IP3 receptor have been described (I, II, and III) (576) that form both homo- and heterotetramers (577). Rat islets express more type III isoforms than types I
and II (578–580), and mouse islets express more type I isoforms than types II and III (581, 582). Type II isoform was, however, undetectable in \( \text{H}_{9252} \)-cells by immunocytochemistry (582). Because of the use of different techniques, it is unclear whether this difference between the rat and the mouse is real or only apparent. The contribution of non-\( \text{H}_{9252} \)-cells in this expression is also unknown.

Studies in various tissues have shown that the three isoforms are differently regulated by cAMP, IP3, ATP, Ca\(^{2+}\), and other factors (583, 584). Type II isoform has a higher affinity for IP3 than types I and III (584–587). Type I isoform contains a regulatory domain for PKA (588, 589), which might explain the observation that cAMP-producing agents enhance the carbachol-induced mobilization of Ca\(^{2+}\) in ob/ob mouse \( \beta \)-cells (347). All isoforms are regulated by Ca\(^{2+}\), possibly through a Ca\(^{2+}\)/calmodulin complex (590). Type I and II isoforms are allosterically modulated by Ca\(^{2+}\) so that the Ca\(^{2+}\)-mobilizing action of IP3 is markedly amplified when [Ca\(^{2+}\)]\(_c\) increases from basal (100 nm) to intermediate levels (typically \( \leq 300 \) nm), whereas it is inhibited when [Ca\(^{2+}\)]\(_c\) reaches higher concentrations (584, 591–593). These positive and negative feedback mechanisms of Ca\(^{2+}\) are considered important for generation of Ca\(^{2+}\) oscillations from IP3-sensitive Ca\(^{2+}\) stores. In contrast, type III isoform is only positively modulated by Ca\(^{2+}\), and this isoform would not be suitable for [Ca\(^{2+}\)]\(_c\) oscillations (584, 594).

The subcellular localization of IP3 receptors has not been firmly established in \( \beta \)-cells, although subcellular fraction-
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FIG. 6. Mechanisms of the effects of ACh on [Ca$^{2+}$]$_{l}$ in mouse pancreatic β-cells. All experiments were performed in the presence of 15 mM glucose. A, Mobilization of intracellular Ca$^{2+}$ in an islet perfused with a Ca$^{2+}$-free medium. Atropine (Atr) suppressed the ACh-induced small sustained elevation of [Ca$^{2+}$]$_{l}$, due to mobilization of intracellular Ca$^{2+}$ in a single cell perfused with a Ca$^{2+}$-free medium. Thapsigargin (Thapsi 1 μM), a specific inhibitor of the SERCA pump, abolished the oscillations by preventing uptake of Ca$^{2+}$ into the endoplasmic reticulum and thereby emptying it of Ca$^{2+}$. [Redrawn from Y. Miura et al.: Biochem Biophys Res Commun 224:67–73, 1996 (510).] C, Mobilization of intracellular Ca$^{2+}$ followed by capacitative Ca$^{2+}$ entry in clusters of cells whose plasma membrane was hyperpolarized with diazoxide (Dz 250 μM). Ca$^{2+}$ mobilization was observed in a Ca$^{2+}$-free medium, and capacitative Ca$^{2+}$ entry occurred upon Ca$^{2+}$ readmission to the medium. A blocker of voltage-dependent Ca$^{2+}$ channels, D-600 (100 μM), was added to the medium to ensure that the sustained [Ca$^{2+}$]$_{l}$ increase that was observed upon Ca$^{2+}$ readmission resulted exclusively from influx through voltage-independent Ca$^{2+}$ channels. D, Sustained [Ca$^{2+}$]$_{l}$ elevation in an islet perfused with a medium containing 2.5 mM Ca$^{2+}$. This sustained rise resulted essentially from the plasma membrane depolarization that ACh produced. It was lower at a high (100 μM) ACh concentration than at a low (1 μM) ACh concentration because the high concentration of the neurotransmitter activates mechanisms of [Ca$^{2+}$]$_{l}$ decrease that oppose to the mechanism of [Ca$^{2+}$]$_{l}$ increase. [Redrawn from P. Gilon et al.: Biochem J 311:259–267, 1995 (545).] E, Sustained decrease of [Ca$^{2+}$]$_{l}$ in islets whose [Ca$^{2+}$]$_{l}$ was raised by depolarizing the plasma membrane with 45 mM K$^{+}$. Diazoxide (Dz 250 μM) was added to the medium to decrease the plasma membrane resistance and prevent ACh from affecting the membrane potential. The initial [Ca$^{2+}$]$_{l}$ peak upon ACh addition reflects Ca$^{2+}$ mobilization from the endoplasmic reticulum, whereas the transient drop induced by atropine (Atrop 10 μM) reflects Ca$^{2+}$ sequestration into the endoplasmic reticulum. [A, D, and E re-drawn from P. Gilon et al.: Biochem J 311:259–267, 1995 (545). © the Biochemical Society.]
endoplasmic reticulum in the presence of ACh (510, 607). It is unclear why ACh is unable to empty the endoplasmic reticulum to the same extent as IP3 itself (50% or more in permeabilized cells) (565, 606, 608). Because desensitization of IP3 receptors does not seem to occur (369, 561, 562, 606, 608), the transient time course of IP3 elevation may be involved.

