Protein Kinase C Activation Antagonizes Melatonin-induced Pigment Aggregation in *Xenopus laevis* Melanophores

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**Abstract.** The pineal hormone, melatonin (5-methoxy N-acetyltryptamine) induces a rapid aggregation of melanin-containing pigment granules in isolated melanophores of *Xenopus laevis*. Treatment of melanophores with activators of protein kinase C (PKC), including phorbol esters, mezerein and a synthetic diacylglycerol, did not affect pigment granule distribution but did prevent and reverse melatonin-induced pigment aggregation. This effect was blocked by an inhibitor of PKC, Ro 31-8220. The inhibitory effect was not a direct effect on melatonin receptors, per se, as the slow aggregation induced by a high concentration of an inhibitor of cyclic AMP-dependent protein kinase (PKA), adenosine 3',5'-cyclic monophosphothioate, Rp-dias-tereoemer (Rp-cAMPS), was also reversed by PKC activation. Presumably activation of PKC, like PKA activation, stimulates the intracellular machinery involved in the centrifugal translocation of pigment granules along microtubules. α-Melanocyte stimulating hormone (α-MSH), like PKC activators, overcame melatonin-induced aggregation but this response was not blocked by the PKC inhibitor, Ro 31-8220. This data indicates that centrifugal translocation (dispersion) of pigment granules in *Xenopus* melanophores can be triggered by activation of either PKA, as occurs after α-MSH treatment, or PKC. The very slow aggregation in response to inhibition of PKA with high concentrations of Rp-cAMPS, suggests that the rapid aggregation in response to melatonin may involve multiple intracellular signals in addition to the documented Gi-mediated inhibition of adenylate cyclase.

Transport of intracellular vesicles and organelles in eukaryotic cells is a ubiquitous phenomenon, essential for a variety of cellular processes including mitosis, exocytosis, endocytosis, fast axonal transport and the spatial organization of the Golgi apparatus, lysosomes and endoplasmic reticulum. Pigment cells of vertebrates are an excellent experimental system for studying intracellular motility. In such cells the movement of pigment granules is rapid, reversible, readily quantitated and under cellular control. The development of methods for the isolation and culture of pigment cells (Ide, 1974a; Jackson et al., 1974) has allowed biochemical studies of the intracellular mechanisms regulating pigment granule transport (Rozdzial and Haimo, 1986a,b). The observation that pigment granule dispersion in goldfish xanthophores is accompanied by the phosphorylation of a 57-kD granule-associated protein by a cyclic AMP-dependent protein kinase (PKA) has suggested a model mechanism by which phosphorylation/dephosphorylation of specific protein(s) can regulate organelle movement (Lynch et al., 1986a,b). Recent evidence indicates that the microtubule-dependent ATPase, kinesin, is the motor protein responsible for centrifugal movement (dispersion) of pigment granules in melanophores (Rodionov et al., 1991), and it seems likely that a second microtubule-dependent motor protein, dynein, is responsible for centripetal movement (aggregation) of pigment granules (Beckerle and Porter, 1982; Clark and Rosenbaum, 1982).

In the melanophores of *Xenopus laevis*, pigment granules disperse within minutes after addition of low concentrations of the pituitary hormone, α-melanocyte stimulating hormone (α-MSH) (Bagnara et al., 1969). Pigment granule dispersion also occurs in response to agents which elevate the concentration of intracellular cyclic AMP such as theophylline (McGuire et al., 1972), forskolin (White et al., 1987) and cell-permeable cyclic AMP analogues (Ide, 1974b). α-MSH has been shown to elevate the intracellular concentration of cyclic AMP in frog skin (Abe et al., 1969) and in isolated melanophores (Daniolos et al., 1990) suggesting that activation of the α-MSH receptor stimulates adenylate cyclase in the melanophore cell membrane resulting in an increase in cyclic AMP, activation of PKA and phosphorylation of specific pigment cell proteins resulting in pigment granule dispersion. The role of protein kinases other than PKA in regulating pigment granule movement in *Xenopus* melanophores is not known.

