British Journal of Pharmacology (1997) 121, 451–458

Specific G_q protein involvement in muscarinic M_3 receptor-induced phosphatidylinositol hydrolysis and Ca^{2+} release in mouse duodenal myocytes

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1 Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) during exposure to acetylcholine or caffeine was measured in mouse duodenal myocytes loaded with fura-2. Acetylcholine evoked a transient increase in $[Ca^{2+}]_i$ followed by a sustained rise which was rapidly terminated after drug removal. Although L-type Ca^{2+} currents participated in the global Ca^{2+} response induced by acetylcholine, the initial peak in $[Ca^{2+}]_i$ was mainly due to release of Ca^{2+} from intracellular stores.

2 Atropine, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, a muscarinic M_3 antagonist), pirenzepine (a muscarinic M_1 antagonist), methoctramine and gallamine (muscarinic M_2 antagonists) inhibited the acetylcholine-induced Ca^{2+} release, with a high affinity for 4-DAMP and atropine and a low affinity for the other antagonists. Selective protection of muscarinic M_2 receptors with methoctramine during 4-DAMP mustard alkylation of muscarinic M_3 receptors provided no evidence for muscarinic M_2 receptor-activated $[Ca^{2+}]_i$ increase.

3 Acetylcholine-induced Ca^{2+} release was blocked by intracellular dialysis with a patch pipette containing either heparin or an anti-phosphatidylinositol antibody and by external application of U73122 (a phospholipase C inhibitor).

4 Acetylcholine-induced Ca^{2+} release was insensitive to external pretreatment with pertussis toxin, but concentration-dependently inhibited by intracellular dialysis with a patch pipette solution containing an anti-α_q/α_{11} antibody. An antisense oligonucleotide approach revealed that only the G_q protein was involved in acetylcholine-induced Ca^{2+} release.

5 Intracellular applications of either an anti-β_{com} antibody or a peptide corresponding to the G$\beta\gamma$ binding domain of the β-adrenoceptor kinase 1 had no effect on acetylcholine-induced Ca^{2+} release.

6 Our results show that, in mouse duodenal myocytes, acetylcholine-induced release of Ca^{2+} from intracellular stores is mediated through activation of muscarinic M_3 receptors which couple with a G_q protein to activate a phosphatidylinositol-specific phospholipase C.

Keywords: Duodenal smooth muscle cells; Ca^{2+} release; muscarinic receptors; G protein; phosphatidylinositol–phospholipase C

Introduction

Pharmacological identification of muscarinic receptors in intestinal smooth muscles has revealed the existence of several receptor subtypes. Although it appears that muscarinic M_3 receptors primarily mediate smooth muscle contraction, the muscarinic M_3 receptor subtype accounts for only 30% of the total muscarinic receptors, whereas the remaining majority of receptors (70%) would be of the muscarinic M_2 receptor subtype (Michel & Whiting, 1990). Both muscarinic M_2 and M_3 receptor subtypes have been shown to mediate phosphatidylinositol hydrolysis, resulting in the generation of inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (Thomas & Ehlert, 1994; Prestwich & Bolton, 1995). $InsP_3$ accumulation has been demonstrated to couple to Ca^{2+} release from intracellular stores, triggering acetylcholine-induced contraction in ileal smooth muscle (Thomas & Ehlert, 1994).

The muscarinic m_1, m_3 and m_5 receptors have been shown to stimulate phospholipase C-β (PLC-β) via pertussis toxin (PTX)-insensitive G proteins of the G_q family, whereas muscarinic m_2 and m_4 receptors couple to PTX-sensitive $G_{i/0}$ proteins (Ashkenazi *et al*., 1989). Both PTX-sensitive and PTX-insensitive G proteins can transduce Ca^{2+} release since the α subunits of the G_q family activate various PLC isoforms, i.e. PLC-β_1, -β_3 and -β_4 (Jhon *et al*., 1993), whereas the $\beta\gamma$ dimers activate PLC-β_2 and -β_3 (Camps *et al*., 1992).

The purpose of the present study was two fold: (1) to investigate the changes in $[Ca^{2+}]_i$ during exposure to acetylcholine and to assess the contribution of Ca^{2+} from various sources in single duodenal myocytes, and (2) to identify the receptors, phospholipids and G proteins involved in the acetylcholine-activated Ca^{2+} release. The results show that the changes in $[Ca^{2+}]_i$ are mediated by muscarinic M_3 receptor activation and correspond to a transient release of Ca^{2+} from the intracellular store, as well as to an entry of Ca^{2+} from outside the cells, partly due to dihydropyridine-sensitive Ca^{2+} channels. Experiments performed with antibodies directed against phosphatidylinositols and different subtypes of G proteins, with antisense oligonucleotides designed to block synthesis of G protein subunits and with synthetic peptides corresponding to the G$\beta\gamma$ binding domain of the β-adrenoceptor kinase 1 (βARK_1) revealed that the muscarinic M_3 receptor-activated Ca^{2+} release from the intracellular store is mediated through activation of a G_q protein and hydrolysis of phosphatidylinositols by a PLC.

