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STIMULUS-SECRETION COUPLING IN PANCREATIC β-CELLS

A patch-clamp study using caged compounds.

Carina Ämmälä

Göteborg, Sweden 1994

STIMULUS-SECRETION COUPLING IN PANCREATIC β-CELLS

A PATCH-CLAMP STUDY USING CAGED COMPOUNDS.

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Carina Ämmälä
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I. ÄMMÄLÄ C, BERGGREN PO, BOKVIST K & RORSMAN P (1992) Inhibition of L-type calcium channels by internal GTP[γS] in mouse pancreatic β cells. *Pflügers Archiv* **420**:72-77.

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ABSTRACT

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The regulation of the stimulus-secretion coupling in single mouse pancreatic β-cells was investigated using the patch-clamp technique in combination with microfluorimetry to record changes in the intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$), capacitance measurements to monitor exocytosis and flash photolysis of caged compounds to produce step increases in the concentration of the substances.

Activation of G-proteins by intracellular application of GTPγS produced a slowly developing inhibition of L-type Ca^{2+}-currents manifested as a slowed time course of activation and a reduction of the peak amplitude. Inclusion of cAMP in the pipette solution failed to counteract the GTPγS-induced inhibition excluding protein kinase A (PKA) as a mediator of the effects. The interaction between the activated G-protein and the Ca^{2+}-channel involves the entry of the Ca^{2+}-channel into distinct closed state from which the channel opens with a slower time-course than in the absence of GTPγS. A fraction of the channels exists in the modified closed state even in the absence of GTPγS as suggested by the finding that pretreatment with pertussis toxin increased the current amplitude under basal conditions.

Rapid membrane potential oscillations, similar to those observed in cells within intact islets, could be recorded from large clusters and were then associated with a transient oscillating outward current. In single β-cell, such rapid oscillations were usually not observed but could be evoked by extracellular application of carbamylcholine (CCh) or dibutyryl-cAMP (db-cAMP), compounds functionally related to hormones and neurotransmitters normally present in the intact islet, or by intracellular infusion with GTPγS. The oscillatory conductance is selective to K^+ and activated by an increase in $[Ca^{2+}]_i$. Tetraethylammonium, charybdotoxin, apamin and tolbutamide, blockers of K^+-channels previously known to be present in the β-cell, had no effect on the current suggesting it flows through a novel type of K^+-channel. The estimated single-channel conductance is 0.1 pS under physiological conditions. The properties of the K^+-channel makes it a possible candidate for a channel involved in the generation of membrane potential oscillations.

The Ca^{2+}-dependent action potentials are associated with a rise in $[Ca^{2+}]_i$. Although $Ins(1,4,5)P_3$ elicited a $[Ca^{2+}]_i$-transient 20-30 times larger than the *average* $[Ca^{2+}]_i$-transients evoked by voltage-clamp depolarizations, the rate of exocytosis produced by the different maneuvers were the same. This suggests that exocytosis, in response to membrane depolarization, requires much higher concentrations than suggested by microfluorimetry and implies the existence of steep Ca^{2+}-gradients within the β-cell. The coupling between an elevation in $[Ca^{2+}]_i$ and the stimulation of the secretion involves activation of Ca^{2+}/calmodulin-dependent protein kinase II and inhibitors of this enzyme suppress exocytosis. In addition, secretion is markedly potentiated by cAMP through activation of PKA. A minor part (20%) of this effect is mediated by an increase in Ca^{2+}-influx through L-type voltage-dependent Ca^{2+}-channels. The major (80%) potentiating effect results from a direct interaction with the secretory machinery, possibly exerted by increasing the Ca^{2+}-sensitivity of the secretory machinery, thus extending the distance from the Ca^{2+}channel over which granules can be recruited for release.

Keywords: β-cells, insulin, exocytosis, calcium, inositol trisphospahte, cyclic AMP
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REPORTS CONSTITUTING THIS THESIS

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CONTENTS

ABBREVIATIONS

ACh	acetylcholine
$[Ca^{2+}]_i$	cytoplasmic free calcium ion concentration
CaM-kinase II	calcium/calmodulin-dependent protein kinase II
cAMP	adenosine 3',5'-cyclic monophosphate
CCh	carbamylcholine
CICR	calcium-induced calcium release
CTX	charybdotoxin
DAG	diacylglycerol
GLP-1	glucagon-like peptide-1
G-protein	GTP-binding protein
Ins(1,4,5)P_3	inositol 1,4,5-trisphosphate
K_{ATP}-channel	ATP-dependent potassium channel
K_{Ca}-channel	large conductance calcium-activated potassium channel
$K_{L,Ca}$-channel	low conductance calcium-activated potassium channel
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTX	pertussis toxin
TEA	tetraethylammonium

INTRODUCTION

Electrical signalling

It has been known for more than two centuries that animal cells can generate electrical signals (GALVANI, 1791). During the 19th century the discovery of electrical activity in nerve cells was made and it was shown that electricity provides the means of carrying signals within and between nerve cells (DU BOIS-REYMOND, 1848; VON HELMHOLTZ, 1850). Propagation of the signals was proposed to be an electrical self-stimulation of the axon by inward currents spreading passively along the length of the nerve cell (HERMANN, 1905). This hypothesis was later proved to be right by HODGKIN (1937a; b), who showed that action potentials propagate electrically. The first recordings of action potentials in axons with intracellular electrodes were made by HODGKIN & HUXLEY (1939, 1945) and CURTIS & COLE (1940, 1942). The underlying ionic components were identified as a K^+- and a Na^+-current (HODGKIN & HUXLEY, 1952a; b; c). Although Na^+- and K^+-fluxes across the plasma membrane constitute the action potential, neither of these is sufficient to maintain transmitter release. By selectively blocking the Na^+- and K^+-conductances, KATZ & MILEDI (1967a; b) found a remaining current carried by Ca^{2+}-ions that is essential for neurotransmitter release.

Electrical excitability was long believed to be restricted to neurones and muscle cells. However, many endocrine cells have subsequently been shown to share this ability. Electrical activity in endocrine cells was first reported by DEAN & MATTHEWS (1968), who demonstrated that pancreatic β-cells generate Ca^{2+}-dependent action potentials under conditions associated with insulin release. Later it have been demonstrated that other hormone-producing cells, such as chromaffin and pituitary cells, are also able to produce action potentials (KIDOKORO et al., 1982; TARASKEVICH & DOUGLAS, 1977).

By the development of the patch-clamp technique it became possible to study the ionic currents flowing through various channels in the plasma membrane of single cells (HAMILL et al., 1981). The application of this technique to the pancreatic islet cells has led to the characterization of many of the of ion channel participating in the generation of the β-cell electrical activity (reviews: ASHCROFT & RORSMAN, 1989; DUNNE & PETERSEN, 1991; HENQUIN & MEISSNER; 1984). A current model for the electrical activity in β-cells suggests that membrane potential is governed by the activity of the ATP-dependent K^+-channel (K_{ATP}-channel). Once this channel

is closed, the reduced K^+-permeability leads to membrane depolarization. The depolarization opens voltage-gated Ca^{2+}-channels and the resulting increase in cytoplasmic free Ca^{2+}-concentration triggers release of insulin to the extracellular environment. Even though the channels underlying electrical activity have been characterized in detail, little is still known about the mechanisms involved in the coupling of electrical activity to insulin release.

Stimulus-secretion coupling

The term "stimulus-secretion coupling" was coined by DOUGLAS (1968) who demonstrated that the release of catecholamines from adrenal chromaffin cells stimulated with acetylcholine (ACh) was caused by an enhancement of Ca^{2+}-uptake into the chromaffin cell (DOUGLAS & POISNER, 1962). Stimulus-secretion coupling refers to all the events occurring in a cell exposed to a stimulus that eventually lead to the release of secretory products. From experiments using isolated chromaffin cells impaled with intracellular microelectrodes, Douglas and co-workers were able to record the membrane potential of isolated chromaffin cells and to demonstrate that they depolarize when exposed to ACh (DOUGLAS et al., 1967). Omission of Ca^{2+}-from the extracellular medium had no effect on the ACh-induced depolarization but abolished secretion (DOUGLAS & KANNO, 1967). These findings confirmed the hypothesis that entry of extracellular Ca^{2+} is a critical event in the stimulus-secretion coupling.

Much of the evidence suggesting a role of electrical activity in the stimulus-secretion coupling of the pancreatic β-cell has been derived from experiments using intracellular electrodes recording the membrane potential from cells within intact islets of Langerhans (HENQUIN & MEISSNER, 1984). It has been shown that the β-cell is electrically silent, with a resting membrane potential of about -70 mV, at sub-stimulatory glucose concentrations. When glucose concentration is raised above the threshold (≥6 mM) for insulin release, the β-cell starts to generate slow oscillations in membrane potential on which bursts of Ca^{2+}-dependent action potentials are superimposed (MATTHEWS & SAKAMOTO, 1975). With the development of Ca^{2+}-sensitive fluorescent dyes it became possible to directly demonstrate that glucose stimulation of insulin release is associated with a rise in $[Ca^{2+}]_i$ and that Ca^{2+} enters through voltage-activated Ca^{2+}-channels (ARKHAMMAR et al., 1987; HELLMAN et al., 1992; RORSMAN et al., 1984; WOLLHEIM & SHARP, 1984).

Upon stimulation, the membrane-bound granules fuse with the plasma membrane and the secretory products are released into the extracellular space, a process named exocytosis

(DE DUVE, 1963). Exocytosis is the principal mode of physiological secretion from a wide variety of cells, excitable and non-excitable, including neurones, neuroendocrine cells, endocrine and exocrine cells, mast cells and blood platelet (RUBIN, 1982). In the pancreatic β-cell, LACY (1961) was the first to suggest that insulin is released by exocytosis. Even though such diverse cell types discharge their different substances in response to a variety of stimuli, they share some common features in the molecular mechanism regulating exocytosis and it is well established that exocytosis is triggered by an increase in $[Ca^{2+}]_i$. However, the processes involved in the actual fusion of the granules with the plasma membrane remain obscure. In neurones, numerous Ca^{2+}-activated proteins, such as *synapsin Ia* and *b*, *synapsin IIa* and *b* and *synaptotagmin*, are associated with the synaptic vesicles, possibly participating in the docking of the vesicles to the plasma membrane (SÜDHOF & JAHN, 1991). However, even though these proteins might well exist in the pancreatic β-cell, there is no evidence that they are associated with the insulin-containing secretory granule.

Insulin secretion

Although the blood glucose concentration is subject to regulation by many hormonal factors, insulin is the only hormone that decreases the blood glucose concentration (MATTHEWS, 1977). It is therefore essential that the secretory machinery of the β-cells responds rapidly and with high sensitivity to changes in extracellular glucose concentrations. A defective secretory response has severe effects on glucose homeostasis and results in non-insulin dependent diabetes mellitus (NIDDM; EFENDIC et al., 1984). Detailed knowledge of the processes underlying the normal secretion of pancreatic hormones may therefore be central for understanding the pathogenesis of NIDDM.

