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

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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.

Keywords: FGF-2, ciliary ganglion, signal transduction pathways, calcium signalling

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 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 a S Fluor 20X 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 24h 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 analyzed 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.

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.

In control experiments, the percentage of cells responsive to FGF-2 was 85.9 ± 5.4 % (n = 149). Mean peak amplitude (ΔR) was 0.43 ± 0.10.

When cells were preincubated for 15 min with the inhibitor of the MAPK pathway, PD98059 (25 μM), subsequent perfusion with 20 ng/ml FGF-2 induced responses in a comparable percentage of cells (Fig. 1B); however, the ΔR was significantly reduced (responding cells: 86.2 ± 3.2 %; n = 182; ΔR = 0.20 ± 0.01, $p < 0.05$; see Fig. 1C).

When cells were challenged with FGF-2 after 15 min of preincubation with 10 nM WM, the inhibitor of the PI3-K enzyme, both the percentage of responsive cells (48.7 ± 9.6 %, n = 269; $p < 0.05$; Fig, 1B) and the peak amplitude (ΔR = 0.21 ± 0.03; $p < 0.05$; Fig. 1C) were significantly reduced.

The time course was similar for control cells and cells preincubated with PD98059 and with WM (Fig. 1A); time to peaks were respectively 355,8 ± 11.6 s (control), 328.9 ± 10.2 s (PD98059) and 332.2 ± 11.6 s (WM).

3.2. Effects of the PLCγ inhibitor U73122

The picture obtained from cells preincubated with the PLCγ inhibitor U73122 (0.5 μM) was more complex. In these conditions, a significant percentage of cells (35%, n = 121 cells out of 346 cells analyzed) showed spontaneous oscillations and stimulation with FGF-2 did not elicit any detectable change in the activity pattern (Fig. 2A); moreover, 87 cells showing a stable baseline did not respond to the factor with an increase in $[Ca^{2+}]_i$. Thus only 138 cells gave a detectable response to FGF-2, yielding a percentage of responding cells of 39.9 ± 9.2 % (Fig. 1B). Of these, nearly half (65 cells) showed only a transient response, with no sustained plateau (Fig. 2B). Even in most of the cells in which the response was sustained, it showed an oscillatory behaviour, see Fig. 2C. Mean

peak amplitude of responding cells was not significantly different from controls ($\Delta R = 0.43 \pm 0.07$; $p < 0.05$; Fig. 1C).

3.3. Characterization of the oscillatory behaviour in cells preincubated with U73122.

The strikingly increased number of cells showing basal $[Ca^{2+}]_i$ oscillations following preincubation with U73122 led us to investigate the mechanism(s) responsible for this perturbation of calcium homeostasis.

As a preliminary step we checked if the oscillations could be ascribed to influx from the extracellular medium or to release from intracellular compartments. When the extracellular Tyrode solution was shifted to a solution containing 0 Ca^{2+} and 0.5 mM EGTA, spontaneous calcium oscillations in cells preincubated with U73122 were completely abolished in 100% of 110 cells, see Fig. 3A, providing evidence for a dependence of the oscillations on calcium influx.

This influx was not ascribable to activation of voltage dependent calcium channels: blockers of L- and N-type voltage dependent calcium channels (that together contribute to more than 90% of the voltage activated calcium currents in these cells, see [5]) did not have any detectable effect on the oscillatory pattern in absence of FGF-2 (n = 103; Fig. 3B).

In order to trace the time course of the changes induced by the PLCγ inhibitor, we recorded $[Ca^{2+}]_i$ in basal, unstimulated conditions, and subsequently U73122 was applied to the extracellular solution. Fig. 4A shows the superimposition of five of these recordings (typical of the behaviour of n = 50 cells). After addition of the PLCγ inhibitor, a slow increase in $[Ca^{2+}]_i$ could be observed, that in about 10 min evolved into an oscillatory behaviour. The same behaviour could be observed when cells were preincubated with the Na^+ channel blocker TTX (thus abolishing the possible onset of electrical activity) prior to perfusion with U73122 (n = 47; Fig. 4B). Moreover, and unexpectedly, calcium influx through voltage-dependent channels was apparently impaired by U73122 pretreatment. Fig. 4C shows that, in parallel with the appearance of spontaneous oscillations, the response to a solution containing 40 mM KCl gradually decreased, being completely abolished in about 30 min. For longer times, the increase in extracellular K^+ concentration actually decreased the calcium signal, pointing to a reduction of the influx through a voltage-independent pathway caused by the decrease in the electrochemical calcium gradient.

Therefore, the block of the PLCγ pathway seems to lead to inhibition of Ca^{2+} VOCs and to the activation of a calcium influx through a different pathway.

4. Discussion

In this paper, we have analyzed the involvement of the three main pathways downstream the activated FGFR1 receptor (ERK/MAPK, PI3K and PLCγ) in the generation of the long lasting $[Ca^{2+}]_i$ increases elicited by FGF-2 in embryonic chick ciliary ganglion neurons, and provided evidence that interfering with each of these pathways by means of pharmacological tools induces different levels of impairment of the somatic calcium signals activated by the factor. This is the first report of the converging involvement of these pathways in FGF-2 induced neuronal calcium signalling, a key step in the neurotrophic action of the factor [4]. The specificity of the inhibitors and the concentrations used in the experiments described above were tested in a previous paper [7] and proved to be effective in selectively interfering with FGF-2 induced neurite outgrowth without affecting the growth in basal conditions. In the same paper it was also shown that the inhibitors of the ERK/MAPK and of the PI3K-AKT pathways, at the same concentrations, significantly interfered with the phosphorilation of the target proteins [7]. The ERK/MAPK inhibitor PD98059 [16], while not influencing the percentage of cells responsive to the factor, reduced the amplitude of the long lasting $[Ca^{2+}]_i$ increases. On the other hand WM, an inhibitor of the PI3-K enzyme [2], reduced both parameters, without altering the time course of the response. Given that this pathway is involved in the promotion of neuronal survival, a process dependent on calcium influx from the extracellular medium [4], the results obtained with the two inhibitors suggest that while the decreased number of cells able to generate an increase in $[Ca^{2+}]_i$ in response to FGF-2 may be related to the increased cell death, the enhancement of neurite growth induced by the factor requires sustained somatic signals above a certain threshold.

