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The Effects of Disruption of A Kinase Anchoring Protein–Protein Kinase A Association on Protein Kinase A Signalling in Neuroendocrine Melanotroph Cells of *Xenopus laevis*

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Key words: AKAP, melanotroph cell, α-MSH, PKA, pituitary, *Xenopus laevis*.

Abstract

The secretory activity of melanotroph cells from *Xenopus laevis* is regulated by multiple neurotransmitters that act through adenylyl cyclase. Cyclic adenosine monophosphate (cAMP), acting on protein kinase A (PKA), stimulates the frequency of intracellular Ca^{2+} oscillations and the secretory activity of the melanotroph cell. Anchoring of PKA near target proteins is essential for many PKA-regulated processes, and the family of A kinase anchoring proteins (AKAPs) is involved in the compartmentalisation of PKA type II (PKA II) regulatory subunits. In the present study, we determined to what degree cAMP signalling in *Xenopus* melanotrophs depends on compartmentalised PKA II. For this purpose, a membrane-permeable stearated form of Ht31 (St-Ht31), which dislodges PKA II from AKAP (thus disrupting PKA II signalling), was used. The effect of St-Ht31 on both secretion of radiolabelled peptides and intracellular Ca^{2+} signalling by superfused *Xenopus* melanotrophs was assessed. St-Ht31 stimulated secretion but had no effect on Ca^{2+} signalling. We conclude *Xenopus* melanotrophs possess a St-Ht31-sensitive PKA II that is associated with the exocytosis machinery and, furthermore, that Ca^{2+} signalling is regulated by an AKAP-independent signalling system. Moreover, our results support a recent proposal that AKAP participates in regulating PKA activity independently from cAMP.

The serine/threonine cyclic adenosine monophosphate (cAMP)-dependent kinase (PKA) phosphorylates different cellular substrates that control multiple cellular functions, including synaptic transmission, ion channel activity and coordination of gene transcription (1, 2). PKA activity is regulated by the second messenger cAMP, produced by adenylyl cyclase, and the PKA holoenzyme consists of two regulatory (R) and two catalytic (C) subunits. Binding of cAMP to the R-subunits dissociates the holoenzyme and releases the C-subunits, allowing them to phosphorylate target proteins (3). There are two major classes of PKA, termed type I PKA and type II PKA (4). The PKA I holoenzyme is soluble in the cytoplasm whereas the PKA II holoenzyme is membrane-anchored (5). PKA II has a low affinity for cAMP compared to PKA I, implying that PKA I is activated by weak cAMP signals whereas PKA II responds to high and persistent cAMP stimulation (6). Most cells possess both type I and type II PKAs, but the expression levels of these different holoenzymes varies greatly among cell types (7).

During the last decade, it has become evident that protein phosphorylation occurs rapidly and efficiently by selective activation of distinct compartmentalised pools of PKA in a cell (8). A kinase-anchoring proteins (AKAPs) form a family of proteins that compartmentalise PKA. By binding regulatory type II subunits (RII), these scaffolding proteins anchor PKA II near its target proteins (9). Numerous AKAP members have been identified on the basis of RII binding assays of cell lysates, as well as by expression library screening (10). AKAPs contain at least two functional motifs, one forming an amphipathic helix that interacts with hydrophobic determinants located in the RII subunits, and another motif acting as a unique targeting domain for keeping AKAPs at the correct subcellular location (11).

Functional studies on AKAP have been facilitated by the development of Ht31, which competes with AKAP for the RII binding site. This peptide, the sequence of which is derived from the sequence of human thyroid RII-anchoring protein, mimics the amphipathic RII binding site of AKAPs (12). Ht31 lacks any targeting domain and, consequently, the

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Ht31-RII complex diffuses into the cytosol and the RII subunit is lost from the signalling compartment. The use of St-Ht31, either by injecting it into cells or by overexpressing it in vector-based systems, has revealed the involvement of AKAP in PKA signalling in a number of cellular processes, such as hormone secretion, ion channel activation and gene expression (5, 13–15). These studies have consistently shown that disruption of RII localisation has similar effects on cellular processes as inhibition of the catalytic activity of PKA, thus demonstrating the importance of AKAP in maintaining the integrity of the PKA signalling compartments.

