Tyrosine Phosphorylation of the \( \kappa \)-Opioid Receptor Regulates Agonist Efficacy*

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To explore the role of highly conserved tyrosine residues in the putative cytoplasmic domains of the seven-transmembrane G protein-coupled opioid receptors, we expressed the rat \( \kappa \)-opioid receptor (KOR) in Xenopus oocytes. KOR activation by the agonist U69593 produced a strong increase in potassium current through coexpressed G protein-gated inwardly rectifying potassium channels (K\(_{\text{IR3.3}}\)). Brief pretreatment with insulin caused a 60% potentiation of the KOR-activated response. The insulin-induced increase in KOR response was blocked by the tyrosine kinase inhibitor genistein. In contrast, insulin had no effect on the basal activity of K\(_{\text{IR3.3}}\), suggesting that KOR is the target of the tyrosine kinase cascade. Mutation of tyrosine residues to phenylalanines in either the first or second intracellular loop of KOR to produce KOR(Y87F) and KOR(Y157F) had no effect on either the potency or maximal effect of U69593. However, neither KOR(Y87F)- nor KOR(Y157F)-mediated responses were potentiated by insulin treatment. Insulin pretreatment shifted the dose-response curve for U69593 activation of KOR by increasing the maximal response without changing the EC\(_{50}\) value for U69593. These results suggest that insulin increases the efficacy of KOR activation by phosphorylating two tyrosine residues in the first and second intracellular loops of the receptor. Thus, tyrosine phosphorylation may provide an important mechanism for modulation of G protein-coupled receptor signaling.

Opioid receptors are widely expressed throughout the nervous system, and opioid drugs affect pain perception, learning and memory, epilepsy, and food intake, among other diverse functions (1). A common mechanism for post-translation regulation of protein function is by phosphorylation, and phosphorylation of opioid receptors is likely to regulate opioid actions. For example, phosphorylation by G protein receptor kinases following agonist treatment has been proposed to play a role in opioid receptor desensitization and tolerance (2). Other serine/threonine kinases such as protein kinase C and calcium/calmodulin-dependent kinase II in some systems may also regulate opioid receptors (3).

To date, few studies have reported any effects of tyrosine kinases on acute opioid receptor function. Tyrosine residues in the putative cytoplasmic domains of G protein-coupled receptors are extremely common, and regulation of opioid receptor signaling by tyrosine kinase cascades would provide a powerful mechanism of cellular coordination. Indeed, a portion of the agonist-induced internalization of the \( \mu \)-opioid receptor is dependent on tyrosine kinase activation (4–6). Opioid receptor activation has been shown to increase tyrosine kinase activity (7, 8), and the agonist-induced internalization mediated by tyrosine kinase could potentially involve a process that is directly activated by opioid receptors. These findings are consistent with data on the \( \beta\)-adrenergic receptor in which tyrosine phosphorylation of the C-terminal tail was shown to play a role in the desensitization and internalization of the \( \beta\)-adrenergic receptor (9). In addition to the inhibitory effects of tyrosine kinases on G protein-coupled receptor function, Valiquette et al. (10) reported that tyrosine phosphorylation of the \( \beta\)-adrenergic receptor on the second intracellular loop potentiated \( \beta\)-adrenergic receptor activation of adenylate cyclases. Although the interactions are not yet clear, these initial studies suggest that tyrosine kinase cascades may regulate G-protein coupled receptors to produce either increases or decreases in functioning.

To explore these mechanisms further, we examined the effect of tyrosine kinase activation on \( \kappa \)-opioid receptor coupling to the G protein-activated inwardly rectifying potassium channel (K\(_{\text{IR3.3}}\)).† To do this, we utilized the Xenopus oocyte expression system and took advantage of the endogenously expressed insulin receptor tyrosine kinase cascade (11).

**EXPERIMENTAL PROCEDURES**

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† The abbreviations used are: K\(_{\text{IR3.3}}\), G protein-activated inwardly rectifying potassium channel; KOR, \( \kappa \)-opioid receptor; BDNF, Brain-derived neurotrophic factor.