The primary initiator of insulin secretion is glucose (HEDESKOV, 1980) but secretion can also be initiated by various amino acids, ketone bodies and fatty acids. Glucose entering the β-cell and is rapidly metabolized, resulting in the generation of intracellular metabolic signals (such as ATP) leading to the depolarization of the cell membrane and Ca^{2+}-entry through voltage-dependent Ca^{2+}-channels. The rise in the intracellular free Ca^{2+}-concentration initiates exocytosis. Once initiated, insulin secretion can be modulated by a number of extracellular signals. The islet of Langerhans receives a rich innervation (WOODS & PORTE, 1974) and neural influences are therefore likely to play an important role in the regulation of insulin secretion

in vivo. Exocytosis is also influenced by many hormones reaching the β-cell either by circulation or through a paracrine route. The potentiating effects of many of these substances on insulin secretion are chiefly mediated either by a Ca^{2+}-independent activation of the PLC-pathway or through activation of the cAMP-messenger system increasing the cAMP-concentration resulting in the activation of protein kinase A. Activation of PLC, possibly mediated by GTP-binding proteins, results in the hydrolysis of membrane lipids, increasing the formation of two second messengers: $InsP_3$ and DAG. $InsP_3$ acts to mobilize Ca^{2+} from intracellular stores whereas DAG may activate PKC and thereby produce protein phosphorylation (reviews: ASHCROFT 6& ASHCROFT, 1992; BERGGREN et al, 1992; PRENTKI & MATSCHINSKY, 1987).

AIMS

The patch-clamp technique and a combination of flash photolysis of caged compounds, microfluorimetry and capacitance measurements were used to investigate the mechanisms involved in the stimulus-secretion coupling in pancreatic β-cells in greater detail. The aims of the study were to:

1) investigate the involvement of GTP-binding proteins in the regulation of voltage-activated L-type Ca^{2+}-channels;

2) explore a mechanism underlying the bursting pattern of glucose stimulated electrical activity;

3) characterize the biophysical and pharmacological properties of a novel oscillatory Ca^{2+}-dependent K^+-channel;

4) examine the importance of Ca^{2+} for the initiation of insulin secretion in individual β-cells; and

5) clarify the mechanisms by which cAMP potentiates Ca^{2+}-induced insulin release.

METHODOLOGY

Patch-clamp measurements

The patch-clamp technique was employed to record whole-cell voltage-clamp currents, to control the membrane potential and to apply the sinusoidal command voltage used for analysis of changes in cell capacitance (described below). All experiments were conducted following the standard procedures described in HAMILL et al. (1981) which delineates four different recording configurations: cell-attached, whole-cell recording, outside-out patch and inside-out patch.

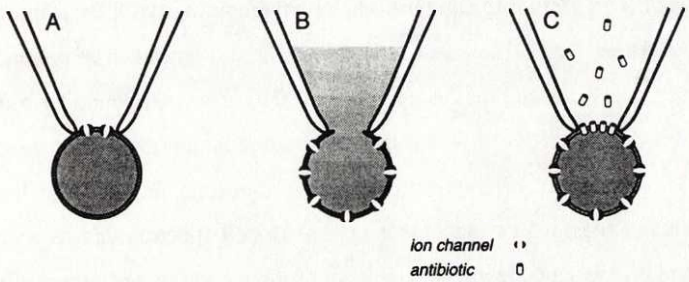


Fig. 1 The three patch-clamp configurations used in this study: A) The cell-attached, B) the whole-cell and C) the perforated-patch whole-cell configuration.

Fig. 1 summarizes the three recording configurations used in this study and here the features of these modes, which are of particular importance for the present study, are considered in greater detail:

Cell-attached: In this configuration (Fig. 1A), the plasma membrane is left intact after the formation of a high-resistance seal (>1 GΩ, a "gigaseal") between the recording pipette and the plasma membrane. The high electrical resistance of the seal reduces the background noise, thus improving the resolution of current recordings. The high resolution permits recordings of the small currents arising from the opening of single ion channels. In this configuration it is also possible to monitor changes in cell capacitance resulting from the fusion of individual secretory granules with the patch of membrane enclosed by the glass electrode. It is important to emphasize that cell-attached is the only of the original recording modes in which the intracellular milieu remains undisturbed.

Whole-cell: After formation of a "gigaseal", the membrane enclosed by the pipette can be ruptured (by a pulse of negative pressure) to establish direct physical contact with the cell interior (Fig. 1B). In small cells, this configuration yields perfect conditions for voltage-clamp recordings of ion currents flowing through channels in the plasma membrane (MARTY & NEHER, 1983). The observed current represent the summed activity of all ion channels in the entire cell membrane. The intracellular milieu can be manipulated since the cytosol is dialyzed by the pipette solution and it is thus possible to add the substance(s) of interest simply by including the compound(s) in the pipette solution. One disadvantage inherent to this configuration is that it is impossible to study processes requiring rapid intracellular application of various substances. Due to diffusional mixing delays, exchange of the cytoplasm for the pipette solution is a slow process, requiring several minutes to be complete (PUSCH & NEHER, 1988). One way to overcome this problem is to combine whole-cell recordings with the technique of flash photolysis of caged compounds (described below), where the substance can be preloaded into the cell as an inactive precursor and then converted into the active compound by a flash of UV-light. Another drawback associated with this configuration is that dialysis of the cell interior may lead to the "wash-out" of important diffusible cytosolic factors. The latter include enzymes and second messengers, which are critical to cell function and as a consequence there may be, and often is, a concomitant rundown of ionic currents and other cellular events, such as exocytosis. This configuration is therefore unsuitable for the study of processes depending on intracellular metabolism and for experiments extending over long periods of time.

Perforated-patch whole-cell: To overcome some of the problems associated with the standard whole-cell configuration, an improved version of the whole-cell configuration developed by HORN & MARTY (1988) can be used (Fig. 1C). In this configuration electrical contact is established by adding a pore-forming antibiotic, for example nystatin or amphotericin B, to the pipette solution. Insertion of the antibiotics in the plasma membrane leads to the formation of pores with a diameter of about 8 Å, allowing monovalent cations and molecules with a molecular weight of <200 D to pass. Bulky substances with a molecular weight larger than 200 D, such as enzymes and regulatory, factors are retained within the cell and cellular metabolism is preserved.

Flash photolysis of caged compounds

The technique of flash photolysis of caged compounds (KAPLAN & SOMLYO, 1989) is a useful tool for the study of biological mechanisms requiring fast application of substrates or messengers. Such studies are often difficult to perform with conventional techniques due to diffusional mixing delays of the active compound when applied to organized systems, such as a cell, that cannot be readily perifused.

Caged compounds are molecules of physiological interest (for example ATP, cAMP, $InsP_3$, GTP) that have been made biologically inactive by chemical modification (GURNEY & LESTER, 1987). The most common way to achieve "inactivity" is to add a photochemically removable 2-nitrophenylethyl group to the substance of interest. Upon exposure to ultraviolet light (UV-light), the labile chemical group is cleaved off, releasing the active compound. Desired properties of caged molecules are that the caged precursor is biologically inactive (or at least several orders of magnitude less active than the photolysis product; NICHOLS et al., 1990; ÄMMÄLÄ et al., 1991) and that the by-products of photolysis are biologically harmless. Finally the rate and efficiency of photolysis should be high and occur at wavelengths >300 nm to ascertain that the illumination as such does not damage the cell.

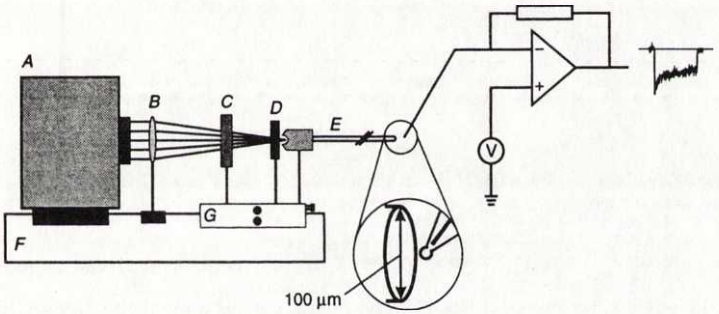


Fig. 2 The experimental set up for flash photolysis of caged compounds. The equipment is described in detail in the text.

When applied in combination with the standard whole-cell patch-clamp configuration, the technique of flash photolysis of caged compounds provides the means to study rapidly activated/inactivated processes in single cells (GOLDMAN et al., 1984; WALKER et al., 1987). The caged precursor is added to the pipette solution and, after establishing contact with the cytosol, the cell is left a few minutes to equilibrate with the pipette solution before illumination by a brief

flash (<1 s) of UV-light releases the active substance. Since recordings of several parameters, for example membrane currents and cell capacitance, can be made before and after alteration of the intracellular milieu, this technique permits the use of each cell as its own control, thus facilitating statistical analysis of the results.

Fig. 2 summarizes the experimental equipment used for flash photolysis of caged compounds in this study (ÄMMÄLÄ et al., 1991). Photorelease of the active compound was achieved by a brief pulse of UV-light from a 200 W mercury lamp (*2A*). The light was focused on the tip of a UV-transmitting optical fibre guide (Ø=100 µm; *2E*) using a quartz glass biconvex lens (*2B*). To obtain light within the range of interest, the light was transmitted through a glass band pass filter (*2C*) selective to wave-lengths between 250 and 500 nm. One end of the light guide was mounted on a XYZ-translational stage (*2G*) to place it in the focus of the lens and the other end was placed in the close vicinity of the cell. A mechanical shutter (*2D*) was used to control the time of exposure. All equipment was mounted on a profile beam (*2F*).

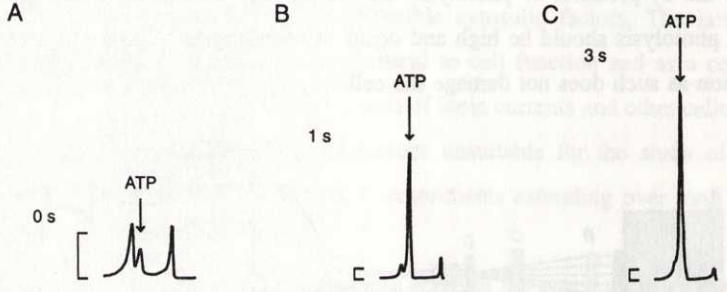


*Fig. 3 HPLC-chromatograms of released ATP: **A**) no illumination, **B**) after 1 s and **C**) after 3s of UV-illumination. Not the different scale bars (arbitrary units) in **A** compared to **B** and **C**.*

To estimate the efficiency of photolysis, a droplet containing 1 mM caged ATP was placed on a coverslip in the focus of the biconvex lens and irradiated for various times. The liberated ATP was measured using high performance liquid chromatography (HPLC). Fig. 3 shows the HPLC-chromatograms of the released nucleotide after 0 s, 1 s and 3 s of UV-irradiation. Liberation after 1 s and 3 s amounted to 75% and >95% respectively. In the subsequent studies, the efficiency of photolysis was assumed to be the same for all caged compound used. This seems as an acceptable approximation since they were all rendered inactive by similar chemical modification.

