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FURTHER CHARACTERIZATION OF THE D_2 DOPAMINE RECEPTOR EXPRESSED IN MMQ CELLS

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Abstract—The D_2 dopamine receptor expressed in the MMQ cell line was characterized by saturation binding using the D_2 dopamine radioligand [^{3}H]spiperone. The K_D for spiperone was 41 pM and the B_{max} for these sites was 34 fmol/mg protein. Inhibition of forskolin-stimulated cAMP accumulation occurred in response to a variety of D_2 agonists, and the agonist effects were reversed by D_2 antagonists. Pertussis toxin pretreatment abolished agonist inhibition of cAMP accumulation. In addition, the α_2-adrenergic agonist UK 14304 inhibited cAMP accumulation; this effect was reversed by an α_2-adrenergic antagonist but not by a D_2 antagonist, indicating the presence of α_2-adrenergic receptors on these cells. Specific oligonucleotide primers were used in the polymerase chain reaction to determine, by restriction enzyme analysis and Southern blotting, that the long form of the two alternatively spliced variants of the D_2 dopamine receptor was the predominant variant expressed in these cells.

The MMQ cell line, derived from the 7315a rat pituitary tumor, has been used as a model system for the study of D_2 dopamine receptor function [1]. Although other cell lines have now been stably transfected to express highly the D_2 dopamine receptor [2], the MMQ cell is one of a small number of cell lines expressing an endogenous, functional D_2 dopamine receptor [3–6], making it an important cell line for the study of D_2 receptor signal transduction and transcriptional regulation. The present study further characterized the D_2 dopamine receptor on these cells by (a) performing radioligand binding studies, (b) pharmacologically characterizing the dopamine inhibition of cAMP accumulation, and (c) analyzing products derived from the polymerase chain reaction (PCR)‖ using MMQ cell cDNA. The results show that functional D_2 dopamine receptors are expressed in the membranes of these cells.

MATERIALS AND METHODS

Tissue culture. MMQ cells were maintained in 162-cm^2 flasks at 37° under 5% CO_2 in RPMI 1640 medium (Sigma, St. Louis, MO) containing 7.5% horse serum (Irvine Scientific, Santa Ana, CA), 2.5% fetal bovine serum (Hyclone, Logan, VT), 2.2 mM glutamine, 20 μg gentamicin, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B (Sigma). The cells were passaged on every third day by centrifugation at 300 *g* in a clinical centrifuge and split 1:15 into fresh growth medium.

Radioligand binding studies. The cells were pelleted by centrifugation as described above. The cell pellet was resuspended in 10 mL of ice-cold binding buffer (50 mM Tris–HCl, 120 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.02% ascorbate, pH 7.4), and a 200-μL aliquot was removed for cell counting. The cell suspension was then homogenized using a Brinkman (Westbury, NY) polytron at setting 5 for 5 sec, and the homogenate was spun at 20,000 *g* at 4° for 10 min. The membrane pellet was resuspended in 25 mL of binding buffer, followed by a 2- to 3-sec polytron burst at setting 5 to assure homogeneity. For saturation analysis, membranes were incubated with increasing concentrations of [^{3}H]spiperone in the presence or absence of 10 μM (+)-butaclamol for 20 min at 37°. Membrane bound radioligand was collected by rapid filtration through glass fiber filters using a Skatron (Sterling, VA) cell harvester. The membranes were washed with approximately 15 mL of ice-cold 0.1× binding buffer, and filter-bound radioactivity was measured by liquid scintillation counting on an LKB Betaplate (Gaithersburg, MD). Nonspecific binding varied from 50 to 80% of total binding.

cAMP accumulation assay. MMQ cells were cultured to a density of 1.5 to 2 × 10^6 cells/mL and then pelleted as described above. The pellet was resuspended in a 37° preincubation medium (RPMI 1640 containing 500 μM 3-isobutyl-1-methylxanthine, 1 μM idazoxan to block α_2-adrenergic effects as described in Results, and 0.02% ascorbate) to a density of approximately 2 × 10^6 cells/mL. The cell suspension was then allowed to preincubate for 5 min at 37° under 5% CO_2. Test compounds (agonists or agonist/antagonist mixtures) were prepared in assay medium (preincubation medium containing 0.6 μM forskolin) in a final volume of 100 μL. Basal levels of cAMP accumulation were

