The Contribution of Protein Kinase C to Cyclopiazonic Acid-Mediated Potentiation of Serotonin-Induced Vascular Calcium Responses

Serotonine Bağlı Vasküler Kalsiyum Yanıtlarının Siklopiazonik Asit-Aracılı Potansiyalizasyonuna Protein Kinaz C'nin Katkısı

Research Article

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ABSTRACT

We previously showed that serotonin (5-Hydroxytryptamine, 5-HT) receptor antagonist methysergide partially inhibited cyclopiazonic acid (CPA)-potentiated and 5-HT-induced contractions suggesting the contribution of protein kinase C (PKC)-mediated receptor internalization in attenuated antagonism. In the present study, the effects of CPA and 5-HT receptor antagonist methysergide and ketanserin on 5-HT-induced calcium responses following PKC inhibition were further investigated. For this purpose, intracellular calcium levels were monitored in A7r5 vascular smooth muscle cells by spectrofluorometry using the fluorescent indicator fura-2. Cells were pre-incubated with specific PKC inhibitor D-sphingosine. In experimental aspects, spontaneous calcium oscillations hindering the monitoring of responses were determined in over confluent cells. 5-HT-induced calcium levels were significantly decreased in D-sphingosine-treated cells compared to control. CPA significantly potentiated 5-HT-induced calcium responses while ketanserin partially but insignificantly inhibited the potentiated responses in the presence of PKC inhibition. In conclusion, increased cell confluency may result in the generation of spontaneous calcium spikes indicating the importance of using optimum cell density for monitoring of intracellular calcium levels. The data suggests that CPA-mediated potentiation and diminished antagonistic effects on 5-HT-induced calcium elevations in vascular smooth muscle cells, are partially mediated by PKC.

Key Words

Calcium, CPA, D-sphingosine, PKC, serotonin.

ÖZ

Önceki çalışmalar 5-HT reseptör antagonisti metiserjidin, siklopiazonik asitin (CPA) potansiyalize ettiği serotonine (5-hidroksitriptamin, 5-HT) bağlı kontraksiyonları kısmen inhibe ettiği gösterilmiş ve azalan antagonistik etkiye protein kinaz C (PKC)-aracılı reseptör internalizasyonunun katkıda bulunduğu önerilmiştir. Bu çalışmada, PKC inhibisyonu varlığında CPA ve 5-HT reseptör antagonistleri metiserjid ve ketanserinin 5-HT'ye bağlı vasküler kalsiyum yanıtları üzerindeki etkileri araştırılmıştır. Bu amaçla A7r5 vasküler düz kas hücrelerindeki hücre içi kalsiyum düzeylerindeki değişimler fluoresan indikatör fura-2 ile spektrofluorometri yöntemi ile belirlenmiştir. Hücreler PKC inhibitörü D-sifingozin ile inkübe edilmiştir. Metodolojik açıdan bakıldığında, aşırı yoğun hücrelerde ajanlara alınan yanıtları gizleyecek düzeyde spontan kalsiyum salınımları belirlenmiştir. 5-HT'ye bağlı kalsiyum yanıtları D-sifingozin ile inkübe edilen hücrelerde kontrol hücrelere göre anlamlı düzeyde azalmıştır. PKC inhibisyonu varlığında CPA, 5-HT'ye bağlı kalsiyum yanıtlarını anlamlı düzeyde potansiyalize etmiştir. Bunun yanı sıra ketanserin, potansiyalize yanıtları kısmen ve anlamlı olmayan düzeyde inhibe etmiştir. Sonuç olarak, hücrelerin aşırı yoğunlukta olması ani kalsiyum tepe (spike) yanıtlarının oluşmasına neden olabilmekte ve hücre içi kalsiyum düzeylerinin izlendiği çalışmalarda hücrelerin optimum yoğunlukta kullanılmasının önemini göstermektedir. Bulgularımız PKC'nin, 5-HT yanıtları üzerindeki CPA-aracılı potansiyalizasyon ve azalmış antagonistik etkiye kısmen aracılık ettiğini göstermiştir.

Anahtar Kelimeler

CPA, D-sifingozin, kalsiyum, serotonin.

