

**Studies of first phase insulin secretion using imposed plasma membrane depolarization**

**Michael Willenborg<sup>1</sup>, Kathrin Hatlapatka<sup>1</sup>, Hany Ghaly<sup>1</sup>, Michael Belz<sup>1</sup>, Uwe Panten<sup>1</sup>, Ingo Rustenbeck<sup>1</sup>**

<sup>1</sup>*Institute of Pharmacology and Toxicology, University of Braunschweig, D-38106 Braunschweig, Germany*

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**1. ABSTRACT**

The first phase of glucose-induced insulin secretion is generally regarded to represent the release of a finite pool of secretion-ready granules, triggered by the depolarization-induced influx of  $Ca^{2+}$  through L-type  $Ca^{2+}$  channels. However, the experimental induction of insulin secretion by imposed plasma membrane depolarization may be more complicated than currently appreciated. A comparison of the effects of high  $K^+$  concentrations with those of  $K_{ATP}$  channel closure, which initiates the electrical activity of the beta cell, suggests that 40 mM  $K^+$ , which is a popular tool to produce a first phase-like secretion, is of suprphysiological strength, whereas the 20 mV depolarization by 15 mM  $K^+$  is nearly inefficient. A major conceptual problem consists in the occurrence of action potentials during  $K_{ATP}$  channel closure, but not during  $K^+$  depolarization, which leaves the  $K^+$  channel conductance unchanged. Recent observations suggest that the signal function of the endogenously generated depolarization is not homogeneous, but may rather differ between the component mainly determined by  $K_{ATP}$  channel closure (slow waves) and that mainly determined by  $Ca^{2+}$  influx (action potentials).

**2. THE STIMULUS SECRETION-COUPPLING OF INSULIN SECRETION: SUBSTRATE SITE HYPOTHESIS VS. RECEPTORS SITE HYPOTHESIS**

The unique characteristic of the pancreatic beta cell is to transform the perceived availability of a limited range of nutrients (glucose, some other carbohydrates and a few amino acids and keto acids) into a suitably conditioned release of a peptide hormone, insulin. The main physiological nutrient to sense is glucose. The insulin release has to be integrated into the requirements of the whole organism, thus under physiological conditions the secretory response to glucose is modified by an array of neuronal and hormonal signals. As a general rule, nutrients are stimulators of the secretion, whereas neurotransmitters and hormones are enhancers, i.e. they require the presence of some nutrient to become effective. While there are inhibitory neurotransmitters and hormones, there is apparently no physiological inhibitory nutrient or nutrient analogue.

In the early days of research on stimulus secretion-coupling in the beta cell, there was a debate whether glucose and the other nutrient secretagogues elicit

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insulin by binding to a plasma membrane receptor or whether the signal for secretion was generated by their intracellular metabolism (1-3). By the early 1980s the latter hypothesis prevailed (4), but there was still a major gap in understanding of how the energy metabolism was coupled to the electrical activity of the beta cell which was apparently responsible for initiating the exocytosis of the insulin-containing large dense core vesicles. This activity was originally described by Dean and Matthews (5), followed by Meissner and Henquin's characterization of the spike and wave pattern typical for the beta cell (6), the mechanisms of which are still incompletely understood. The identification of the ATP-sensitive potassium channel ( $K_{ATP}$  channel) in the beta cell and its clear association with the nutrient-induced depolarization (7-9) permitted for the first time to relate all the essential features of the beta cell in a logical sequence. Thus the sequence of nutrient-induced increase of the ATP-to-ADP ratio, closure of the  $K_{ATP}$  channels, depolarisation-induced opening of voltage-dependent  $Ca^{2+}$  channels and the ensuing  $Ca^{2+}$ -induced exocytosis of insulin granules became generally accepted. It is often referred to as the "consensus hypothesis" and represents the core of textbook knowledge to this day. However, it must be kept in mind that this sequence of events is very elementary and additional regulatory mechanisms are clearly required (see below). E.g. it does not explain one of the very basic features of glucose-induced insulin secretion, the biphasic kinetic.

### 3. THE PHASIC NATURE OF INSULIN SECRETION

The endocrine pancreas responds to a square-wave glucose stimulus in a unique, biphasic dynamic of insulin release rate (10). The first phase, once believed to be an *in vitro* artifact, is now recognized to be of central importance for the tight control of glucose homeostasis (11). Experimental evidence suggests that the efficiency of insulin is dependent on the kinetic of its release. A pulsatile secretion pattern is more effective to lower blood glucose than a constant infusion of the same amount of insulin. Most likely this is due to a preferential effect of high insulin concentration on the liver (12, 13). Thus the first phase secretion is of major physiological importance. Since postprandial glucose concentrations do not increase in a square wave-manner, there is no classical first phase response *in vivo*, however, the fast initial increase in insulin secretion (acute insulin response) can be regarded as the equivalent of the first phase (14).

One of the earliest derangements of type 2 diabetes mellitus is a diminished first phase insulin response to glucose; subsequently as the disease progresses, rates of second phase insulin response decline as well. While there is some debate as to whether the decline of the first phase is actually preceding the decline of the second phase, it is certainly contributing to the progressive impairment of glucose homeostasis (14, 15). Thus a pharmacological reconstruction of the first phase can be considered as a therapeutic option in the treatment of type 2 diabetes. To do this rationally, however, requires an understanding of the mechanisms which have become deficient.

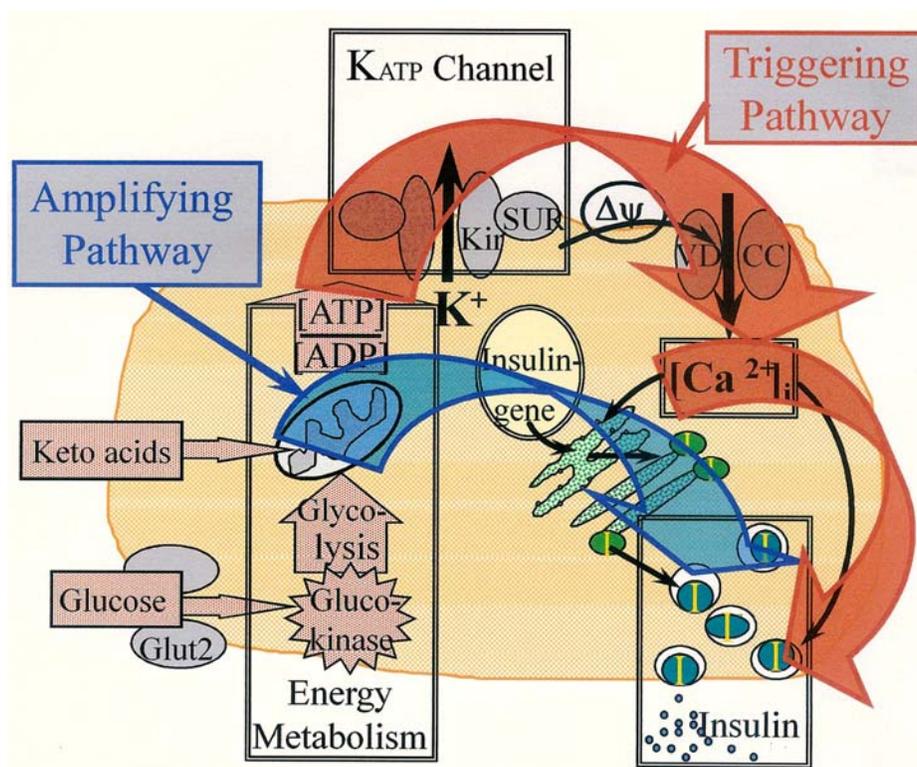
### 4. TRIGGERING AND AMPLIFYING PATHWAYS OF INSULIN SECRETION AND THEIR RELATION WITH THE BIPHASIC KINETICS OF INSULIN SECRETION

Two independent observations paved the way to the realization that the free cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) was not the final common pathway of stimulus secretion-coupling it was originally presumed to be. Glucose was found to further stimulate insulin secretion when a maximally effective concentration of a sulfonylurea had closed virtually all  $K_{ATP}$  channels of the beta cell (16). Similarly, when the  $K_{ATP}$  channels were clamped open by a high diazoxide concentration and the plasma membrane was depolarized by a high external  $K^+$  concentration, the addition of glucose still produced a substantial increase in secretion (17).

