BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 78–82 (1998)
ARTICLE NO. RC988883

Characterization of [^{3}H]MEN 11420, a Novel Glycosylated Peptide Antagonist Radioligand of the Tachykinin NK_2 Receptor

A. R. Renzetti,[1] R-M. Catalioto, M. Criscuoli, P. Cucchi, A. Lippi, M. Guelfi, L. Quartara,* and C. A. Maggi

*Department of Pharmacology and *Department of Chemistry, Menarini Ricerche S.p.A., Via F. Rismondo 12A, I-50131 Florence, Italy*

Received April 14, 1998

[^{3}H]MEN 11420, a radiolabeled glycosylated peptide antagonist of the tachykinin NK_2 receptor, has been investigated in ligand-receptor binding assays using membranes of CHO cells transfected with the human tachykinin NK_2 receptor. [^{3}H]MEN 11420 bound to a single class of high affinity binding sites: its binding was inhibited by natural tachykinins (potency ranking: NKA ⩾ SP ⩾ NKB), as well as by peptide (MEN 11420 > MEN 10376 ⩾ R 396) and nonpeptide (SR 48968 > GR 159897) selective NK_2 receptor antagonists. These data indicate that [^{3}H]MEN 11420 is a potent radioligand for the human tachykinin NK_2 receptor that may represent a useful tool for studying ligand-receptor interactions at the molecular level. © 1998 Academic Press

Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are peptides of the tachykinin family which share the C-terminal sequence Phe-X-Gly-Leu-Met-NH_2. The mammalian tachykinins elicit their effects through three different receptors termed NK_1, NK_2 and NK_3. NKA is the most potent naturally occurring agonist at the tachykinin NK_2 receptor, while SP and NKB are the preferred agonists for the NK_1 and NK_3 receptors, respectively. Studies performed by the use of peptide antagonists, such as the linear peptides MEN 10376 and R 396, in different preparations have led to the suggestion that the NK_2 receptor may be heterogeneous: the existence of two receptor subtypes, NK_{2A} and NK_{2B}, was proposed (1). Following the isolation and pharmacological characterization of the hamster NK_2 receptor protein (2) it was clear that the pharmacological criteria used to define the putative NK_{2A} and NK_{2B} receptor subtypes apply in the pharmacology of the NK_2 receptor to species-related differences. Although many radioligands have been used for the characterization of the tachykinin NK_2 receptor (3), the only radioligands commercially available so far are [^{125}I]-NKA and the non peptide receptor antagonist [^{3}H]SR 48968 (4). By the use of these radioligands it was observed that amino acid substitutions in the first and second extracellular segments and the second transmembrane segment of the human NK_2 receptor led to substantial reduction in NKA affinity without affecting the affinity of the antagonist SR 48968 (5-6). On the other hand, certain mutations in the sixth and seventh transmembrane segments were found to reduce the antagonist affinity substantially (5-6). Therefore the use of different radioligands has proven useful for the study of the residues involved in ligand binding to the tachykinin NK_2 receptor. However the behaviour of peptide-based tachykinin NK_2 receptor antagonists as compared to that of nonpeptide antagonists has been little investigated: it is expected that substantial differences may exist in the mode of binding of peptides vs nonpeptide antagonists to the tachykinin NK_2 receptor (7).

We have recently developed MEN 11420, or cyclo-{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dpr-Leu]cyclo(2β-5β)}, a bicyclic peptide which is a potent and selective tachykinin NK_2 receptor antagonist endowed with interesting *in vitro* and *in vivo* pharmacological properties (8). The aim of the present study was to characterize the tritiated derivative MEN 11420 as a radioligand for the human NK_2 receptor transfected in CHO cells (hNK_2R/CHO).

MATERIALS AND METHODS

Materials

MEN 11420 (cyclo{[Asn(β-D-GlcNAc)-Asp-Trp-Phe-Dpr-Leu]cyclo-(2β-5β)}), [ßAla^8]-NKA(4-10) and MEN 10376 ([Tyr^5, D-Trp^{6,8,9},

[1] Corresponding author. Pharmacology Department, Menarini Ricerche S.p.A., Via Rismondo 12/A, 50131 Florence, Italy. Fax: +39-55-5680419.

