Thiopental-induced insulin secretion via activation of IP3-sensitive calcium stores in rat pancreatic β-cells

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Thiopental-induced insulin secretion via activation of IP3-sensitive calcium stores in rat pancreatic β-cells. Am J Physiol Cell Physiol 302: C796–C803, 2012. First published November 30, 2011; doi:10.1152/ajpcell.00081.2011.–While glucose-stimulated insulin secretion depends on Ca2+ influx through voltage-gated Ca2+ channels in the cell membrane of the pancreatic β-cell, there is also ample evidence for an important role of intracellular Ca2+ stores in insulin secretion, particularly in relation to drug stimuli. We report here that thiopental, a common anesthetic agent, triggers insulin secretion from the intact pancreas and primary cultured rat pancreatic β-cells. We investigated the underlying mechanisms by measurements of whole cell K+ currents, membrane potential, cytoplasmic Ca2+ concentration ([Ca2+]i), and membrane capacitance. Thiopental-induced insulin secretion was first detected by enzyme-linked immunoassay, then further assessed by membrane capacitance measurement, which revealed kinetics distinct from glucose-induced insulin secretion. The thiopental-induced secretion was independent of cell membrane depolarization and closure of ATP-sensitive potassium (KATP) channels. However, accompanied by the insulin secretion stimulated by thiopental, we recorded a significant intracellular [Ca2+]i increase that was not from Ca2+ influx across the cell membrane, but from intracellular Ca2+ stores. The thiopental-induced [Ca2+]i rise in β-cells was sensitive to thapsigargin, a blocker of the endoplasmic reticulum Ca2+ pump, as well as to heparin (0.1 μg/ml) and 2-aminoethoxydiphenyl borate (2-APB; 100 μM). Drugs that inhibit inositol 1,4,5-trisphosphate (IP3) binding to the IP3 receptor, and to U-73122, a phospholipase C inhibitor, but insensitive to ryanodine. Thapsigargin also diminished thiopental-induced insulin secretion. Thus, we conclude that thiopental-induced insulin secretion is mediated by activation of the intracellular IP3-sensitive Ca2+ store.

Inositol 1,4,5-trisphosphate

Thiopental is an ultra-short-acting barbiturate that is commonly used to induce anesthesia. Like other classes of barbiturates, thiopental plays an anesthetic role by directly binding to the GABAA receptor and enhancing its inhibitory action (3). However, besides direct interaction with the GABAA receptor, thiopental has numerous actions on neuronal tissues and muscles, such as the reduction of excitability by inhibition of voltage-dependent currents (15, 31, 35, 38). The effect of thiopental on ion fluxes is also regarded as important with respect to β-cell function, insulin secretion, and plasma glucose levels in vivo (2, 5, 10, 12, 17, 32).

The effects of thiopental on insulin secretion have long been debated, and the underlying mechanisms require further investigation. Decreased insulin secretion by thiopental has been demonstrated in a dog model and may be influenced by an α-adrenergic mechanism (32). On the contrary, thiopental increases insulin release from isolated rat islets and single pancreatic β-cells in the presence of low glucose levels (9, 10, 12, 17). Thiopental may also affect membrane K+ channels as well as insulin secretion. Goncalves et al. (10) found that thiopental inhibits K+ permeability by monitoring the 86Rb+ efflux rate from rat islets.

Intracellular calcium concentration ([Ca2+]i) plays a fundamental role in the regulation of pancreatic β-cell secretion (37). Two intracellular Ca2+ sources are Ca2+ influx from extracellular fluid and Ca2+ release from intracellular Ca2+ stores, such as the endoplasmic reticulum (ER). In general, glucose-elicited insulin secretion is mostly dependent on Ca2+ influx into the cytosol through Ca2+ channels in the plasma membrane. Besides glucose, neurotransmitters, hormones, and drugs can induce insulin secretion in certain contexts (21). For example, in response to vagal nerve stimulation, the released acetylcholine binds to muscarinic M3 receptors on the β-cell membrane, and activates phospholipase C (PLC) to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP3) (1). IP3 acts on specific Ca2+ channels (IP3 receptors) in the ER, causing release of Ca2+ (11, 12). In addition, rat pancreatic β-cells express ryanodine receptors, which also permeate Ca2+ from the ER to the cytosol (14). Although thiopental is known to increase [Ca2+]i in atrial cells and skeletal muscle cells (16, 33), the [Ca2+]i, signaling in β-cells stimulated by thiopental has not been fully explored.