Initially, this additional pathway was referred to as " $K_{ATP}$  channel-independent" pathway (18, 19). Later the term "amplifying pathway" was proposed (20), because under physiological conditions it cannot stimulate insulin secretion as long as the  $K_{ATP}$  channels remain open and thus it is not truly independent on  $K_{ATP}$  channel function. The  $K_{ATP}$  channel-dependent pathway was proposed to produce the indispensable signal for exocytosis, namely the depolarization-induced  $Ca^{2+}$  influx, therefore the term "triggering pathway" was coined (20). While the bifurcation of the nutrient-dependent stimulus secretion coupling is generally recognized (Figure 1), the fact that the mechanism of metabolic amplification has remained elusive up to now suggests that major piece of the puzzle may still be lacking.

The predominant hypothesis to explain the biphasic kinetics of glucose-induced secretion proposes that not all of the ca. 10000 granules in the beta cell are functionally equivalent, but rather that there are different pools of granules at different stages of maturation, only one of which contains secretion-ready granules and is able to react to the depolarization-triggered  $Ca^{2+}$  influx. The nadir separating the first phase from the second phase is explained as the exhaustion of this pool which was estimated to comprise about 50 to 100 granules (21). Since these granules require only the appropriate  $Ca^{2+}$  increase in their vicinity as the final triggering signal, it is a logical consequence of this hypothesis that an activated energy metabolism is not required for the first phase. Experimental evidence has been presented in support of this contention (22). More recently, however, the amplifying pathway was found to affect first phase secretion (10), an observation which does not fit easily into the framework of the above hypothesis.

The second phase would then result from the increasing velocity of granule translocation from the reserve pool and the concomitant granule maturation, which is known to be dependent on activated energy metabolism (23). Thus, the biphasic kinetics might be the expression of two different thresholds for one signal derived from the glucose metabolism (e.g. ATP) or of two different signalling pathways with the amplifying pathway being an obvious candidate to regulate the second phase



**Figure 1.** The triggering and the amplifying pathways of nutrient-induced insulin secretion and their relation to the biphasic kinetics. In rodent beta cells, glucose is taken up by Glut-2 glucose transporters and glucokinase represents the rate-limiting step of glycolysis. The mitochondrial metabolism seems to produce a bifurcation of the signalling cascade into a triggering and an amplifying pathway. The ATP-to-ADP ratio regulates the  $K_{ATP}$  channel activity, the large conductance of which provides overriding control over the plasma membrane potential ( $\Delta\psi$ ).  $K_{ATP}$  channel closure correlates with the slow wave depolarization, which opens voltage-dependent  $Ca^{2+}$  channels and elicits a number of other secondary ion channel activities. The second part of the triggering pathway concerns the transduction of the  $Ca^{2+}$  influx and/or  $[Ca^{2+}]_i$  increase into the fusion of secretory granules. Here, it is assumed that a limited number of secretion-ready granules adjacent to the plasma-membrane make up for the first phase of insulin secretion. This leaves an enhanced mobilization of granules from a more distant reserve pool as a plausible function for the amplifying pathway, which originates from cataplerotic export of Krebs cycle intermediates, but the exact nature of which has remained enigmatic so far. Insulin secretion induced by keto acids (e.g. alpha-ketoisocaproic acid = KIC) is an important tool to test the general validity of hypotheses on nutrient-induced secretion. Recent observations by a number of groups suggest that the contributions of these two pathways to the biphasic kinetics may be more interwoven than is apparent from this sketch.

(24). This sequence of events is at the core of what may be called the “pool” hypothesis of the biphasic kinetics and implies the following testable predictions: 1) A depolarization which is sufficiently strong to induce a  $Ca^{2+}$  influx into the beta cell is sufficient to trigger insulin secretion with a first phase-like characteristic. 2) A block of  $K_{ATP}$  channels leading to depolarization and  $Ca^{2+}$  influx is sufficient to trigger insulin secretion. 3) A drug which blocks  $K_{ATP}$  channels will stimulate insulin secretion at substimulatory glucose concentrations and even in the absence of glucose (provided it does not exert additional, opposing effects more downstream).

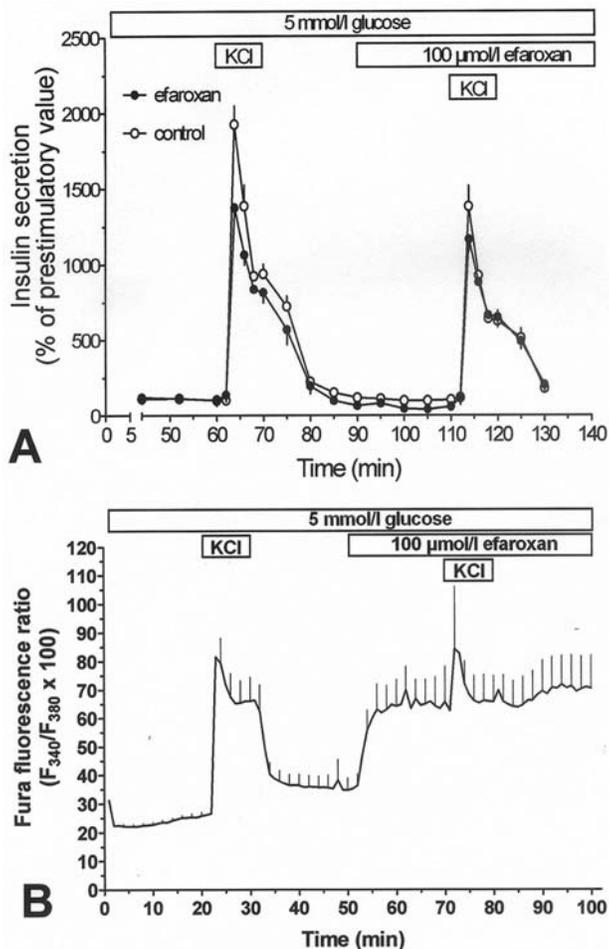
**5. CONSEQUENCES OF  $K_{ATP}$  CHANNEL BLOCK: THE UNSOLVED PROBLEM OF IMIDAZOLINES**

The mechanism of action of imidazolines, a group of investigative antidiabetic drugs, has remained

elusive in spite of considerable research effort, both from basic scientists and pharmaceutical manufacturers. The property which made these compounds interesting is the marked glucose-dependency of the insulinotropic effect, which gave rise to the expectation that an insulinotropic antidiabetic drug without risk of hypoglycaemia could be developed (25). In contrast to sulfonylureas the imidazolines (or, more precisely, several compounds in this group) do not stimulate insulin secretion at a basal glucose concentration, but enhance the efficiency of stimulatory glucose concentrations.

Several observations made while exploring the mechanism of action are not easily explained by the above described hypothesis of stimulus secretion-coupling. While some newer imidazoline derivatives do not affect  $K_{ATP}$  channels, older compounds like alinidine or efaroxan block  $K_{ATP}$  channels by binding to the pore-forming subunit Kir

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**Figure 2.** Dissociation between the insulinotropic effect of 40 mM  $K^+$  and that of efaroxan-induced  $K_{ATP}$  channel closure. (A) Freshly isolated islets were perfused with Krebs-Ringer medium containing 5 mM glucose throughout. From 60 min to 70 min and again from 110 min to 120 min the  $K^+$  concentration was raised to 40 mM. 100  $\mu$ M efaroxan (closed circles) was present from 90 min to 130 min, i.e. the second  $K^+$  depolarization took place in the presence of efaroxan. In the control experiment (open circles) efaroxan was absent throughout. Values are means  $\pm$  SEM of 4 experiments each. (B) Effect of  $K^+$  and efaroxan on  $[Ca^{2+}]_i$  of perfused islets. A Fura 2-loaded islet was perfused with Krebs-Ringer medium containing 5 mM glucose throughout.  $K^+$  was raised to 40 mM first in the absence, then in the presence of efaroxan. Note the efaroxan-induced increase of  $[Ca^{2+}]_i$  in the presence of 5 mM glucose. Values are means  $\pm$  SEM of 5 experiments.