0006-291X/98 $25.00

Arg[10]]NKA (4-10) were synthesized at Menarini laboratories, Florence, Italy, by conventional solid-phase methods. [^{3}H]MEN 11420 (62 Ci/mmol) was synthetized by SibTech, Inc, Elmsford NY, USA; R 396 (Ac-Leu-Asp-Gln-Trp-Phe-Gly-NH_2) was a gift from Prof. D. Regoli, GR 159897, ((R)-1-[2-(5-fluoro-1 *H*-indol -3-yl)ethyl]-4-methoxy-4-[(phenylsulfinyl)methyl]piperidine) was kindly provided by Dr. B.M. Bain (Glaxo Research and Development Ltd., U.K.), SR 48968, ((S) - N - methyl - N[4 - (4 - acetylamino - 4 - phenylpiperidino) - 2 - (3, 4 - dichlorophenyl)butyl]benzamide) was kindly provided by Drs. X. Emonds-Alt and G. Le Fur (Sanofi Recherche, Montpellier, France). NKA, NKB and SP, were from Sigma, St. Louis, MO (USA); senktide and [Sar[9]]SP sulfone were from Peninsula, St. Helens, (England). Other reagents were of the highest purity available from commercial sources.

Methods

Membrane preparation. CHO cells transfected with the human NK_2 receptor were provided by Dr. J.E. Krause (Washington University, School of Medicine, St. Louis, MO). Confluent cells from 4 Petri dishes were harvested in phosphate buffered saline, pelleted by centrifugation at 200 ×g (4°C) and homogenized using a Polytron PT 3000 (Kinematica, Lucerne, Switzerland) at 13,000 rpm for 15 s, in 20 ml of 50 mM Tris-HCl buffer, pH 7.4, containing bacitracin (0.1 mg ml^{-1}), chymostatin (0.01 mg ml^{-1}), leupeptin (5 μg ml^{-1}) and 10 μM thiorphan (buffer A). The homogenate was centrifuged for 1 h at 25,000 ×g (4°C) and the pellet resuspended in the binding buffer, composed of buffer A supplemented with 150 mM NaCl, 5 mM $MnCl_2$ and 0.1% bovine serum albumin, (buffer B) at a protein concentration of circa 0.35 mg ml^{-1} (9) and stored at −70°C until use. Immediately prior to use, frozen membrane aliquots were thawed in fresh binding buffer (buffer B) and gently homogenized to give a homogenous membrane suspension.

Binding assays. Binding assays were performed by incubating membranes (50-90 μg protein/assay) with [^{3}H]MEN 11420 (0.09-15.0 nM in saturation experiments; 0.4-1.0 nM in kinetic and competition experiments) with or without varying concentrations (0.01 nM - 10 μM) of the competing compounds in a final volume of 0.5 ml of buffer B. All the experiments were performed in duplicate or triplicate. 1 μM unlabelled MEN 11420 was used for defining nonspecific binding. After 60 min (or 45 min in the competition studies with SR 48968) of incubation (or variable time in kinetic experiments) at 20°C, bound and free radioactivity were separated through glass-fiber filter sheets (Whatman GF/B) presoaked in 0.3 % polyethylenimine for at least 3 h using a Brandel cell harvester. Filters were washed 3 times with 4 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. The trapped radioactivity was determined by liquid scintillation using a β-scintillation counter (2200 CA, Canberra Packard).

Chemical synthesis of [^{3}H]MEN 11420. The tritiation procedure involves the iodination at the Trp and Phe sites and the exchange for tritium at low temperature (10). No identification of tritiation sites was performed.

[^{3}H]MEN 11420 purity analysis. Radiochemical purity of [^{3}H]-MEN 11420 was checked by HPLC analysis accomplished on a Nucleosil 5 C18 reversed-phase column (25 cm × 4.6 mm I.D.). Mobile phase was composed of aqueous 0.1% TFA, acetonitrile and methanol in the ratio 60/30/10 v/v. Flow rate was set at 1 ml/min. Eluted radioactivity was measured by a Flo-one/Beta Series A-200 radioactivity detector (Radiomatic Instruments, USA), after mixing Flo-Scint A scintillation cocktail (Packard Instruments, USA) with mobile phase in a 3 : 1 ratio. Retention time of MEN 11420 was 11.8± 0.4 min. Radiochemical purity was 95%.

