Plasma membrane depolarization as a determinant of the first phase of insulin secretion

K. Hatlapatka,* M. Willenborg,* and I. Rustenbeck
Institute of Pharmacology and Toxicology, Technical University of Braunschweig, Braunschweig, Germany

Submitted 9 December 2008; accepted in final form 26 May 2009

Hatlapatka K, Willenborg M, Rustenbeck I. Plasma membrane depolarization as a determinant of the first phase of insulin secretion. Am J Physiol Endocrinol Metab 297: E315–E322, 2009. First published May 26, 2009; doi:10.1152/ajpendo.90981.2008.—The role of plasma membrane depolarization as a determinant of the initial phase of insulin secretion was investigated. NMRI mouse islets and β-cells were used to measure the kinetics of insulin secretion. ATP and ADP content, membrane potential, and cytosolic free Ca2+ concentration ([Ca2+]i). The depolarization of metabolically intact β-cells by KCl corresponded closely to the theoretical values. In contrast to physiological (glucose) or pharmacological (tolbutamide) ATP-sensitive K⁺ (KATP) channel block, KCl depolarization did not induce action potential spiking. The depolarization by 15 mM K⁺ (21 mV) corresponded to the plateau depolarization by 50 or 500 μM tolbutamide; that by 40 mM K⁺ (41 mV) corresponded to the action potential peaks. Nifedipine and diazoxide abolished action potentials but not KCl depolarization, suggesting that the depolarizing strength of 15, but not 40 mM K⁺ corresponds to that of KATP channel closure. K⁺ (40 mM) induced a massive secretory response in the presence of 5 mM glucose, whereas 15 mM K⁺, like 50 μM tolbutamide, was only slightly effective, even though a marked increase in [Ca2+]i was produced. Raising glucose from 5 to 10 mM in the continued presence of 15 mM K⁺ resulted in a strongly enhanced biphasic response. The depolarization pattern of this combination could be mimicked by combining basal glucose with 15 mM K⁺ and 50 μM tolbutamide; however, the secretory response to these nonnutrients was much weaker. In conclusion, the initial secretory response to nutrient secretagogues is largely influenced by signaling mechanisms that do not involve depolarization.

It is widely accepted that a block of KATP channels that is sufficient to depolarize the pancreatic β-cell beyond the threshold potential of voltage-dependent Ca2+ channels represents a sufficient stimulus for insulin secretion. Because a number of observations suggest that the first phase of glucose-induced insulin secretion is formed by the Ca2+ influx acting on a limited set of secretion-ready granules (2), it is a logical consequence to hold the triggering pathway entirely responsible for the generation of the first-phase response. According to this model, the second phase results from the increasing velocity of granule translocation from the reserve pool and the concomitant granule maturation, both processes being dependent on the energy metabolism of the β-cell and, more specifically, on the amplifying pathway (2, 27).

While exploring the mechanism of action of KATP channel-blocking imidazoline compounds, we have repeatedly found that the increase of the free cytosolic Ca2+ concentration ([Ca2+]i) caused by some of these compounds was not paralleled by an increased secretion as long as the glucose concentration was basal (3, 4), whereas the depolarization by 40 mM K⁺ reproducibly led to an increase of [Ca2+]i, as well as of insulin secretion. The depolarization by a high-K⁺ concentration is a popular experimental tool because it is perceived to selectively induce insulin secretion via depolarization-induced Ca2+ influx. So the question arose whether the above dissociation was a specific property of the imidazoline compounds or glycolysis via the tricarboxylic acid cycle to ATP synthesis) to stimulated insulin secretion. Specifically, it could be shown that glucose stimulated insulin secretion further when KATP channels were already blocked by sulfonylureas at maximally effective concentrations (26) or when β-cells were depolarized by a high K⁺ concentration concomitantly with a permanent opening of the KATP channels by diazoxide (8, 9).

This additional pathway was originally named the KATP channel-independent signaling pathway. Later the term “amplifying pathway” was proposed (18) because, under physiological conditions, it is unable to increase insulin secretion if the KATP channels remain open and thus cannot be regarded to be truly independent on KATP channel function. The KATP channel-dependent pathway was proposed to be specifically responsible to produce the decisive triggering signal for exocytosis, namely the depolarization-induced Ca2+ influx (18). Currently, the “KATP channel-independent pathway” can be regarded as a descriptive term for one or, possibly, several signaling mechanisms that relate the energy metabolism of fuel secretagogues to the regulation of insulin secretion without eliciting changes in plasma membrane electrical activity of the β-cell. In contrast to the amplifying pathway, this terminology does not imply a specific function in stimulus-secretion coupling.