Article History: Received: Mar 6, 2017; Revised: Aug 22, 2017; Accepted: Oct 20, 2017; Available Online: Dec 25, 2017.

Doi: 10.15671/HJBC.2018.173

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT), a vasoconstrictor which is released from activated platelets, regulates the function of vascular smooth muscle (VSM) cells through activation of its receptors, 5-HT2A and 5-HT1B [1]. 5-HT2A receptor activates phospholipase C leading to accumulation of inositol 1,4,5-trisphosphate (IP3) that causes Ca^{2+} release from internal stores and diacylglycerol which activates protein kinase C (PKC) and voltage-operated Ca^{2+} channels [2]. 5-HT-elevated intracellular Ca^{2+} concentration has been characterized by two steps, a transient phase due to IP3-induced Ca^{2+} release from internal stores and a plateau phase that mainly depends on the extracellular Ca^{2+} influx [3]. Store-operated Ca^{2+} (SOC) entry, which was initially proposed in 1986 [4], and Ca^{2+} release from cyclopiazonic acid (CPA)-sensitive stores also contribute to 5-HT-induced responses [5-6].

In addition to its physiological activation by agonist-induced sarco/endoplasmic reticulum Ca^{2+} depletion, SOC entry is activated experimentally by sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) blockers such as CPA simulating SERCA deficiency. Defects in SERCA function, which regulates intracellular Ca^{2+} levels by pumping Ca^{2+} into sarcoplasmic reticulum, have been associated with cardiovascular pathologies [7]. The impaired SERCA activity is also a characteristic of synthetic VSM cells [8]. CPA leads to endothelium-dependent vascular relaxations via elevation of endothelial Ca^{2+} levels in the presence of endothelium [9] while mediating vascular contraction in the absence of endothelium [10,11].

5-HT vasoconstrictor responses were enhanced in acute hypertension [12] and atherosclerosis [13]. In addition, 5-HT-induced Ca^{2+} responses in platelets from bipolar disorder patients were significantly enhanced compared to control [14]. We previously showed that SERCA blockade by CPA significantly potentiates 5-HT-induced vascular contractions [15]. Furthermore, 5-HT receptor antagonist methysergide partially inhibited CPA-potentiated 5-HT contractions in rat thoracic aorta [15] suggesting the possible SERCA blockade-induced internalization of 5HT2A receptors that are localized on caveolar membranes [16]. PKC has been shown to mediate 5-HT-induced 5-HT2A internalization via receptor phosphorylation [17] which was shown to be followed by recycling back to the plasma membrane by protein phosphatase 2A-mediated-dephosphorylation [18].

Based on the data, the hypothesis of the present study is that PKC mediates SERCA down-regulation-mediated elevation in 5-HT responses as well as decreased receptor antagonism following potentiation. To test this hypothesis, the effects of SERCA blocker CPA and 5-HT receptor antagonist methysergide and ketanserin on 5-HT-induced Ca^{2+} responses have been investigated following PKC inhibition in cultured rat vascular smooth muscle (VSM) cells.

MATERIALS and METHODS

Cell Culture

A7r5 cells derived from embryonic rat thoracic aorta (European Collection of Cell Cultures, ECACC) fed with DMEM containing 10% fetal bovine serum and 2 mM L-glutamine in flasks and maintained in a humidified incubator at 37°C and 5% CO2. When reached 70% confluency, cells were subcultured (1:2) using 0.5% trypsin-EDTA. A detailed culturing protocol for A7r5 cells has been published recently [19].

Intracellular Ca^{2+} Measurements

Intracellular Ca^{2+} levels were measured on cell populations (at passage number 22-24) using a dual wavelength spectrofluorometer (PTI QM8/2005, Photon Technology International, Birmingham, NJ) as described previously [20]. The details of the monitoring system have been reported recently [21]. Briefly, A7r5 cells at passage numbers 22-24 were seeded on round coverslips in 24-well plates at 20000-30000 cells/well density, and then incubated for 24-48 hrs to reach a maximum of 70-80% confluency. For loading of Ca^{2+} indicator, cells were incubated in HEPES buffered saline (HBS, in mM: NaCl 135, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6, $NaHCO_3$ 5.0, glucose 11.5, pH: 7.3) containing 5 μM fura-2 plus 0.02% pluronic F-127 (Molecular Probes) supplemented with 1 mg/ ml BSA for 1 h at room