Analysis of binding data. Kinetic experiments were analysed using the kinetic programme of KELL software for Macintosh (Elsevier Biosoft, Cambridge, UK). Saturation and competition data were processed by the method of Munson and Rodbard (11), by using EBDA and LIGAND programs in sequence. The density of binding sites (Bmax), equilibrium dissociation constant (K_d) and equilibrium inhibition constants (K_i) were obtained. The values are given as means with approximate standard errors, as they were calculated by LIGAND. The goodness of fits was evaluated by the F-ratio.

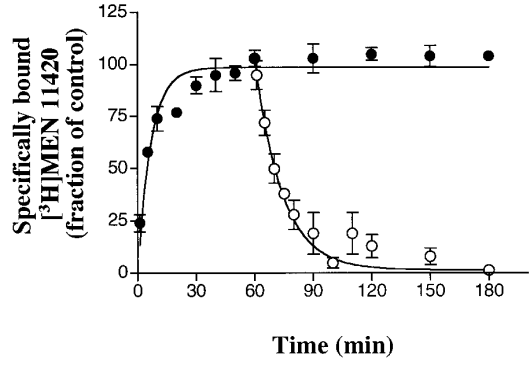

FIG. 1. Association (●) and dissociation (○) time-courses for [^{3}H]-MEN 11420 in hNK_2R/CHO membranes. Association (filled circle) and dissociation (open circle) time-courses in hNK_2R/CHO membranes were plotted against incubation time at 20°C For dissociation studies 1 μM unlabelled MEN 11420 was added to samples after equilibrium was reached (60 min). The data points represent the mean ± S.E.M. of 3 experiments performed in triplicate. Monoexponential curve fiiting was performed by the kinetic program of KELL software.

RESULTS

Binding Characteristics of [^{3}H]MEN 11420 in CHO Cells Transfected with the Human NK_2 Receptor

The specific binding of [^{3}H]MEN 11420 was directly proportional to the membrane concentration (data not shown). At concentrations between 100 and 180 μg ml^{-1}, as used in competition experiments, the specific binding represented approximately 80-90% of the total binding; approximately 3% of the added radioactivity was bound to the membranes.

Kinetic Experiments

The association time course for [^{3}H]MEN 11420 binding to hNK_2R/CHO at 20° C was rapid: the equilibrium was reached within 45 min and remained stable for at least 135 min thereafter (Figure 1). After reaching the equilibrium the radioligand dissociated within 60 min from the addition of unlabelled MEN 11420 (Figure 1). The [^{3}H]MEN 11420 dissociation data could be fitted to a monophasic curve with dissociation rate constants (K_{off}), of 0.13 ± 0.02 min-^{1}. The [^{3}H]MEN 11420 association curve could be fitted to a pseudo first order rate equation. The observed association rate constant (K_{obs} = 0.256±0.044 min^{-1}) was used to determine the actual association rate constant (K_{on}) using the equation K_{on} = (K_{obs}-K_{off})/[L], where [L] is the radioligand concentration. The calculated K_{on} was 1.12 10^8 M^{-1} min^{-1}. The kinetically determined Kd, derived from the ratio K_{off}/

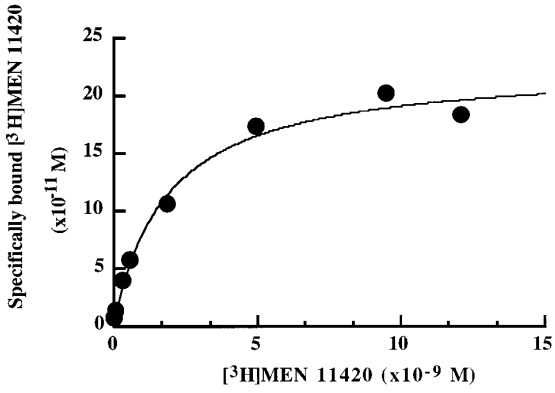

FIG. 2. Saturation isotherm for [^{3}H]MEN 11420 in hNK$_2$R/CHO membranes. The data points are the mean of triplicate determinations and are representative of 8 separate experiments which gave similar results. In the present experiment the calculated K_d, B_{max} and Hill slope were 2.0 nM, 1916 fmol mg^{-1} protein and 0.99, respectively.