However, experimental evidence suggests that KATP channel-mediated signaling is not the only pathway leading from increased energy metabolism (defined as the sequence from

Address for reprint requests and other correspondence: I. Rustenbeck, Institute of Pharmacology and Toxicology, Technical Univ. of Braunschweig, Mendelssohnstr. 1, 38106 Braunschweig, Germany (e-mail: i.rustenbeck@tu-bs.de).

http://www.ajpendo.org 0193-1849/09 $8.00 Copyright © 2009 the American Physiological Society E315
whether it could also be observed by varying the strength of a KCl depolarization.

This study deals with the role of depolarization, either by high KCl or by K<sub>ATP</sub> channel block, as a determinant of insulin secretion, in particular the first-phase response. The loss of the first-phase response appears to play an important role in the pathogenesis of type 2 diabetes (6, 11); therefore, the mechanisms underlying its generation deserve particular attention if a pharmacological restoration is to be attempted.

**MATERIALS AND METHODS**

**Chemicals.** Tolbutamide was obtained from Serva (Heidelberg, Germany), nifedipine from Sigma (Taufkirchen, Germany), and fura PE3-AM from TEF Labs (Austin, TX). Collagenase P was purchased from Boehringer Mannheim/Roche Diagnostics (Mannheim, Germany), cell culture medium RPMI 1640 from GIBCO/Invitrogen (Karlsruhe, Germany), and PCS from PAA (Colbe, Germany). ATP was determined by use of a luciferase luminescence kit (Sigma). All other reagents of analytical grade were from E. Merck (Darmstadt, Germany).

**Tissue and cell culture.** Islets were isolated from the pancreas of NMRI mice by a collagenase digestion technique and hand-picked under a stereomicroscope. Single cells were obtained by incubation of the islets for 10 min in a Ca<sup>2+</sup>-free medium and subsequent vortex-mixing for 1 min. Islets and single islet cells were cultured in cell culture medium RPMI 1640 with 10% FCS (10 mM glucose) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. Animal handling and euthanasia conformed to current German and European Union animal protection laws.

**Measurement of insulin secretion.** Batches of 50 NMRI mouse islets were introduced in a purpose-made perfusion chamber (37°C) and perfused with a HEPS-buffered Krebs-Ringer medium (2 mg BSA/ml) saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, which contained the respective secretagogue. The insulin content in the fractionated effluents was determined by enzyme-linked immunoassay (Mercodia, Uppsala, Sweden).

**Electrophysiological recordings.** The membrane potential of single β-cells was measured by a standard patch-clamp technique (15) using the perforated-patch configuration, which leaves the energy transients yielding the net ADP content.

**RESULTS**

Raising the glucose concentration from 5 to 10 mM in the perfusion medium of isolated mouse islets resulted in a biphasic secretory response. During the prestimulatory phase, [Ca<sup>2+</sup>]<sub>i</sub> values were basal and increased concomitantly with the first-phase secretion. The subsequent elevated [Ca<sup>2+</sup>]<sub>i</sub> levels that resulted from oscillatory changes of [Ca<sup>2+</sup>]<sub>i</sub> in single islets responded with the increased secretion rates but did not reflect the further increase during the second phase (Fig. 1). These observations conform with the view that the first-phase secretion is triggered by the initial Ca<sup>2+</sup> influx into the β-cells, whereas the second phase is largely influenced by amplifying signals in the continuing presence of elevated [Ca<sup>2+</sup>]<sub>i</sub>.

To assess whether a depolarization-induced Ca<sup>2+</sup> influx is sufficient to shape the first phase of glucose-induced insulin secretion, the depolarizing effect of KCl was characterized in more detail. Initially, the conventional whole cell configuration of the patch-clamp technique was used, and the K<sup>+</sup> concentration of the extracellular medium was stepwise elevated from 5.6 mM to 10, 20, 30, and 40 mM (Fig. 2A). The experimental data were compared with those obtained by the Goldman-Hodgkin-Katz equation (12). A close correspondence was found to exist between the theoretically predicted and experimentally obtained values (Fig. 2B). The results obtained with the perforated-patch configuration, which leaves the energy metabolism of the β-cells intact, were only slightly different from those obtained with the whole cell configuration (Fig. 2B). Obviously, KCl affects the β-cell membrane potential proteins were precipitated, and the adenine nucleotides were extracted as described (33). ATP was determined by use of the luciferase method. The ADP content of the extract was converted into ATP by the pyruvate kinase reaction, the difference between both measurements yielding the net ADP content.

