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The potent insulin secretagogue effect of betulinic acid is mediated by potassium and chloride channels

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ABSTRACT

Betulinic acid (BA) has been described as an insulin secretagogue which may explain its potent anti-hyperglycemic effect; however, the exact role of BA as an insulinogenic agent is not clear. The aim of this study was to investigate the mechanism of BA on calcium influx and static insulin secretion in pancreatic islets isolated from euglycemic rats. We found that BA triggers calcium influx by a mechanism dependent on ATP-dependent potassium channels and L-type voltage-dependent calcium channels. Additionally, the voltage-dependent and calcium-dependent chloride channels are also involved in the mechanism of BA, probably due to an indirect stimulation of calcium entry and increased intracellular calcium. Additionally, the downstream activation of PKC, which is necessary for the effect of BA on calcium influx, is involved in the full stimulatory response of the triterpene. BA stimulated the static secretion of insulin in pancreatic islets, indicating that the abrupt calcium influx may be a key step in its secretagogue effect. As such, BA stimulates insulin secretion through the activation of electrophysiological mechanisms, such as the closure of potassium channels and opening of calcium and chloride channels, inducing cellular depolarization associated with metabolic-biochemical effects, in turn activating PKC and ensuring the secretion of insulin.

1. Introduction

Terpenes are isoprenoids formed by six units of isoprene $(C_5H_8)_6$, derived from mevalonic acid [1]. Some pentacyclic triterpenes, such as oleanolic acid, are known to exhibit biological actions, especially in glucose homeostasis, reducing glycemia and acting as an antidiabetic agent, increasing serum insulin and glucose transporter-4 (GLUT4) translocation [2,3]. However, the mechanisms of action of most triterpenes in insulin secretion are not completely elucidated; specifically, betulinic acid (BA) has potent insulin-secreting and antihyperglycemic effects that are mediated by unknown mechanisms [4]. The ionic channels are important targets of action for these compounds in the process of insulin secretion. The classical pathway of glucose-induced insulin secretion is mediated by the ATP-dependent potassium channels (K_{ATP}) and voltage-dependent calcium channels (VDCCs). The increase in calcium influx through the VDCCs leads to an abrupt increase in cytoplasmic calcium concentration, which in turn activates additional effector systems responsible for the exocytosis of insulin granules [5]. Among these effectors are proteins kinases and other ionic channels, such as chloride channels [6,7]. The potassium, calcium and chloride channels are of great physiological and cellular importance, since they associate biochemical metabolism and cellular electrical activity [8].

The various types of chloride channels present differential distributions and functions [9]; of these, the voltage-dependent chloride channels (ClC-3 channel) are suggested to have a critical role in vesicle acidification, due to the presence of a protonic type V electrogenic pump (H^+-ATPase). This effect is important for the vesicular traffic caused by the proton electrochemical gradient [10] and essential for the process of insulin maturation and secretion in response to electrical changes and activation following the stimulus of a ligand to a specific receptor [11].

Another important subgroup of the chloride channel family that can be associated with insulin secretion are the calcium-activated chloride channels (CaCCs), which are found at the plasma membrane of various

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tissues. The opening of these channels leads to depolarization of the cell membrane, followed by opening of VDCCs. CaCCs stimulators are still poorly understood, although it is known that these channels can be activated by increasing the intracellular calcium concentration in response to an agonist after the release of calcium from internal stores and by kinases such as protein kinase calcium/calmodulin-dependent (PKCaMKII) [12]. Due to their cellular depolarization effects, these channels may be involved in vesicular exocytosis [13] and may participate in the process of insulin release.

Diabetes mellitus and insulin resistance are disorders that require careful and controlled care considering the use of specific medications, such as the administration of exogenous insulin (type 1 diabetes) or oral hypoglycemic agents (type 2 diabetes and/or insulin resistance) that stimulate insulin secretion [14]. However, as already described, chronic treatment with oral hypoglycemic agents has adverse effects, such as the chronic use of sulfonylureas, the most commonly administered drug, which can lead to eventual β-cell failure and reduced insulin secretion [15]. Therefore, alternatives such as the use of new exogenous substances, such as compounds from medicinal plants and metabolites of natural compounds, have become an increasingly widespread practice. However, such treatments may be harmful to the patient due to the lack of scientific evidence of the action and mechanism of action of these substances. As BA is a potent insulin secretagogue and an insulinomimetic compound, the aim of the present study was to investigate the intracellular pathways involved in its effects on insulin secretion and on calcium influx in pancreatic islets.

2. Materials and methods

2.1. Materials

Colagenase type V, nifedipine, diazoxide, stearoylcarnitine chloride (ST), 9-anthracenecarboxylic acid, and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) were obtained from Sigma-Aldrich (St. Louis, MO, USA) (Table 1). The ELISA kit for quantitative determination of rat insulin (catalog no. EZRMI-13K) was obtained from Millipore (St Charles, MO, USA). [$^{45}Ca^{2+}$] $CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}), [U−^{14}C]-2-deoxy-d-glucose (^{14}C-DG),sp. act. 9.25 GBq/mmol and biodegradable scintillation liquid were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA, USA).

