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quercetin; pancreatic beta cells; insulin secretion; Ca^{2+} influx; L-type Ca^{2+} currents

BACKGROUND AND PURPOSE
Quercetin is a natural polyphenolic flavonoid that displays anti-diabetic properties *in vivo*. Its mechanism of action on insulin-secreting beta cells is poorly documented. In this work, we have analysed the effects of quercetin both on insulin secretion and on the intracellular calcium concentration ($[Ca^{2+}]_i$) in beta cells, in the absence of any co-stimulating factor.

EXPERIMENTAL APPROACH
Experiments were performed on both INS-1 cell line and rat isolated pancreatic islets. Insulin release was quantified by the homogeneous time-resolved fluorescence method. Variations in $[Ca^{2+}]_i$ were measured using the ratiometric fluorescent Ca^{2+} indicator Fura-2. Ca^{2+} channel currents were recorded with the whole-cell patch-clamp technique.

KEY RESULTS
Quercetin concentration-dependently increased insulin secretion and elevated $[Ca^{2+}]_i$. These effects were not modified by the SERCA inhibitor thapsigargin (1 $\mu mol \cdot L^{-1}$), but were nearly abolished by the L-type Ca^{2+} channel antagonist nifedipine (1 $\mu mol \cdot L^{-1}$). Similar to the L-type Ca^{2+} channel agonist Bay K 8644, quercetin enhanced the L-type Ca^{2+} current by shifting its voltage-dependent activation towards negative potentials, leading to the increase in $[Ca^{2+}]_i$ and insulin secretion. The effects of quercetin were not inhibited in the presence of a maximally active concentration of Bay K 8644 (1 $\mu mol \cdot L^{-1}$), with the two drugs having cumulative effects on $[Ca^{2+}]_i$.

CONCLUSIONS AND IMPLICATIONS
Taken together, our results show that quercetin stimulates insulin secretion by increasing Ca^{2+} influx through an interaction with L-type Ca^{2+} channels at a site different from that of Bay K 8644. These data contribute to a better understanding of quercetin's mechanism of action on insulin secretion.

Abbreviations
Bay K 8644, S-(-)-Bay K 8644; Ca_v, voltage-gated calcium channels; DHP, dihydropyridine; HTRF, homogeneous time time-resolved fluorescence; HVA, high-voltage-activated; KRB, Krebs-Ringer bicarbonate buffer; LVA, low-voltage-activated

Introduction

Plant foods contain flavonoids, polyphenolic compounds with protective effects in several chronic diseases. One of the most widely distributed flavonoids in the human diet, quercetin (3,3′,4′,5,7-pentahydroxyflavone) (Hertog *et al.*, 1993), exhibits numerous beneficial effects in diabetes. The long-term consumption of quercetin appears to control blood glucose levels *in vivo* in animals with streptozotocin-induced diabetes (Coskun *et al.*, 2005; Ramachandra *et al.*, 2005; Adewole *et al.*, 2006; Kim *et al.*, 2011). Quercetin has also been shown to normalize glucose tolerance, reduce oxidative stress markers, regenerate pancreatic islets and preserve the integrity of pancreatic beta cells (Vessal *et al.*, 2003; Mahesh and Menon, 2004; Coskun *et al.*, 2005; Kim *et al.*, 2011). Recently, quercetin was also shown to prevent the inhibition of insulin secretion induced by IL-1β in beta cells of the rat insulinoma cell line RINm5F (Cho *et al.*, 2012).

We have previously reported that quercetin potentiates glucose-induced insulin secretion and protects beta cell function and viability against oxidative damage *in vitro* in the INS-1 beta cell line (Youl *et al.*, 2010). These effects involve ERK1/2 activation, as indicated by the increase in ERK1/2 phosphorylation, and are specific to quercetin, as two other antioxidant compounds (resveratrol and NAC) do not show such effects. In pancreatic beta cells, the exocytosis of insulin is a calcium-dependent process, and an increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) is the main trigger for insulin secretion (Mears, 2004). This increase in $[Ca^{2+}]_i$ follows membrane depolarization, which initiates Ca^{2+} influx through voltage-gated Ca^{2+} channels (Gilon and Henquin, 1992). At sub-stimulating glucose levels, the activity of ATP-sensitive K^+ channels (K_{ATP}) keeps the beta cell membrane hyperpolarized, and $[Ca^{2+}]_i$ remains low. As glucose levels rise, K_{ATP} channels close due to an increase in the cytoplasmic ATP/ADP ratio, resulting in membrane depolarization and activating Ca_v channels, which, in turn, leads to an increase in $[Ca^{2+}]_i$ that triggers the exocytosis of insulin-containing granules (Mears, 2004; Mears and Zimliki, 2004). In addition to Ca^{2+} entry, the mobilization of Ca^{2+} from the endoplasmic reticulum (ER), an internal Ca^{2+} store, is also implicated in the increase in $[Ca^{2+}]_i$ and insulin secretion in beta cells (Kang and Holz, 2003; Dyachok and Gylfe, 2004; Islam, 2010; Ravier *et al.*, 2011).

We have shown that the potentiating effects of quercetin on KCl-induced insulin secretion are correlated with its potentiation of the increase in $[Ca^{2+}]_i$ triggered by this depolarizing agent (Youl *et al.*, 2010). Moreover, in porcine brain microsomes, quercetin appears to inhibit SERCA, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase responsible for pumping Ca^{2+} from the cytosol into intracellular Ca^{2+} stores (Ogunbayo *et al.*, 2008), suggesting that quercetin may also increase $[Ca^{2+}]_i$ by inhibiting SERCA .

In this study, we analysed the effects of quercetin on insulin secretion in relation to $[Ca^{2+}]_i$ modulation in pancreatic beta cells in the absence of any co-stimulating factor, and identified its molecular target. In the INS-1 cell line, quercetin induced an increase in $[Ca^{2+}]_i$ and insulin secretion that were inhibited by nifedipine (an L-type Ca^{2+} channel antagonist) but unchanged by thapsigargin (a SERCA inhibitor), suggesting that the L-type Ca_v is almost certainly involved in its mechanism of action. Patch-clamp experiments confirmed that quercetin directly activated high-voltage-activated (HVA) Ca^{2+} channels in INS-1 cells by interacting with a binding site different from that of the Ca^{2+} channel agonist Bay K 8644. Moreover, quercetin displayed similar effects on rat pancreatic islets.

Methods

The drug/molecular target nomenclature used conforms to the BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

INS-1 cell culture

The insulin-secreting cell line INS-1 (a gift from Professor C.B. Wollheim) was cultured in RPMI-1640 medium supplemented with 10% FCS, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 2 $mmol \cdot L^{-1}$ L-glutamine, 10 $mmol \cdot L^{-1}$ HEPES, 1 $mmol \cdot L^{-1}$ sodium pyruvate and 50 $mmol \cdot L^{-1}$ 2-mercaptoethanol, in a humidified atmosphere (5% CO_2, 37°C), as previously described (Asfari *et al.*, 1992).

Rat pancreatic islets and dispersed cell preparation

All animal care and experimental procedures were performed according to the rules of the Centre National de la Recherche Scientifique Animal Care. Pancreatic islets were isolated from Wistar rats weighing 350–370 g (Janvier, France). Briefly, after anaesthesia with isoflurane and decapitation, the pancreas was filled by ductal injection of collagenase solution, excised, digested at 37°C, and mechanically disrupted with a 14-gauge needle. Pancreatic islets were selected by hand-picking after collagenase digestion of whole pancreas (Bertrand *et al.*, 2002). Isolated cells were prepared by dispersion of islets with trypsin and plated on culture chamber (equivalent to 10 islets per chamber) in RPMI-1640 medium containing 10 mM glucose and the experiments were performed on clusters of cells after 2 days of culture.

