Pigment Dispersion in Frog Melanophores Can Be Induced by a Phorbol Ester or Stimulation of a Recombinant Receptor That Activates Phospholipase C*

(Received for publication, August 27, 1992)

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Pigment dispersion in frog melanophores is classically mediated by receptors that activate protein kinase A via an elevation of intracellular cyclic AMP. Here, 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, is found to induce pigment dispersion. To demonstrate that an increase in cAMP is not required for the melanosome movement, a murine bombesin receptor was expressed in the melanophores. When these cells were treated with bombesin, they accumulated intracellular inositol phosphates but not cAMP and dispersed their pigment. Four agonists, one partial agonist, and two antagonists for the bombesin receptor were compared for their ability to induce or block bombesin-induced pigment dispersion. In all cases, the degree of pigment dispersion followed simple equilibrium reactions. The resulting dose-response curves allowed for the determination of the effective concentration for half-maximal pigment dispersion (EC50) or half-maximal inhibition of bombesin-stimulated pigment dispersion (IC50) for the peptides. As the pigment dispersion assay can rapidly evaluate chemicals for their effects on receptors that activate phospholipase C via a functional assay, it has potential utility for investigations of ligand-receptor interactions and for massive drug screening.

Many vertebrates possess the ability to rapidly change their skin color. Depending on the animal, a variety of chromatophores, including melanophores, xanthophores, erythrophores, and iridophores, are involved in this process. Color changes can be mediated by a variety of stimuli including direct photostimulation, hormonal regulation, and neuronal activity. The best characterized pathway for controlling pigment movement utilizes the cAMP second messenger system. In addition to the role of cAMP in the control of pigment movement, a few recent studies have examined the potential roles of inositol 1,4,5-trisphosphate (1,4,5-IP3) and diacylglycerol (DAG) in this process. In one investigation, 1,4,5-IP3 was found to induce pigment aggregation in melanophores in a species of fish (3). In a second report, the mechanism by which melanin concentrating hormone works in an eel was described (4). In this latter report, 1–10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) induced skin lightening while higher concentrations of up to 10 μM had no effect on coloration.

One of the most popular types of cells for investigating the basis of pigment translocation, as well as chemicals which stimulate melanosome dispersion or aggregation, are melanophores from frogs. From studies with both frog skin and immortalized pigment cells, it is known that an increase in intracellular cAMP leads to pigment dispersion over the course of several minutes. Some examples of agents known both to raise cAMP levels in the frog cells and cause pigment dispersion include α-melanocyte-stimulating hormone (α-MSH), β-adrenergic agents, light, and forskolin (5–9). Melatonin, on the other hand, decreases intracellular cAMP levels and leads to pigment aggregation (6, 10). However, pathways that do not involve modulation of cAMP levels, such as that involving phospholipase C and the generation of inositol phosphates, have not been examined for their potential role in effecting pigment movement. Here we show that pigment dispersion can be induced by TPA, a potent activator of protein kinase C. In addition, we show that activation of a recombinant murine bombesin receptor expressed in melanophores leads to phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis and pigment dispersion in the absence of an elevation in intracellular cAMP.

1. The abbreviations used are: 1,4,5-IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; TPA, 12-O-tetradecanoylphorbol-13-acetate; α-MSH, α-melanocyte-stimulating hormone; BR, bombesin receptor; PIP3, phosphatidylinositol 4,5-bisphosphate; [Leu4,Val14]Bn, [Leu4,Ψ(CH2)5NH]-Leu4bombesin; Ψ, pseudopeptide bond with insertion of CH2NH for CONH; d-F-Phe-pentfluoro-phenylalanine; NH4H2PO4, ammonium dihydrogen phosphate; KHCO3, potassium hydrogen carbonate; PCR, polymerase chain reaction; SBR, Swiss 3T3 murine melanobin/gastrin-releasing peptide receptor; TRH, thyrotropin releasing hormone; PBS, phosphate-buffered saline; EC50, the effective concentration for half-maximal pigment dispersion; IC50, concentration causing half-maximal inhibition of bombesin-stimulated pigment dispersion; HPLC, high performance liquid chromatography; IP3, inositol 1-monophosphate; IP4, inositol 1,4-bisphosphate; IP6, inositol 1,4-bisphosphate; IP3, inositol trisphosphate; GH, clonal rat pituitary tumor cell line; NMB-R, neuropeptide Y preferring bombesin receptor; GRP-R, GRP preferring bombesin receptor; GRP, gastrin-releasing peptide; kb, kilobase; GCSS, General Cell Screening System.