K_{on} was 1.0 ± 0.05 nM. Association kinetics suggested to adopt an incubation time of 60 min for all the experiments at the equilibrium (competitions, saturations). For studying SR 48968 competition we used an incubation time of 45 min, since this compound slowly dissociates from the receptor.

Saturation Experiments

[^{3}H]MEN 11420 showed saturable binding to hNK$_2$R/CHO membranes (Figure 2). The K_d and B_{max} values, calculated from a one-site model for the saturation isotherms were 2.1± 0.4 nM and 1728 ± 450 fmol mg^{-1} protein (n=8), respectively, with Hill slope of 1.01±0.01.

Competition Experiments

The binding inhibition data for all competitors were suitable fitted by the one-site model except for NKA, whose competition curve showed a Hill slope coefficient significantly lower than unity. In fact, NKA competition for the binding of [^{3}H]MEN 11420 was best described according to a two-site model. The K_i values for the high affinity site (41± 10% of the total receptor density) and the low affinity site differed of about 100 fold (10±9.7 vs 1186±872 nM) (Table 1). On the other hand, the selective tachykinin NK$_2$ receptor agonist, [β-Ala^8]NKA(4-10), appeared to compete only for the low affinity site.

SP, NKB and the selective NK$_1$ and NK$_3$ receptor agonists, [Sar^9]SP sulfone and senktide, respectively, were weak displacers of the radioligand from the human NK$_2$ receptor (table 1, figure 3). As shown in table 1 and figure 4 nonpeptide selective tachykinin NK$_2$ receptor antagonists (SR 48968>GR 159897) were more potent displacers than the peptide antagonists (MEN 11420>MEN 10376>R 396).

DISCUSSION

The present data show that [^{3}H]MEN 11420 binds in a reversible and concentration-dependent manner to a single class of high affinity binding sites. The K_d value obtained from saturation studies agreed fairly well with that calculated from association and dissociation kinetics of the radioligand. The observation that the binding of [3H]MEN 11420 and its displacement by tachykinin NK$_2$ receptor antagonists is monophasic is in keeping with the hypothesis that antagonists bind with similar affinities to all receptor conformers/states, regardless to their degree of precoupling with G proteins. On the other hand, NKA, an agonist, was able to discriminate between the low-affinity (not G protein-coupled) and the high-affinity (G protein-coupled) states, in dependence of availability of suitable G proteins. In our system the high affinity NK$_2$ receptors appeared to account for 41 ± 10 % of all receptor population, suggesting a limited degree of precoupling in this cell line. In agreement with the expression of the tachykinin NK$_2$ receptor by CHO cells, SP, NKB and NK$_1$ and NK$_3$ selective agonists showed weak, if any, binding affinity. The finding that the selective NK$_2$ receptor agonist [β-Ala^8]NKA(4-10) is only a weak competitor for the binding of [^{3}H]MEN 11420 to the human NK$_2$ receptor is in agreement with previous studies with the rat receptor (12). Indeed, [β-Ala^8]NKA(4-10), while competing with high nanomolar affinity with [^{125}I]NKA for the binding to rat small intestine mem-

TABLE 1

Inhibition of [^{3}H]MEN 11420 Binding by Tachykinins, NK Selective Agonists, and NK$_2$ Selective Antagonists

	NK selectivity	K_i (nM)
Agonists		
SP	NK$_1$	>1,000
NKA	NK$_2$	10 ± 9.7/1186 ± 872 (3)[a]
NKB	NK$_3$	>10,000
[Sar^9]SP sulfone	NK$_1$	>10,000
[β-Ala^8]NKA(4-10)	NK$_2$	859 ± 272
Senktide	NK$_3$	>10,000
Antagonists		
Peptides		
MEN 10376	NK$_2$	9.0 ± 1.3 (3)
R 396	NK$_2$	292 ± 113 (3)
MEN 11420	NK$_2$	2.1 ± 0.17 (3)
Nonpeptides		
SR 48968	NK$_2$	0.13 ± 0.05 (3)
GR 159897	NK$_2$	1.6 ± 0.2 (3)

Note. The K_i values were calculated from a one-site (slope very close to the unity) binding model applied to inhibition data using LIGAND and are the means ± S.E.M. of 3 experiments performed in duplicate.

