Fluoxetine Inhibits Calcium Influx in MIN6 cells

Ahmed S. Hisab, M.phil.
Medical School, University of Nottingham, Nottingham, UK

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Abstract

Fluoxetine, a widely used antidepressant that primarily acts as a selective serotonin reuptake inhibitor, inhibits various membrane receptors and Ca^{2+} influx by unknown mechanism. In addition, it has been shown that Ca^{2+} is control mitochondrial metabolism and respiration. Here, effect of fluoxetine on the voltage gated Calcium channels in MIN6 cells was examined. Using Ca^{2+} imaging is to assess intracellular calcium. Fluoxetine 30 µM inhibited ca^{2+} levels. These results indicated a tighter binding of fluoxetine to the Calcium than to the resting state of other channels, suggesting a more potent inhibition of Ca^{2+} channels at physiological resting membrane potential. Altogether, these data demonstrate that clinically relevant concentrations of fluoxetine exert a voltage-dependent block of channels that may contribute to this antidepressant's pharmacological effects.

Keywords: Fluoxetine, Ca^{2+} channels, mitochondria, insulin secretion, pancreatic β-cell.

Introduction

Fluoxetine is a psychoactive drug widely prescribed in many psychiatric disorders, including depression, obsessive-compulsive disorder and bulimia nervosa. The therapeutic action of fluoxetine primarily results from the inhibition of serotonin reuptake (Stark, Fuller et al. 1985, Wong, Bymaster et al. 1995), thus enhancing serotoninergic neurotransmission. Besides this mechanism, fluoxetine has several other modulatory effects, such as inhibition of G protein-coupled receptors (Stanton, Bolden-Watson et al. 1993, Pälvimäki, Roth et al. 1996), blockade of monoamine oxidases (Mukherjee and Yang 1999), and modulation of several ionic channels. Indeed, it has been reported that fluoxetine is a potent blocker of K channels (Thomas, Gut et al. 2002, Choi, Choi et al. 2003, Kobayashi, Washiyama et al. 2003, Kennard, Chumbley et al. 2005), Na^{+} channels (Pancrazio, Kamatchi et al. 1998) and Ca^{2+} channels (Deák, Lasztóczi et al. 2000). T-type Ca^{2+} channels, a subgroup of voltage-gated Ca^{2+} channels, are the target of many

antipsychotic drugs. For example, study by Deak et al. (2000) reporting that fluoxetine inhibits T-type Ca^{2+} currents in rat hippocampal pyramidal cells.

The widely accepted key process of glucose stimulated insulin secretion from the beta cell upon glucose stimulation is ATP-sensitive K^+ channel (K^+-$_{ATP}$) dependent. In beta cells, the glut-2 transporter facilitates glucose entry. The glucose is then phosphorylated to glucose -6- phosphate by the enzyme glucokinase (GK), which has a Km of approximately 5 mM for glucose explains the concentration dependence of the β-cell response to glucose in the physiological range to stimulate insulin release (Matschinsky, Liang et al. 1993, Aguilar-Bryan and Bryan 1999). Pyruvate is formed from glucose, which is either transformed to lactate by anaerobic glycolysis or enters the mitochondria and is formed by tricycaboxylic acid (TCA) cycle to activate the respiratory chain in order to generate adenosine triphosphate (ATP). Increased ATP/ADP ratio causes closure of the K^+-$_{ATP}$ sensitive channel, leading to a depolarization of cell membrane (Aguilar-Bryan and Bryan 1999), allowing the L- type Ca^{2+} channels in the cell membrane to open, causing an increase in cytosolic Ca^{2+}. This promotes exocytosis of insulin secretory vesicles.

Therefore, it is of importance to characterize fluoxetine's action on the Ca^{2+} influx and their inhibition by fluoxetine should be investigated for a better understanding of this antidepressant's therapeutic action and side effects. In this article, it has been reported the first electrophysiological study of the inhibitory effects of fluoxetine on Ca^{2+} influx of MIN 6 pancreatic beta cells.

