0026-895X/91/040459-04$03.00/0

MOLECULAR PHARMACOLOGY, 40:459–462

ACCELERATED COMMUNICATION

Immunological Detection of Muscarinic Receptor Subtype Proteins (m1–m5) in Rabbit Peripheral Tissues

FRANK DÖRJE, ALLAN I. LEVEY, and MARK R. BRANN

Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, Bethesda, Maryland 20892 (F.D., M.R.B.), Departments of Neurology and Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (A.I.L.), Department of Pharmacology, University of Frankfurt, D-6000 Frankfurt/M., FRG (F.D.), and Neuroscience Research Unit, Department of Psychiatry, University of Vermont, Burlington, Vermont 05405 (M.R.B.)

Received May 29, 1991; Accepted July 9, 1991

SUMMARY

Employing subtype-specific antisera, we have measured the relative levels of five muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. Immunoprecipitation assays demonstrated the presence of four distinct receptor proteins (m1–m4), which showed notable differences in their tissue distribution. Significant amounts of m5 receptors were not detected in any of the tissues examined. High levels of m1 receptors were found in sympathetic ganglia and submaxillary gland. The m1 receptor was the predominant subtype in vas deferens. Muscarinic receptors of the m2 type were present in varying proportions in all tissues examined, being the major subtype in sympathetic ganglia, ileum, and uterus and the only subtype detected in rabbit atrium. The m3 receptor protein was abundant in submaxillary gland but was not detected at appreciable levels in other tissues studied. Receptors of the m4 type represented a major subtype in peripheral lung and were also present, although at lower levels, in uterine and ileal tissues.

Pharmacological studies have demonstrated the presence of at least three major muscarinic receptor subtypes, designated M_1, M_2, and M_3 (1–3). More recently, radioligand binding studies provided evidence for the presence of pharmacologically defined M_4 receptors in rat striatum and rabbit peripheral lung (4, 5). Molecular cloning studies have identified five distinct muscarinic receptor genes (m1–m5) (6–9) that have been shown to be widely expressed in the brain and in peripheral tissues (8, 10–12). Unfortunately, due to the limited subtype-selectivities of available muscarinic ligands (13–15), it has been difficult to unequivocally assign pharmacologically and genetically defined subtypes. For instance, the antagonist binding profiles of both the m1 and m4 receptors were similar to those of M_1 receptors, suggesting that the putative M_1 receptors may actually be composed of a mixture of these two receptor proteins (13, 14).

Because highly selective ligands for the individual subtypes are not available at present (13–15), the precise tissue distribution of the various receptor proteins has not yet been determined conclusively. Moreover, molecular genetic techniques to evaluate the distribution of muscarinic receptor subtypes are limited to the measurement of mRNAs. The mapping of mRNAs in a given tissue, however, may not necessarily reflect the abundance of the distinct receptor proteins, because individual mRNAs can differ in their translation efficiencies (10, 16). Furthermore, due to different subcellular distributions within neurons, it is important to recognize that receptor proteins but not mRNAs may be present in some tissues if the tissue innervation is extrinsic. Thus, employing fusion proteins of the nonconserved i3 loops as antigens, we have recently generated subtype-specific antisera to directly localize the different muscarinic receptor proteins (17, 18). In the present study, we have used these antisera in quantitative immunoprecipitation assays to evaluate the distribution of each of the five muscarinic receptor proteins among several rabbit peripheral tissues.

Experimental Procedures

Materials. The following compounds were purchased: [^{3}H]NMS (78.9 Ci/mmol), Du Pont-New England Nuclear (Boston, MA); digitonin for use in aqueous solution (lot 119 F 04761), Tris-HCl, cholic acid, and atropine sulfate, Sigma (St. Louis, MO); PMSF, Na_2EDTA, pepstatin, leupeptin, and soybean trypsin inhibitor, Boehringer Mannheim Biochemicals (Indianapolis, IN); and G-25 Sephadex, Pharmacia (Piscataway, NJ).

F.D. was supported by a Kekulé grant from the Stiftung Stipendien-Fonds der Chemischen Industrie. A.I.L. is a recipient of a Career Investigator Development Award NS01387-01 and a Faculty Scholar Award from the Alzheimer's Disease and Related Disorders Association.

ABBREVIATIONS: i3 loop, third cytoplasmic loop of the muscarinic receptors; [^{3}H]NMS, *N*-[^{3}H]methylscopolamine chloride; PMSF, phenylmethanesulfonyl fluoride.

