Neuroscience Letters 523 (2012) 30–34

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Calcium signals induced by FGF-2 in parasympathetic neurons: Role of second messenger pathways

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HIGHLIGHTS

- The PLCγ, PI3-K and MAPK pathways contribute to FGF-2 induced calcium signals in chick parasympathetic neurons.
- Inhibition of PLCγ induces calcium oscillations in chick ciliary ganglion neurons.
- Downregulation of voltage dependent calcium channels following PLCγ inhibition.

ARTICLE INFO

Article history:
Received 22 March 2012
Received in revised form 25 May 2012
Accepted 13 June 2012

Keywords:
FGF-2
Ciliary ganglion
Signal transduction pathways
Calcium signalling

ABSTRACT

Basic Fibroblast Growth Factor, or FGF-2, has been shown to promote neuronal survival and neurite outgrowth in dissociated neurons from the embryonic chick ciliary ganglion; in these effects the three main signal transduction pathways downstream the activated FGFR receptor, i.e. the MAPK, the PI3-K and the PLCγ ones, are differentially involved. While it has been shown that FGF-2 can elicit long lasting elevations in intracellular calcium concentration, $[Ca^{2+}]_i$, the role of the three pathways in this process has not been elucidated. Here we show, by means of pharmacological inhibitors, that all three are involved, at a different extent, in the generation of the $[Ca^{2+}]_i$ increase induced by FGF-2; in particular, inhibition of the PLCγ pathway, in addition to reducing the number of responsive cells, induces, in a significant population of cells, basal calcium oscillations in the absence of the growth factor and interferes with calcium signals elicited by depolarization. We propose that this complex behaviour can be due to a perturbation in PIP_2 levels at the plasmamembrane.

1. Introduction

Basic Fibroblast Growth Factor (bFGF or FGF-2) is a potent neurotrophic factor, that has been shown to determine the fate of several neuronal populations [1,3]. Its action is mediated, downstream of its receptor(s), by different signal transduction pathways, whose differential activation may explain the specificity of action of the factor. The processes in which it has been shown to play a role range from neuronal survival to neurite growth and nerve repair [4,8,13,17,18]. In a well established model of peripheral neurons, cultured E7/E8 embryonic chick ciliary ganglion neurons, we have previously shown that FGF-2 can promote neuronal survival [4] and neurite outgrowth [25] in dissociated and organotypic cultures. Moreover, it induces long lasting changes in $[Ca^{2+}]_i$ through a mechanism mainly dependent on calcium influx from the extracellular medium [4]. We also described the specific contributions to cellular responses of the three main signal transduction pathways downstream of the activated FGFR1 receptor: the PLCγ, the ERK/MAPK and the PI3-K pathways. While the first two are involved in the control of neurite outgrowth but not in the pro-survival action of FGF-2, the third positively affects both processes [7]. However, the involvement of these pathways in FGF-2-induced calcium signalling has not been elucidated. We now describe their specific contribution to the generation of the long lasting somatic increases in $[Ca^{2+}]_i$, providing evidence for an involvement of all these pathways, with a peculiar role for the PLCγ one.

2. Methods

2.1. Cell cultures

Chick ciliary ganglion (CG) neurons were obtained from E7/E8 embryos and maintained for 24 h in a chemically defined N2

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http://dx.doi.org/10.1016/j.neulet.2012.06.035

medium as previously described [4]. Briefly, ganglia were incubated in divalent cation-free phosphate-buffered saline containing 0.06% trypsin at 37 °C for 5 min and, after a gentle trituration, cells were resuspended in N2 medium. Cells were then plated in the middle area of 40 mm glass coverslips coated with poly-D-lysine (100 μg/ml) and laminin (1 mg/ml). Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, MO).

2.2. Calcium imaging

$[Ca^{2+}]_i$ was monitored using the Ca^{2+} indicator dye FURA-2 acetoxymethylester (FURA-2AM, Molecular Probes, Inc.). Cells were loaded for 45 min at 37 °C with 2 μM FURA-2AM in N2 medium and subsequently washed in standard Tyrode solution of the following composition (in mM): NaCl 154, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, glucose 5.5, NaOH to pH 7.34.

After dye loading cells were transferred to a perfusion chamber (Bioptechs, USA) connected to a peristaltic pump and mounted on an inverted fluorescence microscope (Nikon TE-2000-S) equipped with an S Fluor 20× objective (N.A. 0.75), a Xenon lamp illumination system and a CoolSNAP Roper Scientific/Photometrics CCD camera. All experiments were performed at room temperature. A gravity microperfusion system, regulated by electrovalves, was employed to switch from the standard Tyrode solution to the same medium containing human recombinant FGF-2 (20 ng/ml, Alomone Labs, Israel) alone or with the inhibitors of three signalling pathways: PD98059 (25 μM; Calbiochem) for the ERK/MAPK pathway, Wortmannin (WM, 10 nM) for the PI3-K one and U73122 (0.5 μM; Calbiochem, Darmstadt, Germany) for the PLCγ one. Before starting the experiments, cultures were incubated for 15 min in the presence of the inhibitors that were maintained during the perfusion with the growth factor. The concentrations were chosen on the basis of previous observations [7] that showed that these were the optimal doses for interfering with the FGF-2 induced survival and neurite growth while not affecting these parameters in the sole presence of adhesion molecules. All three inhibitors, at the above concentrations, had no effect on neuronal survival (up to 48 h for the PLC and ERK/MAPK inhibitors and up to 24 h for WM).