**Data handling and statistics.** Statistical calculations were performed by Prism and Instat software (GraphPad, San Diego, CA). If not specified otherwise, differences were considered significant if P < 0.05 (unpaired two-sided t-test).

![Fig. 1. Comparison of the effects of a moderate stimulatory glucose concentration on the insulin secretion of 50 batch-perfused and on the cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) of single perfused mouse islets. After the glucose concentration was raised from 5 to 10 mM, the increase of [Ca<sup>2+</sup>]<sub>i</sub>, coincides with the first phase of insulin secretion. Thereafter, an oscillatory pattern of [Ca<sup>2+</sup>]<sub>i</sub> occurred, which is not directly visible in the mean values since the islets did not oscillate in phase. F<sub>340/380</sub>/F<sub>490</sub> ratio of fluorescence at 340 to 380 nm. Values are means ± SE of 6 (insulin secretion) or 5 ([Ca<sup>2+</sup>]<sub>i</sub>) experiments.](http://ajpendo.physiology.org/)

**AJP-Endocrinol Metab • VOL 297 • AUGUST 2009 • www.ajpendo.org**
The effect of 15 and 40 mM K\(^+\) on insulin secretion was characterized in the presence of a substitutary (5 mM) and moderate stimulatory (10 mM) glucose concentration (Fig. 4A). In this situation, the K\(^+\) channel opener diazoxide led to a prompt cessation of action potential spiking but had only a moderate effect on the plateau depolarization (reduction by 5.6 ± 0.8 mV, n = 4, Fig. 3C). The lack of action potentials during KCl depolarization is thus due to a high number of open K\(^+\) channels under this condition.

Fig. 2. Concentration dependency of the depolarizing effect of KCl on pancreatic β-cells as measured in the conventional whole cell mode of the patch-clamp technique. A: after an initial depolarization by 500 μM tolbutamide, the K\(^+\) concentration in the medium was stepwise increased from 5.6 mM (physiological) to 40 mM. Here, the plateau depolarization by tolbutamide looks roughly equivalent to that by 20 mM K\(^+\). B: comparison of the experimental data as measured in the whole cell mode (open circles) with the depolarization as calculated by the Goldman-Hodgkin-Katz equation (diamonds). Both concentration dependencies are closely concordant. With metabolically intact β-cells (perforated-patch mode), the depolarization by 15 mM K\(^+\) was slightly more marked than calculated; that by 40 mM fit exactly (filled circles). The glucose concentration was 0 mM in the whole cell mode and 5 mM in the perforated-patch mode. Values are means ± SE of 4 or 5 experiments.

Only via the Nernst equilibrium and not by inducing secondary biochemical responses.

In contrast to K\(_{\text{ATP}}\) channel blockers like tolbutamide, depolarization by KCl did not normally induce action potential spiking in the presence of 5 mM glucose (Fig. 3A). The effect of 500 μM tolbutamide differed from that of 50 μM in that the action potentials during the first minute were significantly more frequent (1.96 ± 0.26 vs. 1.01 ± 0.33/s), whereas the plateau depolarization, which was in the range of the depolarization by 15 mM K\(^+\), was not significantly stronger (Fig. 3A). Nifedipine (10 μM) strongly reduced the action potential amplitude while leaving the plateau depolarization by tolbutamide intact (Fig. 3B). However, nifedipine had practically no effect (≤2 mV) on the depolarization by 40 mM K\(^+\), suggesting that Ca\(^{2+}\) channel activity is not reflected by action potentials under this condition. Thus 15 mM K\(^+\) (depolarization by 21.3 ± 1.1 mV), but not 40 mM K\(^+\) (depolarization by 41.2 ± 1.5 mV), exerts a depolarization comparable to that typically produced by K\(_{\text{ATP}}\) channel blockade. The action potentials produced by 10 mM glucose originated from a significantly lower plateau depolarization than that established by tolbutamide (−49.9 ± 2.2 vs. −42.7 ± 1.2 mV; P = 0.021, n = 5). Increasing K\(^+\) from 5.6 to 15 mM caused a significant further plateau depolarization by 8.8 ± 1.7 mV (n = 4) with a slightly decreased action potential amplitude (Fig. 3C). In this situation, the K\(^+\) channel opener diazoxide led to a prompt cessation of action potential spiking but had only a moderate effect on the plateau depolarization (reduction by 5.6 ± 0.8 mV, n = 4, Fig. 3C). The lack of action potentials during KCl depolarization is thus due to a high number of open K\(^+\) channels under this condition.