[a] in this case the K_is values were calculated from a two-sites (slope = 0.53 ± 0.04) binding model.

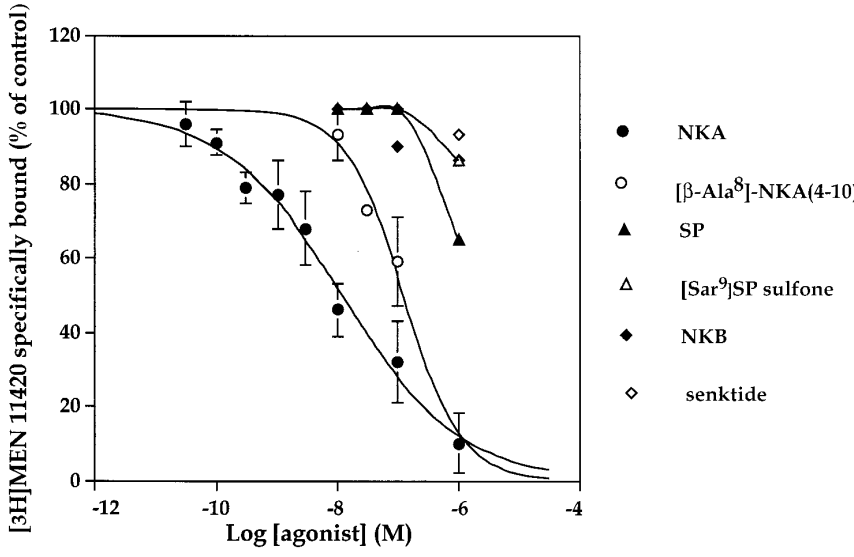

FIG. 3. Inhibition of [^{3}H]MEN 11420 binding to hNK$_2$R/CHO membranes by natural tachykinins, SP (▲), NKA (●), NKB (◆), and selective NK$_1$ (△), NK$_2$ (○), and NK$_3$ (◇) receptor agonists. [^{3}H]MEN 11420 (1nM) and hNK$_2$R/CHO membranes (approximately 120-140 μg ml^{-1} of protein) were incubated as described in the Methods, in the presence of increasing concentrations of displacer. Data represent the mean ± S.E.M of three separate experiments, performed in duplicate.

branes, was able to displace the labelled antagonist [^{3}H]SR 48968 only at micromolar concentrations. The present data suggest that the behaviour of this ligand is independent from the antagonist type and the receptor source. It is worth noting that the affinity of [β-Ala^8]NKA(4-10) for the transfected human NK$_2$ receptor labelled with [^{125}I]NKA is about 40-fold higher (K$_i$ = 21± 8 nM, unpublished data) than that measured against [^{3}H]MEN 11420. Moreover, the agonist potency of [β-Ala^8]NKA(4-10) in producing PGE$_2$ release from CHO cells transfected with the human NK$_2$ receptor agrees (EC50 = 8 nM, unpublished data) with its K$_i$ value against [^{125}I]NKA. The pharmacological effects of [β-Ala^8]NKA(4-10) are potently antagonized by SR 48968 and MEN 11420 (7, 8) and also the PGE$_2$ release induced by [β-Ala^8]NKA(4-10) from the transfected cells is effectively inhibited by both antagonists at nanomolar concentrations (data not shown). These findings are in apparent contrast with the weakness of the agonist in displacing the binding of labelled antagonists from the human NK$_2$ receptor and can be explained by assuming that receptor occupancy and coupling to effector mechanisms by certain synthetic agonists may be differently affected by antagonists. The molecular determinants of the peculiar behaviour of [β-Ala^8]NKA(4-10) are currently under investigation by site-directed mutagenesis of the human NK$_2$ receptor. In agreement with the affinity ratio of MEN 10376 and R 396 as found in functional studies in the human colon (13), MEN 10376 showed a 30 fold higher affinity for the human NK$_2$ receptor than R 396. The nonpeptide NK$_2$ receptor antagonists, SR 48968 and GR 159897, competed for the binding of [^{3}H]MEN 11420 with affinities very similar to those already reported for the human NK$_2$ receptor transfected in CHO cells (3).