Fluorescence measurements

The intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in single β-cells was estimated using dual-emission microfluorimetry with indo-1 or a combination of fluo-3 and fura red as Ca^{2+}-indicator(s). The indicators were added to the cytoplasm by inclusion in the pipette solution. A Xenon arc-lamp was used to excite the Ca^{2+}-indicators and the emitted light was then collected by two photomultipliers at two different wavelengths (GRYNKIEWICZ et al., 1985). One advantage of dual-emission is that the ratio of the intensities at the different wavelengths, representing the Ca^{2+}-concentration, is not dependent on the concentration of indicator used. It should be emphasized that the method reports an *average* of the entire cell and that no information regarding the spatial distribution of $[Ca^{2+}]_i$ can be obtained. It is therefore probable, as discussed below, that *local* Ca^{2+}-transients within the cell exceed the *average* Ca^{2+}-concentration reported by microfluorimetry.

Capacitance measurements

The method of capacitance measurement is a high-resolution patch-clamp technique for time-resolved measurements of exo- and endocytosis in single cells (NEHER & MARTY, 1982). The technique is based on the fact that all biological membranes have a specific capacitance of about 1 $\mu F/cm^2$ (HILLE, 1992). Fusion of secretory granules with the plasma membrane and incorporation of the granular membrane leads to an increase in the surface area of the cell (Fig. 4A). By using circuit-analysis techniques, this change in surface area can be detected as an increase in cell capacitance. In a similar fashion, retrieval of membrane by endocytosis, can be detected as a decrease in membrane capacitance.

The method used for measuring capacitance in this study was a software-based two-phase lock-in amplifier (JOSHI & FERNANDEZ, 1988; LINDAU & NEHER, 1988) in conjunction with the patch-clamp technique. After formation of a "gigaseal", the conductive current transients, resulting from stray capacitances across the pipette wall (C_{st}, see Fig. 4B), are first removed using the compensation circuitry of the patch-clamp amplifier. After establishment of the whole-cell configuration (either standard or perforated patch), the capacitive and conductive current-transients arising from the initial cell surface area and the access conductance of the patch-pipette are compensated for in the same way. A sinusoidal voltage (V) is then added to the

command voltage and the resulting sinusoidal varying current (I) is measured. I has the same frequency ($\omega=2\pi f$) but is phase-shifted with respect to V. Changes in current amplitude will reflect changes in admittance (Y) since $I=V*Y(\omega)$, which in turn reflects changes in the circuit parameters of the recording configuration (i.e. the cell in series with the recording pipette). If the passive electrical properties of the recording mode were described by the cell alone with a resistance ($R_m=1/G_m$) in parallel with a capacitance (C_m), the resistive current component would be in-phase and the capacitive component phase-shifted 90° with respect to the command voltage ($Y=G_m+j\omega C_m$). The patch-clamp electrode, however, introduces an additional resistance ($R_s=1/G_s$) in series with the R_mC_m-net of the cell (Fig. 4B), and so produces an additional phase-shift. The appropriate phase-angle (θ) is therefore different from 90° and, as the circuit parameters differ from cell to cell, has to be determined for each cell separately.

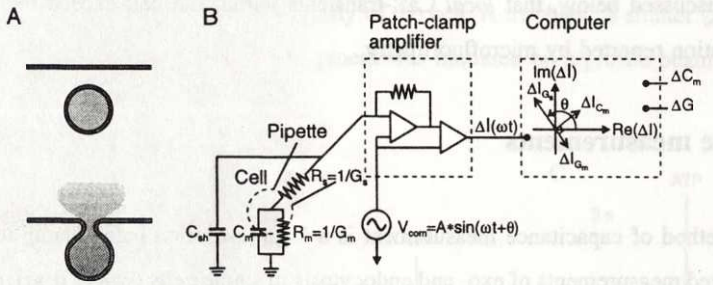


*Fig. 4 **A)** The cell membrane and a secretory granule before (top) and after (bottom) exocytosis. The area of the plasma membrane increases due to incorporation of the granular membrane. **B)** The experimental design of the capacitance measurements. To the left is the electrical equivalent of the cell in series with the recording pipette. The command voltage (V_{com}) controls the sinusoidal membrane potential and the resulting current is recorded and fed into the computer by the patch-clamp amplifier. By choosing the correct phase-angle (θ) the changes in current signal at the two orthogonal vectors, representing the cell capacitance (ΔI_{Cm}) and the conductance (ΔI_{Gs} and ΔI_{Gm}), can be monitored by the computer software.*

The shunt capacitance (C_{sh}) is compensated for at the start of an experiment and remains constant throughout the experiment unless the level of the interface between the air and the bath solution changes. For practical purposes this can be neglected and the admittance of the equivalent circuit of Fig. 4B can be expressed as

$$Y(\omega) = \frac{G_m + j\omega C_m}{1 + \frac{G_m}{G_s} + \frac{j\omega C_m}{G_s}} \qquad \text{Eq. 1}$$

When the changes in circuit parameters are small, the corresponding changes in current may be calculated by linearizing the system as follows

$$\Delta I_{G_m} = V \frac{\partial Y}{\partial G_m} \Delta G_m = VB^2 \Delta G_m \qquad \text{Eq. 2a}$$

$$\Delta I_{C_m} = V \frac{\partial Y}{\partial C_m} \Delta C_m = j\omega VB^2 \Delta C_m \qquad \text{Eq. 2b}$$

$$\Delta I_{G_s} = V \frac{\partial Y}{\partial G_s} \Delta G_s = \left(\frac{G_m}{G_s} + \frac{j\omega C_m}{G_s} \right)^2 VB^2 \Delta G_s \qquad \text{Eq. 2c}$$

Where

$$B^2 = \left(1 + \frac{G_m}{G_s} + \frac{j\omega C_m}{G_s} \right)^{-2} \qquad \text{Eq. 3a}$$

$$\theta = arg\ (\Delta I_{G_s}) = 2 \left[\arctan\left(\frac{\omega C_m}{G_m} \right) - \arctan\left(\frac{\omega C_m}{G_m + G_s} \right) \right] \qquad \text{Eq. 3b}$$

The total change in current, ΔI, is the sum of ΔI_{Gm}, ΔI_{Gs} and ΔI_{Cm}. In Fig. 4B the complex representation (i.e. as vectors with a real and an imaginary portion) of the three components and θ are shown. From Eq. 2a-c, it can be seen that ΔI_{Gm} is orthogonal (90°) to ΔI_{Cm} and almost antiparallel (~180°) to ΔI_{Gs}. Changes in the real component of ΔI reflects changes in current that are in-phase with the applied voltage, while the imaginary component measures changes that are 90° out-of-phase with respect to the command voltage. The change in current amplitude can be measured at arbitrary phase-angles with respect to the command voltage and, by choosing the correct phase-angle (θ), it is possible to monitor the two orthogonal current components, one reflecting changes in capacitance and another reflecting changes in conductance.

The current evoked by the sinusoidal command voltage is sampled and stored in a computer. The sampled response is then analyzed (on-line) by two orthogonal software detectors simply by multiplying the resulting sampled response with a sine- and cosine-function. The angle θ is determined empirically by making small changes in the compensation of G_s (on the patch-clamp amplifier) and adjusting θ until no change is seen in the current trace representing C_m. Calibration of the two signals is then provided by making changes of a known magnitude (i.e. ΔG_s=10-30 nS and ΔC_m=200-500 fF) in the compensation for C_m and G_s.

It may be objected that the method of capacitance measurements merely reports changes in an electrical property of the cell which may, or may not, be associated with exocytosis. The cell capacitance (C) is given by the equation:

$$C_m = \varepsilon \frac{A}{d}$$ Eq. 4

where A is the surface area of the cell, ε the dielectric constant of the membrane and d the thickness of the membrane. It is clear from the equation that an increase in cell capacitance could theoretically result from a thinning of the membrane; for example as a consequence of Ca^{2+}-induced swelling of the cell with a resultant distension of the membrane.

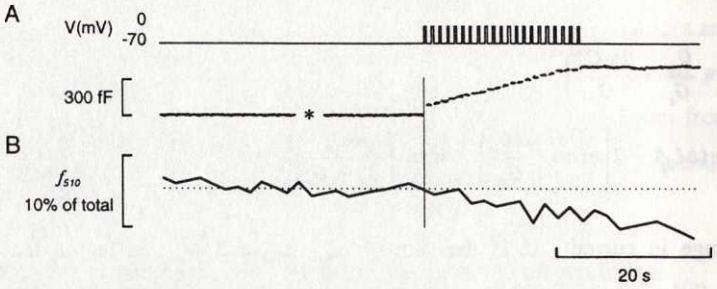


Fig. 5 A) The changes in capacitance induced by Ca^{2+}-currents (not shown) elicited by a train of voltage-clamp depolarization (200 ms, 1 Hz) and B) concomitant decrease in quinacrine fluorescence. At (*), the capacitance trace is interrupted to permit calibration of the capacitance signal.

How can one ascertain that an increase in cell capacitance does indeed reflect exocytosis? To this end we have combined capacitance measurements with microfluorimetric recordings to monitor exocytosis by two independent methods. To label the secretory granules, we have used the indicator quinacrine (LUNDQUIST et al., 1985; PRALONG et al., 1990). This indicator is a weak membrane-permeant base. Once inside the cell, it accumulates in acidic compartments such as the secretory granules (HUTTON et al., 1982; ABRAHAMSSON & GYLFE, 1981). Stimulation of the β-cell (by a train of voltage-clamp depolarizations), with resultant fusion of secretory granules, will consequently be expected to produce a *decrease* in quinacrine fluorescence, whilst increasing the cell capacitance. As shown in Fig. 5, this is exactly what is observed. The train of depolarizations produce an increase in cell capacitance of about 350 fF. From the diameter of the secretory granule (230 nm; DEAN, 1973), it can be predicted that the fusion of a single granule with the plasma membrane will produce a capacitance increase of 1.7 fF. Events of this size have indeed been reported (IV). It can thus be estimated that the increase capacitance corresponds to fusion of about 200 secretory granules with the plasma membrane. This increase in cell capacitance was coincident with a 4% decrease in quinacrine fluorescence and it can thus be estimated, assuming that quinacrine is confined to the

secretory granules (cf. LUNDQUIST et al., 1985), that this β-cell contained 5000 secretory granules. In a series of ten experiments, an average granule content of 11000±2000 was obtained (unpublished observation). The latter value is in reasonable agreement with the 13000 reported in ultrastructural studies (DEAN, 1973). We therefore feel that it may be concluded that changes in cell capacitance do indeed reflect exocytosis of insulin-containing granules.

RESULTS AN DISCUSSION

G-protein modulation of voltage-activated Ca^{2+}-currents (I).

In non-neuronal cells, two families of voltage-activated Ca^{2+}-channel, with properties resembling those previously described for two types of Ca^{2+}-channel in neurones (NOWYCKY et al., 1985), have been characterized: 1) The T-type (Transient) Ca^{2+}-channel, which activates at negative membrane potentials and displays voltage-dependent inactivation; and 2) the L-type (Lasting) Ca^{2+}-channel that activates at more positive voltages with little voltage-dependent inactivation. Available evidence suggests that *mouse* pancreatic β-cell contains exclusively L-type Ca^{2+}-channels (PLANT, 1988; RORSMAN et al., 1988; SMITH et al., 1989). By contrast, studies on *rat* β-cells have revealed the presence of both L- and T-type Ca^{2+}-channels in these cells (ASHCROFT et al., 1990; HIRIART & MATTESON, 1988). Glucose-stimulated insulin release is mediated by a marked increase in the cytoplasmic free Ca^{2+}-concentration ($[Ca^{2+}]_i$) due to Ca^{2+}-entry through voltage-activated Ca^{2+}-channels (GRAPENGIESSER et al., 1989; HELLMAN & GYLFE, 1986; RORSMAN et al., 1984). It seems likely, that even in rat β-cells, this Ca^{2+} enters the cell through L-type Ca^{2+}-channels since the T-type current is almost fully inactivated at the depolarized membrane potential (-40 mV) from which the action potentials originate.