A suitable model system to study the workings of the adenylyl cyclase system, in a neuroendocrine cell, is the melanotroph cell of the amphibian *Xenopus laevis*. This pituitary intermediate lobe cell produces α-melanophore-stimulating hormone (α-MSH), which is responsible for skin darkening during the process of background adaptation. In *Xenopus*, multiple neurotransmitters and neuropeptides act via adenylyl cyclase to regulate α-MSH release (16). Inhibition of adenylyl cyclase is achieved through the neurotransmitter dopamine acting on the dopamine D_2 receptor, GABA working through a $GABA_b$ receptor and by neuropeptide Y action on the Y_1 receptor; stimulation of adenylyl cyclase is induced by corticotrophin-releasing hormone, serotonin and noradrenalin (17). Intracellular Ca^{2+} oscillations are the driving force for secretion in *Xenopus* melanotroph cells and cAMP regulates the frequency of these oscillations via a PKA-dependent mechanism (17–19).

To date, only one study has shown that type I versus type II PKA can have different functions within the same cell. In sensory neurones of the snail *Aplysia,* type I and type II PKAs with distinct but complementary functions are involved in synaptic facilitation (20). The present study aimed to determine to what extent cAMP signalling in *Xenopus* melanotroph cells is dependent on the compartmentalised signalling of type II PKA. For this purpose, the membrane-permeable stearated analogue of Ht31, St-Ht31, was used to disrupt AKAP-dependent signalling. The effect of this treatment on intracellular Ca^{2+} signalling and on secretion of radiolabelled peptides from *in vitro* superfused melanotroph cells was analysed. The results demonstrate that PKA signals in *Xenopus* melanotrophs through both AKAP-dependent and AKAP-independent mechanisms and suggest that there is differential PKA I versus PKA II regulation of components of the secretory machinery.

Materials and methods

Animals

Young-adult (aged 8 months) *Xenopus laevis* were reared in our laboratory under standard conditions. They were kept in filtered tap water at 22 °C at constant illumination and were fed beef heart and trout pellets (Trouvit, Trouw, Putten, the Netherlands). Studies were conducted with animals kept on a black background for 3 weeks. All experiments were performed according to the guidelines of the Dutch law concerning animal welfare.

Cell isolation

Animals were anaesthetised by immersion in tap water containing 1 g/l MS-222 (Sigma Chemical, St Louis, MO, USA) and 1.5 g/l $NaHCO_3$. Blood was removed by perfusing the animals with 95% 0_2/5% CO_2 gassed *Xenopus* Ringer's solution (112 mM NaCl, 115 nM Ultrol Hepes, 2 mM KCl, 2 mM $CaCl_2$, 2 mg/ml D-glucose; pH 7.4) containing 0.25 mg/ml MS-222. Neurointermediate lobes were dissected and washed in *Xenopus* L15 (XL15) medium containing 67% Leibowitz medium (L15, Life Technologies, Paisley, UK), 0.1% kanamycin (Life Technologies) and 0.1% antibiotic/antimycotic solution (Life Technologies) with 0.08 mg/ml $CaCl_2$ and 0.2 mg/ml glucose (pH 7.4). Lobes were incubated in *Xenopus* Ringer's solution without $CaCl_2$ containing 0.25% trypsin (Life Technologies), for 45 min. Trypsin digestion was stopped by addition of 9 ml XL15 containing 10% fetal calf serum (FCS, Life Technologies). Lobes were gently triturated and isolated melanotroph cells were filtered (58 μm mesh) to separate them from tissue fragments. After centrifugation (50 ***g***, 10 min), the cell pellet was resuspended in XL15 or for superfusion experiments in lysine-free XL15 containing 500 μCi/ml ^{3}H-labelled lysine (Amersham, Buckinghamshire, UK).

Secretion studies

Cells were plated on 0.01% poly L-lysine-coated 15-mm Ø glass cover slips. After cells had been allowed to attach for 1 h at 22 °C and an equal volume of lysine-free XL15 + 20% dialysed FCS had been added, they were cultured in a humidified atmosphere, at 22 °C. The next day, 2 ml lysine-free XL15 with 10% dialysed FCS was added and the cells were cultured for another 24 h. After 1 day, cells were rinsed four times with *Xenopus* Ringer's and placed in a four-well plate (Nuclon, Roskilde, Denmark). Then, they were superfused at a rate of 100 μl/min with Ringer's for 1.5 h. Fractions were collected every 2 min and 160 μl scintillation fluid (Optiphase Supermix, Wallac, Loughborough, UK) was added. The amount of radiolabelled peptides in each fraction was determined with a scintillation counter (MicroB, Wallac). Data were collected with Microbeta software (Wallac) and further analysed with Microsoft Excel (Microsoft Corp., Redmond, WA, USA). In each experiment, the average amount of radioactivity in the first 20 fractions was set at 100%. Values of c.p.m. for all fractions were expressed relative to this value. The effects of treatment with various agents, alone or in combination, were determined by introducing the agents to the superfusion medium. Agents tested were the AKAP-disrupting peptide St-Ht31 and the control peptide St-Ht31P, both from Promega (Madison, WI, USA), 8Br-cAMP and the PKA inhibitor H89, both from Sigma Chemical, and sauvagine from Bachem (Basel, Switzerland). Superfusion protocols are given in the Results. The effect of a treatment on secretion was quantified by averaging the amount of released radiolabelled peptides in individual fractions during treatment and comparing this value with the amount of peptides released under untreated condition (averaged over 20 fractions and set at 100%).