In *Xenopus* melanophores with dispersed pigment granules, addition of the pineal hormone, melatonin (5-methoxy

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1. **Abbreviations used in this paper:** PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C.
acetyltryptamine) induces a rapid aggregation of pigment granules around the nucleus. This change in pigment granule distribution is part of the physiological mechanism which adapts skin color to environmental illumination (Rollag, 1988). In melanophore-rich skin of Xenopus (Van de Venderdonk and Konijn, 1970) and in isolated cultured Xenopus melanophores (Daniolos et al., 1990), melatonin inhibits α-MSH-induced changes in cyclic AMP and α-MSH dispersion of pigment. Melatonin-induced aggregation of pigment is also blocked by prior incubation with pertussis toxin, which blocks inhibitory G-protein coupled receptors (Jakobs et al., 1985), both in Xenopus tissue explants (White et al., 1987) and in isolated Xenopus melanophores (Sugden, 1991).

Together these observations suggest that the melanophore melatonin receptor is coupled to an inhibitory G-protein and that activation of the receptor reduces intracellular cyclic AMP resulting in pigment granule aggregation. The melanophore melatonin receptor appears to be pharmacologically identical to the high affinity melatonin binding sites described in the retina and brain of birds, mammals and a marsupial (Krause and Dubocovich, 1991; Sugden and Chong, 1991; Paterson et al., 1992). These binding sites are also G-protein coupled and, in some instances, their activation has been shown to reduce intracellular levels of cyclic AMP in a pertussis toxin-sensitive manner (Carlson et al., 1989; Vanecek and Vollrath, 1989; Morgan et al., 1989).

However, melatonin receptors have also been reported to modulate other transduction systems. Melatonin reduced luteinizing hormone-releasing hormone stimulation of diacylglycerol production (after hydrolysis of phosphatidylinositol), the generation of arachidonic acid, the accumulation of cyclic GMP and the rise in intracellular free Ca2+ ([Ca2+]i) in pituitary explants from immature rats (Vanecek and Vollrath, 1989, 1990; Vanecek and Klein, 1992).

The present study was designed to examine the effect of protein kinase C (PKC) activation on melatonin-induced aggregation and α-MSH-induced dispersion of pigment granules. The results indicate that PKC activation prevents and reverses melatonin-induced aggregation of pigment granules. PKC probably acts by phosphorylating intracellular proteins involved in granule translocation rather than at the melatonin receptor itself. α-MSH-induced dispersion of pigment, which can also antagonize melatonin-induced aggregation, does not involve PKC activation.

Materials and Methods

Culture of Melanophores

Xenopus laevis embryos were obtained from adult frogs induced to lay by injection of human chorionic gonadotrophin (Chorulon, Intervet Laboratories Ltd., UK., 400 IU/male, 600 IU/female). The embryos were reared in tap water until stage 20 assessed using the normal table of Xenopus development (Nieuwkoop and Faber, 1956). The neural plate and underlying notochord and somites from 20 embryos was dissected and dispersed into small aggregates as described previously (Messenger and Warner, 1977) onto collagen-coated (0.2 mg/ml) petri dishes. After 2-3 d, pigment cells were readily visible among the many nerve, muscle and undifferentiated cells. Cells were initially cultured in medium containing (mM): NaCl 100, KCl 2.5, CaCl2 2, MgCl2 2, NaHCO3 5 plus 10% FCS, and penicillin 100 U/ml, and streptomycin 100 µg/ml. After ~7 d the media was changed to Leibovitz L-15 medium (Gibco Laboratories, Lawrence, MA) diluted (1:1) with deionized water containing 10% FCS, 200 µg/ml penicillin, 200 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (Daniolos et al., 1990).

