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Dissociation between the Inhibitory and Stimulatory Effects of Opioid Peptides on cAMP Formation in SK-N-SH Neuroblastoma Cells

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Opioid agonists either potentiate or suppress basal cAMP production in SK-N-SH cells. The inhibitory effect is mediated by PTX-sensitive GTP-binding proteins, while the stimulatory effect involves Ca^{++} entry and calmodulin activation. Both pathways can be activated simultaneously by opioid agonists. Low (nM) concentrations of either mu (DAMGO) or delta (DPDPE) selective opioids potentiate cAMP formation. At higher (100nM) concentrations, however, a net suppression takes over; this suppression can be eliminated by PTX, and the underlying stimulatory effect is disclosed. Micromolar concentrations of either mu or delta selective agonists cross-activate the other (delta or mu) receptors, and augment the stimulatory pathway. The overall outcome (either stimulation or inhibition of cAMP production) is dependent on the balance between the two overlapping pathways, and can be modified by blocking either of the two opposing mechanisms. © 1998 Academic Press

Opioid agonists inhibit adenyly cyclase in various tissues and cells through the activation of Gi GTP-binding proteins (for review see 1). Concurrently with recognizing the inhibitory profile of opioid activity in the 1970′s, a single study reported the stimulation of adenylyl cyclase by morphine in the rat corpus striatum (2). More than a decade later, stimulatory effects of opioids on cAMP accumulation and adenylyl cyclase activity have been detected in different experimental systems (for review see 3). In some studies, opioids induced either inhibition or stimulation, depending on experimental conditions, such as the concentration of the opioid drug(4), pre-exposure of the preparation to opioids (5, 6), activation of adenylyl cyclase by forskolin (5, 7, 8), or the presence of GM1 gangliosides (9, 10).

While the inhibitory activity of opioids is well recognized, the stimulatory effects are less established, and their cellular mechanism is not clear. Recently we suggested that the stimulatory effect of opioids on cAMP formation in SK-N-SH human neuroblastoma is secondary to the mobilization of calcium ions and the activation of adenylyl cyclase by Ca/calmodulin (11). In the present study we describe the multiple effects of opioid peptides on cAMP formation in SK-N-SH cells, and dissociate beteen two different mechanisms which control adenylyl cyclase activity in these cells.

MATERIALS AND METHODS

Materials. [2-^{3}H]adenine was purchased from Amersham (UK). DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol] Enkephalin), DPDPE ([D-Pen^2, D-Pen^5] Enkephalin), cAMP (adenosine 3:5 cyclic monophosphate), Tris, Alumina, IBMX (3-isobutyl-l-methylxanthine) and W-7 (N-(6-aminohexyl)-5-chloro-l-naphthalenesulfonamide hydrochloride) were acquired from Sigma (USA). βFNA (β-funaltrexamide) was from RBI (USA) and naltrindol was contributed by NIDA (USA). PTX (pertussis toxin) was from List (USA) and Dowex AG 50W-X4-H^+ (200-400 mesh) from Bio Rad (USA). All other salts and acids were purchased from BDH and Merck. RPMI 1640 medium (containing L-glutamine), fetal calf serum, penicillin and streptomycin were acquired from Beit Haemek (Israel). Tissue culture flasks (75cm^2) and 24-well plates were obtained from Corning (USA).

Cell culture. SK-N-SH cell line was originally purchased from ATCC (USA). Cells were grown in RPMI 1640 medium containing L-glutamine supplemented with 7.5% fetal calf serum, and 40 units/ml of penicillin and streptomycin, at 37^{0}C in a humidified atmosphere of 5% CO_2-95% air. The cells were grown in 75cm^2 culture flasks and were used 24 hours after sub-cultivation in 24-well plates.