Methodology

Cell line

The MIN6 (Mouse pancreatic beta) cell line is a well-established, time honored, model for the study of pancreatic b-cell function (Miyazaki, Araki et al. 1990, Ishihara, Asano et al. 1993, Daunt, Dale et al. 2006, Cataldo, Cortés et al. 2015). The main advantage of a cell line for pancreatic b-cell research is that it large numbers of phenotypically homogenous cells are available which are devoid of paracrine and neuroendocrine effects that arise from interaction with the other endocrine and neuronal cell types that reside within the pancreatic islet (Ishihara, Asano et al. 1993). Although MIN6 have been reported to contain and secrete other pancreatic endocrine hormones, these cells still remain primarily b-cell in function (Nakashima, Kanda et al. 2009). Cells were grown, and the chronic experiments performed, in Dulbecco's modified Eagle's medium (DMEM) which contained 25mM glucose and was supplemented with: 2mM L glutamine, 10% fetal calf serum, 50 mM 2-mercaptoethanol, and 25mM HEPES. In this study, cells from passage numbers 35–42 were used.

Intracellular Ca^{2+} measurements

To enable imaging (Smith, Proks et al.)$_i$ of MIN6 cells, cells were loaded with the fluorescent Ca^{2+} indicator dye Fluo-4 AM (acetoxymethyl). Fluo-4 is an analog of Fluo-3 with the two chlorine substituents replaced by fluorines. Manufacturers report increased fluorescence excitation at 488 nm resulting in higher fluorescence signal levels (Invitrogen). Fluo-4 AM can be used at lower dye concentrations to generate the same fluorescence signal intensity (Gee, Brown et al. 2000). Lower loading concentrations and dye loading times of Fluo-4 AM make its use in cells a less invasive practice (Gee, Brown et al. 2000).

To monitor changes in the bulk intracellular Ca^{2+}, we used the Ca^{2+} fluorophore Fluo-4, as previously descried (Duchen, Smith et al. 1993, Smith, Duchen et al. 1995). MIN6 cells, they were plated onto 22 mm glass cover-slips. The cells were incubated with Fluo 4-AM (at a final concentration of 1μM in 0.1% BSA (wt/vol), 10 mM glucose, Hank's solution) for 20 minutes in the dark at room temperature (21ºC-22ºC). Loading was performed at room temperature to decrease the likelihood of dye compartmentalisation in accordance with the manufacturer's recommendations (Invitrogen). The cells were washed by perifusion for 10 minutes in Ca^{2+}-containing Hank's solution. To perifuse the microscope chamber, solutions were gravity-fed through polythene tubing. All drug additions were made via bath perifusion. Exchange between solutions was performed using an electrically controlled two-way valve.

The MIN6 cells were unresponsive to the 50 mM K^+ calibration procedure and 10 mM glucose; or if the Ca^{2+} levels continuously decreased throughout the experiment, MIN6 cells that did not respond to extracellular Ca^{2+} removal were also excluded. Data were output as % fluorescence, and expressed as mean ± S.E.M. For each protocol presented at least 4 different MIN6 cell preparations were used and "n" the number of MIN6 cells given as the sum total from these preparations.

Results

Calcium channels are inhibited by fluoxetine in response to glucose

The Previous results suggest that fluoxetine and low Ca^{2+} both inhibit mitochondrial function of MIN6 cells. The aim of these experiments was to assess the effect of fluoxetine on Ca^{2+} influx in presence of 10 mM glucose. At basal glucose concentrations, Ca^{2+} transients were usually absent (Fig. 1A). Addition of 10 mM glucose after a 1 minute delay caused transient fell and followed by a significant increase in intracellular Ca^{2+} ~11±0.83% ($p < 0.0001$, Sidak's multiple comparisons test) compared to the basal (Fig. 1A&B). 30 μM fluoxetine inhibited Ca^{2+} in the presence 10 mM glucose by ~9.4±2.7% ($p < 0.05$, Sidak's multiple comparisons test) which partly

recovered an washout ($p < 0.0001$, Sidak's multiple comparisons test) (Fig. 1A&B). Final addition of 50 mM K^+ (depolarization of cell membrane to –40 mV (Sharp 1996, Aguilar-Bryan and Bryan 1999) caused a rapid increase in Ca^{2+} levels (Fig. 1A). The experiment protocol was repeated but 50 mM K^+ was replaced by perifusion of low Ca^{2+} which mimic the effect of fluoxetine (Fig. 2).