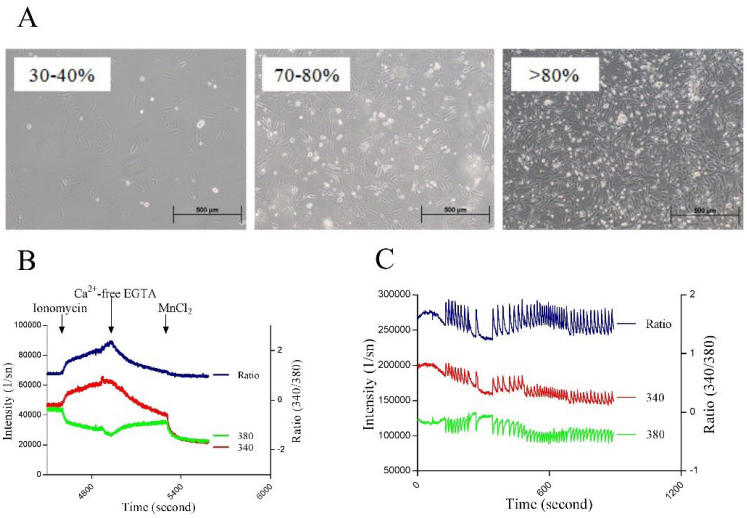

Figure 1. Intracellular Ca^{2+} measurements in VSM cells. Phase contrast images of A7r5 cells in different confluency (30-40%, 70-80% and > 80%) on culture flasks (A) (IX71, Olympus). Tracings of Ca^{2+} calibration protocol used to determine background fluorescence, by quenching the fura-2 fluorescence with MnCl2 (5 mM) in the presence of 10 μM ionomycin in Ca^{2+}-free solution containing 2 mM EGTA (Ca^{2+}-free EGTA), representing the oscillation-free recordings (B). Tracings showing the spontaneous Ca^{2+} oscillations in over-confluent (> 80%) cells (C).

temperature in dark. Fluorescence emission at 510 nm monitored with excitation at 340 and 380 nm and expressed as ratio (340/380). Peak Ca^{2+} responses were evaluated due to time-dependent decays in plateau. Background fluorescence was determined by quenching the fura-2 fluorescence with $MnCl_2$ (5 mM) in the presence of 10 μM ionomycin in Ca^{2+}-free solution containing 2 mM EGTA at the end of the experiment.

Chemicals

All chemicals were from Sigma and dissolved in appropriate solvents as follows: 5-HT (PubChem CID: 164531) (10^{-2} M) in distilled water (DW); CPA (PubChem CID: 54695722) (10^{-1} M) in dimethylsulfoxide (DMSO); methysergide (PubChem CID:5281073) (10^{-2} M) in DMSO; ketanserin (10^{-1} M) in ethanol (EtOH); D-Sphingosine (synthetic) (PubChem CID: 5280335) (10^{-2} M) in EtOH. In order to avoid direct vasorelaxant effects, final DMSO and EtOH concentrations did not exceed 0.1%.

Data Analysis

Data analyses, as well as graphical presentations, were prepared by using GraphPad Prism5. The results were given as mean ± standard error of the mean. "n" represents the number of samples used. The significance of differences was evaluated by Student's t-test for two groups and one-way ANOVA with posthoc Newman-Keuls test for multiple comparisons. $P \leq 0.05$ was considered significant.

RESULTS and DISCUSSION

Cell Density and Intracellular Ca^{2+} Measurements

Cells grown on flasks were seeded on round coverslips in multi-well plates were used to determine intracellular Ca^{2+} levels. Fluctuations in basal Ca^{2+} levels and agonist responses were determined in different measurements. Based on this, the effects of cell seeding density on intracellular Ca^{2+} levels were tested. Cell density was assessed qualitatively by the experimenter and the images of cells in different confluency are given in Figure 1A. Optimum Ca^{2+} measurements were obtained with cells at 70-80% confluency (Figure 1B). In over-confluent (> 80%) cells, spontaneous and persistent Ca^{2+} oscillations, which hindered the monitoring of agonist-induced responses, were observed (Figure 1C).

