Frequency Modulation of Synchronized Ca^{2+} Spikes in Cultured Hippocampal Networks through G-Protein-Coupled Receptors

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Synchronized spontaneous Ca^{2+} spikes in networked neurons represent periodic burst firing of action potentials, which are believed to play a major role in the development and plasticity of neuronal circuitry. How these network activities are shaped and modulated by extrinsic factors during development, however, remains to be studied. Here we report that synchronized Ca^{2+} spikes among cultured hippocampal neurons can be modulated by two small factors that act on G-protein-coupled receptors (GPCRs): the neuropeptide PACAP (pituitary adenylate cyclase-activating polypeptide) and the chemokine SDF-1 (stromal cell-derived factor-1). PACAP effectively increases the frequency of the synchronized Ca^{2+} spikes when applied acutely; the PACAP potentiation of Ca^{2+} spikes requires the activation of the PACAP-specific PAC1 GPCRs and is mediated by the activation of cAMP signaling pathway. SDF-1, on the other hand, significantly reduces the frequency of these Ca^{2+} spikes through the activation of its specific GPCR CXCR4; the inhibitory action of SDF-1 is mediated by the inhibition of cAMP pathway through the G_i component of GPCRs. Taken together, these results demonstrate that synchronized neuronal network activity can be effectively modulated by physiologically and developmentally relevant small factors that act on GPCRs to target the cAMP pathway. Such modulation of neuronal activity through GPCRs may represent a significant mechanism that underlies the neuronal plasticity during neural development and functioning.

Key words: **cAMP; neuropeptide; chemokine; synaptic transmission; Ca^{2+} imaging; Ca^{2+} oscillation**

Introduction

Spontaneous oscillations of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in the forms of waves and spikes are found in many types of neural tissues *in vivo* and *in vitro*, ranging from the hippocampus to the visual system (Shatz, 1990; Robinson et al., 1993; Leinekugel et al., 1999). These spontaneous activities are believed to play a pivotal role in many aspects of neuronal development, including neuronal migration, differentiation, and connection patterning (Shatz, 1990; Spitzer, 1994; Gu and Spitzer, 1995; Katz and Shatz, 1996; Komuro and Rakic, 1996; Feller, 1999; Zhang and Poo, 2001). Synchronized spontaneous Ca^{2+} spikes are often observed among networked neurons without external stimuli *in vitro*, resulting from periodic burst firing of action potentials through excitatory synaptic transmission (Leinekugel et al., 1997; Wang and Gruenstein, 1997; Bacci et al., 1999). It was hypothesized that such synchronized firing can encode information in neural circuits (Lisman, 1997; Bacci et al., 1999) and may play an important role during physiological or pathological events such as epileptic seizures in hippocampus (Traub and Wong, 1982; Miles and Wong, 1983; Traub and Jefferys, 1994). Periodic firing of action potentials is regulated by intrinsic programming of neuronal maturation during development and can be modulated by a variety of extracellular factors (Leinekugel et al., 1997; Przewlocki et al., 1999; Numakawa et al., 2002). Further understanding of the molecular and cellular mechanisms underlying the regulation and modulation of synchronized spontaneous neuronal activities will not only contribute to the understanding of brain development but would also lead to development of potential therapeutic strategies for treatment of abnormal brain activities under pathological conditions.

G-protein-coupled receptors (GPCRs) represent the largest family of surface receptors for a variety of extracellular factors, and GPCR activation often elicits complex and diverse signaling pathways that can give rise to diverse arrays of cellular effects (Neves et al., 2002). Although functions of metabotropic glutamate receptors (GPCRs) in neural transmission have been well studied (Nakanishi, 1994; Woodhall et al., 1999), influences of other ligand–GPCR interactions on neuronal activities are just beginning to be elucidated (Neves et al., 2002). In brain, two novel factors acting on GPCRs have been characterized and are of particular interest: the chemokine stromal cell-derived factor-1 (SDF-1) and the neuropeptide pituitary adenalyte cyclase-activating polypeptide (PACAP). SDF-1 and its sole GPCR CXCR4 are widely expressed in the nervous system (Zou et al., 1998; McGrath et al., 1999) and play an important role in neural development (Asensio and Campbell, 1999; Stumm et al., 2002; Xiang et al., 2002; Zhu et al., 2002). PACAP belongs to the glucagon–secretin–vasoactive intestinal polypeptide (VIP) family

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(Vaudry et al., 2000) and has also been found to play a role in neural development (Jaworski and Proctor, 2000; Vaudry et al., 2000; Waschek, 2002). Both SDF-1 and PACAP, together with their receptors, are expressed in the hippocampus (McGrath et al., 1999; Jaworski and Proctor, 2000; Tham et al., 2001), suggesting a potential role for these two factors in hippocampal development and plasticity. In this study, we found that PACAP and SDF-1, when acutely applied, exhibited profound but opposite effects on synchronized Ca^{2+} spikes in synaptically connected hippocampal neurons in culture. PACAP significantly increased the Ca^{2+} spike frequency, but SDF-1 primarily reduced the frequency; such distinct actions of these two factors on synchronized network activities were mediated by their opposing regulation of cAMP signaling pathway, namely, PACAP activated while SDF-1 inhibited cAMP signaling. Given the wide expression patterns of these two factors and their specific receptors in the developing and mature brains, our findings thus provide evidence for a potential role of these two factors in modulation of neural activities in the brain under normal or pathological conditions.