Insulin secretion studies in INS-1 cells and rat isolated pancreatic islets

INS-1 cells were plated in 24-well plates (4×10^5 cells per well) for insulin secretion studies and were used for experiments after 5 days in culture. Before the treatment, the RPMI medium was removed and the cells were washed twice in HEPES-balanced Krebs–Ringer bicarbonate buffer (KRB) containing (in $mmol \cdot L^{-1}$): NaCl, 120; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 24, with 1 $g \cdot L^{-1}$ BSA (KRB/BSA). For determining the effects of quercetin, KCl or Bay K 8644 (an L-type Ca^{2+} channel agonist), INS-1 cells were incubated for 60 min (5% CO_2, 37°C) in KRB/BSA containing 1.4 $mmol \cdot L^{-1}$ glucose (non-stimulant basal condition for INS-1 cells). When appropriate, pharmacological inhibitors (nifedipine or thapsigargin) were added at the concentrations indicated in the figure legend at the beginning of the incubation period. The INS-1 cells were pre-incubated with the L-type Ca_v antagonist nifedipine for 15 min or the SERCA inhibitor thapsigargin for 60 min at the concentrations indicated, in RPMI medium at 37°C (under 5% CO_2). The corresponding control experi-

ments were performed in the absence of quercetin, KCl, Bay K 8644 or pharmacological inhibitor during the pre-incubation and incubation periods.

Freshly isolated islets were first pre-incubated for 45 min in KRB buffer supplemented with 1 g·L^{-1} BSA and 8.3 mmol·L^{-1} glucose. Batches of 5 islets were then incubated for 30 min in KRB containing 4.2 mmol·L^{-1} glucose (non-stimulant basal condition for rat islets) with or without quercetin as described in the figure legend.

At the end of the incubation periods, samples of the medium were removed and stored frozen at –20°C until the insulin assay was performed by homogeneous time-resolved fluorescence (HTRF), as previously described (Youl *et al.*, 2010). Data are expressed as percentages of the response obtained in non-stimulant basal conditions.

Treatment of INS-1 cells and cells isolated from rat pancreatic islets for image analysis of cytoplasmic Ca^{2+}

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured using the ratiometric fluorescent Ca^{2+} indicator Fura-2 (22 ± 2°C) as previously described (Gysembergh *et al.*, 1999; Youl *et al.*, 2010). Briefly, INS-1 cells sub-cultured for 4 days in Lab-Tek® chambers (VWR International, Fontenay-sous-Bois, France) and cells isolated from rat pancreatic islets were incubated with 2.5 µmol·L^{-1} Fura-2AM plus 0.02% Pluronic F-127. Cells were rinsed with KRB and chambers mounted on a microscope stage (Axiovert, Zeiss, Iena, Germany; 20× objective) during a 15 min waiting period for the de-esterification of Fura-2AM. Media and drugs were then applied directly on top of the imaged cells using a perfusion system, as indicated in the legend to the figures. Images were captured digitally every 2 s with a cooled CCD camera (Photometrics; Roper Scientific, Evry, France). Cells loaded with Fura-2 were illuminated by excitation with a dual UV light source at 340 nm (Ca^{2+}-bound) and 380 nm (Ca^{2+}-free) using a lambda DG-4 excitation system (Sutter Instrument Company, Novato, CA, USA). Fluorescence emission was measured at 510 nm and analysed (Metafluor software, Universal Imaging Corporation, Downingtown, PA, USA). Changes in $[Ca^{2+}]_i$ were deduced from variations in the F340/F380 ratio after correction for background and dark currents. Data were averaged, with *n* representing the number of fields.

Treatment of INS-1 cells for electrophysiological recording

Ca^{2+} channel currents were recorded using the whole-cell patch-clamp technique with a Biologic RK400 amplifier (Biologic, France). Data acquisition and analysis were performed using the pCLAMP system (Axon Instruments, Union City, CA, USA). Currents were recorded with patch pipettes of 2–5 MΩ. Capacitive transients were electronically compensated for. Residual capacitive transient and linear leakage currents were subtracted using a 4 sub-pulse protocol. The extracellular solution contained (in mmol·L^{-1}): 130 NaCl, 5.6 KCl, 1 $MgCl_2$, 11 glucose, 10 HEPES, 5 $BaCl_2$ or $CaCl_2$, adjusted to pH 7.4 with NaOH. The pipette solution contained (in mmol·L^{-1}): 130 CsCl, 10 EGTA, 5 $ATPNa_2$, 2 $MgCl_2$, 10 HEPES, adjusted to pH 7.3 with CsOH. Ba^{2+} was used as the charge carrier so that the T-type Ca^{2+} channel currents could be identified and separated from the L-type Ca^{2+} channel currents because the inactivation kinetics of the latter are much slower than that of T-type currents under these conditions. In some experiments, $BaCl_2$ was replaced by 5 mmol·L^{-1} $CaCl_2$ in the extracellular solution, and EGTA was reduced to 10 µmol·L^{-1} in the presence of 5 µmol·L^{-1} $CaCl_2$ in the pipette solution (free intracellular Ca^{2+} concentration around 50 nmol·L^{-1}). The holding potential (HP) was set at –85 mV, and depolarizing test pulses were applied as described. Drugs were applied to cells by pressure ejection from a glass pipette. All experiments were performed at room temperature.

Statistical analysis

Statistical analysis was performed using GraphPad software (La Jolla, CA, USA). Multiple-group comparisons were based on one-way ANOVA, followed by Fisher's protected least significant difference test. The limit of statistical significance was set at $P < 0.05$.

Materials

RPMI-1640 media and FCS were purchased from Lonza (Levallois-Perret, France). Thapsigargin was purchased from Calbiochem (La Jolla, CA, USA). Quercetin, Bay K 8644, nifedipine and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). Insulin concentrations in cell supernatants were determined using the HTRF insulin assay kit (Cisbio International, Bagnols-sur-Cèze, France). Quercetin, Bay K 8644 and nifedipine were dissolved in DMSO and stored at –20°C. When using compounds dissolved in DMSO, control cells were treated with the solvent at the same concentration. The final concentration of DMSO was less than 0.1% and did not affect insulin secretion or Ca^{2+} measurements (data not shown). Fura-2AM was purchased from TEFlabs (Austin, TX, USA). Pluronic F-127 was purchased from Molecular Probes (Invitrogen, Cergy-Pontoise, France).

Results

Effects of quercetin on the INS-1 beta cell line

In the present study, quercetin was tested under basal conditions, namely in the presence of 1.4 mmol·L^{-1} glucose, a concentration devoid of any stimulating effect on insulin secretion. The effects of quercetin were studied in the concentration range 2 to 20 µmol·L^{-1}. In some experiments, when a single concentration of quercetin was tested, we chose to use 20 µmol·L^{-1}, a concentration previously shown to have a maximal potentiating effect on glucose (or KCl)-induced insulin secretion (Youl *et al.*, 2010).

Effects of quercetin on insulin secretion and intracellular calcium. As shown in Figure 1A, 20 µmol·L^{-1} quercetin induced a significant increase (about 2.2-fold) in insulin secretion from 28.1 ± 1.8 ng·mL^{-1} (basal levels) to 62.1 ± 3.6 ng·mL^{-1}. Under similar experimental conditions, the depolarizing agent KCl (15 mmol·L^{-1}) provoked a 6.2-fold increase (from 28.1 ± 1.8 to 164.5 ± 7.6 ng·mL^{-1}), four times higher than that induced by quercetin. Quercetin increased insulin secretion in a concentration-dependent manner, with an EC_{50} value of 7.5 ± 0.5 µmol·L^{-1} (data not shown).