The modulation of L-type Ca^{2+}-channels in mouse β-cells by hormones and neurotransmitters is largely unknown. Substances such as adrenaline, somatostatin and galanin have been shown to suppress electrical activity, hyperpolarizing the membrane potential and to inhibit insulin secretion (DREWS et al., 1990; HSU et al., 1991; NILSSON et al., 1988; 1989; WÅHLANDER et al., 1991). The effects of these substances are sensitive to pretreatment with pertussis toxin (PTX), suggesting the involvement of an inhibitory GTP-binding protein (G-protein; DOLPHIN, 1990). In mouse pancreatic β-cells, the inhibition of electrical activity can largely be accounted for by the

activation of small conductance K^+-channels (RORSMAN et al., 1991). In addition to this effect, hormones and neurotransmitters also control insulin release at some distal step in the exocytotic machinery (NILSSON et al., 1988; 1989; ULLRICH & WOLLHEIM, 1989). In clonal insulin-secreting HIT cells, adrenalin and somatostatin have been shown to inhibit voltage-dependent Ca^{2+}-currents (HSU et al., 1991; KEAHEY et al., 1989). This suggests that modulation of Ca^{2+}-channels might be a third mode of action by which hormones and neurotransmitters control insulin secretion. The situation in non-tumoural cells is less clear and no such inhibitory action of galanin (AHRÉN et al., 1986; 1989) or adrenalin (BOKVIST et al., 1991) has been observed in tissue-cultured mouse β-cells.

In (I) we address the possibility that modulation of the Ca^{2+}-current by G-proteins is a mechanism by which hormones and neurotransmitters can exert their action on insulin release. We used GTPγS, a non-hydrolyzable GTP-analogue to produce general activation of the G-proteins (DOLPHIN & SCOTT, 1987). Intracellular application of GTPγS was achieved by photorelease from a caged precursor. Although this produces an instant elevation of GTPγS (see METHODOLOGY above), the inhibitory action on the Ca^{2+}-current developed gradually requiring 2 minutes to reach maximum. By analogy with what has been proposed for neurones (DOLPHIN, 1991), we attribute the delayed inhibition of the Ca^{2+}-current by GTPγS to the slow dissociation of GDP from the guanine-binding site of the G-protein. Activation results from an exchange of GDP for GTP bound to the α-subunit of the G-protein; a reaction which is accelerated by the binding of the neurotransmitter to the extracellular receptor. Somewhat surprisingly, the GTPγS-induced inhibition could not be prevented by inclusion of the non-hydrolyzable GDP-analogue GDPβS in the pipette solution. At present we have no explanation to this observation.

Activation of G-proteins is known to inhibit adenylate cyclase (HILDEBRANDT et al., 1983; JAKOBS et al., 1984) and can therefore be expected to reduce the intracellular concentration of cAMP. As Ca^{2+}-channels are under the control of PKA in many tissues (HILLE, 1992) including the β-cell (V), this might be a reason for the effect of GTPγS. To test this hypothesis, we included cAMP in the pipette solution. However, even when added at a high concentration, cAMP failed to counteract the inhibitory effect of GTPγS on the Ca^{2+}-current amplitude, suggesting that inhibition of adenylate cyclase is not the mechanism by which GTPγS blocks the Ca^{2+}-current in mouse β-cells.

Apart from reducing the peak amplitude, GTPγS had at least two other actions on the Ca^{2+}-current. First, in some cells the time course of activation was slowed. Secondly, it

consistently reduced the inactivation of the Ca^{2+}-current. These effects could theoretically result from GTPγS selectively blocking a sub-class of Ca^{2+}-channels (i.e. T-type). To exclude this possibility we investigated the effects of GTPγS on barium (Ba^{2+}-)currents through voltage-gated Ca^{2+}-channels. Consistent with earlier reports, and in keeping with the idea that mouse β-cells contain only L-type Ca^{2+}-channels, substitution of Ca^{2+} for Ba^{2+} removed inactivation. If T-type Ca^{2+}-currents had been present, inactivation of would have persisted (PLANT, 1988) as the inactivation of these channels is voltage-dependent rather than Ca^{2+}-dependent (SATIN & COOK, 1989). However, even when inactivation was abolished, GTPγS inhibited the Ca^{2+}-current. Thus, we can dissociate the inactivation of the Ca^{2+}-current from the inhibition and conclude that the effect is due to GTPγS interfering with the L-type Ca^{2+}-channels.

PTX prevents the coupling of inhibitory G-proteins to activated receptors by ADP-ribosylation of the receptor-binding site on the α-subunit (SCHULTZ et al., 1990). However, pretreatment with PTX did not alter the response to application of GTPγS. This feature of the effect of GTPγS is not readily reconcilable with the concept that G-proteins regulate the β-cell Ca^{2+}-current but it is not entirely without precedent (HESCHELER et al., 1988; SCHULTZ et al., 1990). PTX is believed to prevent the interaction between the receptor and the G-protein, but not GTPγS-induced activation and this might account for the lack of effect on the Ca^{2+}-current. Hence, we feel that the most likely explanation for the effects of GTPγS on Ca^{2+}-currents is an interaction between the channels and the activated G-proteins.

Further support for this idea comes from the observation that GTPγS-induced activation of G-proteins, as already pointed out, slowed the time course of current activation. Similar observations have been made in neurones and were then attributed to the slow recovery from a modified closed ($GTP\gamma S\alpha_o^*C$) state resulting from a voltage-dependent interaction between the activated G-protein and the Ca^{2+}-channel (DOLPHIN, 1991). SCOTT & DOLPHIN (1990) have proposed the following reaction scheme to explain the voltage-dependence of the GTPγS-effect:

$$GTP\gamma S\alpha_o^*C \underset{\alpha_{-k}(V)}{\overset{\alpha_{+k}(V)}{\rightleftarrows}} C \underset{\beta_{-k}(V)}{\overset{\beta_{+k}(V)}{\rightleftarrows}} O$$

The interaction between the Ca^{2+}-channel and the G-protein is a slow process with $\alpha_{+k}(V)<\beta_{+k}(V)$. This would explain the slow onset of the GTPγS-effect. After interaction with GTPγS, the Ca^{2+}-channels exists in the kinetic state $GTP\gamma S\alpha_o^*C$. Upon depolarization, the Ca^{2+}-

channels open (entry into state *O*) with a slower time course than in the absence of GTPγS because more transitions are involved. To test this kinetic model, we applied a two-pulse protocol (cf. GRASSI & LUX, 1989; SCOTT & DOLPHIN, 1990) consisting of a brief pre-pulse to a large depolarizing potential followed by the standard test pulse. The large depolarization shifts the equilibrium towards the open state (*O*). After stepping back to the holding potential, the channels rapidly ($\beta_{-1}(V)$) enters the ordinary, activatable, closed state (*C*). The return of the channels into the modified closed state ($GTP\gamma S\alpha_o{}^*C$) induced by interaction with G-proteins is a slow process ($\alpha_{-1}(V)$) as discussed above. After the large depolarization, many of the channels that were formerly in $GTP\gamma S\alpha_o{}^*C$ will therefore remain in *C* and a second pulse applied shortly after the prepulse will consequently activate a larger current than without the prepulse. An increase in current amplitude, although smaller, was also obtained in the absence of GTPγS. This finding suggests that the Ca^{2+}-current is subject to a partial tonic inhibition in the intact β-cell. This is also indicated by the observation that pretreatment with PTX increased the Ca^{2+}-current amplitude recorded from β-cells under control conditions (i.e. without internal GTPγS).

A novel oscillating Ca^{2+}-activated K^+-conductance (II & III).

Electrical activity in pancreatic β-cells

When stimulated with insulin-releasing glucose-concentrations, mouse pancreatic β-cells start generating action potentials. At intermediate glucose-concentrations (10-15 mM), the β-cell electrical activity consists of a characteristic pattern of slow oscillations in membrane potential between depolarized plateaus, on which the action potentials are superimposed, and repolarized silent intervals (HENQUIN & MEISSNER, 1984). By contrast, single mouse β-cells maintained in tissue culture do not display this type of electrical activity and in these cells, electrical activity consists of either continuous spiking or of very long bursts lasting several minutes (Fig. 6A and SMITH et al., 1990). It has been argued that the failure of the individual β-cell to generate the bursting pattern of electrical activity is simply due to different experimental conditions. Patch-clamp experiments are traditionally performed at subnormal temperatures (30-34°C rather than 37°C as the membrane otherwise becomes labile) and with HEPES-buffered media instead of bicarbonate-buffered media (to facilitate seal formation). However, as shown in Fig. 6B, β-cells are indeed capable of generating oscillatory electrical activity

under precisely these experimental conditions provided that the recording is made from a cell within an intact, freshly isolated, islet. This electrical activity is strikingly similar to that which has been previously recorded with conventional intracellular electrodes (COOK & IKEUCHI, 1989; HENQUIN & MEISSNER, 1984). It may therefore be concluded that the observed differences in electrical activity do not result from the experimental conditions. How then do the differences arise? One possibility we have considered is that the atypical electrical behaviour of cultured β-cells results from the loss of paracrine and neuronal regulation present in the intact pancreatic islet. The intact islet is a highly organized structure with a central core of β-cells surrounded by a mantle of other endocrine cell types (PIPELEERS et al., 1992), such as α-cells (glucagon-secreting), δ-cells (somatostatin-secreting) and PP-cells (pancreatic polypeptide-secreting cells). Although only a small portion of the β-cells are in contact with these cells, their secretory products may influence the β-cell activity via paracrine mechanisms. The islets are also innervated by cholinergic, adrenergic and peptidergic nerves that release their neurotransmitters within the islet. Obviously this provides the background for a complex interplay between the β-cell and its environment.