Tissue and membrane preparation. All tissues were dissected from New Zealand White rabbits (2.5–3.0 kg) and obtained either from Pel-Freez (Rogers, AR) or the National Institutes of Health Small Animal Section (Bethesda, MD). The entire submaxillary gland, vas deferens, atrium, ileum, uterus, brain, and sympathetic ganglia were removed. In the case of lung, only peripheral strips (1–2 cm wide) were dissected. Immediately after collection, tissues were frozen in liquid nitrogen and stored at −70° until use.

At the time of assay, tissues were homogenized for 60 sec using a Polytron (setting 6; Brinkmann Instruments, Westbury, NY), in ice-cold 10 mM Tris·HCl, 1.0 mM EDTA buffer (TE, pH 7.4) supplemented with the following protease inhibitors: 0.2 mM PMSF, 1 μM pepstatin, 1 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor. Homogenates were centrifuged at 300 × *g* for 5 min at 4°, and the pellet was discarded. The supernatant was centrifuged at 20,000 × *g* for 20 min at 4°. The resulting pellet was washed, recentrifuged, and resuspended in TE to give a protein concentration of 1–3 mg/ml. Protein concentrations were determined according to the method of Bradford (19), using a Bio-Rad protein assay kit.

Subtype-specific antisera. Throughout this study, muscarinic receptor subtype-specific antisera generated as described by Levey *et al.* (17, 18) were used. These antisera (α-m1 to α-m5) were raised by immunization of rabbits with recombinant fusion proteins expressed in *Escherichia coli*. The fusion proteins consist of the i3 loops of each of the muscarinic receptors (a region with little or no homology among the subtypes) and glutathione *S*-transferase. Immunoprecipitation assays with detergent-solubilized cloned receptors (m1–m5) verified a high degree of subtype selectivity (cross-reactivity of <3%) and precipitation efficiency for the antisera used in this study (17, 18).

Immunoprecipitation assays. Freshly prepared membrane homogenates were centrifuged and resuspended (1.0–3.0 mg/ml protein) in TE solubilization buffer containing 1% digitonin, 0.1% cholic acid, and protease inhibitors as indicated. The digitonin solution was prepared as described previously (20). The suspensions were left on ice for 60 min and recentrifuged (30,000 × *g* for 20 min). Detergent-solubilized fractions were collected (typically containing 30–50% of total muscarinic binding sites) and labeled with 0.8 nM [^{3}H]NMS. In order to determine antibody binding, antisera to m1–m5 i3 fusion proteins (α-m1 to α-m5) were diluted 1/50 into 96-well microtiter plate wells containing 10–40 fmol of solubilized labeled receptor and were incubated for 4 hr at 4°. Goat anti-rabbit IgG (Pel-Freez) was then added to a final dilution of 1/10. Pilot titration studies were performed to optimize sera concentrations necessary for maximal precipitation. After overnight incubation (18–22 hr), plates were centrifuged at 1000 × *g* for 5 min, immunoprecipitates were washed twice with TE containing 0.1% digitonin and 0.01% cholic acid and were resuspended in 1% sodium dodecyl sulfate, and radioactivity was determined on a LKB Beta counter. Nonspecific trapping of receptor in the immunoprecipitates was determined using control antisera (nonspecific rabbit serum) raised against a non-muscarinic receptor fusion protein (consisting of rat neuronal nicotinic amino terminus β-2 subunit) and averaged 6% of total receptors/assay. Total soluble receptors/assay were determined by gel filtration on G-25 Sephadex and were corrected for nonspecific binding in the presence of 1 μM atropine.

Results and Discussion

Previous studies have evaluated the distribution of muscarinic receptor subtypes using both pharmacological (1, 2, 4, 5, 21–34) and molecular genetic techniques (8, 10–12). Whereas pharmacological approaches are limited by the lack of subtype-selective muscarinic ligands (13–15), the molecular genetic approaches are restricted to the measurement of mRNA levels.

In the present study, therefore, we employed subtype-specific antisera (17, 18) to evaluate the relative levels of the five genetically defined muscarinic receptor subtype proteins (m1–m5) in various rabbit peripheral tissues. The specificity of these antisera has been defined based on several criteria. First, antisera were raised against muscarinic receptor fusion proteins containing large nonconserved segments of the i3 loops (17, 18). Second, on immunoblots, each antiserum was highly specific for the single fusion protein to which it was raised (17) (data not shown). Third, these antisera specifically immunoprecipitated each of the native cloned receptors individually expressed in stable CHO-K1 cell lines (17, 18).