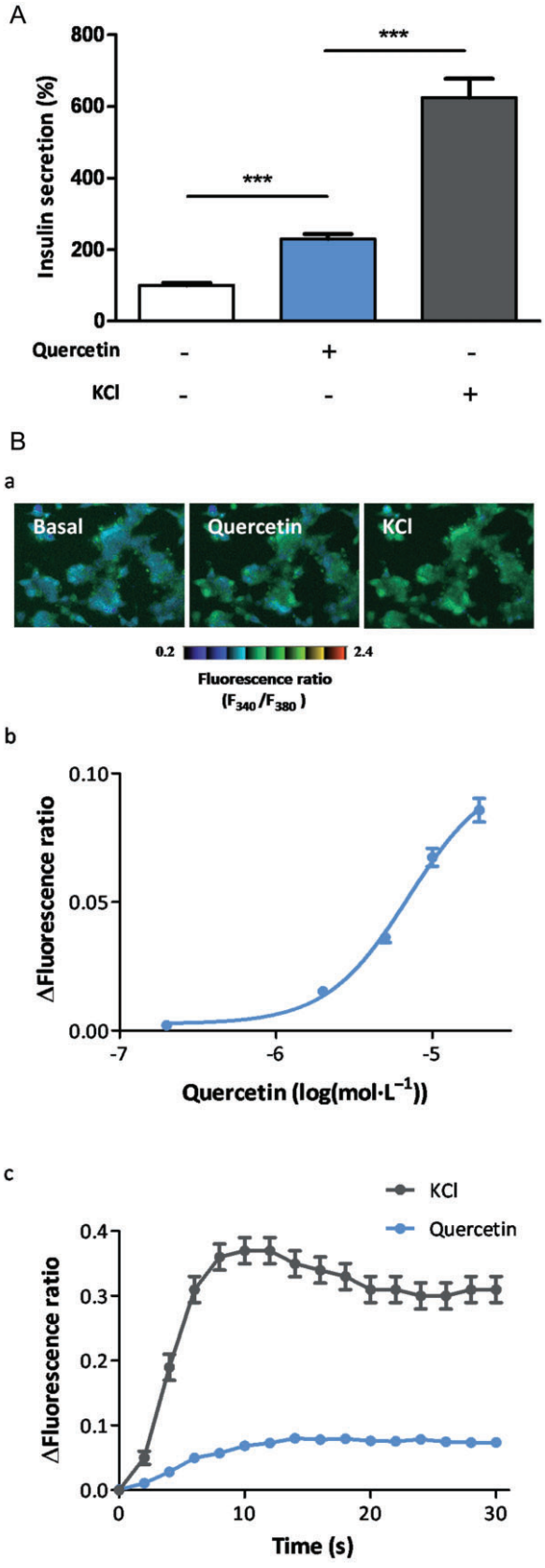

Figure 1

Quercetin stimulates insulin secretion and increases the intracellular calcium ($[Ca^{2+}]_i$) in INS-1 cells. (A) Insulin secretion was stimulated either with 20 µmol·L^{-1} quercetin or with 15 mmol·L^{-1} KCl in the presence of glucose, at a concentration (1.4 mmol·L^{-1}) devoid of any stimulating effect. Values represent the means ± SEM from 10 separate experiments. ***$P < 0.0001$; multiple comparison analysis. (B) $[Ca^{2+}]_i$ variations revealed by false-colour imaging of Fura-2 loaded INS-1 cells: (a) typical cell images illustrating the maximal fluorescence ratio (F_{340}/F_{380}) under basal conditions and after the addition of 20 µmol·L^{-1} quercetin or 15 mmol·L^{-1} KCl. (b) Concentration–response curve of maximal variation in the fluorescence ratio in response to quercetin. Values represent the means ± SEM from five experiments. (c) Time course of variations in the fluorescence ratio in response to quercetin or KCl. Values represent the means ± SEM from 10 experiments.

We then investigated whether, in the absence of any co-stimulating agent (glucose or KCl), quercetin induced a change in $[Ca^{2+}]_i$. We observed that 20 µmol·L^{-1} quercetin triggered a rapid and highly reproducible increase in $[Ca^{2+}]_i$, achieving steady state within 10–15 s. At 2 µmol·L^{-1}, quercetin induced a significant increase in $[Ca^{2+}]_i$, representing 18.6 ± 7.8% of the maximal effect obtained at 20 µmol·L^{-1} (Figure 1B). When INS-1 cells were challenged with the depolarizing agent KCl (15 mmol·L^{-1}), we observed an increase in $[Ca^{2+}]_i$ with a similar time course but with a maximal effect four times higher than that of 20 µmol·L^{-1} quercetin. Both the quercetin- and KCl-induced increases in $[Ca^{2+}]_i$ were maintained for several tens of seconds (Figure 1B).

Mechanism of the quercetin-induced increase in intracellular calcium. We next analysed the mechanisms involved in the increase in $[Ca^{2+}]_i$ induced by 20 µmol·L^{-1} quercetin. First, we determined whether this rise was of extracellular or intracellular origin. In the absence of extracellular Ca^{2+} (Ca^{2+}-free KRB medium containing 0.1 mM EGTA), basal $[Ca^{2+}]_i$ values were lower than those observed with Ca^{2+}-containing KRB, and quercetin failed to increase $[Ca^{2+}]_i$ (Figure 2A). Under these conditions, quercetin was unable to induce insulin secretion (data not shown). As quercetin has been shown to inhibit SERCA (Ogunbayo *et al.*, 2008), we investigated whether the emptying of Ca^{2+} stores by thapsigargin (a SERCA inhibitor) could influence the quercetin response. As expected, fluorescence imaging with Fura-2 showed that 1 µmol·L^{-1} thapsigargin induced an increase in $[Ca^{2+}]_i$ (Figure 2A). The stimulating effect of quercetin was observed even in the presence of thapsigargin. Moreover, the increase in $[Ca^{2+}]_i$ induced by quercetin plus thapsigargin corresponded to the sum of the increase induced by each compound individually (Figure 2A), suggesting that their respective effects occurred via different mechanisms. The same results were obtained with respect to insulin secretion (Figure 2B). Thapsigargin stimulated a twofold increase in insulin secretion when compared with controls (from 28.1 ± 1.8 to 57.7 ± 7.2 ng·mL^{-1}). As observed for the increase in $[Ca^{2+}]_i$, the insulin secretion induced by quercetin plus thapsigargin was higher (91.2 ± 8.6 ng·mL^{-1}) and was comparable to the sum of the effects of each compound separately. Taken together, these results strongly suggest that the effect of quercetin on insulin secre-