HT-induced Ca^{2+} Responses Following PKC Inhibition

D-sphingosine which is a potent and specific inhibitor of PKC [22,23] was used in the study. D-sphingosine at 10 μM, that was previously

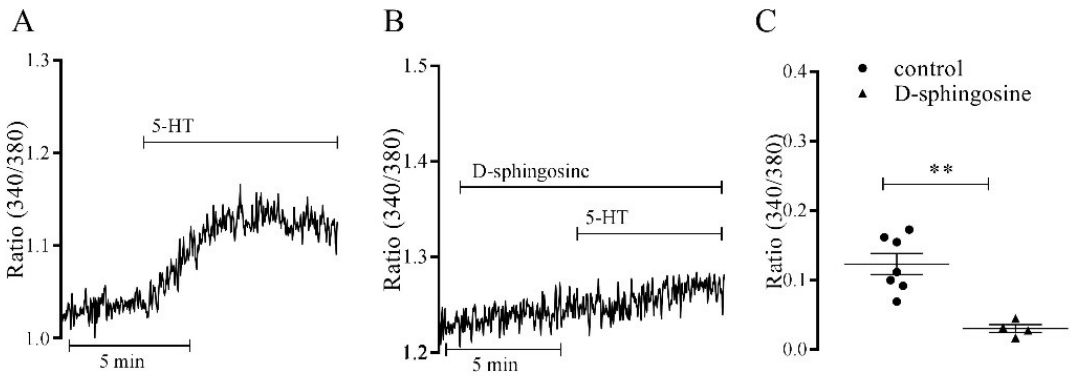

Figure 2. 5-HT-induced Ca^{2+} elevations in the presence of D-sphingosine. Tracings showing 5-HT responses in control (A) and D-sphingosine (10 μM)-incubated (B) cells and the cumulative data ($P \leq 0.01$, n = 4-7) (C). Horizontal lines in box plots represent the mean and standard error of the mean (C).

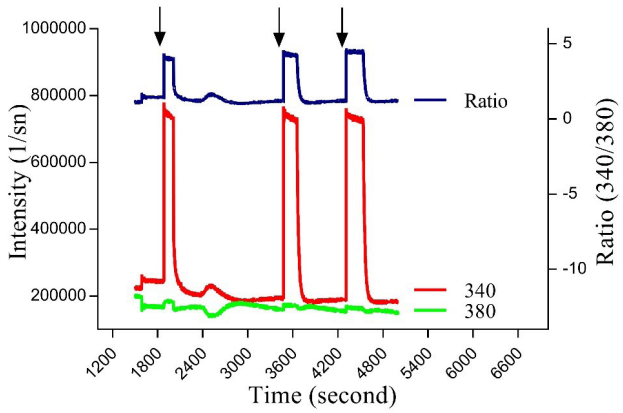

Figure 3. The effects of methysergide on fura-2 fluorescence. The effects of methysergide on emission intensities with 340 and 380 nm excitations and ratio (340/380) levels are given. Arrows indicate three subsequent methysergide (1 μM) applications.

shown to inhibit 5-HT receptor internalization [17], did not affect basal Ca^{2+} levels while resulting in a dramatic increase at 50 μM (not shown). 5-HT was applied at 1 μM concentration previously shown to induce measurable Ca^{2+} elevations in A7r5 cells [20]. First, well-known contribution of PKC to 5-HT responses in rat vascular cells [24] was tested. Following 5 min D-sphingosine (10 μM) incubation, 5-HT responses significantly decreased ($P \leq 0.01$, n = 4-7) compared to control cells (Figure 2), as expected.

Effects of Methysergide on 5-HT-induced Ca^{2+} Elevations

We previously showed that 5-HT receptor antagonist methysergide (1 μM) abolished 5-HT (1 μM)-induced contractions in rat thoracic aorta [15]. Based on this, the effect of methysergide on 5-HT-induced Ca^{2+} elevations were tested. For this purpose, methysergide (1 μM) was applied on 5-HT (1 μM)-induced elevations. Consistent and high levels of increases were observed in both emission intensities and ratio levels following subsequent methysergide applications (Figure 3).