Fig. 3. Comparison of the depolarizing effect by KCl and by tolbutamide on primary pancreatic β-cells as measured in the perforated-patch mode. A: tolbutamide led to a plateau depolarization with superimposed action potential spiking, the amplitude of which varied between 20 and 30 mV. The spiking frequency during the first minute, but not the plateau depolarization by 500 μM tolbutamide, was significantly stronger than that of 50 μM tolbutamide. K\(^+\) at 15 mM produced a plateau depolarization without action potentials. Increasing K\(^+\) to 40 mM established a plateau at a more depolarized level but no spiking. The action potentials during 15 mM K\(^+\) produced by 20 mM K\(^+\) channel blocker, diazoxide, whereas the plateau depolarization by tolbutamide was significantly stronger than that established by tolbutamide (Fig. 3C). Nifedipine had only a minimal effect on the depolarization by 40 mM K\(^+\). Nifedipine had only a minimal effect on the depolarization by 40 mM K\(^+\). Thus the depolarizing effect of Ca\(^{2+}\) channel openings practically did not contribute to the plateau depolarization by 40 mM K\(^+\). Representative trace of 5 experiments. B: action potential spiking induced by glucose could be abolished by 10 μM of the L-type Ca\(^{2+}\) channel blocker, nifedipine (Nif). Nifedipine had only a minimal effect on the depolarization by 40 mM K\(^+\). Thus the depolarizing effect of Ca\(^{2+}\) channel openings practically did not contribute to the plateau depolarization by 40 mM K\(^+\). Representative trace of 5 experiments. C: action potential spiking induced by glucose could be abolished by 300 μM of the K\(^+\) channel blocker, diazoxide, whereas the plateau depolarization by the simultaneous presence of KCl was only slightly affected. Representative trace of 5 experiments.
The presence of 5 mM glucose, 15 mM K⁺ had only a modest stimulatory effect during the first 10 min and was virtually ineffective thereafter. However, the continuing presence of 15 mM K⁺ enhanced the secretory response when the glucose concentration was raised to 10 mM (Fig. 4A). K⁺ at 40 mM, in contrast, induced an immediate and strong secretory response in the presence of 5 mM glucose. After the initial peak, the rate of secretion declined continuously and was only transiently elevated when the glucose concentration was raised to 10 mM (Fig. 4A). At the end of the KCl exposure (120 min), the secretion rate was significantly higher in the presence of 15 mM than of 40 mM K⁺. The difference between the effects of 50 and 500 μM tolbutamide was less impressive (Fig. 4B). Nevertheless, in the presence of 5 mM glucose, there was only a transient increase of secretion by 50 μM tolbutamide, whereas 500 μM caused a sustained increase after the initial peak. Increasing the glucose concentration from 5 to 10 mM in the presence of tolbutamide gave a stronger secretory response in the presence of 50 than of 500 μM tolbutamide, similar to the K⁺ depolarization (Fig. 4B). In contrast to KCl, the presence of tolbutamide did not lead to an overshooting, first phase-like response when glucose was raised to 10 mM.

The effects of 15 and 40 mM K⁺ as well as that of 50 and 500 μM tolbutamide on [Ca²⁺], in islets were explored under the same conditions as used for the secretion measurements (Fig. 5). Both, 15 and 40 mM caused an immediate increase of the fura ratio, which remained elevated throughout the exposure to high K⁺. The increase of the fura ratio by 40 mM was nearly two times as high as that by 15 mM. Raising glucose from 5 to 10 mM did not lead to a further increase of [Ca²⁺], a transient decrease that was also detectable in the experiments with 40 mM K⁺. The extent of the [Ca²⁺], increase by 50 μM tolbutamide was similar to that caused by 15 mM K⁺. Another similarity between the effects of 15 mM K⁺ and 50 μM tolbutamide was that the increase in glucose concentration from 5 to 10 mM did not cause a transient decrease of [Ca²⁺]. In the presence of 50 μM tolbutamide,