The third pharmacological inhibitor, U73122 [22], that blocks PLC enzymes, showed a more complex pattern of effects: on one side, it dramatically increased the percentage of cells showing spontaneous calcium oscillations; on the other it reduced the percentage of cells responsive to FGF-2 and, strikingly, changed the time course of the response, giving only transient, or at most oscillatory, increases in $[Ca^{2+}]_i$.

The first observation may explain why the block of this pathway strongly affects the number of cells responsive to FGF-2 but has no effect on the dependency of neuronal survival from the neurotrophic factor [7]: while cells are less responsive to the factor, many of them show spontaneous calcium oscillations, thus probably bypassing the need for FGF-2 induced calcium influx. The fact that incubation with U73122, that inactivates voltage dependent calcium channels, does not impair survival of ciliary ganglion neurons [7] is in agreement with the previous data [4] showing that Ca^{2+} VOCs are not involved in the FGF-2 induced survival in this experimental model. On the other hand, these findings provide further support to the hypothesis that the lack (or

the reduction) of sustained increases in somatic $[Ca^{2+}]_i$ may in some way impair the growth of neuritic processes, at some stage.

The strong increase in the number of cells showing basal calcium oscillations in the presence of U73122 can be interpreted as evidence that blocking PLC enzymes, even in the absence of the neurotrophic factor, may remove inhibition of a calcium influx pathway through voltage-independent channels.

A direct consequence of the block of PLC activity is an upregulation of PIP_2 levels in the plasmamembrane. PIP_2 is involved in the modulation of several channel types, mainly K^+ channels and cationic, calcium permeable channels of the TRP superfamily [6,9,15,23]. Among the TRP channels that have been shown to be activated/modulated by PIP_2 are several TRPMs [12,20], TRPC6 [10], TRPV5 [11]. TRPV1, one of the best characterized members of the superfamily, has been shown to be both activated and inactivated by PIP_2, depending on the experimental conditions [19]. Finally, Saleh [21] have shown a complex mechanism of activation of TRPC1 channels by PIP_2 and PIP_3, the product of the PI3K-mediated phosphorilation of PIP_2. Therefore, inhibition of the PLCγ pathway may lead to increased PIP_2 levels in the plasmamembrane, with ensuing activation of some of these channels.

The combined activation of cationic and K^+ channels could be responsible for the slow increase in $[Ca^{2+}]_i$ and for the onset of oscillatory behaviour such as observed in our experiments.

Our observation rules out the involvement of voltage dependent channels in the spontaneous oscillations. On the contrary, we observed a complete downregulation of voltage dependent calcium channel activity in neurons preincubated with the PLC inhibitor. This striking observation could be in accordance with the findings of Michailidis [14], who have shown that PIP_2 exerts complex and opposing actions on high voltage activated calcium channels, leading to slow voltage-independent inactivation. As an alternative, L-type channel inhibition due to the rising $[Ca^{2+}]_i$ could be responsible for this effect [24].

In conclusion, the data presented in this paper provide evidence for the involvement of the three main signal transduction pathways activated downstream of FGFR1 in the generation and shaping of somatic calcium signals. These findings are of relevance not only for FGF-2 and CG neurons, but for the general field of neurotrophic factor-induced calcium signalling. The impairment of these signals and, more generally, the perturbations in calcium homeostasis caused by the inhibitors of these pathways may be related to the observed effects on the biological responses of CG neurons to FGF-2.

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Figure legends

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 gray) and 10 nM Wortmannin (light gray). 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, * $p < 0.05$.

Fig. 2

Preincubation with the PLCγ inhibitor U73122 (0.5 μM) induced a strikingly different behaviour both in unstimulated conditions and in the reduced population of cells that responded to FGF-2. A: Example of a cell showing spontaneous oscillations in $[Ca^{2+}]_i$. Stimulation with FGF-2 did not induce a detectable change in the oscillatory pattern. B: Example of quiescent cell that responded to the factor with a transient and short calcium signal. C: A cell that showed a more prolonged but oscillatory increase in $[Ca^{2+}]_i$ in response to the factor.