Measurement of cAMP production. Cultures were incubated in 0.25ml/well of serum-free medium containing 6μCi/ml [^{3}H]adenine for 2hr . The medium was then aspirated and replaced with 0.5ml of serum-free medium containing 0.5mM IBMX and 0.1mg/ml BSA with or without the tested drug for 10min at 37^{0}C. Blockers (naltrindol, βFNA, and $NiCl_2$, $CoCl_2$, $CdCl_2$), when used, were added to the medium 2min prior to the opioid agonist (DAMGO or DPDPE). Incubation was terminated by replacing the medium with 2.5% of ice-cold perchloric acid containing 0.1mM cAMP. After 30min at 4^0 C, the cell extract was neutralized with 4.2M KOH/1M Tris (pH 7) and applied to the two-step column separation as previously described (12). The produced [^{3}H]cAMP was eluted into scintillation vials and counted. A 50μl sample of the cell extract was taken directly for counting as a measure of total [^{3}H]adenine uptake in each well. Percent of cAMP conversion was calculated as [^{3}H] cAMP produced/[^{3}H]adenosine uptake X100.

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Statistics. Each experiment consisted of 12 (treated) vs 12 (control) wells within the same plate. Paired t-test was used to determine the effect of each treatment. Level of significance was set at $p > 0.05$.

RESULTS

Figure 1 illustrates the effect of DAMGO, a mu opioid receptor agonist, on cAMP formation in SK-N-SH cells. A low concentration of DAMGO (10^{-9}M) elevated basal production of cAMP by 29±4.7% (n = 55, $p < 0.001$); a hundred fold higher concentration (10^{-7}M) significantly reduced basal production by 14 ± 4.6%(n = 17, $p < 0.05$); this reduction disappeared and turned into a slight and insignificant elevation (12 ± 7.3%, n = 7) when an even higher concentration of DAMGO (10^{-6}M) was applied.

The inhibitory effect of opioids on cAMP production is known to be mediated by PTX-sensitive Gi GTP-binding proteins. In order to test whether the suppression by 10^{-7}M DAMGO involved Gi proteins, we pretreated SK-N-SH cells with PTX (100ng/ml) for 18 hrs. Under these conditions, 10^{-7}M DAMGO failed to exert its inhibitory effect, and a significant augmentation of 35 ± 4.8%(n = 14, $p < 0.05$) in cAMP production was found (Fig.1).

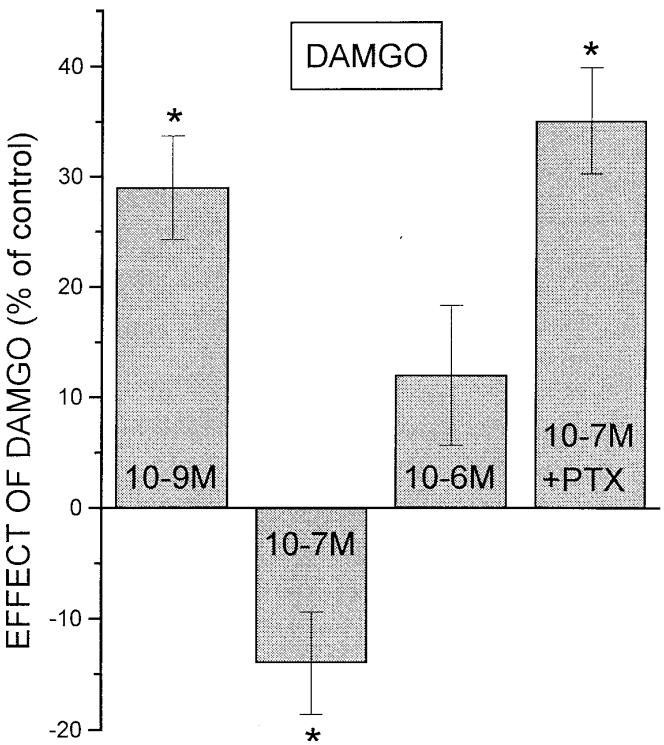

FIG. 1. The effect of the mu-selective agonist DAMGO on cAMP production in SK-N-SH cells. DAMGO 10^{-9}M stimulated production by 29% (n=55), 10^{-7}M attenuated production by 14% (n = 17), and 10^{-6}M elevated cAMP by 12% (n = 7). Following treatment of SK-N-SH cells with pertussis toxin (PTX; 100ng/ml, 18hr), DAMGO 10^{-7}M stimulated cAMP production by 35% (n = 14). Results (mean ± S.E.) are presented as percent of basal cAMP production during the10 min incubation in the absence of DAMGO. Each experiment was consisted of 12 DAMGO vs 12 control wells. The averaged value of [^{3}H]cAMP conversion in all control wells was 0.113 ± 0.019. Asterisks indicate significant differences from matched controls, compared by paired t-test ($p < 0.05$).