The tissue distribution of muscarinic receptor proteins was determined in immunoprecipitation studies. Total brain was included as a control, because all five muscarinic receptor genes are expressed in this tissue (10, 12). The m1–m4 receptor proteins were widely detected and collectively account for the majority of solubilized [^{3}H]NMS binding sites in all tissues studied (Table 1). The m5 receptor could not be detected in any of the tissues studied (Table 1), consistent with the observation that m5 transcripts are extremely rare and can only be found at very low levels in few discrete brain areas (12).

It should be noted that the numbers of specifically precipitated receptors did not add up to 100% in some tissues e.g., rabbit brain (Table 1). This observation may be explained due to slightly less than quantitative precipitation of one or more of the subtypes. On the other hand, although less probable, it cannot be completely ruled out that a failure to precipitate about 10–18% of the total receptors in some tissues might be indicative of the presence of more than the five known muscarinic receptor subtypes in rabbit tissues.

Sympathetic ganglia and vas deferens. Radioligand binding (21–23) as well as functional studies (24, 25) suggest the presence of pharmacologically defined M_1 and M_2 receptors in mammalian sympathetic ganglia. Consistent with these findings, we have directly demonstrated a prevalence of m1 and m2 muscarinic receptor proteins in rabbit sympathetic ganglia (Table 1). Based on the results of previous functional studies, this finding suggests that activation of M_1 (m1) receptors causes slow excitatory postsynaptic potentials (24), whereas M_2 (m2) receptors may mediate slow inhibitory postsynaptic potentials in mammalian sympathetic ganglia (24, 25).

Similar to rabbit sympathetic ganglia, a mixture of m1 and m2 receptors was immunoprecipitated from rabbit vas deferens (Table 1). In agreement with this finding, functional studies have characterized a presynaptic M_1 heteroreceptor mediating inhibition and a postsynaptic M_2 receptor enhancing neurogenic contractions in this tissue (26, 27). It is, therefore, tempting to speculate that the molecularly defined m1 and m2 receptors identified by immunoprecipitation are involved in the mediation of these two opposite muscarinic responses.

Submaxillary gland. Muscarinic receptors in salivary glands have been characterized as M_1 and M_3 subtypes (1, 28, 29). Moreover, m1 and m3 mRNAs have been mapped in glandular tissue (11). Thus, the predominance of m1 and m3 receptor proteins (about equal amounts) in submaxillary gland, as revealed by immunoprecipitation assays (Table 1), is consistent with both pharmacological and molecular biological studies. The small amount of m2 receptor protein detected in this study may account for receptors colocalized with m1 receptors on neuronal-ganglionic structures, which are present in glandular tissue (28).

Atrium, ileum, and uterus. Numerous investigators demonstrated the presence of a homogeneous M_2 (m2) receptor population in the mammalian heart (1, 8, 11, 30). In agreement

TABLE 1

Distribution of muscarinic receptor subtypes (m1–m5) in rabbit tissues

Muscarinic receptors were immunoprecipitated using subtype-specific antisera (α-m1 to α-m5) raised against i3 loop fusion proteins. The receptors were solubilized, labeled with 0.8 nM [^{3}H]NMS, and immunoprecipitated with control (nonspecific rabbit antisera) or i3 loop antisera, as described in Experimental Procedures. Total soluble receptors/assay were determined by gel filtration on G-25 Sephadex columns and were corrected for nonspecific [^{3}H]NMS binding assessed in the presence of 1 μM atropine. Shown are the mean ± standard deviation of triplicate samples in a representative experiment. Numbers in parentheses are the percentage of total receptors specifically bound, given as the mean ± standard deviation of two to three independent experiments (corrected for background values in control immunoprecipitates with nonspecific rabbit serum at equivalent immunoglobulin concentrations).

