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Norepinephrine-induced calcium mobilization in C6 glioma cells[1]

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KEY WORDS glioma; norepinephrine; adrenergic receptors; calcium; protein kinases

ABSTRACT

AIM: To investigate the mechanism underlying the norepinephrine-induced elevation in intracellular calcium concentration ($[Ca^{2+}]_i$) in C6 glioma cells. **METHODS**: Measurement of $[Ca^{2+}]_i$ was carried out using the dual-wavelength fluorescence method with fura-2 as the indicator. **RESULTS**: Norepinephrine was found to induce concentration-dependent increases in $[Ca^{2+}]_i$ through α_1-adrenoreceptors. The $[Ca^{2+}]_i$ elevations were extracellular-calcium independent and not influenced by the treatment of pertussis toxin. Pretreatments with either U73122 or thapsigargin abolished the subsequent cellular calcium responses to norepinephrine. Preincubation with phorbol 12-myristate 13-acetate (PMA) significantly reduced the $[Ca^{2+}]_i$ elevations, while protein kinase C inhibitors Ro31-8220 or GF-109203X completely blocked the inhibitory action of PMA. However, drugs either activating or inhibiting the function of protein kinase A had no effect on the $[Ca^{2+}]_i$ elevations. **CONCLUSION**: Norepinephrine induces calcium mobilization from internal stores by activation of phospholipase C in C6 cells. The $[Ca^{2+}]_i$ elevation is negatively regulated by the activation of protein kinase C.

INTRODUCTION

Astrocytes have a wide variety of important functions in the central nervous system, one of which is removal of neurotransmitters from the synaptic cleft[1]. It has been demonstrated recently that neurotransmitters released from neurons can also bind to the specific receptors on astrocytes, which results in elevations of intracellular calcium concentration ($[Ca^{2+}]_i$) in the cells[2]. The calcium waves trigger the release of certain chemical substances, particularly glutamate, from the astrocytes[3-5], and these substances will serve as astrocyte-to-neuron signaling molecules because they bring about $[Ca^{2+}]_i$ elevations in adjacent neurons and even change the ongoing states of synaptic neurotransmission and neuronal activity[6]. This bidirectional communication between neurons and astrocytes has been demonstrated in many experimental models, and is assumed as a general property of astrocytes[7,8].

It has also been demonstrated that an elevation in $[Ca^{2+}]_i$ is both necessary and sufficient to produce glutamate release from astrocytes, no matter what the stimulation is in nature[3,4]. Nevertheless, depending on the extracellular stimulation intensities and the cellular context, the magnitude of the astrocytic calcium response seems to be graded, which may in turn lead to a graded feedback modulation to the synaptic transmission[7]. It is therefore important to figure out the conditions or factors by which the cellular calcium responses are affected.

Astrocytes are known to express α_1-adrenoreceptors, the activation of which causes $[Ca^{2+}]_i$ increment[9,10]. However, the modulation of the response by protein kinases is not well studied. Rat C6 glioma cells show many characteristics of astrocytes and have been widely employed as model cells for astrocyte research[11,12]. In this study, we investigated the mechanism underlying the norepinephrine (NE)-induced elevation $[Ca^{2+}]_i$ in C6 cells, as well as its modulation by protein kinases.

MATERIALS AND METHODS

Materials Egtazic acid, epinephrine, ionomycin, isoproterenol, NE, *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), phorbol 12-myristate

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13-acetate (PMA), pluronic F-127, prazosin, propranolol, thapsigargin (TG), and U73122 were purchased from Sigma (St Louis, MO). Forskolin, H-89, pertussis toxin (PTX), Ro31-8220, and GF-109203X were obtained from Calbiochem-Novabiochem Co (San Diego, CA). DMEM and *L*-glutamine used for cell culture were from Life Technologies (Gaithersburg, MD). Trypsin was from Amresco Inc (Solon, OH). Fura-2 acetoxymethyl (AM) ester was purchased from Molecular Probes Inc (Eugene, OR). Newborn calf serum was from Sijiqing Co (Hangzhou, China). All other chemicals were of analytical grade.

Cell culture C6 cells, obtained from the cell bank of the Institute of Cell Biology, Chinese Academy of Sciences, were maintained in DMEM supplemented with 10 % newborn calf serum and *L*-glutamine 4 mmol/L, and incubated in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ℃. No antibiotic was added into the media. For $[Ca^{2+}]_i$ measurements, cells were seeded on glass coverslips and used 2 – 3 d thereafter.

