The third intracellular domain of the m3 muscarinic receptor determines coupling to calcium influx in transfected Chinese hamster ovary cells

Dafna Singer-Lahat[a], Jie Liu[b], Jurgen Wess[b], Christian C. Felder[a,*]

[a]*Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD 20892, USA*
[b]*Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892, USA*

Received 23 February 1996

Abstract The m2 and m3 muscarinic acetylcholine receptors were expressed in CHO cells and were shown to couple to the release of calcium from intracellular stores. The m3 receptor, but not the m2 receptor, also coupled to calcium influx. Chimeric m2/m3 receptors were used to determine the structural domain of the m3 receptor linked to the regulation of calcium influx. It was found that the third intracellular loop of m3 receptor plays a fundamental role in regulating Ca^{2+} influx predicted to occur through Ca^{2+} channels located in the plasma membrane in CHO cells.

Key words: Muscarinic receptor; Chimeric receptor; Ca^{2+} influx; CHO-K1 cells

1. Introduction

The five known muscarinic receptors subtypes (m1–m5) are members of the G-protein-coupled receptor family. Structurally, they consist of a single polypeptide containing seven α-helical transmembrane domains which are connected by alternating intracellular and extracellular loops. The m2 and m4 receptors are functionally coupled to the inhibition of adenylate cyclase, weak stimulation of phospholipase C [1,2] and augmentation of phospholipase A_2 activity [3]. The m1, m3 and m5 receptors are coupled to stimulation of multiple signal transduction pathways including phospholipase A_2, phospholipase C, phospholipase D, adenylate cyclase, tyrosine kinase and opening of voltage-independent Ca^{2+} channels [4-10]. The elevation of intracellular calcium after receptor stimulation is biphasic, consisting of an initial rapid spike of intracellular Ca^{2+} release which is followed by a sustained Ca^{2+} influx [11]. The initial spike is the result of calcium release from intracellular stores mediated primarily by IP_3 generation [12]. The receptor-stimulated influx of Ca^{2+} is thought to cross the plasma membrane in part through a family of poorly characterized voltage-insensitive Ca^{2+} channels [13]. These channels can be indirectly activated by the second messengers $InsP_3$, $InsP_4$, intracellular Ca^{2+}, a Ca^{2+} influx factor, or may be independent of second messenger activation [13].

In order to understand which segment of the m3 receptor mediates Ca^{2+} influx, a group of cDNAs encoding m3/m2 receptor chimeras were constructed. These constructs were transiently transfected into Chinese hamster ovary (CHO) cells and evaluated for their Ca^{2+} response following stimulation with the muscarinic agonist, carbachol. The results of this study suggest that the third intracellular loop of the muscarinic m3 receptor plays a dominant role in the regulation of Ca^{2+} influx in CHO cells.

2. Materials and methods

2.1. Chimeric cDNA construct preparation and cell culture

The m2–i4 DNA construct was prepared by replacing the fourth intracellular domain of the wild-type m2 receptor (amino acids 444–466) with the corresponding m3 receptor sequence (amino acids 548–589). All other cDNA constructs were prepared by standard PCR mutagenesis approaches as previously described: (numbers refer to amino acid positions in the human m2 receptor and rat m3 receptor) m2–i1, m2 47–59/m3 91–103; m2–i2, 120–139/m3 164–183; m2–i3, m2 201–225/m3 246–268; m3–i3, m3 246–268/m2 201–225 [14,15].

CHO cells were plated on coverslips coated with poly-L-lysine (10 ng/ml) and Vitrogen (300 μg/ml; Celtrix, Palo Alto, CA) at a density of 100 000 cells per coverslip as described previously [16]. The next day, the coverslips were transferred to 10 cm plates and the cells were transfected with 4 μg of plasmid DNA by the DEAE-dextran method [17]. Two days after the transfection, Ca^{2+} influx measurements were performed.

2.2. Measurement of changes in intracellular Ca^{2+} in individual Fura 2-loaded CHO cells

CHO cells were loaded with 5 μM of the Ca^{2+} sensitive dye, Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min at 37°C in Krebs medium containing 5 mM HEPES. Intracellular Ca^{2+} levels were monitored as the emission wavelength of 510 nm after the cells were illuminated alternately with 350 nm and 380 nm light as described previously [11].