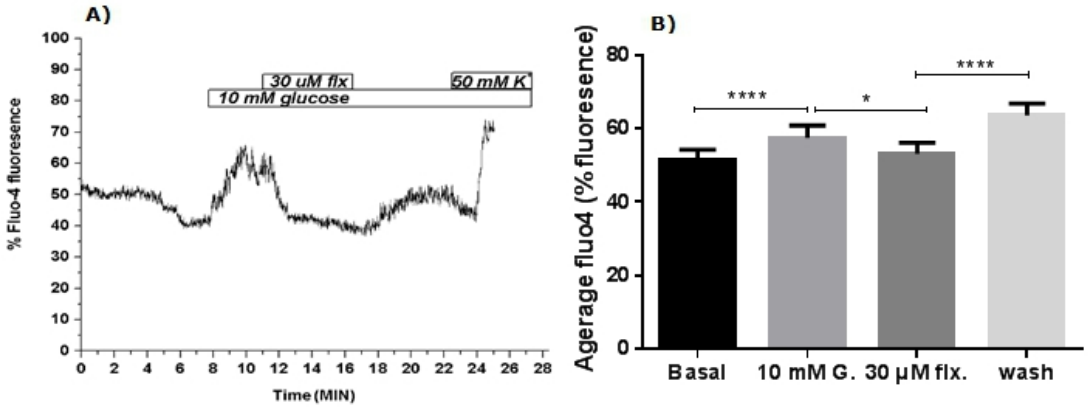

Fig. 1: Effect of 10 mM glucose and 30 µM fluoxetine on the Ca^{2+} response in MIN6 cells. A) Representative effect of 10 mM glucose and 30 µM fluoxetine on the Ca^{2+} influx of a single cell measured by the % change in fluoresence signal relative to 50 mM K^+. B) Mean (±SEM) response to the serial additions as indicated 10 mM glucose (G.) and 30 µM fluoxetine (flx). N= 50 cells collected from 3 experiments where the minimum number of cells is 3 in each one.

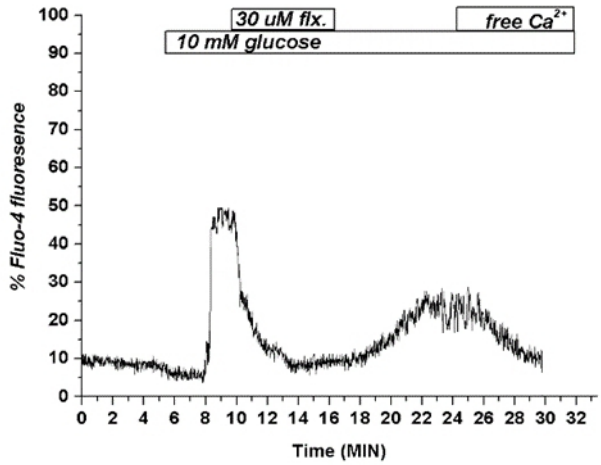

Figure 2: Representative effect of 10 mM glucose and 30 µM fluoxetine on the Ca^{2+} influx of a single cell. N= 40 cells collected from 3 experiments where the minimum number of cells is 3 in each one.

To verify that fluoxetine inhibits voltage calcium channels. The effect of 30 µM fluoxetine in response to 20 µM tolbutamide (Tb) was explored. Since Tb blocks K^+-$_{ATP}$ channel and increases Ca^{2+} influx and reveal any effect that may be seen regarding to direct block of Ca^{2+} voltage dependent channel by fluoxetine. Tolbutamide (20 µM) stimulated Ca^{2+} signal by ~15±1.48% ($P< 0.0001$, Dunnett's multiple comparisons test) after a delay of 1 minute. Subsequent addition of 30 µM fluoxetine abolished the Ca^{2+} influx in the presence of tolbutamide ($P< 0.0001$, Dunnett's multiple comparisons test)(Fig. 3A&B). The vehicle for the fluoxetine, water, had no effect on the Ca^{2+} influx in the presence of tolbutamide (Fig. 3C&D).