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**Agonist Efficacy**

| Agonist | Efficacy |
|---------|----------|
| U69593  | Strong increase in potassium current through coexpressed G protein-gated inwardly rectifying potassium channels (K\(_{\text{IR3.3}}\)). |
| Insulin | Potentiation of the KOR-activated response. |
| Genistein | Block of the insulin-induced increase in KOR response. |

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**References**

1. **Experimental Procedures**

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linearized prior to cRNA synthesis, and the mMESSAGE MACHINE kit (Ambion Inc.) was used to generate capped cRNA.

**Oocyte Culture and Injection**—Defolliculated stage IV oocytes were prepared as described (13) and incubated for 2–3 days after injection of the cRNAs in normal oocyte saline buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with sodium pyruvate (2.5 mM) and gentamycin (50 μg/ml). cRNAs were injected (50 nl/oocyte) with a Drummond microinjector. Each oocyte was injected with 1 ng of KOR cRNA and either 0.1 ng of KIR3.1 and KIR3.2 wild-type potassium channel or 1 ng of KIR3.2(S146T) pore mutant channel cRNA. A few sets of oocytes were injected with KIR3.4 cRNA instead of KIR3.2 as controls for direct effects of insulin-activated cascades on the channel.

**Electrophysiology**—Oocytes were voltage-clamped at −80 mV with two electrodes filled with 3 mM KCl having resistances of 0.3–3.0 megohms using an Axoclamp 2A unit and pCLAMP Version 6 software (Axon Instruments, Inc.). Membrane current traces were recorded using a chart recorder. To facilitate the recording of inward K⁺ currents through the KIR3 channels, the normal oocyte saline buffer was modified to increase the KCl concentration to 96 mM K⁺. The concentration of NaCl was correspondingly decreased to maintain osmolality.

**Data Analysis**—EC₅₀ values and curve fitting were determined using Nfit software (Island Products, Galveston, TX). Confidence intervals were used for comparison of the independent means. Student’s t test was used for comparison of independent means, with values reported as two-tailed p values.

## RESULTS

**Insulin Potentiates the κ-Opioid Activation of KIR3**—Rat KOR was coexpressed in *Xenopus* oocytes with KIR3.1 and KIR3.2 (Fig. 1). When the concentration of potassium was increased in the extracellular recording solution, the basal current through the inwardly rectifying potassium channel was readily detected. Activation of KOR by the κ-opioid-selective agonist U69593 caused an increase in the potassium current, as has been shown previously (14, 15). Brief (12–15 min) pretreatment of the oocytes with 8 μM insulin activated the endogenously expressed insulin receptor and potentiated the response produced by the κ-agonist. In contrast, insulin pretreatment had a small and variable inhibitory effect on the basal KIR3 current (Fig. 2).

**Insulin Does Not Significantly Affect KIR3**—The lack of a robust effect of insulin on the basal current carried by the KIR3.1/KIR3.2 heteromultimer was different from the strong inhibitory actions of other tyrosine kinase cascades on potassium channel conductance. Activation of the endogenously expressed insulin receptor had previously been shown to inhibit the basal activity of a different inwardly rectifying potassium channel (KIR2.1) expressed in oocytes (16). Furthermore, Rogalski et al. (12) showed that activation of the receptor tyrosine kinase TrkB by BDNF inhibited KIR3.1. To distinguish possible effects on the channel from those on the receptor, we tested the effects of insulin pretreatment on three different forms of the channel: KIR3.1 with KIR3.2, KIR3.1 with KIR3.4, and KIR3.2(S146T) alone. The latter is a mutant form of KIR3.2 that allows the 3.2 subunit to function as a homomultimer (17, 18), as has been shown previously (14, 15). Brief (12–15 min) pretreatment of the oocytes with 8 μM insulin activated the endogenously expressed insulin receptor and potentiated the response produced by the κ-agonist. In contrast, insulin pretreatment had a small and variable inhibitory effect on the basal KIR3 current (Fig. 2).

