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Functional Analysis of Cloned Opioid Receptors in Transfected Cell Lines*

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Opioids modulate numerous central and peripheral processes including pain perception, neuroendocrine secretion and the immune response. The opioid signal is transduced from receptors through G proteins to various different effectors. Heterogeneity exists at all levels of the transduction process. There are numerous endogenous ligands with differing selectivities for at least three distinct opioid receptors (μ, δ, κ). G proteins activated by opioid receptors are generally of the pertussis toxin-sensitive Gi/Go class, but there are also opioid actions that are thought to involve Gq and cholera toxin-sensitive G proteins. To further complicate the issue, the actions of opioid receptors may be mediated by G-protein α subunits and/or βγ subunits. Subsequent to G protein activation several effectors are known to orchestrate the opioid signal. For example activation of opioid receptors increases phosphatidyl inositol turnover, activates K^+ channels and reduces adenylyl cyclase and Ca^{2+} channel activities. Each of these effectors shows considerable heterogeneity. In this review we examine the opioid signal transduction mechanism. Several important questions arise: Why do opioid ligands with similar binding affinities have different potencies in functional assays? To which Ca^{2+} channel subtypes do opioid receptors couple? Do opioid receptors couple to Ca^{2+} channels through direct G protein interactions? Does the opioid-induced inhibition of vesicular release occur through modulation of multiple effectors? We are attempting to answer these questions by expressing cloned opioid receptors in GH_3 cells. Using this well characterized system we can study the entire opioid signal transduction process from ligand-receptor interaction to G protein-effector coupling and subsequent inhibition of vesicular release.

KEY WORDS: Opioid receptor; L-type calcium channel; adenylyl cyclase; hormone release; GH_3 cells; G proteins.

INTRODUCTION

Endogenous opioids and their synthetic analogs regulate many neuronal and endocrine processes. Autonomic functions, including respiratory, cardiovascular and digestive systems can be modulated by exogenous opioids (1). In the hypothalamo-pituitary axis opioids inhibit the release of leutenizing hormone and oxytocin from endocrine cells (2–5). Opioids also regulate the immune system (6–8). However, the most extensively investigated action of opioids is their role in pain control (1,9–11). Opioids regulate pain pathways in part by inhibiting neurotransmitter release from dorsal root ganglia (DRG) projections in the dorsal horn of the spinal cord (12–14).

To date, there is evidence for three different classes of opioid receptors (μ, δ, κ) based on selective ligand binding profiles (15) and molecular cloning (16–19). Cloning of opioid receptors confirmed that they belong to the G protein-coupled receptor superfamily, comprised of proteins with seven putative transmembrane domains (20). Activated heterotrimeric G proteins transduce opioid signals directly, or through second messengers, to multiple effector systems. Prior to the cloning

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of the opioid receptors it was known that all three receptors couple to adenylyl cyclase, K^+ and Ca^{2+} channels, and phosphatidyl inositol (PI) turnover (21–24). Pertussis toxin (PTX)-sensitive (Gi/Go) G proteins mediate opioid-induced inhibition of adenylyl cyclase, inhibition of Ca^{2+} channel activity, and activation of inwardly rectifying K^+ channels (20). In some cell types opioid receptors increase free intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels, predominantly through release from intracellular stores (23). In cardiac myocytes this action is mediated by Gq (25), but in smooth muscle this effect is blocked by PTX and therefore presumably involves Gi/Go (26). These G proteins activate phospholipase C, which catalyzes the formation of the cellular messengers inositol triphosphate (IP_3) and diacylglycerol. Subsequently, IP_3 facilitates the release of Ca^{2+} from intracellular stores. Interestingly, in the SH-SY5Y neuroblastoma cell line, opioid receptors can cause Gi/Go-mediated elevation of $[Ca^{2+}]_i$, but activation of Gq proteins by muscarinic receptors appears to be a prerequisite for this phenomenon (27). Opioids also have excitatory actions in the F-11 cell line, derived from the dorsal root ganglia. In these cells opioid receptors stimulate intracellular cAMP accumulation and inhibit K^+ channel activity through cholera toxin-sensitive G proteins (28, 29). Opioid modulation of these effector systems may lead to their modulation of neurotransmitter and hormone release.