In agreement with the widespread localization of the endoplasmic reticulum within the cell, mobilization of Ca2+ by carbachol produces a rather uniform increase in [Ca2+]c, contrary to agents that stimulate Ca2+ influx through voltage-dependent Ca2+ channels and raise [Ca2+]c, preferentially in the periphery of the cell (566, 570, 609, 610). This spatial difference has sometimes been taken as an argument to explain the poor insulinotropic effect of ACh in a Ca2+-free medium. Probably because of close contacts between the endoplasmic reticulum and mitochondria (611, 612), high concentrations of carbachol can also increase the mitochondrial free Ca2+ concentration in clonal β-cells (613).

It is important to emphasize that the process of Ca2+ mobilization by ACh requires relatively high concentrations (≥1 μM) of the neurotransmitter (162, 545). Even in the presence of optimal glucose concentrations, the half-maximal effective concentration of ACh-induced Ca2+ mobilization is approximately 10 μM (545). Stimulation of Ca2+ influx is much more sensitive to ACh (see Section VIII.A.3).

2. Capacitative Ca2+ entry (Fig. 6C). In nonexcitable cells, PLC-linked agonists induce a biphasic rise in [Ca2+]cytosol. The first phase corresponds to mobilization of Ca2+ from intracellular stores, whereas the second phase corresponds to Ca2+ influx through voltage-independent Ca2+ channels belonging to the family of SOCs. The process by which emptying of intracellular Ca2+ pools activates Ca2+ influx has been called capacitative Ca2+ entry (614), but the mechanisms linking Ca2+ pool depletion to Ca2+ influx are still disputed (615, 616).

A capacitative Ca2+ entry has been documented in pancreatic β-cells (522, 550, 617). Indeed, cholinergic agonists and thapsigargin activate a Ca2+ entry sensitive to La3+ but resistant to the blockade of voltage-dependent Ca2+ channels by D-600 (methoxyverapamil) (Fig. 6C) or membrane hyperpolarization with diazoxide. However, the rise in [Ca2+]cytosol that this entry produces is small, approximately 8-fold less than that after the opening of voltage-dependent Ca2+ channels by high K+ (545). Moreover, it decreases when the membrane depolarizes, probably because the driving force for Ca2+ diminishes as the membrane potential approaches the equilibrium potential for Ca2+ (522). Contrary to other systems (615, 618), the capacitative Ca2+ entry in β-cells is not affected by the energy state of the cell, PKC activation, or serine/threonine phosphatase or tyrosine kinase inhibition (550). The situation is different in RINm5F cells in which capacitative Ca2+ entry requires activation of PKC (619).

The concentration dependence of the capacitative Ca2+ entry elicited by ACh has not been precisely studied, but high concentrations of agonists (100 μM) seem to be necessary (550).

3. Ca2+ influx through voltage-dependent Ca2+ channels (Figs. 5 and 6D). Under control conditions, when extracellular Ca2+ is present, the effects of cholinergic agonists on [Ca2+]c, and 45Ca2+ efflux largely depend on the glucose concentration or, more exactly, on the β-cell membrane potential set by the glucose concentration. In the presence of a nonstimulatory glucose concentration, when β-cells are hyperpolarized, ACh induces a biphasic change in [Ca2+]c (Fig. 5A) and 45Ca2+ efflux (162, 285, 480) characterized by an initial slight peak followed by a small sustained elevation. When β-cells are depolarized by a stimulatory or near-stimulatory glucose concentration, cholinergic agonists also induce a biphasic change in [Ca2+]c, and 45Ca2+ efflux, but both phases are now much larger than at low glucose (162, 279, 285, 287, 480, 483, 545, 617, 620, 621) (Figs. 5B–C). When Ca2+ influx is inhibited by the membrane hyperpolarization with diazoxide or by blocking the voltage-dependent Ca2+ channels, the initial peak is only partially reduced, whereas the sustained phase is largely suppressed (Fig. 5A). This indicates that the contribution of Ca2+ influx through voltage-dependent Ca2+ channels is much more important to the sustained phase than the early phase. When Ca2+ influx through voltage-dependent Ca2+ channels is prevented, the residual initial peak results from Ca2+ mobilization from the endoplasmic reticulum, and the small residual sustained phase is caused by continuous mobilization and capacitative Ca2+ entry.

ACh stimulation of Ca2+ influx through voltage-dependent Ca2+ channels is explained by the effects of the neurotransmitter on the membrane potential (described above). In low glucose or in high glucose plus diazoxide, the depolarization by ACh is too small to activate voltage-dependent Ca2+ channels. In contrast, in the presence of high glucose and other depolarizing secretagogues, ACh further activates voltage-dependent Ca2+ channels (279, 480, 545, 546, 617, 622). This constitutes the major mechanism by which ACh, already at low concentrations (~0.01 μM) (545, 546), induces a sustained [Ca2+]c, increase (545).

4. Relative importance and physiological relevance of these three mechanisms. Because the rise in [Ca2+]c resulting from the capacitative Ca2+ entry is very small, requires high concentrations of ACh, and decreases when the membrane depolarizes, its contribution to the overall rise in [Ca2+]c produced by ACh is minimal and will not be discussed further.

