Pergamon

Biochemical Pharmacology, Vol. 49, No. 1, pp. 17–21, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00468-4

MODULATION OF MU OPIOID BINDING BY PROTEIN KINASE INHIBITORS

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(*Received* 31 *May* 1994; *accepted* 6 *September* 1994)

Abstract—Several isoquinolinesulfonamide protein kinase inhibitors, including 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7), inhibited [^{3}H]-[D-Ala^2,MePhe^4, Gly^5-ol]-enkephalin (DAMGO) binding to rabbit cerebellar mu opioid receptors with K_i values similar to those reported for kinase inhibition by these compounds, suggesting that their mechanism of action may involve inhibition of protein kinase activity. However, since the binding assays were performed in the absence of exogenous ATP, it is unlikely that protein phosphorylation is taking place during the binding assay, making it improbable that H7 and its congeners inhibit DAMGO binding by inhibition of protein kinase activity. In support of this hypothesis, K252a, a structurally unrelated, broad spectrum, protein kinase inhibitor, was inactive in modulating DAMGO binding, even at a concentration 5-fold greater than its K_i for inhibiting protein kinase activities. Inhibition of DAMGO binding through inhibition of kinase activity implies a noncompetitive or allosteric mechanism. Scatchard analysis of [^{3}H]DAMGO binding combined with Schild analysis demonstrated that the inhibition of DAMGO binding by the isoquinolinesulfonamides was competitive. These results show that the isoquinolinesulfonamide protein kinase inhibitors directly interact with the mu opioid receptor. Thus, these compounds are unsuitable for the investigation of the potential role of protein phosphorylation in the modulation of mu opioid receptor binding.

Key words: mu opioid receptor; DAMGO; protein kinase inhibitors; H7

Kinase-mediated protein phosphorylation is one mechanism whereby agonist occupied G-protein coupled receptors are desensitized. Several protein kinases, including cAMP-dependent protein kinase, mediate receptor phosphorylation (for a review see Ref. 1). Before initiating experiments designed to test the hypothesis that protein phosphorylation also modulates mu opioid receptors, preliminary experiments were performed to test for possible direct interaction of several protein kinase inhibitors with mu opioid receptors. Rabbit cerebellar membranes were employed as a model system since approximately 80% of the opioid binding in this tissue is comprised of mu opioid receptors [2, 3]. An additional advantage of this tissue is that it contains little or no delta opioid receptors ([2, 3]; Aloyo VJ and Pazdalski PS, unpublished observation), which allows the study of mu receptor binding without possible allosteric interactions with delta receptors [4, 5].

In the present experiments DAMGO†, a highly mu opioid receptor selective ligand [6], was employed to assess mu opioid receptor binding. Several protein kinase inhibitors, differing in their selectivity for cyclic nucleotide-dependent protein kinase and protein kinase C, were tested for their ability to interact with mu opioid receptors and, therefore, modulate DAMGO binding.

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† Abbreviations: DAMGO, [D-Ala^2, MePhe^4, Gly^5-ol]enkephalin; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; H8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride; and HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamidehydrochloride.

MATERIALS AND METHODS

Chemicals. [^{3}H]DAMGO (sp. act. 48.0 Ci/mmol) was obtained from New England Nuclear (Boston, MA), whereas unlabeled DAMGO was obtained from Peninsula Laboratories (Belmont, CA). Naloxone hydrochloride and polyethylenimine were obtained from the Sigma Chemical Co. (St. Louis, MO). H7, H8 and HA-1004 were purchased from Research Biochemicals International (Natick, MA), and K252a was obtained from Calbiochem-Novabiochem (San Diego, CA).

Tissue preparation. Adult female Dutch Belted rabbits were purchased from Myrtle's Rabbitry (Thompson, TN). These studies were conducted in accordance with the principles and procedures outlined by the NIH Guidelines for Care and Use of Experimental Animals and were approved by our Institutional Animal Care and Use Committee. Immediately following killing of the rabbit, the brain was removed rapidly and the cerebellum was dissected free. Only fresh tissue was used in these experiments. The cerebellum was placed immediately in ice-cold homogenization buffer (50 mM Tris–HCl, pH 7.5, at 0°), and all subsequent steps were performed at 0–4°. Tissue was homogenized in 10 vol. (by weight) of homogenization buffer using a Brinkmann Polytron (10 sec at half power). Homogenates were centrifuged at 40,000 *g* for

20 min. The resulting pellet was resuspended in 50 vol. of the homogenization buffer using a Brinkmann Polytron (10 sec at half power), followed by centrifugation as described above. The washed membrane fraction was dispersed in room temperature assay buffer (20 mM Tris–HCl, pH 7.5, at 20°) using a Polytron.