In view of these observations, we investigated the effects of thiopental on insulin secretion by both enzyme-linked immunosorbent assay (ELISA) and membrane capacitance (Cm) measurements. We unambiguously demonstrated cell exocytosis by application of 0.3 mM thiopental. To further understand the underlying mechanism of thiopental-stimulated insulin secretion, we determined the effects of thiopental on whole cell K+ and Ca2+ currents, membrane potential, and [Ca2+]i. By calcium imaging, thiopental-induced [Ca2+]i rises were observed in Ca2+-deficient solution, but diminished when depleting the Ca2+ store or inhibiting IP3-sensitive Ca2+ release from the ER. Our results demonstrate that intracellular IP3-sensitive calcium...
store activation is required for thiopental-induced β-cell insulin secretion.

MATERIALS AND METHODS

Cell isolation and culture. Pancreatic islet β-cells from adult Wistar rats (150–250 g) were isolated and cultured as described previously (4, 26). Briefly, rats were killed by cervical dislocation, and islets were collected from the pancreas by collagenase digestion (3.3 mg/ml collagenase-V for 30 min). Single β-cells were isolated from islets by Dispase-II digestion (0.3 mg/ml for 12 min) and cultured in a standard CO2 incubator for up to 1 wk. β-Cells were identified by their response to glucose and by the presence of AP-sensitive potassium (KATP) channels (22). The animal protocols in this study were reviewed and approved by the Institutional Animal Care and Use Committees of Peking University and Shandong University.

The medium for cell culture contained 10 mg/ml DMEM, 17.72 mM H-HEPES, 7.28 mM Na-HEPES, 44 mM NaHCO3, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 0.6% vitamin C, and 10% fetal bovine serum. The standard bath solution contained (in mM) 138 NaCl, 5.6 KCl, 1.2 MgCl2, 2.6 CaCl2, 3 glucose, and 5 HEPES (pH 7.4). The 0 Ca2+ bath solution was the same except that CaCl2 was removed and 2 mM EGTA was added. To remove K+ currents from the voltage-gated inward currents, 20 mM NaCl in the standard bath solution was replaced by 20 mM TEA-Cl. In some experiments, Ca2+ in the standard bath was replaced by equimolar Ba2+. The standard internal solution contained (in mM) 135 KCl, 10 NaCl, 1.0 MgCl2, 3 MgATP, and 5 HEPES (pH 7.2). For Ca2+ channel recordings, 135 mM KCl in the pipette solution was replaced by equimolar CsCl. DMEM, Dispase-II, fetal bovine serum, and BSA were from GIBCO. Fura-2/AM was from Molecular Probes. Thiopental was from Shanghai New Asiatric Pharmaceutical. All other chemicals were from Sigma.

Insulin secretion measurement by enzyme immunoassay. The rat pancreas was perfused from the abdominal aorta at the level of the celiac artery with modified Krebs-Ringer-bicarbonate buffer (KRBB; in mM: 5 KCl, 120 NaCl, 15 HEPES, pH 7.4, 24 NaHCO3, 1 MgCl2, 2 CaCl2, and 1 mg/ml BSA), and samples were collected from the hepatic portal vein via a cannula. The perfusing fluid was supplied by a pump and the perfusion rate was 1 ml/min. To measure the insulin levels, samples were collected after 30 min preperfusion of the pancreas with KRBB and subjected to a rat/mouse insulin ELISA kit (Millipore, St. Charles, MO).

Primary pancreatic β-cells digested with Dispase-II were cultured in 24-well plates for 2–5 days. β-Cells were washed with KRBB and stimulated with 0.3 mM thiopental or 20 mM glucose prepared in KRBB solution around the cell under study from a given application, provided the application speed was set to 100 μl/min or faster. The pharmacological experiments were set to meet this requirement (34).

Effect of thiopental on insulin secretion. Thiopental stimulates insulin secretion. The direct effects of thiopental on insulin secretion were measured in the intact pancreas. Thiopental induced significant insulin release from the intact pancreas (Fig. 1A, n = 6) at about two-thirds the level of glucose-induced insulin secretion.

To further understand the properties and kinetics of thiopental-induced insulin secretion, we separated rat primary pancreatic β-cells and measured the insulin secretion by both ELISA and membrane capacitance (Cm) (6). In KRBB solution without glucose, application of 0.3 mM thiopental induced insulin secretion similar to the effect of 20 mM glucose (Fig. 1B).

To monitor the thiopental-stimulated insulin secretion kinetics at a high time resolution, we used patch-clamp technique to measure Cm (change) (6), which has a linear relation to the change in the area of plasma membrane as a result of vesicular exocytosis or endocytosis.