* The work was supported by the Burroughs Wellcome Fund, G. D. Searle & Co., the Office of Naval Research, the Medical Scientist Training Program, and the Howard Hughes Medical Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Melatonin, TPA, GRP-14,7 (neuromedin C), litorin, [Leu6,Arg11]Bombesin (B; Peninsula), [Leu6,Arg11]Bombesin (B; Calbiochem), and neuromedin C (B; Peninsula) were purchased from Sigma. The SBR antagonist [d-Phe1]Bombesin methyl ester and [d-Phe1, d-Ala2]Bombesin methyl ester were kindly donated by David H. Coyer. α-MSH was kindly supplied by Aaron Lerner. The Ca2⁺ ionophore A23187 and restriction enzymes were purchased from Roche Applied Science Manhheim, mn-2[H]inositol (2.2-10 ci/mmol) and the [1]H]inositol polyphosphate marker set were purchased from Amer- sham Int. NH4H2PO4 and KClO4 were purchased from Aldrich.

**Cell Culture**—The propagation of Xenopus laevis melanophores and fibroblasts was performed as described earlier (9, 11). The melano- phores used in this paper were derived from a clonal cell line isolated from a primary culture.

**Plasmid DNA Constructs**—The expression vector pJG3.6 was con- structed from pBluescript SK- (–) (Bluescript, Stratagene) and pcDNA1/Neo (Inveritogen) as follows. A 2.0-kb plasmid fragment in Bluescript containing the ampicillin resistance gene and the CoI I origin of replication was amplified by PCR, using Vent DNA polym- erase (New England Biolabs, Inc.), the sense primer from nucleotides 960 to 974 (5'-GACTGCGACGACAACTTGTGTCG-3'). The restriction enzyme sites AatII and SacII were engineered into the 5' end of the former primer and EcoRV and KpnI into the latter to aid with subsequent ligation. A 1.7-kb plasmid fragment encompassing the cytomegalovirus promoter, multicloning site, and poly(A) region in pcDNA1/Neo was am- plified by PCR methodology as described above using the sense primer from nucleotides 1525 to 1539 (5'-GATATCGAGAGGCAAGA- TACGGC-3') and the antisense primer from nucleotides 3179 to 3193 (5'-GACTGCGAGGATCGGATCC-3'). Again, the restriction enzyme sites EcoRV and ClaI were engineered into the 5' end of the former primer and SacI and AatII into the latter. The PCR products were size-fractionated on an agarose gel, and the appropriate bands were cut from the gel and purified following the Geneclean II Kit protocols (Bio 101 Inc.). Both fragments were digested with SacI and ClaI and ligated together to form the expression vector pJG3.6. The ligation mixture was transformed into electrocompetent bacteria DH5α (Gibco Bethesda Research Laboratories) by electroporation (12, 13).

The SBR expression vector (pcDNA1/NeoBR), prepared by cloning the HindIII-digested SBR gene into the HindIII site of the expression vector pcDNA1/Neo, was kindly donated by Jim Battey (14). The SBR gene was subcloned into pJG3.6 (pJG3.6BR) by digesting pcDNA1/NeoBR and pJG3.6 with HindIII, band-purifying the appropriate fragments, and then ligating them together. The vector was then digested with HindIII and SacII to the latter. The restriction enzyme sites EcoRV and ClaI were engineered into the 5' end of the former primer and SacI and AatII into the latter. The PCR products were size-fractionated on an agarose gel, and the appropriate bands were cut from the gel and purified following the Geneclean II Kit protocols (Bio 101 Inc.). Both fragments were digested with SacI and ClaI and ligated together to form the expression vector pJG3.6. The ligation mixture was transformed into electrocompetent bacteria DH5α (Gibco Bethesda Research Laboratories) by electroporation (12, 13).

The substance P receptor gene was cloned into pJG3.6 by digesting both plasmids with HindIII and EcoRV, and purifying the appropriate expression vector plasmid (40 pg) was then added to the cuvette in a volume of 10 µl. The cells were triturated 5 or 10 min after addition of the vector and once again immediately before electroporation (10 or 20 min after vector addition). Immediately after electroporation, the cells were transferred to fibroblast-conditioned growth medium (1.5-12 ml) which consisted of 70% L-15 supple-mented with 20% fetal calf serum (Gibco). Cell death ranged from 40 to 60%, and the range of transient transfection efficiency was 10- 60% but averaged about 30%. SBR transfection efficiency was gen- erally assessed 3 days after electroporation by observing the number of cells that dispersed their pigment in response to 100 nM bombesin.