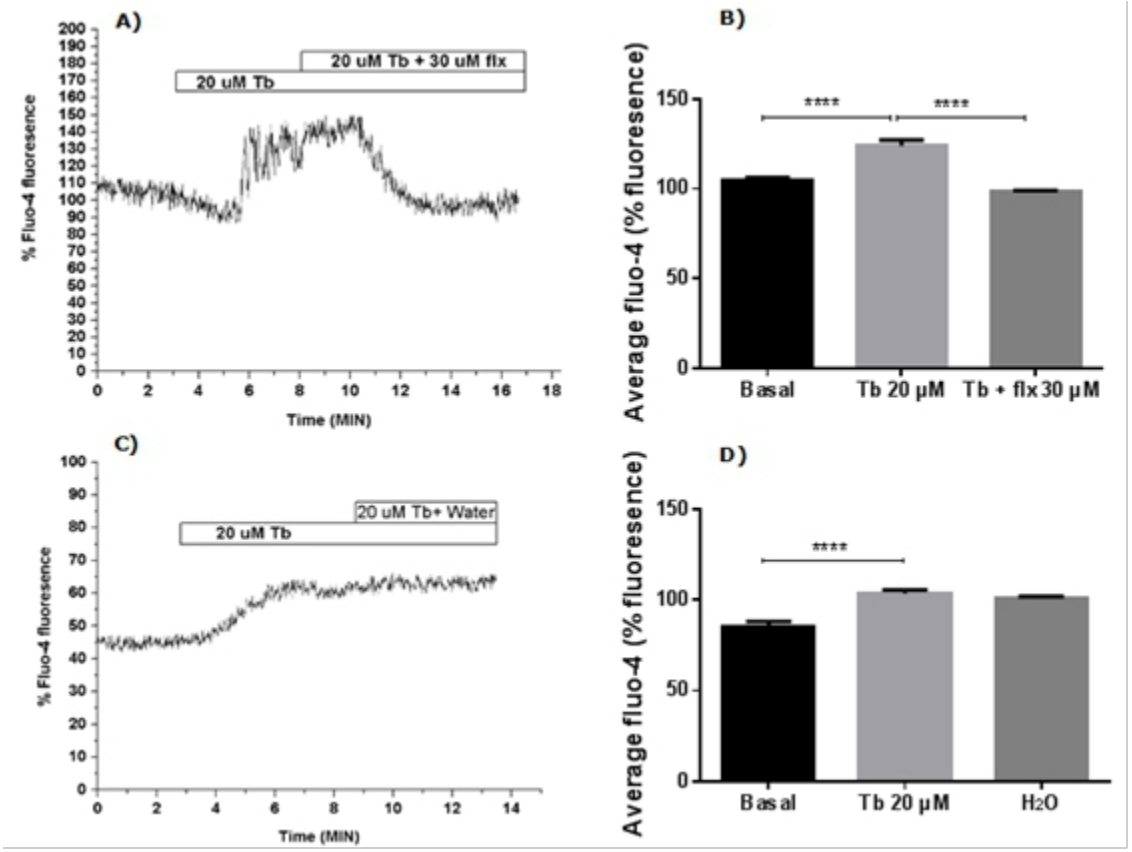

Fig. 3: Effect of 30 µM fluoxetine on Ca^{2+} in presence of 20 µM tolbutamide (Tb). A, C) Representative effect of 20 µM Tolbutamide(Tb) and 30 µM fluoxetine (flx) or water respectively on the Ca^{2+} influx of a single cell monitored by the change in Fluo-4 fluorescence. B, D) Mean (±SEM) of single cells in response to the serial additions as indicated 20 µM Tolbutamide and 30 µM fluoxetine or H_2O respectively. N= 55 cells collected from 5 experiments where the minimum number of cells is 3 in each one.

Figure 4. Shows that the percentage effect of 30 µM fluoxetine in the presence of 20 µM tolbutamide. The data showed that 30 µM fluoxetine

significantly decreased Ca^{2+} influx compared to the vehicle (H_2O) ($P< 0.0001$, Mann Whitney test).

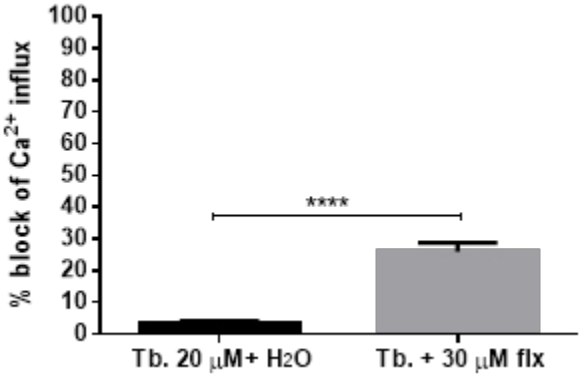

Fig. 4: The percentage block of 30 µM fluoxetine in presence of tolbutamide (Tb) on the Ca^{2+} influx in MIN6 cells. Experimental data for this figure were sourced from figure 14B&D. Values are shown as % change in Ca^{2+} levels compared to the vehicle (H_2O). Data are presented as Mean ± SEM.