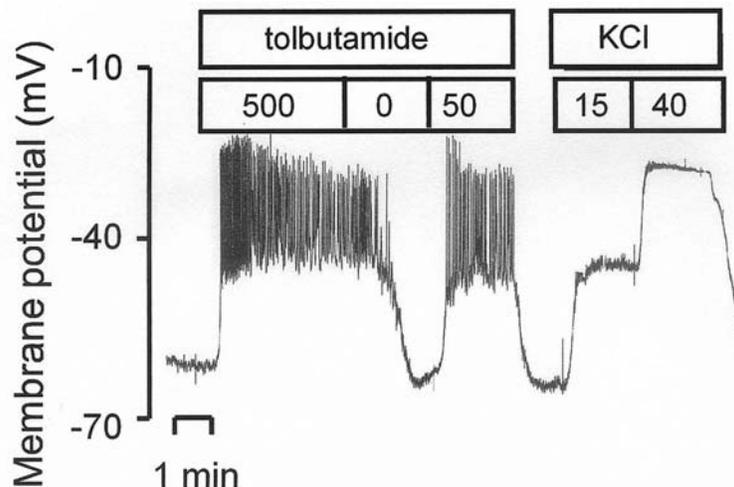
6.2 (26, 27), but are nevertheless without insulinotropic effect in the presence of a basal glucose concentration. This property is unexpected in view of the above predictions. Specifically,  $K_{ATP}$  channel-blocking imidazolines depolarized the plasma membrane, producing action potential spikes and increasing  $[Ca^{2+}]_i$  at a basal glucose concentration but did not increase insulin secretion under the same conditions (28-30). In contrast, the depolarization by 40 mM  $K^+$  reproducibly led to an increase of  $[Ca^{2+}]_i$  as well as of insulin secretion, even in the presence of an imidazoline which had led to such a dissociation between  $[Ca^{2+}]_i$  increase and secretion (Figure 2).

So the question arose whether the above dissociation was a specific property of the imidazoline compounds or whether it could also be observed by varying the strength of the KCl depolarization. The methodology to

address this problem comprises mainly secretion measurements of batch-perfused islets, measurements of  $[Ca^{2+}]_i$  in perfused islets and measurements of the beta cell plasma membrane potential using the perforated patch configuration of the patch-clamp technique (for methodological details see refs 30, 31, 53).

### 6. INSULIN SECRETION BY $K^+$ DEPOLARIZATION: WHICH $K^+$ CONCENTRATION CORRESPONDS TO THE $K_{ATP}$ CHANNEL BLOCK?

The depolarizing effect of an elevated  $K^+$  concentration in the extracellular space can be predicted by the Goldman-Hodgkin-Katz equation. To ascertain the validity of our membrane potential measurements we checked whether the depolarization by stepwise elevating



**Figure 3.** Comparison of the depolarizing effect of  $K^+$  and of tolbutamide on the plasma membrane potential of single mouse beta cells. Tolbutamide led to a plateau depolarization with superimposed action potential spiking, the amplitude of which varied between 20 and 30 mV. During the first minute of exposure to 500  $\mu$ M tolbutamide the spiking frequency was significantly higher than during the first minute of exposure to 50  $\mu$ M tolbutamide. 15 mM  $K^+$  produced a plateau depolarization without action potentials. Increasing  $K^+$  to 40 mM established a plateau at a more depolarized level but again no action potentials. The depolarization by 15 mM  $K^+$  was in the range of the plateau depolarization by tolbutamide, that by 40 mM  $K^+$  in the range of the action potential peaks. Representative trace of 5 experiments. Printed with permission from 31.

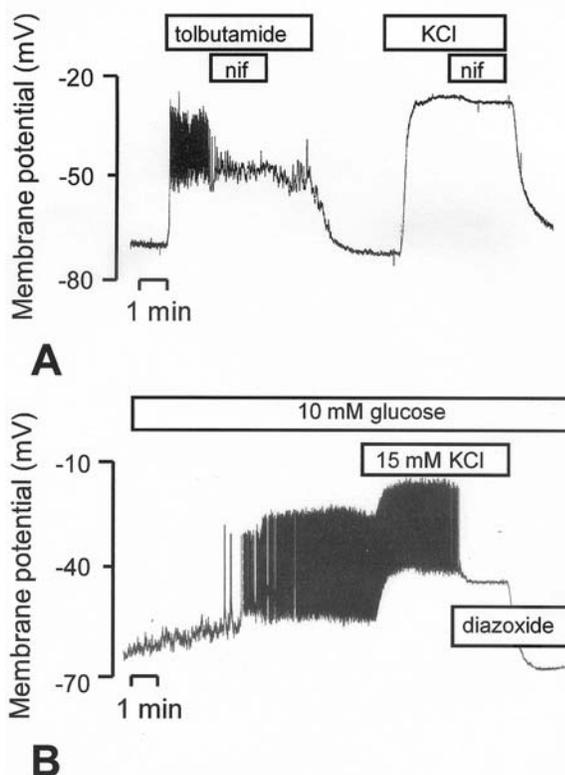
the extracellular  $K^+$  concentration from 5.6 mM to 10, 20, 30 and 40 mM corresponded to the theoretical values. This was indeed the case, not only in the conventional whole cell configuration but also using the perforated patch technique which leaves the cell metabolism intact. Obviously, a  $K^+$  depolarization affects the beta cell membrane potential only via the Nernst equilibrium and not by secondary biochemical responses, at least not within the given time frame (31). This is a clear advantage as compared to pharmacological tools, e.g. sulfonylureas, where additional sites of action, at least in the higher concentration range must be taken into account (32).

In current thinking, the potential of the plasma membrane can be regarded as the central parameter of the triggering pathway, which integrates a variety of ion channel activities to yield the opening signal for the L-type  $Ca^{2+}$  channels, which in turn are linked to the very last step of  $Ca^{2+}$  triggered exocytosis (33). Recently there has been some debate on the role of other voltage-dependent  $Ca^{2+}$  channels (34), but at least during the first phase L-type  $Ca^{2+}$  channels play a decisive role for the initiation of secretion both in rodent and human islets (35, 36).

All other factors being equal, a monotonous relation between the strength of depolarization and rate of exocytosis ought to be expected. So it should be possible to standardize experimentation by using insulin secretagogues at an equi-depolarizing strength. However, the pattern of  $K^+$  depolarization and that of depolarization by  $K_{ATP}$  channel closure is obviously different. While  $K_{ATP}$  channel closure produces a plateau depolarization with superimposed action potentials (corresponding to waves and spikes of the glucose-induced depolarization), only a plateau can be seen during  $K^+$  depolarization (Figure 3).

The question now was as to whether the spike or the plateau value of depolarization by  $K_{ATP}$  channel block corresponds to the constant membrane potential during  $K^+$  depolarization (37). Or, alternatively, is the area under the curve of the action potentials proportional to the opening signal to the  $Ca^{2+}$  channels and should this area be added to the plateau depolarization to give an effective depolarizing strength? To solve this problem we asked whether the processes underlying the action potential are also operative during  $K^+$  depolarization and in case it is so, why they don't become apparent. Since it is known that the action potential spiking results from  $Ca^{2+}$  influx (38, 39) and since it is known that  $K^+$  depolarization leads to  $Ca^{2+}$  influx (both via L-type  $Ca^{2+}$  channels, ref 40), one might presume that at least a part of the depolarization amplitude by 40 mM  $K^+$  corresponds to the action potential amplitude.

So the effect of L-type  $Ca^{2+}$  channel blockade on both types of depolarization was compared (Figure 4A). As expected, 10  $\mu$ M nifedipine abolished action potential spiking, but had a surprisingly modest effect on the depolarization by 40 mM  $K^+$ . Apparently, L-type  $Ca^{2+}$  channel activity contributes only about 5% to the 41 mV depolarization amplitude produced by 40 mM  $K^+$ , even though nifedipine strongly reduced the  $[Ca^{2+}]_i$  increase by 40 mM  $K^+$ . (I.R., unpublished). How then to explain the absence of action potentials during  $K^+$  depolarization? Since  $K^+$  depolarization does not affect  $K_{ATP}$  channel open probability, which determines by far the largest  $K^+$  conductance of the beta cell plasma membrane (41),  $K^+$  ions can freely leave the cell interior. To demonstrate the consequences of open  $K^+$  channels for the depolarization pattern, a beta cell was first depolarized by 10 mM glucose (producing action potentials) then, additionally, by 15 mM  $K^+$ , which shifted the plateau and the action potentials by



**Figure 4.** Effect of an L-type  $\text{Ca}^{2+}$  channel blocker and a  $\text{K}_{\text{ATP}}$  channel opener on action potentials of single mouse beta cells. (A) The action potential spiking induced by tolbutamide could be abolished by  $10 \mu\text{M}$  of the L-type  $\text{Ca}^{2+}$  channel blocker, nifedipine (Nif). Nifedipine had only a modest effect on the depolarization by  $40 \text{ mM K}^+$ . Thus the depolarizing effect of  $\text{Ca}^{2+}$  channel openings did practically not contribute to the plateau depolarization by  $40 \text{ mM K}^+$ . Representative trace of 5 experiments. (B) The action potential spiking induced by glucose could be abolished by  $300 \mu\text{M}$  of the  $\text{K}^+$  channel opener, diazoxide, whereas the plateau depolarization by the simultaneous presence of  $\text{KCl}$  was only slightly affected. Representative trace of 5 experiments. Printed with permission from 31.

ca.  $9 \text{ mV}$  towards zero. Opening of the  $\text{K}_{\text{ATP}}$  channels by diazoxide immediately abolished action potential spiking, but only slightly diminished the plateau depolarization. (Figure 4B). Washout of high  $\text{K}^+$  in the presence of diazoxide re-established the resting membrane potential.

