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The role of calcium in intracellular pathways of rutin in rat pancreatic islets: potential insulin secretagogue effect

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ABSTRACT

Rutin is a flavonol glycoside with multiple biological activities and it has been demonstrated that rutin modulates glucose homeostasis. In pancreatic β-cell, an increase in intracellular calcium concentration triggers exocytosis and thus insulin secretion. The aim of the study reported herein was to investigate the effect of rutin associated intracellular pathways on Ca^{2+} uptake in isolated rat pancreatic islets. We focused on the acute effects of rutin on *in vivo* insulin secretion and the *in vitro* cellular signaling of pancreatic islets related to this effect. The results show that rutin significantly increased glucose-induced insulin secretion in an *in vivo* treatment. Moreover, it was demonstrated that rutin stimulated Ca^{2+} uptake after 10 min of incubation compared with the respective control group. The involvement of L-type voltage-dependent Ca^{2+} channels (L-VDCCs) was evidenced using nifedipine, while the use of glibenclamide and diazoxide demonstrated that the ATP-sensitive potassium (K_{ATP}) channels are not involved in the rutin action in pancreatic islets. In conclusion, rutin diminish glycemia, potentiate insulin secretion *in vivo* and significantly stimulates Ca^{2+} uptake in rat pancreatic islets. A novel cellular mechanism of action of rutin in Ca^{2+} uptake on pancreatic β-cells was elucidated. Rutin modulates Ca^{2+} uptake in pancreatic islets by opening L-VDCCs, alter intracellular Ca^{2+}, PLC and PKC signaling pathways, characterizing K_{ATP} channel-independent pathways. These findings highlight rutin, a dietary adjuvant, as a potential insulin secretagogue contributing to glucose homeostasis.

1. Introduction

Diabetes mellitus is characterized by deranged metabolism and inappropriate hyperglycemia, resulting from defects in the secretion and cellular action of insulin. Treatments aimed at enhancing β-cell function and reducing insulin resistance are therefore key to improving metabolic control and retarding the development of diabetic complications (American Diabetes Association, 2011).

Insulin is the key regulator of glucose uptake in the fed state and is secreted from β-cells located in the islets of Langerhans in the pancreas. Glucose is the primary stimulus for insulin secretion, although there are many other metabolic, endocrine, and neural control mechanisms (Beardsall et al., 2003). Exposure of the pancreatic β-cell to stimulatory glucose concentrations leads to the activation of a cascade of reactions, which ends in the release of stored insulin. Metabolism of glucose in glycolysis and the Krebs cycle results in the generation of ATP. The resulting increase in the cytosolic ATP/ADP-ratio closes ATP-sensitive potassium (K_{ATP}) channels in the plasma membrane, which leads to depolarization of the cell and influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (L-VDCCs). The increase in Ca^{2+} concentration triggers exocytosis and thus insulin secretion (for review Henquin, 2004).

Different mechanisms of action have been reported for therapeutic drugs used in the treatment of diabetes (Sharif, 2011). Some pharmacological agents with insulinotropic properties can directly and positively modulate insulin release (Doyle and Egan, 2003). Recent studies have explored the insulinotropic effects of natural products (Pinent et al., 2008). Several beneficial effects have been reported for flavonoids and published data suggest that there might be direct effects of flavonoids on insulin secretion (Cazarolli et al., 2008a,b; Folador et al., 2010).

Rutin (quercetin-3-*O*-rutinoside) is a flavonol glycoside composed of quercetin and the disaccharide rutinose. Many studies have demonstrated that rutin is a pharmacologically active phytochemical which exhibits multiple biological activities

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(Cazarolli et al., 2008a,b; Pereira et al., 2011). It has also been demonstrated that rutin modulates glucose homeostasis. Rutin can decrease glycemia, increase insulin secretion and inhibit α-glucosidase (Fernandes et al., 2010; Pereira et al., 2011). Although the ability of rutin to improve diabetic status has been reported, studies to determine the exact mechanism of action involved in the regulation of glucose homeostasis are scarce. Thus, it was investigated the *in vivo* effect of rutin on glycemia and insulin secretion as well as the intracellular pathways on Ca^{2+} uptake in isolated pancreatic islets.

2. Materials and methods

2.1. Chemicals

Collagenase Type V, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), (bisindoylmaleimidine IX, 2-{1-[3-(amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl) maleimide methanesulfonate salt (RO-318220), 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), rutin, nifedipine, diazoxide, glibenclamide and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{45}Ca]$CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}) and Optiphase Hisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Massachusetts, USA). All other chemicals were of analytical grade. Enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of rat insulin (catalog no. EZRMI-13K) was purchased from Millipore (St Charles, MO, USA).