Effects of CPA and Ketanserin on 5-HT-Induced Ca^{2+} Elevations Following PKC Inhibition

We previously observed that CPA at 10 μM concentration, which was previously shown to deplete intracellular stores [25], potentiates 5-HT-mediated vascular contractions [15]. In addition, CPA-potentiated responses were partially inhibited by methysergide [15]. In this study, the effects of CPA on 5-HT responses

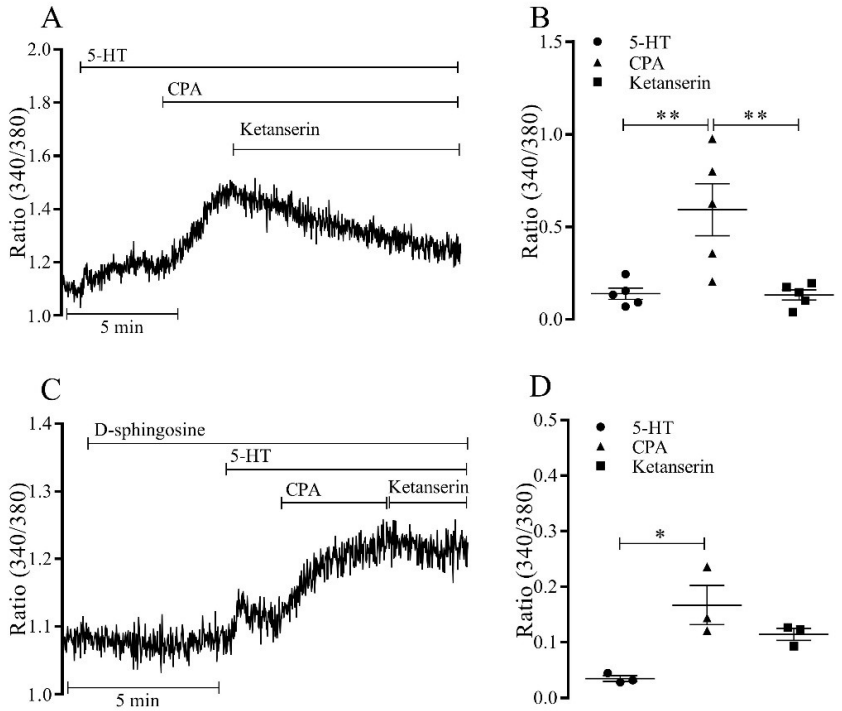

Figure 4. Effects of CPA and ketanserin on 5-HT-induced Ca^{2+} elevations in the presence of D-sphingosine. Serotonin (5-HT, 1 μM), cyclopiazonic acid (CPA, 10 μM) and ketanserin (1 μM) were applied. Cells were incubated with D-sphingosine (10 μM) for 5 min. Tracings and cumulative data in control (A, B) and D-sphingosine-treated (C, D) cells are shown (* $P \leq 0.05$, ** $P \leq 0.01$, n = 3-5). Horizontal lines in box plots represent the mean and standard error of the mean (B, D).

following D-sphingosine incubation were further investigated. Due to structure-related limitations of methysergide, another 5-HT receptor antagonist ketanserin was used to inhibit 5-HT-induced responses.

5-HT-induced Ca^{2+} elevations were significantly potentiated by 10 μM CPA both in control and D-sphingosine-incubated cells ($P \leq 0.01$, $P \leq 0.05$, n = 3-5) (Figure 4). Ketanserin (1 μM) significantly ($P \leq 0.01$, n = 3-5) but not completely inhibited CPA-potentiated 5-HT-induced Ca^{2+} elevations in control cells without any significant effect in D-sphingosine-incubated cells (Figure 4).

DISCUSSION

Spontaneous Ca^{2+} oscillations that hinder the monitoring of agonist-induced responses were determined in over confluent cells in accordance with a previous study in A7r5 cells [26]. Byron et.al. (1993) reported that the spontaneous activity was observed in about 80% of the coverslips monitored. These spontaneous Ca^{2+} spikes were suggested to occur independently of uptake or release of stored Ca^{2+} [26]; however, cell density was not reported in the study. The present data suggests that cell seeding density and resulting confluency are critical in the generation of these spikes indicating the importance of studying with optimum cell density for monitoring of intracellular Ca^{2+} levels. Increased confluency may facilitate the synchronization of Ca^{2+} signals within a population of cells.