Binding assays. The washed membrane fraction (2–3 mg original tissue) was incubated at 25° for 2 hr with 0.2 pmol [^{3}H]DAMGO in 1 mL of assay buffer. Except where indicated, the assay mixture also contained 5 mM $MgCl_2$ (final concentration). Nonspecific binding was determined by the addition of 1 μM naloxone (final concentration). Where indicated, several concentrations of the protein kinase inhibitors H7, H8, HA-1004 or K252a were included in the incubation mixture. K252a was dissolved in DMSO and diluted with assay buffer such that the final concentration of DMSO in the reaction mixture was less than 0.1%, a concentration that did not inhibit DAMGO binding. All other drugs were dissolved directly in assay buffer. Three independent membrane preparations were used to characterize the inhibition of [^{3}H]DAMGO binding of each compound. To determine the K_d, the concentration of [^{3}H]DAMGO was varied between 0.05 and 3.0 nM. As described above, nonspecific binding was determined by the addition of 1 μM naloxone (final concentration). At the K_d concentration of ligand, specific binding accounted for more than 96% of the total [^{3}H]DAMGO bound. To prevent possible interaction of the kinase inhibitors with the membranes before binding occurred, the reaction was initiated by the addition of the membrane fraction. To terminate the reaction and to separate bound from free ligand, the mixture was filtered through Whatman GF/B glass fiber filters [presoaked in 0.15% (w/v) polyethylenimine] and washed with three 4-mL aliquots of ice-cold homogenization buffer. The amount of radioactivity retained on the filter was determined by liquid scintillation counting. Data were analyzed using the nonlinear curve-fitting program EBDA/LIGAND [7] as modified for microcomputers ([8]; Elsevier/Biosoft, Milltown, NJ). One- and two-site binding models were calculated and compared using the F-test. Experiments designed to test the effects of protein kinase inhibitors on the K_d or B_{max} of DAMGO binding were performed such that all tubes within an experiment contained aliquots of a single membrane preparation. Experimental tubes contained a fixed concentration of each test drug, while the control set of tubes contained an equal volume of buffer. For each experiment, the K_d and B_{max} for the control and drug-treated membranes were first analyzed separately. To determine if the presence of the drug resulted in a significant change in either the K_d or B_{max}, the corresponding value derived from the control experiment was substituted as a fixed value in the analysis of the drug experiment. The results were compared using the F-test [7]. The treatment was considered to have a significant effect at $P < 0.05$. K_i values were calculated from the corresponding IC_{50} values assuming that the inhibition was competitive [9].

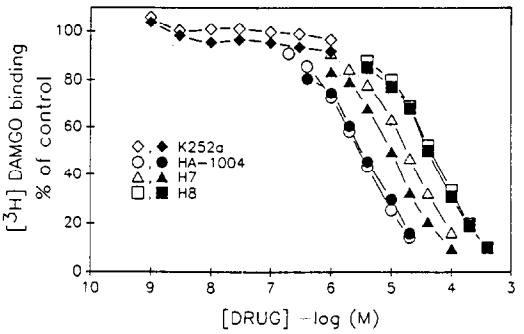

Fig. 1. Effects of protein kinase inhibitors on [^{3}H]DAMGO binding. Cerebellar membranes were incubated with [^{3}H]-DAMGO with (closed symbols) or without (open symbols) the addition of 5 mM $MgCl_2$ plus the indicated concentrations of unlabeled K252a, H7, H8 or HA-1004. The percent of control specific binding (defined as the difference in binding observed with and without 1 μM naloxone) is plotted for triplicate determinations at each drug concentration. Control specific binding in the presence and absence of $MgCl_2$ was 7.2 and 4.4 fmol/mg tissue, respectively. The data presented are from one representative experiment, which was performed a total of three times.