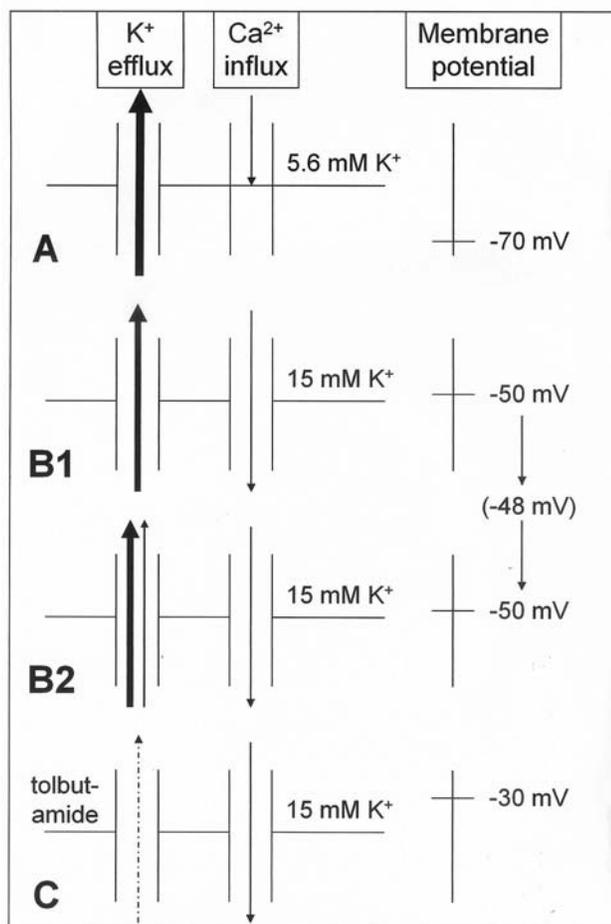
This experiment makes clear that action potentials which represent L-type  $\text{Ca}^{2+}$  channel activity (virtually complete block by nifedipine) can be diminished by increasing  $\text{K}^+$  channel activity. How are these currents related? As shown in the schematic drawing in Figure 5, the depolarization-induced  $\text{Ca}^{2+}$  influx further depolarizes the plasma membrane, probably only by a few millivolts, as is suggested by the above experiment. In doing so, it reduces the electric field which is constantly counteracting the concentration-driven  $\text{K}^+$  efflux. As a rule, the  $\text{K}^+$  efflux is the faster the farther away the actual membrane potential is from the  $\text{K}^+$  equilibrium potential. Apparently, the increased  $\text{K}^+$  efflux quickly and exactly balances the  $\text{Ca}^{2+}$  influx leading to a very smooth depolarization pattern (Figure 5B). Only when  $\text{K}_{\text{ATP}}$  channels are closed (e.g. by a sulfonylurea, Figure 5C, see also Figure 10) does the  $\text{Ca}^{2+}$  influx produce a marked further depolarization which is terminated both by  $\text{Ca}^{2+}$  channel inactivation and opening of voltage-dependent ( $\text{Kv } 2.1$ ) and  $\text{Ca}^{2+}$ -dependent (BK and

SK)  $\text{K}^+$  channels (42, 43). The contribution of the latter to the  $\text{K}^+$  conductance is much lower than that of  $\text{K}_{\text{ATP}}$  channels (41).

To sum up the above: a  $\text{Ca}^{2+}$  influx takes place during  $\text{K}^+$  depolarization, but does not further depolarize the membrane. The conclusion from these considerations for practical experimentation is that, to explore the initial events in glucose-induced insulin, a  $\text{K}^+$  concentration of ca.  $15 \text{ mM}$  rather than  $40 \text{ mM}$  should be used to mimic the depolarizing strength of a  $\text{K}_{\text{ATP}}$  channel closure.

## 7. NUTRIENT STIMULATION OF INSULIN SECRETION AFTER PRIOR DEPOLARIZATION

The use of  $15 \text{ mM}$  instead of  $40 \text{ mM K}^+$  to produce a first phase-like secretion resulted in a dramatically different response. The secretion was only modestly and transiently increased by  $15 \text{ mM K}^+$ , whereas  $40 \text{ mM}$  gives the more familiar view of an immediate and strong response (Figure 6A).  $15 \text{ mM K}^+$  produced the necessary triggering signal by increasing the  $[\text{Ca}^{2+}]_i$  through influx from the extracellular space, even though the response to  $40 \text{ mM}$  was clearly higher (Figure 6B). Another interesting feature was the different response to an



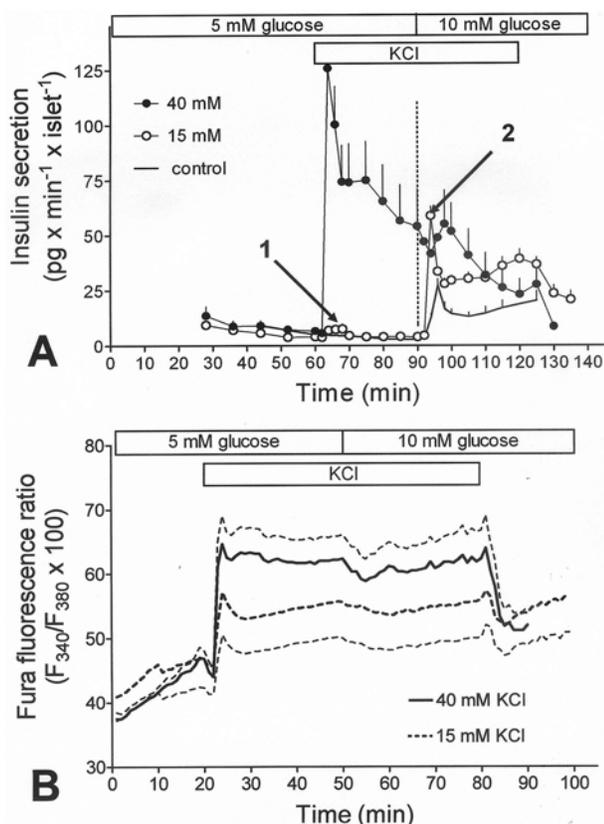
**Figure 5.** Proposed relation between  $\text{Ca}^{2+}$  current,  $\text{K}^{+}$  current and action potential generation. (A) In the resting state, the  $\text{K}^{+}$  conductance is dominant, thus the membrane potential is close to the  $\text{K}^{+}$  equilibrium potential. (B1) The presence of 15 mM  $\text{K}^{+}$  in the extracellular space shifts the membrane potential by ca. 20 mV to more depolarized values. The resultant opening of voltage-dependent  $\text{Ca}^{2+}$  channels produces an influx of positive charge, thus further depolarizing the plasma membrane and reducing the electrical field which opposes  $\text{K}^{+}$  efflux. (B2) Due to the open  $\text{K}_{\text{ATP}}$  channels and the unchanged large  $\text{K}^{+}$  conductance the comparatively modest depolarization by  $\text{Ca}^{2+}$  can be instantaneously offset by an increased  $\text{K}^{+}$  efflux. (C) When  $\text{K}_{\text{ATP}}$  channels are closed, e.g. by tolbutamide, the charge transfer by  $\text{Ca}^{2+}$  influx can no longer be balanced and an action potential develops. The amplitude of the action potential (typical 20 mV) is primarily limited by the onset of  $\text{K}^{+}$  efflux through voltage-dependent  $\text{K}^{+}$  channels and  $\text{Ca}^{2+}$ -dependent  $\text{K}^{+}$  channels (not included in this sketch).

increase of the glucose concentration in the continuing presence of 15 or 40 mM  $\text{K}^{+}$ . Whereas 10 mM glucose can only partially reverse the continuous decline of the secretion rate after the peak secretion established by 40 mM  $\text{K}^{+}$ , the response to 10 mM glucose was clearly enhanced by the presence of 15 mM  $\text{K}^{+}$  with a distinct first phase characteristic. Of note, the modest effect of 10 mM glucose in the presence of 40 mM  $\text{K}^{+}$  is at variance with a published observation (24), but this may be a consequence of a more marked insulinotropic effect of the high  $\text{K}^{+}$  concentration in our experiments, which appears to induce some sort of time-dependent inhibition (I.R., unpublished).