Although much has been learned about the transduction of the opioid signal, the following questions are among those still to be answered. Do different opioid receptors couple to different effectors through distinct G proteins? Which of these effector(s) do opioid receptors utilize to modulate release? Following the cloning of the opioid receptors, we are in a better position to answer these questions, by expressing opioid receptor cDNAs in cell lines, lacking endogenous receptors. Using this approach stably transfected cell lines can be produced containing homogeneous receptor populations enabling biochemical and electrophysiological studies of receptor function. In addition, transfected cells enable site-directed mutagenesis and chimeric receptor studies to elucidate the structural motifs important for ligand and G protein association. Such experiments on cloned opioid receptors in unexcitable cell lines have revealed that the μ-, δ- and κ-receptors have distinct binding properties, and all three inhibit intracellular cAMP production (16–19). While useful for biochemical and pharmacological studies, these unexcitable non-secretory cell lines are not suitable for testing the role of opioid receptors in the modulation of either ion channels or vesicular release.

We have stably expressed cloned opioid receptors in the endocrine GH_3 cell line derived from the rodent anterior pituitary (30,31). GH_3 cells express adenylyl cyclase, phospholipase C, voltage-dependent Ca^{2+} and K^+ channels, and endogenous somatostatin (SRIF) receptors (32–34). Furthermore, these cells release both growth hormone and prolactin (PRL) via large dense-core vesicles.

This review explores the emerging complexity of opioid receptor signal transduction, with a discussion of recent studies of endogenous and recombinant receptors. We present our findings using the GH_3 cell system and include new data exploring the actions of cloned opioid receptors on prolactin release from these cells.

Why Do Opioid Agonists with Similar Binding Affinities Have Different Potencies? Since GH_3 cells do not express opioid receptors (35), but do have the requisite G proteins and effectors for opioid signal transduction, we established two opioid receptor expressing GH_3 cell lines. Cells transfected with rat μ-receptor cDNA (termed GH_3MOR cells) exhibit high affinity specific binding, as assessed by displacement of bound [^{3}H]diprenorphine by the μ-receptor selective ligand DAMGO (Table I). Both DAMGO and the δ-receptor selective ligand DPDPE bind with high affinity to cell membranes of GH_3MOR cells additionally transfected with murine δ-opioid receptor cDNA (GH_3MORDOR cells) (Table I). The affinities of these ligands for their respective receptors are similar to the affinities observed in membrane preparations from brain tissue (36–38), NG108-15 cells (36), and in other transfected cell lines (16–19,39–41).

In GH_3MOR cells DAMGO inhibited adenylyl cyclase and Ca^{2+} channel activities with IC_{50} values of 22 and 105 nM, respectively (Table I). The potencies of DAMGO in these functional experiments are lower than the opioid's apparent affinity (~1 nM) for the receptor, which was assessed in binding assays performed on isolated cell membranes in the absence of Na^+ and guanylyl nucleotides. Such disparities between the results of functional and ligand binding assays have been observed previously; in general, the concentration of DAMGO yielding half-maximal inhibition of adenylyl cyclase activity is between 1 and 2 orders of magnitude higher than its affinity for the μ-receptor measured in membrane binding assays (41,42). In the SH-SY5Y cell line DAMGO bound to the μ-receptor with high affinity (K_d = 3.2 nM) but inhibited Ca^{2+} channel activity with relatively low potency (IC_{50} = 11 nM) (43,44). This disparity between DAMGO's apparent affinity and potency may be due to the different conditions used in these experiments. Membrane fractions are commonly used in binding assays because many agonists bind to opioid receptors with multiple affinities in whole cells (45).