2.2. Animals

The male *Wistar* rats (190–220 g) used in this study were bred in our animal facility and housed in an air-conditioned room (approximately 22 °C) with controlled lighting on a 12:12 h light/dark cycle (lights on from 06:00 to 18:00 h). The animals were maintained with pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil), while tap water was available *ad libitum*. In the *in vivo* treatments rats were deprived of food for at least 16 h but allowed free access to water. In the *in vitro* experiments the animals were not left in fasting. All the animals were monitored and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation. This study was approved by the Committee for Ethics in Animal Research of UFSC (Protocol CEUA PP00398).

2.3. Oral glucose tolerance curve

Fasted rats were divided in groups of four animals for each treatment. Hyperglycemic Group (as control), normal rats that received glucose (4 g/kg; 8.9 M p.o. gavage); Glipizide group, hyperglycemic rats that received glipizide (10 mg/kg; 0.01 M p.o. gavage); Rutin group, hyperglycemic rats that received rutin (50 mg/kg; 0.04 M p.o. gavage). The glucose and insulin levels were measured at zero time (fasted rats). All groups were loaded with glucose 30 min after rutin or glipizide treatment. So, glycemia and insulin levels were determined at 15, 30, 60 min after glucose overload (Folador et al., 2010).

2.4. Insulin serum measurements

The insulin levels were determined at 450 nm with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve. Samples were analyzed in duplicate and results were expressed as ng per ml of insulin serum. The incremental areas under the response curves (AUCs) were calculated. The insulinogenic index (II) was calculated as the ratio between the $AUC_{insulin}$ and $AUC_{glucose}$ (from zero to 60 min) (Frederico et al., 2012).

2.5. Rat islet isolation and Ca^{2+} uptake experiments

Islets were isolated by collagenase digestion as previously described by Lacy and Kostianovsky (1967), with minor modifications (Frederico et al., 2012). The islets were then incubated for 10 min in KRb–HEPES buffer containing 0.1 μCi/ml $^{45}Ca^{2+}$ at 37 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v) without (control) or with rutin. In some experiments channel blockers or kinase inhibitors were added during the last 15 min before the treatment and maintained during the entire incubation period (see figure legends). The following drugs were used: glibenclamide (60 μM), diazoxide (100 μM), nifedipine (1 μM), U-73122 (1 μM) (Frederico et al., 2012), BAPTA-AM (50 μM), RO 31-8220 (20 μM), (Zanatta et al., 2011). Cold buffer with lanthanum chloride (10 mM) was added to the samples at the end of incubation in order to stop Ca^{2+} fluxes (Batra and Sjögren, 1983). Aliquots were taken from each sample for radioactivity measurement in scintillation liquid in an LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA) and were used for protein quantification by the Lowry method (1951).

2.6. Data and statistical analysis

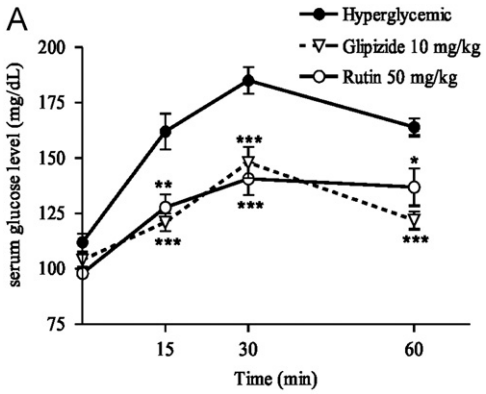
Data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) was carried out followed by the Bonferroni *post hoc* or unpaired Student's *t*-test to determine the significance of differences between groups. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Effect of rutin on oral glucose tolerance curve

Fig. 1A shows the acute effect of rutin on serum glucose levels. Fifteen minutes after the glucose loading the glycemia was significantly increased when compared with zero time on the hyperglycemic control group. The sulfonylurea glipizide (10 mg/kg), an oral hypoglycemic agent, was used as a positive control and produced a typical serum glucose lowering during all periods analyzed (from 15 to 60 min) compared to the hyperglycemic group. With the oral administration of rutin (50 mg/kg) the serum glucose levels were also significantly reduced at 15, 30 and 60 min around 21, 24 and 17% respectively in hyperglycemic rats.