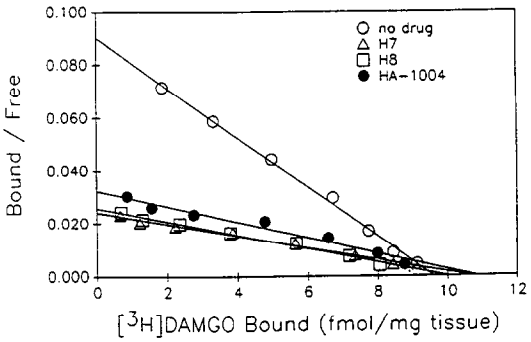

Fig. 2. Scatchard plot of [^{3}H]DAMGO binding in the presence or absence of isoquinolinesulfonamide protein kinase inhibitors. Cerebellar membrane preparation was incubated with various concentrations of [^{3}H]DAMGO (0.05 to 3.0 nM) with 1 μM HA-1004, 10 μM H7 or 40 μM H8. Specific binding was defined as in Fig. 1. Each point is the mean of triplicate determinations. The data presented are from one representative experiment, which was repeated with similar results.

RESULTS

All three isoquinolinesulfonamide protein kinase inhibitors (H7, H8 and HA-1004) inhibited [^{3}H]-DAMGO binding to cerebellar membranes in a concentration-dependent manner (Fig. 1) with K_i values of 4.0 ± 0.3, 15.9 ± 1.4 and 0.6 ± 0.03 μM (mean ± SEM, N = 3), respectively. In contrast, a structurally unrelated protein kinase inhibitor, K252a (up to 1 μM), did not inhibit [^{3}H]DAMGO binding. Omitting $MgCl_2$ from the incubation did not alter the ability of these compounds to inhibit [^{3}H]-DAMGO binding (Fig. 1).

Nonlinear curve-fitting analysis demonstrated that

specific DAMGO binding was consistent with a single-site model with a K_d of 0.14 ± 0.03 nM (mean ± SEM, N = 3) and a B_{max} of 9.1 ± 0.3 fmol/mg tissue (mean ± SEM, N = 3) (Fig. 2). Likewise, [^{3}H]DAMGO binding in the presence of a fixed concentration of each of the three isoquinolinesulfonamide protein kinase inhibitors was also consistent with a single-site model. All three compounds significantly reduced the apparent affinity of DAMGO binding without significantly altering the B_{max} (Fig. 2).

Homologous displacement experiments were performed in which the binding of a fixed concentration of [^{3}H]DAMGO was inhibited by unlabeled DAMGO (0.03 to 100 nM; Fig. 3A). This experiment was performed in the presence of 0, 1, 2, 4 and 10 μM HA-1004. HA-1004 did not alter nonspecific DAMGO binding as defined by the addition of 1 μM naloxone (data not shown). The concentration of unlabeled DAMGO required to achieve 50% inhibition of [^{3}H]DAMGO binding was dose dependently increased by HA-1004 from 0.57 nM in the absence of drug to 0.84 nM at 1 μM HA-1004, 1.04 nM at 2 μM, 1.51 nM at 4 μM, and 3.53 at 10 μM (Fig. 3A). At each HA-1004 concentration, the Hill slope was approximately one (range 0.99 to 1.09; Fig. 3B), indicating that HA-1004 resulted in a parallel shift in the concentration–response curve. The IC_{50} values were plotted as a Schild plot (Fig. 3C), yielding a straight line with a slope of 1.05, demonstrating that HA-1004 is a competitive inhibitor of [^{3}H]DAMGO binding.

DISCUSSION

All three isoquinolinesulfonamide protein kinase inhibitors (H7, H8 and HA-1004) inhibited [^{3}H]-DAMGO binding to cerebellar membranes in a concentration-dependent manner. The calculated K_i values for these compounds to inhibit mu opioid receptor binding were approximately equal to their respective K_i values for the inhibition of at least one of the protein kinases listed in Table 1. For example, H7 which inhibited DAMGO binding with a K_i of 4 μM (Table 1), inhibits cAMP- and cGMP-dependent protein kinases and protein kinase C with K_i values ranging from 3 to 6 μM [10]. Likewise, H8, which inhibits protein kinase C activity with a K_i of 15 μM [10], inhibited DAMGO binding with a K_i of 16 μM. HA-1004 was the most potent inhibitor of DAMGO binding with a K_i of 0.6 μM, similar to its K_i for the inhibition of cAMP- and cGMP-dependent protein kinases (2.3 and 1.3 μM, respectively [10]). The similarities between the potencies for inhibition of DAMGO binding and protein kinase inhibition suggest that these protein kinase inhibitors may modulate DAMGO binding by inhibiting protein phosphorylation.