Table I. Activated Cloned μ- and δ-Opioid Receptors Inhibit Adenylyl Cyclase and Ca^{2+} Channel Activities in Transfected GH_3 Cells

	Ligand Binding Affinity		Adenylyl Cyclase		Ca^{2+} Channel Inhibition	
	K_i (nM)		IC_{50} (nM)		IC_{50} (nM)	
Cell Line	DAMGO	DPDPE	DAMGO	DPDPE	DAMGO	DPDPE
GH_3MOR	1.0 ± 0.6	310 ± 105	21.9 ± 4.1	> 10,000	105	NT*
$GH_3MORDOR$	0.5 ± 0.1	0.7 ± 0.1	174 ± 77	0.5 ± 0.2	NT**	1.6

Saturation binding experiments were performed on GH_3MOR (Piros *et al.*, 1995) and $GH_3MORDOR$ (Piros *et al.*, 1996) cell membranes to verify the presence of μ- and both μ- and δ-opioid receptors, respectively. In these transfected cells μ- and δ-opioids dose-dependently inhibited forskolin-stimulated intracellular cAMP accumulation. In addition, Ca^{2+} channel activity, recorded using the whole-cell configuration of the patch-clamp technique with Ba^{2+} as the charge carrier, was inhibited upon activation of μ- and both μ- and δ-receptors in GH_3MOR and $GH_3MORDOR$ cells, respectively.

*NT, Not tested. DPDPE (1 μM) caused a 3.5 ± 2.1% (n = 9) inhibition of Ba^{2+} current amplitude.

**NT, Not tested. A single dose of DAMGO (1 μM) caused a 17.0 ± 1.4% (n = 5) inhibition of Ba^{2+} current amplitude.

However, whole-cells are generally used for measuring adenylyl cyclase and Ca^{2+} channel activities. In isolated membranes opioid receptors are also present in multiple affinity states if Na^{2+} and GTP are included in the assay buffer. These components are essential for receptor-G protein coupling, and they decrease the observed affinity of the activated receptor for its ligand in membrane preparations (46,47). By omitting Na^+ and guanylyl nucleotides, but including Mg^{2+} in the buffer used in binding assays the receptor is only in the high affinity state (48). Clearly, binding of DAMGO to isolated membranes under these conditions does not mimic the interaction of this ligand with its receptor in functional assays. Yet, the information obtained from such binding assays is useful for confirming receptor expression, and estimating receptor number per cell.

By contrast to DAMGO, the affinity of DPDPE for δ-receptors in membranes from $GH_3MORDOR$ cells and the potency of the agonist as an inhibitor of adenylyl cyclase and Ca^{2+} channel activities in whole-cells are similar (Table I). While in membranes of $GH_3MORDOR$ cells DAMGO and DPDPE bind to their respective receptors with similar affinities, DAMGO has a substantially lower potency than DPDPE as an inhibitor of adenylyl cyclase and Ca^{2+} channel activity (Table I). There are a number of reasons why agonists with similar binding affinities can have differing potencies. If μ- and δ-opioid receptors are identical in their coupling to G proteins and subsequent interactions with effectors, then the difference in agonist potencies could be explained in a number of ways. First, DAMGO and DPDPE could have different affinities for their respective receptors in the conformation required for receptor activation that is perhaps not detectable in membrane binding assays. Second, DAMGO and DPDPE may have differing intrinsic activities. Although these selective opioids cause similar maximal inhibitions of adenyl cyclase and Ca^{2+} channel activities in transfected GH_3 cells (30,31)—suggesting that they could have similar intrinsic activities—the existence of spare receptors leaves this possibility open. The difference in agonist potencies could also be explained by a difference in the number of μ- and δ-receptors. From the Scatchard analyses, $GH_3MORDOR$ cells express approximately ten times more δ- than μ-receptors (31). When spare receptors are present, a smaller fraction of occupied receptors may be required to fully activate the effectors (49). This may explain why the potencies of DPDPE as an inhibitor of adenylyl cyclase and Ca^{2+} channel activities are closer to its affinity for the δ-receptor.