Intracellular calcium measurements

Cells were cultured for 2 days, washed with Ringer's and subsequently loaded with 10 μM fura-2 AM (Molecular Probes, Eugene, OR, USA) and 1 μM Pluronic F127 (Molecular Probes) in Ringer's, for 30 min. Then, they were washed with Ringer's, placed under an inverted microscope (Axiovert 135 TV, Zeiss, Göttingen, Germany) and connected to a superfusion system. Ringer's was pumped over the cells at a rate of 0.6 ml/min. Cells were studied using an ×40 oil-immersion objective N.A. 1.30 (Fluar, Zeiss), and areas of interest were selected. For Ca^{2+}-measurements, every 6 s, the fura-2 probe was alternatingly excited with light of 340 and 380 nm, for 50 ms. The excitation light (340/380 nm) was generated by a 150-W Xenon lamp (Ushio UXL S150, Cypress, CA, USA) connected to a monochromator (Polychrome IV, TILL Photonics, Martinsreid, Germany). The emission light was filtered (Photonics LP440 filter) and collected with a monochrome digital camera (Coolsnap fx, Roper Scientific, Tucson, AZ, USA). Data were collected with Metafluor imaging software (Universal Imaging Corporation, Downingtown, PA, USA) and further processed in Origin 6.0 (Microcal Software Inc., Northampton, MA, USA). Changes in the intracellular Ca^{2+}-concentration were measured as changes in the ratio of 340/380 nm fluorescence intensity.

cAMP radioimmunoassay

Isolated melanotroph cells were incubated in Ringer's for 1 h at 22 °C, and then with 1 μM St-Ht31 and/or 100 nM sauvagine in Ringer's containing 1 mM isobutyl-methyl-xanthine (IBMX) (Sigma Chemical), for 20 min at 22 °C. After washing the cells, cAMP was extracted by incubation in ice-cold 65% ethanol, for 20 min. The extract was centrifuged at 2000 ***g***, for 15 min at 4 °C and the supernatant was transferred to fresh tubes and evaporated under a

stream of nitrogen at 60 °C. Dried extracts were dissolved in milli-Q water and cAMP content was measured with a radioimmunoassay, as described previously (21). Briefly, acetylation was performed with 5 µl acetic anhydride and triethylamine (v/v 1 : 2) to duplicate 100 µl aliquots of each sample. Next, 100 µl anticAMP serum (Sigma Chemical) and 100 µl sodium acetate buffer (pH 6.3) containing ^{125}I-cAMP, 0.5% bovine serum albumin (Sigma Chemical) and 0.2% sodium azide were added. ^{125}I-cAMP was produced by the chloramine-T method and purified according to our standard procedure used for labelling of α-MSH (22). As a standard, nonacetylated cAMP (Sigma Chemical) was used. Bound and free ^{125}I-cAMP were separated by polyethylene glycol/hen egg albumin precipitation. Sensitivity of the assay was 1 fmol cAMP per sample. The assay was performed in polypropylene tubes (Greiner Bio-one, Alphen aan den Rijn, The Netherlands). Previous experiments established that IBMX treatment is necessary to obtain measurable amounts of cAMP (20).

Statistical analysis

Quantitative data were analysed with Student's t-test (α = 5%) and Microsoft Excel software.