**Insulin Effects Are Blocked by Tyrosine Kinase Inhibitors**—The lack of a robust effect of insulin on the basal current carried by the KIR3.1/KIR3.2 heteromultimer was different from the strong inhibitory actions of other tyrosine kinase cascades on potassium channel conductance. Activation of the endogenously expressed insulin receptor had previously been shown to inhibit the basal activity of a different inwardly rectifying potassium channel (KIR2.1) expressed in oocytes (16). Furthermore, Rogalski et al. (12) showed that activation of the receptor tyrosine kinase TrkB by BDNF inhibited KIR3.1. To distinguish possible effects on the channel from those on the receptor, we tested the effects of insulin pretreatment on three different forms of the channel: KIR3.1 with KIR3.2, KIR3.1 with KIR3.4, and KIR3.2(S146T) alone. The latter is a mutant form of KIR3.2 that allows the 3.2 subunit to function as a homomultimer (17, 18), as has been shown previously (14, 15). Brief (12–15 min) pretreatment of the oocytes with 8 μM insulin activated the endogenously expressed insulin receptor and potentiated the response produced by the κ-agonist. In contrast, insulin pretreatment had a small and variable inhibitory effect on the basal KIR3 current (Fig. 2).

**First and Second Intracellular Loops**—To determine whether the effects of insulin were through a tyrosine kinase, we used the nonspecific tyrosine kinase inhibitor genistein (20). Although the mechanism by which genistein inhibits insulin receptor tyrosine kinase activity is unclear (21), this inhibitor is a commonly used to block insulin effects (22, 23). Insulin potentiated the U69593-activated potassium current to 146 ± 12% (n = 12) compared with controls not pretreated with insulin. In contrast, for oocytes pretreated for 25–30 min with 100 μM genistein, insulin had no significant effect on the KOR response (P > 0.05); the response to U69593 after genistein/insulin treatment was 105 ± 12% (n = 11 oocytes) compared with matched controls not pretreated with genistein/insulin. The results suggest that insulin potentiates the opioid-activated response through tyrosine kinase activation.

**Insulin Potentiation Requires Tyrosine Residues in Both the First and Second Intracellular Loops**—As the potentiating effects of insulin required activation of tyrosine kinases, we mutated specific tyrosine residues in KOR to determine whether

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**Table 1**

| Condition | Current (pA) |
|-----------|-------------|
| Basal | 500 nA |
| 1 μM Insulin | 8 μM Insulin |

**Fig. 1.** Insulin potentiates KOR activation of KIR3 channels. Oocytes were injected with 1 ng of rat KOR and 0.1 ng each of KIR3.1 and KIR3.2. Representative traces are shown from an untreated oocyte and an oocyte treated with 8 μM insulin for 12–15 min. This concentration of insulin was found to produce a maximal effect (data not shown). The oocyte membrane potential was clamped at −80 mV while bathed in normal saline buffer containing 2 mM KCl as described under “Experimental Procedures.” Oocytes were then superfused with a saline buffer in which the KCl concentration was increased to 96 mM. This does not activate the channel, but allows the basal inward current to flow through the inwardly rectifying potassium channels at the −80-mV holding potential (Ibasal). After equilibration with the high K⁺ buffer (hK), application of the κ-opioid receptor agonist U69593 (500 nM) increased the inward current (IhK). The increase in inward current through KIR3 channels caused by agonist activation of KOR is defined by the difference evident after addition of naloxone, an effective κ-receptor antagonist. Oocytes injected with KIR3.1 alone did not respond to U69593, and oocytes injected with KOR alone did not show an increase in potassium current (data not shown).
this would attenuate the insulin effect. We substituted phenylalanine for tyrosine at two sites within the KOR sequence, KOR(Y87F) and KOR(Y157F) (Fig. 3A), as a tyrosine residue in the second intracellular loop was previously shown to be responsible for the effects of insulin on the β2-adrenergic receptor (9, 10). U69593 dose-response analysis showed that the EC50 values for activation of wild-type and mutant KORs were not significantly different (Fig. 3B). The EC50 values (with 95% confidence intervals) were as follows: wild-type KOR, 160 (107–243) nM; KOR(Y87F), 138 (77–251) nM; and KOR(Y157F), 76 (48–119) nM. The lack of effect on agonist potency suggests that the tyrosines were not required for U69593 affinity. However, insulin did not potentiate the response to U69593 in oocytes expressing either KOR(Y87F) or KOR(Y157F) (Fig. 3B) compared with matched controls expressing wild-type KOR. The results support the hypothesis that insulin-induced tyrosine phosphorylation occurs at these two sites on KOR. The levels of receptor expression were too low to obtain direct evidence of phosphotyrosine production by Western analysis (data not shown).

