

Full-length article

Inhibition of ATP-induced calcium influx in HT4 cells by glucocorticoids: involvement of protein kinase A¹Jian-zhong HAN, Wen LIN², Yi-zhang CHEN³*Neuroscience Research Institute and Department of Neurobiology, ²Department of Pharmacology, College of Pharmacy, Second Military Medical University, Shanghai 200433, China***Key words**

calcium; glucocorticoids; adenosine triphosphate; protein kinase C; protein kinase A

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Abstract

Aim: In our previous observations, adenosine triphosphate (ATP) was found to evoke immediate elevations in intracellular free calcium concentration ($[Ca^{2+}]_i$) in HT4 neuroblastoma cells of mice. We tried to see if a brief pretreatment of glucocorticoids could inhibit the Ca^{2+} response and reveal the underlying signaling mechanism. **Methods:** Measurement of $[Ca^{2+}]_i$ was carried out using the dual-wavelength fluorescence method with Fura-2 as the indicator. **Results:** Preincubation of HT4 cells for 5 min with corticosterone (B) or bovine serum albumin conjugated corticosterone (B-BSA) inhibited the peak $[Ca^{2+}]_i$ increments in a concentration-dependent manner. Cortisol and dexamethasone had a similar action, while deoxycorticosterone and cholesterol were ineffective. Both extracellular Ca^{2+} influx and internal Ca^{2+} release contributed to ATP-induced $[Ca^{2+}]_i$ elevation. The brief treatment with only B attenuated Ca^{2+} influx. Furthermore, the $[Ca^{2+}]_i$ elevation induced by the P2X receptor agonist adenosine 5'-(β,γ -methylene) triphosphate (β,γ -meATP) was also suppressed. The rapid inhibitory effect of B can be reproduced by forskolin 1 mmol/L and blocked by H89 20 mmol/L. Neither nuclear glucocorticoid receptor antagonist mifepristone nor protein kinase C inhibitors influenced the rapid action of B. **Conclusion:** Our results suggest that glucocorticoids modulate P2X receptor-mediated Ca^{2+} influx through a membrane-initiated, non-genomic and PKA-dependent pathway in HT4 cells.

Introduction

Glucocorticoids (GC) play an important role in the developmental organization and ongoing activities of the nervous system^[1]. At cellular level, they work in a diverse manner. A genomic mechanism where the complex of GC and their receptors acts as nuclear transcription factor has been well elucidated over the past 30 years, and accounts for the long-latency effects of most GC. However, some of their actions take place so rapidly (onset in seconds or minutes) that they do not fit into the paradigm of the genomic mechanism^[2,3]. There are many examples in which GC act through the rapid, non-genomic way in the nervous system, ranging from the regulation of neuronal excitability to the modulation of neurotransmitter secretion and uptake^[1,2].

The HT4 neuroblastoma cell line was derived from the

hippocampal neurons of mice^[4]. In our previous observations, we found an ATP-induced transient in $[Ca^{2+}]_i$ within the cells^[5]. The Ca^{2+} response was decreased by the activation of protein kinase A (PKA) or protein kinase C (PKC) (unpublished data). As it is already known that some of the rapid actions of GC are mediated by protein kinases^[6,7], we wonder whether there is a similar signaling pathway for GC in HT4 cells, which may result in fast modification of the ATP-induced Ca^{2+} response. We provide evidence showing that ATP-induced Ca^{2+} response can be rapidly modified by GC through a membrane-initiated, nongenomic pathway that is PKA dependent.

Materials and methods

Materials ATP, β,γ -meATP, bovine serum albumin con-

jugated corticosterone (B-BSA), cholesterol, corticosterone (B), cortisol, deoxycorticosterone, dexamethasone, egtazic acid, *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES), ionomycin, pluronic F-127, phorbol 12-myristate 13-acetate (PMA), and thapsigargin (TG) were obtained from Sigma (St Louis, MO, USA). Forskolin, H-89, GF-109203X, and Ro31-8220 were obtained from Calbiochem-Novabiochem (San Diego, CA). DMEM and *L*-glutamine used for cell culture from Life Technologies (Gaithersburg, MD, Germany). Trypsin from Amresco Inc (Solon, OH, USA). Fura-2 acetoxyethyl (AM) ester from Molecular Probes (Eugene, OR, USA). Newborn calf serum was from Sijiqing Co (Hangzhou, China). All other chemicals used were of analytical grade.