Calcium co-regulates oxidative metabolism in pancreatic beta cells

The ability of Ca^{2+} to control mitochondrial metabolism and respiration has been mainly attributed to the activation of Ca^{2+}-regulated dehydrogenase (Denton 2009, Griffiths and Rutter 2009), which accelerates oxidative metabolism associated with the formation of the reduced NAD(P)H. The aim of this experiments was to investigate the effect of Ca^{2+} on the NAD(P)H levels. Fig. 5A shows that the effect of 10 mM glucose and Ca^{2+} free on the NAD(P)H levels. Once NADPH levels reached steady-state after glucose stimulation ($p< 0.0001$, Dunn's multiple comparisons test), subsequent addition of free Ca^{2+} decreased NAD(P)H levels in the presence of glucose ($p<0.0001$, Dunn's multiple comparisons test)(Fig. 5B). Re-addition of Ca^{2+} recovered the NAD(P)H levels to the control (glucose) levels ($p< 0.0001$, Dunn's multiple comparisons test)(Fig. 5A). In summary, these results suggest that low Ca^{2+} may inhibit mitochondrial respiration.

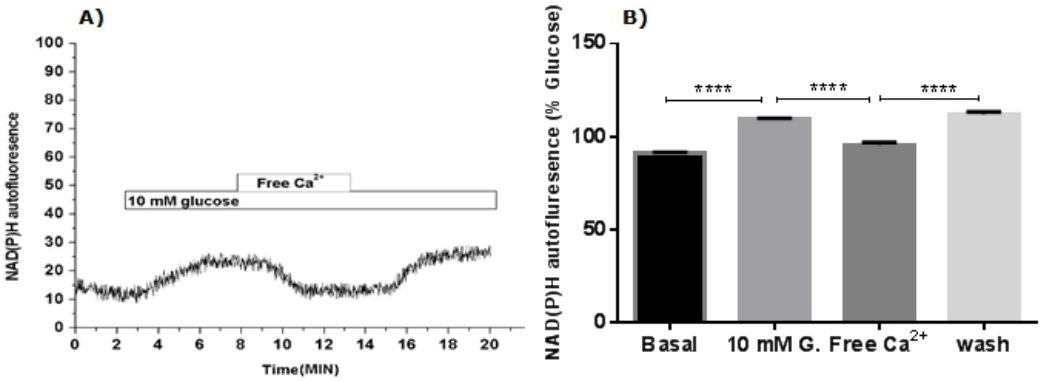

Fig. 5: Effect of calcium signalling on NAD(P)H responses in MIN6 cells. A) Representative effect of 10 mM glucose (G.) and free Ca $^{2+}$ on the NAD(P)H levels of a single cell. B) Mean (±SEM) NADPH fluorescence for single cells in response to the serial additions as indicated. N= 68 cells collected from 4 experiments where the minimum number of cells is 15 in each one.

It has been shown that metabolism affects Ca^{2+} entry in MIN6 cells (Smith, Sellers et al. 2001, De Marchi, Thevenet et al. 2014). The aim of this experiment was to investigate the effect of rotenone on the Ca^{2+} influx. Within a minute of addition, 10 mM glucose produced a large increase in the Ca^{2+} influx which is ~17±1.27% (P< 0.0001, Sidak's multiple comparisons test) compared to the basal (Fig. 6A&B). Subsequent addition of 1 µM rotenone (complex I inhibitor) significantly inhibited Ca^{2+} signal by ~18±2.47% (P< 0.0001, Sidak's multiple comparisons test) in response to 10 mM glucose (Fig. 6A&B). Final addition of 50 mM K^+ (depolarization of cell membrane) considerably increased the Ca^{2+} signal (Fig. 6A).