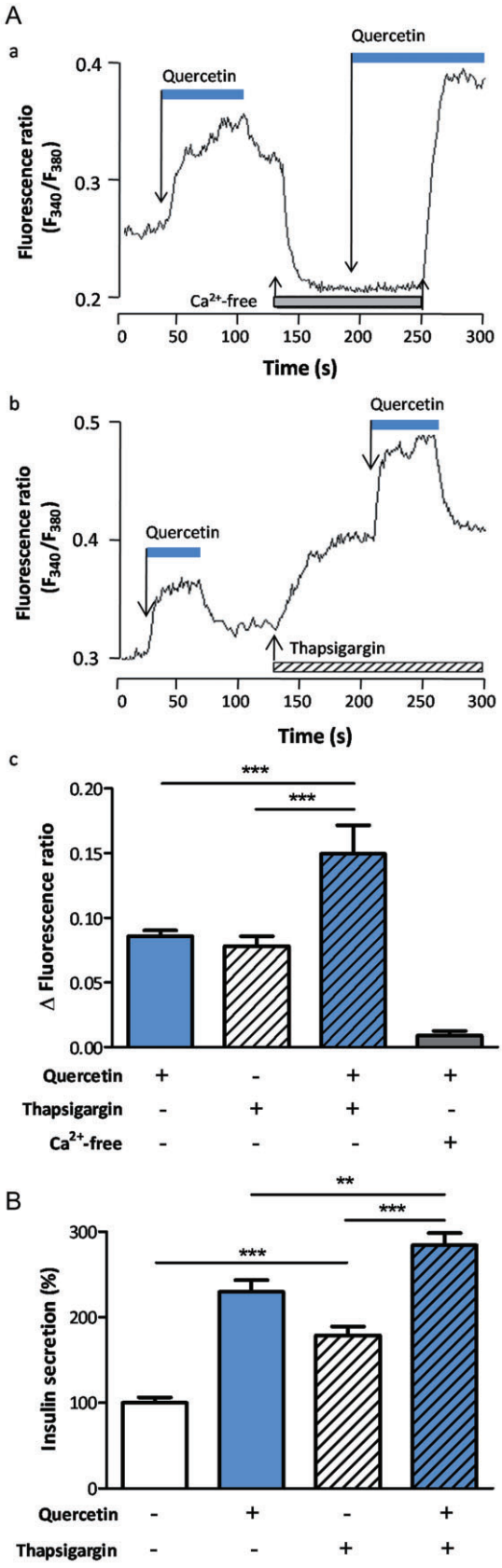

Figure 2

The quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion in INS-1 cells require extracellular calcium but are independent of SERCA activity. (A) Typical recordings of variations in the fluorescence ratio under the different conditions. Cells were stimulated with 20 µmol·L^{-1} quercetin and washed with KRB before further treatment. (a) Cells were then subjected to Ca^{2+} removal by substituting Ca^{2+}-free KRB medium supplemented with 5 mmol·L^{-1} EGTA, the fluorescence was allowed to stabilize before the re-addition of quercetin, and extracellular Ca^{2+} restored. (b) Thapsigargin (1 µmol·L^{-1}) was added in the absence of quercetin, the fluorescence was allowed to stabilize before the re-addition of quercetin, and quercetin was washed out once again. Arrows indicate the starting point of each drug or change of medium. (c) Bar graph representing the maximal variation in the fluorescence ratio induced by quercetin under basal conditions, in the absence of extracellular Ca^{2+} (Ca^{2+}-free) or in the presence of thapsigargin. Results are presented as means ± SEM of five separate experiments. (B) Effects of 20 µmol·L^{-1} quercetin on insulin secretion in the presence or absence of 1 µmol·L^{-1} thapsigargin. Results are presented as means ± SEM from 4–6 separate experiments. ***$P < 0.0001$; **$P < 0.001$; ns: $P > 0.05$; multiple comparison analysis for the different treatment conditions.

tion is due to the entry of extracellular Ca^{2+} rather than to the mobilization of intracellular Ca^{2+} from the ER.

Involvement of L-type Ca^{2+} *channels.* Depolarization-dependent Ca^{2+} influx through voltage-activated Ca^{2+} channels is essential for insulin secretion (Bokvist *et al.*, 1995). Because dihydropyridine (DHP) antagonists, which bind to L-type Ca^{2+} channels, potently suppress insulin secretion, these channels are considered to be crucial for beta cell function (Davalli *et al.*, 1996; Braun *et al.*, 2008). Consequently, we investigated the involvement of these channels in the effect of quercetin, using the L-type Ca^{2+}-channel antagonist nifedipine, and the L-type Ca^{2+}-channel agonist S-(-)-Bay K 8644 (which we will refer to here as Bay K 8644).

Effects of nifedipine on the quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion. Fluorescence imaging experiments showed that nifedipine (1 µmol·L^{-1}), which had no effect on basal $[Ca^{2+}]_i$, reversed the increase in $[Ca^{2+}]_i$ induced by 20 µmol·L^{-1} quercetin (Figure 3A). Moreover, when 1 µmol·L^{-1} nifedipine was applied to the cells before quercetin, it prevented about 90% of the quercetin-induced rise in $[Ca^{2+}]_i$ (Figure 3A). Nifedipine (1 µmol·L^{-1}) had no effect on basal insulin secretion (28.1 ± 1.8 ng·mL^{-1} under basal conditions vs. 26.3 ± 2.4 ng·mL^{-1} in the presence of nifedipine) but potently inhibited quercetin-induced insulin secretion (38.1 ± 4.5 ng·mL^{-1} for quercetin *plus* nifedipine vs. 62.1 ± 3.6 ng·mL^{-1} for quercetin alone) (Figure 3B).

Effects of Bay K 8644 on the quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion. When tested at the maximally active concentration of 1 µmol·L^{-1}, the agonist Bay K 8644 promoted a greater increase in $[Ca^{2+}]_i$ than that induced by 20 µmol·L^{-1}quercetin (Figure 4A). Quercetin continued to increase $[Ca^{2+}]_i$ in the presence of Bay K 8644, and the two drugs had cumulative effects (Figure 4A). At this concentration, the stimulation of insulin secretion by Bay K 8644 (91.3

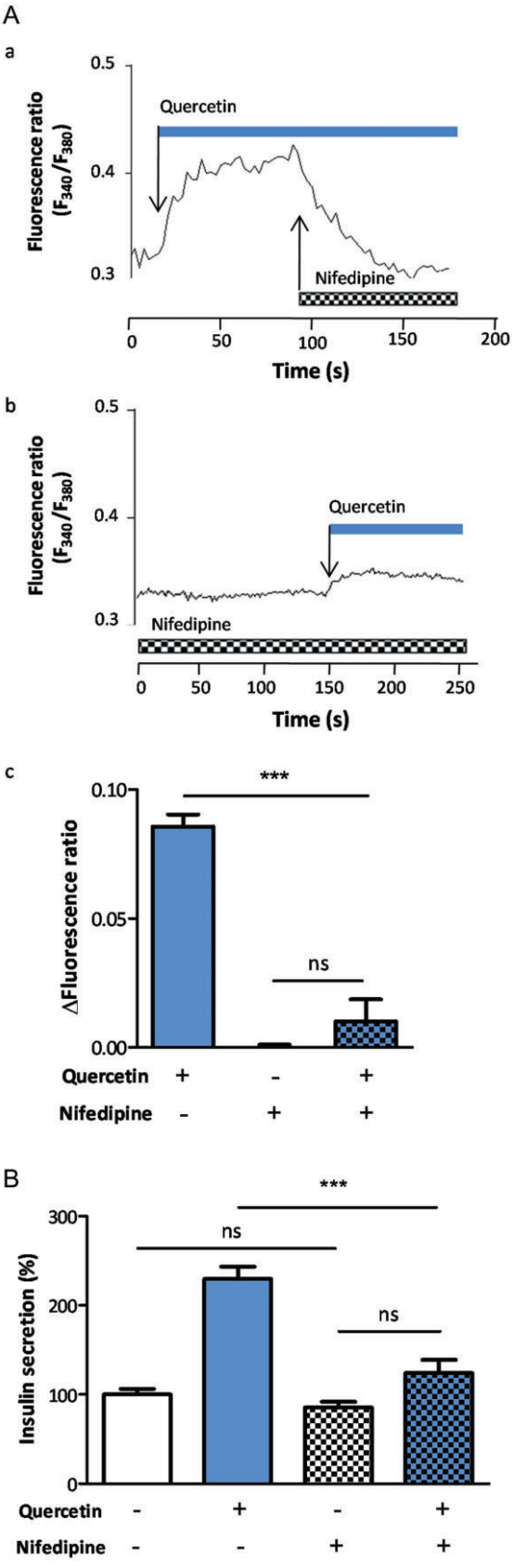

Figure 3

The quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion in INS-1 cells are blocked by the L-type Ca^{2+} channel antagonist nifedipine. (A) Typical recordings of variations in the fluorescence ratio. Arrows indicate the time of application of each drug. (a) Cells were incubated with 20 µmol·L^{-1} quercetin followed by 1 µmol·L^{-1} nifedipine. (b) Cells were incubated with 1 µmol·L^{-1} nifedipine for 3 min before the addition of 20 µmol·L^{-1} quercetin. (c) Bar graph representing the maximal variation in the fluorescence ratio induced by quercetin, nifedipine and quercetin in the presence of nifedipine. Results are presented as means ± SEM of five separate experiments. (B) The effects of 20 µmol·L^{-1} quercetin on insulin secretion in the presence or absence of 1 µmol·L^{-1} nifedipine. Values represent means ± SEM from 4–6 separate experiments. ***$P < 0.0001$; ns: $P > 0.05$; multiple comparison analysis for the different treatment conditions.