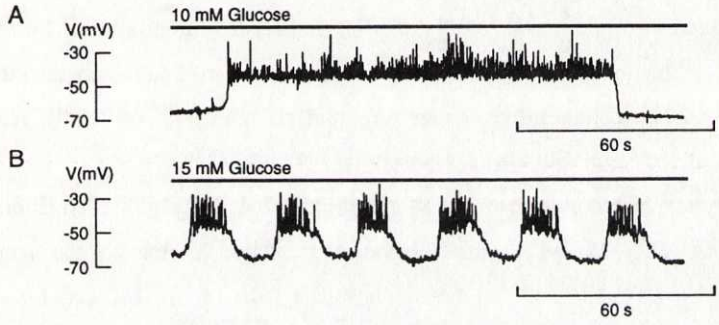


Fig. 6 Electrical activity recorded from ***A)*** *a pancreatic β-cell maintained in tissue-culture and* ***B)*** *a cell within a freshly isolated intact islet. When stimulated with glucose, the cultured cell produces very long bursts (lasting >2 min) whereas the cell within the intact islet produces short lasting bursts (15-20 s) similar to those previously reported with conventional intracellular electrode.*

Oscillatory electrical activity

Consistent with a role of interactions between cells within the intact islet in the generation of β-cell electric activity, spontaneous fast oscillations in membrane potential were observed in a small fraction of cells (5-10%) when the recordings were made from large clusters, but were never seen in individual cells. In the cells exhibiting rapid membrane potential oscillations, a transient spontaneously activating and oscillating outward current was

observed when the cells were subsequently voltage-clamped at -20 mV. As already pointed out, however, the vast majority of β-cells generated continuous electrical or very long bursts even when sitting in large clusters (Fig. 6). It is of interest that in such non-bursting β-cells, rapid oscillations in membrane potential could be induced by application of carbamylcholine (CCh) or dibutyryl-cAMP (db-cAMP), compounds functionally related to hormones and neurotransmitters normally present within the intact islet, such as acetylcholine (ACh), glucagon and glucagon-like peptide-1 (GLP-1; RASMUSSEN et al., 1990). Although it remains unestablished that these oscillations can be equated to those seen in the intact islet, we felt it was important to characterize the molecular mechanisms underlying these oscillations. These studies were facilitated by the observation that in the standard whole-cell configuration, oscillations similar to those evoked by CCh and/or db-cAMP could be induced by intracellular infusion with GTPγS. The frequency of the GTPγS-induced oscillations could be modulated by extracellular application of low concentrations of CCh and they were reversibly abolished by application of a high concentration of CCh, suggesting that CCh and GTPγS act by the same molecular mechanism(s).

Microfluorimetric measurements of Ca^{2+} revealed that the oscillations in membrane potential evoked by CCh and the oscillations in membrane current evoked by GTPγS were paralleled by oscillations in $[Ca^{2+}]_i$. Both the CCh- and GTPγS-evoked membrane current were independent of extracellular Ca^{2+}, suggesting that the elevation of $[Ca^{2+}]_i$ results from mobilization of Ca^{2+} from intracellular stores.

Activation of phospholipase C (PLC) initiates the hydrolysis of membrane-bound inositol lipids (phosphatidyl inositol bisphosphate, PIP_2), leading to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$). DAG acts by stimulating protein kinase C (PKC; NISHIZUKA, 1988), whereas $Ins(1,4,5)P_3$ releases Ca^{2+} from intracellular stores (BERRIDGE, 1987). PLC is known to be regulated by G-proteins (BERRIDGE & IRVINE, 1984; 1989) and it is therefore conceivable that GTPγS operates by activating this enzyme and promoting Ca^{2+}-release through the $Ins(1,4,5)P_3$-pathway. In support of this idea, membrane current oscillations similar to those obtained by infusion of GTPγS could be induced by photorelease of $Ins(1,4,5)P_3$ from a caged precursor. $Ins(1,4,5)P_3$ was, however, not able to initiate the repetitive pattern and only one large oscillation was evoked subsequent to flash photolysis of caged $Ins(1,4,5)P_3$. Furthermore, infusion of the non-hydrolyzable $InsP_3$-analogue inositol 2,4,5-trisphosphate did not initiate large oscillations but evoked small and rapid fluctuations in membrane

conductance. This raises the interesting possibility that fluctuations in the $Ins(1,4,5)P_3$-concentration are important for the oscillations in K^+-current (cf. HAROOTUNIAN et al., 1991).

Application of $Ins(1,4,5)P_3$ had different effects on the membrane conductance depending on whether it was released before or after a GTPγS-induced oscillation. When applied at the end of a GTPγS-induced oscillation, $Ins(1,4,5)P_3$ had no effect on the membrane current, whereas a large current could be evoked when it was liberated just before an oscillation. Furthermore, $Ins(1,4,5)P_3$ was also able to reset the periodicity of the oscillations. These findings constitute strong evidence that GTPγS acts by an $Ins(1,4,5)P_3$-dependent mechanism.

Pharmacological properties

The pharmacological and biophysical properties of the Ca^{2+}-activated K^+-conductance were also investigated. At a membrane potential of 0 mV, a major part of the GTPγS-induced current is carried by large-conductance Ca^{2+}-activated (K_{Ca}) channels, as evidenced by the high sensitivity to low concentrations of tetraethylammonium (TEA; BOKVIST et al., 1990; FATHERAZI & COOK, 1991) and the more selective blocker charybdotoxin (CTX; MILLER et al., 1985). At the more physiological membrane potential of -40 mV (i.e. the plateau potential of the β-cell), neither TEA nor CTX had any effect on the current. This is in keeping with the steep voltage dependence of the K_{Ca}-channel gating in the β-cell (COOK et al., 1984). Clearly, the TEA- and CTX-resistant current flows through a K^+-conductance distinct from the K_{Ca}-channels. In order to identify this channel, we explored the effects of other K^+-channel blockers. However, the current remained unaffected by tolbutamide, a blocker of the K_{ATP}-channel (TRUBE et al., 1986) and apamin, a blocker of small-conductance Ca^{2+}-activated K^+-channels (BLATZ & MAGLEBY, 1986) whether applied at 0 or -40 mV. The channel is highly selective for K^+ as evidenced by the observation that the changes in reversal potential induced by varying the extracellular K^+-concentration ($[K^+]_o$) are in excellent agreement with those predicted by the Nernst equation for a perfectly K^+-selective channel. These observations suggest that the oscillations reflects the activation of a K^+-channel with pharmacological properties distinct from those reported for any other K^+-channel previously documented in the β-cell.

Biophysical properties

We next proceeded to investigate the biophysical properties of this channel. The single-channel conductance is a useful "fingerprint" of an ion channel. As already pointed out

in METHODOLOGY, the cell-attached configuration is the only single-channel recording mode where the cell interior is left intact. This configuration also facilitates the detection of small single-channel events. This is because the noise produced by the patch membrane decreases with increasing resistance and decreasing capacitance. In the cell-attached configuration, the area of the membrane enclosed by the pipette is small and consequently the patch resistance is high and the patch capacitance low. The resolution is therefore principally limited by the noise originating from the current measurement circuitry (LEVIS & RAE, 1992). Characterization of the single-channel properties is also favoured by the rather trivial fact that there are fewer channels in a small patch of membrane than in the whole cell. However, although current oscillations similar to those observed in the whole-cell experiments could be elicited by extracellular application of CCh, it was not possible to detect any unitary events. Nevertheless, the fact that CCh could initiate oscillations, even when it was absent from the pipette solution, suggests that the effect is mediated by some diffusible intracellular factor, presumably Ca^{2+}.

As we were unable to record the individual channel openings, the single-channel conductance had to be estimated by stationary fluctuation analysis (see box and HILLE, 1992) of the excess noise associated with the development of the current oscillations induced by GTPγS. The estimated amplitude at -70 mV was determined as 35 fA (fA≡10^{-15} Ampere) in the presence of high $[K^+]_o$. With a reversal at 0 mV, as indicated by the current-voltage (I-V-)relationship, this corresponds to a single-channel conductance of 0.5 pS and to 0.1 pS with physiological ionic gradients.

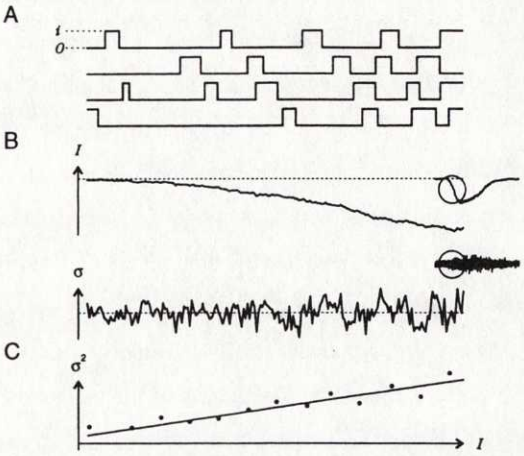
The high turnover rate of ion channels is regarded as an important diagnostic criterion when distinguishing between ion transport through channels and that mediated by other processes such as pumps and carrier (HILLE, 1992). As the estimated single-channel conductance of this channel is much lower than that of most ion channels, one might wonder whether the current reflects ion flux through ion channels. An indication that the current does indeed flow through ion channels comes from the following considerations: If this channel is responsible for repolarizing the membrane from the depolarized plateau of -40 mV to about -70 mV, the single-channel conductance (0.1 pS) predicts a single-channel amplitude at the plateau potential of 4 fA. This corresponds to a rate of transport through the channel of approximately 10^4 ions per second. This is still more than 10-100 times faster than pumps and carrier which typically operate at a rate of 100-1000 ions per second (HILLE, 1992).

Stationary fluctuation analysis

i = single-channel amplitude

I = whole-cell current = Nip

σ^2 = current noise = Ni^2pq



A) Four idealized current traces representing single openings of ion channels. **B**) A segment of the whole-current resulting from the summed activity of all channels present in the cell. The current shown is an expansion of the falling (encircled) part of a GTPγS-induced current oscillation shown in total to the right. **C**) High-pass filtering, 2 Hz, removes the slow current component, the remaining current fluctuations represent the noise associated with the whole-cell current (σ). The trace displayed is an enlargement of trace shown to the right **D**) A schematic relationship between the whole-cell current (I) and the variance (σ^2). The assumption that p is small (and N is large) is valid if the correlation is linear. The single-channel amplitude can be estimated from Eq. 5 and is identical to the slope coefficient of the σ^2-I-curve.

$$i = \frac{\sigma^2}{I(1-p)} \qquad \text{Eq. 5}$$

Abbreviations used:
N = total number of channels p = open probability (< 1) q = closed probability = $(1-p)$

Functional significance

The electrophysiological mechanisms regulating the membrane potential oscillations are not known but several hypotheses have been proposed. One model that has been implicated in the process involves the accumulation of Ca^{2+} entering the β-cell during the action potentials with the subsequent activation of K_{Ca}-channels (ATWATER et al., 1983; COOK et al., 1984). The recent evidence that electrical activity is unaffected by CTX (KUKULJAN et al., 1991), a specific inhibitor of K_{Ca}-channels, suggests that this hypothesis is incorrect.

A second possibility originates from the observation that inactivation of the whole-cell Ca^{2+}-current consists of a rapid and a slow component where the slow inactivation occurs over a time scale similar to the burst duration. The plateau is believed to result from persistent activation of the voltage-dependent Ca^{2+}-channels (ASHCROFT & RORSMAN, 1989; COOK et al., 1991). When this depolarizing Ca^{2+}-current becomes too small to balance the background hyperpolarizing K^+-current, the β-cells starts to repolarize. This model provides an explanation to the observation that current injected into the cells resets the periodicity of the bursts (COOK et al., 1991). One major difficulty with this model is to understand why cultured mouse β-cells, even though they show slow inactivation of the Ca^{2+}-current, do not exhibit the characteristic pattern of electrical activity.

The third hypothesis involves cyclic variations in the activity of K_{ATP}-channels. When $[Ca^{2+}]_i$ is elevated, ATP in the vicinity of the membrane is consumed as a consequence of the activation of the Ca^{2+}-ATPase that extrudes Ca^{2+} from the cell. The associated decrease in ATP/ADP-ratio has been proposed to be sufficient to activate K_{ATP}-channels and thereby repolarize the membrane (COOK & IKEUCHI, 1989; HENQUIN, 1988; 1990). This idea would be consistent with the finding that tolbutamide, a blocker of K_{ATP}-channels, converts oscillatory electrical activity to continuous spiking, but is difficult to reconcile with the observation that low concentrations of the sulphonylurea evoke membrane potential oscillations similar to those of glucose, as tolbutamide is unlikely to produce an variable block of K_{ATP}-channels.