Although Ca2+ mobilization is by far the most widely studied effect of ACh on [Ca2+]c, its importance in the electrically excitable β-cell must be qualified. In the absence of glucose or in the presence of low concentrations of the sugar (<3 mM), ACh has almost no effect on [Ca2+]c, because little Ca2+ can be mobilized from nearly empty intracellular Ca2+ pools, and because the membrane depolarization is insufficient to open Ca2+ channels. At glucose concentrations (3–6 mM) that allow refilling of intracellular stores with Ca2+ (565, 623) but remain below the threshold for generation of electrical activity (7 mM), mobilization of Ca2+ is the major mechanism by which ACh increases [Ca2+]c. At near-stimulating glucose concentrations (~6–7 mM), the depolarization that ACh produces also triggers Ca2+ influx through voltage-dependent Ca2+ channels. At stimulating glucose concentrations, this mechanism contributes even more than the mobilization of Ca2+ to the overall increase in [Ca2+]c, brought about by ACh. Moreover, the different concentra-
tion dependencies of Ca\(^{2+}\) mobilization and Ca\(^{2+}\) influx for ACh at stimulating glucose concentrations reinforce the role of the depolarization in the \([\text{Ca}^{2+}]_c\) rise. Indeed, Ca\(^{2+}\) influx is already stimulated by low concentrations of the neurotransmitter (~0.1 \(\mu\)M), whereas Ca\(^{2+}\) mobilization requires higher ACh concentrations (~1 \(\mu\)M).

The relative contribution of each mechanism to the action of ACh in vivo is difficult to evaluate, but two reasons reinforce the view that the changes in membrane potential play a predominant role. First, without knowing what concentration of ACh can be reached in the vicinity of Ca\(^{2+}\)-secreting cells (393, 631), it is reasonable to assume that the effect observed with the lower concentrations is likely to be physiological. Second, because of the influence of non-glucose stimuli (which are not present in the experimental buffers), the threshold glucose concentration that triggers depolarization of \(\beta\) cells is lower in vivo than in vitro (624).

\section*{B. Mechanisms by which ACh decreases [Ca\(^{2+}\)]\textsubscript{c}}

When the effects of various concentrations of ACh on \([\text{Ca}^{2+}]_c\) were compared in glucose-stimulated islets, it was unexpectedly found that the steady-state \([\text{Ca}^{2+}]_c\), was higher in the presence of low concentrations (0.1–1 \(\mu\)M) of ACh than in high (~10 \(\mu\)M) concentrations (Figs. 5, B and C, and 6D). This suggests that ACh might also decrease \([\text{Ca}^{2+}]_c\), an effect that is clearly demonstrated in islets steadily depolarized with high K\(^+\) (545) (Fig. 6E). The \(45\text{Ca}^{2+}\) efflux measurements indicate that a slight acceleration of \([\text{Ca}^{2+}]_c\) efflux contributes to this effect. This acceleration may be ascribed to PKC stimulation because phorbol esters also promote \(\text{Ca}^{2+}\) efflux (407, 625, 626) by activating the plasma membrane \(\text{Ca}^{2+}\)-ATPase (627) or the Na\(^+\)/\(\text{Ca}^{2+}\) exchanger (628). PA and DAG, which increase in the presence of muscarinic agonists, stimulated \(\text{Ca}^{2+}\)-ATPase activity in an islet cell plasma membrane-enriched fraction (629).

However, membrane potential measurements also revealed that whereas low ACh concentrations increased the electrical activity elicited by glucose, high concentrations of the neurotransmitter decreased the amplitude of the spikes (Fig. 4B). Because spikes reflect \(\text{Ca}^{2+}\) influx through voltage-dependent \(\text{Ca}^{2+}\) channels, this observation suggested that high concentrations of ACh might inhibit these channels (545). This was confirmed by patch-clamp experiments (630) (Fig. 4D). ACh dose dependently inhibited the whole-cell voltage-dependent \(\text{Ca}^{2+}\) current of the L-type. Maximum inhibition was produced by approximately 100 \(\mu\)M ACh and reached about 35%, whereas the 50\% inhibitory concentration was observed at 5 \(\mu\)M ACh. This effect was mediated by a pertussis- and cholera toxin-inhibitable G protein. It is unlikely to involve DAG-sensitive PKCs, because phorbol esters increase voltage-dependent \(\text{Ca}^{2+}\) currents in insulin-secreting cells (393, 631–634). The inhibitory effect of ACh on the \(\text{Ca}^{2+}\) current is compatible with the inhibition of the L-type current by photorelease of guanosine 5’-\(\gamma\)-thio] triphosphate in \(\beta\) cells (635). Inhibition of L-type current by muscarinic receptors has also been observed in smooth muscle (636) and neuronal cells (637–641). In contrast to the situation found in normal \(\beta\) cells, the muscarinic agonist bethanecol increased the L-type \(\text{Ca}^{2+}\) current by activating PKC in HIT-T15 cells (634). This discrepancy might be related to the very different responses to muscarinic agents between normal and insulin-secreting cell lines (see Section IX.D).