± 3.1 ng·mL^{-1} or 3.2 times basal levels) was higher than that induced by quercetin (62.1 ± 3.6 ng·mL^{-1}), which may reflect the difference in their effects on $[Ca^{2+}]_i$. These results confirm the previously reported effects of Bay K 8644 on $[Ca^{2+}]_i$ and insulin secretion in INS-1 cells (Adisakwattana *et al.*, 2011). In addition, in the presence of Bay K 8644, not only was the stimulating effect of quercetin on insulin secretion still observed, but insulin concentrations increased substantially, from 28.1 ± 1.8 ng·mL^{-1} (basal levels) to 275.4 ± 4.7 ng·mL^{-1}.

Effect of quercetin on the L-type Ca^{2+} *current.* As both quercetin and Bay K 8644 increased $[Ca^{2+}]_i$ and insulin secretion, we analysed the ability of quercetin to modulate the L-type Ca^{2+} current in INS-1 beta cells. Current clamp studies showed that the average resting membrane potential of these cells was –54.5 ± 3.4 mV, which did not change much following quercetin application (–2.7 ± 0.6 mV). Thus, we used the whole-cell configuration of patch-clamp technique, with Ba^{2+} as the charge carrier instead of Ca^{2+} as described in the Methods section. Figure 5A shows typical examples of Ba^{2+} currents: (i) an L-type Ba^{2+} current activated at high voltage (e.g. –10 mV) with slow inactivation; (ii) a T-type current activated at low voltage (e.g. –30 mV) with fast inactivation; and (iii) a mix of both currents (a fast transient followed by delayed slow inactivation). The T-type current is best recorded at small step depolarizations (–30 mV), as the L-type current is only weakly activated at this potential. As illustrated in Figure 5B, quercetin (20 µmol·L^{-1}) increased the L-type Ba^{2+} current but had no effect on the T-type Ba^{2+} current. This former effect of quercetin was concentration-dependent and was observed within the micromolar range (EC_{50} = 1.57 ± 0.18 µmol·L^{-1}) (Figure 5C). Replacing the extracellular Ba^{2+} (the charge carrier) with Ca^{2+}, the physiological cation, had no effect on the response to quercetin (20 µmol·L^{-1}). This response was also resistant to depolarization, being observed even when the HP was set at –60 mV, a depolarized membrane potential known to inactivate the T-type Ca^{2+} current (Figure 5C). The effect of quercetin developed rapidly, reaching steady state within 20 s and remaining stable thereafter (Figure 5D). This enhancing effect of quercetin (20 µmol·L^{-1}) was observed at potentials between –40 and 0 mV (Figure 6A). Quercetin not only increased the peak current but also the sustained component of the current, which excluded an effect on the T-type Ca^{2+} channel current, as T-type currents are inactivated after

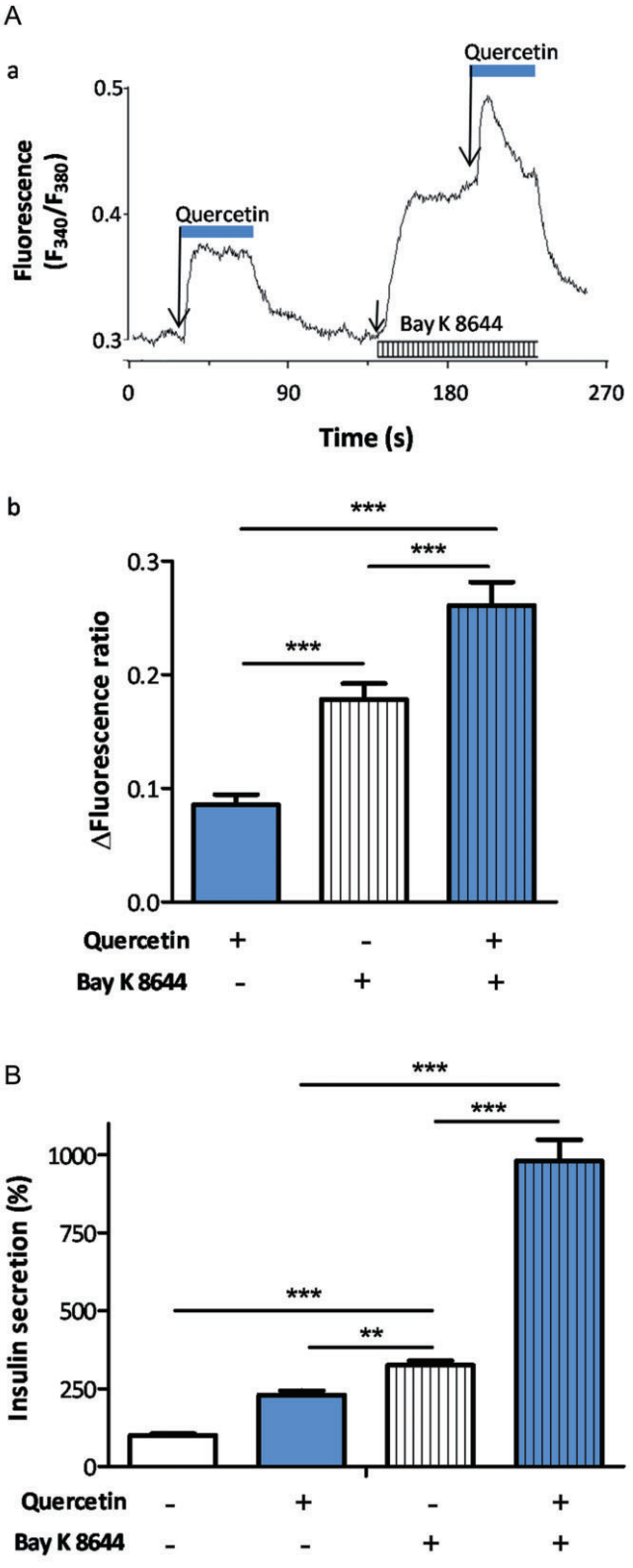

Figure 4

Effects of the L-type Ca^{2+} channel agonist Bay K 8644 on quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion in INS-1 cells. (A) Typical recordings of variations in the fluorescence ratio. Arrows indicate the time of application of each drug. (a) Cells were stimulated with 20 µmol·L^{-1} quercetin and, after a wash-out period with KRB, 1 µmol·L^{-1} Bay K 8644 was added. Fluorescence was allowed to stabilize before the addition of 20 µmol·L^{-1} quercetin, which was subsequently washed out. (b) Bar graph representing the maximal variation in the fluorescence ratio induced by quercetin, Bay K 8644 and quercetin in the presence of Bay K 8644. Results are presented as means ± SEM from five separate experiments. (B) The effects 20 µmol·L^{-1} quercetin on insulin secretion in the presence or absence of 1 µmol·L^{-1} Bay K 8644. Results are presented as means ± SEM from 4–6 separate experiments. ***$P < 0.0001$; **$P < 0.001$; multiple comparison analysis for the different treatment conditions.

150 ms of depolarization, leaving only L-type currents active. Consequently, quercetin shifted the potential at half-maximal activation ($V_{0.5}$) of the high-voltage-activated current to the left (–7 ± 1.2 mV; $n = 6$). Importantly, quercetin induced the appearance of a current at negative voltages (between –40 and –30 mV) (Figure 6A) at which the channels are not normally activated. Both this leftward shift of the threshold of channel activation and the time course of the quercetin effect were similar to the effects of the L-type Ca^{2+} channel agonist Bay K 8644. Indeed, Bay K 8644 also potentiated the L-type current at voltages between –40 and 0 mV, and shifted the $V_{0.5}$ of the current to the left (–8.5 ± 1.1 mV; $n = 3$) (Figure 6B), with no effect on the T-type current, as expected (data not shown). In the presence of Bay K 8644, the effect of quercetin was unchanged (Figure 6C), and the effects of the two drugs on voltage-dependent activation were additive (data not shown), suggesting that quercetin and Bay K 8644 have distinct modes of action.