Quantitation of Pigment Aggregation

On the day of the experiment, cells were washed with fresh diluted L-15 medium. Variable numbers of melanophores were found in each dish (10-100) but they were easily recognized by their large size (50-100 µm diam), characteristic shape and dark appearance due to the presence of many melanin-containing pigment granules (Fig. 1A). In most of the melanophores in the cultures, the pigment granules were dispersed, that is they were evenly distributed throughout the cell and the cell boundary was well-defined. Typically, the responses of eight individual melanophores, chosen at random, were measured in each dish. Melanophores were viewed under bright field illumination using a trinocular Swift microscope, a 10× Olympus objective and video camera (NCD 108, Norbain, UK). Under these conditions, essentially only the pigment mass of the cell is visible. The area occupied by pigment in individual melanophores was quantitated be-
fore and after various drug treatments using computer-assisted analysis of the digitized melanophore image with the DIGIT program (Hayes and Fitzke, 1987).

The changes in pigmented area are expressed as a percentage of the initial area occupied by pigment in each cell. For statistical comparisons of data Student's t test for multiple groups was used.

Drugs were prepared in MeOH or DMSO at a concentration of 10 mM and stored at -30°C. Drugs were diluted with deionized water just before use and added from 100X concentrated stock solutions. The maximal concentrations of MeOH and DMSO added to cultures (0.1% vol/vol) did not alter the area of the cell occupied by pigment. The details of drug additions in each experiment are given in the figure legends.

**Drugs**

Collagen (type I, acid soluble), melatonin, α-MSH, mezerein, 1,2 dioctanoyl-glycerol and all phorbol esters except 4α-phorbol 12,13-dibutyrate (4αPDBu) were purchased from Sigma Chemical Co. (Poole, UK). 4αPDBu was obtained from LC Services Corporation (Woburn, MA). Adenosine 3',5'-cyclic monophosphothioate, Rp-diastereomer (Rp-cAMPS) was obtained from Biolog (Bremen, Germany). Ro 31-8220 was obtained from Dr. J. S. Nixon, Roche Products Ltd., Welwyn Garden City, UK.

**Results**

**Effect of PKC Activation on Melatonin-induced Pigment Aggregation**

Addition of melatonin to *Xenopus laevis* melanophores resulted in a dramatic condensation of pigment granules around the center of the cell (Fig. 1 B). The response to melatonin was rapid, reaching a maximum after 15 min, could be readily quantitated and at a maximal concentration of melatonin (10⁻⁹ M) was manifest as a large (70-80%) reduction in the area of the cell occupied by pigment. The response to melatonin was related to the concentration added to the culture (Fig. 2). The concentration of melatonin producing a 50% maximal response was 36 pM. Treatment of melanophores with 4α phorbol 12-myristate 13-acetate (4αPMA, 10⁻⁷ M) did not itself significantly alter the area of the cell occupied by pigment (Table I) but prevented the expected response to melatonin (Fig. 2). 4αPMA was also able to reverse pigment aggregation induced by melatonin (Fig. 1 C); this reversal of the melatonin response was related to the concentration of 4αPMA used (Fig. 3). Reversal of melatonin-induced pigment aggregation was time dependent (Fig. 4); a maximal effect was seen after ~60 min.

Treatment of melanophores with PKC activators, in addition to dispersing aggregated pigment granules, produced some changes in the morphology of the melanophores. In some, but not all, control melanophores an area around the perimeter of the nucleus appeared to be devoid of pigment granules (Fig. 1 A). Upon treatment of melanophores with 4αPMA (Fig. 1 C) pigment granules appear to be dispersed throughout the cell and no peri-nuclear halo can be distinguished. Furthermore, in the cluster of cells shown (Fig. 1), the margins of contiguous control melanophores (Fig. 1 A) contain pigment granules such that most cells can
Figure 4. Time-course of the reversal of melatonin-induced aggregation by 4β phorbol 12-myristate 13-acetate (4βPMA). Melatonin (MEL, 10^{-8} M, 15 min) was used to aggregate pigment and the time-course of 4βPMA reversal of aggregation determined by repeated measurement of pigmented area during the next 90 min. Each column represents the mean response of six cells and vertical bars show SEM. * significantly different from the melatonin-treated group, p < 0.005.