![Figure 4](https://example.com/figure4.png)

**Fig. 4.** Concentration-dependent changes in the insulinotropic characteristics of KCl and tolbutamide. A: freshly isolated islets were perfused with Krebs-Ringer medium containing 5 mM glucose for 90 min. From 60 to 120 min, the medium contained either 40 mM K⁺ (filled circles), 15 mM K⁺ (open circles), or unchanged 5.6 mM K⁺, which was the control (solid line, identical with the secretion data shown in Fig. 1). From 90 to 140 min, the glucose concentration was raised from 5 to 10 mM. Values are means ± SE of 5 or 6 experiments. B: freshly isolated islets were perfused with Krebs-Ringer medium containing 5 mM glucose for 90 min. From 60 to 120 min, the medium contained either 500 (filled circles) or 50 (open circles) μM tolbutamide. From 90 to 140 min, the glucose concentration was raised from 5 to 10 mM. Only 50 but not 500 μM tolbutamide had a lasting stimulatory effect in the presence of basal glucose (5 mM). Values are means ± SE of 4 or 5 experiments.

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Comparison of the effect of KCl and of tolbutamide on the [Ca²⁺], in islets were explored under the same conditions as used for the secretion measurements (Fig. 5A). Tolbutamide (500 μM) established an elevated [Ca²⁺], plateau that was slightly lower than that of a preceding depolarization by 40 mM K⁺. Raising glucose from 5 to 10 mM did not lead to a further increase of [Ca²⁺], but caused a transient decrease that was also detectable in the experiments with 40 mM K⁺ (Fig. 5A). The extent of the [Ca²⁺], increase by 50 μM tolbutamide was similar to that caused by 15 mM K⁺. Another similarity between the effects of 15 mM K⁺ and 50 μM tolbutamide was that the increase in glucose concentration from 5 to 10 mM did not cause a transient decrease of [Ca²⁺].
there was even a moderate increase that resulted in the same steady-state \([\text{Ca}^{2+}]\), levels as those established by 500 \(\mu\text{M}\) tolbutamide (Fig. 5B).

The increase of secretion when glucose was raised from 5 to 10 mM in the presence of 15 mM \(\text{K}^+\) had a marked biphasic kinetic (Fig. 4A). It was thus of interest to define whether the enhanced first phase-like response was due to a \(\text{K}_{\text{ATP}}\) channel-dependent or -independent mechanism. The ATP and ADP content of islets exposed for 30 min to 15 mM and to 40 mM \(\text{K}^+\) in the presence of 5 mM glucose was not significantly different from control. Rather, the ATP-to-ADP ratio decreased moderately, but significantly (Fig. 6A), with increasing \(\text{K}^+\) concentration (5.5 \(\pm\) 0.6, 4.5 \(\pm\) 0.6, and 3.8 \(\pm\) 0.5, respectively; \(P = 0.0085\), paired Friedman test). Thus a more complete closure of the \(\text{K}_{\text{ATP}}\) channels is unlikely to account for the enhanced response. When the islets were incubated as described and additionally for 30 min in the presence of 10 mM glucose (Fig. 6B), a significantly increased ATP content was noted in the presence of all three \(\text{K}^+\) concentrations; in the presence of 40 mM \(\text{K}^+\), there was even an increase in the ATP-to-ADP ratio. Apparently, the inability of 10 mM glucose to produce a lasting increase in secretion when preceded and accompanied by a strong KCl depolarization is not because of an inhibition of oxidative phosphorylation.

The mechanism underlying the enhanced effect of 10 mM glucose in the presence of 15 mM \(\text{K}^+\) was further explored by simulating the depolarizing effect of 10 mM glucose and 15 mM \(\text{K}^+\) with a combination of 5 mM glucose, 15 mM \(\text{K}^+\), and 50 \(\mu\text{M}\) tolbutamide as a nonnutrient \(\text{K}_{\text{ATP}}\) channel blocker. Using the perforated-patch technique, it was ascertained that the depolarization patterns produced by these two combinations were not significantly different (Fig. 7, A and B). The addition of tolbutamide to the combination of basal glucose and 15 mM \(\text{K}^+\) had a clear effect on secretion, but was markedly inferior to the combination of 10 mM glucose and 15 mM \(\text{K}^+\) [area under the curve (AUC) from 90 to 120 min: 18.9%]. Of note, both the initial and the second-phase response were higher in the presence of the nutrient stimulus (Fig. 8A).

