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# Calcium oscillations in melanotrope cells of *Xenopus laevis* are differentially regulated by cAMP-dependent and cAMP-independent mechanisms

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**Summary** Intracellular Ca<sup>2+</sup> oscillations play an important role in the induction of  $\alpha$ -MSH release from pituitary melanotrope cells of *Xenopus laevis*. Oscillatory, secretory and adenylyl cyclase activities are all inhibited by dopamine, neuropeptide Y (NPY) and baclofen (a GABA<sub>B</sub> receptor agonist) and stimulated by sauvagine. In this study, we test the hypothesis that these neural messengers regulate the Ca<sup>2+</sup> oscillations via a cAMP/protein kinase A (PKA)-dependent mechanism. To this end, video-imaging microscopy was applied to single *Xenopus* melanotropes loaded with the Ca<sup>2+</sup> indicator Fura-2. The cAMP-dependent PKA inhibitor H89 blocked Ca<sup>2+</sup> oscillations as well as the stimulatory actions of 8-Br-cAMP and sauvagine. Treatment of cells inhibited by baclofen with either 8-Br-cAMP or sauvagine led to a reappearance of Ca<sup>2+</sup> oscillations. A similar result was found for cells inhibited by NPY. Neither 8-Br-cAMP nor sauvagine induced Ca<sup>2+</sup> oscillations in cells inhibited by dopamine. Depolarizing dopamine-inhibited cells with high potassium also failed to induce oscillations, but combining 8-Br-cAMP with membrane depolarization induced oscillations. It is concluded that sauvagine, baclofen and NPY work primarily through a cAMP/PKA-pathway while dopamine inhibits Ca<sup>2+</sup> oscillations in a dual fashion, namely via both a cAMP-dependent and a cAMP-independent mechanism, the latter probably involving membrane hyperpolarization.

## INTRODUCTION

In lower vertebrates, melanophore-stimulating hormone ( $\alpha$ -MSH) controls the adaptation of skin colour to the light intensity of the background [1–3]. This adaptation process is being used as a model to study neural and endocrine information processing mechanisms [4].  $\alpha$ -

MSH is produced by the neuroendocrine melanotrope cells in the pars intermedia of the pituitary gland. The hormone causes pigment dispersion in skin melanophores and thereby skin darkening. In the clawed toad *Xenopus laevis*, the release of  $\alpha$ -MSH is under control of several neural factors. Dopamine, neuropeptide Y (NPY) and  $\gamma$ -aminobutyric acid (GABA) inhibit secretion [5–9] whereas thyrotropin-releasing hormone (TRH) [5,10], corticotropin-releasing hormone (CRH) and sauvagine, an amphibian peptide related to CRH, stimulate secretion [5,11]. The inhibitory factors occur in nerve terminals that make synaptic contacts with the melanotrope cells [12]. CRH and TRH have been demonstrated immunocytochemically to be present in nerve terminals in the pars nervosa [11] and presumably diffuse to the pars intermedia to act on the melanotrope cells.

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It is established that calcium plays an important role in the regulation of a variety of cellular activities, including the process of hormone secretion. *Xenopus* melanotropes display spontaneous  $\text{Ca}^{2+}$  oscillations [13–15]. While electrophysiologically we have shown the presence of both N- and L-type voltage-operated  $\text{Ca}^{2+}$  channels on *Xenopus* melanotropes [16], the oscillations are completely dependent on calcium influx through the  $\omega$ -conotoxin GVIA-sensitive N-type  $\text{Ca}^{2+}$  channels [14]. Confocal laser scanning microscopy has shown that this  $\text{Ca}^{2+}$  entry triggers mobilization of intracellular  $\text{Ca}^{2+}$  so that the oscillation is propagated to the nucleus [17]. The oscillations most likely trigger  $\alpha$ -MSH secretion because, as in the case of the oscillations, secretion depends on calcium influx through  $\omega$ -conotoxin GVIA-sensitive N-type channels [16] and secreto-inhibitors block  $\text{Ca}^{2+}$  oscillations, whereas the secreto-stimulators induce or enhance oscillatory activity [13,15]. The mechanisms through which these neural messengers control oscillatory activity in *Xenopus* melanotropes are largely unknown. Possibly, adenosine 3',5'-cyclic monophosphate (cAMP) is involved, as this second messenger stimulates  $\alpha$ -MSH secretion from *Xenopus* melanotropes [18–20]. Furthermore, activation of the dopamine  $D_2$  receptor [8,21], the NPY  $Y_1$  receptor [7,8] or the  $\text{GABA}_B$  receptor [20,21] decreases cAMP production by inhibiting adenylyl cyclase in this cell type, whereas sauvagine stimulates cAMP production [20,21]. On the basis of these data, we hypothesize that the regulatory neural factors control  $\alpha$ -MSH secretion by controlling oscillatory activity via a cAMP-dependent mechanism. To test this hypothesis, video-imaging microscopy was used to visualize the cytosolic  $\text{Ca}^{2+}$  dynamics in single melanotropes, loaded with the fluorescent  $\text{Ca}^{2+}$  indicator Fura-2/AM, that were treated with dopamine, NPY, baclofen (a  $\text{GABA}_B$  receptor agonist), sauvagine, 8-Br-cAMP, the protein kinase A (PKA) inhibitor H89 [22,23] or 20 mM  $\text{K}^+$  as a depolarizing stimulus. The results obtained in this study show that the melanotrope cell of *Xenopus laevis* possesses both cAMP-dependent and cAMP-independent mechanisms for the regulation of  $\text{Ca}^{2+}$  oscillatory activity.

## MATERIALS AND METHODS

### Animals

Young-adult *Xenopus laevis*, bred in our department according to standard procedures, were adapted, under continuous illumination at 22°C, to a dark background for at least 3 weeks prior to the experiments.

### Cell preparation

After anaesthetization with 1% MS222 (Sigma, St Louis, MO, USA), the animals were perfused with *Xenopus*

Ringer's solution (*see* below) to remove blood cells. After decapitation, neurointermediate lobes of the pituitary gland were rapidly dissected and rinsed 4 times in culture medium (*see* below). Subsequently, the lobes were transferred to 1 ml dissociation medium composed of *Xenopus* Ringer's solution without  $\text{CaCl}_2$  and with 0.25% (w/v) trypsin (Gibco, Renfrewshire, UK) and incubated for 45 min at 20°C. At the end of the incubation period, the lobes were triturated with a siliconized Pasteur pipette and 9 ml culture medium was added. The resulting cell suspension was filtered, followed by centrifugation for 10 min at 500 rpm. After removal of the supernatant, the cell pellet was resuspended in an adequate volume of culture medium (100  $\mu\text{l}$ /lobe equivalent). Then, cells were plated on cover slips coated with poly-L-lysine (Sigma; MW > 300 kDa), in aliquots equivalent to 1 lobe per cover slip, yielding approximately 10,000 cells/cover slip. Cell attachment was allowed for 16 h in an incubator, at 20°C. Then 2 ml culture medium was added to each culture dish and cells were incubated for another 3 days, at 20°C. Melanotrope cells were readily identified on the basis of their characteristic round shape.