To ensure that transfection efficiency was homogenous when prepar- ing identical experiments, electroporations were pooled together before plating.

**Microtiter Plate Assays**—After electroporation, cells were seeded into either flat bottom 96-well tissue culture plates (Falcon) or GCSS 96-well tissue culture plates (SLT Lab Instruments). The plates were seeded either at or slightly below confluency (5,000-7,000 cells/GCSS well or 12,000-15,000 cells/flat bottom well) using a volume of 100 µl/well. After the cells had attached to the well surface (1-2 h), the medium was removed and replaced with 100 µl of conditioned growth medium. In some instances, this procedure was performed the next day. The cells were incubated at 27 °C for 2 days following electroporation. Except as noted, the medium was removed by aspiration after 45 h and the cells washed with 100 µl of 70% PBS or 95% PBS containing 5% calf serum (Gibco) and 1% penicillin and streptomycin (Sigma). The cells were then washed once and incubated in 100 µl of 70% PBS, and quenched with 70% L-15 (Sigma) containing 20% (v/ v) calf frog serum (Gibco). Peptide solutions in water were aliquoted and used only once. When a subset of the plate was chosen for analysis, the remaining wells received water as the control. To ensure that pigmentation was homogenous, the wells were washed with 100 µl of 70% PBS before the assay, the cells were washed with and incubated in 100 µl of EX-CELL. Biosassays were performed as described below.

Prior to adding ligands, 5 µl of 21-nM melatonin in EX-CELL (1 nm final concentration) was added to each well to induce pigment aggregation. The cells were incubated in the dark in the presence of melanin for 2 h then drugs were added to the wells in 5-µl aliquots at 22 times their final concentration under a red light in the dark. Drug addition was performed in this manner in order to avoid pigment dispersion due to photostimulation. All drug solutions were prepared in EX-CELL and contained 1 nm melatonin. When the peptide concentration was assumed to be 70%. Melatonin was prepared from a 10 mm stock solution in ethanol stored at -20 °C. TPA was prepared as a concentrated stock solution in ethanol and diluted accordingly with EX-CELL. The final concentration of ethanol used in the assay was never more than 0.1%. Concentrations of either less than or equal to 10 µM or 10 mM do not initiate pigment dispersion (19). Concentrated peptides were occasionally prepared in dimethyl sulfoxide but care was taken to ensure that the final concentration of dimethyl sulfoxide
was less than or equal to 1.4 mM (0.01%, v/v) for the assays. Concentrations of dimethyl sulfoxide above 1.4 mM initiate pigment dispersion in a dose-dependent manner (data not shown).

Phototransmission was measured at 620 nm using a 340 ATTC microtiter plate reader (SLT Lab Instruments) or at 690 nm using a GeneRuler (SLT Lab Instruments). Transmission readings were taken 2 h after adding 1 mM melatonin (T). Drugs were immediately added and additional readings were made at various time points as specified in the text (T). Measurements were made using the agglutination mode of the SOFT 2000 program (SLT Lab Instruments) (7) or the General Cell Screening System Version 1.06e or 1.06d (SLT Lab Instruments). When using the General Cell Screening System Version 1.06e (SLT Lab Instruments) the agglutination mode acquired 40 separate transmission measurements for each well of which the first and last five were discarded. The remaining 30 transmission readings were averaged and taken as an individual value. Data was directly transferred from General Cell Screening System to Microsoft Excel software for reduction.

Data was curve fitted with the equation for pKd determinations (see below) described by the nonlinear regression program EnzFitter (BioSoft),

\[ y = y_{\text{min}} + \frac{(y_{\text{max}} - y_{\text{min}}) (10^x)^{1/10}}{10^x + 1} \quad (\text{Eq. 1}) \]

where \( x = \log[\text{ligand}] \) and \( \log EC_{50} \) or IC\(_{50}\), \( y_{\text{min}} = \) minimum plateau value of \( y \), and \( y_{\text{max}} = \) maximum plateau value of \( y \). The nonweighted analyses were performed using the software program KaleidaGraph (Synergy Software). The error listed for each \( EC_{50} \) or \( IC_{50} \) value is calculated from the standard error for each.