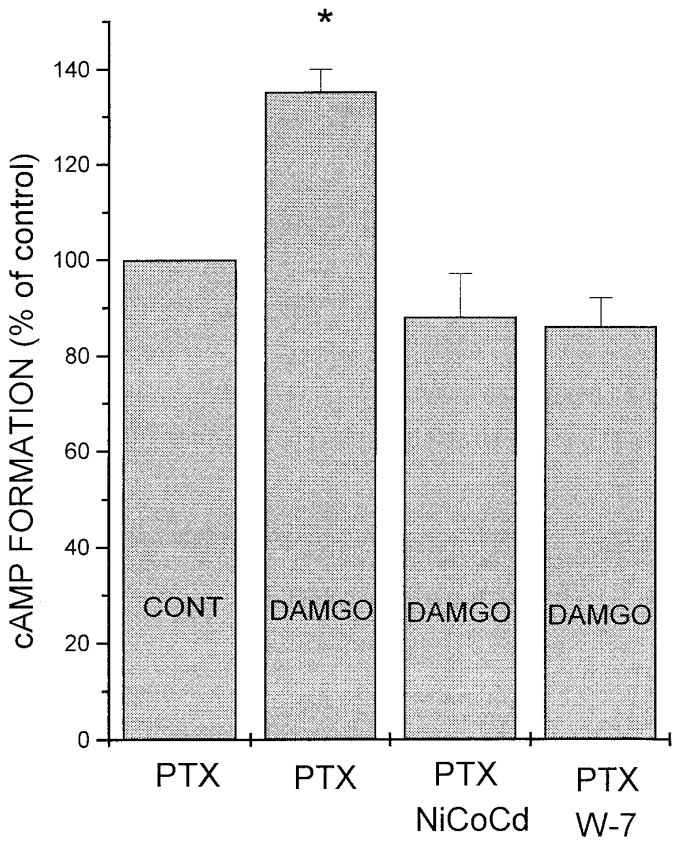

FIG. 2. The stimulatory effect of DAMGO 10^{-7}M on cAMP production in PTX-treated cells is abolished by blocking either Ca^{++} entry or calmodulin activity. SK-N-SH cultures were exposed to pertussis toxin (100ng/ml) for 18 hrs. In 10 experiments, $NiCl_2$ (1mM), $CoCl_2$ (1mM) and $CdCl_2$ (0.2mM) were added to the incubation medium of both DAMGO and control wells. In 9 other experiments, W-7 (30μM) was added. Each experiment was consisted of 12 DAMGO vs 12 control wells. Results are presented as percentage of basal production. *$p<0.05$, compared to matched controls by paired t-test.

We previously showed that the potentiation of cAMP formation by a low concentration (10^{-9}M) of DAMGO can be eliminated either by blocking Ca^{++} entry to the cells, or by blocking the activation of Ca/calmodulin. Here we tested the involvement of calcium ions in the stimulatory effect of DAMGO 10^{-7}M in PTX-treated cells: in the presence of the calcium channel blockers Ni^{++} (1mM),Co^{++} (1mM) and Cd^{++} (0.2mM), DAMGO 10^{-7}M failed to potentiate basal cAMP production. Similarly, exposing the PTX-treated cells to the calmodulin inhibitor W-7 (30μM, 30min) completely abolished the stimulatory effect of DAMGO (Fig.2).