### Solutions and chemicals

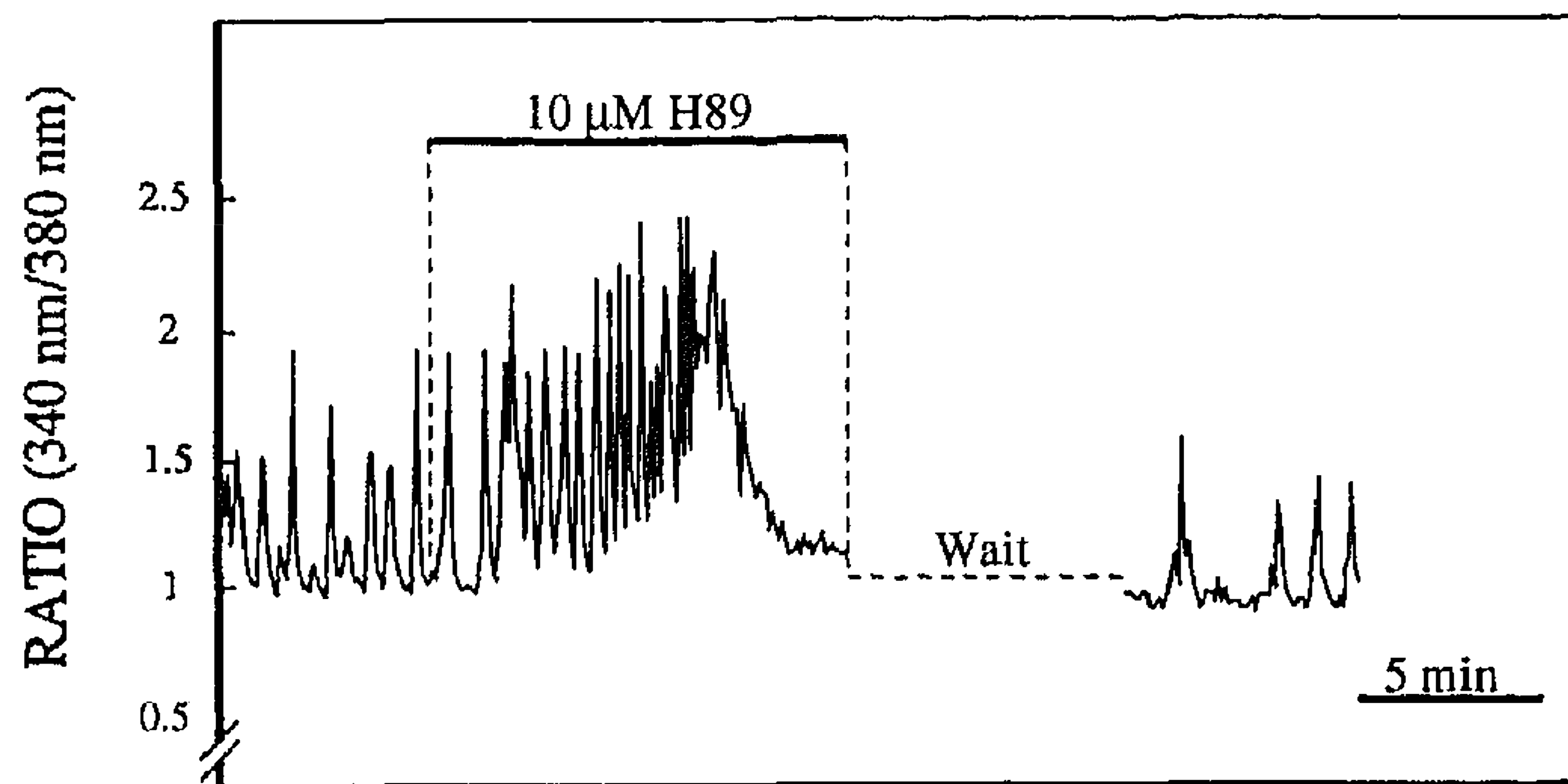
*Xenopus* Ringer's solution contained 112 mM NaCl, 15 mM Ulral HEPES (Calbiochem, La Jolla, CA, USA; pH = 7.4), 2 mM KCl and 2 mM  $\text{CaCl}_2$ , and was gassed with carbogen (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ). Then the pH was adjusted to 7.4 with NaOH.

The culture medium consisted of 76% (v/v) L15 medium (Gibco), 1% (v/v) kanamycin solution (Gibco), 1% (v/v) antibiotic/antimycotic solution (Gibco), 10% fetal calf serum (Gibco), 2 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 10 mM glucose (pH = 7.4). Before use the culture medium was sterilized by ultrafiltration using a 0.2  $\mu\text{m}$  filter (Schleicher & Schuell, Dassel, Germany).

Solutions of baclofen (RBI, Natick, MA, USA), NPY (American Peptides, Sunnyvale, CA, USA), dopamine (Sigma), 8-Br-cAMP (Sigma), sauvagine (Bachem, Bubendorf, Switzerland), H89 (Calbiochem),  $\omega$ -conotoxin GVIA (Peninsula, Belmont, CA, USA) or nifedipine (Sigma) were prepared with *Xenopus* Ringer's solution and administered to cell preparations (*see* below) using a perfusion pump. Concentrations used are stated in the Results.