Cyclic AMP Quantitation—Cyclic AMP studies were performed essentially as described by Daniolos et al. (9). Cells were electroporated with the appropriate vector (either pCDNA/NeoBR or pON260) and seeded in 35-mm tissue culture dishes (Falcon) to an approximate density of 170,000 cells/dish. The cells were allowed to attach to the dish for at least 1.5 h before the medium was removed and replaced with 2 ml of conditioned growth medium. After incubation at 27 °C for 48 h, the medium was removed by aspiration and the cells washed with 70% PBS (2 ml). The cells were then incu- bated overnight in EX-CELL (1.5 ml) and assayed the next day. A working concentration of 1 mM melatonin (15 μl of 100 mM melatonin in 70% PBS) was added to each dish to induce pigment aggregation. After incubating the cells in the dark for 30 min, bombesin or α-MSH was added at various concentrations and incubated for an additional 30 min. Drugs were prepared in 70% PBS at 100-fold the working concentration and were added to the medium and cAMP extracted by adding 5% (v/v) trichloroacetic acid (0.9 ml) followed by incubation at 4 °C for 30 min. The trichloroacetic acid was transferred to a polypropylene tube (15 ml, Falcon) and the cells washed with an additional 0.9 ml of 5% (v/v) trichloroacetic acid. The extracts were combined and extracted with water-saturated ether (3 × 10 ml). The aqueous phases were removed and to dryness. The aqueous layer was applied to the cells. To monitor any potential pigment aggregation, cells were transfected with pJG3.6 or pJG3.6BR via electroporation and seeded in 35-mm tissue culture dishes (Falcon) to an approximate density of 170,000 cells/dish. The cells were incubated for 30 min to induce pigment aggregation. After incubation for 30 min the medium was removed and 1.5 ml of conditioned growth medium was added at various concentrations and incubated for an additional 30 min. Phototransmission immediately before adding TPA and that at selected subsequent time points was measured using a GeneRuler (SLT Lab Instruments). Once the evolution of carbon dioxide had dissipated, the solution was mixed thoroughly with a vortex (pH approximately 7.5 after neutralization) and centrifuged for 1 min in a microcentrifuge at maximum speed (15,000 × g). The aqueous layer was transferred to a new microcentrifuge tube, briefly centrifuged at 15,000 × g, and injected onto the HPLC.

Separation and Identification of Inositol Phosphates by HPLC—Resolution of the inositol phosphates was performed by amion-exchange chromatography using a Pharmacia Smart System. The procedures were essentially those described by Balla et al. (22). Samples (200 μl) were injected onto a Mono-Q PC 1.6/5 column (Pharmacia, 1.6 × 50-mm, 0.1 ml) over a period of 3.3 min at a flow rate of 60 μl/min. The sample was then washed for 2.7 min with water followed by elution with a linear gradient of 0–0.63 mM NH₄H₂PO₄ (pH 3.35) from 30 to 31 min. After washing for 10 min with water at a flow rate of 100 μl/min, the collected fractions were mixed with 200 μl of scintillation fluid (OptiPhase “HiSafe” 3, LKB Wallac) and the radioactivity was acquired 40 separate transmission measurements for each well of the microtiter plate reader (SLT Lab Instruments) or at 690 nm using a Microplate Reader (SLT Lab Instruments). The peak labeled as IP₃ coeluted with [3H]1,4,5-IP₃. The lack of more identified peaks are listed as di- or tri-inositol phosphates even though the peaks were coeluted with IP₃ at a flow rate of 60 μl/min. The aqueous fractions were collected in microcentrifuge tubes beginning at 0.21 M NH₄H₂PO₄. The column was then washed with a linear gradient of 0.63–0.7 M NH₄H₂PO₄ (pH 3.35) from 30 to 31 min followed by 19 min of washing at 0.7 M NH₄H₂PO₄. Preceding each injection, the column was washed for 10 min with water at a flow rate of 100 μl/min. The collected fractions were mixed with 200 μl of scintillation fluid (OptiPhase “HiSafe” 3, LKB Wallac) and the radioactivity was determined as counts per min (Wallac 1410 Liquid Scintillation Counter). Peak identification was determined by coelution and comigration with the radiolabeled standard compounds [3H]IP₃, [3H]1,4-IP₃, and [3H]1,4,5-IP₃. In the text (see Fig. 2 and Table 1), the identified peaks are listed as di- or tri-inositol phosphates even though the peaks were coeluted with IP₃ and in both controls the peak labeled as IP₃ coeluted with [3H]1,4-IP₃ and [3H]1,4,5-IP₃. The lack of more detailed study precluded absolute identification of the peaks because of the potential for coelution of other untested isomers.