Methods

Cell preparation

Swiss mice (20–25 g) were killed by cervical dislocation. The longitudinal layer of the duodenal smooth muscle was cut into several pieces and incubated for 10 min in low Ca^{2+} (40 μM)

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physiological solution, then 0.8 mg ml^{-1} collagenase, 0.2 mg ml^{-1} pronase E and 1 mg ml^{-1} bovine serum albumin were added at 37°C for 20 min. After this time, the solution was removed and the pieces of duodenum were incubated again in a fresh enzyme solution at 37°C for 20 min. Tissues were then placed in enzyme-free solution and triturated with a fire-polished Pasteur pipette to release cells. Cells were maintained in short-term primary culture in medium M199 containing 2% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 20 u ml^{-1} penicillin and 20 μg ml^{-1} streptomycin and kept in an incubator gassed with 95% air, 5% CO_2 at 37°C and used within 72 h.

Fluorescence measurements

Cells were loaded by incubation in physiological solution containing 1 μM fura-2-acetoxymethylester (fura-2AM) for 30 min at room temperature. These cells were washed and allowed to cleave the dye to the active fura-2 compound for at least 1 h. Fura-2 loading was usually uniform over the cytoplasm and compartmentalization of the dye was never observed.

Measurement of intracellular Ca^{2+} concentration was carried out by the dual-wavelength fluorescence method, as previously described (Leprêtre *et al*., 1994a). Briefly, fura-2-loaded cells were mounted in a perfusion chamber and placed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). A single cell was alternately excited with u.v. light of 340 nm and 380 nm through a 100 × oil-immersion objective (Nikon, 1.3 NA), and emitted fluorescent light from the Ca^{2+}-sensitive dye was collected through a 510 nm long-pass filter with a Charge-Coupled Device camera (Hamamatsu Photonics, Hamamatsu City, Japan). The signal was processed (Hamamatsu Photonics DVS 3000) by correcting each fluorescence image for background fluorescence and calculating 340/380 nm fluorescence ratios on a pixel-by-pixel basis. Averaged frames were usually collected at each wavelength from a single cell every 0.5 s. $[Ca^{2+}]_i$ was calculated from mean ratios by use of a calibration curve for fura-2 determined in loaded cells. Some experiments were carried out in the presence of 1 μM oxodipine (a light-stable dihydropyridine derivative) in order to inhibit voltage-dependent Ca^{2+} channels. All measurements were made at 25 ± 1°C.

Patch-clamp and fluorescence measurements

Voltage-clamp and membrane current recordings were made with a standard patch-clamp technique by use of a List EPC-7 patch-clamp amplifier (Darmstadt-Eberstadt, Germany). The whole-cell recording mode was performed with patch pipettes of 1–3 MΩ resistance. Membrane potential and current records were stored and analysed with an IBM-PC computer (P-clamp system, Axon, Foster City, CA). Simultaneous measurements of intracellular calcium concentration were carried out, as previously described (Leprêtre *et al*., 1994b). Briefly, 100 μM fura-2 was added to the pipette solution and so entered into the cells following establishment of the whole-cell recording mode. A steady fluorescence was obtained within 3 min. $[Ca^{2+}]_i$ was estimated from the 340/380 nm fluorescence ratio by use of a calibration determined within cells. All experiments were carried out at 25 ± 1°C.

The results are expressed as means ± s.e.mean. Significance was tested by means of Student's *t* test. *P* values of < 0.05 were considered as significant.

Antibodies

Antibodies were added to the pipette solution to allow dialysis of the cell after a break through in whole-cell recording mode for at least 5–8 min, a time longer than that expected theoretically for diffusion of substance in solutions. Purification and specificity of the autoantibody directed against phosphatidylinositol (anti-PtdIns) has been shown previously (Leprêtre *et al*., 1994a,b). The anti-$\alpha_{q/11}$ antibody was raised to the C-terminal amino acids (LQLNLKEYNLV) of $G\alpha_{q/11}$ subunit. The anti-β_{com} antibody was raised to the C-terminal amino acids (TDDGMAVATGSWDSFLKIWN) of $G\beta_1$ subunit.