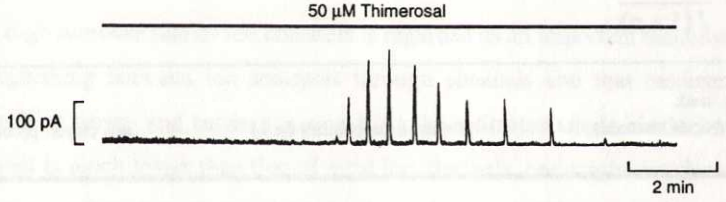


Fig. 7 Oscillations in membrane conductance evoked by intracellular application of thimerosal. The whole-cell configuration was established ~20 s before the beginning of the recording.

Based on the experiments presented in II & III, we propose that activation of low-conductance Ca^{2+}-activated K^+-channels ($K_{L,Ca}$-channels) underlies the bursting behaviour of the β-cell. Although activation of this current by Ca^{2+} derived by mobilization from internal stores is clearly *one* mechanism that evokes membrane potential oscillations (and indeed the only one that has so far been shown to do so experimentally), it remain possible that in the freshly isolated intact islet, Ca^{2+}-influx through voltage-gated Ca^{2+}-channels triggers openings

of such channels. Such a concept would account for the finding that the frequency can be reset by current injection.

Another possibility we have considered is that the Ca^{2+}-influx associated with the bursts of action potentials leads to a localized increase in $[Ca^{2+}]_i$. When the Ca^{2+}-concentration in this region of the β-cell rises above a certain threshold it triggers a further elevation of $[Ca^{2+}]_i$ by activation of Ca^{2+}-induced Ca^{2+}-release (CICR). ISLAM and co-workers (1992) have provided some evidence for the existence of CICR in insulin-releasing cells using the thiol reagent thimerosal, activating CICR (SWANN, 1991). Furthermore, as shown in Fig. 7, thimerosal elicits repetitive oscillations in membrane current similar to those obtained with GTPγS.

Fig. 8 presents a model for oscillatory electrical activity in the β-cell involving the novel Ca^{2+}-activated K^+-conductance. When exposed to glucose, the enhanced metabolism leads to an increased intracellular ATP-concentration of the cell which promotes the closure of the K_{ATP}-channels. The cell membrane then depolarizes, the voltage-activated Ca^{2+}-channels open (*i*) and electrical activity is initiated. A burst of action potentials produce an increase in $[Ca^{2+}]_i$ which in itself, subsequent to the release of Ca^{2+} by CICR (*ii*) or by Ca^{2+}-induced activation of PLC that leads to the generation of $Ins(1,4,5)P_3$ and mobilization of Ca^{2+} from intracellular stores (*iii*), opens the $K_{L,Ca}$-channels. The increased K^+-permeability repolarizes the cell membrane, and the voltage-activated Ca^{2+}-channels are closed.

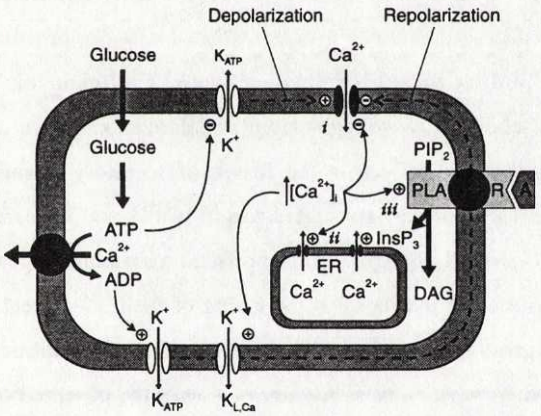


Fig. 8 A model for the regulation of the membrane potential involving the novel low-conductance K^+-channel ($K_{L,Ca}$-channel). The model is described in the text.

It is clear that the mechanisms involved in the generation of the bursting pattern remain unestablished. However, many of the properties of the $K_{L,Ca}$-channel makes it an

attractive candidate for a channel involved in the regulation of the bursting activity. The role of Ins(1,4,5)P_3-induced oscillations in the control of the β-cell electrical activity remains controversial. It is possible that in the intact islet, activation of $K_{L,Ca}$-channels by Ca^{2+}-entering the cell during action potentials and/or mobilized from intracellular stores, inactivation of Ca^{2+}-current and Ca^{2+}-induced oscillations in the intracellular ATP/ADP-ratio all contribute to the generation of the membrane potential oscillations.

Ca^{2+}-dependence of insulin secretion (IV).

As discussed above (II & III), glucose-stimulated insulin secretion is associated with electrical activity consisting of Ca^{2+}-dependent actions potentials resulting in an elevation of $[Ca^{2+}]_i$ (RORSMAN et al., 1992; SANTOS et al., 1991; THELER et al., 1992). The changes in $[Ca^{2+}]_i$ are of importance for insulin secretion and a direct correlation between elevation of $[Ca^{2+}]_i$ and exocytosis has been demonstrated (GILON et al., 1993; PRALONG et al., 1990; ROSARIO et al., 1986). However, the underlying molecular and cellular processes have only been partially elucidated. The recently developed technique for capacitance measurements of exocytosis (JOSHI & FERNANDEZ, 1988; LINDAU & NEHER, 1988) has opened possibilities to monitor secretion from single cells with a high temporal resolution. In (IV) we have combined the patch-clamp technique with microfluorimetry and capacitance measurements to explore the role of Ca^{2+} in the process of insulin secretion.

Exocytosis and Ca^{2+}-influx through voltage-activated Ca^{2+}-channels

Ca^{2+}-currents elicited by voltage-clamp depolarizations were associated with step increases in cell capacitance reflecting the fusion of secretory granules with the plasma membrane. The peak Ca^{2+}-current, associated rise in $[Ca^{2+}]_i$ and increase in cell capacitance displayed the same U-shaped voltage-dependence with maximum responses around +20 mV. When allowance is made for the fact that the gating of the Ca^{2+}-current activation is shifted by ≈20 mV to more positive voltages under these experimental conditions relative that seen in the intact islet cells (RORSMAN & TRUBE, 1986; SMITH et al., 1993), the observed relationship indicates that secretion is maximally activated within the voltage range of the β-cell action potential (ASHCROFT & RORSMAN, 1989; HENQUIN & MEISSNER, 1984). Depolarization *per se* is not sufficient to evoke exocytosis as application of cobalt (Co^{2+}), a blocker of Ca^{2+}-channels (RORSMAN & TRUBE, 1986)

inhibited both the Ca^{2+}-current and exocytosis. Exocytosis was initiated when the *average* $[Ca^{2+}]_i$ exceeded a threshold of 0.5 μM and was markedly increased above 0.7 μM.

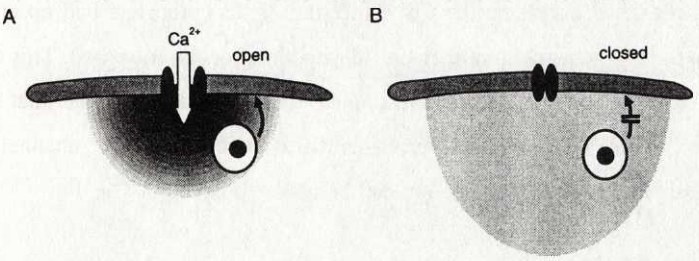


Fig. 9 Regulation of exocytosis by the local Ca^{2+}-concentration in the vicinity of the Ca^{2+}-channel. A) When the channel opens, Ca^{2+}immediately reaches a high concentration in the active zone and exocytosis is initiated. B) Upon closure of the channel, the local Ca^{2+}-transient collapses and exocytosis stops although a fluorescent Ca^{2+}-indicator may continue to report an elevated $[Ca^{2+}]_i$ for some time.

Ca^{2+}-gradients within the β-cell

Experiments on the squid giant synapse have demonstrated that neurotransmitter release is resistant to injection of Ca^{2+}-buffers, such as EGTA, to concentrations as high as 80 mM and that the latency between the arrival of the electric impulse to the synapse and release is only 0.2 ms, most of which is required for the Ca^{2+}-channels to open (ADLER et al., 1991; AUGUSTINE et al., 1985). These observations have led to the proposal that neurotransmitter release is determined by Ca^{2+} in the immediate vicinity of the Ca^{2+}-channels and that the concentration there changes too rapidly and too much to be buffered by the chelator. Neurotransmitter release can therefore be expected to mirror Ca^{2+}-channel activity with the release being coincident with the opening of the Ca^{2+}-channel. Our observation that exocytosis stops immediately upon repolarization would be consistent with a similar arrangement in the β-cells. During Ca^{2+}-channel openings, the Ca^{2+}-concentration in the vicinity of the Ca^{2+}-channels (=active zones) rises to very high concentrations and exocytosis is initiated (Fig. 9A). When the channel closes, the local Ca^{2+}-transient that controls exocytosis, collapses and exocytosis ends (Fig. 9B). It is only during repetitive stimulation, when high Ca^{2+}-concentrations have been attained throughout the cytoplasm, that exocytosis proceeds in the intervals between the pulses but then only at a much lower rate than during the pulses (AUGUSTINE & NEHER, 1992; NEHER & AUGUSTINE, 1992).

It appears, however, that although β-cell exocytosis is determined by the Ca^{2+}-concentration in the vicinity of the Ca^{2+}-channel(s), some diffusion of Ca^{2+} is necessary. This is suggested by our observation that exocytosis in the β-cell was abolished by millimolar concentration of EGTA. Increasing the Ca^{2+}-buffering of the cytoplasm had no effect on the inactivation of the Ca^{2+}-channel (another Ca^{2+}-dependent process; PLANT, 1988). This observation suggests that the diffusion path required to initiate exocytosis is longer than that involved in the inactivation of the Ca^{2+}-channel. During its diffusion from the Ca^{2+}-channel to the site where exocytosis is initiated, the Ca^{2+}-ion can be chelated by EGTA (Fig. 10).

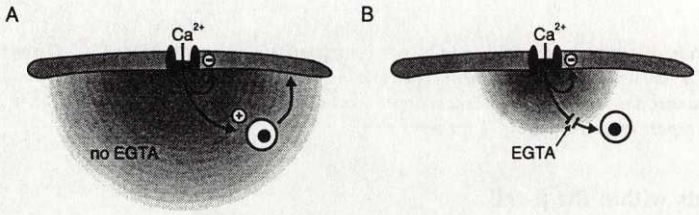


Fig. 10 A) In the absence of chelator (EGTA) Ca^{2+}-influx through the open channel both inactivates the Ca^{2+}-channel and initiates exocytosis. B) In the presence of EGTA, Ca^{2+} still inactivates the channel but, due to a longer diffusion path, the Ca^{2+}-ion can be chelated before it reaches the secretory site and exocytosis is abolished.