Serum insulin levels in fasted rats were determined after an oral glucose loading (4 g/kg) as shown in Fig. 1B. As expected, a sulfonylurea agent, glipizide, stimulated insulin secretion by 295, 152 and 191% at 15, 30 and 60 min after glucose loading, respectively, compared to the hyperglycemic control group. The rutin (50 mg/kg) potentiated insulin secretion induced by glucose about 155% at 15 min after glucose loading. The *in vivo* treatment with rutin resulted in 1.5-fold increase in II (0.64 ng/mg), compared with hyperglycemic control group (0.44 ng/mg) (Fig. 1C)



C

	Control	Rutin 50 mg/kg	Glipizide 10 mg/kg
II	0.44	0.64	1.07

Fig. 1. Acute effect of rutin on (A) serum glucose levels, (B) insulin levels, and (C) insulinogenic index (II; ng/mg). Values are expressed as mean ± S.E.M.; $n=4$ in duplicate for each treatment. Statistically significant difference compared to the corresponding hyperglycemic group, $^{***}P<0.001$; $^{**}P<0.01$ and $^{*}P<0.05$.

3.2. Mechanism of action of rutin associated with Ca^{2+} uptake in isolated rat pancreatic islets

Calcium is well known to be involved in insulin secretion by β-cells. Thus, in order to study the mechanism of action of rutin on insulin secretion, *in vitro* studies were carried out. After the Ca^{2+} equilibrium obtained through 60 min of islet incubation with $^{45}Ca^{2+}$, Ca^{2+} uptake was then monitored for 1, 5 and 10 min without stimuli (control) and in the presence of rutin (10^{-14} M), in isolated pancreatic islets. The treatment with rutin augmented the Ca^{2+} uptake after 10 min (58%). Although the ascendant Ca^{2+} uptake profile observed on Fig. 2, no significant effect of rutin on Ca^{2+} uptake was detected at 1 and 5 min.

To study the involvement of K_{ATP} channels in the stimulatory effect of rutin on Ca^{2+} uptake, glibenclamide a K^+ channel blocker and, diazoxide a K^+ channel activator, were used. As expected, the treatment with glibenclamide significantly increased the Ca^{2+} uptake in isolated pancreatic islets after 10 min. Also, in the presence of glibenclamide an additional effect on Ca^{2+} uptake stimulated by rutin was observed (Fig. 3A). Furthermore, Fig. 3B shows that diazoxide significantly decreased the Ca^{2+} uptake. On the other hand, the presence of diazoxide did not influence the stimulatory effect of rutin on Ca^{2+} uptake in isolated pancreatic islets (Fig. 3B).

In addition to verify the participation of L-VDCCs in the mechanism involved in the stimulatory effect of rutin on $^{45}Ca^{2+}$ uptake, nifedipine was used. Fig. 4A shows that nifedipine did not change the basal level of Ca^{2+} uptake. However, nifedipine inhibited the effect of rutin.

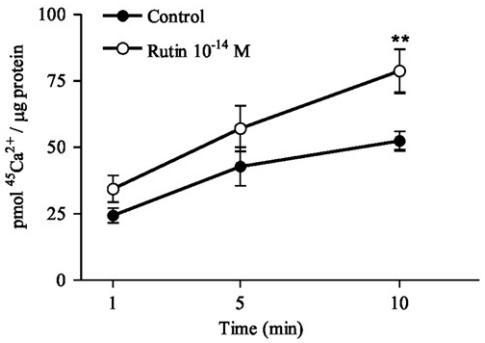


Fig. 2. Time-course of rutin effect on $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min in the presence of 0.1 µCi/ml of $^{45}Ca^{2+}$. The pancreatic islets were incubated with/without 10^{-14} M rutin for 1, 5 and 10 min. Values are means ± S.E.M. (quadruplicate analysis for each group). $^{**}P<0.01$ compared with control group.

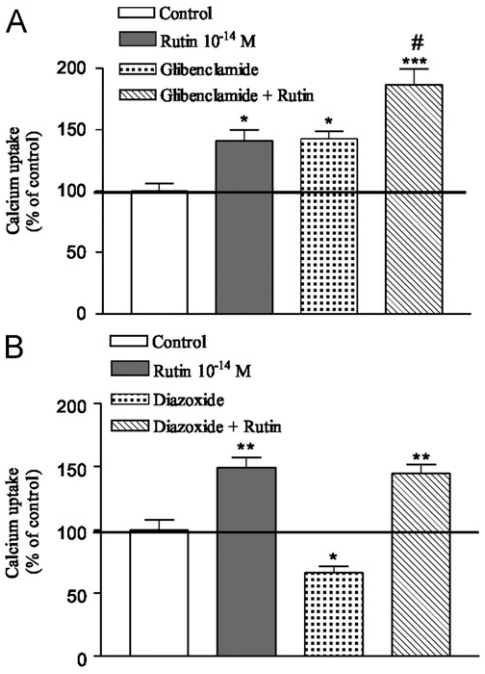


Fig. 3. Influence of K^+ channel on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min with 0.1 µCi/ml of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation (A), glibenclamide 60 µM (blocker) and (B), diazoxide 100 µM (activator) was added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to control group. $^{\#}P<0.05$ compared to rutin group.