The decrease in \([\text{Ca}^{2+}]_c\), occurring in the presence of high concentrations of ACh might constitute a protective mechanism against deleterious \(\text{Ca}^{2+}\) overload. As will be discussed below, it is not accompanied by an equivalent decrease in insulin secretion.

\section*{IX. Mechanisms of the Stimulation of Insulin Secretion by ACh}

ACh brings into operation at least two types of \(\text{Ca}^{2+}\)-dependent mechanisms: the first one involves a rise in \([\text{Ca}^{2+}]_c\), and the second one increases the efficacy of \(\text{Ca}^{2+}\) on exocytosis.

\subsection*{A. The rise in [Ca\(^{2+}\)]\textsubscript{c} by ACh triggers exocytosis}

When the stimulation by ACh is applied in the presence of diazoxide or a voltage-dependent \(\text{Ca}^{2+}\) channel blocker, or in a \(\text{Ca}^{2+}\)-free medium, there exists a tight temporal parallelism between the rise in \([\text{Ca}^{2+}]_c\), and insulin secretion. Indeed, insulin release is stimulated only during the transient elevation of \([\text{Ca}^{2+}]_c\), (compare trace with open circles of Fig. 1B with middle panel of Fig. 5A). This indicates that ACh triggers exocytosis by increasing \([\text{Ca}^{2+}]_c\). Two effects of \(\text{Ca}^{2+}\) may be involved: a direct action of \(\text{Ca}^{2+}\) on the exocytotic machinery close to the zone of fusion of secretory granules with the plasma membrane (642–645), and a \(\text{Ca}^{2+}\)-mediated acceleration of granule movements to sites of release (646–648). This second effect, which may serve to amplify exocytosis upon subsequent stimulation, could be independent from PKC activation but might involve a \(\text{Ca}^{2+}\)-calmodulin-dependent protein kinase (376), either myosin light chain kinase (647) or \(\text{Ca}^{2+}\)/calmodulin-dependent kinase II (648).

The amplitude of the transient secretory peak in a \(\text{Ca}^{2+}\)-free medium depends on the glucose concentration (162). Two reasons may explain this glucose-dependence: mobilization of \(\text{Ca}^{2+}\) is greater in the presence of glucose (see Section VIII.A.1), and \(\text{Ca}^{2+}\)-induced insulin secretion is increased by glucose through its amplifying pathway (for a given \([\text{Ca}^{2+}]_c\), more insulin is secreted at high glucose than at low glucose) (280, 649).

\subsection*{B. ACh increases the efficacy of \text{Ca}^{2+}\) on exocytosis}

In a \(\text{Ca}^{2+}\)-containing medium, the effects of ACh on insulin secretion result from a balance between multiple mechanisms that increase or decrease \([\text{Ca}^{2+}]_c\), and amplify the efficacy of \(\text{Ca}^{2+}\) on exocytosis. Indeed, there is no good temporal or quantitative relationship between the sustained changes in \([\text{Ca}^{2+}]_c\), and insulin secretion induced by ACh in the presence of 15 mm glucose. During the first minutes of stimulation, both \([\text{Ca}^{2+}]_c\), and insulin responses (initial peaks) increase with the concentration of the neurotransmitter (Fig. 7). However, during steady-state stimulation, concentrations of ACh that barely increase \([\text{Ca}^{2+}]_c\), strongly potentiate glucose-induced insulin secretion. This indicates that one or several mechanisms other than the rise in \([\text{Ca}^{2+}]_c\),
become operative and increase the efficacy of Ca\(^{2+}\) on the secretory machinery. In patch-clamp experiments using membrane capacitance measurements in which the intracellular Ca\(^{2+}\) concentration is artificially clamped (650), it has been clearly demonstrated that ACh sensitizes the secretory machinery to Ca\(^{2+}\). This sensitization is also evident in islets depolarized with high K\(^+\) and diazoxide. Under these conditions, ACh exerts no or minor effects on the membrane potential of \(\beta\)-cells (279, 545), lowers [Ca\(^{2+}\)]\(_e\) (545), but potentiates insulin secretion (651).

1. **The PKC pathway plays a major role.** Whereas accumulation of phosphoinositols per se is devoid of any stimulatory effect on insulin secretion (311, 319, 341), activation of PKC sensitizes the secretory machinery to Ca\(^{2+}\) (326, 625, 632, 652–655).

   Involvement of PKC in the stimulation of insulin secretion by ACh is suggested by experiments using various PKC inhibitors, including bisindolylmaleimide, H-7, and staurosporine (400, 408, 656–658). However, these experiments are not conclusive because the inhibitors are nonspecific kinase inhibitors or exert nonspecific effects (376, 659). Synthetic pseudosubstrate peptide inhibitors permit more specific inhibition of certain PKC isoforms. The insulin response of rat islets to carbachol was completely prevented by an inhibitory peptide corresponding to the consensus sequence of the pseudosubstrate regions of the PKC isoforms \(\alpha\) and \(\beta\) (660). Down-regulation of PKC by prolonged exposure (>20 h) of \(\beta\)-cells to phorbol esters strongly inhibited the insulin response to a subsequent cholinergic stimulation (163, 290, 399, 573, 661, 662). Because the treatment with the phorbol ester down-regulated DAG-sensitive PKC isoforms, with the surprising exception of PKC\(\beta\II\) isoform in 