Insulin Increases the Maximal Response to the κ-Agonist U69593 without Changing the Affinity for KOR—To examine the mechanism by which insulin caused a potentiation of the KOR response, we generated U69593 dose-response curves either before or after insulin treatment. There was no significant change in the EC50 value for U69593 in oocytes pretreated with insulin compared with untreated oocytes (p > 0.05). The control EC50 value (with 95% confidence intervals) was 160 (107–243) nM, and the EC50 value for U69593 was 192 (127–289) nM in oocytes pretreated with insulin. However, the maximal response to the κ-agonist was increased by insulin pretreatment at U69593 doses above 100 nM (Fig. 5). An increase in agonist efficacy could have been caused either by a functional increase in receptor number in the plasma membrane or by an increase in efficiency of G protein activation.
Tyrosine phosphorylation of KOR regulates efficacy

FIG. 4. Mutation of intracellular tyrosine residues prevents insulin potentiation of the KOR response. Oocytes were injected with the following cRNAs: 1 ng of wild-type KOR (KOR WT), KOR(Y87F), or KOR(Y157F) and 0.1 ng of both KIR3.1 and KIR3.2. The bar graph shows K-agonist-induced responses following insulin treatment normalized to responses of untreated oocytes. Each bar represents the mean ± S.E. calculated from 14 to 22 separate oocytes from at least two different donors (*, p < 0.01 compared with matching control oocytes).

DISCUSSION

The major finding of this study is that insulin treatment potentiates the activation of the inwardly rectifying potassium channel by the K-agonist receptor. This potentiation may require tyrosine kinase activation, as the insulin receptor is a tyrosine kinase, and the effects in this study were blocked by genistein. Furthermore, the regulation caused by insulin requires specific tyrosine residues in the first and second intracellular loops of the K-agonist receptor. In addition, analysis of the dose-response curves demonstrated that the potentiation results from an increase in K-agonist efficacy. These results indicate that the insulin potentiation of the K-agonist response is caused by a change in the phosphorylation state of the receptor.

Several possible cellular mechanisms might underlie the insulin potentiation of the K-agonist response. First, insulin receptor activation might cause a direct phosphorylation of specific tyrosine residues in KOR. Activated insulin receptors have been shown to phosphorylate other receptor proteins (24).

Second, the complex cascade activated by insulin receptors might be indirectly altering the K-opioid response by activation of a tyrosine phosphatase (25). Third, the effects of insulin on the opioid receptor could be mediated through an auxiliary protein that requires Tyr87 and Tyr157 for its association with the K-opioid receptor. The latter alternative is least likely, as the mutations did not cause a gross alteration in receptor conformation, evident by the lack of U69593 affinity change. Direct measures of phosphorylation of specific residues in KOR were not feasible; we obtained insufficient 32P incorporation into KOR expressed in oocytes to resolve the phosphopeptide fragments derived from the immunoprecipitated receptor. Thus, the results do not distinguish between potentiation through an increase in phosphotyrosine or a decrease in basal tyrosine phosphorylation. However, the data indicate that tyrosine phosphorylation regulates KOR signaling.

Serine/threonine phosphorylation of opioid receptors has been shown to inhibit the coupling of opioid receptors to their effectors (for review, see Ref. 2). This is the first demonstration that regulation of tyrosine phosphorylation of KOR modulates receptor function. A previous study by Pak et al. (4) showed that phosphorylation of tyrosine residues is required for a portion of agonist-induced internalization. Furthermore, tyrosine kinase inhibitors have also been shown to inhibit agonist-induced internalization (5, 6). The effects of insulin on the acute responses to K-receptor activation seen in this study suggest a different regulatory role for tyrosine phosphorylation than that documented in the desensitization studies. We are studying the effect of insulin on the initial coupling of KOR, not agonist-dependent desensitization and internalization. In this study, we have shown that other signaling pathways can affect opioid receptor coupling through modulation of tyrosine residues, illustrating a novel mechanism of cross-talk between opioid receptor signaling and tyrosine kinase cascades.