### Fluorescence measurements of intracellular $\text{Ca}^{2+}$

For fluorescence measurements, cells were loaded with 2  $\mu\text{M}$  Fura-2/AM (Molecular Probes, Eugene, OR, USA) in *Xenopus* Ringer's solution containing 1  $\mu\text{M}$  Pluronic F127 (Molecular Probes) [24], for 20 min at 20°C. In previous experiments it had been found that Pluronic F127 greatly enhances the trapping of the dye into the melan-



**Fig. 1** Effect of the PKA inhibitor H89 on spontaneous  $\text{Ca}^{2+}$  oscillations in *Xenopus* melanotropes. H89 initially has a stimulatory effect on spontaneous  $\text{Ca}^{2+}$  oscillations but after 5–10 min the oscillations become suppressed.  $\text{Ca}^{2+}$  oscillations reappeared after returning to normal *Xenopus* Ringer's solution. The indicated waiting period corresponds to the time scale.

otrope cytoplasm without affecting the  $\text{Ca}^{2+}$  oscillations [24]. After loading, cells were washed with *Xenopus* Ringer's solution in a Leiden perfusion chamber (volume 800  $\mu\text{l}$ ) [25], at a flow rate of 1 ml/min, for 25 min, to remove excess of non-hydrolysed Fura-2/AM. Unattached cells were sucked off. The perfusion chamber was placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) and the cells were studied using an epifluorescent 40 $\times$  magnification oil immersion objective. The light from a 100 W xenon lamp was directed through a quartz neutral density filter (ND 2, Ealing Electro-Optics, Holliston, MA, USA) to reduce bleaching of the intracellularly trapped dye. Video-imaging microscopy was carried out as described previously [26] using the MagiCal hardware and TARDIS software of Joyce Loeb (Dukesway, Team Valley, Gateshead, Tyne and Wear, UK). The fluorescence emission ratio at 492 nm (average of 16 images) was monitored as a measure of the concentration of intracellular free calcium ions ( $[\text{Ca}^{2+}]_i$ ) after excitation at 340 and 380 nm [27]. The interframe interval between two successive ratio frames was 6.4 s, yielding a maximal sampling time of 32 min.

## RESULTS

All data were obtained from single *Xenopus* melanotrope cells loaded with Fura-2/AM, of which about 80% spontaneously showed  $\text{Ca}^{2+}$  oscillations. The  $n$  values in the results indicate the total number of single oscillating cells observed under the experimental paradigm. Each experiment was repeated at least 3 times. Non-oscillating cells were excluded from consideration. Unless indicated otherwise, all cells behaved in an identical manner.

### Effects of H89 on spontaneous and 8-Br-cAMP- or sauvagine-enhanced $\text{Ca}^{2+}$ oscillations

In order to investigate the role of cAMP and PKA in the regulation of  $\text{Ca}^{2+}$  oscillations, the effects of the selective PKA inhibitor H89, 8-Br-cAMP and sauvagine were studied. Upon addition of 10  $\mu\text{M}$  H89 to the melanotropes an initial stimulatory effect on  $\text{Ca}^{2+}$  oscillations was observed which lasted 5–10 min. Subsequently,  $\text{Ca}^{2+}$  oscillations disappeared (Fig. 1,  $n = 17$ ). Returning to normal *Xenopus* Ringer's solution slowly reintroduced oscillatory activity, showing that the cells were still viable and responsive.

Under suppression of oscillatory activity by H89, 10 nM sauvagine (Fig. 2A,  $n = 10$ ) or 1 mM 8-Br-cAMP (Fig. 2B,  $n = 14$ ) alone or in combination with a depolarizing  $\text{K}^+$  pulse (20 mM KCl) did not induce  $\text{Ca}^{2+}$  oscillations. Also in cells in which the frequency of the  $\text{Ca}^{2+}$  oscillations was increased, H89 completely blocked the oscillatory activity. In the presence of H89, 20 mM KCl together with 10 nM sauvagine (Fig. 2C,  $n = 14$ ) or with 1 mM 8-Br-cAMP (Fig. 2D,  $n = 8$ ) did not induce  $\text{Ca}^{2+}$  oscillations.

### Effects of 8-Br-cAMP and sauvagine, alone and in combination with H89, on $[\text{Ca}^{2+}]_i$ in baclofen-, dopamine- and NPY-inhibited melanotropes

#### Baclofen

In order to investigate whether a cAMP-dependent mechanism is involved in the inhibition of  $\text{Ca}^{2+}$  oscillations by baclofen, the effects of 8-Br-cAMP and sauvagine were studied in cells treated with baclofen. H89 was used to test the possibility of PKA being involved in the mechanism of action of baclofen.

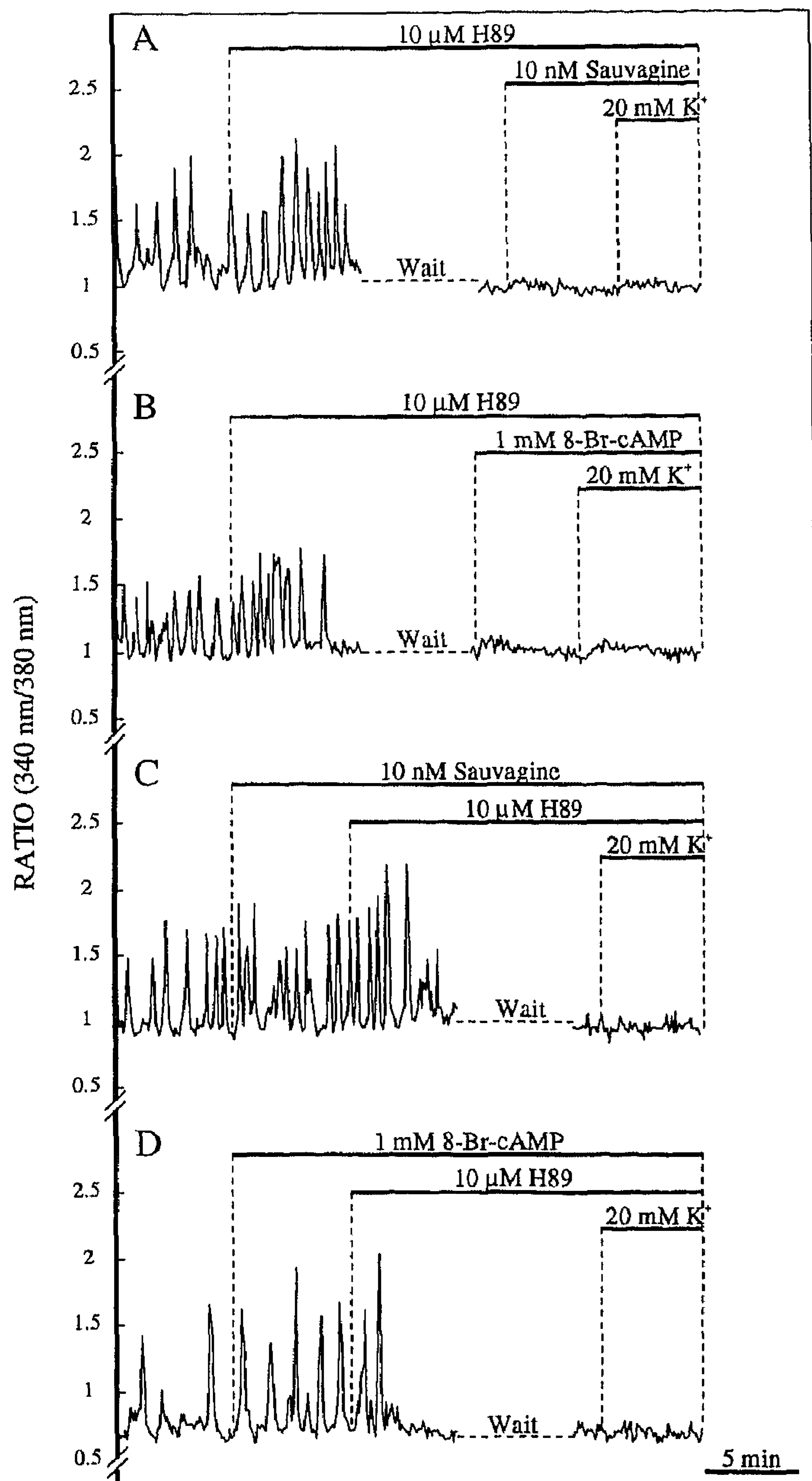
Within a few seconds following the addition of 3.3  $\mu\text{M}$  baclofen, spontaneous  $\text{Ca}^{2+}$  oscillations were blocked in all cells (Fig. 3,  $n = 36$ ). Subsequent addition of 1 mM 8-Br-cAMP led to reappearance of  $\text{Ca}^{2+}$  oscillations in most cells (Fig. 3A,  $n = 23$  out of 24), and in some cases ( $n = 7$  out of 24) the frequency of the oscillations was even higher than before baclofen treatment. The same experiment with addition of 10 nM sauvagine instead of 8-Br-cAMP showed a similar result (Fig. 3B,  $n = 12$ ); the frequency of the reappeared  $\text{Ca}^{2+}$  oscillations in Figure 3B is clearly higher than the frequency of the spontaneous  $\text{Ca}^{2+}$  oscillations before inhibition by baclofen.