   FIG. 7. Comparison of the effects of various concentrations of ACh on [Ca\(^{2+}\)]\(_e\), and insulin secretion measured during the first minutes of stimulation (integrated over 2 min for [Ca\(^{2+}\)]\(_e\), and 4 min for insulin secretion) and the steady-state stimulation (integrated over 3 min for [Ca\(^{2+}\)]\(_e\), and 6 min for insulin secretion) with ACh. The results are presented as percentages of control values, which were computed by integrating [Ca\(^{2+}\)]\(_e\), and insulin secretion during the last 3 and 6 min, respectively, before addition of ACh. The glucose concentration of the medium was 15 mM throughout. All data were obtained with cultured mouse islets. (Derived from Ref. 545 for [Ca\(^{2+}\)]\(_e\) experiments.)

   the major isoform expressed in normal \(\beta\)-cells, it is likely that most of the PKC-dependent effects of ACh on insulin secretion are mediated by this isoform.

   A small residual stimulation of insulin secretion by muscarinic agonists was observed in islets with down-regulated PKC (163, 290, 662, 663). It probably results from the increase in [Ca\(^{2+}\)]\(_e\), that cholinergic agents still produce in such islets (290) and from the activation of PKC-independent pathways. It is important to stress here that translocation of PKC to the plasma membrane by carbachol does not stimulate insulin secretion when [Ca\(^{2+}\)]\(_e\) is low (163, 657). The PKC-dependent stimulation of insulin secretion only occurs when [Ca\(^{2+}\)]\(_e\) is elevated (Figs. 1, A and B). It is therefore teleologically understandable that ACh brings into operation separate mechanisms that simultaneously increase [Ca\(^{2+}\)]\(_e\) (depolarization) and stimulate PKC.

   As described above, PKC activation exerts a negative feedback control on the signal transduction linked to PLC, which might explain the biphasic time course of accumulation of arachidonate-enriched DAG upon stimulation by cholinergic agents. However, this feedback control does not determine the time course of insulin secretion; cholinergic agonists can induce a sustained insulin secretion for relatively long periods (30–60 min) without any sign of desensitization (161, 162, 263, 286, 288, 664, 665). This suggests that the decrease in PLC-derived DAG levels is probably not accompanied by a parallel decrease of PKC activation. Low levels of PLC-derived DAG levels might be sufficient to maintain a sustained PKC activation. Other phospholipid-derived products formed during stimulation by ACh (arachidonic acid, lyso-phosphatidate, phosphatidate, various DAGs, and probably several other metabolites) may, alone or in synergy, stimulate PKC (297, 453) and support the sustained secretion of insulin. Such a time-dependent, multifactorial activation of PKC has been reported in various systems (381).

2. **The role of the PLA\(_2\) pathway is uncertain.** Whereas the role of the PLC-PKC pathway in the insulinotropic effect of ACh is
firmed that the linotropic effect of cholinergic agonists is still unsettled. Exogenous arachidonic acid inhibited, had no effect, or stimulated insulin secretion by mouse or rat islets depending on the glucose concentration used (435, 440, 446, 451, 668, 669). It also induced insulin release from permeabilized islets (670, 671). Arachidonic acid-stimulated insulin secretion has been reported to involve PKC (446), but it has also been reported not to involve PKC (668, 671, 672). At the concentrations that induce insulin secretion, arachidonic acid may also exert toxic effects in islets and inactivate PKC (672). Its insulinitropic effect is not blocked by norepinephrine (670), which, in contrast, prevents ACh-induced insulin secretion (673). Because of all these controversies, the contribution of the PLA2 pathway to the insulinitropic effect of cholinergic agonists is still unsettled.

C. Delayed effects of ACh on insulin secretion

It has been suggested that cholinergic agonists also exert long-lasting effects on insulin secretion. The phenomenon, referred to as time-dependent potentiation or priming, consists in the enhancement of the β-cell secretion response to various stimuli, including glucose, GIP, cholecystokinin, and tolbutamide, by prior transient stimulation with cholinergic agonists (164, 342, 366, 674, 675). This effect has been observed in the rat and the mouse (658) and might play a role during the preabsorptive phase (see Section III.B.2.a). Because it was mimicked by phorbol 12-myristate 13-acetate (PMA) (658, 676, 677), it has been ascribed to a persistent activation of PKC, which can then be readily activated by the rise in [Ca2+]c that glucose produces. However, in the perfused rat pancreas, the phenomenon could be induced by PMA (678), but not by carbachol (679). Comparison of the effects of both agents is not easy because PMA, unlike carbachol, exerts irreversible activation of PKC even after short application.

Whereas brief stimulation with ACh amplifies insulin secretion, prolonged stimulation might exert adverse effects. Exposure of rat islets to 10 μM carbachol for 3.5 h has been reported to desensitize β-cells to subsequent stimulation by glucose and cholinergic agonists (263, 665, 680). This desensitization might result from an impaired phosphoinositide pathway (342). Ubiquitination is a process whereby ubiquitin, a 76-residue protein, is associated with certain proteins to make them recognizable by the proteasome pathway that degrades them (681). Prolonged exposure (6 h) to carbachol has recently been shown to down-regulate IP3 receptors in mouse islet by the ubiquitin/proteasome pathway (582).