2.2. Isolation and chemical characterization of betulinic acid

BA was isolated and characterized as described [4]. *Rosmarinus officinalis* L. was collected in Santo Amaro da Imperatriz, Santa Catarina, Brazil and identified by Dr. Daniel de Barcelos Falkenberg (Department of Botany, UFSC). A voucher of the specimen (No. 34,918) was deposited in the herbarium of the Department of Botany, UFSC, Santa Catarina, Brazil. The hydroalcoholic extract of aerial parts of *R. officinalis* L. was obtained as described by Ref. [16]. BA was identified by IR, MS and NMR analysis.

2.3. Animals

Male Wistar rats, aged 50–55 days (180–210 g), were used. Rats were bred in the animal facility and housed in an air-conditioned room (21 ± 2 °C) with controlled lighting (lights on from 6:00 a.m. to 6:00 p.m.). The animals were kept with food (Nuvital, Nuvilab CR1, protein 22%, gross energy 3976 kcal/kg, Curitiba, PR, Brazil) and water *ad libitum*. All animals were carefully monitored and maintained in accordance with the local Ethics Committee for Animal Use (CEUA-Protocol UFSC PP00414).

2.4. Pancreatic islet isolation

The rat pancreas was accessed through a central abdominal incision. The bile duct was obstructed in the duodenal portion (after pancreatic duct) and cannulated at the beginning of the bile duct. Krebs Ringer-bicarbonate buffer (KRb), supplemented with 8 mM HEPES (KRb-HEPES), was introduced through the bile duct by a syringe until the pancreas became clearly distended. The pancreas was removed and then cut into small slices in Petri dishes containing KRb-HEPES. The tissue was incubated in KRb-HEPES supplemented with collagenase (3 mg/mL). After incubation, the mixture was transferred to a conical tube, resuspended in 10 mL of collagenase-free medium and centrifuged at room temperature for 3 min at 45 x g in an Excelsa Baby Centrifuge (model 206, FANEM, São Paulo, SP, Brazil). The supernatant was discarded and the pellet resuspended in fresh KRb-HEPES. This washing procedure was repeated five times. Aliquots (100 µL) of the final pellet (containing isolated islets) were transferred to Eppendorf tubes with the KRb-HEPES incubation medium [17,18].

2.5. Studies of $^{45}Ca^{2+}$ influx

The isolated islets were preincubated for 60 min in a Dubnoff metabolic incubator in KRb-HEPES buffer containing 5.6 mM glucose and 0.1 µCi/mL $^{45}Ca^{2+}$ at 37 °C, pH 7.4 and aerated with O_2: CO_2 (95%: 5%; v/v) in order to balance the intra- and extracellular concentrations of $^{45}Ca^{2+}$. The islets were incubated in the absence (basal) or presence of BA in KRb-HEPES. In some assays a blocker, channel activator or protein kinase inhibitor was added during the last 15 min of incubation, prior to treatment, and maintained throughout the incubation period. The following drugs were used: 250 µM diazoxide, 20 µM glibenclamide [19], 1 µM nifedipine [19,20], 200 µM DIDS [21,22], 1 µM 9-AC [23] and 0.1 µM stearoylcarnitine (ST) [23]. The incubation was stopped (1 mL with lanthanum chloride) using the technique described by Ref. [24] with modifications [19]. As such, the islets were added to 0.5 M NaOH and subjected to alkaline digestion (100 °C). Aliquots of 50 µL from each sample were used for the quantification of radioactivity in a liquid scintillation Optiphase Hisafe III (Wallac Oy, Turku, Finland) using a spectrometer of beta radiation (Model LS 6500; multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA) and aliquots of 5 µL were used for quantification of protein by Lowry's Method [25].

2.6. ^{14}C-deoxyglucose uptake studies in pancreatic islets

Isolated pancreatic islets from rats were assayed for their 2-deoxy-D-glucose (^{14}C-DG) uptake studies. The islets were pre-incubated (30 min) and incubated (15 min) in KRb-HEPES (37 °C, pH 7.4, 5.6 mM glucose) bubbled with O_2/CO_2 (95%:5%, v/v). ^{14}C-DG (0.1 µCi/mL; 0.12 nM)

Table 1
Compounds, abbreviation and action of each agonist/antagonist used in this study.

Compound	Abbreviation	Action/Reference
4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid	DIDS	CLC Chloride Channel Inhibitor [24,25]
9-anthracenecarboxylic acid	9-AC	Calcium-activated chloride current inhibitor [26]
Diazoxide	Diazo	ATP-sensitive potassium channel activator [22]
Glibenclamide	Glibe	Inhibitor of ATP-sensitive K+ channels [22]
Nifedipine	Nife	L-type voltage-dependent Ca^{2+} channels blocker [22,23]
Stearoylcarnitine chloride	ST	PKC inhibitor [26]