Vasoconstrictor responses to 5-HT in the isolated perfused rat lung were shown to be reduced following administration of PKC inhibitor staurosporine [27]. In addition, PKC inhibition significantly attenuated 5-HT-induced platelet intracellular Ca^{2+} mobilization in normal and major depressive disorder patients without affecting those in bipolar disorder patients [14]. In the presence of D-sphingosine, 5-HT-induced responses were significantly inhibited supporting the contribution of PKC in 5-HT-mediated vascular Ca^{2+} responses in rat VSM cells [24].

We previously demonstrated that CPA potentiates 5-HT contractile responses and attenuates 5-HT receptor antagonism in endothelium-denuded rat thoracic aorta [15]. Furthermore, CPA-induced intracellular Ca^{2+} elevation in VSM cells was suggested to be coupled to contraction in the presence of PKC activation [28]. CPA significantly elevated 5-HT-induced Ca^{2+} responses both in control and PKC inhibitor-treated cells in accordance with our previous results in rat thoracic aorta [15]. In contrast to our findings, Saini et.al. (2003) reported a small but significant decrease in 5-HT-induced Ca^{2+} responses following treatment with thapsigargin and CPA in primary cultured rat aortic smooth muscle cells [29]. This discrepancy may result from the presence of residual endothelial cells in primary VSM cell culture, which results in CPA-induced endothelium-dependent relaxations [9] hindering the potentiation of 5-HT responses. Supporting this, we previously showed that CPA inhibits 5-HT- and phenylephrine-induced contractions in endothelium-intact rat thoracic aorta whereas potentiating those in endothelium-denuded [15]. The potentiation of 5-HT-induced Ca^{2+} responses by CPA in the presence of PKC inhibition, in the present study, further suggests the partial involvement of PKC in CPA-mediated potentiation of agonist responses. Voltage-operated Ca2+ channels possibly do not contribute to CPA-mediated potentiation since verapamil previously has been reported to have no effect on CPA-induced Ca2+ elevations in cultured VSM cells [20].

5-HT-induced vascular contractions were shown to be significantly inhibited by methysergide in our earlier report [15]. Moreover, the antagonistic effect of methysergide on 5-HT responses significantly decreased following SERCA inhibition [15]. In the present study, the effects of methysergide, a semi-synthetic derivative of ergometrine used for migraine prophylaxis [30], on 5-HT-induced vascular Ca^{2+} elevations could not be tested due to the obstruction of fura-2 fluorescence by methysergide in accordance with an earlier report [31]. The ineffectiveness of methysergide on 5-HT induced responses was also reported in a more recent study, without mentioning technical difficulties or representing the tracings [29]. Another 5-HT receptor antagonist ketanserin significantly but partially decreased 5-HT Ca^{2+} responses in accordance with diminished 5-HT receptor antagonism on aortic contractions [15]. Following PKC inhibition, the partial inhibitory effect of ketanserin on CPA-potentiated 5-HT-induced Ca^{2+} elevations was insignificant which suggest the contribution of other cell signalling mechanisms as well as PKC to diminished antagonism in the presence of SERCA inhibition. The possible role of RhoA/Rho kinase and Src kinase, which have previously been shown to mediate enhancement of 5-HT-induced contractions in mesenteric arteries from type II diabetic mice [32], in attenuated 5-HT receptor antagonism needs further investigation.

CONCLUSIONS

In experimental aspects, spontaneous Ca^{2+} oscillations were observed in over-confluent VSM cells emphasising studying with optimum cell density for monitoring of intracellular Ca^{2+} levels. In addition, the effects of methysergide on intracellular Ca^{2+} levels cannot be tested due to the disruption of fura-2 fluorescence. The data suggests that diminished antagonistic effect on 5-HT-induced Ca^{2+} elevations in the presence of SERCA inhibition may partially be mediated by PKC activation. Further investigation is required to confirm the role of PKC in potentiated 5-HT responses and attenuated 5-HT antagonism following SERCA-down-regulation.

ACKNOWLEDGMENTS

This work was performed in Ege University, Faculty of Pharmacy, Department of Pharmacology Research Laboratory and supported by Ege University Research Project (BAP-14ECZ022) to Çiğdem Selli.

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