The influence of intracellular Ca^{2+} in the stimulatory effect of rutin on Ca^{2+} uptake was investigated using BAPTA-AM, an intracellular Ca^{2+} chelator. Fig. 4B shows that BAPTA-AM did not alter the basal level of Ca^{2+} uptake. However, in the presence of BAPTA-AM the stimulatory effect of rutin on $^{45}Ca^{2+}$ uptake was inhibited.

Glucose-induced insulin release is controlled by a balance between protein phosphorylation and dephosphorylation (Ammala et al., 1994) and involves the activation of protein kinase C (PKC) (Mendez et al., 2003). Thus, the involvement of phospholipase C (PLC) and protein kinase C (PKC) in rutin-induced Ca^{2+} uptake was investigated. To this aim, isolated pancreatic islets were incubated in the presence or absence of rutin with/without specific inhibitors—U-73122 and RO-318220. These

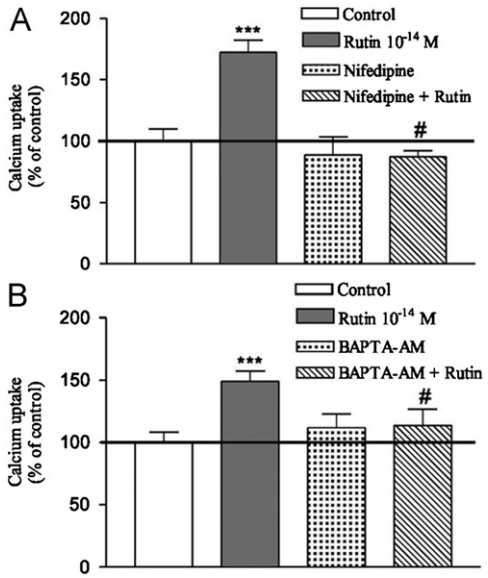


Fig. 4. Influence of L-type voltage-dependent Ca^{2+} channel (L-VDCC) and intracellular Ca^{2+} on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min with 0.1 μCi/ml of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation (A), nifedipine 1 μM (L-VDCC blocker) and (B), BAPTA-AM 50 μM (intracellular Ca^{2+} chelator) were added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). $^{***}P < 0.001$ compared to control group. $^{\#}P < 0.05$ compared to rutin group.

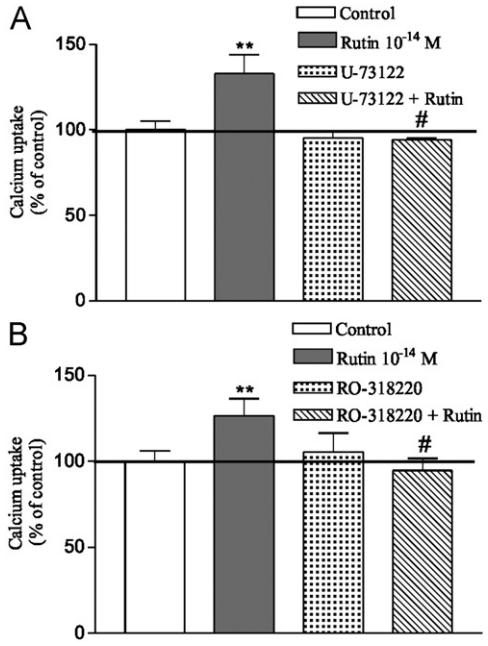


Fig. 5. Influence of PLC and PKC on stimulatory effect of rutin in $^{45}Ca^{2+}$ uptake in isolated rat pancreatic islets. The pancreatic islets were pre-incubated for 60 min with 0.1 μCi/ml of $^{45}Ca^{2+}$. In the last 15 min of pre-incubation, (A), U-73122 1 μM (PLC inhibitor) and (B), RO-318220 1 μM (PKC inhibitor) was added to the incubation medium. The tissue was then treated with or without 10^{-14} M rutin for 10 min (incubation). Values are means ± S.E.M. (quadruplicate analysis for each group). $^{**}P < 0.01$ compared to control group. $^{\#}P < 0.05$ compared to rutin group.

results showed that the inhibitors used did not change the basal level of Ca^{2+} uptake. On the other hand, U-73122 and RO-318220 completely prevented the rutin stimulatory effect (Fig. 5A and B).