Fig. 3

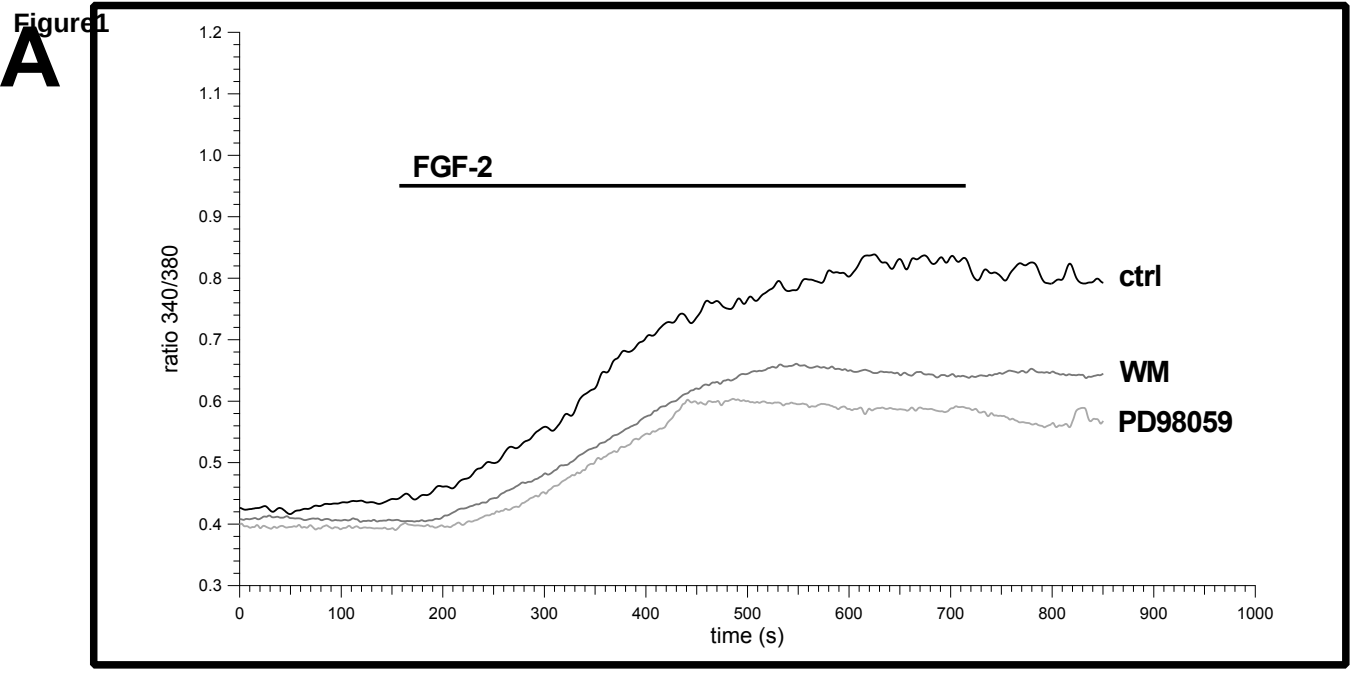
Spontaneous calcium oscillations in cells preincubated with U73122 are dependent on calcium influx and independent from activation of voltage dependent calcium channels. A: In the presence of a calcium-free extracellular medium, oscillations were completely abolished. B: Oscillating behaviour was unaltered in the presence of 10 μM nifedipine and 2 μM ω-conotoxin, blockers of L- and N-type calcium channels, respectively.

Fig. 4

Perturbations in calcium signalling mechanisms induced by incubation with U73122. A: The slow increase in $[Ca^{2+}]_i$ and onset of the oscillatory behaviour observed following addition of the inhibitor to the extracellular medium. Five representatives traces are shown superimposed. B: A

similar behaviour was present when cells were preincubated with the Na^+ channel blocker tetrodotoxin (TTX) prior to U73122 administration. Four traces are shown, representative of 47 recordings. C: Responses to chemical depolarization with 40 mM KCl decreased in amplitude with a similar time course. After about 30 min, voltage dependent calcium influx was no longer detectable and after about 50 min a decrease in $[Ca^{2+}]_i$ could be observed in response to KCl.