The situation may be more complex, μ- and δ-receptors may couple to the same effectors through different G proteins and this may also affect the efficiency of signal transduction. The ability of the μ-receptor to activate its respective G protein(s) may be inferior to the δ-receptor, which could explain the lower potency observed in functional assays. In transfected GH_3 cells μ- and δ-receptors inhibit Ca^{2+} channels through PTX-sensitive G proteins, it is uncertain which of the numerous subtypes are preferred by which receptors. So far, G protein α subunits arising from sixteen genes have been cloned (50), and four of them are alternatively spliced, giving rise to twenty different α subunits. Seven α subunits are sensitive to PTX treatment, and at least four of these (α_{i2}, α_{i3}, α_{o1}, α_{o2}) are present in GH_3 cells (51). Adding to the complexity, five different β and six γ subunits are available to combine with an α subunit to form a heterotrimeric complex (50). Multiple permutations of G protein heterotrimers may be available to transduce receptor signals in the same cell. For instance, in GH_3 cells, M4 muscarinic and somatostatin receptors appear to inhibit voltage-dependent Ca^{2+} channels via different G proteins, $\alpha_{o1A}\beta_3\gamma_4$ and $\alpha_{o2B}\beta_1\gamma_3$, respectively (52). Opioid receptors, also exhibit specificity

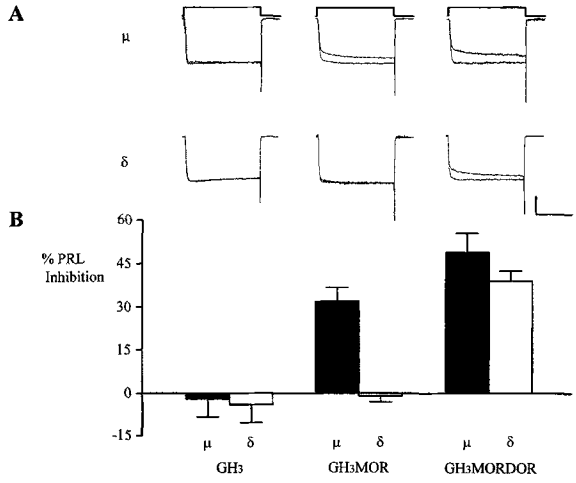

Fig. 1. Cloned μ- and δ-opioid receptors inhibit Ca^{2+} channel activity and prolactin release in transfected GH_3 cells. A. Whole-cell Ba^{2+} current inhibition by activation of μ- and δ-opioid receptors with DAMGO (1 μM) and DPDPE (1 μM), respectively. Ba^{2+} currents, activated by depolarization from −80 to 0 mV, were recorded using the whole-cell configuration of the patch-clamp technique. Superimposed current traces recorded from GH_3, GH_3MOR and GH_3MORDOR cells (from left to right) are shown before and during agonist application. The vertical calibration bar for currents in the top row from left to right indicates 360 pA, 300 pA and 175 pA, in the lower row the calibration bar represents (from left to right) 450 pA, 280 pA and 200 pA. In all cases the horizontal bar represents 50 ms.
B. Inhibition by opioids of basal prolactin (PRL) release from transfected GH_3 cells. PRL levels, secreted from GH_3 cells, were measured by a competitive ELISA before and subsequent to a 30 minute exposure to DAMGO (1 μM) and DPDPE (1 μM), respectively. Results are expressed as the percent inhibition of the control PRL levels secreted prior to treatment.

towards selective G proteins. Endogenous μ-receptors in SH-SY5Y cells couple more efficiently to α_{i3}, while δ-receptors prefer α_{i1} and α_{o1} in the same cell line (53). It is possible that μ- and δ-receptors utilize different subtypes of G protein complexes to inhibit Ca^{2+} channels in transfected GH_3 cells.