When 500 \(\mu\text{M}\) tolbutamide was used instead of 50 \(\mu\text{M}\), the initial phase of secretion was closely similar to the one elicited by glucose, but the AUC from 90 to 120 min was still inferior (78.2%). Under this condition, however, the depolarization patterns were different. The further depolarization by adding tolbutamide to the preexistent KCl depolarization was now significantly higher than that caused by adding 10 mM glucose (9.2 \(\pm\) 1.1 vs. 3.8 \(\pm\) 0.8 mV; \(P = 0.026\); Fig. 7, C and D). Also, the action potential frequency was higher by a marginal significance (\(P = 0.06\)). Because the action potentials were also significantly more frequent than those by 50 \(\mu\text{M}\) tolbutamide in the presence of 15 mM \(\text{K}^+\) (\(P = 0.039\)), this difference is most likely not by chance. The most clear-cut difference between the insulinotropic mechanisms of action of 500 \(\mu\text{M}\) tolbutamide and of 10 mM glucose was the effect on \([\text{Ca}^{2+}]\). The addition of 500 \(\mu\text{M}\) tolbutamide produced a transient further increase above the plateau established by 15 mM \(\text{K}^+\) alone, whereas the increase in glucose from 5 to 10 mM had no immediate effect (Fig. 8B). The latter observation concurs with a preceding experiment (compare Fig. 5A). Apparently, the closely similar first phase-like secretory responses resulted from different mechanisms.

**DISCUSSION**

The current view of how the depolarization of the \(\beta\)-cell initiates glucose-induced insulin secretion is largely influenced by experiments employing \(\text{K}^+\) depolarization. The experiments of this study, comparing moderate and strong \(\text{K}^+\) depolarization with a depolarization by moderate or strong inhibition of \(\text{K}_{\text{ATP}}\) channels, suggest that some revision may be necessary.

A strong \(\text{K}^+\) depolarization by 30 or 40 mM \(\text{K}^+\) is widely known to elicit a marked insulin secretion from perfused mouse islets in the presence of a basal glucose concentration and even in the absence of glucose (20). This response is often regarded as being equivalent to the first phase of glucose-induced insulin secretion (31), which conforms with the view that the first phase is due to a limited pool of secretion-ready granules requiring only one final signal that is provided by the \([\text{Ca}^{2+}]\) elevation in the vicinity of voltage-sensitive \(\text{Ca}^{2+}\) channels (2, 27).

Our membrane potential values as obtained with the conventional whole cell mode fitted closely to the theoretical values as calculated by the Goldman-Hodgkin-Katz equation.

---

**Fig. 6. Effect of a KCl depolarization on the ATP and ADP contents of statically incubated mouse islets.** As in the perifusion experiments (Fig. 4A), the medium contained either 40 mM \(\text{K}^+\), 15 mM \(\text{K}^+\), or 5.6 mM \(\text{K}^+\) (control). A: incubation time was 30 min; the glucose concentration was 5 mM glucose. In a paired comparison (Friedman test), the increase of the \(\text{K}^+\) concentration led to a significant decrease of the ATP-to-ADP ratio. Values are means \(\pm\) SE of 6 experiments. B: incubation time was 30 min in the presence of 5 mM glucose; next, the glucose concentration was increased to 10 mM, and the incubation was continued for another 30 min. At all \(\text{K}^+\) concentrations, the ATP concentration was increased significantly compared with A. There was no \(\text{K}^+\) concentration-dependent change in the ATP-to-ADP ratio. Values are means \(\pm\) SE of 7 experiments.
The depolarization by 15 mM K⁺ was in the range of the plateau depolarization by 50 or 500 μM of the sulfonylurea, tolbutamide. In contrast, the depolarization by 40 mM K⁺-established values was comparable to the peak value of the action potentials, which appear superimposed on the plateau depolarization elicited by glucose, sulfonylureas (19, 30), and other KATP channel blockers (3). The suppression of action potential spiking by 10 μM nifedipine confirmed that it is caused by L-type Ca²⁺ channel activity (22). The minimal effect of nifedipine on the depolarization by 40 mM K⁺ shows that, under this condition, Ca²⁺ channel activity is only weakly transduced into membrane potential changes. The most likely explanation is that the depolarizing influx of positive charge through Ca²⁺ channels is offset by an increased efflux of positive charge through the open K⁺ channels. This explanation is supported by the observation that the K⁺ channel opener diazoxide immediately abolished action potential spiking produced by the combination of glucose and 15 mM K⁺, but left the plateau depolarization by K⁺ practically unchanged.