Now, the theoretical problem is whether the first phase corresponds to the secretion increase produced by the depolarization-stimulated  $\text{Ca}^{2+}$  influx in the presence of a basal glucose concentration or whether the increase caused

by the elevation of glucose is the “true” first phase response. If we were to follow the hypothesis outlined above, the response to the  $\text{K}^{+}$  depolarization ought to represent the first phase. But if so, why is it so small when a physiologically relevant strength of depolarization is applied? On the other hand, the insulin release caused by 40 mM  $\text{K}^{+}$  is much more pronounced than the one during the first 10 min after raising the glucose concentration from 5 to 10 mM. If we stick to the original description of the first phase as a consequence of a square wave-glucose stimulation (44, 45), then we have to state that glucose was able to produce a first phase response even though a prior increase in  $[\text{Ca}^{2+}]_i$  had taken place.

This raises the question as to whether a finite pool of granules can be responsible for the phasic nature of insulin secretion. Ideally, this information could be

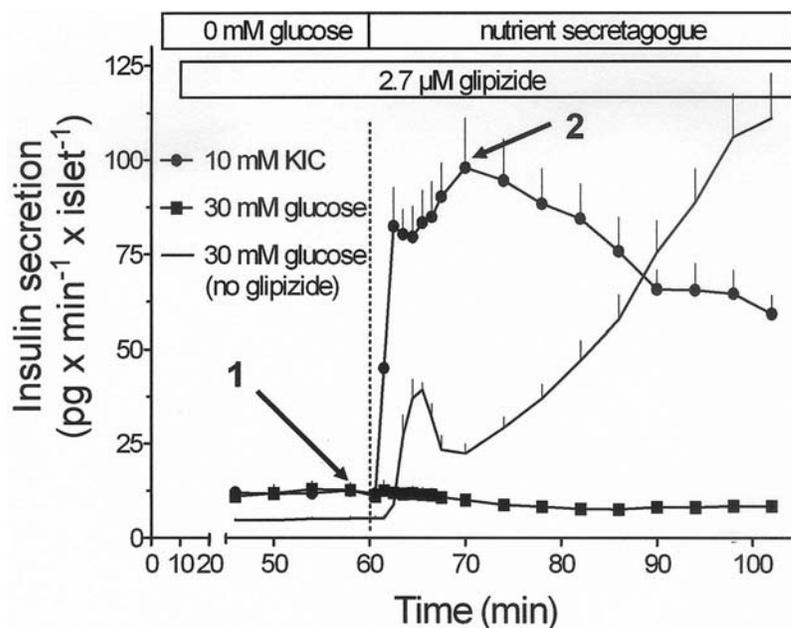


**Figure 6.** Concentration-dependent changes in the insulinotropic characteristics of a K<sup>+</sup> depolarization (A) Freshly isolated mouse islets were perfused with Krebs-Ringer medium containing 5 mM glucose for 90 min. From 60 min to 120 min the medium contained either 40 mM K<sup>+</sup> (closed circles), 15 mM K<sup>+</sup> (open circles) or unchanged 5.6 mM K<sup>+</sup> = control (solid line). From 90 min to 140 min the glucose concentration was raised from 5 to 10 mM. Note the modest secretory response to 15 mM K<sup>+</sup> (arrow 1) in the presence of 5 mM glucose and the enhanced response to 10 mM glucose in the presence of 15 mM K<sup>+</sup> (arrow 2). Values are means  $\pm$  SEM of 5 or 6 experiments. (B) Fura-2/PE3-loaded isolated mouse islets were perfused with Krebs-Ringer medium containing 5 mM glucose for 50 min. From 20 min to 80 min the medium contained either 40 mM K<sup>+</sup> (solid line) or 15 mM K<sup>+</sup> (dotted line). From 50 min to 100 min the glucose concentration was raised to 10 mM. At both K<sup>+</sup> concentrations an immediate increase in the Fura fluorescence ratio was visible. Values are means plus or minus SEM of 4 or 5 experiments. Printed with permission from 31.

provided by TIRF microscopy, which can selectively detect submembrane granules (46). However, the calculated number of granules in this pool makes up less than 10% of the total submembrane granules (21), making a clear-cut detection of a pool-depletion by optical techniques quite difficult. On the other hand, the pool concept is primarily based on functional evidence, thus it can be tested by functional experiments. Since 15 mM K<sup>+</sup> produced a [Ca<sup>2+</sup>]<sub>i</sub> increase (Figure 6B) which was not inferior to that by K<sub>ATP</sub> channel closure (31) a pool depletion analogous to the situation at the end of the first phase was to be expected, but instead of a blunted first phase in response to the subsequent glucose stimulus, there was an enhanced and apparently accelerated secretory response (Figure 6A). A similar problem occurs when considering the following experiment which involved a prolonged depolarization by K<sub>ATP</sub> channel closure prior to nutrient stimulation. Here, it was originally intended to characterize the amplifying signal produced by glucose or KIC (47, 48). To do so, the K<sub>ATP</sub> channels were closed by a maximally effective

concentration of the sulfonylurea glipizide. Since the exposure to a nutrient secretagogue influences the response to a subsequent stimulus (49, 50), beta cells were perfused for one hour with 2.7  $\mu$ M glipizide in the absence of glucose. The response to 30 mM glucose was blunted by this pre-treatment, which might lead to the conclusion that an emptying of the pool of release-ready granules had occurred. However, such islets were still able to respond to 10 mM KIC, which is similarly effective as 30 mM glucose as a nutrient secretagogue (Figure 7).

So did glipizide really increase insulin secretion in the absence of glucose? A lack of effect is not just a theoretical possibility, given the dependence of the sulfonylurea efficacy on the glucose concentration (51, 52). A moderately increased rate of secretion could be documented (Figure 7) which concurs with the demonstration of an increase of [Ca<sup>2+</sup>]<sub>i</sub> by glipizide under this condition (48). In terms of the current theory, an increased rate of secretion by K<sub>ATP</sub> channel block without



**Figure 7.** Effect of a depolarization by  $K_{ATP}$  channel closure in the absence of glucose on the insulinotropic characteristics of a subsequent nutrient stimulation. Freshly isolated mouse islets were perfused with Krebs-Ringer medium containing  $2.7 \mu\text{M}$  glipizide and  $0 \text{ mM}$  glucose for  $60 \text{ min}$ . From  $60 \text{ min}$  to  $100 \text{ min}$  the medium contained additionally either  $30 \text{ mM}$  glucose (closed squares) or  $10 \text{ mM}$  KIC (closed circles). The response to  $30 \text{ mM}$  glucose after  $60 \text{ min}$  perfusion in the absence of glucose or glipizide (no symbols) is shown as a control to demonstrate the typical biphasic response pattern and the effect of glipizide in the absence of glucose (arrow 1). Mechanistically, this response ought to correspond to the first phase secretion (depolarization but no nutrient) but this explanation is not compatible with the brisk increase in response to KIC (arrow 2). Values are means  $\pm$  SEM of 5 to 8 experiments.

increased energy metabolism should mechanically correspond to the first phase secretion. However, not only the first phase of glucose-induced secretion was abolished by this pretreatment, but the second phase response was suppressed as well. Vice versa, the preserved secretory response to KIC was not only visible during the first  $10 \text{ min}$  but during the whole exposure time. Our conclusions from these data are that i) the metabolic amplification may be different for glucose and KIC and ii) the metabolic amplification affects both phases of secretion (assuming that the initial response to KIC is equivalent to the first phase of glucose-stimulated secretion).