Results

Effect of St-Ht31 on peptide release

To examine the possible role of subcellular PKA localisation by AKAP in the control of α-MSH release, the effect of the AKAP inhibitor St-Ht31 on peptide secretion was tested. The rate of ^{3}H-lysine-labelled peptide release is an accurate measure for α-MSH release by *Xenopus* melanotrophs (23). Isolated melanotrophs were superfused with different concentrations of St-Ht31 or with the control peptide St-Ht31P (Fig. 1). St-Ht31 at 1 µM maximally stimulated peptide release (153 ± 10%), whereas higher concentrations were less effective, suggesting some desensitisation to the treatment was occurring (Fig. 1A). After termination of St-Ht31 treatment, peptide secretion returned to control level (Fig. 1A), whereas St-Ht31P did not alter secretion (Fig. 1B). Because calcium influx through voltage-operated calcium channels (VOCC) is a prerequisite for α-MSH secretion by *Xenopus* melanotrophs (24), we tested whether the St-Ht31-stimulated effect on secretion is dependent on calcium influx. In the presence of the general VOCC blocker $NiCl_2$ (5 mM), there was only a slight stimulation by 1 µM St-Ht31 (to 109 ± 0.11%, n = 3) (Fig. 2A) and no stimulation was found in the presence of another general VOCC blocker $CoCl_2$ (Fig. 2B).

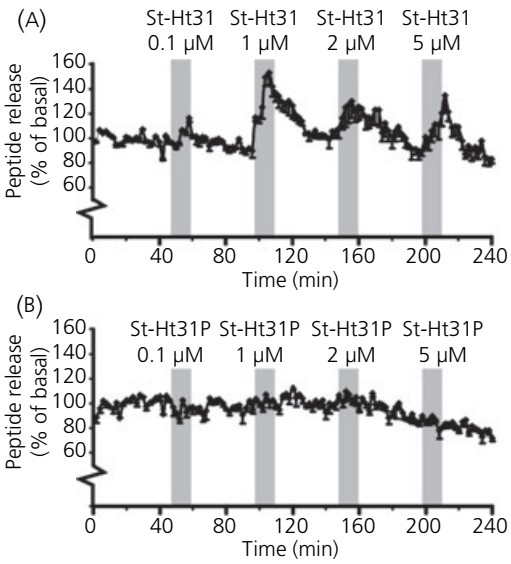

FIG. 1. Effects of A kinase-anchoring protein (AKAP) inhibitor St-Ht31 and control peptide St-31P on radiolabelled peptide release from superfused melanotrophs of black-adapted *Xenopus laevis*. Different concentrations of St-Ht31 (A) stimulate peptide release. St-Ht31P is without effect on peptide release (B). The average amount of peptide release in the first 20 fractions was set as 100% (basal) and the amount of release of all other fractions was expressed relative to this value. Data are mean ± SEM of separate superfusion experiments (n = 4).

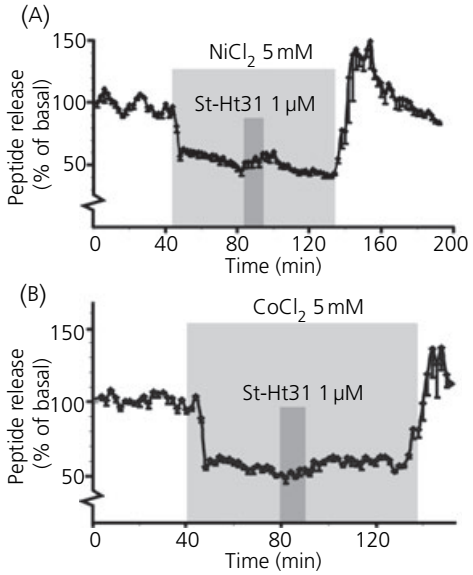

FIG. 2. Effects of the general voltage-operated calcium channels blockers $NiCl_2$ (A) and $CoCl_2$ (B) on St-Ht31 induced stimulation of release of radiolabelled peptides from superfused melanotroph cells. Data are mean ± SEM of separate superfusion experiments ($NiCl_2$ n = 3; $CoCl_2$ n = 4).

Effect of H89 on St-Ht31-induced secretion

To investigate whether the action of St-Ht31 is PKA-dependent, we used H89, an ATP analogue that specifically blocks the catalytic subunit activity of PKA (25). Figure 3 shows the result of H89 incubation on St-Ht31-stimulated secretion. The control group (Fig. 3A) was treated for 10 min with 1 µM St-Ht31, and the test group was treated with 10 µM H89 for 120 min prior to adding 1 µM St-Ht31 for 10 min (Fig. 3B). Total peptide release induced by St-Ht31 in H89-treated cells is plotted in Fig. 3(C). H89 incubation reduced secretion stimulated by St-Ht31 to 33 ± 7% of the control level ($P < 0.05$, n = 5).

Effect of St-Ht31 on cAMP production

A cAMP-radioimmunoassay was used to test whether St-Ht31 changed the level of cAMP (Fig. 4). Treatment with 1 µMSt-Ht31 did not increase cAMP production, whereas sauvagine, which is known to stimulate adenylate cyclase (21), substantially increased cAMP production (155 ± 16%; $P < 0.05$, n = 4).