Materials and Methods

Cell culture. Hippocampal neurons from embryonic rats (E18) were obtained according to the method previously described (Banker and Cowan, 1977). In brief, hippocampal tissues from 18 d fetal rats were dissected and treated with 0.25% trypsin and 0.25 mg/ml DNase (Sigma, St. Louis, MO) in Ca^{2+}–Mg^{2+}-free HBSS (Invitrogen, Carlsbad, CA) at 37°C for 15 min; they were then dissociated by trituration with a glass Pasteur pipette and plated in 35 mm culture dishes with glass bottom (MatTek, Ashland, MA) for culture and subsequent microscopy. The glass surface in each dish (~15 mm diameter) was pretreated with poly-D-lysine (Sigma) for 2 hr (500 μg/ml in borate buffer), washed three times, and air-dried before cell plating. Approximately 35,000 cells were plated in the glass area of each dish in DMEM (Invitrogen, San Diego, CA) containing 5% fetal bovine serum and 5% horse serum (Hyclone, Logan, UT). On the second day after plating, the culture medium was replaced by serum-free Neurobasal medium containing B27 supplement and 500 μM Glutamax (Invitrogen) for reduced glial growth. Cells were maintained in a CO_2 incubator at 37°C, and one-half volume of the culture medium was replaced with fresh Neurobasal medium every 3 d. Unless indicated otherwise, all the experiments were performed on cultures of 1–2 weeks after plating.

Ca^{2+} imaging. Hippocampal cells were loaded with 6 μM Fluo-3-AM (Sigma) in Krebs–Ringer's saline (in mM: 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4) (Bacci et al., 1999) at 37°C for 30 min, followed by three washes and a 15 min incubation period for further de-esterification of fluo-3-AM before imaging. Cells grown on the glass bottom in 35 mm dishes were directly imaged on a Nikon (Tokyo, Japan) inverted microscope (TE300) using a 40× numerical aperture, 1.30 oil immersion Plan Fluor objective. A Lambda DG-4 high-speed wavelength switcher (Sutter Instruments, Novato, CA) was used for fluo-3 excitation at 480 nm, and a cooled CCD camera (CoolSnap FX; Roper Scientific, Princeton, NJ) was used for image acquisition. MetaFluor imaging software (Universal Imaging Corporation, Downingtown, PA) was used for hardware control, image acquisition, and image analysis. Hippocampal cells were maintained at 35–37°C during imaging through the use of a heater positioned nearby the microscope stage. Typically, time-lapse recording of Ca^{2+} signals in hippocampal neurons was performed for 2 min control period before and 10 min period after the application of different chemicals; the sampling rate was one frame every 2 sec with a typical exposure time of 50 msec when CCD binning of 4 × 4 was used.

Quantitative analysis of synchronized Ca^{2+} spikes. Quantitative measurements of changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were done by obtaining average fluo-3 fluorescence intensity of a 3 × 3 $pixel^2$ analysis box placed at the center of the cell body; the intensity values were then subtracted of average background intensity measured in cell-free regions. Changes of $[Ca^{2+}]_i$ in each cell were then represented by the changes of relative fluo-3 fluorescence ($\Delta F/F_0$) where F_0 was the baseline intensity obtained from the 2 min control period. Ca^{2+} spikes were defined as rapid elevation of $\Delta F/F_0$ equal to or >20%. Under our imaging settings, fields of 3–10 neurons were typically recorded and subsequently analyzed. To determine the frequency of Ca^{2+} spikes, we counted the number of Ca^{2+} spikes over a 2 min period of the recording at a defined time point. As a result, the 2 min control period yielded only one frequency value, whereas the experiment period (10 min) resulted in five frequency values at different time points after bath application of a specific molecule. To assess the change in the spike frequency, these five frequency values after the drug application were normalized to the control frequency value and expressed as percentages, with a value of 100% indicating no change. Although we quantified and examined the changes in the spike frequency through the entire 10 min period after bath application, we typically used the normalized changes from the 2 min period starting at 5 min after bath application (5–6 min) for assessing the effects of a particular molecule on the spike frequency. Data from at least three dishes from different batches of cultures were pooled together and analyzed for statistical significant differences using paired Student's *t* test. Compiled data are expressed and graphed as mean ± SD, with *n* denoting the number of neurons studied.

Bath application of different factors. To examine the effects of PACAP and SDF-1 on spontaneous Ca^{2+} spikes, a 2× working concentration of the factor was made in Krebs–Ringer's solution and was applied to the cells to achieve the desired final concentration through 1:1 dilution (v/v). Specifically, we first recorded Ca^{2+} activities for 2 min control period in 1 ml of Krebs–Ringer's solution, removed 0.5 ml from the bath, added 0.5 ml of the 2× solution, and subsequently recorded for 10 min to examine the effects on spontaneous Ca^{2+} spikes. For control, we simply performed the same procedure to apply Krebs–Ringer's solution to determine that there was no artifact of this application method. Different agonists and antagonists were normally preincubated with the cultures for 20 min before the start of experiments. However, pertussis toxin (PTX) was preincubated with hippocampal cultures for 16 hr before the experiments. These agonists and antagonists were purchased from Sigma unless otherwise specified.

Electrophysiology. Synaptic currents were recorded from the somatic region of pyramidal-like hippocampal neurons using the whole-cell voltage-clamp technique. Similar to Ca^{2+} imaging, the culture medium was replaced with Krebs–Ringer's solution buffer before recording. The patch pipettes (4–6 MΩ resistance) were filled with an intracellular solution (in mM: 140 KCl, 10 HEPES, and 10 EGTA, pH 7.2). Whole-cell path-clamp recordings were made at room temperature (21–23°C) with the Axopatch-200B amplifier (Axon Instruments, Foster City, CA) in conjunction with pClamp8 software (Axon Instruments). The signals were filtered with a current filter at 5 kHz. Similar to Ca^{2+} imaging, two periods of recording were performed, one before and one after the addition of PACAP or SDF-1, and the frequency of spikes was determined from a 2 min period.