3. Results

Application of carbachol (100 μM) to CHO cells transiently expressing the wild-type m3 receptor resulted in a rapid increase in intracellular Ca^{2+} that declined to baseline following removal of the agonist (Fig. 1A). In most of the cells, carbachol had no effect on untransfected CHO cells (Table 1). In order to observe carbachol-activated Ca^{2+} influx, independent of intracellular release, ATP (10 μM) was applied to activate purinergic receptor-dependent store depletion prior to carbachol stimulation. In the absence of extracellular Ca^{2+}, carbachol failed to stimulate an increase in intracellular calcium concentration following application of ATP, indicating complete depletion of carbachol-sensitive Ca^{2+} stores (Fig. 1B). These results indicate that carbachol and ATP share the same intracellular Ca^{2+} stores. Following ATP-mediated Ca^{2+} pool depletion, carbachol stimulated an extracellular Ca^{2+}-dependent increase in intracellular calcium that declined to baseline following removal of carbachol (Fig. 1A). Using the same protocol, wild-type m2 receptor failed to stimulate Ca^{2+} influx following store depletion with ATP (Fig. 1C). Similar to wild-type m3 receptor, carbachol stimulated Ca^{2+} influx in cells expressing the chimeric m2–i3 receptor (m2 receptor in which the third cytoplasmic loop was exchanged with the corre-