Facilitation and depression

An increase in $[Ca^{2+}]_i$ initiates the release of the secretory granules. The number of granules released during stimulation (for example by a Ca^{2+}-current elicited by a voltage-clamp depolarization) is influenced by the previous stimulation. For example, during repetitive high-frequency stimulation, a second or a third depolarization often produce a larger response than the first pulse (Fig. 11, left part). Using the terminology developed to describe neuromuscular transmission, we refer to this as *facilitation* (KATZ, 1966). Facilitation can be explained in terms of the interval between two successive depolarizations being insufficient for $[Ca^{2+}]_i$ to return to the prestimulatory level, and the individual Ca^{2+}-transients therefore summate to generate a higher $[Ca^{2+}]_i$. As exocytosis is steeply Ca^{2+}-dependent, the second depolarization therefore produces a larger exocytotic response than the first.

When subject to repetitive high-frequency stimulation, the exocytotic capacity of the cell usually declines (Fig. 11, to the right), a behaviour similar to *depression* seen in neurones. This we interpret as the depletion of the readily releasable pool of secretory granules. In order

for the secretion to proceed, the releasable pool must be replenished; a process which may require several minutes (GILLIS & MISLER; 1992).

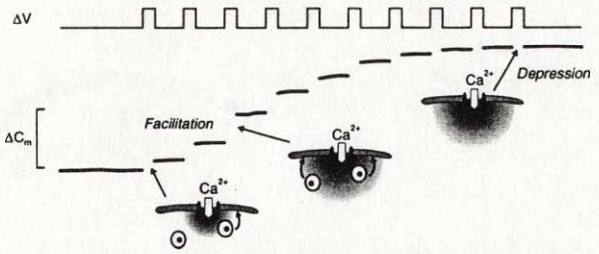


Fig. 11 Schematic illustration of facilitation and depression of the exocytotic response. Voltage-clamp depolarizations (ΔV) give rise to Ca^{2+}-currents (not shown) and thus stimulating exocytosis. As a consequence of the fusion of secretory granules with the plasma membrane, secretion can be monitored as a change in cell capacitance (ΔC_m) reflecting changes in the total cell area. Facilitation can be explained as the summation of two $[Ca^{2+}]_i$-transients and $[Ca^{2+}]_i$ rises to exocytotic levels in a larger part of the cell than in response to a single depolarization and more granules can consequently be recruited for release. Depression we explain as depletion of the releasable pool of granules.

Regulation of exocytosis by Ca^{2+}-domains

If exocytosis is regulated by the *local* Ca^{2+}-concentration in the vicinity of the Ca^{2+}-channels, it follows that exocytosis should be dependent on (but *not* initiated by) changes in membrane potential since the L-type Ca^{2+}-channel amplitude (and thus the number of Ca^{2+}-ions entering the cell through the channel) is dependent on the voltage. The whole-cell Ca^{2+}-current is the product of the single-channel amplitude (i) and the channel activity (Np; N=total number of channels available for activation, p=open probability). Both i and the product Np show voltage dependence, illustrated schematically in Fig. 12A (upper and middle panel). Because of the inwardly rectifying properties of the Ca^{2+}-channel (SMITH et al., 1993), the single-channel amplitude decreases, whereas the channel activity increases with depolarization. The whole-cell Ca^{2+}-current therefore displays a U-shaped I-V-relationship (Fig 12A, lower panel). At negative membrane potentials, fewer channels are open, but the current flowing through the individual channel is larger than that at more positive voltages. If exocytosis is determined by the *local* rather than the *global* Ca^{2+}-concentration, the domain theory of Ca^{2+}-entry (CHAD & ECKERT, 1984), illustrated in Fig. 12B, would predict that $[Ca^{2+}]_i$ is more effective as an initiator of exocytosis at negative than at positive potentials. This is indeed, what we observed, and despite *average*

Ca^{2+}-transients of the same magnitude, a depolarization to 0 mV was more effective at evoking exocytosis than a pulse to +40 mV (Fig. 5 in IV).

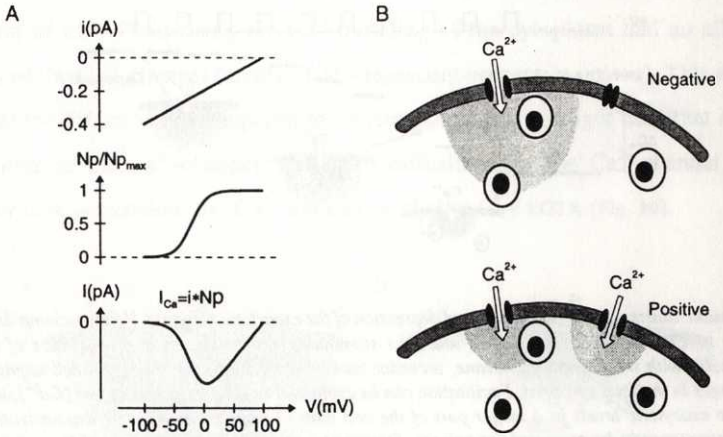


*Fig. 12 **A)** The voltage-dependence of the single-channel amplitude (top), the channel activity (middle) and the whole-cell Ca^{2+}-current (bottom). **B)** At negative membrane potentials (top) fewer channels, but with larger amplitude, are activated than at more positive voltages (bottom). The number of Ca^{2+}-ions entering the cell per channel is therefore larger at the more negative membrane potential and the $[Ca^{2+}]_i$-transient can thus be envisaged to extend deeper into the cell (shaded areas) thus promoting the release of more secretory granules.*

Estimation of the local Ca^{2+}-concentration at the exocytotic site

As outlined above, much evidence indicates that exocytosis is regulated by the *local* Ca^{2+}-concentration in the vicinity of the Ca^{2+}-channels and that $[Ca^{2+}]_i$ there is much higher than that suggested by the microfluorimetric recordings, which report the *average* concentration of the entire cell. In an attempt to estimate the true Ca^{2+}-concentration within the active zones, we tried to induce a *global* rather than a localized increase of Ca^{2+} by release from intracellular stores using caged $Ins(1,4,5)P_3$.

As shown in Fig. 13, photorelease of $Ins(1,4,5)P_3$ (15 μM) from a caged precursor evoked a Ca^{2+}-transient which was considerably larger (>10 times) than that elicited by a 500 ms voltage-clamp depolarization to 0 mV. $Ins(1,4,5)P_3$ also evoked large exocytotic responses. Interestingly, the rates of the capacitance increase (dC/dt; dotted lines in Fig. 13) were roughly the same whether exocytosis was elicited by $Ins(1,4,5)P_3$ or by voltage-clamp depolarization, although measured $[Ca^{2+}]_i$-levels were vastly different. The simplest explanation to account for this observation is that the Ca^{2+}-concentration at the release site, that controls exocytosis, is the same under both experimental conditions. This implies that the local $[Ca^{2+}]_i$ in the

vicinity of the Ca^{2+}-channels is as high as 10-20 μM, i.e. 20-30 times higher than that actually measured by microfluorimetry. The reason why the microfluorimetric measurements underestimate the true $[Ca^{2+}]_i$-transients at the secretory site is that only a fraction of the indicator is located in the active zone.

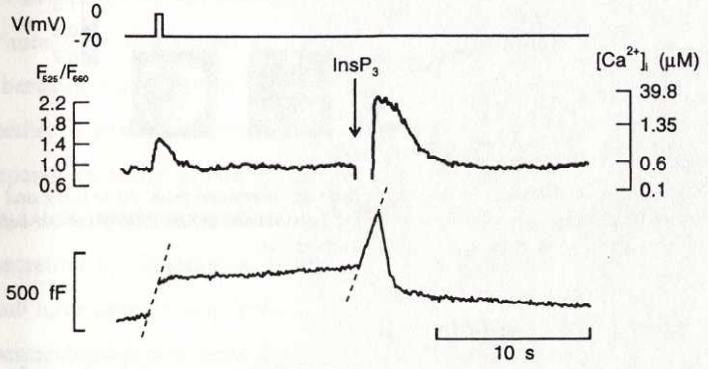


Fig. 13 The Ca^{2+}-transients (top panel) and capacitance changes (bottom) associated with a voltage-clamp depolarization and photorelease of Ins(1,4,5)P_3 (15 μM) from a caged precursor. Although giving rise to a substantially larger Ca^{2+}-transient, the rate of exocytosis (dotted lines) induced by Ins(1,,4,5)P_3 is the same as that elicited by the voltage-clamp depolarization. The decrease in capacitance following the Ins(1,4,5)P_3-induced increase probably reflects retrieval of secreted membrane by endocytosis.

The cell shown in Fig. 13 also exhibited a rapid decrease in cell capacitance following the Ins(1,4,5)P_3-induced capacitance increase. We interpret this decrease in capacitance as retrieval of secreted membrane (NEHER & ZUCKER, 1993) suggesting that Ca^{2+} might also be involved in the regulation of endocytosis.

Recent experiments utilizing digital imaging of fluorescence from fura-2 have indeed revealed the existence of *localized* increases in $[Ca^{2+}]_i$ in response to voltage-clamp depolarization. Fig. 14A shows the Ca^{2+}-current elicited by a 200 ms voltage-clamp pulse and the associated increase in cell capacitance. Images of the spatial distribution of Ca^{2+} at various times after the onset of the depolarization are shown in Fig. 14B. It is clear that $[Ca^{2+}]_i$ varies substantially within the cell. The increase in Ca^{2+}-dependent fluorescence is particularly pronounced in a region close to the plasma membrane. It is also of interest, that the increase is restricted to the left part of the cell with other parts being relatively unaffected. This indicates an uneven distribution of Ca^{2+}-channels in the plasma membrane and it is tempting to speculate that these regions might correspond to "hot spots" of exocytosis.

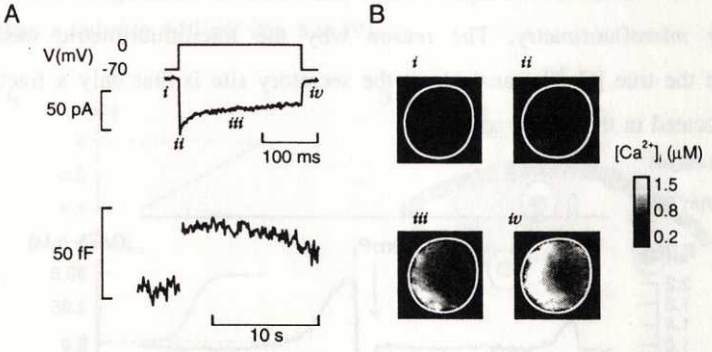


Fig. 14 A) The Ca^{2+}-current (top) elicited by a voltage-clamp depolarization from -70 to 0 mV and the resulting increase in capacitance (bottom). B) Digital images of the Ca^{2+}-distribution at four different times: before (i), in the beginning (ii), in the middle (iii) and at the end of a Ca^{2+}-current (iv).