To Which Ca^{2+} Channel Subtypes Do Opioid Receptors Couple? All three subtypes of endogenous opioid receptors couple to a variety of voltage-activated Ca^{2+} channels in various cell types derived from the periphery and the brain (22,23). In primary cultured DRG preparations, opioid receptors inhibit N-(54–56), T-(54) and P/Q-type (56) Ca^{2+} channels. Opioid receptor-mediated inhibition of both N- and P/Q-type Ca^{2+} channels occurs in acutely dissociated nucleus tractus solitarius (NTS) neurons (57). In the NG108-15 (58) and SH-SY5Y neuroblastoma cell lines (44) activated opioid receptors inhibit N-type channels. Subsequent to the cloning of opioid receptors, Tallent et al. (59) and Morikawa et al. (60) observed inhibitory coupling between κ- and μ-receptors and N-type Ca^{2+} channels in transfected PC-12 and NG108-15 cell lines, respectively. Coupling of opioid receptors to dihydropyridine-(DHP) sensitive L-type Ca^{2+} channels could not be demonstrated in these neuronal cell lines. It is worth noting that N- and/or P/Q-type channels are more abundantly expressed than L-type channels in all of the aforementioned preparations. Therefore, interactions between G protein coupled receptors and L-type channels may be overshadowed by more robust coupling to N- and P/Q-type channels. In bovine chromaffin cells (61) and in ventricular myocytes (62), where DHP-sensitive L-type Ca^{2+} channels predominate, enkephalins inhibit these channels. Alternatively, opioid receptors may couple to a subset of L-type Ca^{2+} channels expressed only in certain cell types.

Now that opioid receptors and several voltage-activated Ca^{2+} channels are cloned, the specificity of their coupling can be examined in heterologous expression systems. To date six Ca^{2+} channel α_1 (α_{1A}-α_{1E}, and α_{1S}) subtypes, three β subunits and an $\alpha_2\delta$ dimer have been identified (63). Adding to the heterogeneity, the α_{1C} subunit has three splice variants. Expression of homomeric α_1 subunits produces functional Ca^{2+} channels with distinct biophysical and pharmacological properties. Recently, Bourinet et al. (64) coexpressed μ-receptors and different Ca^{2+} channel α_1 subunits (along with ancillary α_2 and β_4 subunits) in *Xenopus* oocytes. They found that ligand-activated μ-receptors inhibit currents mediated by α_{1A} (P/Q-type) and α_{1B} (N-type) channels, but did not modulate α_{1C} (L-type) and α_{1E} (R-type?) channel activity. The other previously cloned DHP-sensitive L-type Ca^{2+} channel α_1 subunits (α_{1D} and α_{1S}) were not tested in this study.

GH_3 cells express predominantly L- and, to a lesser extent, T-type Ca^{2+} channels. The presence of these channels has been confirmed by pharmacological means (30,65) and on the basis of their current deactivation kinetics (66). In addition, RNAse protection assays indicate the presence of DHP-sensitive Ca^{2+} channel α_{1C} and α_{1D} subunit mRNAs in GH_3 cells (67). In these cells, activation of cloned μ- and δ-receptors inhibits DHP-sensitive L-type Ca^{2+} channel activity (30, 31, Fig. 1A.). It is presently unclear whether the opioid receptors couple to the α_{1C}, α_{1D}, or an as yet unidentified DHP-sensitive Ca^{2+} channel subtype. In the light of the findings of Bourinet et al. (64), coupling to the α_{1C} subunit is unlikely, but it cannot be excluded since the α_{1C} gene has three splice variants. It is possible that in GH_3 cells and *Xenopus* oocytes alternative splicing gives rise to different isoforms of Ca^{2+} channels, with differential susceptibility to modulation by opioid receptors. Alternatively, the transduction from opioid receptors to L-type

Ca^{2+} channels may utilize G proteins available in GH_3 cells, but not present in *Xenopus* oocytes.

In summary, endogenous opioid receptors inhibit Ca^{2+} entry into neuroblastoma cells, and into peripheral and central NTS neurons via mostly N- and P/Q-type Ca^{2+} channels. Native opioid receptors in chromaffin cells and in the heart, and cloned opioid receptors in GH_3 cells inhibit L-type channels. These findings suggest that, depending on the cellular environment, opioid receptors are able to interact with a variety of Ca^{2+} channel subtypes. Further studies are required to determine which of these channels are important in the central actions of opioids.