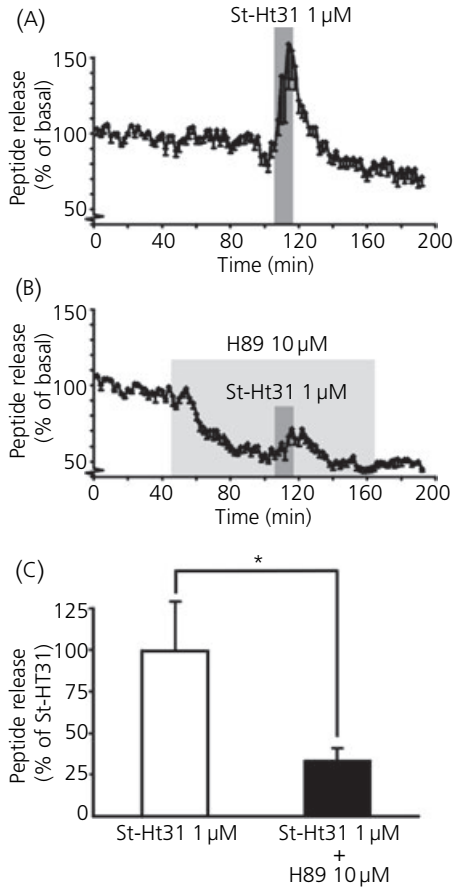

FIG. 3. Effects of protein kinase A (PKA) inhibition on St-Ht31-induced radiolabelled peptide release from superfused melanotrophs cells of black-adapted *Xenopus laevis*. 1 μM St-Ht31 stimulates peptide release (A). The PKA inhibitor H89 inhibits basal secretion as well as the stimulatory effect of St-Ht31 (B). Total amount of peptide release induced by St-HT31 is plotted for H89-treated cells and control cells (C). Data are mean ± SEM of separate superfusion experiments (n = 5). *Statistical difference (P < 0.05).

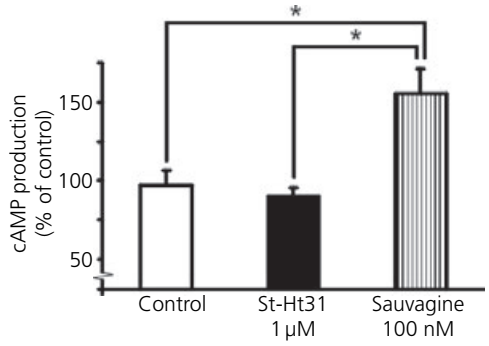

FIG. 4. Effects of St-Ht31 on cyclic adenosine monophosphate (cAMP) production in isolated melanotroph cells of black-adapted *Xenopus laevis*. Cells were incubated for 20 min with 1 μM ST-Ht31 or 100 nM sauvagine. cAMP production is plotted relative to the control group. Data are mean ± SEM of separate experiments (n = 4). *Statistical difference with control (P < 0.05).

Effect of St-HT31 on Ca^{2+} oscillations

In *Xenopus* melanotroph cells, peptide release is driven by intracellular Ca^{2+} oscillations and cAMP-dependent mechanisms increase the frequency of these oscillations (17). Because St-Ht31 stimulated peptide release, the effect of this peptide on the frequency of Ca^{2+} oscillations was tested. St-Ht31 had no effect on the frequency of intracellular Ca^{2+} oscillations as illustrated in a representative cell (Fig. 5A), nor on the average frequency of 33 cells (Fig. 5B) ($P > 0.05$, $n = 33$). St-Ht31 also had no effect on the amplitude of the Ca^{2+} oscillations, nor did it have an effect on basal $[Ca^{2+}]$.

Effect of St-Ht31 on 8Br-cAMP induced secretion

To test whether St-Ht31 could increase secretion in cells maximally stimulated by cAMP, we first tested different concentrations of 8Br-cAMP and showed that a concentration of 1 mM 8Br-cAMP induced a maximal increase in secretion (Fig. 6, insert; n = 4). Next, we tested if St-Ht31 would stimulate secretory activity of cells in which PKA had been activated maximally by 8Br-cAMP. Peptide release was stimulated by 136 ± 8% following treatment with the cyclic nucleotide alone (Fig. 6), whereas subsequent addition of 1 μM St-Ht31 further increased secretion to 164 ± 8% ($P < 0.001$, n = 3).