Results

Synchronized spontaneous Ca^{2+} spikes in cultured hippocampal networks

Hippocampal neurons from 1 to 3 weeks in culture were used for this study. Consistent with previously reported studies (Bartlett and Banker, 1984; Benson et al., 1994), our cultures contained an enriched population of pyramidal glutamatergic neurons, whereas a low percentage of inhibitory GABAergic cells could be found; glial growth was significantly reduced through the use of serum-free Neurobasal medium (Brewer et al., 1993). It has been shown that these primary hippocampal cultures mature after ~1 week in culture and form functional synaptic connections to exhibit spontaneous synaptic transmission (Verderio et al., 1999a). Spontaneous synaptic activities of these hippocampal neurons were examined by Ca^{2+} imaging using calcium-sensitive dye fluo-3 (Minta et al., 1989). Because each imaging field normally contained 3–10 neurons, we were able to not only examine the

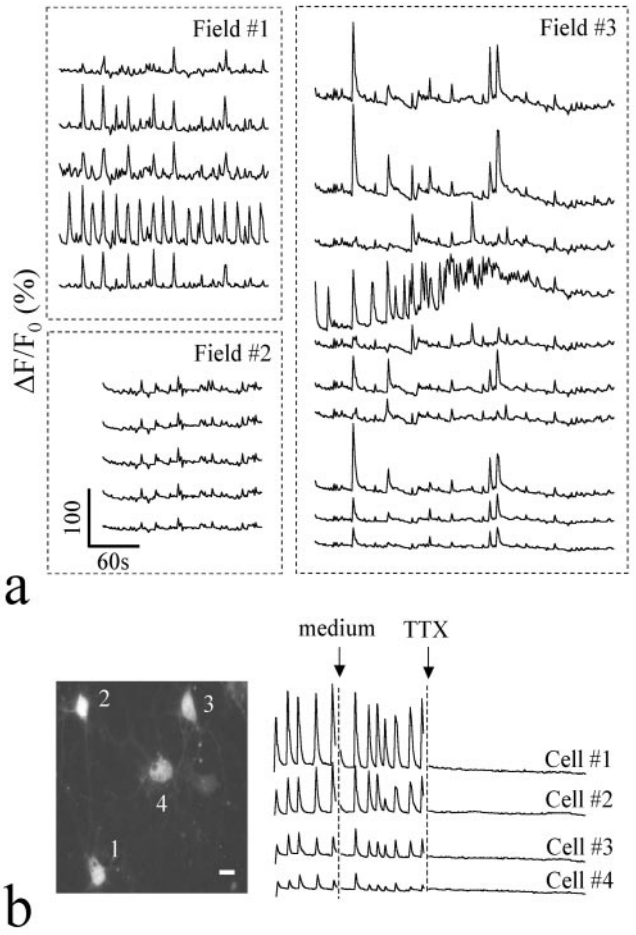

Figure 1. Synchronized spontaneous Ca^{2+} spikes in cultured hippocampal neurons. *a*, Representative recordings of synchronized spontaneous Ca^{2+} spikes from hippocampal neurons 2 weeks in culture. Traces indicate relative changes of fluo-3 intensity ($\Delta F/F_0$) over time in neurons from three microscopic fields randomly selected from the same culture dish. Each trace represents $\Delta F/F_0$ of an individual neuron acquired every 2 sec (see Materials and Methods). *b*, Blockade of spontaneous synchronized Ca^{2+} spikes by TTX. The fluorescent image on the left shows four hippocampal neurons loaded with fluo-3. Scale bar, 5 μm. Traces on the right depict Ca^{2+} spikes in these cells under control conditions, after application of control medium, and subsequent application of TTX (1 μM final).

spontaneous activity of individual neurons but also spatial and temporal patterns of Ca^{2+} activities among those interconnected neurons in the field. We found that many hippocampal neurons exhibited periodic, spontaneous spike elevation of $[Ca^{2+}]_i$ after 1 week in culture (Fig. 1*a*), and these spikes appeared to be primarily synchronized among the local group of cells. Neurons in different regions of the same culture dish were found to exhibit slightly different patterns of synchronized Ca^{2+} spikes. For example, Ca^{2+} spikes from three imaging fields in different regions of a single dish from 2 week culture displayed slightly different temporal patterns and amplitudes (Fig. 1*a*). Although synchronization is clearly observed among neighboring cells, some neurons did exhibit Ca^{2+} spikes with different temporal patterns. Furthermore, some neurons in a local region exhibited relatively smaller Ca^{2+} oscillations (Fig. 1*a*, field 2). Such variations in the spatiotemporal pattern of Ca^{2+} spikes may reflect the heterogeneity of connections among these cultured neurons, partially because of the nonuniform cell density in different regions of the dish. Because we were interested in neuronal activities of synaptically connected network, we focused mainly on regions of neurons exhibiting synchronized Ca^{2+} spikes hereafter.

Periodic, spontaneous Ca^{2+} spikes in cultured CNS neurons have been reported previously to result from action potential firing among synaptically connected neurons (Bacci et al., 1999; Przewlocki et al., 1999; Verderio et al., 1999b; Numakawa et al., 2002). Furthermore, concurrent recordings of membrane potentials and $[Ca^{2+}]_i$ revealed that each Ca^{2+} spike correlates to a burst of action potentials (Bacci et al., 1999; Verderio et al., 1999b). To confirm that Ca^{2+} spikes observed in our hippocampal cultures were driven by membrane depolarization through action potentials, we bath-applied tetrodotoxin (TTX) to block neuronal action potentials in culture. We found that TTX at 1 μM (final concentration in bath) completely abolished all Ca^{2+} spikes (Fig. 1*b*), indicating that these spontaneous Ca^{2+} spikes were the direct result of membrane depolarization from action potentials among synaptically connected neurons. These findings demonstrate that cultured hippocampal neurons formed functional synaptic connections and exhibited periodic firing of action potentials that could be readily detected as Ca^{2+} spikes by Ca^{2+} imaging. Furthermore, our results further support the use of Ca^{2+} imaging as an efficient method to examine the temporal and spatial patterns of neuronal activities among a small network of interconnected neurons.