Effects of quercetin on rat isolated pancreatic islets and dispersed cells

To confirm the effect of quercetin in a physiological model, we performed experiments on rat isolated pancreatic islets and dispersed cells. In clusters of cells, quercetin induced a concentration-dependent rise in $[Ca^{2+}]_i$, with a significant increase at 2 µmol·L^{-1} representing 15.7 ± 3.6% of the maximal augmentation obtained with 20 µmol·L^{-1} (Figure 7A). In isolated pancreatic islets, quercetin increased insulin secretion in a concentration-dependent manner. In the presence of a non-stimulating glucose concentration (4.2 mmol·L^{-1}), a significant increase was observed with 10 µmol·L^{-1} quercetin, about 2.2-fold versus basal (Figure 7B).

Discussion and conclusions

In a previous study, we showed that quercetin potentiates the insulin secretion induced by various secretagogues such as glucose, glibenclamide or KCl in the INS-1 pancreatic beta cell line (Youl *et al.*, 2010). In the present work, using INS-1 cells, we demonstrated that quercetin *per se* can promote insulin secretion, in the absence of any stimulation (glucose or cell depolarization by KCl). Quercetin-induced insulin secretion involved a rise in $[Ca^{2+}]_i$, mediated mainly by a direct effect on L-type Ca^{2+} channels. The underlying mechanism is based on a shift in the voltage-dependent activation

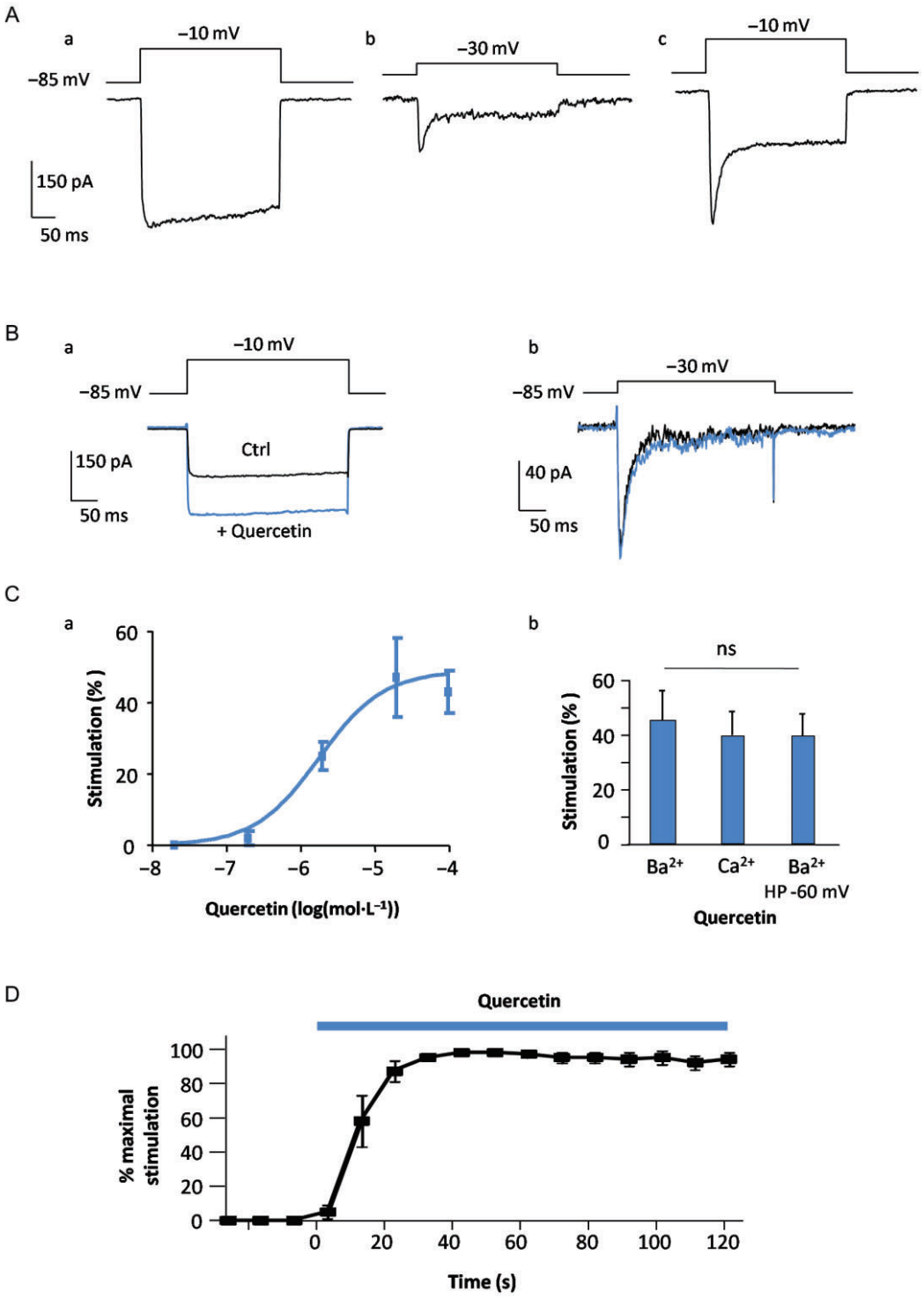

Figure 5

Quercetin affects high- but not low-voltage-activated (LVA) Ca^{2+} channel currents carried by Ba^{2+} in INS-1 cells. (A) Typical traces of (a) a HVA Ba^{2+} current recorded at a test pulse (tp) of –10 mV and characterized by slow inactivation; (b) a predominantly LVA (T-type) current recorded at low depolarization (tp of –30 mV) and (c) a mixed LVA (with a fast-inactivating component) and HVA current (with a slow-inactivating component). The holding potential (HP) was –85 mV. (B) The effects of 20 µmol·L^{-1} quercetin on (a) the HVA Ba^{2+} current and (b) the LVA current. (C) (a) Concentration–response curve for the effects of quercitin on the HVA Ba^{2+} current recorded at –10 mV. (b) Bar graph representing the effects of 20 µmol·L^{-1} quercetin on the HVA Ba^{2+} current, Ca^{2+} current and the Ba^{2+} current recorded at an HP of –60 mV (step depolarization at –10 mV, HP –80 mV or –60 mV). Results are presented as means ± SEM (n = 5–11 cells). ns: $P > 0.05$; multiple comparison analysis for the different conditions. (D) Time course of the effect of 20 µmol·L^{-1} quercetin on a HVA Ba^{2+} current (–10 mV, HP = –80 mV; frequency of stimulation 0.1 Hz). Data points indicate means ± SEM (n = 5 cells).