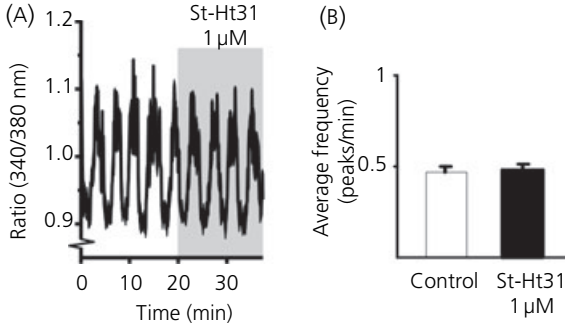

FIG. 5. Effects of 1 μM St-Ht31 on Ca^{2+} oscillations in melanotroph cells of black-adapted *Xenopus laevis*. Ca^{2+} oscillations of a representative cell treated with 1 μM St-Ht31 for 20 min (A). Average Ca^{2+} oscillation frequencies in Ringer's (control) and during treatment with 1 μM St-Ht31 (B) (n = 33). No statistical difference between control and St-Ht31 treatment was found.

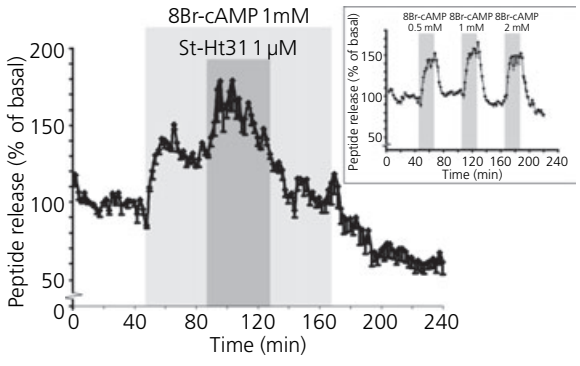

FIG. 6. Effects of St-Ht31 on radiolabelled peptide release from superfused melanotroph cells during stimulation by 1 mM 8Br-cAMP. Data are mean ± SEM of separate superfusion experiments (n = 3). Inset: dose–response for 8Br-cAMP to establish that 1 mM gives maximum stimulation of secretion (n = 4).

Discussion

Using St-Ht31 to dislodge the type II R subunit from its binding site with AKAP, we demonstrate that this membrane permeable peptide causes a dramatic, transitory stimulation of secretion by *Xenopus* melanotroph cells. To test the specificity of St-Ht31 treatment on melanotroph cells, St-Ht31-P, an analogue that possesses a proline substitution in the RII-binding motif (and thus fails to bind RII), has been used and was without effect on secretion when applied at the same concentrations as St-Ht31. Furthermore, the St-Ht31-induced melanotroph peptide release was dramatically reduced by both the PKA blocker H89 and the general VOCC blocker Ni^{2+}. The Ca^{2+} dependency of St-Ht31 demonstrates this peptide acts in a physiological fashion because melanotroph cell secretion is fully dependent on Ca^{2+} influx (24). Because the St-Ht31 induction of peptide release is also dependent on PKA activity, we assume that St-Ht31 acts on AKAP-bound type II PKA in the *Xenopus* melanotrophs, as described in previous studies (20, 26).

The pronounced stimulation of melanotroph secretion by St-Ht31 is remarkable. In all other studies using Ht31 to examine AKAP-dependent PKA, this peptide has led to attenuation of PKA activity (5, 13–15). In *Xenopus* melanotrophs, regulated secretion is positively correlated with cAMP production (16) and therefore an inhibition of peptide release would be expected as a result of disruption of the interaction between AKAP and PKA RII. It is worth noting that the present study examines short-term and dynamic effects rather than the tonic effects of Ht31 on PKA regulated processes. Recent studies on human HeLa cells and rat neonatal ventriculocytes have shown that PKA anchored to a muscle specific AKAP can stimulate phosphodiesterase (PDE) to reduce local cAMP levels (27). Extrapolating this mechanism to *Xenopus* melanotrophs, we considered the possibility that St-Ht31 treatment would lead to a disruption of a PKA-PDE signalling complex, thus leading to elevated cAMP and elevated secretion. However, our results clearly established that St-Ht31 has no effect on cAMP levels in *Xenopus* melanotrophs.