Regulation of exocytosis by CaM-kinase II

The Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) has been shown to play an essential role in the regulation of neurotransmitter release in the squid giant synapse (LLINÁS et al., 1991). This enzyme has been proposed to act by phosphorylating the synaptic vesicle-protein *synapsin I*. This results in the release of the synaptic vesicles from the cytoskeleton, thus facilitating the transport to the membrane and/or docking with the release sites. CaM-kinase II has been identified in the β-cell, but little is known about its physiological role (HARRISON & ASHCROFT, 1982). In this context, it is of particular interest that inhibitors of the kinase reduce nutrient-stimulated insulin secretion and block the potentiating effect of forskolin (HARRISON et al., 1986; LI et al., 1992). Pretreatment of the cells with KN-62, an inhibitor of CaM-kinase II (TOKUMITSU et al., 1990), reduced depolarization-induced exocytosis and Ca^{2+}-current by 50% compared to control. The latter effect complicates the interpretation of the data as the inhibition may simply result from the reduced Ca^{2+}-influx. However, inclusion of the calmodulin-binding domain of CaM-kinase II, a specific inhibitor of the kinase (PAYNE et al., 1988) was also able to reduce exocytosis whilst not affecting the Ca^{2+}-current. This suggests that activation of CaM-kinase II is indeed involved in the cascade of events that links the elevation of Ca^{2+} to the initiation of exocytosis but the substrate(s) of this enzyme in the β-cell remain(s) to be identified. It is unlikely to be *synapsin I*, however, as this protein is neurone-specific and there is no evidence for its association with the insulin containing secretory granule.

cAMP dependent potentiation of Ca^{2+}-induced insulin release (V).

Insulin secretion is potentiated by hormones such as glucagon (PIPELEERS et al., 1982; 1985) and GLP-1 (HOLST et al., 1987; MOJSOV et al., 1987), which act by elevating the intracellular concentration of cAMP (RASMUSSEN et al., 1990), but the mechanisms involved are not completely clear. For example, from experiments on intact islet or suspensions of β-cells, it is not even possible to distinguish between the possibility that cAMP acts by recruitment of previously non-secreting cells or whether it potentiates secretion in every cell. It is well established that insulin-secretion depends on a rise in $[Ca^{2+}]_i$ (PRENTKI & MATSCHINSKY, 1987). The simplest explanation to account for cAMP-dependent potentiation of secretion is therefore that these substances potentiate secretion by elevation of $[Ca^{2+}]_i$. However, there is evidence that agents which increase cAMP have little (or any) effect on $[Ca^{2+}]_i$ (RORSMAN & ABRAHAMSSON, 1985). Furthermore, studies in permeabilized cells have demonstrated that cAMP is able to potentiate exocytosis even when the Ca^{2+}-concentration is held constant (JONES et al., 1989; 1992). In (V) we have attempted to clarify the mechanism of cAMP-induced potentiation of exocytosis by using the patch-clamp technique in combination with microfluorimetry and capacitance measurements.

When recorded in the perforated-patch configuration under basal conditions, Ca^{2+}-currents elicited by voltage-clamp depolarizations, evoked relatively small increases in cell capacitance. However, extracellular application of either the membrane permeable cAMP-analogue 8-bromo-cAMP or forskolin, an activator of adenylate cyclase, markedly potentiated the exocytotic response. The Ca^{2+}-current increased by 50% and 70% and exocytosis was potentiated by 360% and 580% after exposure to 8-bromo-cAMP or forskolin, respectively. The effects of forskolin were mediated by activation of protein kinase A (PKA) and both the augmentation of the Ca^{2+}-current and stimulation of capacitance could be abolished by Rp-cAMPS, a specific inhibitor of PKA (DE WIT et al., 1984).

Since exocytosis is a Ca^{2+}-dependent process and cAMP increased the Ca^{2+}-currents, the simplest explanation to account for the effects of exocytosis is that it results from the enhancement of Ca^{2+}-influx. To investigate this possibility we used the dihydropyridine BAY K8644 to promote Ca^{2+}-influx. This compound increases the Ca^{2+}-current by increasing the open-time of individual L-type Ca^{2+}-channels (HESS et al., 1984; RORSMAN et al., 1988). At the concentration used (1 μM), BAY K8644 doubled the Ca^{2+}-current and potentiated exocytosis to the same extent. Subsequent inclusion of forskolin in the continued presence of BAY K8644

produced a further 3-fold enhancement of exocytosis with no further action on the Ca^{2+}-current. Comparison of the effects of BAY K8644 and forskolin on the Ca^{2+}-current and exocytosis, indicates that only about 20% of the potentiation of secretion can be accounted for by the enhancement of Ca^{2+}-influx. It was therefore concluded that the major effect of cAMP is exerted at some step(s) distal to the elevation of Ca^{2+}.

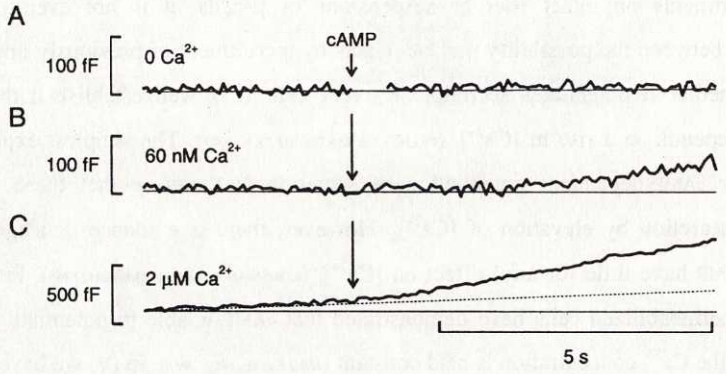


Fig. 15 Stimulation of exocytosis by photorelease of cAMP (80 µM) from a caged precursor. The cells were infused with Ca^{2+}-EGTA mixtures yielding free Ca^{2+}-concentrations of: A) 0, B) 60 nM and C) 2 µM Ca^{2+}.

To investigate the nature of such a distal step we next performed experiments using the standard whole-cell configuration. In this recording mode, application of cAMP by photorelease from a caged precursor during a train of depolarization-evoked Ca^{2+}-currents (0.1 Hz), dramatically and rapidly (<1 s) increased the exocytotic response (+200%) with only a small effect on the amplitude of the Ca^{2+}-transient (+20%). From the relationship between Ca^{2+} and exocytosis, we estimate that the rise in $[Ca^{2+}]_i$ produced by cAMP would only stimulate exocytosis by 20%. This was only about 10 % of that actually observed suggesting that the main action of cAMP was exerted at steps distal to the elevation of $[Ca^{2+}]_i$. Further evidence for such a direct effect of cAMP comes from experiments where mixtures of Ca^{2+} and EGTA, to clamp $[Ca^{2+}]_i$ at fixed concentrations and to abolish influence by the cell's own Ca^{2+}-homeostasis, were infused into the cell. As shown in Fig. 15, photorelease of cAMP (80 µM) in cells dialyzed with a high concentration of Ca^{2+} (2 µM; Fig. 15C) and voltage-clamped, to -70 mV to preclude activation of voltage-activated Ca^{2+}-channels, resulted in a marked acceleration of exocytosis. Interestingly, cAMP failed to stimulate exocytosis in the complete absence of Ca^{2+} (Fig. 15A). These observations supports the concept that cAMP is a potentiator rather than an initiator of exocytosis, and acts by sensitizing the secretory machinery to Ca^{2+}

(HUGHES et al., 1989). Indeed, at a sub-stimulatory Ca^{2+}-concentration (0.06 μM), application of cAMP was able to initiate exocytosis (Fig. 15B). The concept that cAMP acts by increasing the sensitivity of the secretory machinery might also provide an explanation to the observation that photorelease of cAMP re-initiated exocytosis in a cell were secretion had been exhausted by repetitive stimulation. By increasing the Ca^{2+}-sensitivity of the secretory machinery, the distance from the Ca^{2+}-channel over which secretory granules can be recruited for release is substantially extended in the presence of cAMP (Fig. 16).

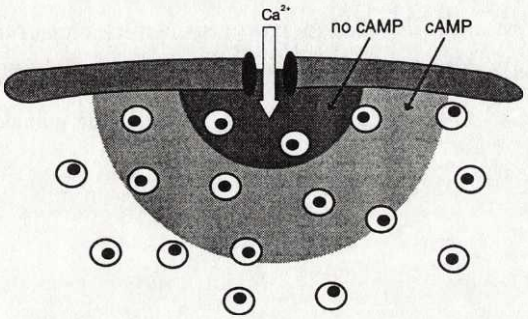


Fig. 16 Stimulation of exocytosis by cAMP increases the distance from the Ca^{2+}-channel over which the secretory granules can be recruited for release by sensitizing the secretory machinery to Ca^{2+}. The dark sector indicates the zone in which Ca^{2+} is sufficient to evoke exocytosis in the absence of cAMP. The lighter area indicates the zone in which secretory granules can be recruited for release in the presence of cAMP.

The molecular processes by which cAMP control exocytosis remain largely unknown. Activation of PKA produces phosphorylation of several β-cell proteins (JONES et al., 1988) but which of these regulates exocytosis has not been determined. In this context, it is of interest that recent experiments have indicated that the exocytotic response is also dramatically increased by activation of PKC or inhibition of protein phosphatases. These effects also occurred without any enhancement of Ca^{2+}-influx (ÄMMÄLÄ et al., 1994). This suggests that Ca^{2+}-independent processes also mediate the action of potentiators of exocytosis acting via these pathways. An interesting observation is that although large Ca^{2+}-currents could be evoked under basal conditions (i.e. absence of kinase activators or phosphatase inhibitors), the exocytotic responses were almost invariably small. These findings suggests that although Ca^{2+} is certainly required to initiate exocytosis, its role may be permissive rather than decisive. Phosphorylation and dephosphorylation of certain regulatory proteins by protein kinases and

phosphatases might be of much greater *quantitative* importance in the modulation of exocytosis

PERSPECTIVES

Although much information is now available regarding the ionic channels in the pancreatic β-cells and the way they co-operate to produce electrical activity, little is known about the molecular processes involved in exocytosis. An important future area of research will be the identification and characterization of the proteins involved in exocytosis and the mechanisms linking Ca^{2+} to the release of the insulin-containing granules.

CONCLUSIONS

1. Voltage-gated L-type Ca^{2+}-channels are modulated by GTP-binding proteins and can be inhibited by GTPγS via a pertussis toxin-insensitive mechanism. The inhibitory effect of GTPγS is not mediated by adenylate cyclase.

2. Activation of an oscillatory Ca^{2+}-dependent K^+-conductance, either by extracellular application of carbamylcholine or intracellular infusion of GTPγS, produces a transient membrane repolarization sufficient to interrupt action potential firing. The effect is mediated by mobilization of Ca^{2+} from $Ins(1,4,5)P_3$-sensitive intracellular Ca^{2+}-stores.

3. The oscillating membrane current induced by GTPγS is highly selective to K^+. The single-channel conductance is 0.5 pS when recorded at nonphysiological ionic gradients and estimated to 0.1 pS at physiological ionic gradients. This novel K^+-conductance is insensitive to tetraethylammonium, charybdotoxin, apamin and tolbutamide.

4. Exocytosis is dependent on a rise in $[Ca^{2+}]_i$ and is initiated when the *average* concentration exceeds 0.5 μM. However, the concentration at the secretory site is likely to be much higher and estimated to exceed 10 μM. Ca^{2+}/calmodulin-dependent protein kinase II is involved in the coupling between elevation of $[Ca^{2+}]_i$ and the activation of the secretory machinery.

5. In single pancreatic β-cells, cAMP potentiates insulin release by activation of protein kinase A. A small fraction (20%) of the cAMP-dependent potentiation is accounted for by an increase in Ca^{2+}-influx through voltage-activated Ca^{2+}-channels. The major effect (80%) is due to a direct interaction with the secretory machinery, possibly exerted by increasing the Ca^{2+}-sensitivity of the secretory machinery.

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