Figure1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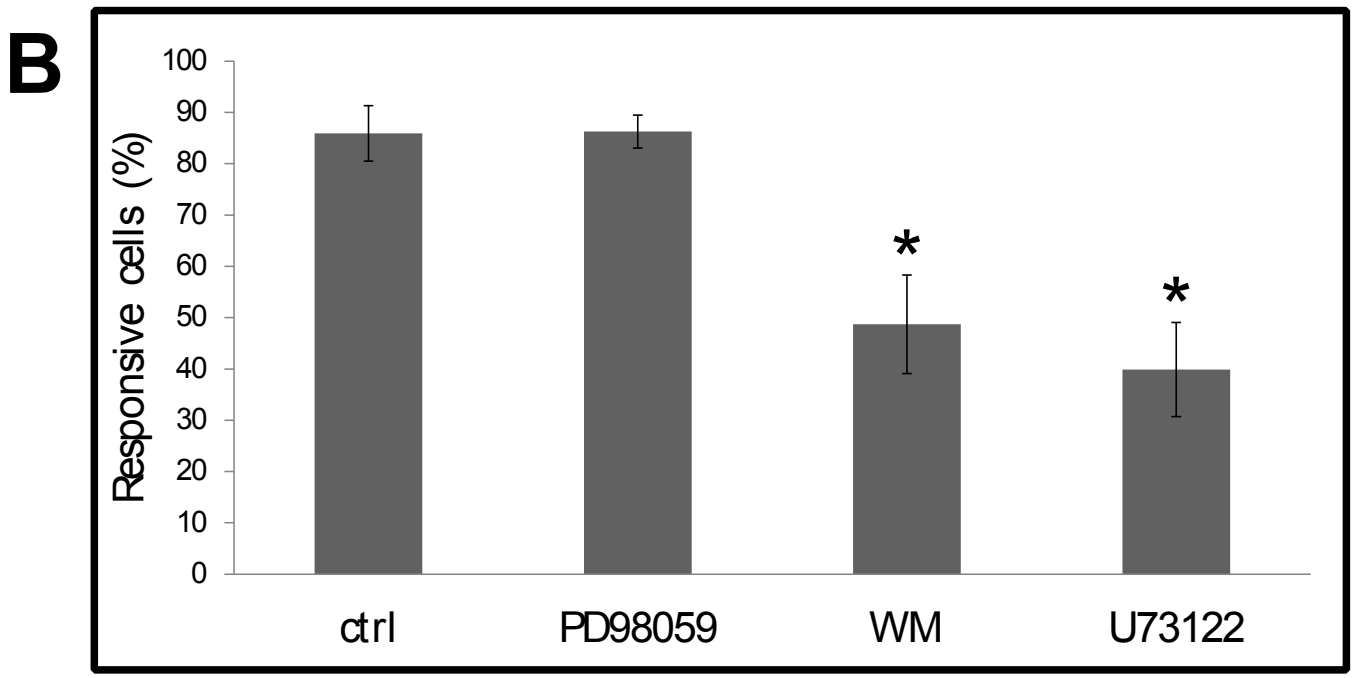
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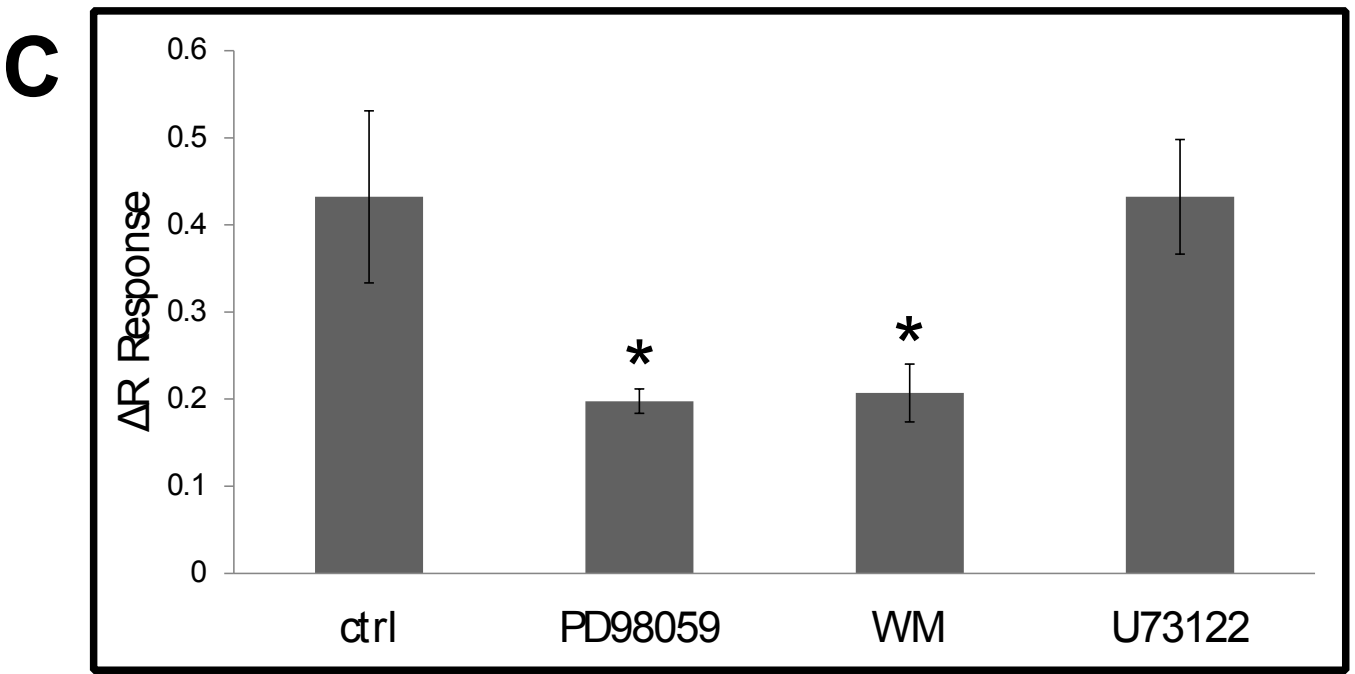
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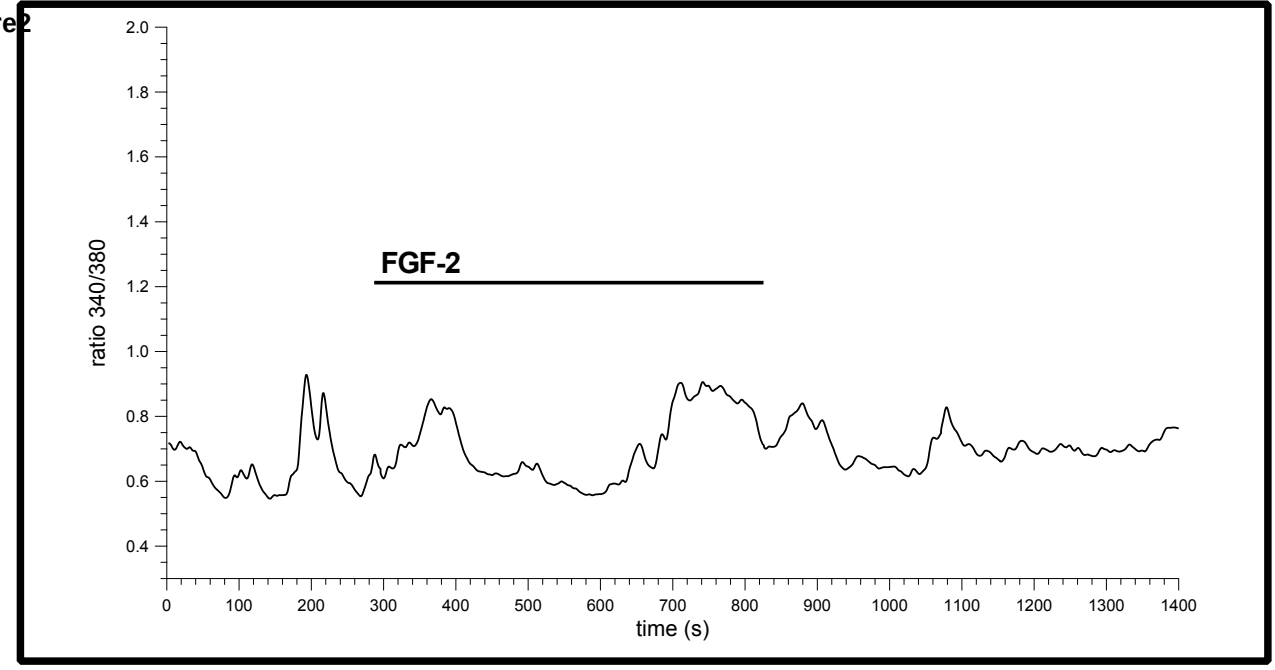
Figure2