Effect of PKC Activation on Pigment Aggregation Induced by Rp-cAMPS

To determine if the PKC activators specifically blocked the pigment aggregating effects of melatonin, the ability of 4βPMA to reverse pigment aggregation induced by Rp-cAMPS, a competitive inhibitor of PKA (Parker Botelho et al., 1988) was tested. Rp-cAMPS (500 μM) induced a modest degree of pigment aggregation in Xenopus melanophores (≈50%) with aggregation occurring slowly over several hours (Fig. 5 A). Addition of increasing concentrations of Rp-cAMPS produced a concentration-related aggregation of pigment (Fig. 5 B). Addition of 4βPMA (10^{-7} M) reversed Rp-cAMPS-induced aggregation of pigment granules (Fig. 5, A and B).

Role of PKC in αMSH-induced Pigment Aggregation

α-MSH is known to produce pigment granule dispersion in Xenopus melanophores (Bagnara et al., 1969). Like the activators of PKC, α-MSH produced a concentration-related reversal of melatonin-induced pigment granule aggregation (Fig. 6). However, unlike the effect of 4βPMA, the effect of α-MSH could not be antagonized by the PKC inhibitor, Ro 31-8220 (Fig. 6).

Discussion

4βPMA, an activator of PKC, did not significantly alter the pigment distribution in fully dispersed melanophores but it did prevent the expected aggregation induced by melatonin and also reversed aggregation induced by melatonin in a concentration- and time-dependent manner. Several lines of evidence indicate that the effect of 4βPMA is due to its ability to activate PKC. First, the concentration of 4βPMA producing a 50% reversal of aggregation was ≈2.5 × 10^{-4} M which is close to the concentration required in vitro.

Table I. Reversal of Melatonin-induced Pigment Granule Aggregation by Activators of PKC

<table>
<thead>
<tr>
<th>PKC activator</th>
<th>Concentration (M)</th>
<th>% of initial pigment area</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Melatonin</td>
<td>10^{-8} M</td>
<td>30.9 ± 2.7</td>
<td>(30)</td>
</tr>
<tr>
<td>+ 4βPMA</td>
<td>10^{-7} M</td>
<td>81.0 ± 7.2*</td>
<td>(14)</td>
</tr>
<tr>
<td>+ 4βPDBu</td>
<td>10^{-6} M</td>
<td>64.7 ± 17.2*</td>
<td>(8)</td>
</tr>
<tr>
<td>+ 4αPDBu</td>
<td>10^{-6} M</td>
<td>24.4 ± 4.4</td>
<td>(8)</td>
</tr>
<tr>
<td>+ 4αPDD</td>
<td>10^{-6} M</td>
<td>36.5 ± 11.5</td>
<td>(8)</td>
</tr>
<tr>
<td>+ 4βPhorbol</td>
<td>10^{-6} M</td>
<td>22.7 ± 3.1</td>
<td>(8)</td>
</tr>
<tr>
<td>+ DOG</td>
<td>10^{-4} M</td>
<td>84.6 ± 10.1*</td>
<td>(8)</td>
</tr>
<tr>
<td>+ Mezerein</td>
<td>10^{-4} M</td>
<td>74.2 ± 10.0*</td>
<td>(8)</td>
</tr>
</tbody>
</table>

Cultures were treated with melatonin (10^{-8} M) for 30 min to aggregate pigment. As the responses to melatonin were not significantly different between experiments all melatonin-treated groups were pooled. Activators of PKC were then added for 60 min. Data shown are the mean ± SEM responses from the number of cells given in parentheses. * significantly different from melatonin-treated group, p < 0.005.