RESULTS

TPA Induces Dose-dependent Pigment Dispersion within Melanophores from Xenopus laevis—As a first step to determine whether outward melanosome movement within melanophores might be induced independently of a rise in cAMP concentration, TPA, a potent activator of protein kinase C, was applied to the cells. To monitor any potential pigment movement, cells were seeded into a 96-well microplate at a concentration of approximately 15,000 cells/well and grown for 3 days. Melatonin was then added to the wells such that the pigment cells were exposed to a 1 nM concentration of the indole for 2 h. After melatonin treatment, during which the cells aggregated their pigment, TPA was added such that cells were exposed to concentrations ranging from 0.1 to 1000 nM. A total of 11 concentrations of TPA were applied to quadruplicate wells. Immediately following the addition of TPA, and at 17 subsequent time points over the course of 2 h, the ability of the wells to allow transmission of light at 620 nm was determined with a microplate reader. Figure 1A demonstrates the results of plotting the acquired data according to the formula 1/(Tf/Ti) versus time, where Ti is the initial measured phototransmission immediately before adding TPA and Tf is that at selected subsequent time points (7). Pigment dispersion within melanophores and concentric darkening within each cell in a dose-dependent manner after which the pigment was displayed as an upward displacement along the y axis. Beginning at 10 nM, TPA induces pigment dispersion which remains constant over the several hour duration of the experiment. Except for 1 μM TPA, the greatest concentration of the phorbolester to which the cells were exposed, the higher the concentration of drug used to treat the cells, the greater their overall pigment dispersion. While 1 μM TPA causes a more rapid initial rate of cell darkening than any of the lower

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Stimulation of a Murine SBR Expressed in Frog Melanophores Leads to the Generation of Inositol Phosphates but Not cAMP—To determine whether the pigment cells would functionally express the rodent SBR, cells were transiently transfected via electroporation with the plasmid pJG3.6BR (see "Experimental Procedures"). Three days later, the cells were incubated with 1 nM melatonin for 30 min. Bombesin (100 nM) was then added to the cultures and, 30 s later, the cells were extracted and their levels of inositol bisphosphate (IP$_2$) and inositol trisphosphate (IP$_3$) assessed by anion-exchange HPLC. Fig. 2 compares the quantities of inositol phosphates within SBR expressing recombinant cells versus control ones in which the cells were transfected with the same vector lacking cDNA coding for the receptor (pJG3.6). The large peak eluting between fractions 12 and 21 is IP$_2$ and the peak eluting between fractions 45 and 75 (see inset) is IP$_3$. These results, which are quantitated in Table I, show that there is approximately a 3-fold difference in inositol phosphate levels between the SBR expressing cells and the control population not expressing the SBR when both are exposed to bombesin. Table I also contains data from an additional control experiment: cells transfected with pJG3.6BR do not generate increased amounts of inositol phosphate when incubated in the absence of bombesin. Because these experiments used cells that were transiently but not permanently transfected, the ability to generate inositol phosphates in response to SBR stimulation depends on plasmid transfection efficiencies. For the two separate experiments reported for melanophores transfected with pJG3.6BR, transfection efficiencies were 10% for those cells treated with 100 nM bombesin and 30% for the control that had not been exposed to bombesin (see "Experimental Procedures"). Had all the cells expressed the SBR, inositol phosphate accumulation in response to bombesin would have possibly attained a value 20-30 times the wild type base-line levels. cAMP measurements were made to determine if SBR ac-
tivation caused levels of the nucleotide to rise within the melanophores. 170,000 cells were plated into triplicate 35-mm tissue culture dishes and after allowing the cells to grow for 3 days, they were exposed to 1 nM melatonin for 30 min. Cells were then treated with between 0 and 100 nM bombesin for 30 min, collected, and their intracellular cAMP levels were measured by radioimmunoassay. Bombesin treatment of recombinant melanophores expressing the SBR (73 ± 3 pmol of cAMP/mg of protein) or β-galactosidase (66 ± 7 pmol of cAMP/mg of protein) did not show any accumulation of cAMP versus the controls with no bombesin treatment. However, both the SBR (202 ± 11 pmol of cAMP/mg of protein) and β-galactosidase (206 ± 8 pmol of cAMP/mg of protein) expressing cells generated cAMP in response to 100 nM α-MSH. The transfection efficiency of SBR in these experiments was 32%. This level of receptor expression would have been sufficient to see increases in cAMP if receptor-mediated pigment dispersion were initiated by an increase in cAMP (8).