A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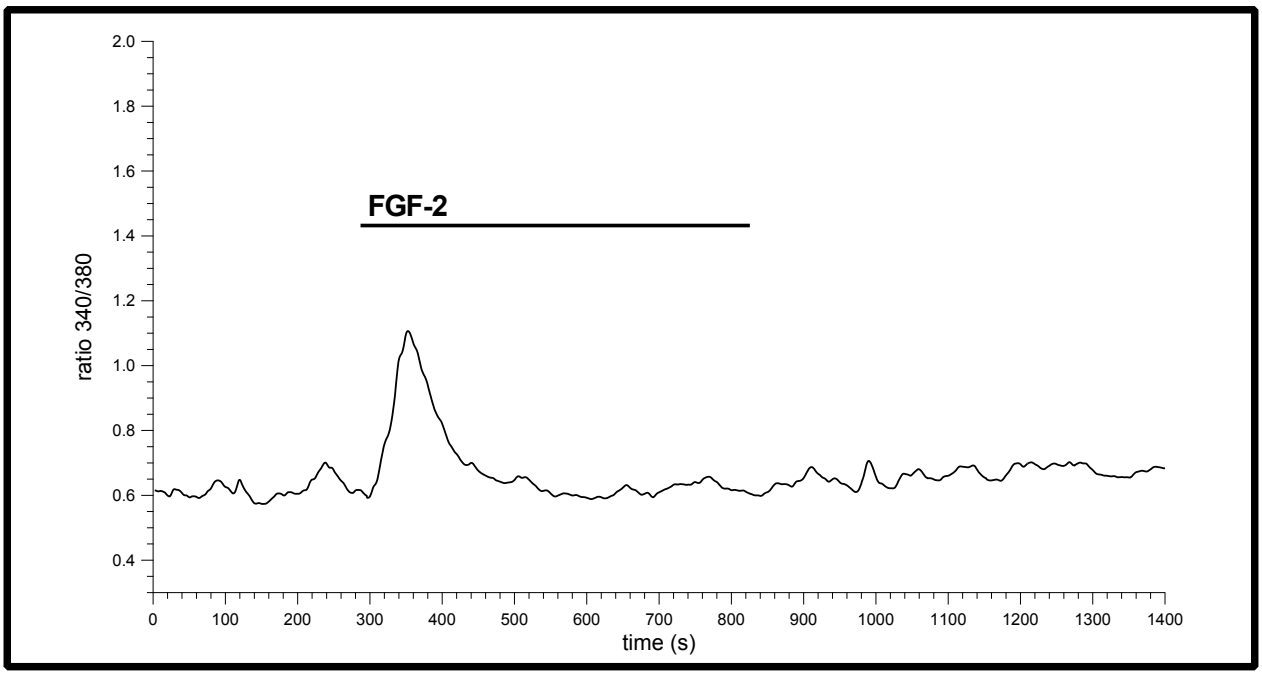
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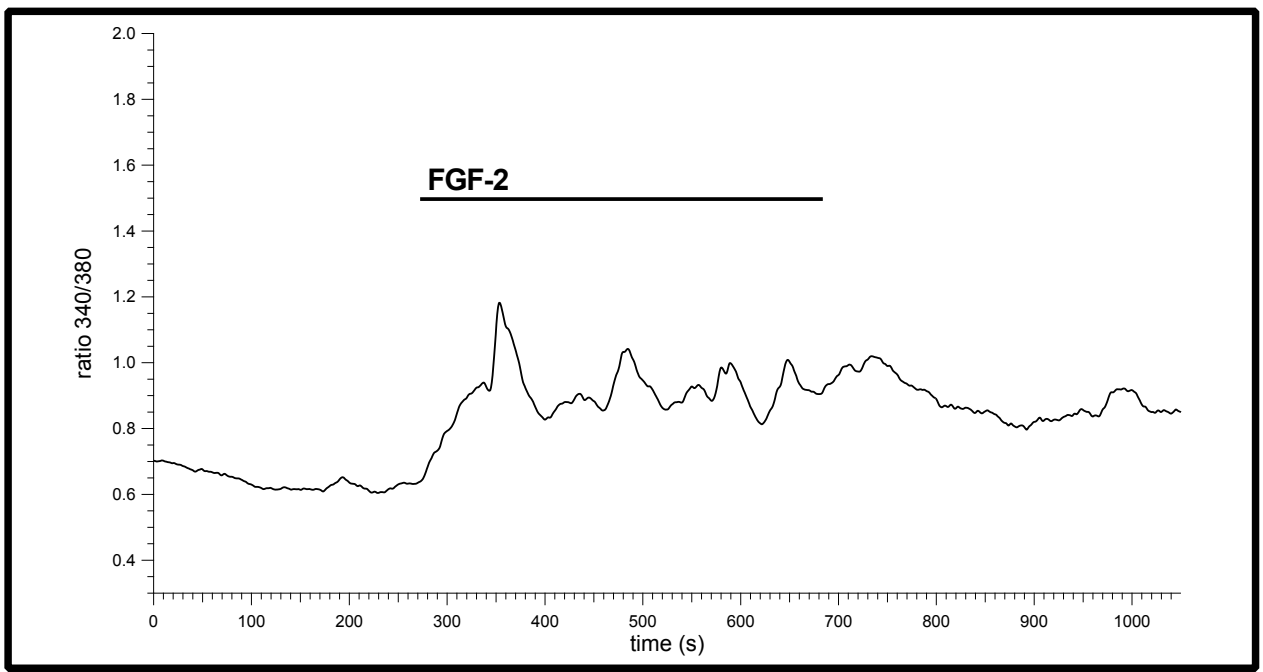