Increasing the tolbutamide concentration from 50 to 500 μM did not result in a further significant increase of the plateau depolarization, but in a significantly higher frequency of action potentials. Similarly, it was shown that increasing the glucose concentration from a moderate to a strong stimulatory value through Ca²⁺ influx as such is only weakly effective to stimulate secretion, and the first phase may require an activated energy metabolism to fully develop. In marked contrast, raising glucose from 5 to 10 mM in the continuous presence of 40 mM K⁺ was only moderately and transiently effective to increase secretion. Apparently a strong, but not a moderate, K⁺ depolarization has an inhibitory effect on nutrient-induced secretion, which is reminiscent of the earlier demonstration that a

The depolarization by 15 mM K⁺, which led to a prompt increase in [Ca²⁺], had a surprisingly small effect on secretion in the presence of a substimulatory glucose concentration. On the other hand, when a moderate glucose stimulus (10 mM) was applied during the continuous presence of 15 mM K⁺, the secretory response to glucose was markedly enhanced with a biphasic characteristic. It appears that, in contrast to the general perception, Ca²⁺ influx as such is only weakly effective to stimulate secretion, and the first phase may require an activated energy metabolism to fully develop. In marked contrast, raising glucose from 5 to 10 mM in the continuous presence of 40 mM K⁺ was only moderately and transiently effective to increase secretion. Apparently a strong, but not a moderate, K⁺ depolarization has an inhibitory effect on nutrient-induced secretion, which is reminiscent of the earlier demonstration that a

---

**Fig. 7.** Comparison of the depolarizing effect of a nonnutrient (50 or 500 μM tolbutamide) in the presence of 5 mM glucose and 15 mM K⁺ with that of a nutrient (10 mM glucose) in the presence of 15 mM K⁺. The membrane potential was measured in the perforated-patch mode using primary pancreatic β-cells. Both tolbutamide and glucose induced action potential spiking together with a moderate further increase in the plateau depolarization as established by 15 mM K⁺. A: representative trace of 5 experiments comparing the effect of 50 μM tolbutamide with that of 10 mM glucose. B: mean values ± SE of 5 experiments for the conditions as indicated by the nos. in A. There was no significant difference between the values representing conditions 1–4 (50 μM tolbutamide) and the corresponding values for conditions 5–8 (10 mM glucose). Thus the triggering signal for both combinations of depolarizing agents can be regarded as equal. C: representative trace of 5 experiments comparing the effect of 500 μM tolbutamide with that of 10 mM glucose. D: mean values ± SE of 5 experiments for the conditions as indicated by the nos. in C. The depolarization by adding tolbutamide (3 vs. 2) was significantly stronger than that by adding glucose (7 vs. 6). Also, the plateau established by 500 μM tolbutamide was significantly higher than that established by glucose (3 vs. 7). The triggering signal produced by 500 μM tolbutamide plus 15 mM K⁺ is different from that produced by 10 mM glucose plus 15 mM K⁺.
nonnutrient stimulation of insulin secretion decreased the response to a subsequent glucose stimulus (23, 24). This delayed inhibitory action is a further argument against the view that the strong secretory response to 40 mM KCl is mechanistically equivalent to the first phase of glucose-induced insulin secretion.

The insulinotropic effect of 50 μM tolbutamide in the presence of a basal glucose concentration was comparable to that of 15 mM KCl in that there was only a moderate transient response during the first 10 min of perfusion. This observation concurs with earlier secretion measurements showing that, in the presence of a basal glucose concentration, 60 μM tolbutamide was weakly effective and 25 μM tolbutamide was entirely ineffective, even though the KATP channel activity in intact β-cells was reduced to ~20% by 30 μM tolbutamide under closely similar conditions (25). Tolbutamide (500 μM) elicited a sustained secretory response in the presence of basal glucose; however, this increase was much smaller than that produced by 40 mM KCl.