When speculating on the mechanism by which St-Ht31 induces PKA-dependent secretion, it is of interest to note that AKAP has been reported to be capable of functioning as an inhibitor of PKA activity. Jackson and Berg (28) have shown that, during oogenesis in *Drosophila,* over-expression of a mutant AKAP, unable to bind PKA RII, leads to the cells displaying an actin morphology that is similar to the actin morphology brought about by over-expression of a constitutively active PKA C-subunit (which is unable to bind a PKA R-subunit). They suggest that, under normal conditions, RII subunits bind to AKAP to create a RII-binding surface that acts as an inhibitory 'sponge' for PKA C-subunits. Interestingly, Kopperud *et al.* (29) demonstrated that over-expression of R-subunits decreases PKA signalling, even under cAMP saturating conditions, suggesting that regulation of the expression of R-subunits represents a novel

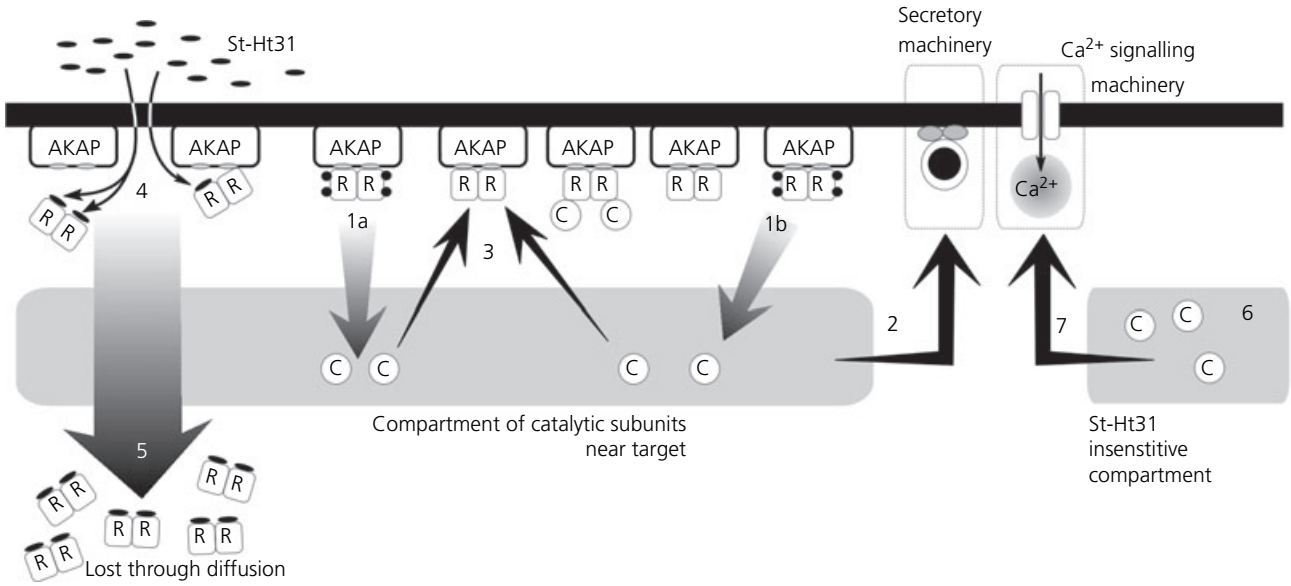

FIG. 7. Model to explain the generation of a protein kinase A (PKA) signal in *Xenopus* melanotrophs through disruption of A kinase anchoring protein (AKAP) function. Cyclic adenosine monophosphate, indicated as black circles, binds to the R subunit of PKA and consequently the C subunits of the enzyme join the pool of active kinases (1a and 1b). These catalytic subunits can act on effectors within the secretory machinery (2), such as SNAP-25, and thus promote secretion; alternatively they can be inactivated through binding to anchored R subunits (3). The concentration of active C subunits in this pool is dependent on their rate of production (steps 1a, b) and rate of binding to anchored R subunits (step 3). In this scenario, AKAP holds, via its specific R subunit binding sites (indicated as grey ovals on the AKAP), an excess of R subunits in the signalling complex. This carpet of R subunits drives the [R] + [C] ⇆ [R – C] equilibrium in the direction of the catalytically inactive R–C complex. St-Ht31 (black ovals) dislodges R subunits from their AKAP binding sites (4) and, consequently, these are lost from the signalling compartment through diffusion (5). This has positive consequences to the secretory process because the (excess) R subunits are no longer available to inactivate the C subunits and thus the C subunits become trapped in the active state. The model shows the presence of a St-Ht31-insensitive pool of catalytic subunits (6) that would be generated from sources of PKA not associated with AKAP, probably PKA type I. These could phosphorylate other components of the machinery associated with secretion, such as protein(s) of the voltage-operated Ca^{2+} channels (7), and thus stimulate secretion independent of the AKAP-associated signalling system.