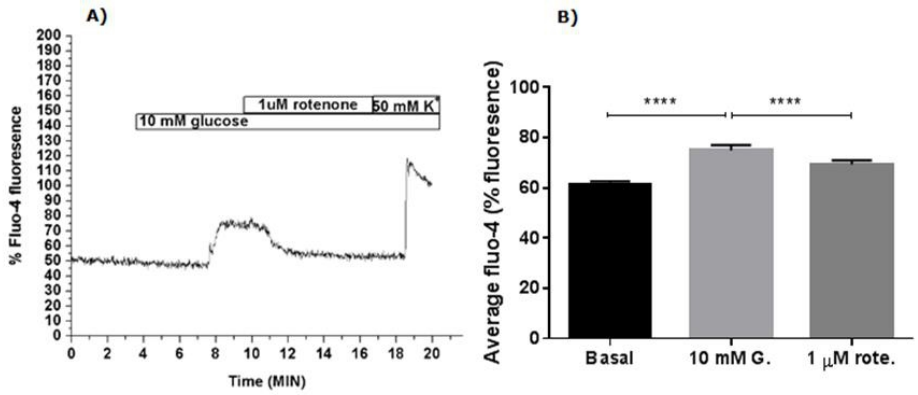

Fig. 6: Effect of 1 µM rotenone on the Ca^{2+} in response to 10 mM glucose. A) Representative effect of 1 µM rotenone (rote) in the presence 10 mM glucose on the Ca^{2+} influx of a single cell monitored by the % change in Fluo-4 fluorescence relative to 50 mM K^+ (%100). B) Mean (±SEM) of single cells in response to the serial additions as indicated 1 µM rotenone (rote) in the presence 10 mM glucose (G.). N= 76 cells collected from 3 experiments where the minimum number of cells is 3 in each one.

Discussion

Effect of glucose and fluoxetine on Ca^{2+} influx

Fluoxetine significantly inhibited Ca^{2+} influx by either a direct inhibition of L- type voltage dependent Ca^{2+} channels or an indirect impairment of mitochondria of pancreatic beta cells. In the present experiments, the 11% stimulation in the level of Ca^{2+} produced by 10 mM glucose for MIN6 cells; these results are consistent with a central role for mitochondrial oxidative phosphorylation in coupling changes in glucose concentration with insulin release. For example, it has been found that, glucose-stimulated hyperpolarization of $\Delta\psi m$ by increasing ATP/ADP ratio, leads to closure of K^{+}-$_{ATP}$ channels, followed by depolarization cell membrane, leading to open L-Type Voltage Ca^{2+} channels and increased Ca^{2+} (Duchen, Smith et al. 1993, De Marchi, Thevenet et al. 2014). However, 30 µM fluoxetine significantly but reversibly inhibits Ca^{2+} levels. Isotonic replacement of Na^{+} (by 50 mM K^{+}) caused a rapid increase Ca^{2+} influx of these cells; an observation consistent with depolarization the cell membrane potential and activation of voltage-dependent Ca^{2+}channels (Duchen, Smith et al. 1993).

Fluoxetine blocks L- type voltage dependent Ca^{2+} channels

In present study, we found that 20 µM tolbutamide increased Ca^{2+} influx; an observation consistent with previous finding which indicates that tolbutamide, stimulate insulin release from pancreatic β-cells predominantly by blocking the activity of ATP-sensitive K^{+} (K^{+}-$_{ATP}$) channels in the plasma membrane (Duchen, Smith et al. 1993, Smith, Proks et al. 1999). This causes membrane depolarization, activation of voltage-gated Ca^{2+} channels, increased Ca^{2+} influx, an increase in the cytosolic Ca^{2+} and insulin secretion.in the presence of tolbutamide. 30 µM fluoxetine significantly decreased Ca^{2+}. These data suggest that fluoxetine inhibits the L-type voltage dependent Ca^{2+} channel by direct mechanism in MIN6 cells. These results are comparable with The IC_{50} values obtained with fluoxetine for blocking of other ionic channels in different preparations. For example, the IC_{50} value of fluoxetine block of T-type voltage calcium channels is 6.8 µM and both L- and N- type voltage calcium channels is 3 µM in rat hippocampal pyramidal cells (Deák, Lasztóczi et al. 2000) and (Pancrazio, Kamatchi et al. 1998) found that 20 µM fluoxetine inhibited N-type voltage Ca^{2+} channel in bovine adrenal chromaffin cells. (Traboulsie, Chemin et al. 2006) indicated that 30 µM fluoxetine blocked T-type voltage calcium channel in human embryonal kidney cells. To our knowledge, this is the first evidence for the inhibition of calcium channels by fluoxetine in MIN6 cell lines.

Fluoxetine inhibits Ca^{2+} entry by blocking metabolism

In the present study, 10 mM glucose increased level of Ca^{2+} influx in MIN6 cells. This can be accounted for by central role of mitochondrial oxidative phosphorylation in coupling changes in glucose concentration with insulin release (Duchen, Smith et al. 1993, De Marchi, Thevenet et al. 2014). It has been found that inhibition of respiration by inhibiting ATP synthesis with rotenone rapidly decreased intracellular Ca^{2+} in MIN6 cells. Our results underline the importance of continuous mitochondrial ATP synthesis to maintain beta cell Ca^{2+} signalling.

Conclusion

These data suggest that fluoxetine inhibited Ca2+ influx by two mechanisms: by direct block of voltage-dependent Ca2+ channels or by direct inhibition of mitochondrial function.

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