INCREASE IN $[\text{Ca}^{2+}]_i$ AND SUBSEQUENT INSULIN RELEASE FROM $\beta$TC3-CELLS WITH THE L-TYPE Ca$^{2+}$-CHANNEL ACTIVATOR, FPL 64176

Janne Springborg, Jesper Gromada, Peter Madsen, Annemarie R. Varming, and Jannie Fuhlendorff

Novo Nordisk A/S
Novo Allé, DK-2880 Bagsvaerd, Denmark

INTRODUCTION

The regulation of calcium entry is central in the control of insulin secretion from the pancreatic $\beta$-cell. Glucose is the primary physiological stimulator of insulin secretion and a principal action of glucose is consequently to depolarize the $\beta$-cell by closing $K^+$-channels sensitive to the ATP produced during metabolism of the sugar$^{1,2}$. This will lead to membrane depolarization and activation of the voltage-dependent L-type Ca$^{2+}$-channels leading to increase in intracellular calcium concentration, $[\text{Ca}^{2+}]_i$, and insulin secretion. Pharmacological important secretagogues such as sulphonylureas (e.g. glibenclamide) close the ATP-sensitive $K^+$-channels leading to depolarization of the $\beta$-cell membrane, activation of voltage gated Ca$^{2+}$-channels by opening the channels and initiation of repetitive action potentials. The resulting calcium influx increases the $[\text{Ca}^{2+}]_i$ and thereby triggers insulin release. Compounds interfering with this process also regulate the insulin secretion and therefore the L-type Ca$^{2+}$-channel might, like the ATP-sensitive $K^+$-channel, be a suitable target for therapeutic intervention in treatment of Non-Insulin Dependent Diabetes Mellitus (NIDDM). The ligands for L-type Ca$^{2+}$-channels can be divided into three categories: dihydropyridines, phenylalkylamines and benzothiazepines. Bay K 8644 has become the prototype for the class of dihydropyridines. Some of the dihydropyridines are chiral and resolution of these have revealed the (−)-enantiomers as potent activators, whereas the (+)-enantiomers are weak in-activators$^3$. Analysis of the calcium current activation and deactivation kinetics shows that Bay K 8644 increases the mean open time

* Author for correspondence at address $\beta$-Cell Biology 6B.3.99, Novo Nordisk, Novo Alle, DK-2880 Bagsvaerd, Denmark.
while it leaves the mean closed times of the Ca\textsuperscript{2+}-channel unchanged\textsuperscript{4}. While Bay K 8644 acts as an activator, other dihydropyridins such as nitrendipine and nifedipine acts as inactivators like the phenylalkylamine, verapamil. Recently, a new benzoyl pyrrole compound, FPL 64176 has been synthesized and shown to have properties consistent with Ca\textsuperscript{2+}-channel activation\textsuperscript{5}.

We have tested FPL 64176 for insulin release in monolayers of βTC3 cells and compared the potency with various drugs affecting L-type Ca\textsuperscript{2+}-channels and ATP-sensitive K\textsuperscript{+}-channels. The effect of FPL 64176 on [Ca\textsuperscript{2+}], is examined by the use of the calcium fluorescent indicator fura-2/AM. Here, for the first time, we describe the action of a non-dihydropyridine Ca\textsuperscript{2+}-channel activator, FPL 64176, on the insulin secretion.

**MATERIALS AND METHODS**

**Synthesis**

FPL 64176 was synthesized at Novo Nordisk essential as described\textsuperscript{6}. Bay K 8644 (RBI Research Biochemicals) was resolved by HPLC (multiple injections of 100 μg, on a column of Chiracel OD, 4.6 × 250 mm, eluted with hexane: 2-propanol (9:1), with a flow of 0.45 ml/min. and detected at 254 nm) to give (−)-(S)-Bay K 8644 and (+)-(R)-Bay K 8644 > 99.2 and 92% ee, respectively.

**Cell Culture**

All experiments were carried out with the insulin secreting cell line, βTC3, which was kindly provided by Cold Spring Harbor Laboratory. The βTC3 cell line is derived from insulinomas of transgenic mice that express the simian virus 40 T antigen under control of the insulin promoter\textsuperscript{7}. Cells were plated in Nunclon culture flasks (800 ml) and grown in Dulbecco’s modified Eagle’s medium (5.5 mM glucose, GIBCO), (DMEM) in the presence of 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin in an atmosphere of 5% CO\textsubscript{2} in air at 37°C. Once a week the cells were split 1:7 and the medium was changed after 4 days.

**Insulin Release**

The insulin release experiments were carried out in monolayer cultures in batch type incubation at 37°C. After trypsination the cells were plated in 12-wells dishes (area 4.5 cm\textsuperscript{2}/well, approximately 4 × 10\textsuperscript{5} cells/well) and cultured until 70–90% confluency (2 days). The cells were washed twice to remove dead or floating cells with Hepes Krebs Ringer (Hepes K.R.) medium consisting of 10 mM Hepes, pH 7.4, 119 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl\textsubscript{2}, 1.19 mM MgSO\textsubscript{4} · 7H\textsubscript{2}O, 25 mM NaHCO\textsubscript{3}, 0.1% human serum albumin and 0.15 mM glucose. The cells were then incubated for one hour with the medium whereafter the compounds were added in fresh Hepes K. R. medium. After another hour incubation the medium from each well was transferred to minisorb vials which were centrifuged at 190 × g for 5 min at 20°C. The supernatant was used in a double sided enzyme-linked-immunosorbant-assay (ELISA) to determine the insulin released. The wells were washed twice with PBS and added 1 ml 0.1 M NaOH. After 20 min the NaOH containing the cells were transferred to minisorb vials and the content of protein was determined using Pierce protein assay.