Do Opioid Receptors Couple to Ca^{2+} Channels Through Direct G protein Interactions? Pertussis toxin pretreatment reversed the inhibitory actions of activated μ-and δ-opioid receptors on L-type Ca^{2+} channels in GH_3 cells, indicating that Gi/Go types of G proteins mediate the opioid action (30,31). G proteins inhibit Ca^{2+} channel activity by either directly interacting with the channel subunits (membrane-delimited action) or by the use of diffusible intracellular second messengers, such as cAMP (68). There is evidence for membrane-delimited coupling between activated opioid receptors and Ca^{2+} channels. By recording from single cells simultaneously with two configurations of the patch-clamp technique Wilding *et al.* (69) demonstrated that DAMGO's inhibition of Ca^{2+} channel activity appears to occur through closely associated receptors, G proteins and Ca^{2+} channels. Using the cell-attached patch and whole-cell configurations in the same cell, Ca^{2+} channel activity in a small patch and on the remaining cellular membrane can be simultaneously recorded. Bath application of DAMGO did not cause an inhibition of Ca^{2+} channels isolated by the cell-attached electrode, but did inhibit the whole-cell Ca^{2+} current. These data suggest that opioid receptors can only modulate closely associated Ca^{2+} channels, perhaps by a direct interaction between activated G proteins and the adjacent channels.

There is similar evidence for membrane-delimited interactions between other G protein coupled receptors and N-type Ca^{2+} channels. A distinguishing feature of the putative direct interaction between G proteins and N-type Ca^{2+} channels is reversibility during depolarization (68,70–73). It is thought that the binding of several activated G protein subunits places each channel in a state in which it is "reluctant" to open (i.e. the channel is inhibited). Strong depolarization drives G protein subunits off the channel, making it more "willing" to open (70). In GH_3 cells, depolarizing prepulses completely reverse both μ- and δ-receptor-induced L-type Ca^{2+} channel inhibitions (31). Similar observations were made by Keja and Kits (74) in pituitary melanotropes on the voltage-dependence of D2 receptor-induced L-type Ca^{2+} channel inhibition. Therefore, taken together, the results of the studies discussed here suggest that opioid receptors can couple to either N-(58,72), P/Q-(63) or L-type Ca^{2+} channels (31) in a membrane-delimited voltage-dependent manner. Which subunit(s) of heterotrimeric G proteins mediate this inhibitory action? Initial experiments investigating the function of G proteins suggested that activated α subunits were responsible for transducing signals from G protein-coupled receptors to various effectors (68,75–78). According to these studies, the βγ dimer would only have a passive role of terminating the response by reassociating with the α subunit. However, several laboratories have demonstrated that the βγ subunit itself can inhibit adenylyl cyclase activity, stimulate phospholipase C, and increase openings of inwardly rectifying K^+ channels (reviewed in refs 78 and 79). Recent evidence suggests that βγ subunits are important in the actions of G protein coupled receptors on Ca^{2+} channels. Overexpression of the βγ subunit in sympathetic neurons (80) and in tsA-201 cells (81) attenuated N- and P/Q-type Ca^{2+} channel activity, respectively. These βγ subunit-induced inhibitions could be reversed by depolarizing prepulses. The inhibition of Ca^{2+} channels by norepinephrine was significantly reduced in sympathetic neurons, subsequent to overexpression of βγ subunits, suggesting that α_2-adrenergic receptors utilize βγ subunits in their coupling to Ca^{2+} channels (80,81). Therefore, the following scheme for coupling between the receptor and Ca^{2+} channel is emerging: The receptor binds an appropriate ligand leading to the liberation of G protein βγ subunits which directly interact with Ca^{2+} channels in a voltage-dependent fashion (Fig. 2). It is not clear what the role of the α subunit is in the transduction of this signal. It is possible that α subunits may also interact with Ca^{2+} channels causing inhibition. Whether such a mechanism exists and if so, whether it is voltage-dependent, remains to be determined. There is a voltage-independent component of Ca^{2+} channel inhibition that may be mediated by G protein α subunits (68). Interestingly, in *Xenopus* oocytes consistent coupling of μ-opioid receptors to cloned Ca^{2+} channels requires the coexpression of α_0 G protein subunits (64).