way to regulate PKA activity. With the above studies in mind, we propose that the AKAP-dependent PKA compartment associated with the secretory machinery in *Xenopus* melanotrophs possesses an excess of AKAP-bound R-subunits that are in dynamic equilibrium with dissociated C-subunits (Fig. 7). The degree of dissociation of the R-C complex in the dynamic [R] + [C] ⇆ [R − C] equilibrium depends on the concentration of the R subunits and the level of cAMP. St-Ht31 strips R-subunits from the AKAPs and these subunits subsequently diffuse away from the signalling compartment. The decrease in [R] consequently drives the [R] + [C] ⇆ [R − C] equilibrium in favour of the free (active) C-subunits. As a result, PKA-dependent stimulation of secretion takes place. Of further interest is the observation that St-Ht31 increases secretion even under cAMP-saturated conditions (created by adding the cell permeant 8Br-cAMP). In their studies of HEK 293 cells, Kopperud *et al*. (29) have proposed the existence of an inactive or nondissociated pool of PKA enzymes in the presence of a saturated level of cAMP. Under conditions of high R-subunit expression, the size of this inactive pool would be enhanced, thus lowering overall PKA activity (28). Extending these findings to the *Xenopus* melanotroph cell, it is possible that St-Ht31, by stripping R-subunits away from the AKAP surface, allows nondissociated PKA enzymes to become dissociated, thus promoting PKA signalling.

Another noteworthy finding in our analysis is that St-Ht31 did not affect intracellular Ca^{2+} oscillation frequency, whereas it led to a strong stimulation in peptide release. Extensive studies have shown that Ca^{2+} oscillations are the driving force for secretion in melanotroph cells (17). Moreover, it has been established that stimulatory factors that work through the adenylyl cyclase system regulate the frequency of Ca^{2+} oscillations in *Xenopus* melanotrophs, as does treatment with membrane permeable cAMP analogues (19, 30). Therefore, St-Ht31 treatment produces an apparent anomaly of strongly stimulated secretion in the absence of a change in Ca^{2+} oscillatory behaviour. An explanation for this discrepancy may come from the possibility that the strength of the secretory activity of the melanotroph could partly be set by the phosphorylation status of PKA targets in the exocytosis machinery. Candidate targets include SNAP-25 (31), snapin (32), cysteine string protein (CSP) (33) and syntaphilin (34). Phosphorylated by PKA, SNAP-25, snapin and syntaphilin all stimulate exocytosis, whereas phosphorylated CSP inhibits exocytosis. Nagy *et al*. (31) have shown that PKA-dependent phosphorylation of SNAP-25 increases the size of the releasable vesicle pool and stimulates secretion by chromaffin cells. A similar phenomenon possibly occurs in an AKAP-sensitive, type II PKA signalling compartment in *Xenopus* melanotrophs. The PKA phosphorylation site of *Xenopus* SNAP-25 is conserved (35). A St-Ht31-induced increase in active C-subunits near the cytoplasmic exocytotic machinery may therefore increase exocytosis efficiency independently from any effect on Ca^{2+} signalling. We have previously shown that the general PKA blocker H89 completely eliminates Ca^{2+} oscillations in *Xenopus* melanotrophs, thus establishing that Ca^{2+} signalling is this cell type is PKA-dependent (30). The fact that St-Ht31 treatment had no effect on Ca^{2+} signalling indicates that the Ca^{2+} signalling machinery would, for the most part, be regulated by an St-Ht31 insensitive PKA, probably PKA type I.

In conclusion, we suggest that St-Ht31 treatment, by stripping R subunits away from the AKAP surface, disrupts the dynamics of R and C subunit interactions in favour of the free catalytic subunits (Fig. 7). Moreover, we propose that this AKAP-associated PKA type II signalling is occurring in a compartment distinct from the PKA system responsible for regulating Ca^{2+} signalling in *Xenopus* melanotrophs (Fig. 7). To date, there has been only one study devoted to distinguishing between different compartments of PKA signalling. Liu *et al*. (20) demonstrated a subcellular division of labour with respect to the function of type I versus type II PKA. In sensory neurones of *Aplysia californica*, type I PKA mainly occurs in the cell body whereas type II is associated with AKAPs in nerve endings. Because both PKA types are required for synaptic facilitation, they likely have distinct but complementary functions (20). The present study extends this concept of complementary functions of the two types of PKA. We present functional data indicating that the *Xenopus* melanotroph cell possesses two compartments of PKA signalling, one compartment being AKAP-associated PKA type II and the other likely involving type I PKA. Our data indicate that these two kinase systems have distinct functions in regulating the components of the secretory machinery (i.e. one being concerned with the secretory machinery and the other with Ca^{2+} signalling).

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