Nevertheless, the depolarization by tolbutamide and KCl has in common that raising glucose to a stimulatory level elicited an ascending second phase-like response only when the secretion rate before the nutrient stimulation was as low as the level of the nutrient secretagogue, again suggesting that a depolarization without increased nutrient availability produces not only stimulatory but also longer-lasting inhibitory signals. Both KCl and tolbutamide led to a more marked increase of [Ca^{2+}]i when used at the higher concentration. In both cases, the increased nutrient availability by raising glucose from 5 to 10 mM was accompanied by a transient decrease of [Ca^{2+}]i. Because this decrease, which may be due to an ATP-dependent Ca^{2+} sequestration or Ca^{2+} extrusion, was not visible during exposure to the lower concentration of KCl and tolbutamide, it may be related to the weaker insulinotropic effect of glucose under this condition. The straightforward explanation that the high [Ca^{2+}]i produced by 40 mM KCl or 500 μM tolbutamide has an uncoupling effect on mitochondrial energetics is obviously not appropriate, since 10 mM glucose could be shown to produce a clear increase of islet ATP content in the presence of the physiological as well as of both elevated KCl concentrations.

The enhancement of both secretion phases when glucose was raised from 5 to 10 mM in the continuous presence of 15 mM KCl led to the question as to whether triggering (KATP channel-dependent) or amplifying (KATP channel-independent) mechanisms were mainly involved. Simulating the depolarization pattern of the stimulatory glucose concentration plus KCl by a basal glucose concentration plus KCl plus 50 μM tolbutamide defined two stimuli with equal triggering signals. Thus the markedly stronger secretory response elicited by the nutrient (glucose) compared with the combination of two nonnutrients has to be ascribed to KATP channel-independent, amplifying signals. This reasoning also applies to the initial phase of the glucose-stimulated secretion. This observation lends further support to the view that amplifying signals are able to influence the first phase of glucose-induced insulin secretion (21). In this context, the earlier observation that the inhibition of oxidative phosphorylation by oligomycin nearly abolished the secretory response to 500 μM tolbutamide or 40 mM KCl, even though both stimuli were still able to markedly increase [Ca^{2+}]i (28), suggests that either the readily releasable pool is much smaller than the first phase or there may be an energy-dependent step in addition to Ca^{2+} influx (32).

Interestingly, the enhanced first-phase response to glucose in the presence of 15 mM KCl could be mimicked when 500 μM tolbutamide instead of 50 μM was used together with 15 mM KCl. Here, however, the depolarization pattern was different from that of glucose in that the plateau depolarization was stronger and the action potentials were more frequent. Also, the combination of a moderate depolarization plus metabolic amplification as well as from an increased depolarization-dependent Ca^{2+} influx in the presence of basal glucose.

This conclusion does not contradict an indispensable role of membrane depolarization. According to the SNARE hypothesis of neuronal exocytosis (10) and extrapolations of this model for the β-cell (27), synaptotagmin is the Ca^{2+} sensor for stimulated exocytosis, and consequently only Ca^{2+} influx...
through plasma membrane channels can serve the trigger function. Taking into account the much slower kinetics of insulin secretion, however, additional mechanisms may well become important for the full expression of the first-phase response. In this regard, the present investigation concurs with other recent observations (16) that [Ca\textsuperscript{2+}], levels are less direct regulators of insulin secretion than widely assumed.

Finally, we wish to emphasize that the extent of plasma membrane depolarization is not of uniform relevance for stimulus-secretion coupling in the \( \beta \)-cell. The plateau depolarization by \( K_{\text{ATP}} \) channel closure represents a signal for Ca\textsuperscript{2+} influx, whereas the action potential spiking is the indirect expression of this Ca\textsuperscript{2+} influx but does not appear to be a signal in its own right. Also, the spike amplitude is only loosely related to the strength of Ca\textsuperscript{2+} influx, depending on the membrane permeability to other ions. Thus, a depolarization by increasing the extracellular K\textsuperscript{+} concentration or by injecting currents should not exceed values produced by \( K_{\text{ATP}} \) channel closure (20–25 mV), if physiological mechanisms of stimulus secretion coupling are to be investigated. Conversely, a depolarization by 40 mM K\textsuperscript{+} (28, 31) or by an electrical depolarization from −70 to 0 mV (14) may elicit effects not normally produced by nutrient stimuli.

ACKNOWLEDGMENTS

The skilful technical assistance by Angela Hahlbom, Verena Lier-Glaubitz, and Sabine Warmbold is gratefully acknowledged.

GRANTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Ru 368/5-1), the Deutsche Diabetes Gesellschaft, and the Deutsche Diabetes Stiftung.

REFERENCES