*Corresponding author. Fax: (1) (301) 402 5050;
E-mail: feldercc@CODON.NIH.GOV

PII S0014-5793(96)00398-5

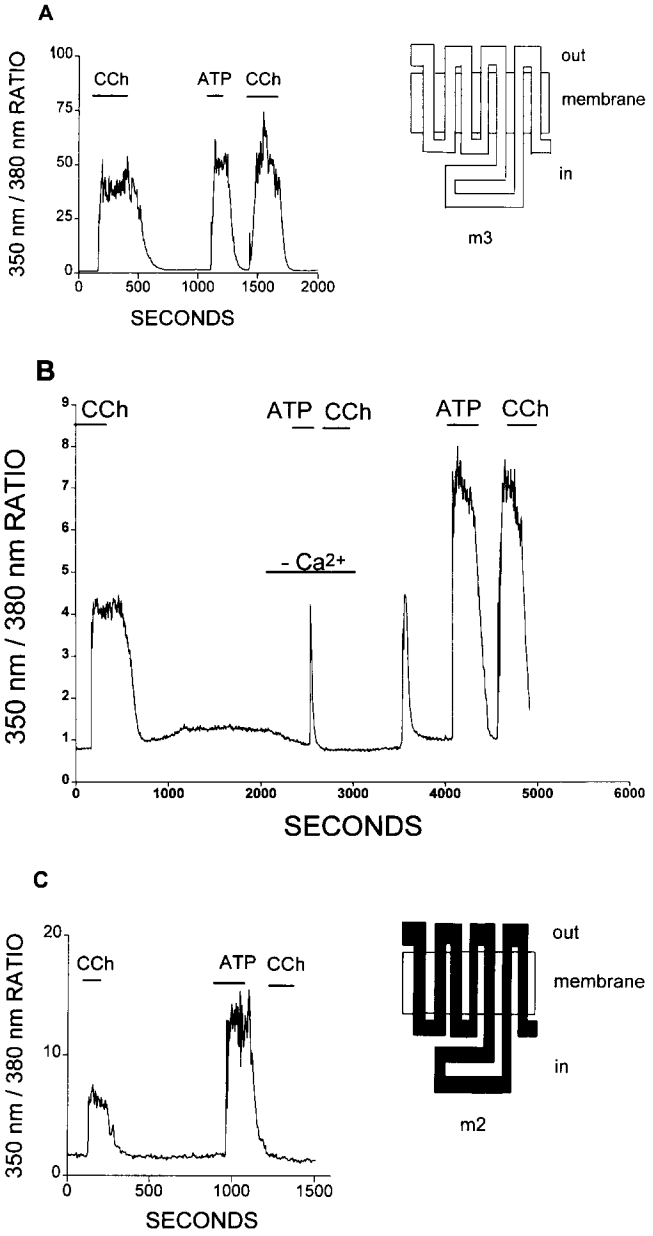

Fig. 1. Carbachol- and ATP-stimulated intracellular Ca^{2+} mobilization in CHO m3 and CHO m2 cells. Single cells were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2 and changes in intracellular Ca^{2+} were monitored as indicated in Section 2. Data shown are representative of data collected from at least 15 cells. A: CHO-K1 cells transiently expressing m3 receptor were exposed to carbachol (100 μM) or ATP (10 μM). B: CHO-K1 cells transiently expressing m3 receptor were exposed to carbachol (100 μM) or ATP (10 μM) in the presence or absence of extracellular Ca^{2+}. C: CHO-K1 cells transiently expressing m2 receptor were exposed to carbachol (100 μM) or ATP (10 μM).

sponding domain of the m3 receptor; Fig. 2C, Table 1). Chimeric muscarinic receptors, in which the first, second or fourth intracellular domain of m2 receptor was exchanged with the corresponding m3 receptor region, did not couple to carbachol-stimulated Ca^{2+} influx (Fig. 2), similar to the wild-type m2 receptor (Table 1). In addition, cells expressing m3–i3 hybrid receptors (m3 receptor in which the third cytoplasmic loop was exchanged with the corresponding m2 receptor domain) did not show carbachol-mediated Ca^{2+} influx following ATP-dependent store depletion (Fig. 2 and Table 1). Table 1 presents a comparison between the efficiency of the m3 receptor and the various chimeric muscarinic receptors to activate Ca^{2+} influx.

In most of the non-transfected, as well as sham-transfected cells, carbachol treatment had no effect. However, in a small cell population (<10%), carbachol activated extracellular Ca^{2+}-dependent changes in intracellular Ca^{2+} levels (7.4 ± 6.5% and 6.25 ± 6.25%, respectively; Table 1 and Fig. 3).

4. Discussion

The purpose of this study was to evaluate the potential contribution of each cytoplasmic loop of the m3 receptor in regulating Ca^{2+} influx in CHO cells. This was approached systematically by substituting each of the cytoplasmic loops of the m3 receptor into the structure of the m2 receptor which is not coupled to Ca^{2+} influx.

Exchange of the third intracellular loop of the m2 receptor with the corresponding m3 receptor domain (m2–i3 chimeric receptor) conferred on the m2 receptor the ability to couple to Ca^{2+} influx. In contrast, exchanging any other intracellular region of the m2 receptor with the corresponding m3 receptor domain did not result in coupling to Ca^{2+} influx. Conversely, exchange of the third intracellular loop of the m3 receptor with the corresponding m2 receptor domain (m3–i3) resulted in a loss of the ability of the chimeric receptor to activate Ca^{2+} channels (Fig. 2 and Table 1), similar to wild-type m2 receptors. Although most cells expressing m2–i1, m2–i2, m2–i4 or m3–i3 did not show a change in intracellular calcium concentrations after carbachol application, a very low percentage of the cells responded by a mono- or bi-phasic Ca^{2+} elevation. This was also observed in non- or sham-transfected CHO-K1 cells, indicating that the observed calcium response in cells transfected with m2–i1, m2–i2, m2–i4 or m3–i3 was mediated by the low expressed endogenous receptors. It has been shown before in COS-7 cells that the levels of expression of m2–i1, m2–i2, and m2–i3 [14,15] and m2–i4 (unpublished

Table 1
Comparison of carbachol-stimulated Ca^{2+} influx mediated by wild-type m3 receptor to different chimeric muscarinic receptors transiently expressed in CHO-K1 cells

Receptor construct	Carbachol-stimulated calcium influx (% of cells)
m3 receptor (wild-type)	39.4 ± 9 (n=7)
m2 receptor (wild-type)	7.2 ± 0.05* (n=5, P=0.02)
m2–i1 receptor	9.6 ± 6* (n=6, P=0.03)
m2–i2 receptor	5.5 ± 2* (n=10, P=0.001)
m2–i3 receptor	23.9 ± 6 (n=5, P=0.39)
m2–i4 receptor	2.8 ± 2.8* (n=4, P=0.02)
m3–i3 receptor	6.2 ± 3.8* (n=5, P=0.02)
non-transfected cells	7.4 ± 6.5* (n=4, P=0.04)
sham-transfected cells	6.2 ± 6.2* (n=8, P=0.01)

CHO-K1 cells were transfected with wild-type m2, m3 or different chimeric m2/m3 receptors and the ability of carbachol to activate Ca^{2+} influx was measured. In addition, carbachol-activated Ca^{2+} influx was tested in native CHO-K1 cells and in sham-transfected CHO-K1 cells. The values shown are percentages of the selected cell population that responded to carbachol (mean ± S.E.M.). The P-value was determined with the one tailed t-test.
*Significant difference from wild-type m3 receptor.