In cells treated with baclofen, the selective PKA inhibitor H89 (10  $\mu\text{M}$ ) prevented induction of  $\text{Ca}^{2+}$  oscillations by 1 mM 8-Br-cAMP (Fig. 3C,  $n = 16$ ).

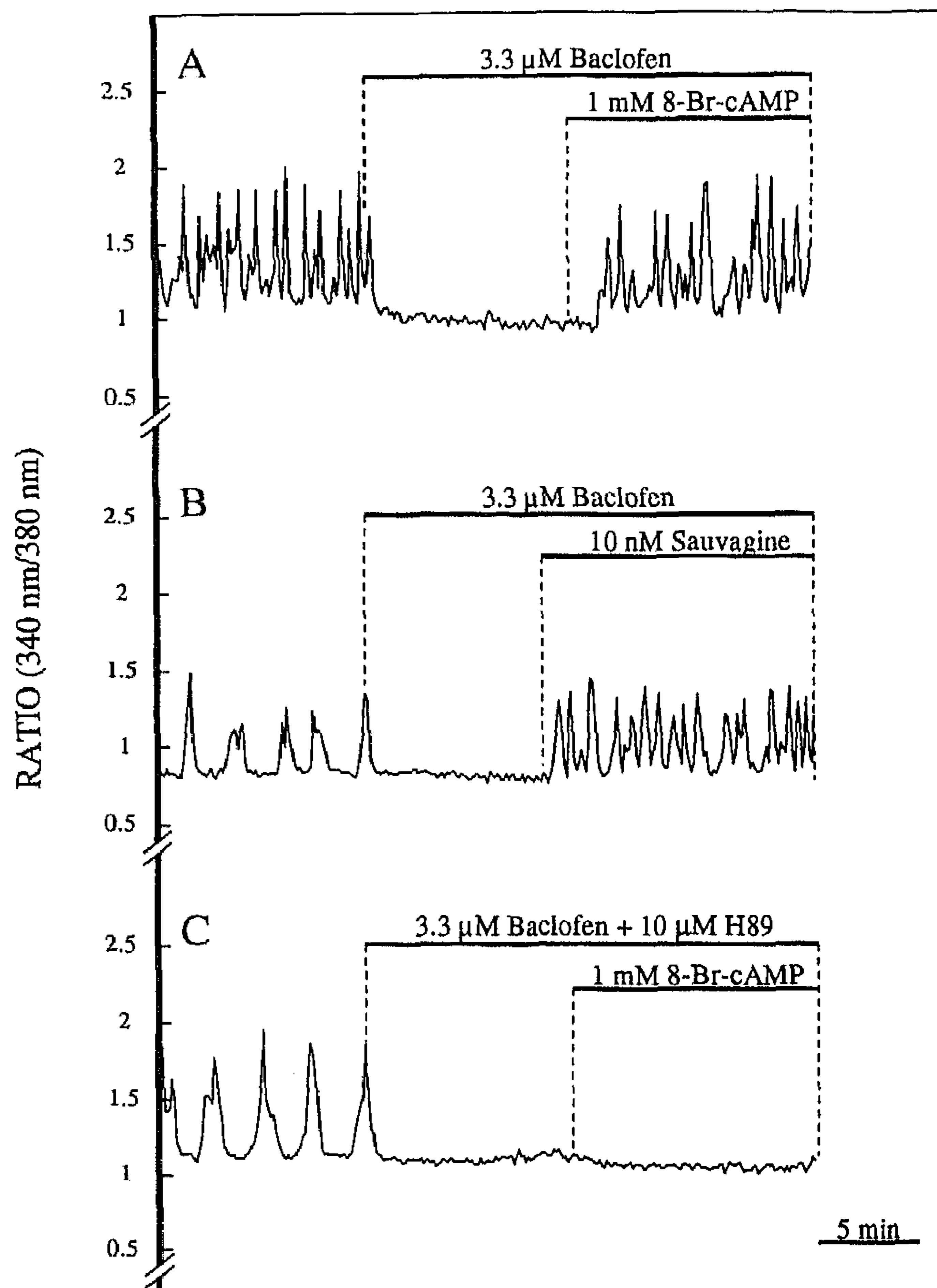
#### Dopamine

Dopamine (10 nM) blocked  $\text{Ca}^{2+}$  oscillations within a few seconds in all cells (Fig. 4,  $n = 42$ ). Neither 1 mM 8-Br-cAMP (Fig. 4A,  $n = 12$ ) nor 10 nM sauvagine (Fig. 4B,  $n =$

30), nor 20 mM  $K^+$  (Fig. 4C,  $n = 28$ ) restored  $Ca^{2+}$  oscillatory activity in such cells. On the other hand, in all cells, 20 mM  $K^+$  caused an increase in basal  $[Ca^{2+}]_i$ , often starting with a transient peak increase ( $n = 14$  out of 28). A



**Fig. 2** Effect of the PKA inhibitor H89 on  $Ca^{2+}$  oscillations and on treatments of sauvagine or 8-Br-cAMP with 20 mM  $K^+$ . (A) H89 initially has a stimulatory effect on spontaneous  $Ca^{2+}$  oscillations but 5–10 min after addition the  $Ca^{2+}$  oscillations become suppressed. Under H89 inhibition sauvagine alone or together with 20 mM  $K^+$  is not able to restore  $Ca^{2+}$  oscillations. (B) As in A but with 8-Br-cAMP instead of sauvagine. (C) H89 suppresses  $Ca^{2+}$  oscillations with an increased frequency enhanced by sauvagine. Under this condition 20 mM  $K^+$  has no effect on  $[Ca^{2+}]_i$ . (D) As in C but with 8-Br-cAMP instead of sauvagine.