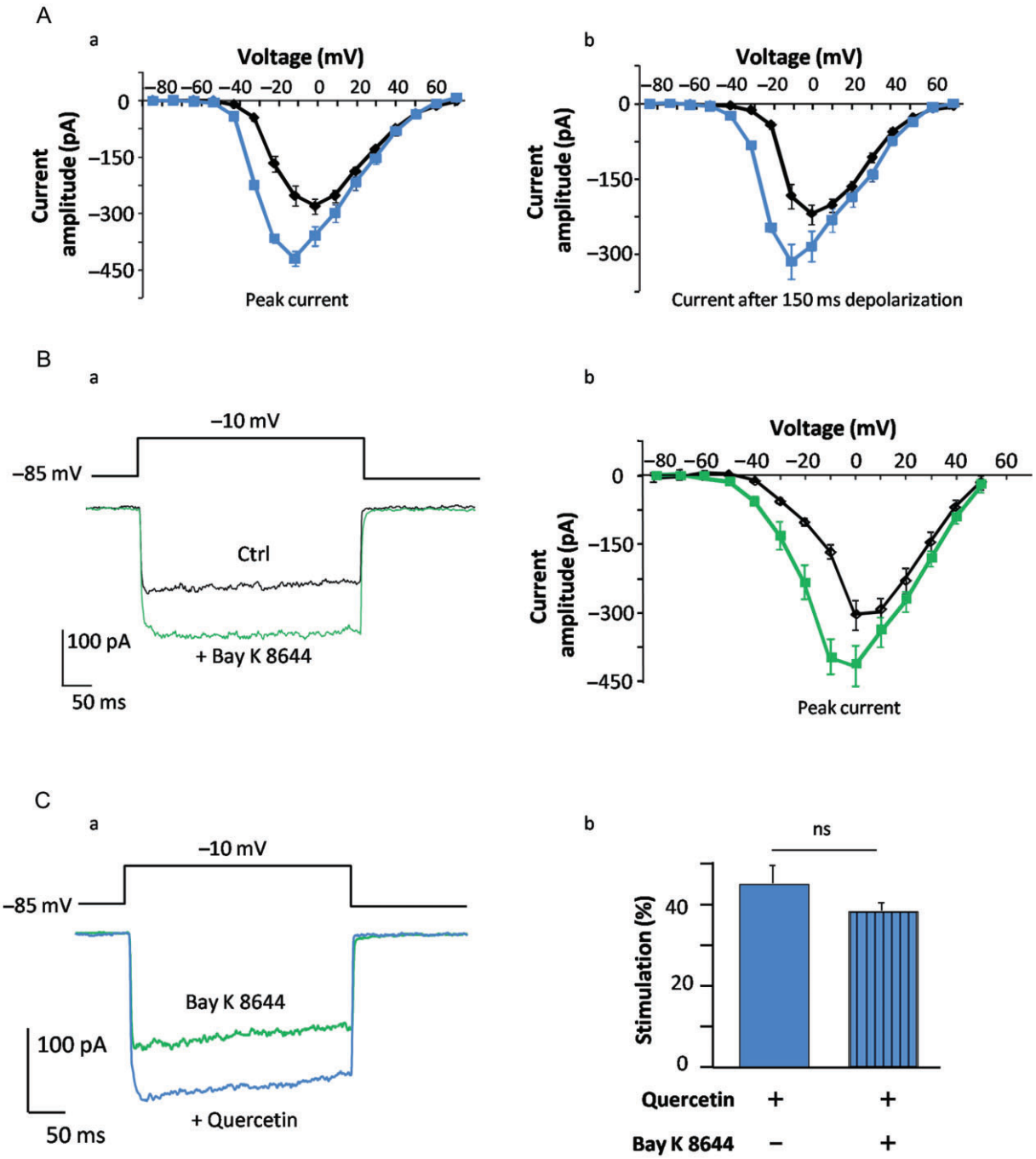

Figure 6

Effects of quercetin and Bay K 8644 on the HVA Ca^{2+} channel current carried by Ba^{2+} in INS-1 cells. (A) Effect of quercetin on the HVA current at all voltages of the current-to-voltage (*I*/*V*) relationship; (a) peak current and (b) the sustained component measured after 150 ms of depolarization, under control conditions (black line) or in the presence of 20 µmol·L^{-1} quercetin (blue line). Data points indicate means ± SEM ($n = 6$ cells). (B) Effect of 1 µmol·L^{-1} Bay K 8644 on (a) the HVA Ba^{2+} current and (b) the peak HVA current at all voltages of the current-to-voltage relationship, under control conditions (black line) or in the presence of 1 µmol·L^{-1} Bay K 8644 (green line). Data points indicate means ± SEM ($n = 3$ cells). (C) (a) Effect of 20 µmol·L^{-1} quercetin on the HVA Ba^{2+} current in the presence of 1 µmol·L^{-1} Bay K 8644. (b) Bar graph representing the effects of quercetin on the HVA Ba^{2+} current in the presence or absence of Bay K 8644. Results are presented as means ± SEM ($n = 5$ cells). ns: $P > 0.05$; Student's *t*-test for paired samples.

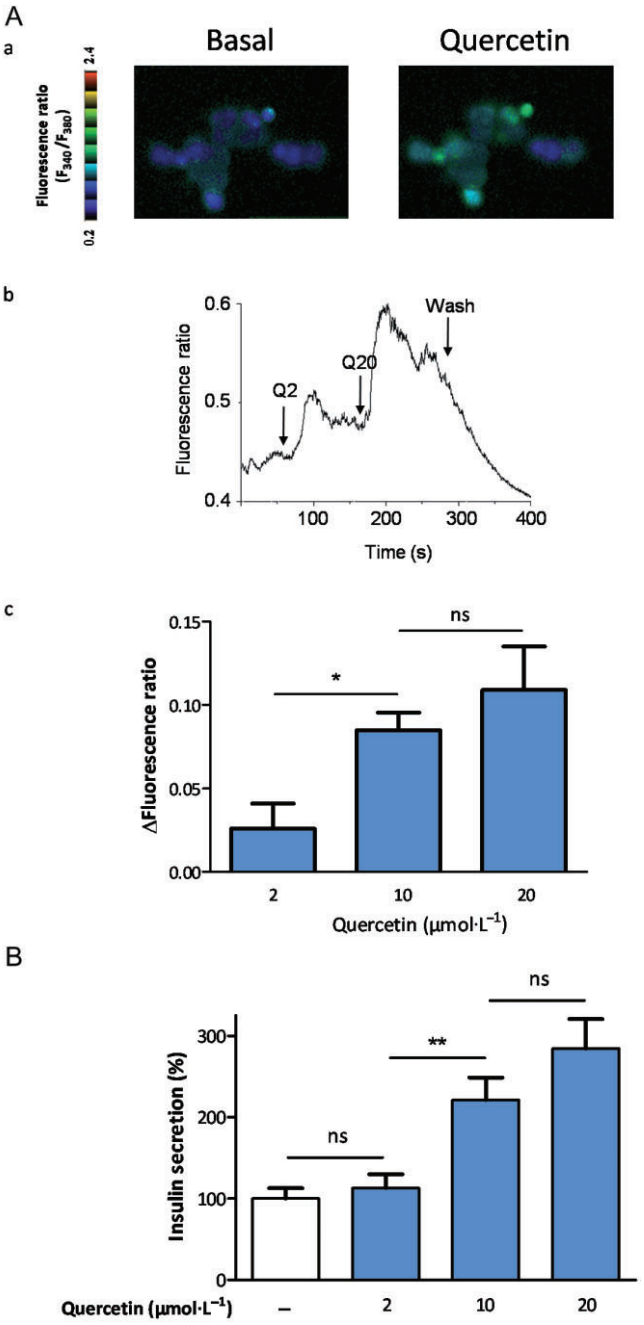

Figure 7

Quercetin increases $[Ca^{2+}]_i$ and stimulates insulin secretion in rat isolated pancreatic beta cells and islets respectively. (A) Effect of quercetin on the fluctuations in $[Ca^{2+}]_i$ in rat isolated pancreatic beta cells: (a) typical cell images illustrating the maximal fluorescence ratio (F_{340}/F_{380}) under basal conditions and after the addition of 20 µmol·L^{-1} quercetin. (b) Typical recordings of fluctuations in the fluorescence ratio. Arrows indicate the time of application of quercetin. Cells were stimulated with 2 µmol·L^{-1} quercetin (Q2) and fluorescence was allowed to stabilize before the addition of 20 µmol·L^{-1} quercetin (Q20), which was subsequently washed out. (c) Bar graph representing the maximal variation in the fluorescence ratio induced by increasing concentrations of quercetin (2, 10 and 20 µmol·L^{-1}). Results are presented as means ± SEM from five separate experiments. (B) Effect of increasing concentrations of quercetin (2, 10 and 20 µmol·L^{-1}) on insulin secretion in rat pancreatic islets. Results are presented as means ± SEM from three separate experiments. ***$P < 0.0001$; **$P < 0.001$; ns: $P > 0.05$; multiple comparison analysis for the different treatment conditions.

of the Ca^{2+} channels, favouring their opening at the resting cell membrane potential. Both in INS-1 cells and in the more physiological model of rat pancreatic islets, we observed that quercetin was active at concentrations previously observed in humans treated with this drug (2–5 µmol·L^{-1}) (Williamson and Manach, 2005).