Rabbit tissue	Total receptors	Immunoprecipitates					
		Nonspecific rabbit serum	α-m1	α-m2	α-m3	α-m4	α-m5
	fmol/assay	*fmol/pellet*					
Total brain	41.1 ± 2.1	0.5 ± 0.0	15.4 ± 1.1 (31.0 ± 7.0)	14.1 ± 3.9 (31.5 ± 2.1)	1.0 ± 0.1 (1.5 ± 0.7)	8.1 ± 0.4 (18.5 ± 0.7)	0.5 ± 0.0 (0.0 ± 0.0)
Sympathetic ganglia	10.6 ± 0.4	0.7 ± 0.1	2.9 ± 0.2 (17.5 ± 3.5)	7.5 ± 0.9 (70.5 ± 9.1)	0.7 ± 0.1 (0.5 ± 0.7)	1.1 ± 0.2 (3.0 ± 0.0)	0.6 ± 0.1 (0.0 ± 0.0)
Vas deferens	10.3 ± 0.4	0.9 ± 0.1	7.4 ± 0.6 (65.6 ± 3.9)	4.1 ± 0.4 (30.3 ± 2.0)	0.9 ± 0.1 (1.3 ± 1.5)	0.9 ± 0.1 (1.0 ± 0.0)	0.8 ± 0.2 (0.7 ± 0.6)
Submaxillary gland	13.1 ± 0.3	0.7 ± 0.1	5.7 ± 0.4 (36.3 ± 2.3)	1.8 ± 0.1 (12.3 ± 2.5)	6.1 ± 0.9 (42.0 ± 4.0)	1.2 ± 0.4 (6.8 ± 3.0)	0.8 ± 0.2 (1.6 ± 1.5)
Atrium	18.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.2 (0.5 ± 0.7)	17.9 ± 2.1 (88.0 ± 5.6)	1.3 ± 0.2 (1.0 ± 0.0)	1.3 ± 0.2 (0.5 ± 0.7)	1.0 ± 0.3 (0.0 ± 0.0)
Peripheral lung	10.1 ± 0.3	0.7 ± 0.1	1.3 ± 0.0 (5.5 ± 0.7)	4.7 ± 0.5 (40.0 ± 1.4)	1.0 ± 0.2 (3.0 ± 0.0)	5.0 ± 0.3 (40.5 ± 2.9)	0.8 ± 0.1 (0.5 ± 0.7)
Uterus	12.3 ± 0.5	1.0 ± 0.3	1.3 ± 0.2 (2.5 ± 0.7)	9.9 ± 0.6 (65.0 ± 11.0)	1.5 ± 0.1 (4.5 ± 0.7)	2.6 ± 0.3 (15.0 ± 2.8)	1.1 ± 0.1 (1.5 ± 0.7)
Ileum	11.7 ± 0.5	0.8 ± 0.1	1.1 ± 0.1 (2.5 ± 0.7)	9.3 ± 1.5 (68.5 ± 6.0)	1.1 ± 0.1 (3.5 ± 0.7)	2.1 ± 0.1 (12.0 ± 0.5)	0.7 ± 0.2 (0.0 ± 0.0)

with these studies, only m2 receptor protein was detected in rabbit atrium (Table 1).

The majority of receptors that could be immunoprecipitated from ileal and uterine tissues were of the m2 type. Binding studies have indicated the presence of M_2 and M_3 receptor subtypes in ileal smooth muscle tissue (31–33). The more abundant is the M_2 receptor, which mediates inhibition of adenylate cyclase (33), and the less abundant is the M_3 receptor, which appears to be involved in triggering smooth muscle contraction (33, 34) and phosphoinositide hydrolysis (33). Northern blot analysis of the porcine small intestine revealed a relatively large amount of m2 RNA and a small amount of m3 RNA (11). Somewhat surprisingly, our study did not reveal any appreciable amounts of m3 receptor proteins in either smooth muscle tissue or rabbit total brain (Table 1). One possible explanation for this phenomenon may be the relatively low level of m3 receptor protein in these tissues. In this respect, it should be borne in mind that, given the sensitivity of our assay system, it seems difficult to detect very small populations of receptors with sufficient accuracy. Furthermore, it is also conceivable that the m3 receptor protein might undergo post-translational modifications that hinder efficient solubilization or antibody binding, thus decreasing the sensitivity of the assay. Interestingly, although less abundant, a considerable amount of m4 receptor protein was detected in both smooth muscle tissues studied (Table 1). This finding represents the first direct evidence of the presence of m4 receptors in ileal and uterine smooth muscle tissues.