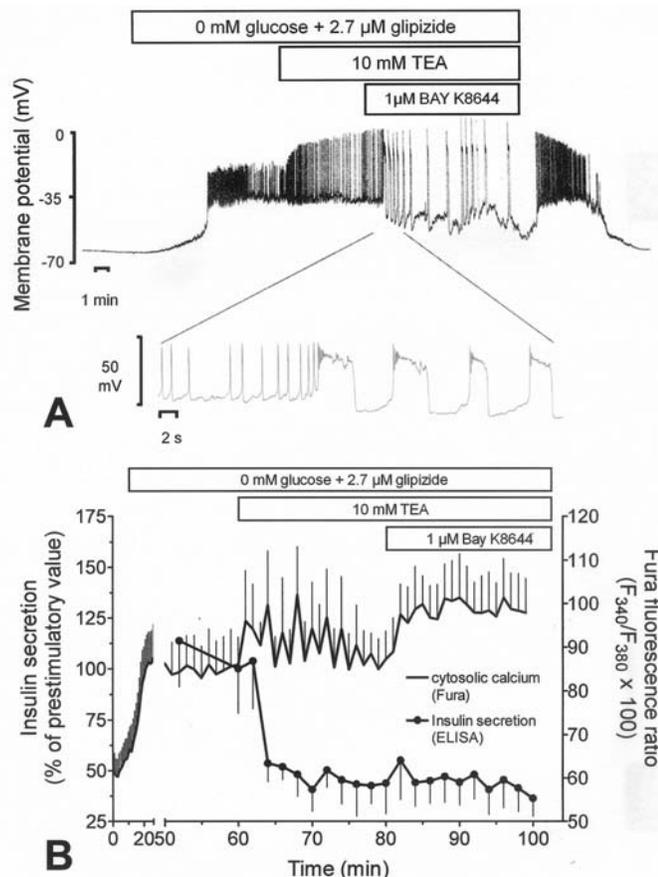
## 8. SIGNALLING ROLE OF THE ACTION POTENTIAL FOR INSULIN SECRETION

Both, pre-exposure to depolarizing  $K^+$  concentrations and to  $K_{ATP}$  channel blockers yield secretory responses which cannot be easily explained within the framework of current theory. There are two ready objections against these experimental protocols. One is that the long-term exposure to a sulfonylurea without a nutrient depletes the energy reserves of the beta cells. The other is that  $15 \text{ mM}$   $K^+$  cannot be the equivalent of a physiologically relevant depolarization-strength since it does not produce action potentials. Concerning the first argument, measurements of islet contents of ATP and ADP have in fact shown a reduction, but it was of moderate extent and clearly less than the reduction produced by the

uncoupler, CCCP (53). Also, glucose and KIC were still able to induce an increase of the NAD(P)H autofluorescence with unchanged characteristics (53). The second objection infers a specific signalling role for the beta cell action potentials.

An unexpected observation on the effect of TEA was the reason for us to re-examine the role of action potentials. TEA is known to block voltage-dependent  $K^+$  channels and, at higher concentrations, a number of other  $K^+$  channels including  $K_{ATP}$  channels (54, 55). In doing so, it retards the repolarization of the action potential, thereby increases  $Ca^{2+}$  influx and, consequently, insulin secretion (56-58). However, the presence of glucose seems to be necessary for the TEA enhancement of secretion, since TEA, when added to glipizide in the absence of glucose actually decreased insulin secretion (53). By measuring the membrane potential in the perforated patch mode, it became clear that  $10 \text{ mM}$  TEA significantly increased action potential amplitude and (less clearly visible) action potential duration under this condition (Figure 8A). Of note, both types of stimulated secretion were reduced by more than 50%: the modest secretion by glipizide in the absence of glucose, which in current terminology stands for triggering and the strong secretory response to added KIC, which stands for amplification (53).

The effect on whole cell membrane current was tested using hyperpolarizing and depolarizing steps of  $10$

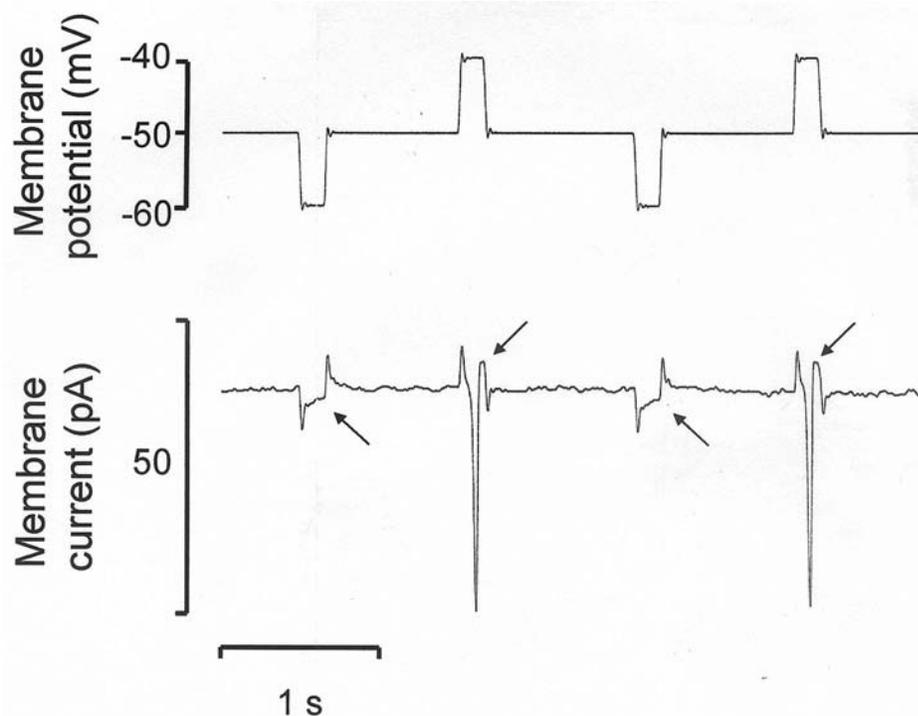


**Figure 8.** Synopsis of the effects of glipizide, TEA and Bay K8644 on action potentials,  $[Ca^{2+}]_i$  and insulin secretion of perfused primary mouse beta cells and islets. (A) Original recording of the combined effect of TEA and Bay K8644 on action potential amplitude and duration. The depolarization was initiated by 2.7  $\mu$ M glipizide in the absence of glucose, then TEA and Bay K8644 were successively added. While 10 mM TEA increased primarily the action potential amplitude, the additional presence of 1  $\mu$ M Bay K8644 strongly increased action potential duration which was followed by an undershooting repolarization and a strong reduction of action potential frequency (lower trace). Representative trace of 4 experiments. (B) Single Fura-PE3-loaded isolated islets were perfused with Krebs-Ringer medium containing 0 mM glucose throughout. From 10 min on the medium contained 2.7  $\mu$ M glipizide, to which 10 mM TEA was added from 60 min on and 1  $\mu$ M Bay K8644 from 80 min on. Values are means  $\pm$  SEM of 4 experiments (black trace). To measure secretion 50 freshly isolated islets were perfused with Krebs-Ringer medium containing 0 mM glucose and 2.7  $\mu$ M glipizide throughout. From 60 min to 80 min the medium contained additionally 10 mM TEA and from 80 min to 100 min 10 mM TEA plus 1  $\mu$ M Bay K8644. Values are means  $\pm$  SEM of 3 experiments.

mV at a holding potential of -50 mV. 2.7  $\mu$ M glipizide led to an 83 % reduction of inward and outward currents. In this situation, the addition of 10 mM TEA produced a strong inward current. In intermittent current-clamp registrations this appeared as an increase of action potential amplitude. Both, the inward current and the action potential amplitude could be abolished by 10  $\mu$ M nifedipine, suggesting that they result from L-type  $Ca^{2+}$  channel activity (53). On closer inspection (Figure 9) it can be recognized that the  $Ca^{2+}$  currents are triggered by the depolarizing step. The inward current developed after a lag time of about 50 ms and ceased completely within 150 ms, leaving time for an outward current to develop before the holding potential was switched back to -50 mV. This outward current had a slightly larger magnitude than the inward current which developed in response to the hyperpolarizing step (Figure 9) and probably represents the

residual  $K_{ATP}$  channel current. From these currents the input conductance can be estimated to be between 0.3 and 0.4 nS, which is in reasonable agreement with earlier measurements on single beta cells (59). The inward current triggered by the 10 mV depolarization had an amplitude of about 40 pA, which is close to the 37 pA  $Ca^{2+}$  current produced by a depolarizing step from -70 mV to -30 mV (41). Of note, in this range of depolarization there is no significant difference between the  $Ca^{2+}$  currents of single beta cells and those measured in beta cells within islets (41).