When a single β-cell was perforated and whole cell voltage-clamped at −70 mV, 0.3 mM thiopental induced a gradual increase in Cm while depolarization evoked an instantaneous jump in Cm (Fig. 1C, top trace). The vesicles in pancreatic β-cells have an average diameter of 0.45 μm (29), which corresponds to a 2-fF change (6), which has a linear relation to the change in the area of plasma membrane as a result of vesicular exocytosis or endocytosis.

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Effect of thiopental on ion channels and cell membrane. To explore the mechanism underlying thiopental-stimulated insulin secretion, we next determined the effect of thiopental on membrane Ca2+ channels and β-cell membrane depolarization, which are key mediators of insulin secretion. The effects of thiopental on Ca2+ channels, K+ channels, and membrane potential in β-cells at basal (3 mM) glucose concentration are shown in Fig. 2. In cells voltage-clamped at −70 mV, 0.3 mM thiopental markedly suppressed the Ca2+ currents induced by depolarization to 20 mV (Fig. 2A), and the suppression was recovered after washing. We used Ba2+ as a carrier to monitor the Ca2+ currents here. By comparing peak current amplitude under conditions of puffing thiopental with control, we found that, with depolarizations to 20 mV, 0.3 mM thiopental re-
duced whole cell inward currents to 60%, suggesting an inactivation of the Ca\(^{2+}\) channels.

A similar suppression of K\(^{+}\) currents was found with application of thiopental. The inhibition of whole cell K\(^{+}\) currents was 40% (Fig. 2B). This inhibition of K\(^{+}\) channels is consistent with previous reports (10, 16).

Because closure of K\(_{ATP}\) channels is coupled to insulin secretion from \(\beta\)-cells, we investigated the effect of thiopental on K\(_{ATP}\) channels. Using the cell-attached configuration, a typical burst of K\(_{ATP}\) channel currents was seen when the pipette potential was held at 0 mV. As expected, 0.2 mM tolbutamide significantly inhibited the K\(_{ATP}\) channels while 0.3 mM thiopental only partially suppressed them to 80% (Fig. 2C). These results indicate that thiopental does not inhibit K\(_{ATP}\) channels.

To determine whether thiopental induces cell depolarization, we transferred to the current-clamp method and puffed 40 mM KCl and 0.3 mM thiopental sequentially onto a single cell (Fig. 2D). The high concentration of KCl induced depolarization to −40 mV in all cells, while thiopental slightly depolarized 20% of the cells to −50 mV. In most of the cells, 0.3 mM thiopental did not depolarize the cell membrane, and even caused hyperpolarization in some cells, which suggests that thiopental barely evokes voltage-dependent Ca\(^{2+}\) influx.
the whole cell configuration, we readily detected a $C_{m}$ increase when puffing 0.3 mM thiopental for 30 s, while $[Ca^{2+}]_{i}$ also increased significantly. These observations indicate that thiopental-induced exocytosis is accompanied by an increase in $[Ca^{2+}]_{i}$.

Thiopental induces insulin secretion through ER calcium store release. To identify the source of the thiopental-induced $[Ca^{2+}]_{i}$ increase, we examined the effects of extracellular $[Ca^{2+}]_{i}$ and selective calcium store antagonists on thiopental responses.

Removal of extracellular $Ca^{2+}$ did not affect the thiopental-induced $[Ca^{2+}]_{i}$ increase in β-cells (Fig. 4A). The resulting amplitude was similar to that in the presence of extracellular $Ca^{2+}$ and comparable to the methacholine (MCh)-induced $[Ca^{2+}]_{i}$ increase. These observations indicate that thiopental-induced $Ca^{2+}$ release was from an intracellular $Ca^{2+}$ store, which provided an explanation for thiopental-triggered insulin secretion despite the inhibition of cell membrane $Ca^{2+}$ channels. Next, we used pharmacological antagonists to determine the intracellular calcium store on which thiopental acts. First we used thapsigargin, a blocker of the $Ca^{2+}$ pump in the ER by irreversible inhibition of Ca-ATPase. In a $Ca^{2+}$-containing bath, thiopental induced a $[Ca^{2+}]_{i}$ increase repeatedly, while after puffing 1 μM thapsigargin for 10 min, the effect of thiopental on $[Ca^{2+}]_{i}$ was completely blocked (Fig. 4B). Puffing 40 mM KCl at the end of experiments induced a $[Ca^{2+}]_{i}$...
THIOPENTAL INDUCES INSULIN SECRETION

Insulin secretion was triggered by Ca\textsuperscript{2+} increase and cell membrane capacitance rise. Combined measurements show that TP induced a \( \Delta C_m \) with a detectable change in \([\text{Ca}^{2+}]_i\). TP still induced a \([\text{Ca}^{2+}]_i\) increase when the cell was voltage-clamped, and TP induced a \( \Delta C_m \) synchronous with \([\text{Ca}^{2+}]_i\) store release (n = 3).