Recombinant Melanophores Expressing SBRs Disperse Their Pigment in Response to Bombesin and Related Agonist Peptides in a Dose-dependent Manner—Fig. 3A demonstrates the results of applying different concentrations of bombesin and three related SBR peptide agonists, GRP18-27, litorin, and neuromedin B, to SBR-expressing melanophores. For all of the agonists, pigment dispersion reached a maximum after approximately 30 min and remained constant for the duration of the experiment. As with TPA before, the dose-response relationships between concentrations of all four peptides and pigment dispersion in SBR expressing melanophores can be described by a simple hyperbolic function. All of the peptides follow established rank order potencies for their ability to stimulate the SBR (23). For comparison, the figure also provides the results of applying the same peptides to wild type cells (Fig. 3B), and reveals no signals above background. The data shown in Fig. 3A was used to generate EC50 values (Table II).

Results in Fig. 4 reveal that [Leu11,Val13-14]Bn is a partial agonist with respect to pigment dispersion in melanophores transiently expressing the SBR but does not induce pigment dispersion in wild type melanophores. With an EC50 of 13 nM (Table II), it has approximately 66% of the intrinsic activity of bombesin in inducing melanophore pigment dispersion as determined by 1 - (Tc/Ta). The results reveal that the pigment dispersion assay provides a sensitive and reproducible measure of the ability of agonists to interact with the SBR.

SBR Receptor Antagonists Block Agonist-induced Pigment Dispersion in a Dose-dependent Manner—Fig. 5A demonstrates the ability of two analogs of bombesin, [D-Phe3]Bn1-13 methyll ester (24) and [D-F-Phe6,d-Ala11]Bn1-13 methyl ester (25), to inhibit bombesin-induced pigment dispersion. Neither peptide induced pigment dispersion when applied to wild type cells (Fig. 5B). The experiments were performed by initially incubating the melanophores in 1 nM melatonin for 2 h to preset their melanosomes in a pigment-aggregated state. Next, the cells were simultaneously treated with 0.2 nM bombesin, essentially the EC50 value for bombesin as determined above, plus different concentrations of the antagonist peptides for 30 min. As with the application of agonists to cause pigment dispersion, the dose-response relationships for the two antagonists can be described by the same hyperbolic function described earlier. With IC50 values for [D-Phe3]Bn1-13 methyl ester and [D-F-Phe6,d-Ala11]Bn1-13 methyl ester of 17 and 23 nM, respectively (Table II), both peptides are potent antagonists for the SBR when it is expressed in melanophores.

**Discussion**

Initially, two questions arose, given the result that the protein kinase C activator, TPA, induced pigment dispersion. Could pigment dispersion be induced in the absence of a rise

**Table I**

| Vector transfected | 100 nM bombesin | IP3 | IP1 | cpm/assay |
|-------------------|-----------------|-----|-----|----------|
| pJG3.6            | 8,200 ± 478     | 1,550 ± 36 |
| pJG3.6BR          | 10,400 ± 794    | 1,400 ± 117 |
| pJG3.6BR          | 23,000 ± 508    | 5,120 ± 36 |

**Fig. 3.** Pigment dispersion in wild type or SBR-expressing melanophores in response to bombesin and three related peptides. A, dose-response curves of melanophores transiently expressing the SBR and stimulated for 30 min with bombesin (x), litorin (o), GRP18-27 (c), or neuromedin B (m). Cells were plated in 96-well plates and assayed 3 days after electroporation. Data was derived by determining the agonist-induced change in phototransmission through cells. Each point in the graph is the mean from quadruplicate samples with error bars representing the corresponding standard errors of the mean. B, dose-response curves of wild type melanophores stimulated for 30 min with bombesin (x), litorin (o), GRP18-27 (c), or neuromedin B (m). The data was derived as outlined in A and as detailed under "Experimental Procedures."
Potencies of bombesin receptor agonists and antagonists on SBR transfected melanophores as determined using the pigment dispersion assay

TABLE II

| Ligand                              | EC<sub>50</sub> nM | IC<sub>50</sub> nM |
|-------------------------------------|-------------------|-------------------|
| Bombesin                           | 0.18 ± 0.04 (0.2, 5) |                |
| Litorin                             | 0.20 ± 0.05 (0.4, ND)<sup>a</sup> |               |
| GRP<sub>25,27</sub>                 | 0.23 ± 0.03 (0.4, 110) |              |
| Neuremedin B                        | 1.4 ± 0.4 (6, 4) |                 |
| [Leu<sup>14</sup>,Ψ13-14]Bn        | 13.0 ± 3 |                      |
| [d-Phe<sup>1</sup>]Bn<sub>6-13</sub> methyl ester | 17 ± 6 (1, NI)<sup>b</sup> |             |
| [d-F,Phe<sup>2</sup>,d-Ala<sup>5</sup>]Bn<sub>6-12</sub> methyl ester | 25 ± 10 (1, NI) |              |

<sup>a</sup> ND, no data.
<sup>b</sup> NI, no inhibition.