Cell culture HT4 cells, a kind gift from Dr B AMES (University of California at Berkeley), were maintained in DMEM containing 10% newborn calf serum and 4 mmol/L *L*-glutamine in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. No antibiotic was added to the media. For [Ca²⁺]_i measurements, cells were seeded on glass coverslips and used within 3–4 d.

Fura-2 loading In the present study, fluorescent dye Fura-2 was used to indicate the [Ca²⁺]_i. The cells on the coverslips were loaded *in situ* at 37 °C for 30–60 min in HEPES-buffered saline (in mmol/L: NaCl 140, KCl 5, MgCl₂ 1, CaCl₂ 2.5, glucose 11.5, HEPES 10, pH 7.2–7.4) containing Fura-2 AM 2 μmol/L and 0.04% pluronic F-127. The Fura-2-loaded cells were then rinsed three times with saline and mounted in a perfusion chamber for immediate [Ca²⁺]_i measurements. Alternatively, they were kept in dark conditions at room temperature for up to 2 h. Fura-2 loading was uniform over the cytoplasm, and compartmentalization of the dye was seldom observed.

[Ca²⁺]_i measurements Measurement of [Ca²⁺]_i was carried out by the dual-wavelength fluorescence method as described previously^[8] with some modifications. Fura-2-loaded cells were mounted in a perfusion chamber and placed under 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 (UAp0/340, 40×/0.90). The resulting fluorescent emission from the Ca²⁺-sensitive dye was collected through a 510 nm-long pass filter (Chroma) with a cooled 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 °C.

Images acquired were corrected for background fluorescence and shading across the field of view. Conversion of the ratio to [Ca²⁺]_i was carried out using the equation: [Ca²⁺]_i = $K_d \times [(R - R_{\min}) / (R_{\max} - R)] \times (F_{\min}^{380} / F_{\max}^{380})^{[8]}$, 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, $F_{\min}^{380} / F_{\max}^{380}$ is the ratio of Ca²⁺-free and Ca²⁺-saturated fluorescence signals at the excitation wavelength of 380 nm, and K_d is the dissociation constant of the Fura-2/Ca²⁺ complex (224 nmol/L). R is the acquired ratio value at each time point.

Perfusion chamber and drug applications The custom-built chamber, with a coverglass at the bottom, 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 the experiments and applied directly to the cells using a computer/manual operated 7-barrel local superfusion system for desired lengths of time. The tip (100 μm inside diameter) of the outlet 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).

Data analysis [Ca²⁺]_i values were expressed as means ± SD. Statistical analysis was performed using *t*-test or analysis of variance (ANOVA) depending on data types. $P < 0.05$ was considered to be significant.

Results

Inhibition of ATP-induced [Ca²⁺]_i increase by GCs In HT 4 cells, the basal level of [Ca²⁺]_i was 54 ± 8 nmol/L^[5]. ATP 100 μmol/L induced an immediate rise in [Ca²⁺]_i, which was significantly attenuated by pre-incubation with B (1 μmol/L) for 5 min (Figure 1A). The original Ca²⁺ response recovered 5–10 min after B was washed out (data not shown). The inhibitory effect was concentration-dependent (0.1–100 μmol/L). B-BSA was also effective in suppressing the Ca²⁺ response with a similar potency compared to B (Figure 1B), while BSA had no effect on either basal or stimulated [Ca²⁺]_i levels (data not shown). To further elucidate the rapid action of B, we studied the effect of mifepristone (a GR antagonist) on the response. Pretreatment with mifepristone (1 μmol/L) for 30 min did not influence the inhibitory effect