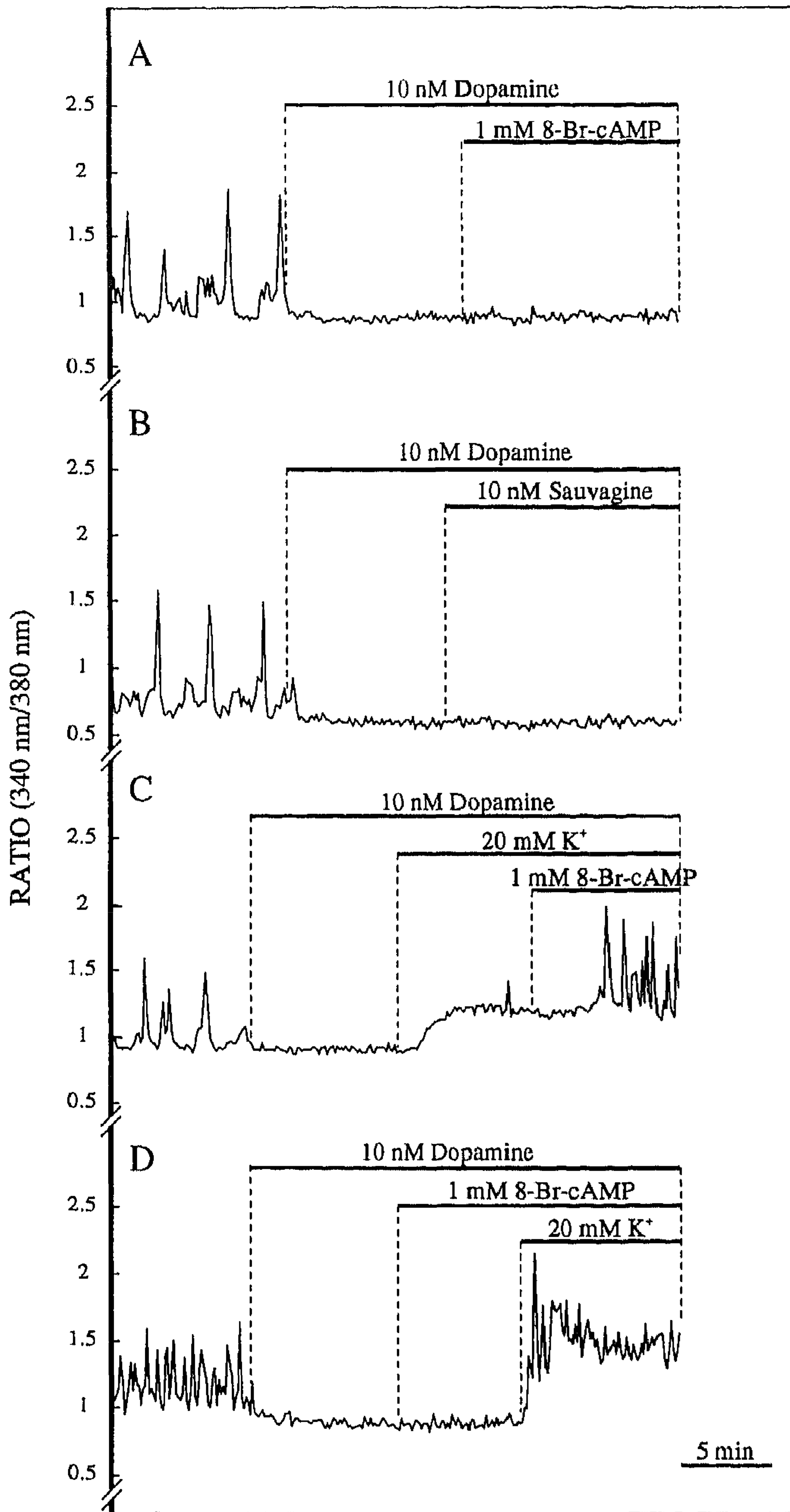


**Fig. 3** Effect of 8-Br-cAMP or sauvagine under baclofen inhibition. (A) 8-Br-cAMP restores  $Ca^{2+}$  oscillations under baclofen inhibition. (B) Sauvagine also restores  $Ca^{2+}$  oscillations under baclofen inhibition. (C) H89 prevents the reappearance of  $Ca^{2+}$  oscillations induced by 8-Br-cAMP under baclofen inhibition.

combination of 20 mM  $K^+$  and 1 mM 8-Br-cAMP effectively reintroduced  $Ca^{2+}$  oscillations in cells inhibited by dopamine (Fig. 4C,  $n = 18$  out of 28). Giving first 8-Br-cAMP and then 20 mM  $K^+$  led to a similar response (Fig. 4D,  $n = 23$ ). The N-type channel blocker  $\omega$ -conotoxin GVIA prevented the reappearance of  $Ca^{2+}$  oscillations (Fig. 5A,  $n = 34$ ), whereas the L-type channel blocker nifedipine did not (Fig. 5B,  $n = 19$ ). However, nifedipine blocked the increase in basal  $[Ca^{2+}]_i$  (Fig. 5B). In cells treated with dopamine, H89 prevented 20 mM  $K^+$  from increasing basal  $[Ca^{2+}]_i$ , and it also prevented 20 mM  $K^+$  in combination with 1 mM 8-Br-cAMP from restoring  $Ca^{2+}$  oscillations (Fig. 5C,  $n = 22$ ).