Table II. Pretreatment with a PKC Inhibitor Prevents the Inhibition of Melatonin-induced Pigment Aggregation Produced by 4βPMA

<table>
<thead>
<tr>
<th>Aggregation</th>
<th>% of initial pigment area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Melatonin</td>
<td>34.1 ± 6.1</td>
</tr>
<tr>
<td>4βPMA</td>
<td>110.5 ± 11.9</td>
</tr>
<tr>
<td>Ro 31-8220</td>
<td>112.3 ± 8.8</td>
</tr>
<tr>
<td>Melatonin + PMA</td>
<td>106.3 ± 12.3*</td>
</tr>
<tr>
<td>Melatonin + PMA + Ro 31-8220</td>
<td>28.1 ± 2.5*</td>
</tr>
</tbody>
</table>

Cultures were treated with melatonin (10^{-8} M) for 15 min, 4βPMA (10^{-7} M) for 60 min, and Ro 31-8220 (3 × 10^{-4} M) for 30 min. * significantly different from melatonin-treated group, p < 0.001. † significantly different from melatonin + PMA group, p < 0.001. Eight cells were measured in each group.
Figure 5. (A) Time course of pigment aggregation in response to the cyclic AMP antagonist Rp-cAMPS. The area occupied by pigment granules was determined in 10 melanophores after addition of Rp-cAMPS (500 μM) to the culture medium. Each point represents the mean with vertical bars showing SEM. When aggregation appeared to have reached a maximum (300 min) 4β-phorbol 12-myristate 13-acetate (4βPMA, 10⁻⁷ M) was added and pigment area determined 60 min later. (B) Response to increasing concentrations of Rp-cAMPS. Rp-cAMPS was added to the medium and pigment area measured 60 min after each addition. 60 min after the highest concentration added (1 mM), 4βPMA (10⁻⁷ M) was added and pigment area measured 60 min later. Each point represents the mean of 10 cells with vertical bars showing SEM * significantly different from the maximal response to Rp-cAMPS p < 0.005.

to activate PKC. Second, 4βPDBu, another phorbol ester able to activate PKC in vitro, also reversed melatonin-induced pigment aggregation (Table I). Phorbol esters which do not activate the kinase (4αPDBu, 4αPDD, 4βphorbol) did not significantly reverse the melatonin response. Third, mezelein, an activator of PKC chemically distinct from the phorbol esters, also reversed pigment aggregation (Table I). Fourth, 1,2-diocatnolglycerol, a synthetic diacylglycerol able to mimic the endogenous cellular activators of PKC, dispersed aggregated pigment granules in melatonin-treated melanophores (Table I). Finally, Ro 31-8220, an antagonist of PKC, blocked the ability of 4βPMA to reverse melatonin-induced aggregation (Table II). Unlike many commonly used inhibitors of PKC Ro 31-8220 has been reported to have considerable specificity for PKC both in vitro and in vivo (Davis et al., 1989). This, in fact, is demonstrated by these experiments in melanophores as Ro 31-8220 itself did not cause pigment aggregation, an effect which would be expected if the drug was inhibiting PKA at the concentrations used.