Fig. 3. TP induces both transient cytoplasmic \([\text{Ca}^{2+}]_i\) increase and cell membrane capacitance rise. Combined \( C_m \) and \([\text{Ca}^{2+}]_i\), measurements show that TP induced a \( \Delta C_m \) with a detectable change in \([\text{Ca}^{2+}]_i\). TP still induced a \([\text{Ca}^{2+}]_i\) increase when the cell was voltage-clamped, and TP induced a \( \Delta C_m \) synchronous with \([\text{Ca}^{2+}]_i\) store release (n = 3).

Effect of thiopental on plasma membrane. Studies of thiopental effects on insulin secretion have yielded conflicting results and interpretations. Our results showed that thiopental acts on \( \beta \)-cells by affecting both ion channels and intracellular \([\text{Ca}^{2+}]_i\) stores. The intracellular \([\text{Ca}^{2+}]_i\) store is important in thiopental-induced insulin secretion, which was blocked by application of thapsigargin. Early investigations focused on ion channels on the plasma membrane, especially K\textsuperscript{+} channels (10, 17). These studies indicated that the depression of K\textsuperscript{+} currents by thiopental is responsible for insulin secretion. Though we confirmed the inhibition of whole cell K\textsuperscript{+} channels, we recorded only a weak inhibition of K\textsubscript{ATP} currents and did not record membrane potential depolarization to above \(-40\) mV with application of thiopental. Here we used primary cultured rat pancreatic \( \beta \)-cells, which are different from the CRI-insulinoma cell line used by Kozlowski and Ashford (17). The observed discrepancy is probably due to the cell source difference (23). As well as K\textsubscript{ATP} channels, Kv1.4 (A-current)

increase, which demonstrated that the fura-2 concentration used in the experiment had enough buffering capacity and that fura-2 was sensitive to \([\text{Ca}^{2+}]_i\) change. This result indicates that the thiopental-induced \([\text{Ca}^{2+}]_i\) is largely from ER \([\text{Ca}^{2+}]_i\) stores.

To monitor the contribution of intracellular calcium stores to thiopental-induced insulin release, we measured primary cell insulin secretion with or without thapsigargin and found that thapsigargin totally blocked thiopental-induced insulin secretion (Fig. 4C). This result suggests that the thiopental-induced insulin secretion was triggered by \([\text{Ca}^{2+}]_i\) through ER calcium store release.

\( IP_3 \)-sensitive \([\text{Ca}^{2+}]_i\) increase. The ER \([\text{Ca}^{2+}]_i\) stores include both \( IP_3 \) receptors and ryanodine receptors. Application of either antagonist of the \( IP_3 \) receptor, heparin (0.2 mg/ml) or 2-aminoethoxydiphenyl borate (2-APB; 100 \( \mu \)M), inhibited the effect of thiopental by 90\% (Fig. 5). Consistently, when we prepuffed the PLC antagonist U-73122 (2 \( \mu \)M) to inhibit the PLC/IP3 pathway, both MCh-induced and thiopental-induced \([\text{Ca}^{2+}]_i\) signals were completely blocked, while the caffeine-induced ryanodine-sensitive \([\text{Ca}^{2+}]_i\) increase was slightly diminished (Fig. 6, A and B).

The above experiments demonstrated that the \( IP_3 \)-sensitive calcium store contributed to the thiopental-induced \([\text{Ca}^{2+}]_i\) increase. We then analyzed the effects of ryanodine on the thiopental response. In a single cell, application of MCh, caffeine, or thiopental elevated the calcium levels (Fig. 6C). After application of 100 \( \mu \)M ryanodine, the caffeine-induced \([\text{Ca}^{2+}]_i\), elevation was almost totally blocked while the MCh- or thiopental-induced calcium increase persisted. These results suggest that an \( IP_3 \)-sensitive \([\text{Ca}^{2+}]_i\) store, rather than a ryanodine-sensitive calcium store, is responsible for thiopental-induced insulin secretion.

**DISCUSSION**

Here, we investigated the direct effect of thiopental on rat pancreatic \( \beta \)-cells and its underlying mechanism. We revealed that thiopental induced insulin secretion with a slow and rising kinetics, which was dependent on \([\text{Ca}^{2+}]_i\) release from intracellular \([\text{Ca}^{2+}]_i\) stores.