Fura-2 loading In this study, fluorescent dye fura-2 was adopted to indicate the intracellular Ca^{2+} concentration. The cells grown on the coverslips were loaded *in situ* in HEPES-buffered saline (NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2.5, glucose 11.5, HEPES 10 mmol/L, pH 7.2 – 7.4) containing fura-2 AM 2 μmol/L and 0.04 % pluronic F-127 for 30 – 60 min at 37 ℃. Afterwards, fura-2-loaded cells were rinsed three times with saline and mounted in a perfusion chamber for immediate $[Ca^{2+}]_i$ measurements. Alternatively, they could be kept in dark at room temperature for up to 2 h. Fura-2 loading was uniform over the cytoplasm, and compartmentalization of the dye was seldom observed.

$[Ca^{2+}]_i$ measurements Measurement of intracellular Ca^{2+} concentration was carried out with the dual-wavelength fluorescence method as described previously[13] with some modifications. Briefly, fura-2-loaded cells were mounted in a perfusion chamber and placed on the stage of an inverted microscope (IX70, Olympus). Light emitted from a 75-W xenon arc lamp (AH2-RX, Olympus) passed through an excitation filter set (Chroma) to generate ultraviolet monochromatic waves of 340 and 380 nm. With the aid of a computerized filter wheel (Lambda 10-2, Sutter Instruments), the cells in the chamber were alternately exposed to the two waves through an Olympus objective (UApo/340, 40×/0.90). The resulting fluorescent emission from the Ca^{2+}-sensitive dye was collected through a 510 nm-long pass filter (Chroma) with a charge-coupled device camera (MicroMax 5 MHz system, Princeton Instruments). All image acquisition was computer-controlled by MetaFluor Imaging program (v 4.01, Universal Imaging Corporation). Images were acquired at 3-s intervals to reduce photobleaching. All measurements were made at 22 – 25 ℃.

Images acquired were corrected for background fluorescence and shading across the field of view. Background fluorescence was determined by placing a data collection circle in an area without cells; this value was subtracted from each data point before calculating the ratio of the fluorescent emission intensities at each excitation wavelength (340 nm/380 nm). Conversion of the ratio to $[Ca^{2+}]_i$ was carried out using the equation: $[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)] \times (F^{380}{}_{min}/F^{380}{}_{max})$[13], where R_{max} and R_{min} are the maximum and minimum ratio obtained by the addition of ionomycin (10 μmol/L) and egtazic acid (10 mmol/L) respectively, $F^{380}{}_{min}/F^{380}{}_{max}$ the ratio of Ca^{2+}-free and Ca^{2+}-saturated fluorescence signals at the excitation wavelength of 380 nm and K_d dissociation constant of the fura-2/Ca^{2+} complex (224 nmol/L). R is the fluorescence ratio value.

Perfusion chamber and chemical applications The custom-built chamber, bottomed with a coverglass, was continuously perfused with HEPES-buffered saline during measurements. The perfusion rate was set at 2 mL/min to aid drug removal. All drugs were diluted in saline from their stock solutions immediately before experiments, and applied directly to the cells using a computer/manual operated seven-barrel local superfusion system for desired lengths of time. The tip (100 μm inside diameter) of the outlet of the superfusion system was placed approximately 200 μm away from the cells, and the gravity force was adjusted to achieve rapid drug application while avoiding any mechanical disturbance of the cells. The time delay for arrival of drugs at the cells was less than 100 ms (data not shown).

Statistics $[Ca^{2+}]_i$ values were expressed as $\bar{x} \pm s$. Statistical analysis was performed using Student's *t*-test or analysis of variance (ANOVA) depending on data types. $P < 0.05$ was considered as statistically significant difference.