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‖ Abbreviations: PCR, polymerase chain reaction; and SDS, sodium dodecyl sulfate.

determined in the absence of forskolin. Then 200 μL/tube of the cell suspension were added, the tubes were placed back into the incubator, and the reaction was terminated after 15 min with a 1-mL addition of 0.1 N HCl. The released cAMP was acetylated by the addition of 50 μL of acetylation mixture containing triethylamine and acetic anhydride (2.5:1). The cAMP concentrations of the samples were determined using an automated radioimmunoassay system (Attoflow from Atto Instruments, Potomac, MD). Antagonist K_i values were derived from IC_{50} values by the Cheng–Prusoff equation [7].

Pertussis toxin treatment. One 162-cm^2 flask containing approximately 2×10^6 cells/mL was split into two 75-cm^2 flasks. One flask was treated with 50 μL vehicle (RPMI 1640), while the other flask received pertussis toxin dissolved in an equal volume of RPMI 1640 to achieve a final concentration of 100 ng pertussis toxin/mL. The cells were incubated for approximately 18 hr before pelleting by centrifugation for use in the cAMP accumulation assay as described above.

RNA isolation. RNA was prepared from MMQ cells by the RNAzol method (Biotecx Laboratories, Houston, TX). Briefly, cells were homogenized in RNAzol solution (2 mL/1 × 10^7 cells) containing guanidinium thiocyanate, phenol and 2-mercaptoethanol, and the RNA was extracted following addition of chloroform and centrifugation. The aqueous phase was removed and the RNA precipitated by isopropanol at −20°.

Oligonucleotide synthesis, PCR and identification of PCR products. Oligonucleotides were synthesized by the Corporate Molecular Biology Group of Abbott Laboratories using the β-cyanoethyl phosphoramidite method on DNA synthesizers from Applied Biosystems (models 380A and 380B). The upstream primer sequence was

5′-AATCTACATCGTCCTCCGGAAGCGCC-3′

and the downstream primer was

5′-TGGGATGGATCAGGGAGAGTGAGCTG-3′.

These primers span an alternatively spliced insert in the third cytoplasmic loop of the D_2 dopamine receptor and, in the presence of D_2 dopamine receptor DNA, produce PCR products of 305 and 215 bp depending on the presence or absence of the insert, respectively [8]. Total MMQ cell RNA was reverse transcribed, and the PCR reaction was performed according to the Perkin–Elmer Cetus (Norwalk, CT) protocol for the GeneAmp RNA PCR kit. An aliquot of the PCR products was digested with *Sac*I according to standard protocols in Sambrook *et al.* [9] and cut and uncut products were resolved on a 6% polyacrylamide gel containing 2× TBE (180 mM Tris-borate, 4 mM EDTA) and visualized by ethidium bromide staining. For Southern analysis, cut and uncut products were resolved on a 4% NuSieve agarose (FMC, Rockland, ME) gel containing 1× TAE (40 mM Tris-acetate, 1 mM EDTA). Following electrophoresis, the separated products were transferred overnight to Gene Screen Plus (New England Nuclear, Wilmington, DE) according to the manufacturer's protocol. Following a 3-hr prehybridization at 42°, the blot was hybridized with a *Bam*HI/*Stu*I fragment encoding much of the D_2 dopamine receptor coding region ^{32}P-labeled by random priming (Boehringer-Mannheim). The hybridization solution contained 50% formamide, 6× SSPE (1× SSPE contains 0.3 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA), 1.0% sodium dodecyl sulfate (SDS), 200 μg/mL denatured salmon sperm DNA, and 0.2% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. The blot was washed with 0.2× SSC (1× SSC contains 0.15 M NaCl and 0.015 M sodium citrate), 1.0% SDS once for 30 min at room temperature and twice for 30 min at 67°, and exposed to X-Omat (Kodak, Rochester, NY) film with intensifying screens.