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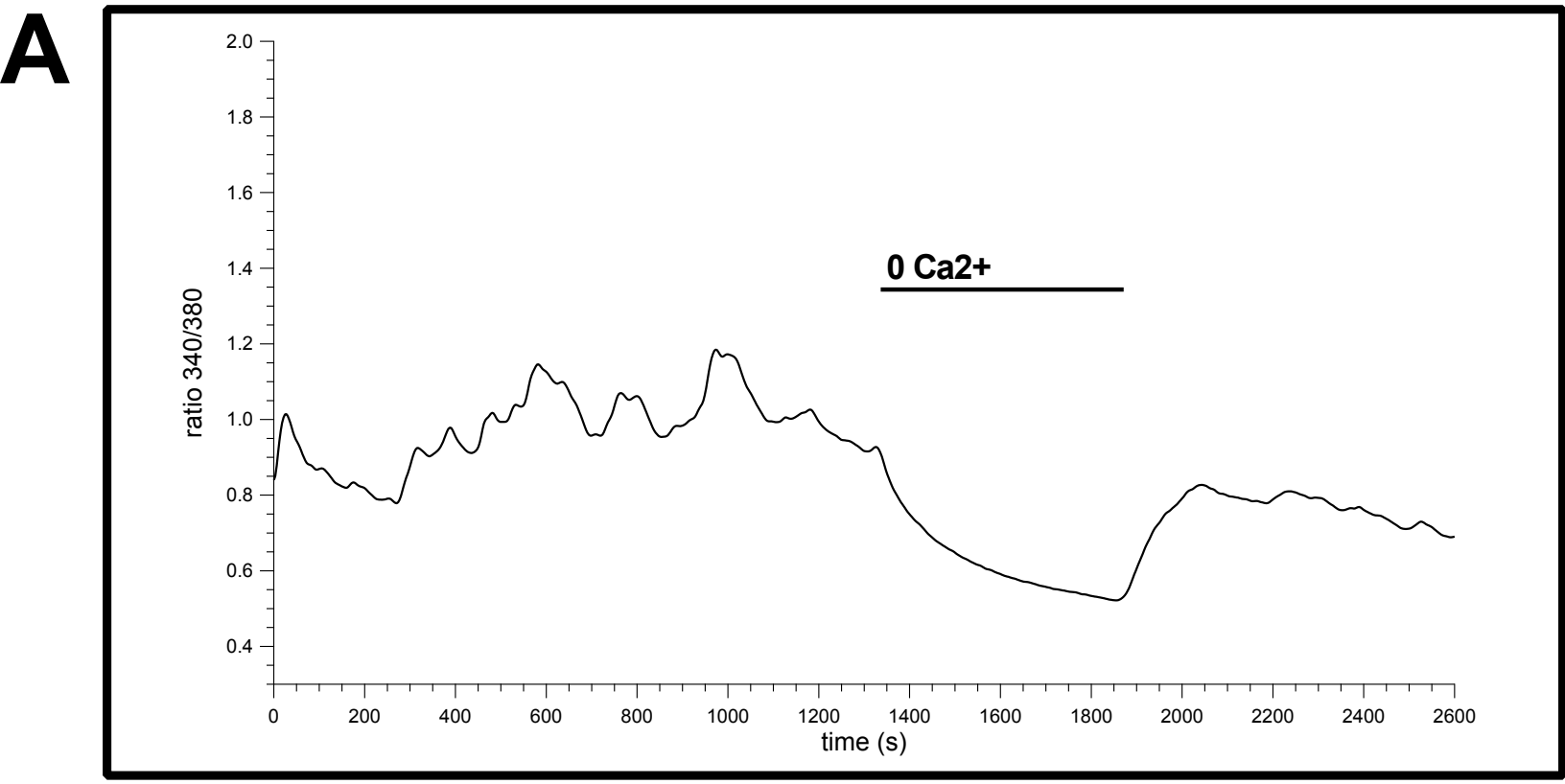
Figure3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