RESULTS

NE-induced $[Ca^{2+}]_i$ elevations In C6 cells,

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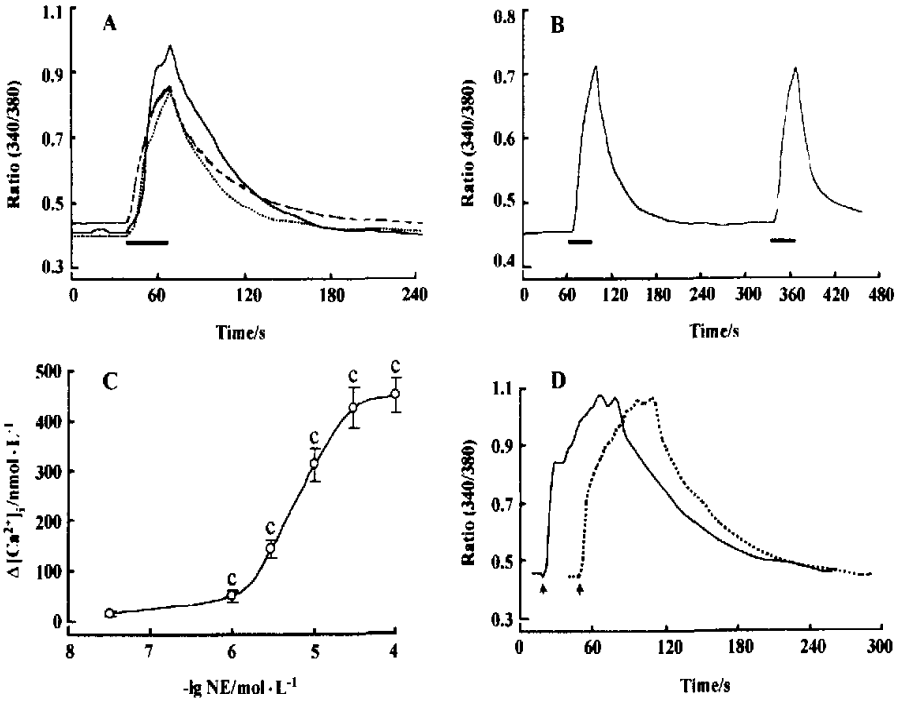

Fig 1. $[Ca^{2+}]_i$ **elevations induced by NE in C6 glioma cells. A: an increase in** $[Ca^{2+}]_i$ **was observed in response to NE 10 μmol/L. Representative traces from three cells in a field of view are presented. The bars indicate drug application. B: repetitive treatments with NE did not significantly influence the peak increments. C: NE-induced** $[Ca^{2+}]_i$ **elevations** ($\Delta[Ca^{2+}]_i$) **as a function of NE concentrations. Each point represents the average of three determinations.** $\bar{x} \pm s$. $^cP < 0.01$ *vs* **control (NE 0 μmol/L). D: extracellular** Ca^{2+} **was not required for NE-induced** $[Ca^{2+}]_i$ **increase. The traces are superimposed for ease of comparison (continuous trace: plus extracellular** Ca^{2+}**, dotted trace: extracellular** Ca^{2+} **depleted). The arrows indicate when NE was applied and each application lasted 30 s.**

NE 10 μmol/L induced an immediate increase in $[Ca^{2+}]_i$ as measured by fura-2 fluorescence ratiometric imaging ($n = 280$ cells) (Fig 1A). Each application lasted 30 s and the interval between stimulations was 3 – 4 min allowing to wash out the agonist and ensure a complete recovery of $[Ca^{2+}]_i$ to baseline. There was no receptor desensitization occurring after repeated application of NE 10 μmol/L ($n = 100$ cells) (Fig 1B). A concentration-response curve was derived by expressing NE-induced $[Ca^{2+}]_i$ peak elevations as nanomolar above basal level (61 ± 9) nmol/L (Fig 1C). The lowest NE concentration that gave a detectable response was around 100 nmol/L.

Receptors mediating the $[Ca^{2+}]_i$ elevations

As seen in Fig 2A, epinephrine 10 μmol/L elicited an elevation in $[Ca^{2+}]_i$ comparable to that induced by NE 10 μmol/L, but isoproterenol at the same concentration had no effect ($n = 70$ cells). Further analysis with adrenoreceptor antagonists showed that prazosin, an α_1 antagonist completely blocked the NE-induced $[Ca^{2+}]_i$ elevations at a concentration of 10 μmol/L ($n = 130$ cells) (Fig 2B), while β adrenoreceptor antagonist propranolol had no influence. Taken together, the results above suggested an involvement of α_1-adrenoreceptor (α_1-AR) in NE-induced $[Ca^{2+}]_i$ elevations in C6 cells.