Frequency increase of synchronized Ca^{2+} spikes by the neuropeptide PACAP

To examine how synchronized neuronal activities in networked neurons can be modulated by G-protein signaling through cAMP pathway, we examined the effects of PACAP on synchronized Ca^{2+} spikes. PACAP belongs to the family of VIPs and has been shown to exert a number of profound influences on the nervous system (Lindholm et al., 1998; Waschek, 2002). To test the effects of PACAP on synchronized Ca^{2+} spikes, we bath-applied PACAP to hippocampal cultures and performed Ca^{2+} imaging before and after application. We found that PACAP (10 nM in bath) induced a marked increase in the frequency of Ca^{2+} spikes, although the amplitude of each Ca^{2+} spike was not significantly affected (Fig. 2*a*). To quantify the frequency enhancement of PACAP on Ca^{2+} spikes, we determined the spike frequency at various time points after PACAP application and compared it to that of the control period. We found that at 5 min after PACAP application, the spike frequency was increased to 178 $\pm$ 28% (mean $\pm$ SD) of that of the control period. On the other hand, the spike amplitude was not significantly affected by PACAP because the average spike amplitude from the 5–6 min period after PACAP addition was 106 $\pm$ 60% ($n = 24$) of that of the control period. Bath application of the control saline buffer (control) did not affect the frequency of Ca^{2+} spikes (Fig. 2*b*), indicating that no artifact was produced by the bath application method. We also performed electrophysiological recordings using the whole-cell voltage-clamp method. Consistent with Ca^{2+} imaging results, hippocampal neurons exhibited periodic firing that was recorded as EPSCs, which corresponded to Ca^{2+} spikes. Bath application of 10 nM PACAP significantly increased the frequency of EPSCs without affecting the amplitude (Fig. 3*a*). We quantified the frequency of spontaneous firing by counting the number of EPSCs over a 2 min time period and found that PACAP gradually increased neuronal firing within minutes after bath addition (Fig. 3*b*). The enhancing effect of PACAP on periodic spikes was most evident at 5–6 min after PACAP addition (Fig. 3*c*), a finding that supports our Ca^{2+} imaging results. We thereafter used the spike frequency from the 5–6 min period after PACAP application for future statistical comparison and analysis.

PACAP and the closely related VIP act on a distinct family of

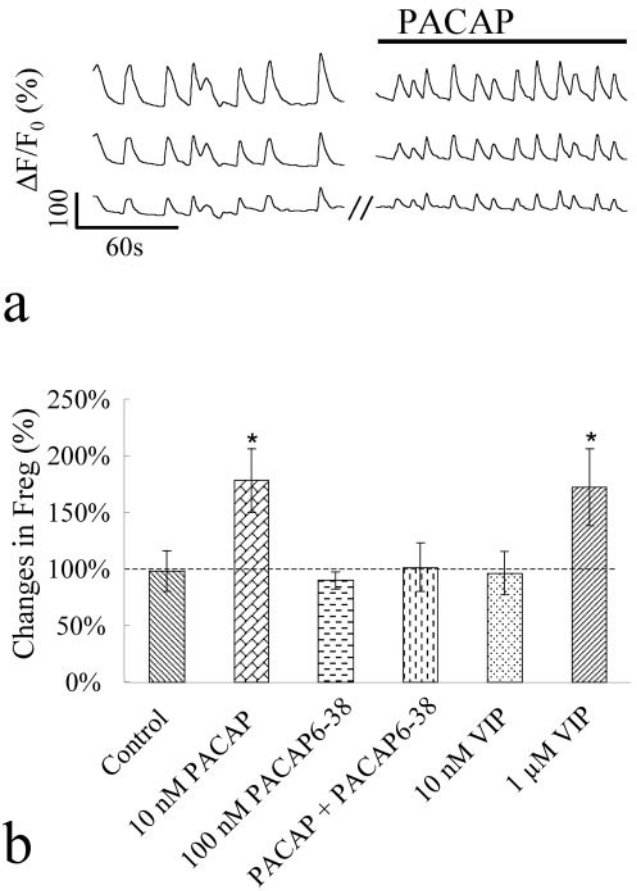

Figure 2. Increase in the frequency of synchronized Ca^{2+} spike firing induced by PACAP. *a,* Traces show synchronized Ca^{2+} spikes in three neurons randomly selected from a group of synchronically firing cells before and after bath addition of PACAP (10 nM). The time gap (//) is 5 min. *b,* Changes of the synchronized Ca^{2+} spike frequency 5–6 min after bath application of a different molecule or molecules. The change of the Ca^{2+} spike frequency was quantified by normalizing the spike number from a 2 min period (5–6 min) after bath application to the spike number in the control period and presented as the percentage. Data are presented as the mean ± SD from many cells (PACAP: $n = 24$; PACAP6–38: $n = 21$; PACAP + PACAP6–38: $n = 22$; 10 nM VIP: $n = 22$; 1 μM VIP: $n = 23$, PACAP6–38). Asterisks indicate significance against the control ($p < 0.005$, *t* test).