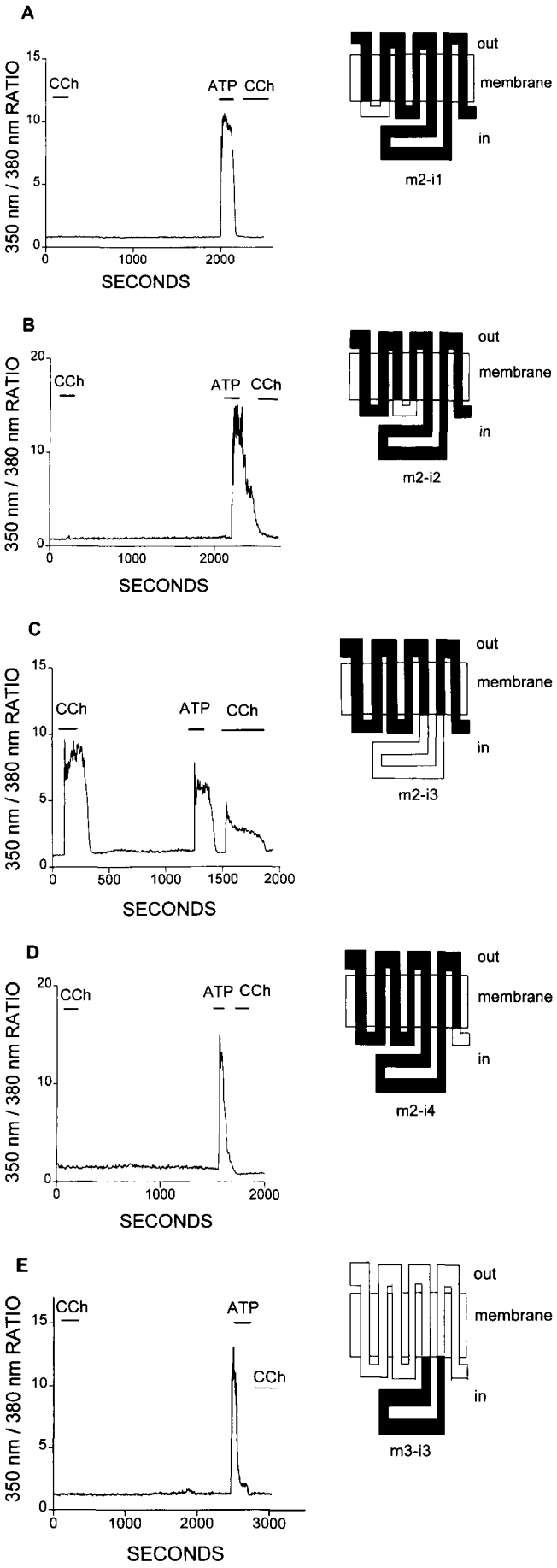

Fig. 2. Carbachol- and ATP-activated intracellular Ca^{2+} mobilization in CHO-K1 cells expressing different chimeric muscarinic m2/m3 receptors. Data are from representative cells. In each section of the figure there is a schematic representation of the muscarinic receptor that mediated the intracellular Ca^{2+} response in the presence of carbachol and is shown on the left side of the figure. Carbachol (100 μM) stimulation of m2 muscarinic receptor containing the m3 first cytoplasmic loop (A) or m3 second cytoplasmic loop (B) or m3 third or fourth (D) cytoplasmic loop. E: Carbachol (100 μM) stimulation of the m3 muscarinic receptor containing the m2 third cytoplasmic loop.

data) chimeras are similar to the wild-type m2 and m3 receptors. This argues against the possibility that m2–i1, m2–i2, or m2–i4 chimeras do not mediate calcium influx because of lower density of the expressed receptors.