NE-induced Ca^{2+} mobilization in C6 cells

The $[Ca^{2+}]_i$ elevations induced by NE 10 μmol/L were insensitive to extracellular Ca^{2+} depletion (calcium-free solutions plus egtazic acid 2 mmol/L), suggesting that Ca^{2+} release from intracellular stores should be responsible for the $[Ca^{2+}]_i$ elevations ($n = 85$ cells) (Fig 1D). No $[Ca^{2+}]_i$ transient was observed when extracellular Ca^{2+} was re-added into the bath solution (data not shown). In Ca^{2+}-deprived solution, NE-induced $[Ca^{2+}]_i$ elevations were blocked by preincubation of the cells either with U73122 5 μmol/L for 10 min, or

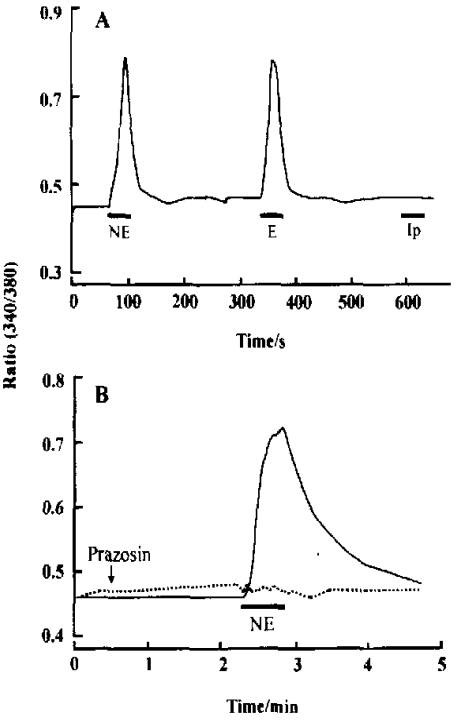

Fig 2. Characterization of the receptors mediating the NE-induced $[Ca^{2+}]_i$ elevations in C6 glioma cells. A: Cells were treated with NE 10 μmol/L, epinephrine (E), and isoproterenol (Ip) successively. B: Exposure of a cell to prazosin (10 μmol/L), completely abrogated the $[Ca^{2+}]_i$ elevation (dotted trace). Prazosin was added 2 min before NE 10 μmol/L (arrow) and present continuously through the test. The traces are superimposed for ease of comparison.

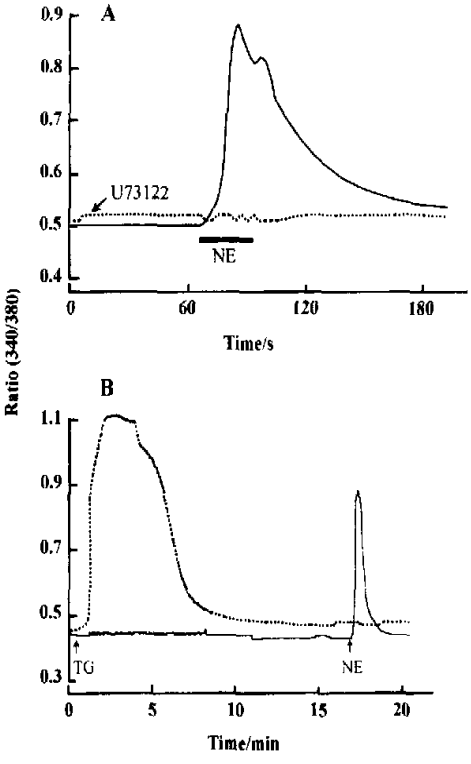

Fig 3. NE-induced Ca^{2+} mobilization in C6 glioma cells. The $[Ca^{2+}]_i$ elevations elicited by NE 10 μmol/L in Ca^{2+}-free solution was abolished either by pretreatment of the cells with U73122 5 μmol/L (dotted trace) (A), or with TG 1 μmol/L (dotted trace) (B). Representative traces are superimposed for ease of comparison.

TG 1 μmol/L for 15 – 20 min (n = 100 and 60 cells respectively) (Fig 3). U73122 had no effect on basal $[Ca^{2+}]_i$ levels by itself, while treatment of C6 cells with TG induced an increase in $[Ca^{2+}]_i$ with greater magnitude than that caused by NE. The elevated $[Ca^{2+}]_i$ levels returned to baseline slowly though TG was applied continuously (Fig 3B). These findings implied that an intracellular TG-sensitive, IP_3-dependent Ca^{2+} store was responsible for the NE-induced Ca^{2+} mobilization in C6 cells.

Effect of PTX on NE-induced Ca^{2+} increment To investigate the effect of PTX pretreatment on NE-induced Ca^{2+} increment, C6 cells were incubated in normal culture medium for 24 h with PTX 100 μg/L, and then $[Ca^{2+}]_i$ was measured in HEPES-buffered saline. As shown in Fig 4, PTX had no effect on NE-induced Ca^{2+} increment.