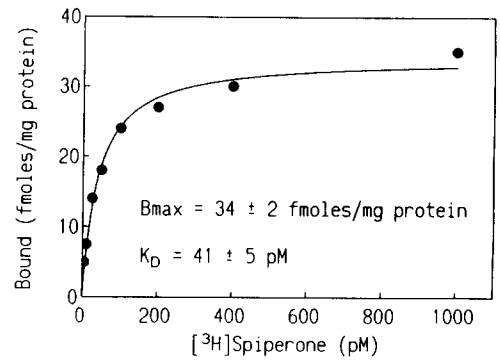

Fig. 1. Saturation binding of [^{3}H]spiperone to MMQ cell membranes. The data are from two experiments in which each point was determined from triplicate tubes. Results were analyzed by non-linear least squares regression analysis using the GraphPAD (San Diego, CA) Inplot program.

Compounds. Quinpirole HCl, *R*(−)- and *S*(+)-apomorhine, spiperone, (+)- and (−)-butaclamol HCl, *S*-(−)- and *R*(+)-sulpiride, haloperidol and yohimbine were purchased from Research Biochemicals, Inc., Natick, MA. Dopamine HCl and bromocryptine mesylate (2-bromo-α-ergocryptine methane sulfonate) were purchased from the Sigma Chemical Co. YM 09151-2 was a gift from Yamanouchi Pharmaceuticals, Tokyo, Japan. N-0437 was a gift from Whitby Pharmaceuticals, Richmond, VA. UK 14304 was obtained in-house. Pertussis toxin was purchased from List Biological Laboratories, Campbell, CA. Forskolin was purchased from Calbiochem, San Diego, CA. [^{3}H]Spiperone was purchased from Amersham, Arlington Heights, IL, and ^{32}P was purchased from New England Nuclear, Boston, MA.

RESULTS AND DISCUSSION

As shown in Fig. 1, there was specific and saturable [^{3}H]spiperone binding to MMQ cell membranes, consistent with the presence of D_2 dopamine receptors. However, the relatively low density of receptor sites resulted in low specific binding (20–50% of total bound), making radioligand binding a

Table 1. Activity of D_2 dopaminergic compounds on MMQ cells

	EC_{50} (nM)	N	K_i (nM)	N
Agonists				
Dopamine	514 ± 72	5		
LY 171555	162 ± 26	8		
N-0437	21 ± 7	4		
Bromocryptine	65 ± 13	4		
R(−)-Apomorphine	144 ± 45	4		
S(+)-Apomorphine	>10,000	3		
Antagonists				
Spiperone			0.10 ± 0.07	4
YM 09151-2			0.08 ± 0.03	3
Haloperidol			0.34 ± 0.16	3
(+)-Butaclamol			2.41 ± 1.12	4
(−)-Butaclamol			>10,000	3
S(−)-Sulpiride			5.27 ± 2.72	3
R(+)-Sulpiride			>1,000	2

Agonist EC_{50} values were determined using agonist inhibition of forskolin-stimulated cAMP accumulation as described in Materials and Methods. Antagonist K_i values were derived from IC_{50} values according to the Cheng–Prusoff equation [7]. The activity of antagonists was measured as antagonism of LY 171555 (10 μM)-induced inhibition of forskolin-stimulated cAMP accumulation. The EC_{50} and K_i values, with a few exceptions, are means ± SEM from N separate concentration–response curves in which each point in the curve was determined from triplicate wells.

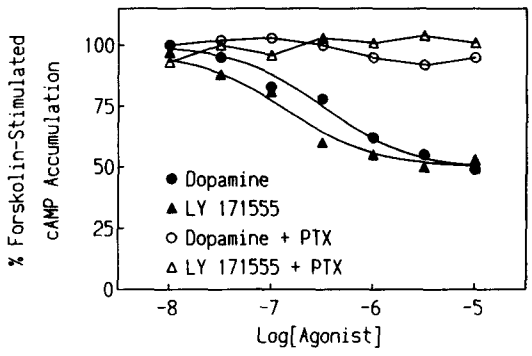

Fig. 2. Blockade of D_2 dopamine agonist inhibition of forskolin-stimulated cAMP accumulation in MMQ cells by pretreatment with pertussis toxin (PTX). The data are from two experiments in which each point was determined in triplicate. The EC_{50} values for dopamine and LY 171555 in the absence of pertussis toxin treatment were 327 ± 38 and 139 ± 18 nM, respectively. Results were analyzed by non-linear least squares regression analysis using the GraphPAD Inplot program.