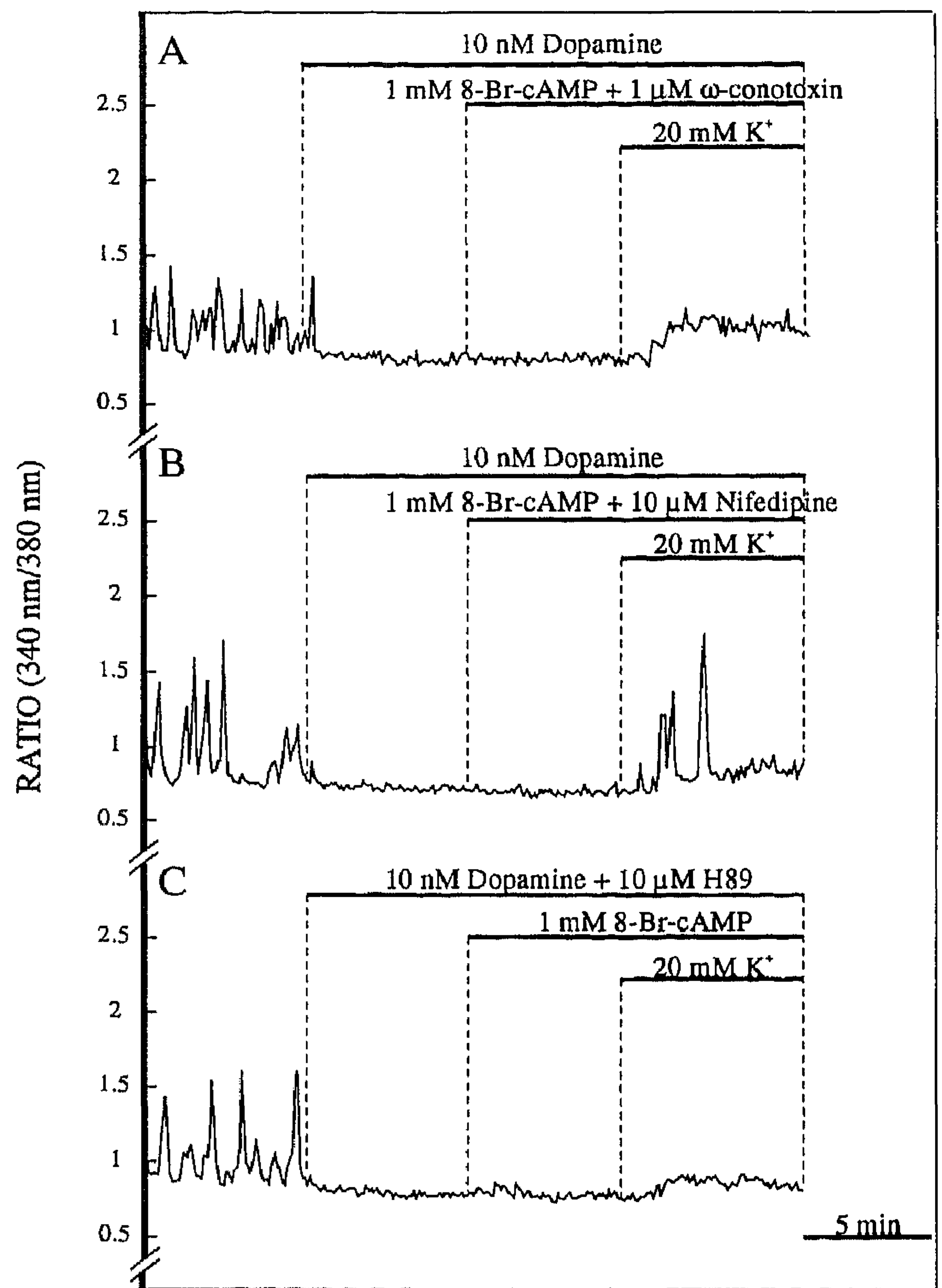
#### NPY

After addition of 2 nM NPY,  $Ca^{2+}$  oscillations were suppressed within a minute in all cells (Fig. 6,  $n = 49$ ). The



**Fig. 4** Effect of 8-Br-cAMP and sauvagine under dopamine inhibition. (A) 8-Br-cAMP fails to restore  $\text{Ca}^{2+}$  oscillations under dopamine inhibition. (B) Sauvagine fails to restore  $\text{Ca}^{2+}$  oscillations under dopamine inhibition. (C) 20 mM  $\text{K}^+$  alone fails to induce  $\text{Ca}^{2+}$  oscillations under dopamine inhibition but causes an increase in basal  $[\text{Ca}^{2+}]_i$ . A combination of 20 mM  $\text{K}^+$  and 8-Br-cAMP restores  $\text{Ca}^{2+}$  oscillations under dopamine inhibition. (D) As in C but with 20 mM  $\text{K}^+$  and 8-Br-cAMP reversed.

majority of cells inhibited by NPY displayed  $\text{Ca}^{2+}$  oscillations again when 1 mM 8-Br-cAMP (Fig. 6A,  $n = 26$  out of 33) or 10 nM sauvagine (Fig. 6B,  $n = 12$  out of 16) were added. When cells inhibited by NPY were treated with 10