In a very small percentage of non-transfected cells carbachol activated changes in intracellular Ca^{2+} concentration, which were extracellular and intracellular Ca^{2+}-dependent (Table 1 and Fig. 3B,C). By applying ATP prior to carbachol we could observe that in some cells carbachol evoked only release of Ca^{2+} from intracellular stores and in others a biphasic response. However, when the endogenous receptor responding to carbachol in untransfected cells activated both phases of Ca^{2+} responses, there was a transient increase in the intracellular concentration of calcium that declined to the sustained phase. These data suggest that the endogenous receptor is coupled to phospholipase C more strongly than to the Ca^{2+} influx pathway. This rare effect of carbachol on CHO-K1 cells may reflect a low expression of a novel subtype of muscarinic receptor which binds to carbachol and functionally couples to Ca^{2+} mobilization. Since the numbers of responding cells among CHO m3 cells and CHO cells expressing m2–i1, m2–i2, m2–i4 or m3–i3 were significantly different (Table 1), we assume that carbachol-activated intracellular calcium elevation in the latter group was a result of activation of these unknown receptors and did not correlate with the responses observed when chimeric receptors were expressed by the cell.

This study indicates that the third intracellular segment of the m3 muscarinic receptor plays a pivotal role in linking the receptor to Ca^{2+} channels in the plasma membrane of CHO cells. Coupling of muscarinic receptors to respective G-proteins has been shown to occur primarily but not exclusively through interactions involving the third cytoplasmic loop [15,18,19]. Therefore, it is possible that a G-protein is involved in the m3 receptor-calcium influx interaction. Further experiments will have to be carried out to understand better the interaction between the receptor and the channel and whether a G-protein is indeed involved. Our results are in contrast with previous findings in A9 cells [11] in which the m3 receptor-mediated calcium influx was proved to be independent of the third cytoplasmic loop. This controversy may reflect differences in the cell lines used in these studies. It is also possible that in both cell lines the expressed m3 receptor is linked to Ca^{2+} channels through different signal transduction pathways.

References

[1] Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J. and Capon, D.J. (1988) Nature 334, 434–437.