In summary, by analogy with the α_2 adrenergic receptor, voltage-dependent, membrane-delimited coupling between opioid receptors and N-, P/Q- and L-type Ca^{2+} channels is probably mediated by βγ G protein subunits. However, experimental data supporting this hypothesis are required.

Does the Opioid-Induced Inhibition of Vesicular Release Occur through Multiple Effectors? Opioids in-

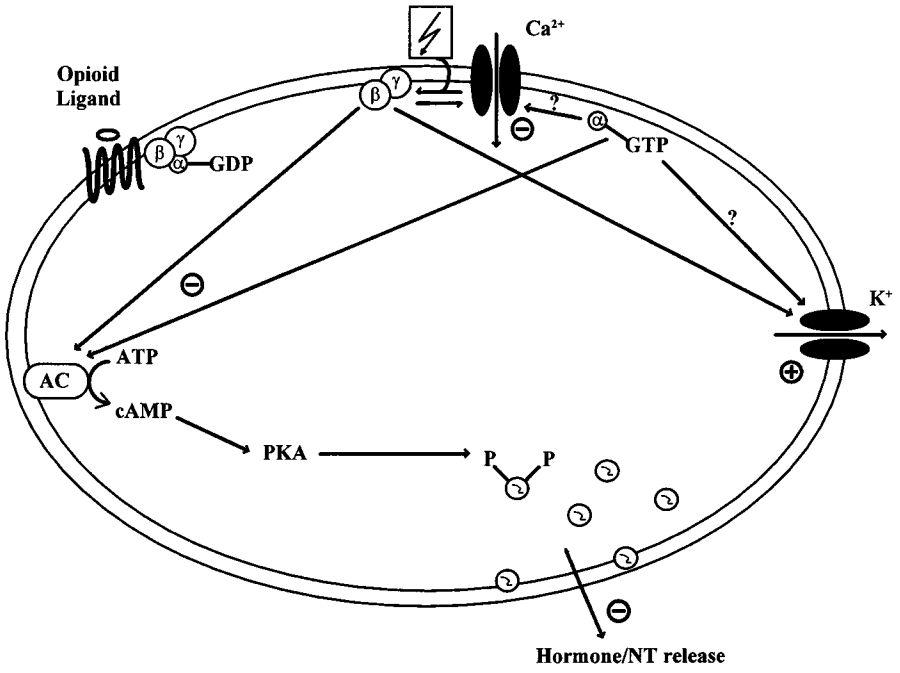

Fig. 2. Schematic diagram of the proposed mechanisms for the opioid-mediated modulation of hormone release from transfected GH_3 cells. In GH_3MOR and $GH_3MORDOR$ cells activated opioid receptors inhibit voltage-gated Ca^{2+} channel and adenylyl cyclase (AC) activities via heterotrimeric G proteins. The interaction between activated G proteins and L-type Ca^{2+} channels is inhibited by depolarization. We therefore propose that βγ subunits are involved (see text). Opioid receptors may also activate K^+ channels and modulate inositol triphosphate production in GH_3 cells. Modulation of one or more of these effector(s) leads to inhibition of vesicular release.