The conclusion from the membrane potential measurements is that the paradox inhibitory effect of TEA on insulin secretion is not caused by a change in its ion channel-blocking property. This view is supported by the induction of an oscillatory  $[Ca^{2+}]_i$  pattern when TEA was



**Figure 9.** Membrane current evoked in mouse beta cells by a -50 to -40 mV depolarization in the presence of 2.7  $\mu\text{M}$  glipizide and 10 mM TEA. Only in the combined presence of glipizide and TEA depolarizing 10 mV steps, starting from a holding potential of -50 mV, led to a marked inward current with a maximal value of about 40 pA. Since this current could be completely inhibited by 10  $\mu\text{M}$  nifedipine, it is likely to represent an L-type  $\text{Ca}^{2+}$  channel activity. The 10 mV hyperpolarizing steps led to an inward current of about 3.0 pA (lower arrows), similarly, after cessation of the inward current in response to the depolarizing step, an outward current of about 3.5 pA could be observed (upper arrows). These currents correspond to an input conductance of about 0.3 to 0.35 nS. Representative trace of 4 experiments.

added to glipizide in the absence as well as in the presence of glucose (Figure 8B). Even the further addition of the opener of L-type  $\text{Ca}^{2+}$  channels, Bay K8644, which led to a drastic prolongation of the action potentials (Figure 8A) and to a continuous elevation of  $[\text{Ca}^{2+}]_i$ , did not revert the inhibition of secretion by TEA (Figure 8B). It may well be that this uncoupling of secretion from  $\text{Ca}^{2+}$  influx is due to a direct interaction between Kv2.1 channels and components of the exocytotic machinery, e.g. SNAP-25 (60-62).

From these data no stringent relation between the parameters of action potential spiking and insulin secretion can be recognized. The amplitude is determined by the relation between  $\text{Ca}^{2+}$  influx and  $\text{K}^+$  efflux, and thus cannot be taken as a measure of  $\text{Ca}^{2+}$  influx. As exemplified by the  $\text{K}^+$  depolarization, there may be a strong induction of secretion without any obvious action potentials, i.e. with a zero amplitude. The duration and the frequency are linked to changes in the  $[\text{Ca}^{2+}]_i$  but in a seemingly counterintuitive way: the prolonged and less frequent action potentials caused by TEA plus Bay K8644 led to the transformation of an oscillatory  $[\text{Ca}^{2+}]_i$  pattern into a plateau-like elevation, suggesting a relevant modulatory role of intracellular  $\text{Ca}^{2+}$  transport. Since even this increase did not raise the secretion rate, one may speculate that changes in the  $[\text{Ca}^{2+}]_i$  may accompany changes in secretion but need

not be causally related. Or to be more positive, they may be causally related to secretion in beta cells only under a limited set of conditions requiring the presence of a nutrient secretagogue.

#### 9. DEPOLARIZATION BY CURRENT INJECTION: HOW TO RECONCILE MEMBRANE CAPACITANCE MEASUREMENTS WITH SECRETION MEASUREMENTS.

The above described experiments suggest that corrections may be due in our view on the mechanisms underlying the first phase of insulin secretion. On the other hand, the currently prevailing hypothesis is based on a large number of biophysical data, which by themselves are unlikely to be incorrect. To find a way to reconcile the seemingly opposite points of view some of these data are considered in more detail.

The preferred technique to measure insulin granule exocytosis with a single granule resolution is the membrane capacitance measurement (Neher-Lindau technique, 63). Data obtained with this technique suggest that the first phase results from a limited pool of secretion-ready granules: as a functional, not morphological definition the term “readily releasable pool” was coined, analogous to what has been described in the chromaffin cell

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(64). The spontaneous deceleration of the high initial rate of exocytosis (21, 65, 66) was interpreted to represent the depletion of the pool of secretion-ready granules and to correspond to the end of the first phase. It is assumed that only one signal is required to elicit the exocytosis of these granules, namely an increase in the  $\text{Ca}^{2+}$  concentration in the immediate vicinity of the granules, sufficient to be sensed by synaptotagmin. Synaptotagmin is the established  $\text{Ca}^{2+}$  sensor protein among the SNARE-proteins of exocytosis, the subtypes synaptotagmin 7 and 9 have been described to be relevant for insulin secretion (67).

Based on the rather low number of  $\text{Ca}^{2+}$  channels and a high initial rate of exocytosis, a privileged communication between the inner orifice of a  $\text{Ca}^{2+}$  channel and the surrounding granules has been hypothesized (68, 69). The existence of such microcompartments of  $\text{Ca}^{2+}$  channels and insulin granules (70) should lead to a virtual "all or nothing" response characteristic, since the requirement for  $\text{Ca}^{2+}$  influx is minimized. However, the cellular response could still be graded as a result of the number of calcium channels that open, which is a testable hypothesis. The possible existence of these microcompartments is a relevant argument against data showing a dissociation between changes in  $[\text{Ca}^{2+}]_i$  as conventionally measured by the Fura-technique and changes in secretion (e.g. 30, 31, 71-73). However, if stimuli are selected which increase the Fura ratio by  $\text{Ca}^{2+}$  influx through voltage-dependent  $\text{Ca}^{2+}$  channels the hypothetical microcompartments ought to be affected. In this regard it is important that the  $\text{Ca}^{2+}$  increase critical for the initiation of secretion is produced by a  $\text{Ca}^{2+}$  influx via L-type channels both in rodent and in human beta cells (36, 41). Interestingly, synaptotagmin has been proposed to arrange secretory granules in the vicinity of L-type  $\text{Ca}^{2+}$  channels (74). So, openers and blockers of L-type  $\text{Ca}^{2+}$  channels can be regarded as sufficiently selective tools to affect the critical  $\text{Ca}^{2+}$  compartment for the initiation of insulin secretion, whatever its precise nature (the characteristics of submembrane  $\text{Ca}^{2+}$  increase have been reported to differ depending on the strength of depolarization, see ref 75, however, other investigators could not identify specific submembrane microcompartments in beta cells, see ref 76).

From a methodological point of view two sources of uncertainty can be identified which may lead to misinterpretations. First, the exocytotic response of a beta cell is routinely evoked by a train of square wave-depolarizations (e.g. 65, 66), which differ from the spike and wave-pattern of the beta cell. Second, by analogy with neuroendocrine cells it is assumed that the granule content is completely discharged with each vesicle fusion, so the insulin release is presumed to be proportional to the capacitance increase, at least as long as endocytosis remains slow. While this may apply to chromaffin cells where a good correlation between the capacitance data and exocytosis as measured by TIRF-microscopy was found (77), the relation between granule fusion and insulin release is likely to be more complex (78).

Concerning the train of depolarization the question is whether the parameters usually employed are adequate for beta cells. The depolarization amplitude is derived from the resting value of the membrane potential

which, as is known since the days of microelectrode measurements, is around -70 mV and the peak of the action potentials which is somewhere between -20 and 0 mV (6, 38, 79). However, the standard depolarizing pulse from -70 to 0 mV with a duration of several hundred milliseconds is clearly no good fit to an endogenously produced depolarization pattern (Figure 10).

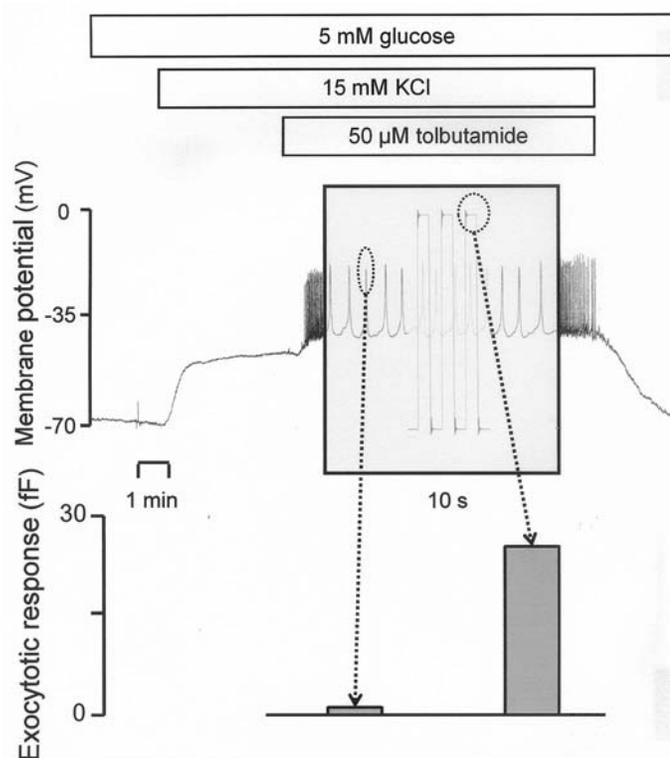
The consequences of using less depolarizing pulses (from -70 to -20 mV instead of 0 mV) or shorter pulses (50 or 100 ms instead of 500 ms) have been characterized in detail (80). In each case the response was reduced to about 20%, so in combination, the capacitance increase is most likely less than 5 % of that produced by the standard pulse protocol (Figure 10). Such a modest response size comes close to our above described measurements of secretion when a moderately depolarizing stimulus (15 mM  $\text{K}^+$  or  $\text{K}_{\text{ATP}}$  channel closure by 50  $\mu\text{M}$  tolbutamide) was employed in the presence of a basal glucose concentration. To conclude, there may be no intrinsic contradiction between our present observations and the biophysical data on beta cell exocytosis, if reference is made to the same strength of stimulation.