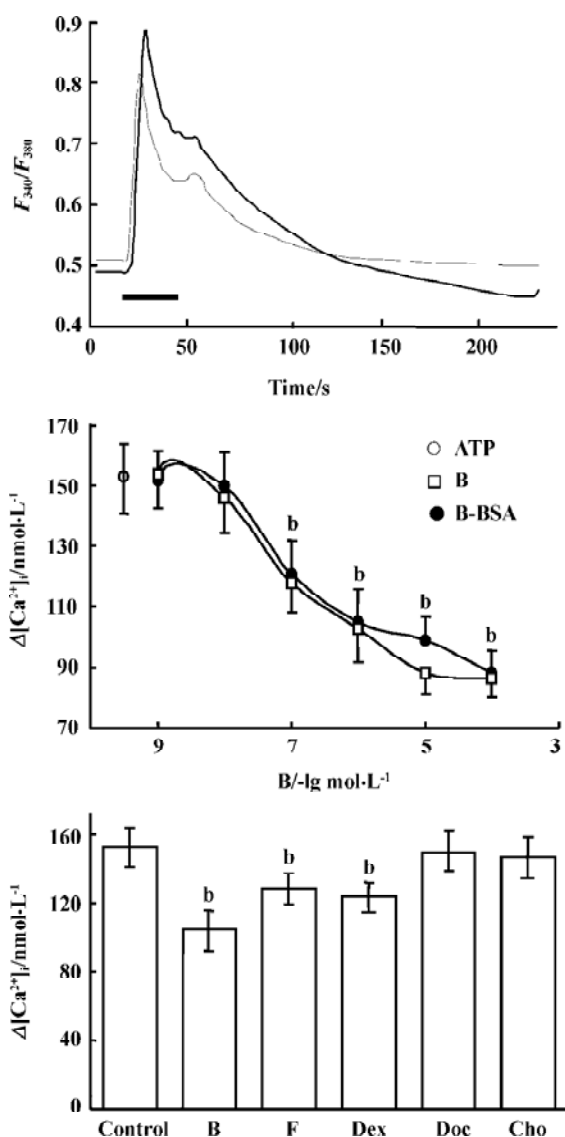


Figure 1. Inhibition of ATP-induced $[\text{Ca}^{2+}]_i$ increase by GCs in HT4 cells. (A) the $[\text{Ca}^{2+}]_i$ increase elicited by 100 $\mu\text{mol/L}$ ATP in HEPES-buffered saline was measured after a 5-min pre-incubation with or without 1 $\mu\text{mol/L}$ B. Traces from representative cells are shown and superimposed for ease of comparison (continuous trace: ATP only, dotted trace: B pretreated). The horizontal bar represents drug application. (B) Dose-response relationships of the B and B-BSA induced inhibition. (C) $[\text{Ca}^{2+}]_i$ increases elicited by 100 $\mu\text{mol/L}$ ATP were measured after a 5-min pre-incubation with cortisol (F), dexamethasone (Dex), deoxycorticosterone (Doc), and cholesterol (Cho), respectively. All steroids were at 1 $\mu\text{mol/L}$ in concentration. $n=3$ determinations. Mean \pm SD. ^b $P<0.05$ vs control (100 $\mu\text{mol/L}$ ATP only).

of B (1 $\mu\text{mol/L}$) (data not shown). Thus, the findings above imply that the action of B in HT4 cells is initiated at the plasma membrane and may not involve the nuclear receptors.

Other GCs or analogs, such as cortisol and dexamethasone, were also tested and shown to be effective using the same experimental protocol. However, deoxycorticosterone and cholesterol were not effective (Figure 1C).

P2X receptor-mediated Ca^{2+} influx was affected by B

In the absence of extracellular Ca^{2+} , ATP (100 $\mu\text{mol/L}$) was also capable of elevating $[\text{Ca}^{2+}]_i$ but with smaller amplitude. The $[\text{Ca}^{2+}]_i$ level dropped gradually to baseline even if ATP was not withdrawn. The subsequent introduction from the local superfusion system of 2.5 mmol/L Ca^{2+} -containing ATP solution to the same cells gave rise to a secondary calcium elevation (Figure 2A). These results suggest the generation of both Ca^{2+} influx across plasma membrane and Ca^{2+} release from intracellular stores upon ATP treatment. Using this experimental protocol, we investigated which component of the Ca^{2+} response was affected by the brief treatment of B. As shown in Figure 2A, the Ca^{2+} release evoked by ATP was not affected while the $[\text{Ca}^{2+}]_i$ increment during Ca^{2+} influx was significantly smaller after the pre-incubation of B (1 $\mu\text{mol/L}$). β, γ -meATP is a specific agonist for P2X receptors that has little effect on P2Y receptors^[9]. The $[\text{Ca}^{2+}]_i$ increase induced by β, γ -meATP 10 $\mu\text{mol/L}$ in HT4 cells was also inhibited by the pre-incubation of B (1 $\mu\text{mol/L}$) (Figure 2B and 2C). Taken together, the above results suggest that Ca^{2+} influx through P2X receptors was affected by B.