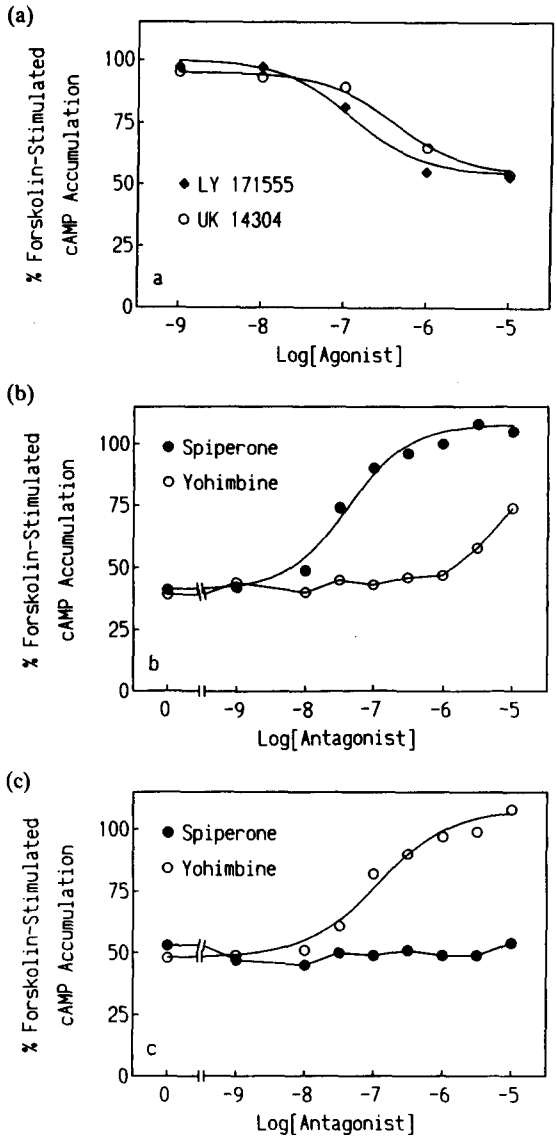

Fig. 3. D_2 dopamine and α_2-adrenergic inhibition of forskolin-stimulated cAMP accumulation in MMQ cells. (a) LY 171555 and UK 14304 inhibition of cAMP accumulation. The EC_{50} values for these curves were 123 ± 21 and 416 ± 53 nM for LY 171555 and UK 14304, respectively. (b) Spiperone (IC_{50} = 28 ± 2 nM) and yohimbine (IC_{50} = 12 ± 2 μM) reversal of the LY 171555 (10 μM) inhibition of forskolin-stimulated cAMP accumulation. (c) Yohimbine (IC_{50} = 102 ± 17 nM) but not spiperone reversals of UK 14304 (10 μM) inhibition of forskolin-stimulated cAMP accumulation. The data are from duplicate experiments in which each point was determined in triplicate. Results were analyzed by non-linear least squares regression analysis using the GraphPAD Inplot program.

less than ideal method for the characterization of these sites.

It should first be mentioned that the cAMP response to agonist was somewhat variable and was often lost in passages greater than 30. Data in the present report were taken only from experiments in which agonists reversed forskolin-stimulated acumulation by at least 40%. Agonist responses exhibited a pharmacological profile typical of D_2 receptor activation and the response to the D_2-selective agonist LY 171555 (quinpirole) was blocked by D_2-selective antagonists (Table 1). Dopamine