Fig. 4. Pigment dispersion in wild type or SBR-expressing melanophores in response to bombesin or [Leu<sup>14</sup>,Ψ13-14]Bn. Dose-response curves of melanophores transiently expressing the SBR and stimulated for 30 min with bombesin (○) or [Leu<sup>14</sup>,Ψ13-14]Bn (●). Included in the graph is the dose-response curve of wild type cells in the presence of [Leu<sup>14</sup>,Ψ13-14]Bn (×). The displayed data was generated from recombinant cells derived from one set of electroporations to ensure that the observed differences in maximal response was not due to differential transfection efficiency. The day before the assay, the cells were not rinsed with EX-CELL before incubation in serum-free medium overnight. Data was derived by determining the agonist-induced change in phototransmission through cells. Each point in the graph is the mean from quadruplicate samples with error bars representing the corresponding standard errors of the mean.

Fig. 5. Responses of SBR-expressing cells in the presence of 0.2 nM bombesin or wild type melanophores to SBR antagonists. A, dose-response curves of melanophores transiently expressing the SBR and stimulated for 30 min with 0.2 nM bombesin and [d-Phe<sup>1</sup>]Bn<sub>6-13</sub> methyl ester (○) or [d-F,Phe<sup>2</sup>,d-Ala<sup>5</sup>]Bn<sub>6-13</sub> methyl ester (●). Cells were plated in 96-well plates and assayed 3 days after electroporation. Data was derived by determining the antagonist-induced inhibition of change in phototransmission through cells. Each point in the graph is the mean from quadruplicate samples with error bars representing the corresponding standard errors of the mean. B, dose-response curves of wild type melanophores stimulated for 30 min with [d-Phe<sup>1</sup>]Bn<sub>6-13</sub> methyl ester (○) or [d-F,Phe<sup>2</sup>,d-Ala<sup>5</sup>]Bn<sub>6-13</sub> methyl ester (●) but without 0.2 nM bombesin. The data was derived as outlined in A and detailed under "Experimental Procedures."
tion with bombesin leads to the rapid formation of intracellular inositol phosphates, presumably catalyzed by phospholipase C. By definition, there must be a concomitant production of DAG. However, bombesin stimulation does not induce a rise in intracellular cAMP. A similar observation was reported for a clonal rat pituitary tumor cell line (GH4Cl) containing an endogenous BR. In this instance as well, GH4Cl cells stimulated with bombesin did not increase cellular cAMP levels.

The SBR displays appropriate pharmacological behavior when it is expressed in melanophores based on the characteristics for the two distinct BR subtypes. Two general classes of BR have been identified and can be differentiated on the basis of ligand binding and biological potencies. 

A rise in intracellular CAMP. A similar observation was reported for a clonal rat pituitary tumor cell line (GH4Cl) containing an endogenous BR. In this instance as well, GH4Cl cells stimulated with bombesin did not increase cellular cAMP levels. Compared to NMB-R, these receptors display greater potency and selectivity towards the NMB-R. The second class of bombesin receptor, the GRP-R, is found in Swiss 3T3 cells, pancreatic acini, rat gastric muscle strips, and guinea pig gastric smooth muscle cells. Compared to NMB-R, these receptors display greater binding affinities for GRP18-27 than for GRP18-27 and poorer abilities to bind neureomedin B. When GRP-R-stimulated amylase release is measured, bombesin, litorin, and GRP18-27 are found to be equipotent agonists, whereas neureomedin B is about 8-fold less effective (Table II). Meanwhile, the antagonist [D-Phe6]bombesin6-13 methyl ester (24), a derivative of [D-Phe6]bombesin6-13 ethyl ester, is a potent inhibitor of bombesin-stimulated pigment dispersion. These results are in good agreement with the published data for GRP-R receptor subtypes.