Effect of protein kinase A (PKA) on NE-induced Ca^{2+} increment The possible involvement of PKA in the regulation of NE-induced Ca^{2+} increment was examined in C6 cells using forskolin and H-89. Pretreatment with forskolin 1 μmol/L or H-89 20 μmol/L had no effect on both the basal and NE-stimulated $[Ca^{2+}]_i$ levels (Fig 4).

Effects of protein kinase C (PKC) on NE-induced Ca^{2+} increment Preincubation with PMA 1 μmol/L for 3 min significantly reduced NE-induced Ca^{2+} increment (Fig 4). PKC inhibitors Ro31-8220 (0.5 μmol/L) or GF-109203X (0.5 μmol/L), when applied to cells before PMA, completely blocked the inhibition by PMA (Fig 4). The results implied that increase in PKC activity negatively regulated the $[Ca^{2+}]_i$ increment induced by NE in C6 cells. Neither the PKC activator or inhibitors changed the basal $[Ca^{2+}]_i$ levels in C6 cells (data not shown).

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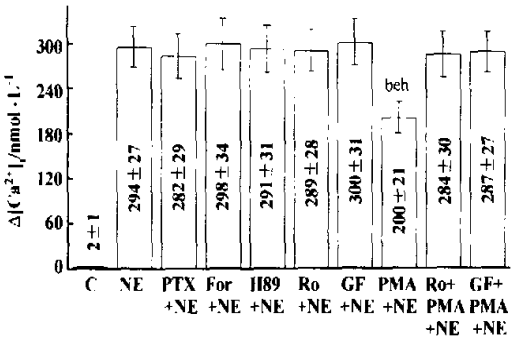

Fig 4. Modulation of NE-induced Ca^{2+} increment in C6 glioma cells. The cells were first incubated with different reagents for certain periods respectively, and then NE 10 μmol/L was added. PTX + NE: PTX 100 μg/L for 24 h; For + NE: forskolin 1 μmol/L for 10 min; H-89 + NE: H-89 20 μmol/L for 20 min; Ro + NE: Ro31-8220 0.5 μmol/L for 20 min; GF + NE: GF-109203X 0.5 μmol/L for 5 min; PMA + NE: PMA 1 μmol/L for 3 min; Ro + PMA + NE: Ro31-8220 0.5 μmol/L for 20 min, and then PMA 1 μmol/L for 3 min; GF + PMA + NE: GF-109203X 0.5 μmol/L for 5 min, and then PMA 1 μmol/L for 3 min; C: saline control; NE: NE 10 μmol/L alone. Each column represents the average of three determinations. $x \pm s$. $^bP < 0.05$ *vs* NE. $^eP < 0.05$ *vs* Ro + PMA + NE. $^bP < 0.05$ *vs* GF + PMA + NE.

DISCUSSION

C6 cells have been documented to possess β-adrenergic receptors by many research groups. Treatments of the cells with epinephrine or isoproterenol lead to a dramatic rise in cAMP level[14,15]. However, there are few reports referring to the question whether α-adrenergic receptors are expressed in C6 cells. Until 1998, Kyo *et al*, using a $[Ca^{2+}]_i$ measurement method, suggested that C6 cells possessed functional α_1-adrenergic receptors (AR). However, they did not analyze the Ca^{2+} response in detail[16]. In this study, we investigated the issue of NE-induced $[Ca^{2+}]_i$ elevations in depth, focusing on the underlying signaling mechanisms and its modulation. To our knowledge, this is the first comprehensive description about the alpha adrenergic effect in C6 cells.

The observed Ca^{2+} response to NE was characterized by a rapid peak elevation and a following sustained increase in $[Ca^{2+}]_i$. The sustained phase could last for several minutes until the agonist was removed (data not shown). However, in order to minimize the effect of homogenous desensitization, we generally exposed the cells to the agonist for no more than 30 s. Under this condition, the peak $[Ca^{2+}]_i$ elevations after repetitive stimulations were almost unchanged. This nature of the reaction facilitated the subsequent experiments and rendered the results obtained by repetitive treatments of a cell readily comparable.