Calcium measurements were performed exciting FURA-2AM every 0.8 s alternatively at 340 nm and 380 nm, and emission was recorded at 510 nm. For each experiment about 30 ROIs (Regions of Interest) corresponding to the cell bodies were chosen. Images were visualized on a computer with the dedicated acquisition software Metafluor (Universal Imaging Corporation, PA).

2.3. Statistical analysis

Each experiment was performed on four different cell cultures for each experimental condition. By this way sampling distributions were drawn for each condition and for every parameter of interest (i.e. number of responsive cells, peak amplitude of the response and time to peak) allowing data to be represented as mean ± standard error, even in case of the response percentages (Fig. 1). Normality of the residuals distribution has been verified through Shapiro–Wilk normality test and variances resulted to be quite homogeneous over each condition (Levene's test of homogeneity of variances gave a p-value > 0.30). For these reasons one-way ANOVA was a suitable method for significance assignment. Statistical analysis was carried out using SPSS software and other self-made code, while raw data were analysed with IGOR Pro software (6.03a version): in this context, peak values were calculated subtracting the baseline value for each trace from the maximum value reached during drug treatment.

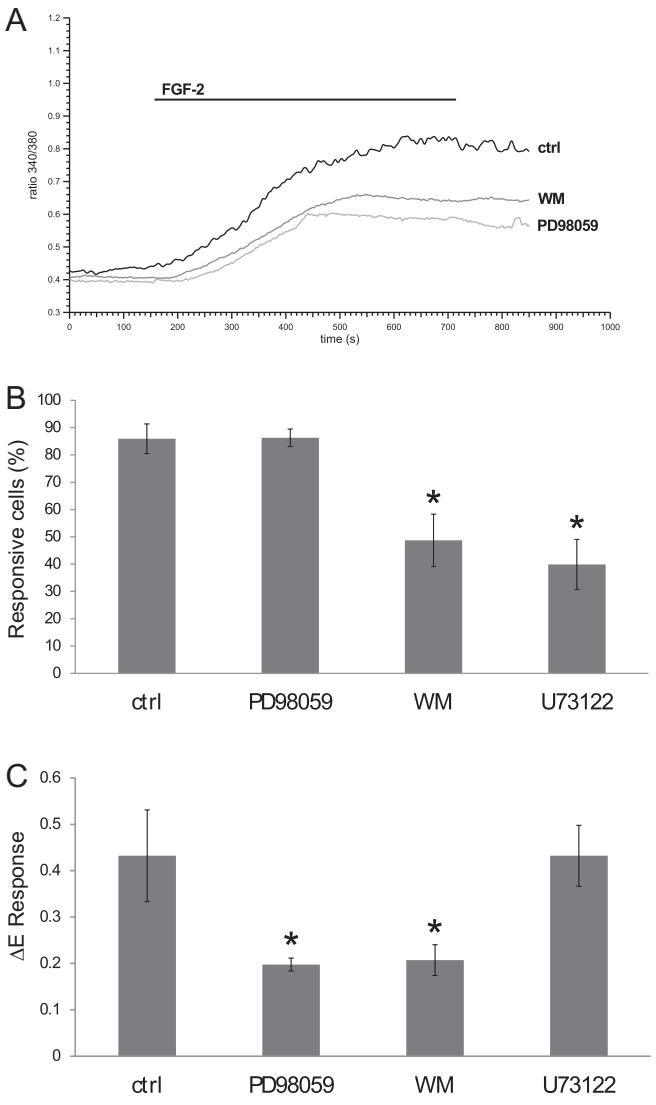

Fig. 1. Inhibitors of the MAPK, PI3-K and PLCγ pathways differentially affect the long lasting increase in $[Ca^{2+}]_i$ induced by FGF-2. (A) Superimposed responses of three typical responses to the factor, in control conditions (black trace), and in the presence of 25 μM PD98059 (dark grey) and 10 nM Wortmannin (light grey). (B) Percentage of responsive cells with FGF-2 alone or in cells preincubated with the three inhibitors (U73122: PLCγ inhibitor; 0.5 μM). n = 149 for control cells, n = 182 for cells preincubated with PD98059, n = 269 for cells preincubated with WM, n = 346 for cells preincubated with PLCγ. (C) Peak response amplitudes in the same conditions as in B. n = 128 for control cells, n = 157 for cells preincubated with PD98059, n = 131 for cells preincubated with WM, n = 138 for cells preincubated with U73122. All data were obtained from 4 separate experiments. In B and C, $^*p < 0.05$.

3. Results

3.1. Effects of the inhibitors of the ERK/MAPK and PI3-K signal transduction pathways on FGF-elicited calcium signals

We have previously shown that the increases in somatic $[Ca^{2+}]_i$ induced by stimulation of E7 CG neurons with 20 ng/ml FGF-2 are characterized by a great variability in the time course, with a long lasting plateau preceded in some cases by a transient phase (Fig. 4 of Ref. [4]). This heterogeneity made it difficult to evaluate the role of inhibitors of the signal transduction pathways in acute experiments; for this reason, we used preincubation protocols and quantified the effects of the pharmacological agents on several parameters: number of responsive cells, peak amplitude of the response, time course.