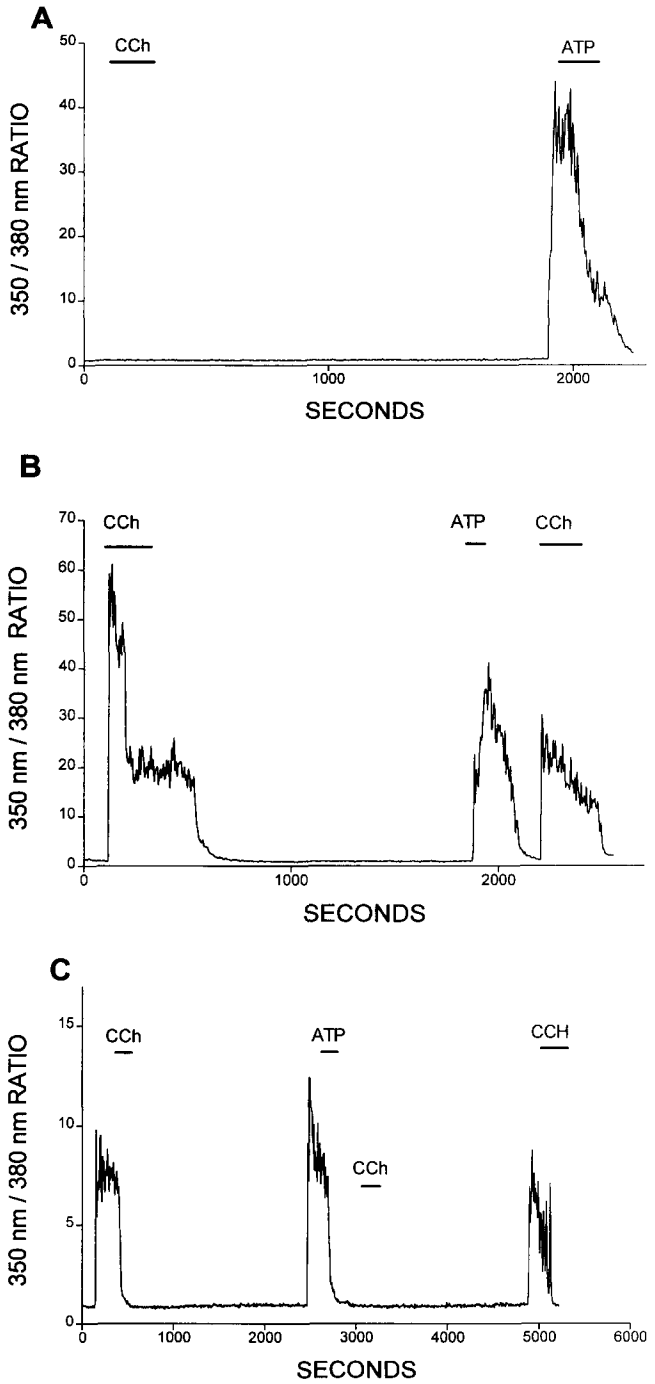

Fig. 3. Carbachol- and ATP-activated intracellular Ca^{2+} mobilization in sham transfected CHO-K1 cells. Cells were treated with DEAE-dextran and quinoline in the absence of plasmid DNA as indicated in Figs. 1 and 2. Two days after the transfection, carbachol had no effect on intracellular Ca^{2+} mobilization in most of the cells tested (A). Rarely, carbachol evoked the release of Ca^{2+} from intracellular stores (B) or evoked both intracellular calcium release as well as Ca^{2+} influx (C).

[2] Novotny, E.A. and Brann, M.R. (1989) Trends Pharmacol. Sci. 10 (Suppl.), 116.
[3] Felder, C.C., William, H. and Axelrod, J. (1991) Proc. Natl. Acad. Sci. USA 88, 6477–6480.
[4] Bonner, T.I., Young, A.C., Brann, M.R. and Buckley, N.J. (1988) Neuron 1, 403–410.
[5] Shapiro, R.A., Scherer, N.M., Habecker, E.M., Subers, E.M. and Nathanson, N.M. (1988) J. Biol. Chem. 263, 18397–18403.
[6] Conklin, B.R., Brann, M.R., Buckley, N.J., Ma, A.L., Bonner, T.I. and Axelrod, J. (1988) Proc. Natl. Acad. Sci. USA 85, 8698–8702.
[7] Felder, C.C., Dieter, P., Kinsella, J., Tamura, K., Kanterman, R.Y. and Axelrod, J. (1991) J. Pharmacol. Exp. Ther. 255, 1140–1147.
[8] Felder, C.C., Kanterman, R.Y., Ma, A.L. and Axelrod, J. (1989) J. Biol. Chem. 264, 20356–20362.
[9] Felder, C.C., MacArthur, L., Ma, A.L., Gusovsky, F. and Kohn, E.C. (1993) Proc. Natl. Acad. Sci. USA 90, 1706–1710.
[10] Felder, C.C., Ma, A.L. and Kohn, E.C. (1991) J. Pharmacol. Exp. Ther. 257, 967–997.
[11] Felder, C.C., Poulter, M.O. and Wess, J. (1991) Proc. Natl. Acad. Sci. USA 89, 509–513.
[12] Berridge, M.J. (1993) Nature 361, 315–325.
[13] Felder, C.C., Singer-Lahat, D. and Mathes, C. (1994) Biochem. Pharmacol. 48, 1997–2004.
[14] Blin, N., Yun, J. and Wess, J. (1995) J. Biol. Chem. 270, 17741–17748.
[15] Wess, J., Brann, M.R. and Bonner, T.I. (1989) FEBS Lett. 258, 133–136.
[16] Singer-Lahat, D., Ma, A.L. and Felder, C.C. (1996) Biochem. Pharmacol. (in press).
[17] Cullen, B.R. (1987) Methods Enzymol. 152, 684–704.
[18] Wess, J., Bonner, T.I., Dorje, F. and Brann, M.R. (1990) Mol. Pharmacol. 38, 517–523.
[19] Lechleiter, J., Hellmiss, R., Durson, K., Ennulat, D., David, N., Clapham, D. and Peralta, E. (1990) EMBO J. 9, 4381–4390.