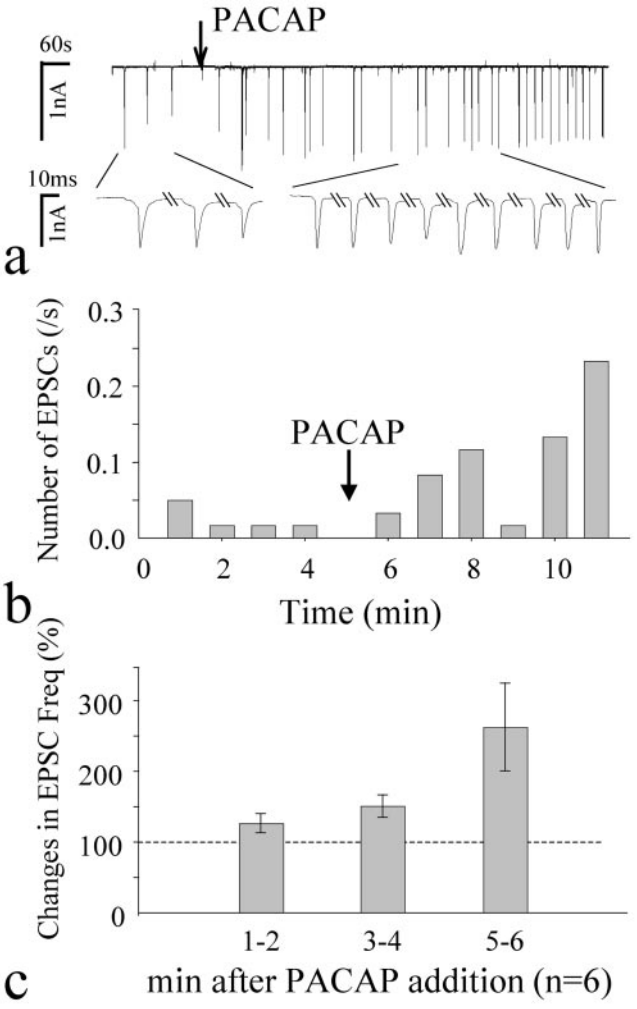

Figure 3. Increased frequency of firing of hippocampal neurons by PACAP, as recorded by the whole-cell patch-clamp technique. *a,* Effects of PACAP on EPSCs in a hippocampal neuron from a 1 week culture. The trace represents the spontaneous EPSCs of a hippocampal neuron from a 1 week culture before and after bath application of 10 nM PACAP. The shape of each EPSC is depicted by the expanded traces. To determine the changes of the EPSC number over time, we counted the numbers of EPSCs every 1 min before and after the addition of PACAP (*b*). *c,* The increase in the number of EPSCs after PACAP addition was quantified by normalizing EPSC number from a 2 min period to that of the control period. Data represent mean ± SD.

G-protein-coupled receptors to induce a variety of cellular effects. To determine the receptors involved in PACAP potentiation of Ca^{2+} spikes, we first used the antagonist PACAP6–38 to block PACAP action (Robberecht et al., 1992; Beaudet et al., 2000). Bath application of 100 nM PACAP6–38 alone slightly inhibited the Ca^{2+} spike frequency, suggesting that there might be endogenous activation of PACAP receptors in untreated hippocampal cultures. Adding PACAP6–38 before PACAP completely blocked PACAP increase of Ca^{2+} spikes, indicating the requirement of activation of PACAP receptors (Fig. 2*b*). To further determine what specific type of receptors mediated the PACAP potentiation of Ca^{2+} spikes, we tested the effects of VIP on synchronized Ca^{2+} spikes. Although the PACAP-selective PAC1 receptor can be activated by nanomolar PACAP, it can only be activated by VIP at micromolar concentrations. On the contrary, VPAC GPCRs (VPAC1 and VPAC2) can be activated equally by PACAP and VIP at nanomolar concentrations (Vaudry et al., 2000). Two different concentrations of VIP, 10 nM and 1 μM, were used to examine the effects of VIP on synchronized Ca^{2+} spikes. We found that VIP at 10 nM did not affect the frequency of Ca^{2+} spikes, whereas VIP at 1 μM significantly increased the frequency (Fig. 2*b*). The results indicate that activation of PAC1 receptors likely mediated the potentiation of synchronized Ca^{2+} spikes in hippocampal networks. Taken together, these results demonstrate that PACAP can increase the frequency of synchronized Ca^{2+} spikes in hippocampal networks through the activation of mainly PAC1, not VPAC GPCRs.

Activation of cAMP signaling pathway mediates PACAP potentiation effects

One major intracellular signaling pathway downstream of PACAP and PAC1 receptor interaction is the activation of adenylate cyclases to elicit cAMP signaling cascades (Ishihara et al., 1992; Hashimoto et al., 1993; Lutz et al., 1993; Vaudry et al., 2000). To verify the involvement of cAMP pathway in PACAP-induced potentiation of network Ca^{2+} spikes, a number of pharmacological agents known to affect cAMP signaling pathway were tested, including specific antagonists of protein kinase A KT5720 and Rp-cAMP as well as adenylate cyclase activator for-

skolin. Bath application of KT5720 (1 μM) or Rp-cAMP (200 μM) alone was found to reduce the frequency of spontaneous Ca^{2+} spikes (Fig. 4*a,b*), suggesting the involvement of cAMP pathway in regulation of synchronized Ca^{2+} spikes in hippocampal networks. Furthermore, for neurons that we first bath-applied Rp-cAMP, subsequent application of PACAP failed to increase the spike frequency, leaving it below the control level (Fig. 4*b*). The complete blockade of PACAP frequency-enhancing effects was better illustrated by experiments in which hippocampal neurons were preincubated with either KT5720 or Rp-cAMP (Fig. 4*c,d*). The presence of either antagonist resulted in no change of Ca^{2+} spike frequency in response to PACAP application. These results provide direct evidence that PACAP-induced increase of Ca^{2+} spike frequency required activation of cAMP pathway. Finally, to test whether activation of cAMP pathway was sufficient to increase the frequency of synchronized network Ca^{2+} spikes, we bath-applied forskolin to activate adenylate cyclase to elevate intracellular cAMP levels. Bath application of 15 μM forskolin greatly increased the synchronized Ca^{2+} spike frequency to an extent similar to that induced by PACAP (Fig. 4*b*). These results demonstrate that cAMP pathway is necessary and sufficient to regulate the synchronized Ca^{2+} spikes in neuronal networks, and it directly mediates PACAP enhancement effects on neuronal activities.