To test the hypothesis that DAMGO binding is modulated by inhibition of protein phosphorylation, a structurally unrelated protein kinase inhibitor, K252a [11], was tested in the mu opioid receptor binding assay. This broad spectrum, potent kinase inhibitor was inactive in modulating DAMGO binding even at a concentration 5-fold greater than its K_i for inhibition of the cAMP- and cGMP-

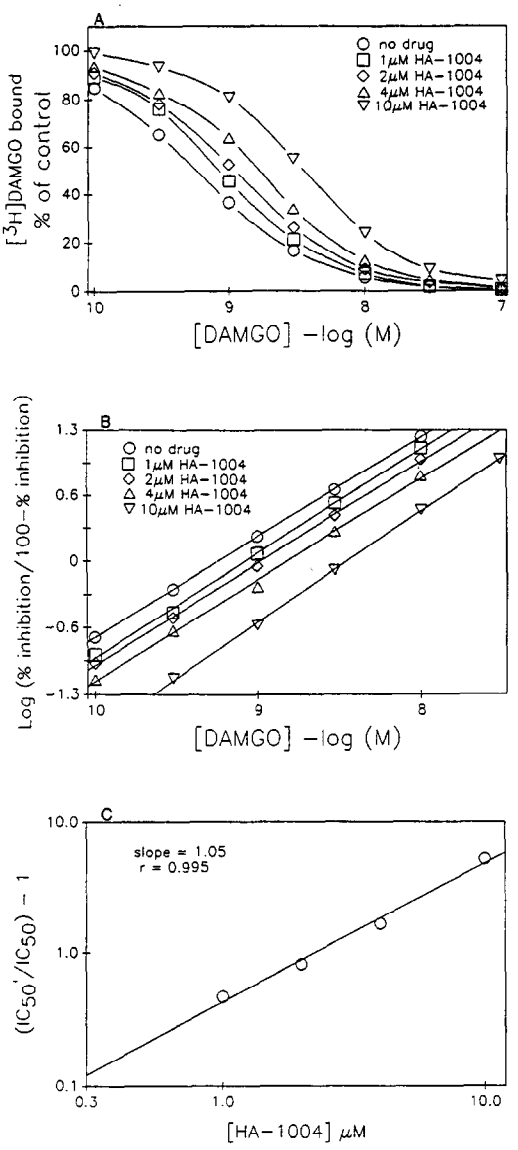

Fig. 3. Homologous displacement of [^{3}H]DAMGO binding: effects of HA-1004. (A) Concentration–response curves. Membranes were incubated with 0.4 nM [^{3}H]DAMGO plus the indicated concentration of unlabeled DAMGO, at each concentration of HA-1004. In the absence of unlabeled DAMGO, the amount of specifically bound [^{3}H]DAMGO was 4790 dpm in the absence of HA-1004, 3480 dpm at 1 μM HA-1004, 2780 dpm at 2 μM HA-1004, 1880 dpm at 4 μM HA-1004 and 1020 dpm at 10 μM HA-1004. The specifically bound [^{3}H]DAMGO in the absence of unlabeled DAMGO at each HA-1004 concentration was considered the control (100%). The percent of control binding in the presence of each HA-1004 concentration for triplicate determinations is plotted at each DAMGO concentration. (B) Hill plot analysis. The data shown above are replotted as a Hill plot. The lines are drawn by linear regression (r values > 0.998) with slopes approximately equal to 1 (range 0.99 to 1.09). (C) Schild plot analysis. The IC_{50} values at each HA-1004 concentration were calculated from the data shown in panels A and B. The log of the ratio of the IC_{50} in the presence of HA-1004 (IC_{50}') divided by the IC_{50} in the absence of HA-1004 is plotted as a function of the log of the HA-1004 concentration. The line is drawn by linear regression.