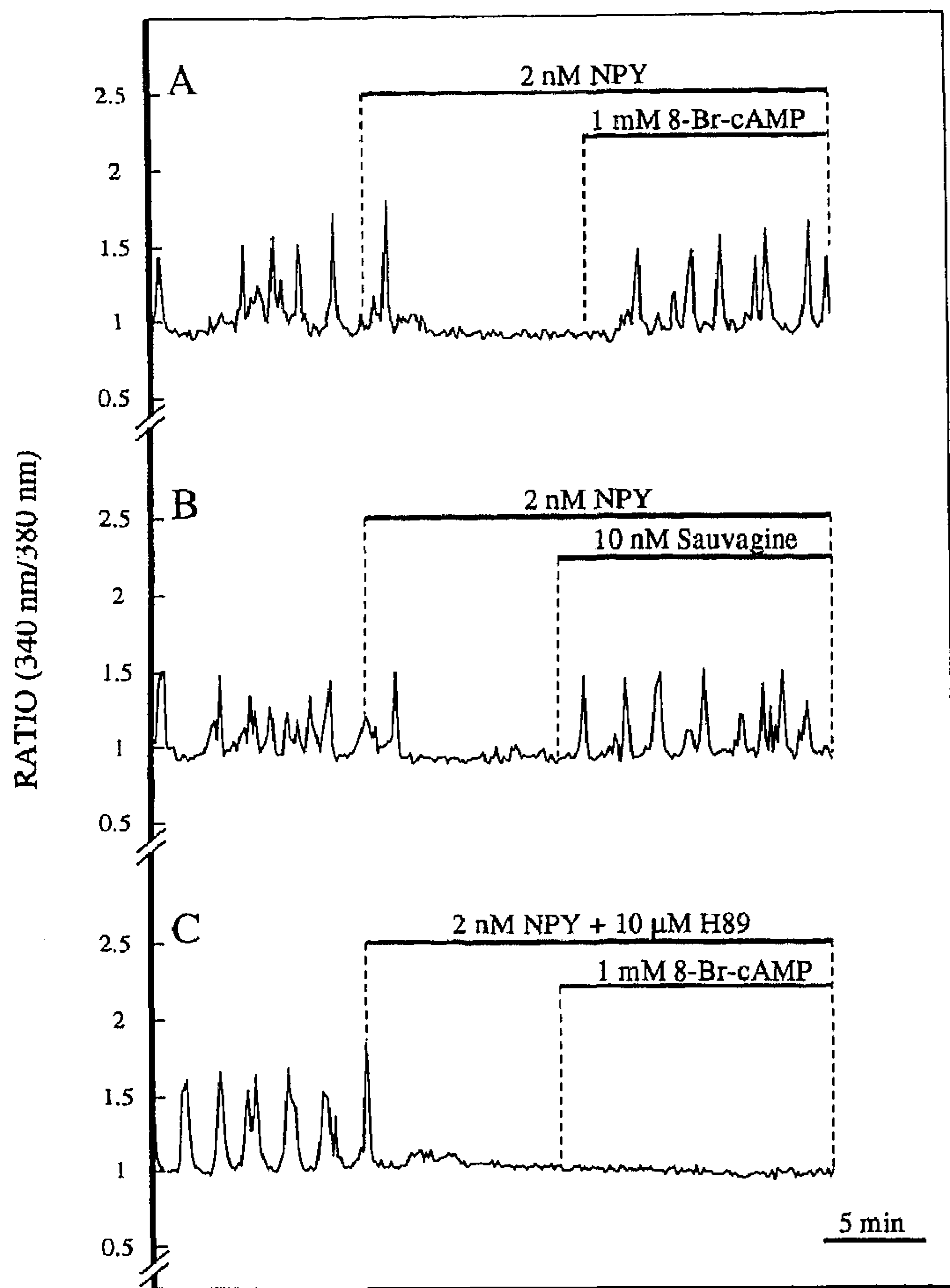
**Fig. 5** Effect of  $\omega$ -conotoxin GVIA, nifedipine and H89 on the responses of 8-Br-cAMP under dopamine inhibition. (A) As in Figure 4D but with in addition  $\omega$ -conotoxin GVIA.  $\omega$ -conotoxin GVIA prevents reappearance of  $\text{Ca}^{2+}$  oscillations whereas basal  $[\text{Ca}^{2+}]_i$  is still increased. (B) As in A but with nifedipine instead of  $\omega$ -conotoxin GVIA.  $\text{Ca}^{2+}$  oscillations are induced by 8-Br-cAMP and 20 mM  $\text{K}^+$  in the presence of nifedipine but the increase in basal  $[\text{Ca}^{2+}]_i$  is blocked by nifedipine. (C) Under dopamine inhibition H89 prevents the reappearance of  $\text{Ca}^{2+}$  oscillations and the increase in basal  $[\text{Ca}^{2+}]_i$  induced by 8-Br-cAMP and 20 mM  $\text{K}^+$ .

$\mu\text{M}$  H89, 1 mM 8-Br-cAMP did not restore oscillatory activity (Fig. 6C,  $n = 23$ ).

## DISCUSSION

### cAMP and PKA are necessary for $\text{Ca}^{2+}$ oscillations

In this study, it is shown that, in *Xenopus* melanotropes, the selective PKA inhibitor H89 reversibly abolishes spontaneous  $\text{Ca}^{2+}$  oscillations. This result supports an earlier H89 study on *Xenopus* melanotropes [28]. H89 is



**Fig. 6** Effect of 8-Br-cAMP or sauvagine under NPY inhibition. (A) 8-Br-cAMP restores  $\text{Ca}^{2+}$  oscillations under NPY inhibition in a majority of the cells. (B) As in A with sauvagine instead of 8-Br-cAMP. (C) H89 prevents the reappearance of  $\text{Ca}^{2+}$  oscillations induced by 8-Br-cAMP under NPY inhibition.

reported to be a highly selective PKA inhibitor [22,23] and thus these results indicate that the spontaneous  $\text{Ca}^{2+}$  oscillations are dependent on protein phosphorylation through a cAMP/PKA-dependent mechanism. We have no definite explanation for the initial stimulatory effect on  $\text{Ca}^{2+}$  oscillations during H89 treatment, but possibly, this transitory effect is a response to a temporary disturbance of the balance between phosphatase and kinase activities. Since voltage-operated  $\text{Ca}^{2+}$  channels are known to possess several serine and threonine phosphorylation sites [29], the N-type  $\text{Ca}^{2+}$  channel may be the substrate for PKA [30]. Phosphorylation of these sites by PKA increases channel activity, whereas dephosphorylation reduces channel activity. The fact that oscillations reappear after exposing cells to normal *Xenopus* Ringer's solution indicates that the cells remain viable during H89 treatment.

### Sauvagine activates through a cAMP/PKA-dependent mechanism

Under H89 inhibition neither sauvagine, a peptide which stimulates intracellular cAMP production [20,21] and  $\alpha$ -MSH secretion [11], nor 8-Br-cAMP were able to induce  $\text{Ca}^{2+}$  oscillations. This demonstrates that 8-Br-cAMP and sauvagine stimulate  $\text{Ca}^{2+}$  oscillations by activating PKA and, therefore, that sauvagine activates  $\text{Ca}^{2+}$  oscillatory activity through a cAMP/PKA-dependent mechanism. Even a combination of 8-Br-cAMP (or sauvagine) with a 20 mM  $\text{K}^{+}$  pulse, which normally causes  $\text{Ca}^{2+}$  entry through the voltage-operated  $\text{Ca}^{2+}$  channels [28], failed to induce  $\text{Ca}^{2+}$  oscillations in melanotropes treated with H89, indicating that, in *Xenopus* melanotrope cells, PKA activity is a prerequisite for  $\text{Ca}^{2+}$  channel opening.