Peripheral lung. A recent study of muscarinic receptors in rabbit peripheral lung identified a homogeneous population of muscarinic binding sites that exhibited a unique pharmacology, most similar to that of M_4 (m4) binding sites in NG108-15 cells (5). The authors concluded that rabbit peripheral lung contains muscarinic sites of the M_4 subtype. This conclusion was further supported by the mapping of m4 mRNA in the rabbit lung (5). Our data indicate the preponderance of m2 and m4 receptor proteins in rabbit peripheral lung (Table 1), thus providing evidence for the presence of at least two muscarinic receptor subtypes in this tissue. It is important to recognize that the lung is a heterogeneous tissue containing multiple cell types and multiple muscarinic receptor subtypes. At present, however, the cellular location and function of m2 and m4 receptors identified as major muscarinic receptor subtype proteins in peripheral lung are unknown and remain to be determined. Future immunocytochemical studies will be necessary to localize the distinct subtypes to a specific cell type.

In conclusion, using subtype-specific antisera, we have directly characterized the major muscarinic receptor subtype proteins in a variety of rabbit peripheral tissues. The precise knowledge of the identity and distribution of these receptor proteins should be of considerable interest for future pharmacological studies and the targeting of new drugs.

Acknowledgments

The authors thank Drs. Ernst Mutschler, Günter Lambrecht, and Jürgen Wess for helpful discussions and critical reading of the manuscript.

References

1. Doods, H. N., M. J. Mathy, D. Davidesko, K. J. van Charldorp, A. De Jonge, and P. A. van Zwieten. Selectivity of muscarinic antagonists in radioligand and *in vivo* experiments for the putative M_1, M_2 and M_3 receptors. *J. Pharmacol. Exp. Ther.* **242**:257–262 (1987).
2. Mitchelson, F. Muscarinic receptor differentiation. *Pharmacol. Ther.* **37**:357–423 (1988).
3. Levine, R. R., and N. J. M. Birdsall (eds.). Subtypes of muscarinic receptors. IV. *Trends Pharmacol. Sci.* **10**(suppl.):1–119 (1989).
4. Waelbroeck, M., M. Tastenoy, J. Camus, and J. Christophe. Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain. *Mol. Pharmacol.* **38**:267–273 (1990).
5. Lazareno, S., N. J. Buckley, and F. Roberts. Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. *Mol. Pharmacol.* **38**:805–815 (1990).
6. Bonner, T. I., N. J. Buckley, A. C. Young, and M. R. Brann. Identification of a family of muscarinic acetylcholine receptor genes. *Science* (*Washington D. C.*) **237**:527–533 (1987).
7. Bonner, T. I., A. C. Young, M. R. Brann, and N. J. Buckley. Cloning and

expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1**:403–410 (1988).
8. Peralta, E. G., A. Ashkenazi, J. W. Winslow, D. H. Smith, J. Ramachandran, and D. J. Capon. Distinct primary structures, ligand-binding properties and tissue specific expression of four human muscarinic acetylcholine receptors. *EMBO J.* **6**:3923–3929 (1987).
9. Akiba, I., T. Kubo, A. Maeda, H. Bujo, J. Nakai, M. Mishina, and S. Numa. Primary structure of porcine muscarinic acetylcholine receptor III and antagonist binding studies. *FEBS Lett.* **235**:257–261 (1988).
10. Buckley, N. J., T. I. Bonner, and M. R. Brann. Localization of a family of muscarinic receptor mRNAs in rat brain. *J. Neurosci.* **8**:4646–4652 (1988).
11. Maeda, A., T. Kubo, M. Mishina, and S. Numa. Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett.* **239**:339–342 (1988).
12. Weiner, D., A. I. Levey, and M. R. Brann. Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. *Proc. Natl. Acad. Sci. USA* **87**:7050–7054 (1990).
13. Buckley, N. J., T. I. Bonner, C. M. Buckley, and M. R. Brann. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.* **35**:469–476 (1989).
14. Dörje, F., J. Wess, G. Lambrecht, R. Tacke, E. Mutschler, and M. R. Brann. Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.* **256**:727–733 (1991).
15. Hulme, E. C., N. J. M. Birdsall, and N. J. Buckley. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **30**:633–673 (1990).
16. Brann, M. R., R. M. Collins, and A. Spiegel. Localization of mRNAs encoding the α-subunits of signal-transducing G-proteins within rat brain and among peripheral tissues. *FEBS Lett.* **222**:191–198 (1987).
17. Levy, A. I., T. M. Stormann, and M. R. Brann. Bacterial expression of human muscarinic receptor fusion proteins and generation of subtype-specific antisera. *FEBS Lett.* **275**:65–69 (1990).
18. Levey, A. I., C. A. Kitt, W. F. Simonds, D. L. Price, and M. R. Brann. Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci.*, in press.
19. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
20. Repke, H., and H. Matthies. Biochemical characterization of solubilized muscarinic acetylcholine receptors. *Brain Res. Bull.* **5**:703–709 (1980).
21. Hammer, R., and A. Giachetti. Muscarinic receptor subtypes: M_1 and M_2: biochemical and functional characterization. *Life Sci.* **31**:2991–2998 (1982).
22. Watson, M., W. R. Roeske, P. C. Johnson, and H. I. Yammamura. [^{3}H]-Pirenzepine identifies putative M_1 muscarinic receptors in human stellate ganglia. *Brain Res.* **290**:179–182 (1984).
23. Feifel, R., J. F. Rodrigues de Miranda, C. Strohmann, R. Tacke, A. J. Aasen, E. Mutschler, and G. Lambrecht. Selective labelling of muscarinic M_1 receptors in calf superior cervical ganglia by [^{3}H]-(±)-telenzepine. *Eur. J. Pharmacol.* **195**:115–123 (1991).
24. Newberry, N. R., and T. Priestly. Pharmacological differences between two muscarinic responses of the rat superior cervical ganglion *in vitro*. *Br. J. Pharmacol.* **92**:817–826 (1987).
25. Yarosh, C. A., A. C. Olito, and J. H. Ashe. AF-DX 116: a selective antagonist of the slow inhibitory postsynaptic potential and methacholine-induced hyperpolarization in superior cervical ganglion of the rabbit. *J. Pharmacol. Exp. Ther.* **245**:419–425 (1988).
26. Eltze, M. Muscarinic M_1- and M_2-receptors mediating opposite effects on neuromuscular transmission in rabbit vas deferens. *Eur. J. Pharmacol.* **151**:205–221 (1988).
27. Eltze, M., G. Gmelin, J. Wess, C. Strohmann, R. Tacke, E. Mutschler, and G. Lambrecht. Presynaptic muscarinic receptors mediating inhibition of neurogenic contractions in rabbit vas deferens are of the ganglionic M_1-type. *Eur. J. Pharmacol.* **158**:233–242 (1988).
28. Martos, F., E. Monferini, E. Giraldo, A. M. De Paoli, and R. Hammer. Characterization of muscarinic receptors in salivary and lacrimal glands of the rat. *Eur. J. Pharmacol.* **143**:189–194 (1987).
29. Mei, L., W. R. Roeske, K. T. Izutsu, and H. I. Yamamura. Characterization of muscarinic acetylcholine receptors in human labial salivary glands. *Eur. J. Pharmacol.* **176**:367–370 (1990).
30. Waelbroek, M., M. Gillard, P. Robberecht, and J. Christophe. Muscarinic receptor heterogeneity in rat central nervous system. I. Binding of four selective antagonists to three muscarinic receptor subclasses: a comparison with M2 cardiac muscarinic receptors of the C type. *Mol. Pharmacol.* **32**:91–99 (1987).
31. Giraldo, E., E. Monferini, H. Ladinsky, and R. Hammer. Muscarinic receptor heterogeneity in guinea pig intestinal smooth muscle: binding studies with AF-DX 116. *Eur. J. Pharmacol.* **141**:475–477 (1987).
32. Michel, A. D., and R. L. Whiting. Methoctramine reveals heterogeneity of M_2 muscarinic receptors in longitudinal ileal smooth muscle membranes. *Eur. J. Pharmacol.* **145**:305–311 (1988).
33. Candell, L. M., S. H. Yun, L. L. P. Tran, and F. J. Ehlert. Differential coupling of subtypes of the muscarinic receptor to adenylate cyclase and phosphoinositide hydrolysis in the longitudinal muscle of the rat ileum. *Mol. Pharmacol.* **38**:689–697 (1990).
34. Lambrecht, G., R. Feifel, U. Moser, M. Wagner-Röder, L. K. Choo, J. Camus, M. Tastenoy, M. Waelbroeck, C. Strohmann, R. Tacke, J. F. Rodrigues de Miranda, J. Christophe, and E. Mutschler. Pharmacology of hexahydrodifenidol, hexahydro-sila-difenidol and related selective muscarinic antagonists. *Trends Pharmacol. Sci.* **10**(suppl.):61–64 (1989).

Send reprint requests to: Frank Dörje, Department of Pharmacology, University of Frankfurt, Theodor-Stern-Kai 7, Geb 75A, D-6000 Frankfurt/M, Federal Republic of Germany.