D. Muscarinic responses are often abnormal in insulin-secreting cell lines

Insulin-secreting cell lines have been used extensively to study stimulus-secretion coupling. They can be useful when responses occurring in non-β-cells of the islets complicate interpretation of the results, when large amounts of cells are needed for biochemical determinations, and for transfection experiments. Their use has yielded interesting data that can sometimes be extrapolated to normal β-cells. However, it is important to bear in mind that they are, in many respects, different from normal β-cells. A major difference between normal β-cells and some cell lines that were established long ago is a markedly different glucose dependence (682). Described below are some important differences regarding cholinergic effects.

RINm5F cells (a clonal rat β-cell line) are not depolarized by cholinergic agonists (311, 607), but are depolarized by phorbol esters that activate PKC (607, 654, 683, 684). This is exactly opposite to the situation in normal β-cells, in which ACh depolarizes the plasma membrane, whereas PMA lacks this effect (407, 625). In RINm5F cells, carbachol induces a transient increase in [Ca2+]c by mobilizing intracellular Ca2+, but causes a sustained secretion of insulin that is independent from a rise in [Ca2+]c, and persists after depleting the Ca2+ content of the endoplasmic reticulum with thapsigargin (311, 607). The effect of ACh on insulin secretion is poorly glucose dependent (685), and PKC down-regulation or inhibition does not affect (400, 607) or paradoxically enhances (397) insulin secretion in response to carbachol in RINm5F cells. Some of these peculiar effects might be explained by the fact that carbachol also translocates the phorbol ester-insensitive ζ-isoform of PKC (400).

In MIN6 (a mouse β-cell line) and HIT cells (a clonal hamster β-cell line), ACh and carbachol induce a transient rise in [Ca2+]c, which mainly results from Ca2+ influx. Surprisingly, they stimulate insulin secretion even when [Ca2+]c has returned to basal levels (573, 686). The latter effect is markedly reduced by PKC down-regulation (163, 399, 573). In MIN6 (686) and HIT cells (685), the insulinitropic effect of ACh is poorly dependent on the glucose concentration.

X. Nature of the Muscarinic Receptor Activated by ACh

With the exception of two reports from the same group (687, 688), there is general agreement that all direct effects of ACh on insulin-secreting cells are exclusively mediated by muscarinic receptors (288, 480, 486, 621, 689, 690).

Muscarinic receptors belong to the family of receptors with seven transmembrane domains connected by three cytoplasmic loops and three extracellular loops (691–694). Five muscarinic receptor subtypes, which elicit classical responses, have been cloned so far: the M1, M3, and M5 subtypes are linked to G proteins of the Go class and activate PLC, and the M2 and M4 are linked to pertussis toxin-sensitive G proteins of the Gi or Go class and initiate several processes such as inhibition of adenylate cyclase or of voltage-dependent Ca2+-channels and activation of the atrial cardiac K+ channel by M2 (314, 416, 513, 637, 638, 640, 693–696). However, classification of muscarinic receptors on the basis of the signal transduction is unreliable because of the overlap between the transduction pathways activated by the different subtypes (692, 697, 698).

Three strategies have been used to identify the muscarinic receptor subtypes present in β-cells: pharmacological blockade of physiological responses by selective antagonists, binding studies of selective ligands, and molecular biology studies.
A. Pharmacological studies

More than a decade ago, only three muscarinic receptor subtypes were identified and classified as neuronal M1 (high affinity for pirenzepine), cardiac M2 (M2pr, low affinity for pirenzepine/high affinity for AF-DX 116), and glandular M4 (M4pr, low affinity for both pirenzepine and AF-DX 116). A first study comparing the effects of atropine and pirenzepine on insulin secretion from the perfused rat pancreas suggested that the receptor present in β-cells was different from the M1 receptor (664). Subsequent experiments testing atropine, pirenzepine, and AF-DX 116 on insulin release, 86Rb⁺ efflux, and Ca²⁺ efflux ruled out the presence of M4 and cardiac M2 receptors in mouse islets and suggested that the receptor present in β-cells was a glandular M2 subtype (699). This was confirmed by studying the effect of other agonists or antagonists on the electrical activity (486) and insulin release (700). Later, when gene receptor analysis revealed the existence of 5 muscarinic subtypes (691, 692, 701), it clearly appeared that the glandular M2 receptor corresponded to a new subtype, the M3 subtype (702, 703). The observations that the insulin response to cholinergic agonists is mediated by a M3 subtype (699) were confirmed in vitro in RINm5F cells (704) and rat islets (288) and in vivo in the mouse (705) with more specific antagonists.

B. Binding studies

The presence of muscarinic receptors in the endocrine pancreas was clearly demonstrated by measuring the specific binding of the muscarinic antagonists [³H]-methylscopolamine or [³H]quinuclidinyl benzilate (QNB) to rat (321, 700, 706–709), mouse (46, 485), and guinea pig islets (710), or to insulin-secreting tumoral RINr cells (312) and INS-1 cells (711). Scatchard plot analysis revealed a single population of high affinity binding sites without any obvious low affinity binding sites (312, 485, 706, 709). Displacement of the binding of [³H]-methylscopolamine by various antagonists indicated the presence of M3 receptors in rat islets (700).