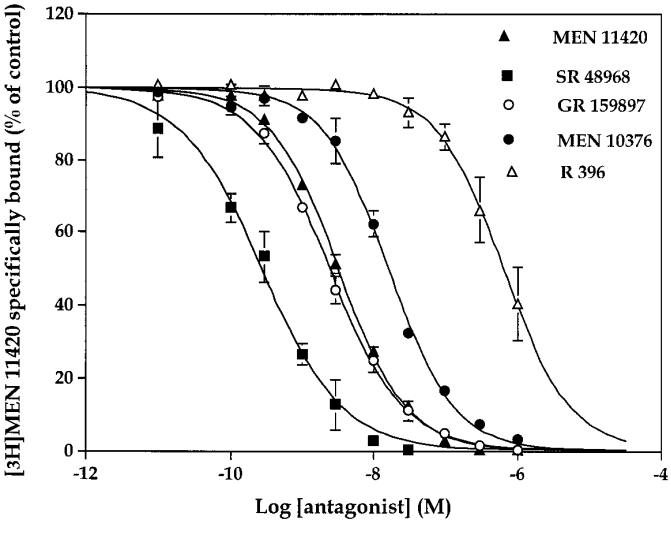

FIG. 4. Inhibition of [^{3}H]MEN 11420 binding to hNK$_2$R/CHO membranes by selective peptide, MEN 11420 (▲), MEN 10376 (●), R 396 (△) and nonpeptide, SR 48968 (■), GR 159897 (○) NK$_2$ receptor antagonists. [^{3}H]MEN 11420 (1nM) and hNK$_2$R/CHO membranes (approximately 120-140 μg ml^{-1} of protein) were incubated as described in the Methods, in the presence of increasing concentrations of displacer. Data represent the mean ± S.E.M. of three separate experiments, performed in duplicate.

Taken together, these results dimonstrate that [^{3}H]-MEN 11420 is a new radioligand endowed with high affinity for the human NK$_2$ receptor which might be

helpful, in combination with the other available radioligands, for the identification of the residues involved in ligand binding to the human tachykinin NK_2 receptor.

REFERENCES

1. Maggi, C. A., Patacchini, R., Rovero, P., and Giachetti, A. (1993) *J. Auton. Pharmacol.* **13,** 23–93.
2. Aharony, D., Little, J., Thomas, C., Powell, S., Berry, D., and Graham, A. (1994) *Mol. Pharmacol.* **45,** 9–19.
3. Beresford, I. J. M., Sheldrick, R. L. G., Ball, D. I., Turpin, M. P., Walsh, D. M., Hawcock, A. B., Coleman, R. A., Hagan, R. M., and Tyers, M. B. (1995) *Eur. J. Pharmacol.* **272,** 241–248.
4. Emonds-Alt, X., Golliot, F., Pointeau, P., Le Fur, G., and Breliere, J. C. (1993) *Biochem. Biophys. Res. Commun.* **191,** 1172–1177.
5. Huang, R-R. C., Vicario, P. P., Strader, C. D., and Fong, T. M. (1995) *Biochemistry* **34,** 10048–10055.
6. Bhogal, N., Donnelly, D., and Findlay, J. B. C. (1994) *J. Biol. Chem.* **269,** 27269–27274.
7. Patacchini, R., De Giorgio, R., Giachetti, A., and Maggi, C. A. (1994) *Eur. J. Pharmacol.* **271,** 111–119.
8. Catalioto, R-M., Criscuoli, M., Cucchi, P., Giachetti, A., Giannotti, D., Giuliani, S., Lecci, A., Lippi, A., Patacchini, R., Quartara, L., Renzetti, A. R., Tramontana, M., Arcamone, F., and Maggi, C. A. (1998) *Br J. Pharmacol* **123,** 81–91.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
10. Merkushev, E. B. (1988) *Synthesis* 923–937.
11. Munson, P. J., and Rodbard, D. (1980) *Anal. Biochem.* **107,** 220–239.
12. Renzetti, A. R., Criscuoli, M., and Maggi, C. A. (1997) *Naunyn-Schm. Arch. Pharmacol* **356,** 139–144.
13. Giuliani, S., Barbanti, G., Turini, D., Quartara, L., Rovero, P., Giachetti, A., and Maggi, C. A. (1991) *Eur. J. Pharmacol.* **203,** 365–370.