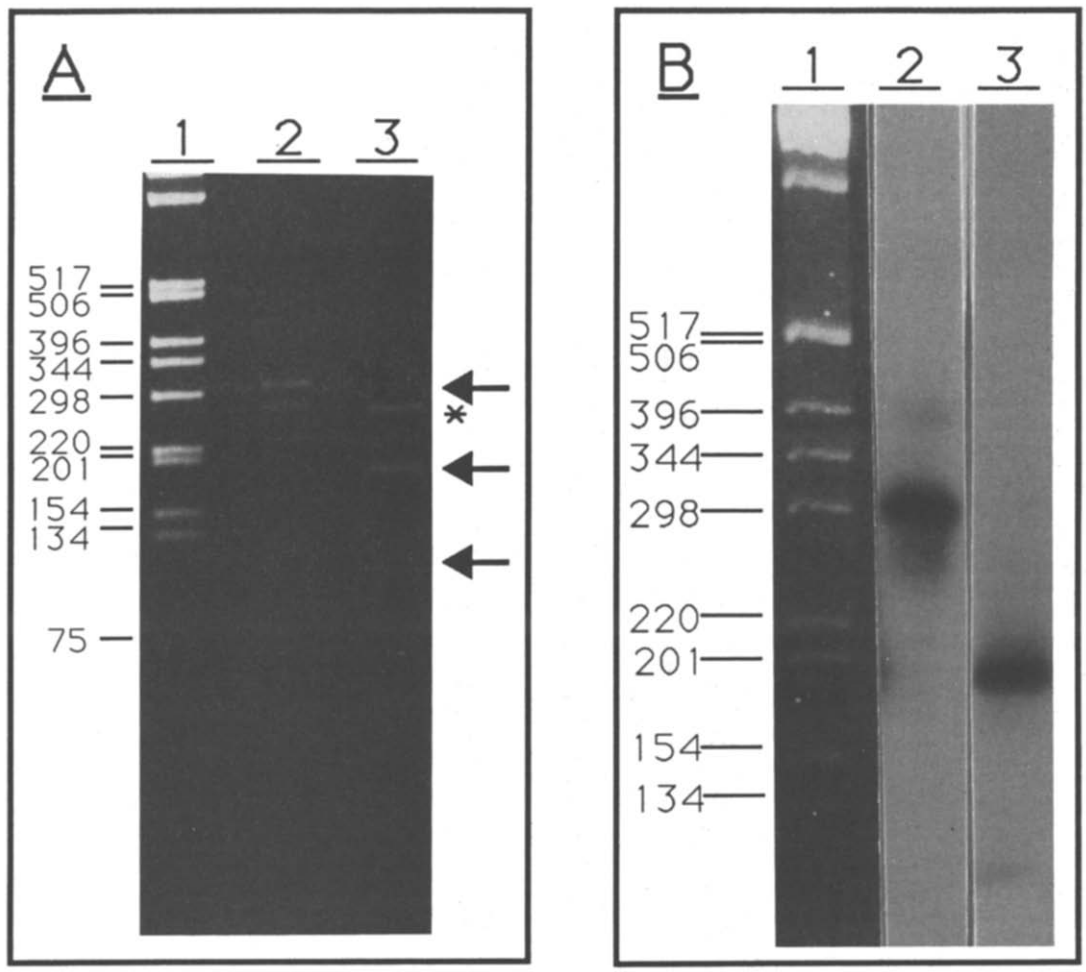

Fig. 4. Identification of PCR products by restriction digest and Southern blotting. (A) Ethidium bromide stained gel with 1 kb ladder standards (BRL, Gaithersburg, MD) in lane 1; PCR products derived from MMQ cell cDNA in lane 2; same products following a *Sac*I digest in lane 3. Note that the lower band (*) of lane 2 was not cut by *Sac*I. (B) Lane 1 shows ethidium bromide stained 1 Kb ladder standards. Southern blot shows that the labeled D_2 receptor probe only hybridized to the upper band of the uncut PCR products (lane 2) and to the lower two bands of the cut PCR fragments (lane 3).

and LY 171555 inhibited forskolin-stimulated cAMP accumulation in MMQ cells and this effect was blocked by pretreatment with pertussis toxin (Fig. 2), presumably via toxin-catalyzed ADP-ribosylation of the α-subunit of the G-protein inhibitory to adenylyl cyclase, G_i [10]. LY 171555 inhibition of cAMP accumulation (Figs. 2 and 3a) was reversed by spiperone, a D_2 antagonist, and was only weakly affected by yohimbine, an α_2-adrenergic antagonist (Fig. 3b). Surprisingly, the specific α_2-adrenergic agonist UK 14304 was also able to inhibit cAMP accumulation (Fig. 3a) and this effect was reversed by yohimbine but not spiperone (Fig. 3c), suggesting the presence of α_2-adrenergic as well as D_2 dopamine receptors on these cells. The recent demonstration of D_2 dopamine and α_2-adrenergic agonist inhibition of prolactin release from MMQ cells [11] agrees well with the results in Fig. 3.