C. Molecular identification of the receptor subtypes

Using RT-PCR or ribonuclease protection assays, RNA encoding M3 and M4 receptor subtypes was detected in rat islets (288, 711). These two receptor subtypes were much more expressed than the M3 receptor subtype (711). Although similar results were obtained in INS-1 cells (711), this type of determination does not prove that the three types of receptors are expressed in β-cells because isolated islets contain at least four endocrine cell types (β-cells, α-cells, δ-cells, and PP-cells) as well as vascular muscle and endothelial cells. Immunocytochemical experiments using a specific antibody against the M3 receptor subtype indeed indicated that both central (mainly β-cells) and peripheral cells (mainly non-β-cells) express the M3 receptor (711). On the other hand, M3 (704, 711) and M4 receptor subtypes (704, 711), but not M1 (711), were detected in RINm5F cells. Interestingly, although several studies reported the presence of M1 and non-M1 receptors, two of these (288, 704) suggested that only M3 receptors are involved in the secretion of insulin in response to cholinergic agonists.

D. One or several receptor subtypes for several transduction pathways?

On the basis of pharmacological, binding, and RT-PCR studies, it is clear that the M3 receptor plays a central role in β-cells. The idea that this sole subtype activates several transduction pathways is supported by the observation that three different parameters of the β-cell function (insulin secretion, ⁸⁶Rb⁺ efflux, and Ca²⁺ efflux) displayed a similar antagonistic profile (699).

Activation of multiple transduction pathways by a single class of muscarinic receptors is not a unique feature of the pancreatic β-cell. Another example of complexity is found in ventricular myocytes in which cholinergic agonists, likely acting solely on the M2 subtype, inhibit L-type Ca²⁺ current through an inhibition of adenylate cyclase activity and activate a Na⁺ current (512, 513). Activation of two different transduction pathways by two different parts of the M3 receptor has also been documented in A9 fibroblast cells (712). The diversity of the effects mediated by ACh not only depends on the nature of the muscarinic receptor subtype involved, but also on posttranslational modifications (glycosylation, phosphorylation, etc.), which might be different from one cell type to another (314) or on the nature of the effector system present in the cells (315, 512, 695, 697). Indeed, when heart M2 muscarinic receptors, which classically inhibit adenylate cyclase, are expressed in CHO cells, their activation also produces nonclassical effects such as phosphoinositide breakdown (713). Similar results were found for the M1 receptor (697). Activation of all five muscarinic receptor subtypes expressed in NIH 3T3 cells has recently been shown to inhibit L-type current of this cell type (714). This suggests that each receptor subtype elicits preferential rather than specific effects, depending on the cell type in which it is expressed.

In the same line of ideas, the different concentration dependencies of the multiple effects of ACh in β-cells do not necessarily imply that several muscarinic receptors are involved. They might result from different sensitivities of the effector systems to G protein activation or from other unidentified mechanisms. Activation of transduction pathways with different concentration dependencies has recently been reported for the M3 receptor expressed in CHO cells. Moderate concentrations of carbobal (1–10 μM) elicited maximal capacitative Ca²⁺ influx, whereas higher concentrations were necessary to activate an inward monovalent cation current that depolarizes the plasma membrane (521).

XI. Summary and Conclusions

A. The physiological role of ACh

ACh is released by intrapancreatic nerve endings under the control of the vagus nerves during the preabsorptive, cephalic, and enteric phases of feeding and, very likely, also during the absorptive phase. Vagal stimulation occurs after activation of cephalic sensory organs including those of the
oral cavity and the visual and olfactory systems, and after activation of glucoreceptors in the gut, brain, and liver. ACh stimulates insulin secretion in a glucose-dependent manner, becoming more and more effective as the plasma glucose concentration increases. This stimulation appears to be important to ensure optimal glucose tolerance during the periods of feeding.

Several animal models of type 2 diabetes are characterized by an alteration of the autonomic nervous system with an increased ratio of the parasympathetic over sympathetic activities leading to hyperinsulinemia. Hyperinsulinemia is a characteristic of obesity, and the kinetics of insulin secretion is often altered in type 2 diabetes, but it is unclear to which extent these abnormalities result from an impaired activity of the autonomic nervous system. Because their effects on insulin secretion are glucose dependent, cholinergic agonists might theoretically be helpful to improve insulin secretion and glucose homeostasis in certain type 2 diabetic patients (46, 715). Although supported by some animal studies (46), this idea has not been largely tested because of insufficient selectivity of the available muscarinic agents for β-cells.

B. The mechanisms of action of ACh in β-cells

At the β-cell level, ACh binds to M3 receptors and activates several transduction pathways (Fig. 3); one of the major pathways is PLC, which mainly generates IP3 and diacylglycerol, a potent PKC activator. ACh also stimulates PLA2, probably secondary to the [Ca^{2+}]c rise. This leads to accumulation of arachidonic acid and lysophosphatidylcholine. ACh might also activate PLD by a mechanism that possibly depends on PKC activation. Many of the phospholipid-derived messengers are also, alone or in synergy with other lipid messengers such as diacylglycerol, activators of PKC (Fig. 3). Besides these complex effects on lipid metabolism, ACh also depolarizes the plasma membrane of β-cells by a Na^{+}-or nonspecific cationic-dependent mechanism (Fig. 8A, pathway 3), and possibly also by a mechanism involving SOCs activated by intracellular Ca^{2+} pool emptying (Fig. 8A, pathway 4). This depolarization is small and reaches the threshold for the activation of voltage-dependent Ca^{2+} channels only if the plasma membrane is already depolarized by secretagogues such as glucose. The glucose dependence of this depolarization largely contributes to the glucose-dependence of ACh effects on insulin secretion.