Effect of B on Ca^{2+} influx through SOC

As shown earlier, ATP increases Ca^{2+} release from intracellular stores in HT4 cells. The depletion of the Ca^{2+} stores has been reported to trigger the opening of store-operated channels (SOC)^[10,11]. We therefore examined whether B might exert its inhibitory effect by reducing Ca^{2+} influx through SOC. Thapsigargin (TG), a specific inhibitor of endoplasmic reticulum Ca^{2+} -ATPase, is widely used to activate SOC by depleting Ca^{2+} stores. In HT4 cells, exposure of TG (1 $\mu\text{mol/L}$) caused an initial spike followed by a sustained elevation at lower level that was indicative of the activation of SOC. Our results showed that the TG-induced plateau was insensitive to treatment of B (1 $\mu\text{mol/L}$), while ATP-induced further increase in $[\text{Ca}^{2+}]_i$ at this stage was still attenuated by B (Figure 3).

Rapid action of B was PKA-dependent

Our previous observation found that ATP-induced $[\text{Ca}^{2+}]_i$ increase was negatively modulated by PKA or PKC in HT-4 cells^[5]. Therefore we examined whether PKA or PKC related signaling pathways were involved in the rapid action of B. Effects of forskolin (1 $\mu\text{mol/L}$), PMA (0.5 $\mu\text{mol/L}$) on the $[\text{Ca}^{2+}]_i$ increase were studied and compared with that of B (1 $\mu\text{mol/L}$). There was no significant difference in the degrees of inhibition among groups pretreated with forskolin or B, or both, while the effect of a combination of PMA and B was addi-

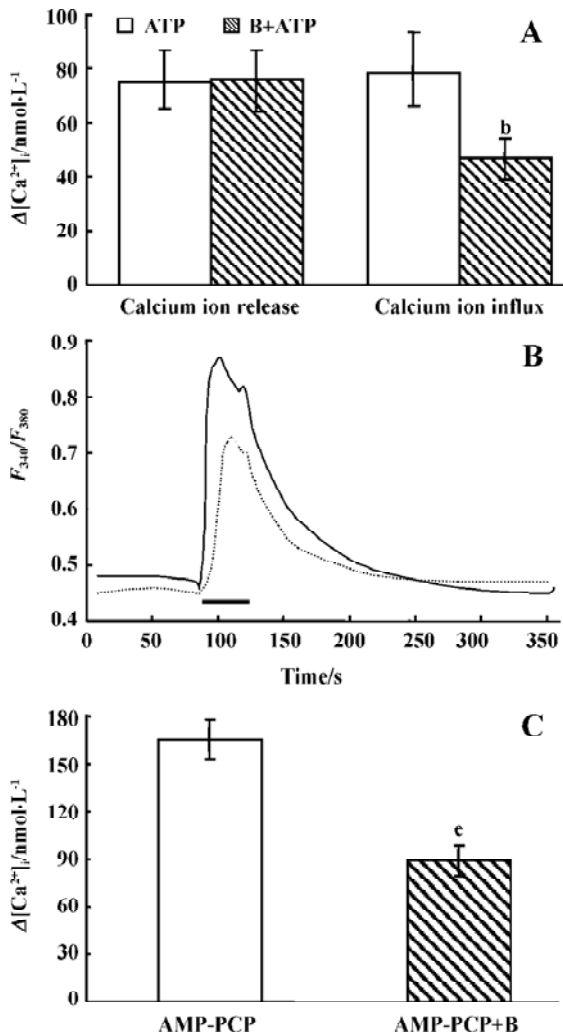


Figure 2. P2X receptor-mediated Ca²⁺ influx was affected by B. (A) using a Ca²⁺-free and Ca²⁺-reintroduction protocol, the ATP-evoked Ca²⁺-influx was found to be suppressed by 5-min pretreatment of B (1 μmol/L). n=3 determinations. Mean±SD. ^bP<0.05 vs control (10 μmol/L β,γ-meATP only). (B) β,γ-meATP (10 μmol/L) caused a rapid increase in [Ca²⁺]_i (continuous trace), which was inhibited by pretreatment of the cells for 5 min with 1 μmol/L B (dotted trace). The horizontal bar represents drug application. (C) n=3 determinations. Mean±SD. ^cP<0.05 vs AMP-PCP group.