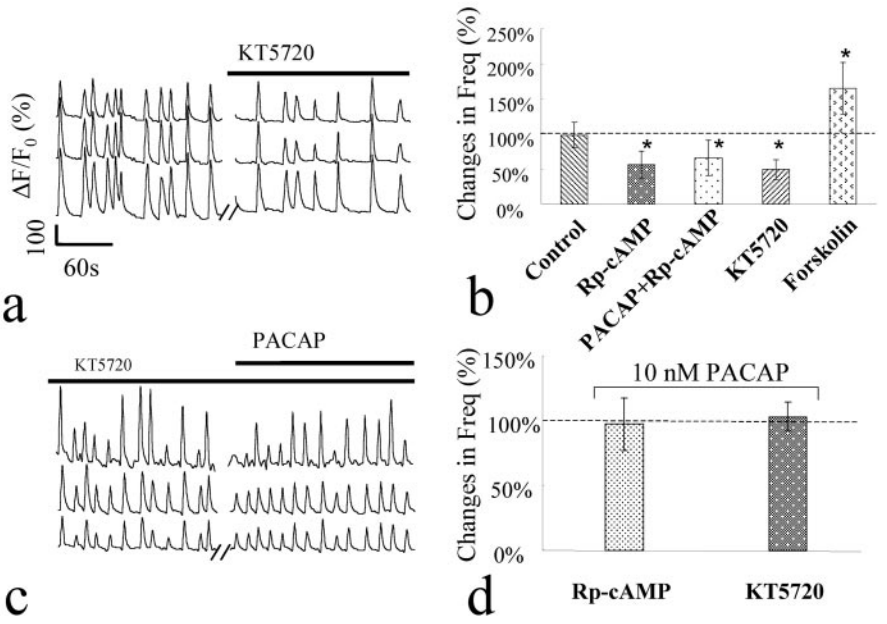

Figure 4. cAMP dependence of PACAP enhancement of Ca^{2+} spike frequency. *a,* Modulation of the frequency of spontaneous Ca^{2+} spikes by manipulation of cAMP signaling pathway. Traces show representative recordings of synchronized Ca^{2+} spikes in three hippocampal neurons randomly selected from the imaging field under baseline and after bath application of KT5720 (1 μM). *b,* Effects of different PAK antagonists and agonist on the frequency of the synchronized Ca^{2+} spikes. As described previously, the modulation was quantified by normalizing the number of spikes in 5–6 min after bath application to that of the control period. Data represent mean ± SD from populations of cells (1 μM KT5720: $n = 9$; 200 μM Rp-cAMP: $n = 21$; 10 nM PACAP + Rp-cAMP: $n = 18$; 15 μM forskolin: $n = 20$). *$p < 0.005$ to the control group treated by control medium (*t* test). *c,* Traces indicate the Ca^{2+} spikes in three hippocampal neurons in the presence of 1 μM KT 5720 before and after PACAP application (10 nM). *d,* Bar graph summarizes the blockade of PACAP effects on Ca^{2+} spike frequency by KT5720 or Rp-cAMP.

Chemokine SDF-1 inhibits synchronized spontaneous Ca^{2+} spikes

Both SDF-1 and its sole GPCR CXCR4 are widely expressed in the nervous system, including hippocampus, and SDF-1 and CXCR4 interactions have been shown to activate G_i signaling pathway (Asensio and Campbell, 1999). We therefore tested whether and how SDF-1 was able to affect the network spike activities. Acute application of 100 nM SDF-1 markedly reduced the frequency of synchronized Ca^{2+} spikes (Fig. 5*a*). We have quantitatively analyzed the inhibitory effects of two concentrations of SDF-1, 50 and 100 nM, on the frequency of Ca^{2+} spikes, and both concentrations were able to significantly decrease the Ca^{2+} spike frequency with higher concentration being more effective (Fig. 5*b*). Because previous studies have shown that activation of CXCR4 GPCRs by SDF-1 led to the activation of Gi signaling pathway to inhibit adenylate cyclase (Asensio and Campbell, 1999), we tested this possibility by using PTX. The $G\alpha_i$ subunits contain a conserved COOH-terminal cysteine residue that is the site of ADP ribosylation catalyzed by PTX, and this irreversible, covalent modification uncouples the G-proteins from its activating receptor to block its effect (Morris and Malbon, 1999). We found that pretreatment of hippocampal neurons with PTX (200 ng/ml for 16 hr) primarily abolished the inhibition of SDF-1 on synchronized Ca^{2+} spikes (Fig. 5*b*), demonstrating the involvement of Gi signaling pathway in SDF-1 inhibitory effects on synchronized network activity.

Because the Gi pathway directly inhibits adenylate cyclases to reduce cAMP production, thus inhibiting cAMP signaling pathway, we examined whether SDF-1 inhibition on synchronized spikes was a result of inhibition of cAMP pathway. To experimentally test this hypothesis, a potent cell-permeant cAMP agonist, dibutyryl-cAMP (db-cAMP), was used to activate PKA. Whereas bath application of SDF-1 (100 nM) significantly reduced the frequency of synchronized Ca^{2+} spikes, subsequent application of db-cAMP (500 μM) rapidly abolished the inhibition and restored the spike frequency to that of the control period (Fig. 6). Together with our findings that inhibition of the cAMP pathway by KT5720 or Rp-cAMP resulted in decrease of spike frequency (Fig. 4*a,b*), these results demonstrate that SDF-1 decreased the frequency of synchronized network activity (Ca^{2+} spikes) by inhibiting the cAMP pathway.