hibit presynaptic neurotransmitter release (12–14,23) and hormone secretion (2–4). However, the mechanisms by which opioids inhibit secretion are poorly understood. The inhibitory action of endogenous SRIF receptors on hormone release is well characterized in GH_3 cells (82). SRIF and opioid receptors share high structural homology (16), which may imply that these receptors couple to similar G proteins and therefore have similar mechanisms for their modulation of hormonal secretion. Indeed, in NG108-15 cells Ca^{2+} channel inhibition by both SRIF and opioid receptors can be mediated by the G_{oA} subtype of G proteins (83). Cloned μ- and δ-opioid receptors, like endogenous SRIF receptors, couple to Ca^{2+} channels and adenylyl cyclase in GH_3 cells (30,31). Reduction of intracellular Ca^{2+} and cAMP levels by SRIF receptors leads to decreased prolactin (PRL) and growth hormone secretion from this cell line (82). Recently, we developed an enzyme-linked immunosorbent assay (ELISA) to measure PRL release from GH_3, GH_3MOR and $GH_3MORDOR$ cells (84). As expected, opioid ligands had no effect on PRL release from untransfected GH_3 cells. However, DAMGO (1 μM) inhibited PRL secretion by 32% and 49% in GH_3MOR and $GH_3MORDOR$ cells, respectively (Fig. 1B). The δ-receptor specific ligand DPDPE also inhibited PRL release from the cotransfected $GH_3MORDOR$ cell line in a dose-dependent manner (IC_{50} = 3.8 nM), while it was without effect in the μ-receptor expressing GH_3MOR cells (Fig. 1B). The inhibitory actions of DAMGO and DPDPE were attenuated by PTX treatment (84).

From these results we conclude that both μ- and δ-receptors inhibit PRL release from transfected GH_3 cells via PTX-sensitive G proteins. Which effectors mediate the opioid-induced inhibition of hormone release? In view of the crucial role of Ca^{2+} entry during vesicular release the most obvious candidate is the Ca^{2+} channel (Fig. 2). Lower $[Ca^{2+}]_i$ leads to decreased Ca^{2+} -dependent hormone release. Although direct (G protein-mediated) inhibition of Ca^{2+} channels lowers $[Ca^{2+}]_i$, it is also possible that these voltage-activated Ca^{2+} channels are indirectly inhibited due to hyperpolarization of the cell. Hyperpolarization could occur as a consequence of K^+ channel activation. Another candidate for mediating inhibition of release is cAMP, which activates several kinases required for the phosphorylation of proteins involved in the secretory process (85). Therefore, a reduction of intracellular cAMP levels may lead to decreased hormone secretion.

The inhibition of PRL release from transfected GH_3 cells by opioids is relatively large, when compared to

their inhibition of Ca^{2+} channel activity (Fig. 1). This may suggest that additional mechanisms, such as attenuation of adenylyl cyclase activity, play a role in the modulation of release by opioids. Alternatively, there may be a non-linear relationship between Ca^{2+} entry and subsequent release of PRL. It is likely, that voltage-activated Ca^{2+} channels are localized in regions of GH_3 cells from which hormones are released. This concept of "active zones" in neuroendocrine cells is supported by pulsed-laser Ca^{2+} imaging experiments on chromaffin cells (86). If PTX-sensitive G proteins were also localized at these putative active zones, then inhibition of Ca^{2+} entry may occur predominantly at the sites most likely to affect hormone release. Using imaging techniques it may be possible in the future to establish whether cloned opioid receptors, G proteins and L-type Ca^{2+} channels are associated with active zones in GH_3 cells.

Future Directions

There are several unanswered questions about how opioid receptors function. Does the pharmacology of an opioid receptor remain the same when coupled to different G proteins? Which G proteins do opioid receptors utilize to interact with different effectors? Do the α and $\beta\gamma$ subunits of G proteins activate different effectors? And perhaps most importantly, what is the relevance of coupling to these effectors to the modulation of vesicular release? Cell lines that express transfected opioid receptors, have well characterized endogenous G proteins, have voltage-activated ion channels and that are equipped for vesicular release are useful for addressing these questions.

CONCLUSIONS

The complexity of opioid receptor signal transduction was appreciated well before the cloning of these receptors. Opioid receptors modulate intracellular accumulation of cAMP, IP_3, and both Ca^{2+} and K^+ channel activity via heterotrimeric G proteins. Cloning has allowed the expression of individual opioid receptors in well characterized cell lines. When these cell lines are both excitable and equipped for vesicular release, then a wide spectrum of opioid receptor functions can be studied. Novel findings, such as the coupling of opioid receptors to multiple effectors and a possible direct interaction between activated G proteins and L-type Ca^{2+} channels, provide a more thorough understanding of the complex mechanisms through which opioid receptors transduce their signals.

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