tive (Figure 4). These results imply that the forskolin-activated pathway is involved in the signaling of B-induced inhibition. However, PMA and B exerted their modulatory effects through distinct pathways. PKA inhibitor H89 (20 μmol/L), when applied 20 min before B, completely abrogated the inhibitory effect of B (1 μmol/L), while PKC inhibitors Ro31-8220 (0.5 μmol/L) and GF-109203X (0.5 μmol/L) had little influence (Figure 4). None of the drugs changed the basal [Ca²⁺]_i level in HT4 cells (data not shown).

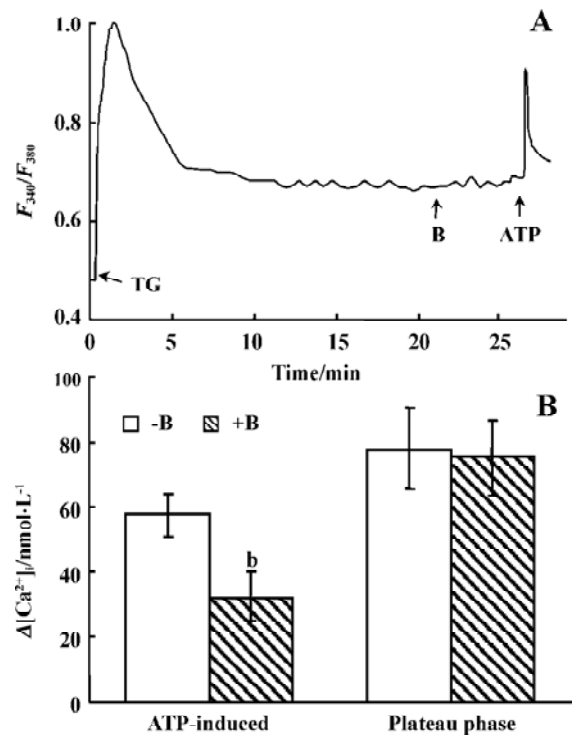


Figure 3. Effect of B on ATP-induced [Ca²⁺]_i increase after TG treatment. HT-4 cells were first exposed to TG (1 μmol/L) and subsequently stimulated with ATP (100 μmol/L) 25 min later. B (1 μmol/L) was applied 20 min after TG when the [Ca²⁺]_i levels reached a plateau. (A) a representative trace is presented to show the experimental protocol. (B) Δ[Ca²⁺]_i values recorded immediately before ATP application represents the plateau phase Ca²⁺ levels. n=3–4 determinations. Mean±SD. ^bP<0.05 vs control (100 μmol/L ATP after TG treatment).

Discussion

Pre-incubation with B inhibited the ATP-induced [Ca²⁺]_i increase in HT4 cells in a concentration-dependent manner. The minimum effective dose was 100 μmol/L, which falls below the peak-free B concentration (750 μmol/L) in serum during stress^[12]. The pre-incubation time for B adopted in most of our experiments was 5 min. However, a 3-min pretreatment was found to be enough for B to take effect (data not shown). The very short latency is consistent with the criteria to define a rapid effect of steroid hormones^[11]. Moreover, the rapid action was insensitive to treatment with nuclear receptor antagonist, further supporting the notion that this is a nongenomic effect of B.