## 10. SUMMARY AND HYPOTHESES

A re-examination of the concentration dependency of  $\text{K}^+$  depolarization as a means to stimulate insulin secretion has led us to observe that 15 mM  $\text{K}^+$  comes close to the depolarizing strength (20 mV) of a  $\text{K}_{\text{ATP}}$  channel closure, which produces the slow wave depolarization in response to glucose. 40 mM  $\text{K}^+$  depolarizes by ca. 42 mV and comes close to the peak value of the action potential spikes which appear superimposed on plateau depolarisation established by  $\text{K}_{\text{ATP}}$  channel closure, so it has a depolarizing strength which corresponds to that of waves and spikes combined.

When 40 mM  $\text{K}^+$  was used to stimulate insulin secretion in the presence of a basal glucose concentration, a very strong secretory response occurred which peaked at 4 min and receded thereafter. This is often regarded as the equivalent of the first phase of glucose induced insulin secretion. When 15 mM  $\text{K}^+$  was used to stimulate insulin secretion in the presence of a basal glucose concentration there was only a modest transient response which corresponded to less than 10 % of the one produced by 40 mM  $\text{K}^+$ . However, a subsequent moderate glucose stimulus (10 mM) in the continuing presence of 15 mM  $\text{K}^+$  evoked an enhanced biphasic response with marked first phase peak. This observation is not easily explained by the hypothesis that first phase is due to the release of secretion-ready granules by a depolarization-triggered  $\text{Ca}^{2+}$  influx (21, 23).

The hypothesis that the first phase results from a limited pool of granules adjacent to L-type channels combines earlier hypotheses by Grodsky (81, 82) and the generally accepted sequence of exocytosis in neuronal and neuroendocrine cells, where a pool of immediately releasable granules was shown to exist in the vicinity of  $\text{Ca}^{2+}$  channels (33, 83, see however 84). Since the release of



**Figure 10.** Comparison of action potentials by  $K_{ATP}$  channel closure and of a train of depolarizations by current injection as a typical stimulus to measure exocytosis at a single granule resolution. Initially, the beta cell was depolarized by 15 mM  $K^+$ , then 50  $\mu$ M tolbutamide was added to elicit action potentials (upper panel). Such action potentials have an amplitude of ca. 20 mV and a duration at half-maximal amplitude of less than 100 ms, as can be estimated from the high resolution segment in the frame. Projected upon this action potential spiking is a sequence of three 500 ms depolarisations with a 70 mV amplitude to illustrate the different quality of the stimuli. While a 500 ms, 70 mV- depolarization train by current injection has been reported to produce an increase in membrane capacitance by ca 25 fF (lower panel), a depolarization with the characteristics of a real wave and spike potential can be estimated to give a capacitance increase of about 5 % of this value (estimate based on data in ref (90)).

insulin proceeds at much slower pace, it may well be that, in spite of the same basic exocytotic machinery, additional mechanisms may be involved in shaping the first phase of insulin secretion. As an alternative to Grodsky's early model Cerasi and colleagues have suggested that the biphasic kinetics is the net result of stimulatory and inhibitory effects emanating from the glucose metabolism with different latencies (85). The main problem with this model and its later versions (50) is that the nature of the inhibitory signals has remained unclear.

The main problem in identifying the contribution of depolarization to the secretory response may lie in the definition of what constitutes the depolarization caused by glucose. The depolarisation is a composite signal not only with respect to its generation (which is well known), but in our view also with respect to its function. The slow wave is the net expression of  $K_{ATP}$  channel closure in the presence of a hitherto ill-defined inward leak current (possibly by TRPM4, see 86, 87). The spike depolarization is the net expression of  $Ca^{2+}$  influx via voltage-dependent  $Ca^{2+}$  channels and of  $K^+$  efflux via voltage- and  $Ca^{2+}$ -dependent  $K^+$  channels, provided the majority of  $K_{ATP}$  channels is

already closed. In the presence of open  $K_{ATP}$  channels no action potential can be generated, but nevertheless there is a  $Ca^{2+}$  influx (31).

The plateau depolarization (or the glucose-induced slow wave) has an obvious trigger function for  $Ca^{2+}$  influx, whereas the more variable action potential depolarization may not have a trigger function in itself (except for  $K_v$  channel opening). A prolonged action potential more than an increased amplitude appeared to be related to the increase of  $[Ca^{2+}]_i$ . In consequence, 40 mM  $K^+$  or a 70 mV depolarization by repeated current injection for 500 ms may thus elicit effects not normally occurring in nutrient-induced insulin secretion. To mimic a physiological strength of depolarization in beta cells a depolarization from -70 mV to about -40 or -30 mV appears more appropriate. Interestingly, the strength of the  $Ca^{2+}$  current thus produced (ca 40 pA, ref 41) is quite similar to the one evoked by depolarizing 10 mV steps starting from a plateau of -50 mV (Figure 9), .

The beta cell action potential, in contrast to the neuronal action potential, may not represent a unit of

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information in its own right. In neurons, the uniform action potential serves as a fast messenger to cover the comparatively large distances between the sites of signal generation and those of signal transmission to the neighbouring cells. The action potential frequency, not its shape, codes for an exactly matching transmitter release and the subsynaptic neuron recodes the information provided by a number of transmitters into another action potential frequency (88). Obviously, this is not the role of electrical activity in an islet of 150  $\mu\text{m}$  diameter containing a network of electrically coupled beta cells (79, 89). However, there may be other roles for the ion channels involved in shaping the action potential. It has been suggested by others that Kv 2.1 and also Cav 1.2 are coupled to exocytosis in beta cells in a way not requiring their ion conducting function (60-62, 90). Some of the above observations of ours point in the same direction (53). This raises the possibility that the respective ion conductances may not be as directly linked to the regulation of exocytosis as currently assumed.

Finite or exhaustible pools of secretory granules (readily releasable or immediately releasable) may well exist in the submembrane space of the beta cells and may be released during the first phase of glucose-induced insulin secretion, but our conclusion from the above experiments is that their release does not constitute the correlate of this unique feature of hormone secretion. TIRF-microscopy has provided data showing that so-called newcomer insulin granules fuse with the plasma membrane, apparently without establishing a definite docked state (91, 92). This would be in keeping with the current hypothesis if such granules would constitute a highly  $\text{Ca}^{2+}$ -sensitive pool due to the high  $\text{Ca}^{2+}$  sensitivity of synaptotagmin 7 as compared to the more conventional low  $\text{Ca}^{2+}$  sensitivity of synaptotagmin 9 (93). However, synaptotagmin 7 null mice showed reductions in both phases of insulin secretion, not only in the second phase, as was originally expected (94). In line with our above observation (Figure 7) this suggests that the release mechanisms may not be as fundamentally different as currently assumed. In fact, it has been observed earlier that the beta cell energy metabolism affects secretion by mechanisms acting “distal” to  $\text{Ca}^{2+}$  influx (95, 96), which concurs with the view the first phase is influenced by amplifying signals circumventing plasma membrane depolarization (10).

## 11. ACKNOWLEDGMENTS

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**Abbreviations:**  $[Ca^{2+}]_i$ : cytosolic free calcium concentration; Kv channel: voltage-dependent  $K^+$  channel, KATP channel: ATP-sensitive  $K^+$  channel, KIC:  $\alpha$ -ketoisocaproic acid; TEA: tetraethylammonium

**Key Words:** Cytosolic calcium concentration; Insulin secretion; Kv channel;  $K_{ATP}$  channel; Pancreatic islets; Plasma membrane potential, Review

**Send correspondence to:** Ingo Rustenbeck, Institute of Pharmacology and Toxicology, University of Braunschweig, Mendelssohnstr. 1, 38106 Braunschweig, Tel. 49-531-391-5670, Fax: 49-531-391-8287, E-mail: i.rustenbeck@tu-bs.de

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