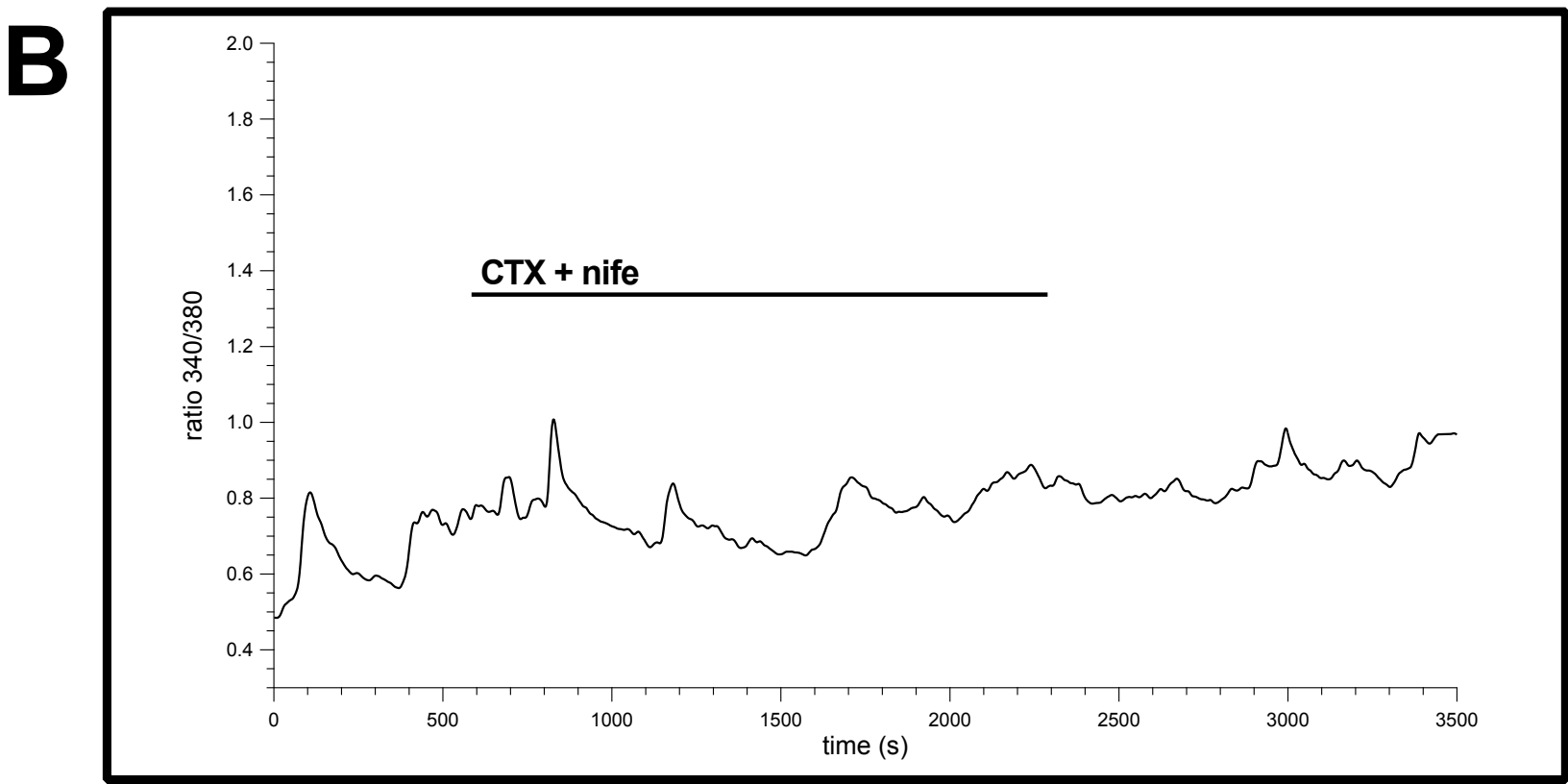
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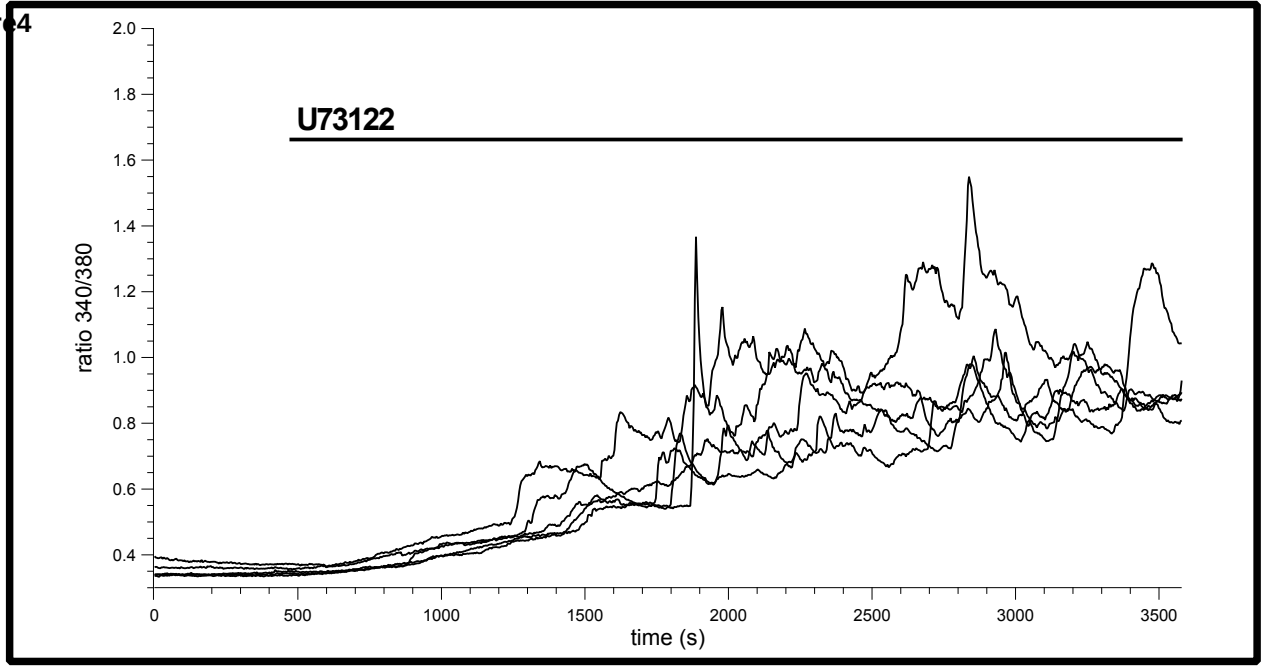
2500
3000
3500
time (s)