All these transduction pathways modulate [Ca^{2+}]c in β-cells (Fig. 8). ACh transiently increases [Ca^{2+}]c, by mobilizing Ca^{2+} from IP3-sensitive stores mainly in the endoplasmic reticulum (Fig. 8A, pathway 1). ACh induces a sustained increase in [Ca^{2+}]c, by stimulating Ca^{2+} influx by two pathways: through voltage-independent Ca^{2+} channels that open upon intracellular Ca^{2+} pool emptying (capacitative Ca^{2+} entry; Fig. 8A, pathway 2) and through voltage-dependent Ca^{2+} channels that are activated by depolarization (Fig. 8A, pathways 3 and 4). ACh decreases [Ca^{2+}]c under certain circumstances (Fig. 8B). This effect, which is detectable only after the initial phase of intracellular Ca^{2+} mobilization and only when [Ca^{2+}]c is sustained, requires higher ACh concentrations than those de-polarizing the plasma membrane. It results from a stimulation of Ca^{2+} efflux that likely involves PKC (Fig. 8B, pathway 6) and a G protein-mediated inhibition of Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Fig. 8B, pathway 5). It might protect β-cells against deleterious Ca^{2+} overload.

The insulinotropic effect of ACh largely depends on the glucose concentration and Ca^{2+} influx. When no Ca^{2+} influx can occur, ACh induces a transient, small, monophasic stimulation of insulin secretion, provided a high concentration of glucose is present. The tight temporal parallelism between the rises in [Ca^{2+}]c and insulin secretion that occur under these conditions indicates that ACh triggers exocytosis by increasing [Ca^{2+}]c. When Ca^{2+} influx can occur, ACh induces a biphasic stimulation of insulin secretion, the amplitude of which, again, largely depends on the glucose concentration. However, there is no good temporal and quantitative relationship between changes in [Ca^{2+}]c and insulin secretion because, in the steady state, a large stimulation of insulin secretion occurs with only a moderate increase in [Ca^{2+}]c (Fig. 7). This suggests that an additional mechanism becomes operative and increases the efficacy of Ca^{2+} on the secretory machinery. The most important amplifying mechanism involves PKC. This PKC-dependent mechanism increases insulin secretion only when [Ca^{2+}]c is sufficiently elevated above basal levels. Thus, the insulinotropic effect of ACh results from two Ca^{2+}-dependent mechanisms, one that involves a rise in [Ca^{2+}]c, and another that increases the efficacy of Ca^{2+} on exocytosis (Fig. 3).

Although the mechanisms of action of ACh have been extensively studied, many remain incompletely understood. How PKC increases insulin secretion is unclear. Because of the transient accumulation of the PLC-derived arachidonate and the multiple interactions between PKC and various phospholipid-derived products, it is not known which routes require PKC or lead to PKC activation. Interactions between PLA2, PLC, and PLD-derived products and PKC are not well defined. Moreover, it is unclear which PKC isoform is activated by ACh, whether the neurotransmitter translocates specific isoforms to different targets, and which proteins are phosphorylated by PKC. Phorbol esters not only stimulate insulin secretion, they also activate early genes and stimulate cell proliferation (403). It is unknown whether ACh could exert such effects physiologically. Many other questions await clear answers: What are the precise roles of PLA2 and PLD in the action of ACh? What is the identity of the channel involved in the depolarization produced by ACh? Is the ACh-induced inhibition of the Ca^{2+} current mediated by a cytosolic diffusible messenger or by a direct interaction with Ca^{2+} channels? How can a single subtype of receptor activate so many different transduction pathways with different concentration dependencies for ACh? Answers to this last question might be provided by the use of the recently developed model of muscarinic receptor-knockout mice (253, 641, 716) and of systems expressing truncated muscarinic receptors subtypes.
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Address all correspondence and requests for reprints to: Dr. Patrick Gilon, Unité d’Endocrinologie et Météabolisme, UCL 55.30, Avenue Hippocrate 55, B-1200 Brussels, Belgium. E-mail: gilon@endo.ucl.ac.be

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**JOURNÉES INTERNATIONALES D’ENDOCRINOLOGIE CLINIQUE**

**Henri-Pierre Klotz**

**Société Française d’Endocrinologie**

**First announcement**

The 45th Journées Internationales d’Endocrinologie Clinique will be held in Paris on May 23–24, 2002 and will be devoted to: “A decade of advances in thyroidology.”

Program will include 20 state-of-the-art lectures and a limited number of selected free communications for oral or poster presentation.


Information: Dr. G. Copinschi, Laboratory of Experimental Medicine, Brussels Free University - CP 618, 808 Route de Lennik, B-1070 Brussels, Belgium. Fax: +32 2 5556239; E-mail: klotz@ulb.ac.be