The second phase of cAMP stimulation by DAMGO was achieved when a very high concentration (10^{-6}M) of this mu opioid agonist was used (Fig.1). Such a high concentration of DAMGO is expected to also activate non-mu opioid receptors. SK-N-SH cells express, in addition to the abundance of mu receptors, a considerable amount of delta opioid receptors (13,14). We tested whether delta receptors in these cells stimulate cAMP production as well: a low concentration (10^{-9}M) of the selective delta agonist DPDPE significantly elevated basal cAMP formation in SK-N-SH cells by 18±7.9% (n=10, $p<0.05$; Fig.3). In order to examine whether the stimulatory effect of DAMGO 10^{-6}M was due to marginal activation of delta receptors by the mu agonist, delta receptors were blocked by naltrindole $5x10^{-9}$M. Under these conditions, DAMGO 10^{-6}M failed to stimulate cAMP production (data not shown). A mirror image situation was observed with DPDPE: while 10^{-6}M of this delta agonist did not significantly affect cAMP formation, the same concentration of DPDPE significantly inhibited (14 ± 3%; n = 6, $p < 0.05$) cAMP formation when mu receptors were blocked by 10^{-8}M of the mu selective antagonist βFNA (Fig.3).

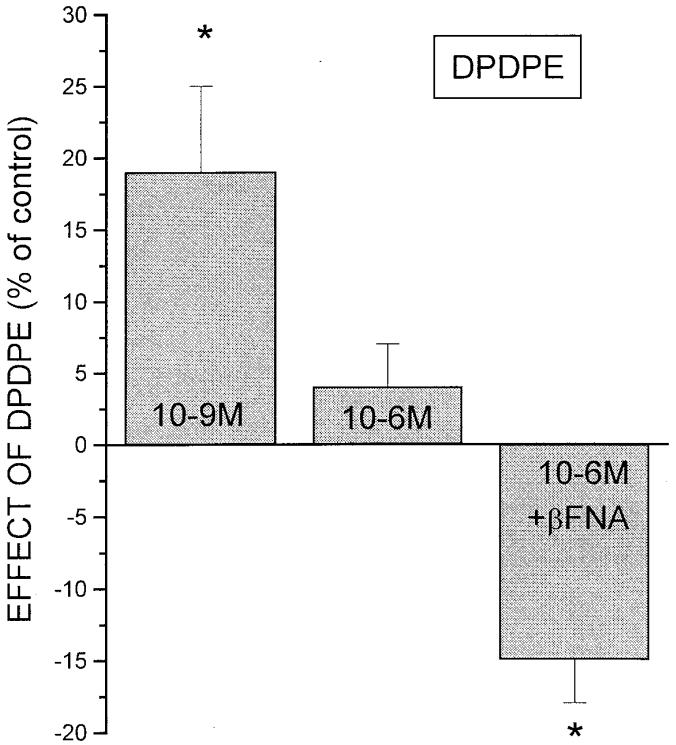

FIG. 3. The effect of the delta-selective agonist DPDPE on cAMP production in SK-N-SH cells. DPDPE 10^{-9}M stimulated production by 18% (n=10), while 10^{-6}M elevated cAMP by only 4% (n = 7). In the presence of the mu-selective antagonist βFNA, DPDPE 10^{-6}M suppressed cAMP production by 14% (n = 6). Results (mean ± S.E.) are presented as percent of basal cAMP production during the 10 min incubation in the absence of DPDPE. Each experiment was consisted of 12 DPDPE vs 12 control wells. Asterisks indicate significant differences from matched controls, compared by paired t-test ($p < 0.05$).

DISCUSSION

The present study describes opposite effects of opioid peptides on cAMP formation in SK-N-SH neuroblastoma cells. The stimulatory effect was induced by nanomolar concentrations of either mu- or delta-selective agonists, and turned into the "conventional" opioid inhibition when the concentration of the drug was elevated. The two phenomena involved two different cellular mechanisms: the inhibitory one was mediated by PTX-sensitive GTP-binding proteins, as is expected for opioid inhibition (1); the "unconventional" stimulation, on the other hand, was resistant to PTX. Similar stimulation of cAMP formation by opioids through a PTX-resistant mechanism was previously found in neuroblastoma-sensory neuron hybrid F11 cells (5) and guinea pig myenteric plexus (4). The stimulation of cAMP production by nanomolar concentrations of opioids in SK-N-SH cells was recently shown by us to be mediated by calcium: it was eliminated by removing Ca^{++} from the external medium, by blocking Ca^{++} infux through voltage-dependent calcium channels, and by suppressing calmodulin activity (11). The present study illustrates that the same stimulatory pathway is also activated by higher concentrations of the opioid ligand; however, the stimulatory effect is masked by a concomitant inhibitory activity; only after removing the inhibitory effect by PTX, the stimulatory counterpart is disclosed. The stimulation of cAMP formation by DAMGO 10^{-7}M in PTX-treated cells was abolished in the presence of inorganic ions (Ni^{++},Co^{++},Cd^{++}), which blocked Ca^{++} entry, and in the presence of W-7, which blocked calmodulin activity (Fig.2).