To determine if the antagonism of the melatonin response was due to a desensitization of the melatonin receptor, the effect of 4βPMA on aggregation induced by another agent was tested. Rp-cAMPS is a cell-permeable analogue of cyclic AMP (Parker Botelho et al., 1988), resistant to hydrolysis by phosphodiesterases (Braumann et al., 1986), which competitively inhibits PKA by preventing the dissociation of the holoenzyme (Rothermel and Parker Botelho, 1988). In melanophores, Rp-cAMPS consistently induced aggregation of pigment granules. This was completely reversed by 4βPMA indicating that the effect of PKC activation is most likely not a specific effect on the melatonin receptor. Rather, PKC may activate the intracellular mechanism(s) which are responsible for the centrifugal movement of pigment granules. Pigment aggregation in response to Rp-cAMPS required a high concentration (EC50, concentration required to produce a half-maximal aggregation in 60 min >1 mM) and was very slow, taking 5 h to reach a maximum (Fig. 5A). This, of course, could simply reflect very poor and/or slow penetration of Rp-cAMPS in these cells, although in some mammalian cells, Rp-cAMPS is an effective antagonist of PKA activation at much lower concentrations (Parker Botelho et al., 1988). Alternatively, the concentration of intracellular cyclic AMP in these melanophores may be particularly high as it appears to be in melanophores from the angelfish (Pterophyllum scalare) (Sammak et al., 1992). The slow response to Rp-cAMPS is in stark contrast to the rapid aggregation response to melatonin which can be detected within a few minutes (Sugden, 1991). This difference is surprising as melatonin, by reducing intracellular cyclic AMP, is thought to act essentially by the same mechanism, an inhibition of PKA activity. Further studies are required to determine if changes in intracellular cyclic AMP do mediate the
aggregation induced by melatonin or if there are other transduction mechanisms activated by melatonin which may also participate.

There is strong evidence that pigment granule aggregation in the erythrophores and melanophores of some teleosts is controlled by the concentration of [Ca$^{2+}$]. In erythrophores of the squirrel fish (Holocentrus), pigment aggregation is induced by ionophores which elevate [Ca$^{2+}$] (Luby-Phelps and Porter, 1982) and by Ca$^{2+}$ itself in cells stripped of their plasma membrane (McNiven and Ward, 1988). Also in melanophores of the African cichlid (Tilapia mossambica) pigment aggregation is triggered by an elevation of [Ca$^{2+}$], (Thaler and Haimo, 1990). Furthermore, direct measurements of [Ca$^{2+}$] in melanophores and erythrophores of the platyfish, Xiphophorus maculatus with the fluorescent probe fura-2 have shown an increase on pigment aggregation (Oshima et al., 1988). However, intracellular cyclic AMP, and not the rise in [Ca$^{2+}$], triggered by an aggregating stimulus, regulates pigment movement in angelfish, Perophyllum scalare melanophores (Sammak et al., 1992). These findings suggest that even among teleosts the intracellular second messengers which mediate pigment granule aggregation vary depending, perhaps, upon the species, the type of pigment cell and the aggregating stimulus.

$\alpha$-MSH stimulates pigment granule dispersion and reverses melatonin-induced aggregation in a similar manner to activators of PKC, although this occurs somewhat more quickly with $\alpha$-MSH (complete in 30 min) than after PKC activation (60 min). Interestingly, recent work has suggested that at least some of the actions of $\alpha$-MSH may be mediated by activation of PKC. For example, $\alpha$-MSH appears to exert its stimulatory effect on steroidogenesis through the activation of phospholipase C (Kapas et al., 1991) and in murine B16 melanoma cells $\alpha$-MSH translocates PKC activity to the membrane fraction (Buffey et al., 1992). It is clear, however, that in Xenopus melanophores pigment dispersion in response to $\alpha$-MSH does not involve activation of PKC as Ro 31-8220, did not prevent $\alpha$-MSH-induced dispersion, yet did prevent dispersion induced by direct activation of PKC with 4$\beta$PMA.

In conclusion, it appears that two distinct intracellular signaling mechanisms can activate the intracellular machinery which regulates pigment granule dispersion: the first involves an elevation of intracellular cyclic AMP, such as that occurring after activation of $\alpha$-MSH receptors; the second involves activation of PKC. It is not known if there are any receptors on melanophores which are linked to the Ca$^{2+}$/ phosphatidylinositol system and PKC. Conceivably both PKA and PKC may regulate pigment dispersion by phosphorylating the same protein(s) and activating the same microtubule-dependent motor mechanism. Presumably the activity of the motor proteins involved must be regulated or the interaction of the motor protein with pigment granules or the microtubule itself is controlled. Further studies are required to determine if PKC- or PKA-dependent phosphorylation of specific melanophore proteins accompanies granularity.

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