Being large and predominantly hydrophilic molecules, steroid-BSA conjugates are thought to be membrane impermeable within 30 min^[13]. However, they are biologically active and can reproduce the actions of free steroids^[14]. In our study, B-BSA was found to be effective in inhibiting

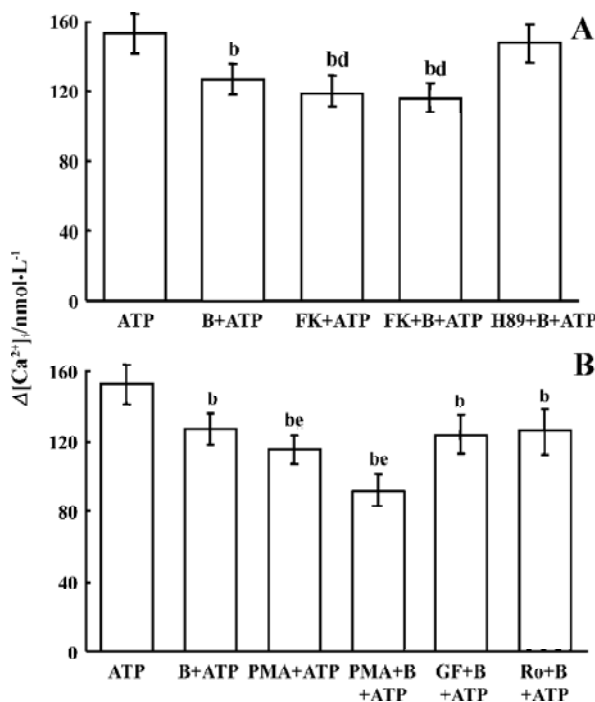


Figure 4. Rapid action of B was PKA-dependent. (A) before ATP (100 μmol/L) stimulation, HT4 cells were pretreated with B (1 μmol/L) for 5 min or forskolin (FK, 1 μmol/L) for 10 min or in combination. In experiments using H89 (20 μmol/L), the drug was applied 20 min before the addition of B. (B) before ATP (100 μmol/L) stimulation, HT4 cells were pretreated with PMA (0.5 μmol/L) for 5 min alone or in combination with B. In experiments using Ro31-8220 (Ro, 0.5 μmol/L) or GF109203X (GF, 0.5 μmol/L), the drugs were applied 20 min before the addition of B. *n*=3–4 determinations. Mean±SD. ^b*P*<0.05 vs control (100 μmol/L ATP only). ^d*P*>0.05, ^e*P*<0.05 vs B+ATP group.

ATP-induced [Ca²⁺]_i increase, suggesting its action at the plasma membrane, where a signal was generated and transduced into the cells. Until now, several specific membrane proteins have been reported as the receptors that mediate the rapid actions of GC^[15,16]. It is interesting to note that they are different not only in molecular weight, but also in genetic identity. Being a splice variant of the nuclear GR, one membrane receptor has been reported to have cross-antigenicity with its nuclear counterpart, while others do not^[17]. The identity of the membrane target in HT4 cells is not clear and needs to be elucidated.

The potency of B-BSA in this experimental model is almost the same as that of B. Previous work in our lab has revealed that both B and B-BSA activate PKC in PC12 cells, but the dose-dependence curve of the latter shifts rightward (ie, with lower potency)^[6]. The reason for the difference is also unclear.

ATP, being a normal metabolite, exists in all living cells.

In the nervous system, it is an important signaling molecule belonging to a class of fast excitatory transmitter in the brain^[18,19]. Its signaling is mediated through the metabotropic P2Y and ionotropic P2X receptors^[9]. In our experiment, the stable ATP analog β,γ-meATP was shown to be a potent agonist in HT4 cells. This result strongly implies actions at P2X receptors. Modulation of P2X purinoceptor-mediated Ca²⁺ responses by protein kinases has been well investigated^[10,20–22]. In PC12 cells, for example, ATP-induced Ca²⁺ influx through P2X receptors is inhibited when PKC activity is enhanced^[10].

It has been reported that PKA is involved in the GC rapid, nongenomic actions in many cell models^[2]. It is interesting to note that the models are all pituitary-originated, such as rat pituitary cells in culture^[23], cichlid fish pituitary *in vitro*^[24], and mouse corticotroph tumor cell line AtT20^[25]. In contrast, studies on rat brain synaptosomes, rat hippocampal neurons, PC12 cells, and glial cell lines reveal an alternative PKC pathway^[2,26]. This prompted us to speculate that the protein kinases involved in GC rapid action could be predicted by cell type^[2]. However, the present data we found in HT4 cells, a mouse hippocampus-derived cell line, show that such generalization is premature and open to further consideration.

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