Quercetin-induced insulin secretion is mediated by L-type Ca^{2+} channels

Flavonoids have been reported to inhibit the activity of the intracellular sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) in porcine brain microsomes at micromolar concentrations (Ogunbayo *et al.*, 2008). In pancreatic beta cells, SERCA pumps are responsible for Ca^{2+} uptake from the cytosol into the ER (Váradi *et al.*, 1996). Here, we found that thapsigargin, a natural plant-derived compound considered to be a tight-binding inhibitor of SERCA (Treiman *et al.*, 1998), had no inhibitory action on the effects of quercetin on INS-1 cells. While thapsigargin rapidly increased $[Ca^{2+}]_i$ as expected from the Ca^{2+} uptake blockade and subsequent depletion of Ca^{2+} in the ER (Ravier *et al.*, 2011), and induced insulin secretion (Johnson *et al.*, 2004), it did not modify the effects of quercetin on either parameter measured. The thapsigargin-resistant stimulating effect of quercetin, both on $[Ca^{2+}]_i$ and on insulin secretion, therefore occurred independently of SERCA activity (blunted) and of the amount of Ca^{2+} stored in the ER. Moreover, the absence of an effect of quercetin on $[Ca^{2+}]_i$ and insulin secretion in Ca^{2+}-free conditions provides further confirmation that Ca^{2+} entry is involved in its response.

In pancreatic beta cells, Ca^{2+} entry via voltage-gated Ca^{2+} channels is crucial for insulin secretion (Mears, 2004; Yang and Berggren, 2006). One striking result of our study was the major inhibition (approximately 70–90%) of the stimulating effect of quercetin on both $[Ca^{2+}]_i$ and insulin secretion by the DHP antagonist nifedipine. Indeed, DHP-sensitive L-type Ca^{2+} channels account for 50–90% of the global voltage-dependent Ca^{2+} current in mouse beta cells (Plant, 1988; Gilon *et al.*, 1997) and are responsible for the long-lasting elevation of $[Ca^{2+}]_i$ required for insulin secretion and beta cell function (Satin *et al.*, 1995; Horvath *et al.*, 1998; Mears, 2004; Drews *et al.*, 2010). Our patch-clamp experiments revealed that quercetin had no depolarizing effect on resting membrane potential but rather promoted a concentration-dependent direct increase in the HVA Ca^{2+} channel current present in INS-1 cells. In contrast, the fast-inactivating T-type Ca^{2+} current, also present in these cells, was insensitive to quercetin, as previously demonstrated in vascular smooth muscle Ca^{2+} channels (Saponara *et al.*, 2002). T-type Ca^{2+} currents are therefore unlikely to contribute to the effects of quercetin on insulin secretion.

We cannot definitively exclude the possibility that HVA channels other than L-type Ca^{2+} channels are also targeted by quercetin. Indeed, we observed a small nifedipine-resistant

component both to the quercetin-induced increase in $[Ca^{2+}]_i$ and insulin secretion, reflecting either the incomplete blockade of L-type Ca^{2+} channels or the contribution of another type of Ca^{2+} channel of the HVA family. SNX-482, an R-type Ca^{2+} channel antagonist, slightly inhibits glucose-mediated insulin secretion (Vajna *et al.*, 2001). P/Q-type Ca^{2+} currents have also been reported in INS-1 cells, but do not contribute significantly to the glucose-induced increase in $[Ca^{2+}]_i$ in these cells (Horvath *et al.*, 1998; Mears, 2004). However, it should be pointed out that if quercetin does activate non–L-type Ca^{2+} channels, these channels would contribute only moderately to the elevation of $[Ca^{2+}]_i$ and insulin secretion when compared to the contribution of DHP-sensitive L-type Ca^{2+} channels demonstrated here.

Quercetin lowers the voltage threshold for the opening of L-type Ca^{2+} channels

Our study shows that quercetin operates as a direct activator of L-type Ca^{2+} channels without the need for cell depolarization, and its effects are reminiscent of those of the DHP agonist Bay K 8644. Quercetin not only increased the peak L-type Ca^{2+} current at negative voltages but also shifted the threshold of activation of these channels towards more negative potentials by about 7 mV, which is perfectly in line with the data reported for rat tail artery smooth muscle cells (Saponara *et al.*, 2002). The enhancement of the peak current is probably only a consequence of this leftward shift, which reflects the increased sensitivity of the Ca^{2+} channels to negative voltages. In other words, in the presence of quercetin, L-type Ca^{2+} channels become more sensitive to a given depolarization and are activated at more negative potentials, potentials at which they are usually closed. This mechanism explains the induction of DHP-sensitive Ca^{2+} entry and insulin secretion by quercetin in the absence of KCl or glucose.

Apart from its effects on the beta cells used in our study, quercetin also behaves as an L-type Ca^{2+} channel activator in vascular cells (Saponara *et al.*, 2002; 2008; 2011). In our study, quercetin imitated the effects of the L-type Ca^{2+} channel activator Bay K 8644 in several respects: the shift in the voltage-dependent activation of the L-type Ca^{2+} current, the time course of the increase in both the Ca^{2+} current and $[Ca^{2+}]_i$, and the insulin secretion. However, despite these similarities at the functional level, the possibility that both of these compounds bind to the same site was excluded, as a maximally active concentration of Bay K 8644 did not prevent the effects of quercetin and *vice versa* (data not shown). Another flavonoid, myricetin, has been shown to activate L-type Ca^{2+} channels by binding to a site different from that of Bay K 8644 in vascular smooth muscle (Fusi *et al.*, 2003). At the functional level, in INS-1 cells, the two agonists induced a cumulative leftward shift in the voltage-dependent activation of L-type Ca^{2+} channels, which could explain their cumulative effect on $[Ca^{2+}]_i$.

In pancreatic beta cells, Ca^{2+} channels are co-localized with mature secretory granules in microdomains (Islam, 2010). Indeed, exocytosis is controlled by $[Ca^{2+}]_i$ just below the inner mouth of Ca^{2+} channels (Rorsman *et al.*, 2012). We observed that Bay K plus quercetin have additive effects on $[Ca^{2+}]_i$, whereas insulin secretion induced by Bay K plus quercetin was more than the sum of the effect of each compound alone. This co-operative effect of Bay K 8644 and quercetin on exocytosis could be related to a co-localization of Ca^{2+} channels with mature secretory granules and the formation locally of an increased $[Ca^{2+}]_i$ in microdomains. In line with this hypothesis, knowing that thapsigargin mobilizes Ca^{2+} from the ER and increases global cytosolic Ca^{2+}, we demonstrated that quercetin and thapsigargin had additive effects on both $[Ca^{2+}]_i$ and insulin secretion.

In conclusion, in the present study we showed that quercetin, on its own, is capable of stimulating insulin secretion by increasing $[Ca^{2+}]_i$. This effect is mediated by the direct activation of L-type Ca^{2+} channels; quercetin changes the voltage sensitivity of these channels by interacting with them at a site different from that of Bay K 8644. Quercetin probably enhances the intrinsic efficiency of glucose, which may explain the potentiating effect of quercetin on glucose-induced insulin secretion that we previously observed (Youl *et al.*, 2010). In the light of these findings, the potential use of quercetin in the control of type 2 diabetes deserves further investigation. This drug may be a useful pharmacological tool to further study the regulation of L-type Ca^{2+} channels in beta cells. Indeed, although many drugs are known to block these channels, only a few ligands (including the synthetic Bay K 8644) have been shown to activate them.

Conflict of interest

None declared.

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