It is well known that a functionally active heterotrimeric G protein includes one α, β, and γ subunit. Up to now, approximately 17 different α, 4 different β, and 7 different γ subunits have been identified, and a great number of heterotrimers composed of distinct α, β, and γ subunits may exist and be involved in signal transduction pathways[17]. In our study, we tested the G protein species according to α subunit. As the treatment of pertussis toxin did not influence the evoked $[Ca^{2+}]_i$ elevations, the PTX-sensitive $G_{i/o}$ subtypes could be excluded from the signaling pathway[17]. It has been documented that α_1-AR utilize two different G_α subunits to increase $[Ca^{2+}]_i$: $G_{\alpha q}$ activates phosphatidylinositol 4,5-bisphosphate hydrolysis and induces Ca^{2+} mobilization from intracellular stores; $G_{\alpha 11}$ enhances the Ca^{2+} influx through calcium release-activated channels (CRAC), which replenish intracellular Ca^{2+} stores[18]. As shown above, the evoked $[Ca^{2+}]_i$ elevations in C6 cells were independent of extracellular calcium and abolished by the pretreatments of the cells with phospholipase C inhibitor U73122 or endoplasmic reticulum Ca^{2+}-ATPase inhibitor TG. These data are in agreement with previous reports[18,19] and provide evidence supporting the existence of a $G_{\alpha q}$-PLC-IP_3-Ca^{2+} mobilization pathway in C6 cells. On the other hand, the α_1-AR-$G_{\alpha 11}$ coupling was not substantiated by our results, since no Ca^{2+} influx through CRAC was observed following the re-addition of extracellular calcium to the bath solution.

It is generally accepted that α_1-AR's function and cellular distribution (internalization/recycling) are modulated by phosphorylation/dephosphorylation processes[19]. The possible involvements of different serine-threonine kinases specific for G protein-coupled receptors (GRK) and second messenger-regulated kinases (like PKA and PKC) in such events have been extensively studied but remain unclear, and some aspects of their roles are still controversial[19]. Phosphorylation of α_1-ARs seems to be a pivotal regulatory step for the shutdown of their functions, and takes place after the activation of second messenger-regulated kinases, such as PKC[20]. In the present study, the regulatory effect of PKC on alpha adrenergic effect has been confirmed in C6

cells. PKC inhibitors applied alone did not influence the evoked $[Ca^{2+}]_i$ elevations, suggesting that PKC activation upon NE-stimulated phosphatidylinositol hydrolysis, if any, does not suffice to modulate the Ca^{2+} response, either because of low degree of activation or intracellular signaling compartmentalization[21].

In conclusion, our results indicate that 1) functional α_1-adrenergic receptors are expressed in C6 glioma cells. 2) activation of the receptor by its agonist NE will result *in a rapid* $[Ca^{2+}]_i$ *transient through a* $G_{\alpha q}$-PLC-IP_3-Ca^{2+} mobilization signaling pathway. 3) activation of PKC down-regulates the $[Ca^{2+}]_i$ increment.

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去甲肾上腺素引起 C6 胶质瘤细胞中钙的动员[1]

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关键词 神经胶质瘤; 去甲肾上腺素; 肾上腺素受体; 钙; 蛋白激酶类

目的: 研究去甲肾上腺素引起大鼠 C6 神经胶质瘤细胞中钙离子浓度($[Ca^{2+}]_i$)增加的机理. **方法**: 以荧光染料 fura-2 为指示剂, 采用双波长荧光比值成像

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的方法测定细胞中钙离子浓度. **结果**: 通过激活细胞上的 α_1 肾上腺素能受体, 去甲肾上腺素剂量依赖地使 C6 细胞中钙离子浓度增加. 这种反应不依赖细胞外钙, 且不受百日咳毒素(PTX)处理的影响. 将细胞与磷酯酶 C (PLC)抑制剂 U73122 或内质网 Ca^{2+}-ATP 酶抑制剂 thapsigargin 预孵育, 去甲肾上腺素引起的胞内钙反应则消失; 蛋白激酶 C 激动剂佛波醇酯(PMA)预处理细胞可以使去甲肾上腺素引起的胞内钙离子浓度升高幅度降低, 而佛波醇酯的效应能够被蛋白激酶 C 抑制剂 Ro31-8220 或 GF-109203X 完全阻断. 但是, 改变胞内蛋白激酶 A 活性的药物对去甲肾上腺素的作用没有影响. **结论**: 通过激活胞内的磷酯酶 C, 去甲肾上腺素使 C6 细胞的胞内钙库释放钙离子. 去甲肾上腺素引起的细胞内钙离子浓度增加受蛋白激酶 C 的负性调节.

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