The modulation of KOR signaling reported here is through conserved tyrosines in the first and second intracellular loops. This supports the emerging theory that G protein-coupled receptors may generally be regulated by tyrosine phosphorylation. Previously, β2-adrenergic receptor coupling to adenyl cyclase was shown to be potentiated by insulin in HEK293 cells, and this potentiation of β2-adrenergic receptor coupling by insulin was also attenuated by mutation of a tyrosine in the second intracellular loop (10). However, Morris and Malbon (9) report that insulin activation of either the insulin receptor or the insulin-like growth factor-1 receptor inhibits β2-adrenergic receptor signaling through modulation of tyrosine residues in the cytoplasmic tail and second intracellular loop, respectively. Furthermore, they showed that phosphorylation leads to interactions with Grb2 and other proteins and to eventual internalization of the receptor (9). The basis for the discrepancy between these two reports is not clear, but presumably is due to the different conditions used. However, it appears that regulation of G protein-coupled receptors through modulation of specific tyrosine residues is likely to be found in more members of the G protein-coupled receptor superfamily.

The results of this study also show that insulin has no significant effect on the basal channel activity of K3. This finding is in contrast to the reported effects of insulin on another inwardly rectifying potassium channel (K3.2) (16). The lack of effect of insulin is also in contrast to the large inhibitory effect of BDNF on K3.3 through activation of TrKB receptors (12). These findings suggest that K3.3 may be differentially modulated by receptor tyrosine kinases. Another possible explanation is the level of activation of tyrosine kinases. In this study, insulin acted through an endogenously expressed recep-
tor, whereas BDNF acted through the exogenously expressed TrkB receptor. The higher levels of tyrosine kinase expression under the latter conditions could lead to a higher kinase activity, although this was not specifically examined.

The insulin receptor is known to activate a large number of signaling cascades (for review, see Ref. 19). Our results show that the potentiating effect of insulin requires activation of tyrosine kinases; however, the tyrosine kinase that directly phosphorylates KOR was not identified. The Xenopus oocyte expresses both the insulin receptor and the insulin-like growth factor-1 receptor, which bind and are activated by insulin; but in our study, we did not address which of these receptors mediates the effects of insulin (for review, see Ref. 11). However, the fact that a conservative mutation of specific tyrosine residues to phenylalanines attenuates the effects of insulin (for review, see Ref. 11) argues that the regulation is through direct regulation of tyrosine phosphorylation of KOR.

The increase in KOR coupling seen here appears to be due to an increase in efficacy and not a shift in the affinity of $K_\text{m}$3. This increase in efficacy could be due to an increase in the total number of receptors or to an increase in the ability of each receptor to activate its effector, in this case, $K_\text{m}$3. These studies were carried out under conditions in which there were no spare $\kappa$-receptors (15), and we did not distinguish between these two possible mechanisms for an increase in agonist efficacy in this study.

The critical tyrosine residues are located in the first and second intracellular loops of KOR. This region is thought to be involved with G protein binding along with the third intracellular loop and the C-terminal tail (26, 27). It is possible that regulation of the phosphorylation state of these residues alters the affinity of KOR for the GTP-bound state of the G protein, and so more receptors are associated with G proteins and available to activate the downstream effectors. Interestingly, both Tyr$^{87}$ and Tyr$^{157}$ were required for the effect of insulin, suggesting that regulation of both tyrosine residues is important for the changes in efficacy and that the contribution of both residues is not additive.

The physiological relevance of these findings remains to be determined. The insulin receptor kinase is a member of a family of receptor kinases that include growth factor, cytokine, and insulin-like growth factor-1 receptors. It is possible that other members of this family of receptor tyrosine kinases also could modulate opioid receptor function in the same way. Opioid receptors are expressed along with growth factors and cytokines in many different regions both centrally and peripherally. This mechanism could therefore have the potential for cross-talk with growth factors and cytokines in diverse opioid functions such as in the regulation of pain, epilepsy, and learning and memory.

In conclusion, this study shows that insulin potentiates the coupling of KOR to $K_\text{m}$3. This potentiation is through activation of a tyrosine kinase and requires specific tyrosine residues in the first and second intracellular loops of KOR. These results suggest the possibility of a novel mechanism of cross-talk between receptor tyrosine kinases and opioid receptors.

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