There are two alternatively spliced variants of the D_2 dopamine receptor, which differ by an 87 bp insert located in the third cytoplasmic loop [12]. Primer pairs for PCR reactions were chosen to span the insert and therefore amplify templates derived from either long or short forms of the D_2 dopamine receptor mRNA resulting in fragment sizes of 302 and 215 bp, respectively [8]. Using RNA from MMQ cells, only the long form of the receptor was detected (Fig. 4A, lane 2, upper arrow). This fragment was cut by *Sac*I into fragments of 189 and 113 bp (Fig. 4A, lane 3) as predicted for D_2 receptor cDNA and the fragments were hybridized to the labeled D_2 receptor cDNA probe (Fig. 4B, lanes 2 and 3). The amplified PCR fragment, which ran as the lower band in Fig. 4A, lane 2, did not hybridize with the D_2 receptor probe and remains unidentified.

We have quantified the minimum ratio of mRNA long form to short form, given the parameters of the experiments employed, as follows. We estimated

that approximately 350 pg of D_2 long form PCR product was electrophoresed on the gel used for Southern blotting. Since the sensitivity of Southern blotting is between 0.1 and 10 pg [9], and since no short form was detected by Southern blotting, there is, at the very minimum, 35 times more long form than short form mRNA present in the MMQ cells. This is a very conservative estimate based upon the parameters of the experiments employed, and it is quite possible that there was no D_2 short form mRNA present in these cells.

Taken together, results from the present work confirm previous use of the MMQ cell as a model for D_2 dopamine action by demonstrating the presence of D_2 radioligand binding receptors on these cells and by extending the pharmacological characterization of the inhibitory cAMP response to more standard D_2 agonists and antagonists. Moreover, it is most likely the long form of the D_2 receptor that is expressed in these cells since only transcripts for the long form of the receptor were amplified.

REFERENCES

1. Judd AM, Login IS, Kovacs K, Ross PC, Spangelo BL, Jarvis WD and MacLeod RM, Characterization of the MMQ cell, a prolactin-secreting clonal cell line that is responsive to dopamine. *Endocrinology* **123**: 2341–2350, 1988.
2. Vallar L, Muca C, Magni M, Albert P, Bunzow J, Meldolesi J and Civelli O, Differential coupling of dopaminergic D_2 receptors expressed in different cell types. *J Biol Chem* **265**: 10320–10326, 1990.
3. Ivins KJ, Luedtke RR, Artymyshyn RP and Molinoff PB, Regulation of dopamine D2 receptors in a novel cell line (SUP1). *Mol Pharmacol* **39**: 531–539, 1991.
4. Courtney ND, Howlett AC and Westfall TC, Dopaminergic regulation of dopamine release from PC12 cells via a pertussis toxin-sensitive G protein. *Neurosci Lett* **122**: 261–264, 1991.
5. Missale C, Castelletti L, Boroni F, Memo M and Spano P, Epidermal growth factor induces the functional expression of dopamine receptors in the GH3 cell line. *Endocrinology* **128**: 13–20, 1991.
6. Monsma FJ, Barton AC and Sibley DR, Expression of functional D_2 dopamine receptors following differentiation of Y-79 human retinoblastoma cells. *J Neurochem* **54**: 1200–1207, 1990.
7. Cheng Y-C and Prusoff WH, Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22**: 3099–3108, 1973.
8. Rao DD, McKelvy J, Kebabian J and MacKenzie RG, Two forms of the rat D_2 dopamine receptor as revealed by the polymerase chain reaction. *FEBS Lett* **263**: 18–22, 1990.
9. Sambrook J, Fritisch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
10. Katada T and Ui M, Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc Natl Acad Sci USA* **79**: 3129–3133, 1982.
11. Judd AM and MacLeod RM, Dopamine receptor and adrenoceptor agonists inhibit prolactin release from MMQ cells. *Eur J Pharmacol* **195**: 101–106, 1991.
12. Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD and Seeburg PH, The dopamine D_2 receptor: Two molecular forms generated by alternative splicing. *EMBO J* **8**: 4025–4034, 1989.