In previous studies we have shown that opioids potentiate $^{45}Ca^{++}$ uptake (15), and elevate cytoplasmic Ca^{++} level (15,16) in SK-N-SH cells. These effects were resistant to PTX, indicating that Gi/Go proteins were not involved. The stimulatory effects of opioids on intracellular Ca^{++} is not unique for SK-N-SH cells. In many neuronal (15-20), as well as non-neuronal (21,22) cells, opioids were shown to elevate $[Ca^{++}]_{in}$ either by stimulating Ca^{++} influx (15,17,19) or by mobilizing intracellular stores (17,20,21). Interestingly, some of these effects were resistant to PTX, while others were abolished by the toxin (for review see 3). Thus, opioid receptors can activate various cellular pathways that lead to calcium elevation. The elevation of intracellular Ca^{++} concentration by opioids may result in the activation of several intracellular components, including certain isoforms (type I,III,VIII) of adenylyl cyclase. An indirect stimulatory effect of opioids on adenylyl cyclase which is mediated by calcium ions, as found by us in SK-N-

SH cells, is not necessarily exclusive. A membrane delimited activation of adenylyl cyclase by opioid agonists was previously found in the rat olfactory bulb (21,22); this stimulatory effect was blocked by PTX (21), indicating a direct activation of adenylyl cyclase by Gi/Go proteins, probably through $\beta\gamma$ subunits.

The multiplicity of mechanisms by which opioids regulate cAMP production leads to heterogeneity in the response of various cells to these agents. In some cells (e.g. NG108-15), opioid agonists reduce basal formation of cAMP along all the range of concentrations (24); in others (F11 and SK-N-SH), a significant augmentation is induced by at least certain concentraions (5,11); this concentration-dependency probably reflects the relative efficacy of the various processes. In this regard it is interesting to notice that maximal opioid inhibition of cAMP production in NG108-15 cells (40-70%; ref 25,26) exceeded that in SK-N-SH cells (20-30%; ref 13,27). The relative preference of opioid receptor coupling to Gi (and, hence, inhibition of adenylyl cyclase), as compared to other GTP-binding proteins (which lead to stimulation), may result from differences in membrane ganglioside composition of the various cells. It was shown that GM1, but not other gangliosides, promoted the coupling of opioid receptors to the stimulatory pathway of cAMP production at the expense of their inhibitory activity (8,9). Moreover, even the elevation of $[Ca^{++}]_{in}$ by opioids may result in either stimulation or inhibition of different isoforms of adenylyl cyclase; thus, the relative cellular content of calcium-inhibitable (type V and VI) and calcium-stimulated (type I,III and VIII) isoforms can determine the modality of opioid regulation of cAMP production.

The bimodal activity of opioid agonists is even further complicated by the co-presence of various opioid receptors. In this study, the selective mu (DAMGO) and delta (DPDPE) opioid agonists cross-activated the other receptor when used in micromolar concentrations. Since the affinity of each selective agonist to the other receptor is lower by 2-3orders of magnitude (28), micromolar concentrations are equivalent to nanomolar concentrations of the selective agonist, and may add to the stimulatory activity of the drug. Only when the other receptor is blocked by a selective antagonist, 10^{-6}M of the agonist can induce a net inhibition of cAMP formation (Fig.3).

In conclusion, both pharmacological (selectivity of drugs and heterogeneity of receptors) and biochemical (two opposing cellular pathways) factors determine the overall effect of opioid agonists on cAMP production in neuronal cells. The final outcome of the activation of an opioid receptor is dependent on the balance between various overlapping cellular processes. This balance can differ between different cells, or even between different states of the same cell (5,16).

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