### Baclofen inhibits through a cAMP/PKA-dependent mechanism

In *Xenopus* melanotropes, it is established that  $\text{GABA}_B$  receptor activation decreases intracellular cAMP production through inhibition of adenylyl cyclase [20,21]. The results show that  $\text{Ca}^{2+}$  oscillations are inhibited by baclofen and that sauvagine and 8-Br-cAMP are both able to induce  $\text{Ca}^{2+}$  oscillations under baclofen inhibition. It is also shown that inhibition of PKA by H89 prevents the reappearance of these oscillations. From these results we conclude that baclofen inhibits spontaneous  $\text{Ca}^{2+}$  oscillations by the inhibition of adenylyl cyclase with the consequent inhibition of PKA activity leading to inhibition of  $\text{Ca}^{2+}$  channels. Recently, it was found that PKA participates in desensitization of a rat  $\text{GABA}_B$  receptor expressed in *Xenopus* oocytes [31]. The  $\text{GABA}_B$  receptor-mediated response was suppressed by dibutyryl-cAMP through activation of PKA. In view of the importance of the adenylyl cyclase system in the maintenance of  $\text{Ca}^{2+}$  oscillations in *Xenopus* melanotropes, it would seem unlikely that such a desensitization mechanism would be functioning in this cell type.

### Dopamine inhibits through cAMP/PKA-dependent and cAMP/PKA-independent mechanisms

Several  $\text{D}_2$ -receptor mechanisms have been described. Some act through inhibition of cAMP production [8,21], some act through a direct modulation of  $\text{Ca}^{2+}$  channels [32,33], whereas other mechanisms involve  $\text{D}_2$ -receptor activation of voltage-operated  $\text{K}^{+}$  channels [34–38] and/or  $\text{D}_2$ -receptor inhibition of voltage-operated sodium channels [39,40] causing closure of voltage-operated  $\text{Ca}^{2+}$  channels through membrane hyperpolarization. Because dopamine  $\text{D}_2$ -receptor activation in *Xenopus* melanotropes is known to inhibit adenylyl cyclase activity

[8,21], we first tested the hypothesis that dopamine inhibits Ca<sup>2+</sup> oscillations exclusively through a cAMP/PKA-dependent pathway. When cells were inhibited by dopamine, the addition of 8-Br-cAMP or sauvagine did not reintroduce Ca<sup>2+</sup> oscillations, thus indicating that dopamine is not inhibiting simply through its action on adenylyl cyclase. However, combining 8-Br-cAMP with a depolarizing pulse of 20 mM K<sup>+</sup>, which alone also could not reintroduce Ca<sup>2+</sup> oscillations, restored Ca<sup>2+</sup> oscillations in such cells. The possibility that application of 8-Br-cAMP simply depolarizes the membrane further above that elicited by 20 mM K<sup>+</sup> is ruled out by the fact that even 60 mM K<sup>+</sup>, while increasing basal [Ca<sup>2+</sup>]<sub>i</sub>, fails to elicit oscillations in cells inhibited by dopamine (unpublished observations). Therefore, it can be concluded that dopamine inhibits Ca<sup>2+</sup> oscillations via both a cAMP/PKA-dependent and a cAMP/PKA-independent mechanism, the latter probably involving a membrane hyperpolarization step.

In view of the complex responses found in the dopamine experiments, in which 20 mM K<sup>+</sup> pulses were given (apparent changes in both oscillatory behaviour and basal [Ca<sup>2+</sup>]<sub>i</sub>), additional experiments with specific channel blockers were conducted. These showed that ω-conotoxin GVIA prevents reappearance of Ca<sup>2+</sup> oscillations during cAMP/K<sup>+</sup> treatment whereas nifedipine prevents the increase in basal [Ca<sup>2+</sup>]<sub>i</sub>. This indicates that the N-type Ca<sup>2+</sup> channel is involved in generating oscillations and, furthermore, establishes the participation of L-type Ca<sup>2+</sup> channels in setting basal [Ca<sup>2+</sup>]<sub>i</sub>. Both induction of Ca<sup>2+</sup> oscillations and increase in basal [Ca<sup>2+</sup>]<sub>i</sub> appear to be prevented by inhibition of PKA by H89, giving rise to the idea that phosphorylation of both N- and L-type Ca<sup>2+</sup> channels is essential for their opening.

#### NPY inhibits through a cAMP/PKA-dependent mechanism

As in the case of inhibition by baclofen, 8-Br-cAMP and sauvagine were able to induce Ca<sup>2+</sup> oscillations in melanotropes inhibited by NPY. Activation of the NPY Y<sub>1</sub>-receptor in *Xenopus* melanotropes inhibits adenylyl cyclase activity [7,8]. Apparently, NPY inhibits Ca<sup>2+</sup> oscillations through a cAMP/PKA-dependent mechanism. This conclusion is supported by the fact that in NPY-inhibited cells, 8-Br-cAMP cannot induce Ca<sup>2+</sup> oscillations when PKA is blocked by H89. A subpopulation of NPY-treated cells did not respond with oscillatory activity to 8-Br-cAMP treatment. Therefore, in some cells, NPY may act via an other mechanism, for example via inhibition of the N-type Ca<sup>2+</sup>-channels directly through a G-protein [41,42] or via activation of a hyperpolarizing potassium current [43]. These possibilities are currently being studied in electrophysiological experiments on *Xenopus* melanotrope cells.

## CONCLUSIONS

### Physiological implications

The present data reveal that sauvagine, baclofen, NPY and dopamine differentially control Ca<sup>2+</sup> oscillatory activity in *Xenopus* melanotropes. Sauvagine apparently stimulates Ca<sup>2+</sup> oscillations through a cAMP/PKA-dependent mechanism, possibly involving phosphorylation of N-type Ca<sup>2+</sup> channels, whereas the main mechanism by which baclofen and NPY inhibit oscillations is by inhibiting this mechanism. Dopamine inhibition not only involves a cAMP/PKA-dependent mechanism but also a mechanism independent of cAMP and PKA, probably involving a membrane hyperpolarization step. Physiologically, these differential actions may be important in determining the circumstances under which α-MSH is released from melanotropes. The secreto-inhibitors GABA, dopamine and NPY coexist in nerve terminals making synaptic contacts on the *Xenopus* melanotropes [12], GABA is stored within electron-lucent vesicles and dopamine and NPY are costored within electron-dense vesicles [12]. The cell bodies of the neurons that form the inhibitory synapses on the melanotropes are located in the suprachiasmatic nucleus [44]. The present studies demonstrate that a melanotrope cell which is inhibited through GABA<sub>B</sub> receptors can be easily stimulated if there is activation of sauvagine receptors. However, if the inhibitory synapses release dopamine from the dense core vesicles the melanotrope cell would be inhibited by dual intracellular pathways and thus be insensitive to sauvagine stimulation. Whether they are able to release electron-lucent and electron-dense vesicles in a differential way, for instance in response to changing environmental conditions the animal lives in, remains to be investigated. If so, the melanotrope cells of the pars intermedia would be able to respond in a flexible way to hypothalamic regulatory factors, which in turn, are under the control of external conditions such as background light intensity, temperature and stressors.

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