Figure4

A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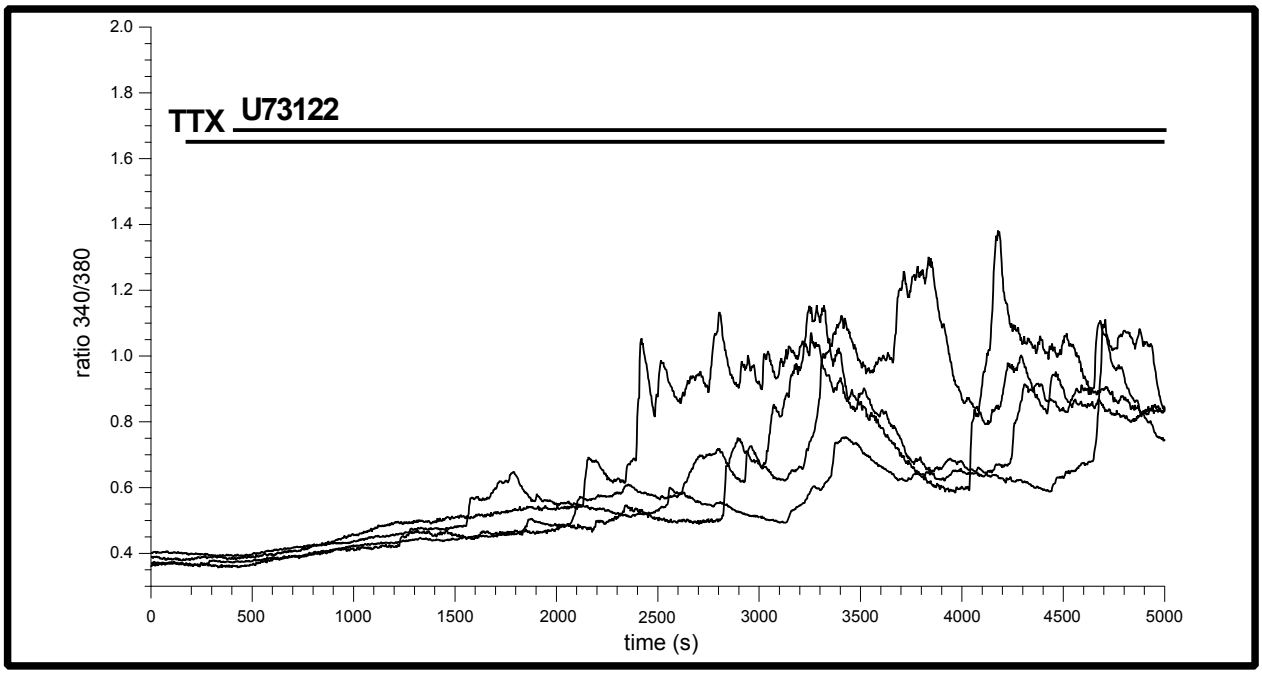
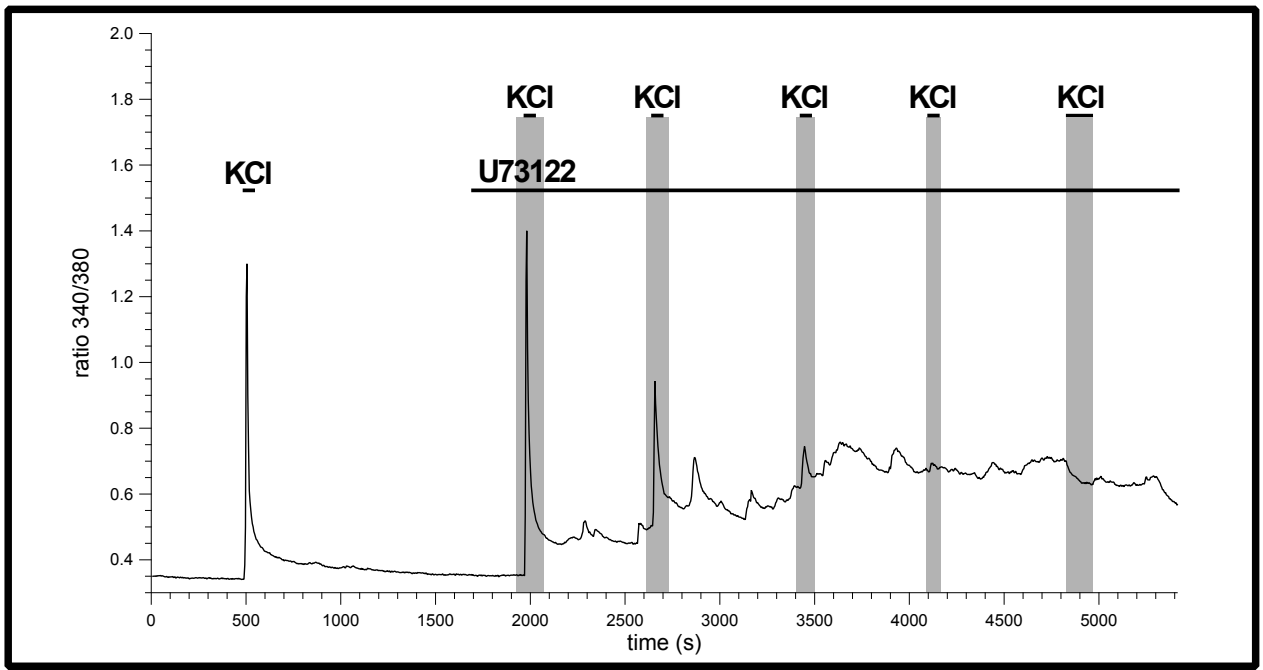
time (s)

B


TTX
U73122
ratio 340/380
time (s)

C


KCl
KCl
KCl
KCl
KCl
KCl
U73122
ratio 340/380
time (s)