Discussion

In this study, we report profound modulatory effects of PACAP and SDF-1 on spontaneous network activities of developing neuronal circuits in culture. Although both molecules have previously been shown to exert numerous influences on the nervous system, our findings demonstrate, for the first time to our knowledge, that these two molecules can oppositely modulate the frequency of synchronized spike activities in cultured hippocampal circuits. Specifically, we show that the frequency of synchronized, spontaneous Ca^{2+} spikes occurring in networked hippocampal neurons can be effectively increased and inhibited by PACAP and SDF-1, respectively. We then demonstrate that both molecules exert their distinct actions through the activation of their G-protein-coupled receptors. Finally, we provide evidence that the cAMP pathway mediates the opposite effects of these two

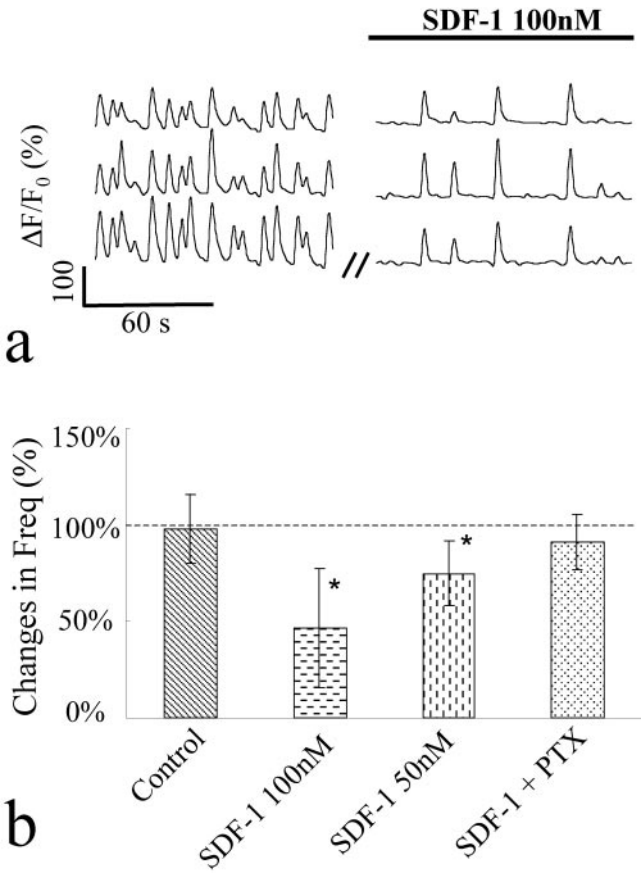

Figure 5. Inhibition of synchronized Ca^{2+} spikes by SDF-1. *a,* Representative recordings of synchronized Ca^{2+} spikes in three hippocampal neurons randomly selected in the imaging field before and after addition of SDF-1 (100 nM). The time gap (//) indicates ~5 min. *b,* The decrease of the Ca^{2+} spike frequency by SDF-1 is quantified by normalizing the number of spikes from 5–6 min after SDF-1 application to the control period. Data represent mean ± SD from populations of cells (100 nM SDF-1: $n = 35$; 50 nM SDF-1, $n = 15$). For PTX experiments, hippocampal cultures were pretreated with 200 ng/ml PTX for 16 hr before Ca^{2+} imaging ($n = 11$). *$p <$ 0.005 to the control values treated by control medium (*t* test).

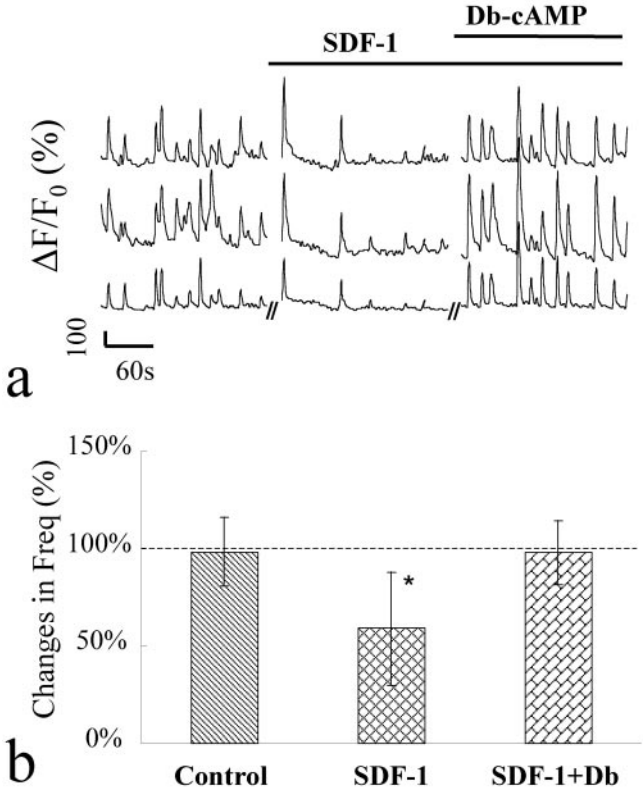

Figure 6. cAMP dependence of SDF-1 inhibition of synchronized spontaneous Ca^{2+} spikes. *a,* Representative recordings of synchronized Ca^{2+} spike in three hippocampal neurons under baseline control conditions, in the presence of SDF-1 (100 nM), and after subsequent addition of 500 μM dibutyryl cAMP. *b,* The bar graph depicts the normalized frequency changes of control (saline application), SDF-1 addition, and after subsequent addition of db-cAMP. Data represent mean ± SD from 13 neurons of three different dishes. *$p < 0.005$ to the control values treated by control medium (*t* test).

molecules on network spikes: PACAP activates, but SDF-1 inhibits cAMP signaling, probably through their regulation of adenylate cyclase activity. Taken together, these results demonstrate that neuronal activities can be effectively modulated by molecules acting through G-protein-coupled receptors and suggest a potential role for PACAP and SDF-1 in the development of neuronal circuitry.