4. Discussion

In the study reported herein, rutin as much as glipizide was effective on serum glucose lowering after acute oral treatment in hyperglycemic rats. Pereira et al. (2011) also evidenced the antihyperglycemic effect of rutin (50 mg/kg) in the similar experimental model. Numerous reports have demonstrated that flavonoids can act as insulin secretagogues (Cazarolli et al., 2008a,b; Folador et al., 2010). Here, rutin significantly increased insulin secretion with an II about 56% of the glipizide effect. Kamalakkannan and Prince (2006) also demonstrated that rutin increase insulin levels after *in vivo* treatment. The authors suggested that rutin act as an antioxidant compound protecting β-cells in diabetic rats.

It is reported that the most important pathway for quantitative insulin release from islet interstitial is regulated by ionic Ca^{2+} (for review Henquin, 2004). Our results showed that rutin is able to alter Ca^{2+} uptake in isolated pancreatic islets (Fig. 2). In agreement with Hii and Howell (1985), the exposure of rat isolated islets to certain flavonoids such as epicatechin or quercetin (aglycone of rutin) enhance insulin release and increase the intracellular Ca^{2+}.

Glibenclamide directly blocks K_{ATP} channels of the β-cell membrane and increase intracellular Ca^{2+}. In our experimental conditions, as expected glibenclamide increased Ca^{2+} uptake in pancreatic islet. In addition, when rutin was present into incubation medium, an additive effect on Ca^{2+} uptake was observed. The co-treatment with diazoxide did not alter the stimulatory effect of rutin on Ca^{2+} uptake, suggesting that rutin effect is independent of plasma membrane K_{ATP} channels activation. In agreement with these data, Adisakwattana et al. (2011) demonstrated that a cinnamic acid derivative, *p*-methoxycinnamic acid, stimulated insulin secretion from pancreatic β-cells by increasing Ca^{2+} influx via the L-VDCC, but not through the closure of K_{ATP} channels.

It is well known that the depolarization of the plasma membrane induced by sulfonylureas activates L-VDCCs in pancreatic islets (for review Yang and Berggren, 2005). The stimulatory effect of rutin on the Ca^{2+} uptake was abolished in the presence of nifedipine. Moreover, the stimulatory effect of rutin was reduced by the presence of an intracellular Ca^{2+} chelator, BAPTA-AM. The calcium dynamic is critical since it depends on the equilibrium of Ca^{2+} transport and buffer cellular systems (Friel and Chiel, 2008). So, it is difficult to predict how rutin handles the Ca^{2+} levels. However our results indicate a strong influence of intracellular Ca^{2+} on stimulatory effect of rutin on Ca^{2+} uptake.

The results presented so far suggest that increased Ca^{2+} uptake by rutin can be independent of K_{ATP} channels. Following, Ca^{2+} uptake can also be produced or enhanced by mechanisms that are independent of plasma membrane depolarization induced by K_{ATP} channel activity (Henquin, 2004; MacDonald and Wheeler, 2003). Fig. 5A showed that in the presence of a PLC inhibitor (U-73122) the stimulatory effect on Ca^{2+} uptake generated by rutin was blocked. In addition, other studies have indicated that the PLC plays a significant role in maintaining the quantity of intracellular Ca^{2+} (Jing et al., 2005; Yang and Berggren, 2005). In a whole, the effect of RO-318220 on Ca^{2+} uptake stimulated by rutin and PKC involvement is according with that reported by Arkhammar et al. (1994) concerning with the Ca^{2+} uptake through VDCC that diminish dramatically after deprivation of PKC, in mouse pancreatic β-cells.

Taking together, our results point that rutin may be acting through two signaling pathways to increase Ca^{2+} uptake in β-cells. One pathway through the activation of Ca^{2+} channels directly, since that Ca^{2+} also acts as cofactor for the activation

of the PLC and is required to PKC activation. Another way can be by activation of Gq protein-coupled receptors (Ahrén, 2009).

5. Conclusions

The rutin diminish glycemia, potentiate insulin secretion *in vivo* and significantly stimulates Ca^{2+} uptake in rat pancreatic islets. A novel cellular mechanism of action of rutin in Ca^{2+} uptake on pancreatic β-cells was elucidated. Rutin modulates Ca^{2+} uptake in pancreatic islets by opening L-VDCC, alter intracellular Ca^{2+}, PLC and PKC signaling pathways, characterizing K_{ATP} channel-independent pathways. These finds highlight rutin, a dietary adjuvant, as a potential insulin secretagogue contributing to glucose homeostasis.

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