Table 1. Potencies of several compounds for inhibiting protein kinase activity and [^{3}H]DAMGO binding

Drug	K_i (μM)				
	DAMGO binding	cGMP-dependent kinase*	cAMP-dependent kinase*	Protein kinase C*	Myosin light chain kinase*
H7	4	5.8	3.0	6.0	97
H8	16	0.5	1.2	15	68
HA-1004	0.6	1.3	2.3	40	150
K252a	$\gg$1	0.020	0.018	0.025	ND

* Values for the potencies for protein kinase activity were obtained from Refs. 10 and 11. ND = not determined.

dependent protein kinases and protein kinase C. Since K252a and the isoquinolinesulfonamide-type protein kinase inhibitors (H7, H8, HA-1004) are all competitive with respect to ATP [10, 11], the observed lack of inhibition by K252a strongly suggests that inhibition of protein kinase activity is not the mechanism by which H7 and its congeners inhibit DAMGO binding.

The hypothesis that H7 and its congeners attenuate DAMGO binding via inhibition of protein kinase activity would require ongoing protein phosphorylation during the receptor binding assay. Protein phosphorylation requires not only an appropriate phosphate acceptor protein (in this case presumably the mu opioid receptor), but also ATP–Mg^{2+} complex as the phosphate donor. However, Wiegant and co-workers [12] have demonstrated that incubation of ATP with neuronal membranes under conditions similar to those employed in these mu opioid receptor binding assays results in the very rapid degradation of ATP. Therefore, in the present experiments it is highly unlikely that protein kinase activity continues for more than a few seconds before all endogenous ATP is depleted, whereas the binding assay continues for 2 hr.

Additional evidence supporting the lack of continuous protein phosphorylating activity during the binding assay is derived from experiments where the $MgCl_2$ was omitted from the binding assay. Since this divalent cation is required to complex with ATP to form the kinase substrate (ATP–Mg^{2+} complex), a decreased concentration of $MgCl_2$ would result in a decreased rate of phosphorylation and, consequently, an attenuation of the effects of H7. However, omitting $MgCl_2$ from the assay did not alter the inhibition of DAMGO binding by H7 and its congeners. Therefore, it is improbable that H7 and its congeners inhibit DAMGO binding by inhibition of protein kinase activity.

Scatchard analysis of [^{3}H]DAMGO binding combined with Schild analysis was used to investigate the mechanisms of action of the isoquinolinesulfonamide protein kinase inhibitors [13]. Saturation experiments performed in the presence of a fixed concentration of each of the three isoquinolinesulfonamide protein kinase inhibitors demonstrated that all three compounds significantly reduced the apparent affinity of DAMGO binding without significantly altering the B_{max}, thus ruling out a noncompetitive mechanism and suggesting competitive inhibition. However, since Scatchard analysis performed at a single inhibitor concentration cannot rule out possible allosteric interactions [13], HA-1004 was chosen for further study. HA-1004 concentration dependently increased the IC_{50} value for homologous DAMGO inhibition of [^{3}H]-DAMGO binding. At each concentration of HA-1004, the Hill slope was approximately equal to 1, indicating that [^{3}H]DAMGO bound to a single class of receptors even in the presence of this kinase inhibitor. A plot of the log of the dose ratio (the IC_{50} in the presence of HA-1004 divided by the IC_{50} in the absence of drug) versus the log of the HA-1004 concentration in a manner analogous to a Schild plot [13] had a slope of 1, indicating that HA-1004 is a competitive inhibitor of DAMGO binding and thus ruling out allosteric interactions [13].

In conclusion, the commonly employed protein kinase inhibitors (H7, H8, and HA-1004) appear to be competitive inhibitors of DAMGO binding to mu opioid receptors. Since their ability to inhibit DAMGO binding is independent of their protein kinase inhibiting activity, these compounds are not suitable for the investigation of the potential role of protein phosphorylation in the regulation of mu opioid receptors.

Acknowledgements—This work was supported, in part, by USPHS Grant MH16841 to Dr. John A. Harvey, who is thanked for his continuing support.

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