Considering that the hippocampal area is the locus of mammalian learning and memory (Traub and Wong, 1982; Abel and Kandel, 1998) and where both SDF-1 and PACAP, as well as their receptors CXCR4 and PAC1, are expressed during development and adult time (McGrath et al., 1999; Jaworski and Proctor, 2000; Tham et al., 2001), it is conceivable that PACAP and SDF-1 could play an important role in hippocampal circuitry formation and plasticity. Effects of PACAP and SDF-1 on neurotransmission have been reported previously (Zhong and Pena, 1995; Asensio and Campbell, 1999; Limatola et al., 2000; Roberto and Brunelli, 2000). However, it is not known how these two factors may affect and modulate neuronal network activity. Our results here provide the direct evidence that both molecules can effectively modulate the frequency of synchronized Ca^{2+} spikes resulted from action potential firing of hippocampal networks in culture. Significantly we show that the two molecules can oppositely modulate the spike frequency of the network, PACAP increases, whereas SDF-1 inhibits network firing. Spontaneous neuronal activities are believed to shape developing neuronal circuits by facilitating the establishment of regional specific connections and refining initial projections to precise connectivity in the mature network (Goodman and Shatz, 1993). Although how the opposite effects of PACAP and SDF-1 on spontaneous activities observed here contribute to the early circuitry development *in vivo* remains to be determined, our findings should provide interesting avenue for future research. Moreover, because long-term potentiation (LTP) and depression (LTD) of neuronal circuitry are believed to be the cellular mechanisms underlying learning and memory, the modulatory effects of these two molecules on neuronal activity may play a part in normal neuronal functions. In the hippocampus, knock-out of PACAP and its receptors has been shown to lead to impairment of LTP (Otto et al., 2001). Therefore, it would be of specific interest to investigate how these two molecules may contribute to LTP and LTD underlying learning and memory.

Both PACAP and SDF-1 act on G-protein-coupled receptors to exert their functions. Unlike SDF-1, which binds to its only GPCR, CXCR4 (Zou et al., 1998; McGrath et al., 1999), PACAP is capable of binding to three different GPCRs, PAC1, VPAC1, and VPAC2, with high affinity (Vaudry et al., 2000; Waschek, 2002). Our results provide strong evidence that PAC1, the PACAP-selective GPCR, mediates the enhancing effects of PACAP on Ca^{2+} spikes: 10 nM VIP did not affect the synchronized Ca^{2+}

spikes, however, 1 μM VIP was found to significantly increase the frequency of synchronized Ca^{2+} spikes, a result that is consistent with the fact that VIP activation of PAC1 receptors requires micromolar concentrations. Together with the blockade of PACAP potentiation by PACAP6–38, our results indicate that the PAC1 GPCR mediates PACAP effects.

Our results show that the opposing modulation on the frequency of synchronized Ca^{2+} spikes in cultured hippocampal networks by PACAP and SDF-1 is mediated by opposing regulation on cAMP levels. This is consistent with previous findings that PAC1 is coupled to Gs (Vaudry et al., 2000), whereas CXCR4 is linked to Gi signaling pathway to target on adenylate cyclases (Asensio and Campbell, 1999). The cAMP signal transduction pathway is one of the pivotal regulators of synaptic neurophysiology (Anholt, 1994). The most likely mechanism of cAMP to mediate its effect is through the activation of cAMP-dependent protein kinase A (PKA), which has a wide range of neuromodulatory actions from ion channel modification to gene expression (Johnson et al., 1994; Impey et al., 1996; Gray et al., 1998). The blockade of PACAP potentiation by PKA antagonists suggests that other downstream signal pathways other than PKA might not play a significant role in mediating the potentiation effects of PACAP. This notion is further supported by our findings that direct alteration of PKA activity could also change the frequency of spontaneous Ca^{2+} spikes. Because considerable amount of information is known about the modulatory effects of PKA on various receptors and channels activity ranging from the L-type Ca^{2+} channel to $GABA_A$ receptors (Moss et al., 1992; Bunemann et al., 1999), modulation of spike frequency by PACAP and SDF-1 might result from actions of PKA on a variety of downstream targets, especially including the ionic permeability of channels. It can be reasonably inferred that phosphorylation of voltage-dependent calcium channels and potassium channels would increase the probabilities of action potential firing, thus finally increasing the frequency of synchronized Ca^{2+} spikes. It is also conceivable that other mechanisms might contribute to the modulatory effects on synchronized spike activity by these types of ligand–GPCR interactions. For example, direct regulation through nucleotide-gated channels (Kaupp and Seifert, 2002) and G-protein-linked voltage-sensitive channels (Zamponi and Snutch, 1998; Dascal, 2001) can modulate the synaptic excitability to influence the spike frequency. Further investigation of the molecular and cellular mechanisms underlying the modulation by these two molecules is clearly required.

The role of cAMP in synaptic transmission as well as learning and memory has been extensively studied (Kandel and Schwartz, 1982). For instance, application of membrane-permeable analog of cAMP was shown to enhance the number of active presynaptic terminals, leading to synaptic potentiation (Qi et al., 1996; Ma et al., 1999). However, cAMP-mediated modulation of synchronized spontaneous Ca^{2+} spikes in network activity has not been well studied. In this study, we present direct evidence that the cAMP pathway mediates modulation of spike frequency of synchronized spontaneous Ca^{2+} spikes in hippocampal circuits in culture by PACAP and SDF-1 acting on GPCRs. Because spontaneous neuronal activities have been shown to regulate synaptic strength and patterning, and vice versa (O'Donovan and Chub, 1997; Rutherford et al., 1998), our findings suggest that PACAP and SDF-1 may have a role in neuronal development *in vivo*. Furthermore, our findings on the modulation of Ca^{2+} spike frequency are of significance because many cellular events during neuronal differentiation appear to depend primarily on the frequency of Ca^{2+} signals, including the activation of distinct sets of transcriptional factors and the expression of different genes (Gu and Spitzer, 1995; Fields et al., 1997; De Koninck and Schulman, 1998; Dolmetsch et al., 1998; Li et al., 1998); potentially the changes in gene expression would subsequently alter the distribution and function of membrane receptors to affect the synaptic activities, ultimately leading to refined neuronal connectivity. Therefore, it is reasonable to speculate that the changes in Ca^{2+} spike frequency (presumably the action potential firing frequency) induced by endogenous or exogenous factors through G-protein signaling during development could effectively alter the connectivity as well as synaptic strength, allowing the formation of distinct neuronal circuits.

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