Fig. 4. TP-induced calcium signal is largely from calcium store. A: in \([\text{Ca}^{2+}]_i\)-deficient bath, TP induced a \([\text{Ca}^{2+}]_i\) increase (n = 30). B: puffing 1 \( \mu \)M thapsigargin (Tg) for 10 min inhibited the TP-induced \([\text{Ca}^{2+}]_i\) transient, while a high KCl (40 mM)-evoked \([\text{Ca}^{2+}]_i\) transient was detectable (n = 5). C: pretreatment with 1 \( \mu \)M Tg for 10 min inhibited the TP-induced insulin secretion. **P < 0.001 compared with 0 mM glucose; #P < 0.001 compared with 0.3 mM TP.
and Kv2.1 (delayed rectifier) channels also reside in rat pancreatic β-cells but do not open at the resting potential (19, 20). The Kv1.4 and Kv2.1 channels may be inhibited by thiopental upon depolarization. In our studies, thiopental stimulated insulin secretion when the membrane potential was clamped at −70 mV. These results demonstrate that inhibition of whole cell K⁺ channels by thiopental is not important in the mediation of thiopental-stimulated insulin secretion in basal glucose conditions.

Role of calcium stores in thiopental-induced secretion. Two fundamental pathways contribute to [Ca²⁺]i increases: one is Ca²⁺ influx through voltage-dependent calcium channels (VDCCs), and the other is Ca²⁺ release from intracellular Ca²⁺ stores, such as IP₃ receptors and ryanodine receptors. For example, the principal Ca²⁺ signal in nerve terminals, controlling transmitter release, is Ca²⁺ influx through VDCCs (40), while in pancreatic acinar cells, secretion of digestive enzymes is controlled by Ca²⁺ release from the ER mediated by IP₃ and other messengers (24). In β-cells, Ca²⁺ release from the ER is important (34), but more attention has been paid to extracellular Ca²⁺ influx through VDCCs (7, 28, 30). While calcium influx has been shown to influence insulin secretion (8, 28), activation of intracellular Ca²⁺ stores by appropriate secretagogues also contributes to insulin secretion in β-cells. By recording Ca²⁺ images and Cm simultaneously, we were able to study the function of Ca²⁺ stores during cell secretion. Our current study revealed that thiopental stimulated considerable secretion via a significant elevation of [Ca²⁺].

In our studies, thiopental induced a transient [Ca²⁺]i increase in extracellular Ca²⁺-deficient solution, suggesting that intracellular Ca²⁺ stores mediate the effect of thiopental on pancreatic β-cells. Meanwhile, thapsigargin abolished both thiopental-induced insulin secretion and Ca²⁺ signals by blocking the Ca²⁺ pump in the ER. In further studies of the intracellular Ca²⁺ signals, we showed that thiopental induced IP₃-sensitive Ca²⁺ release, but not ryanodine-sensitive Ca²⁺ release. These results demonstrate that the ER, especially the IP₃ receptor in the ER, plays an essential role when β-cells are exposed to thiopental in basal glucose or in resting conditions.
Implication of clinical thiopental application. Thiopental is a short-term intravenous anesthetic in the induction of anesthesia, to stop seizures, and to treat status epilepticus, particularly in children (27). It has been reported that thiopental may disturb glucose metabolism in dogs, but the mechanism is unknown (32). In the present work, direct application of thiopental to the rat intact pancreas or cultured pancreatic β-cells promoted insulin secretion through a K<sub>ATP</sub> channel-independent, but IP<sub>3</sub> calcium store-dependent, mechanism. Remarkably, the insulin secretion in response to thiopental was comparable to glucose-induced secretion. Thus, like another antiepileptic drug, valproic acid (25), thiopental treatment might increase fasting serum insulin. Our results suggest caution when using thiopental with epileptic patients, especially in fasting conditions such as hypoglycemic seizures.

The PLC inhibitor U-73122 blocks thiopental-induced intracellular calcium increase, suggesting that thiopental may work on IP<sub>3</sub> production upstream, most likely a G<sub>2/P</sub>-coupled seven transmembrane receptor. Future work is needed to determine the molecular targets of thiopental on the IP<sub>3</sub> pathway and to develop a thiopental derivative for drug discovery in diabetes.

Conclusions. Our results revealed that thiopental induced insulin secretion from isolated resting pancreatic β-cells by initiating Ca<sup>2+</sup> release from intracellular IP<sub>3</sub>/Ca<sup>2+</sup> stores, rather than by triggering it from cell membrane depolarization.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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