The finding that [Leu14,Ψ(CH2NH)-Leu15] bombesin ([Leu14,Ψ(CH2NH)-Leu15] bombesin) is a partial agonist for the SBR when it is expressed in frog melanophores was unexpected based on previous literature (Fig. 4). [Leu14,Ψ(CH2NH)-Leu15] BN is the first reported BR antagonist with a binding affinity of less than 100 nM (48). It has a Kd of 65 nM for the SBR in Swiss 3T3 murine embryonal fibroblasts and has been shown to inhibit the growth of these cells as measured by its ability to prevent bombesin-stimulated incorporation of [3H]thymidine (IC50 = 18 nM) (49). Based on these findings, [Leu14,Ψ(CH2NH)-Leu15] BN was expected to antagonize bombesin-induced pigment dispersion. However, Spindel et al. (41) reports that although [Leu14,Ψ(CH2NH)-Leu15] BN shows substantial agonist activity in oocytes expressing the SBR, it also exhibited partial agonist activity. In this particular study, bombesin stimulation was assayed by measuring light emission in oocytes that had been coinjected with RNA coding for the SBR and the calcium photoprotein aequorin. Although the possibility exists that [Leu14,Ψ(CH2NH)-Leu15] BN or the receptor behaves somewhat differently in Swiss 3T3 cells compared with frog melanophores, the discrepancy may reflect the distinct assay methods and not the different cellular environments.

Receptor number and assay method probably influence measurements of EC50 and IC50. For example, the melanophore pigment dispersion system provides an EC50 value for bombesin of 0.2 nM and a minimum dose of 10 nM is required to observe a maximal biological response. Meanwhile, measurements of bombesin-induced amylase release from rat and guinea pig pancreatic acini or [3H]thymidine uptake into Swiss 3T3 cells show EC50 values of about 0.2 and 1 nM, respectively (25, 44, 48, 50). Furthermore, in the latter assay, 3 nM bombesin provides a maximum biological response. Also, the melanophore assay reveals that [D-Phe6]BN6-13 methyl ester has an IC50 of 17 nM while both classic assays show IC50 values near 1 nM (23-25). The results of the different assays can be affected by factors such as the numbers of BRs expressed per cell and the cell type of cellular response being measured following the addition of chemicals. Regardless of the differences in the absolute EC50 and IC50 values seen with the three assays, the rank order potencies of the various agonists and antagonists are comparable.

It has been demonstrated that pigment dispersion in frog melanophores can be mediated by a cAMP-independent pathway. This alternative mechanism appears to utilize DAG as the second messenger for three reasons. First, TPA, a potent activator of protein-kinase C causes dose-dependent pigment dispersion. Second, when bombesin was added to recombinant cells expressing SBR, IP3 was produced while intracellular cAMP levels remained constant and pigment dispersion occurred. In addition, the substance P and thyrotropin-releasing hormone (TRH) receptors, which, like the SBR, activate phospholipase C following exposure to agonists (51, 52), were expressed in melanophores. Substance P and TRH induced pigment dispersion with EC50 values of 3 ± 1 and 0.09 ± 0.03 nM, respectively, when applied to recombinant cells expressing the appropriate receptors (data not shown). Since DAG is a mandatory coproduct of 1,4,5-IP3 resulting from the cleavage of PIP2, it appears that DAG is the second messenger responsible for the induction of pigment movement following SBR stimulation. Third, 1,4,5-IP3, which typically functions to elevate intracellular calcium, does not appear to play a significant role in inducing centrifugal pigment movement. If it were a contributing factor, then raising intracellular calcium concentrations by treating the melanophores with the ionophore A23187 might be expected to induce pigment dispersion. When the cells were exposed to up to 10 μM of the Ca2+ ionophore, there was no observable dispersion of melanosome lamellae (data not shown).

Support for this conclusion comes from a recent publication in which it has been demonstrated that Ca2+ is not involved in pigment vesicle movement in fish melanophores (53). In summary, receptor-mediated DAG generation leads to pigment dispersion in a manner that appears to be as closely controlled as that accomplished via receptor-mediated cAMP production. This suggests that frog melanophores may have endogenous receptors that function via DAG to control the state of pigment disposition, and experiments to address this issue are in progress.

Finally, the ability of melanophores to disperse their pigment in response to the accumulation of DAG should provide a powerful tool for investigations into ligand-receptor interactions. Beside its utility for basic research, the assay described here may be useful for the rapid screening of drugs for their abilities to functionally interact with specific G-protein-coupled receptors that activate phospholipase C.

Acknowledgments—We thank Alison Roby-Shemkovitz and Linda Golovan for technical assistance and Tim McClintock for suggestions during revision of the manuscript. We are also grateful to Mark Quillan for contribution of the TRH data, including subcloning of the TRH receptor into pJG3.6.

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