Microinjection of oligonucleotides

Myocytes were seeded at a density of about 10^3 cells per mm^2 on glass slides imprinted with squares for localization of injected cells and maintained in short-term primary culture in medium M199 containing 2% foetal calf serum, 2 mM glutamine, 1 mM pyruvate, 20 u ml^{-1} penicillin, and 20 μg ml^{-1} streptomycin; they were kept in an incubator gassed with 95% air, 5% CO_2 at 37°C. The sequences of the oligonucleotides used in this study were determined by sequence comparison and multiple alignment by use of Mac Molly Tetra software (Soft Gene, Berlin, Germany). Injection of oligonucleotides was performed into the nucleus of myocytes by a manual injection system (Eppendorf, Hamburg, Germany). The injection solution contained 10 μM oligonucleotides in water; approximately 10 fl were injected with commercially available microcapillaries (Femtotips, Eppendorf) with an outlet diameter of 0.5 μm. In some control experiments, myocytes were injected only with water and tested in comparison with non-injected cells and cells injected with antisense oligonucleotides. The myocytes were cultured for 3 days in culture medium and the glass slides were transferred into a perfusion chamber for intracellular Ca^{2+} measurements. The sequences of the anti-α_q and anti-α_{11} oligonucleotides have been published previously (Dippel *et al*., 1996). The sequence of anti-$\alpha_{q/11com}$ is ATGGACTCCAGAGT, of sense $\alpha_{q/11com}$ is ACTCTGGAGTCCAT corresponding to nt 4–17 of α_q cDNA (Strathmann & Simon, 1990) and of scrambled anti-$\alpha_{q/11com}$ is TACGGTCCAGAGTA corresponding to a scrambled sequence of nt 4–17 of α_q cDNA (Strathmann & Simon, 1991).

Solutions

The normal physiological solution contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11 and HEPES 10, pH 7.4 with NaOH. The basic pipette solution contained (in mM): KCl 130, HEPES 10, pH 7.3 with NaOH. Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and by adding 0.5 mM EGTA. Muscarinic agonists and caffeine were applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the records. Before each experiment a fast application of physiological solution was tested and cells with movement artefacts were excluded.

Chemicals and drugs

Collagenase was obtained from Worthington (Freehold, NJ); pronase (type E), bovine serum albumin, acetylcholine, carbachol, oxotremorine, pilocarpine, pirenzepine, gallamine, thapsigargin, pertussis toxin (PTX), heparin and chondroitin sulphate were from Sigma (St Louis, MO). M199 medium was from Flow Laboratories (Puteaux, France). Foetal calf serum was from Flobio (Courbevoie, France). Streptomycin, penicillin, glutamine and pyruvate were from Gibco (Paisley, U.K.). 4-Diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP), atropine and methoctramine were from RBI (Natick, MA, U.S.A.). Oxodipine was a gift from Dr Galiano (IQB, Madrid, Spain). Caffeine was from Merck (Nogent sur Marne, France). Fura-2AM, fura-2 and ryanodine were from Calbiochem (Meudon, France). Synthetic peptides corresponding to the $G\beta\gamma$ binding domain of βARK_1 (peptide G) or to a region outside the $G\beta\gamma$ binding site (peptide A) were from Genosys (Cambridge, U.K.). 1-(6-((17β-3methoxystra-1,3,5 (10) - trien - 17 - yl) amino) hexyl) - 1H - pyrrole - 2,5 - dione (U73122) and 1-(6-((17β-3methoxystra-1,3,5(10)-trien-17-yl) amino)hexyl)-1H-pyrrolipine-dione (U73343) were from Biomol (Plymouth Meeting, PA, U.S.A.). Anti-β_{com} antibody (SC378) was from Santa Cruz Biotechnology (Santa Cruz, CA,

U.S.A.). Anti-$\alpha_{q/11}$ antibody was a gift from G. Guillon (Montpellier, France) and oligonucleotides were a gift from F. Kalkbrenner (Berlin, Germany).

Results

Effects of acetylcholine and caffeine on $[Ca^{2+}]_i$ in duodenal myocytes

In single myocytes isolated from mouse duodenum, ejection of 10 μM acetylcholine for 10 s caused a clear biphasic Ca^{2+} response (Figure 1Aa). On average, $[Ca^{2+}]_i$ increased rapidly from a resting level of 48 ± 2 nM to a peak of 215 ± 26 nM, which was followed by a sustained $[Ca^{2+}]_i$ elevation of 79 ± 4 nM ($n = 14$). The rapid, initial increase in $[Ca^{2+}]_i$ was still present in Ca^{2+}-free external solution for 1 min but was decreased by $40 \pm 6\%$ ($n = 5$), whereas the sustained phase was removed (Figure 1Ab). This indicates that the transient increase in $[Ca^{2+}]_i$ is largely due to release of Ca^{2+} from intracellular stores whereas the later sustained Ca^{2+} response is maintained by Ca^{2+} entry into the cell from the extracellular space. In the presence of 1 μM oxodipine (a light-resistant dihydropyridine) for 5 min, the transient Ca^{2+} response induced by 10 μM acetylcholine was reduced by $25 \pm 5\%$ (Figure 1B; $n = 9$) and the sustained Ca^{2+} response (measured at the end of acetylcholine ejections) was decreased by $49 \pm 6\%$ (Figure 1B; $n = 9$) whereas the resting Ca^{2+} level was unchanged. These results suggest that part of the transient and sustained Ca^{2+} response is due to activation of voltage-dependent L-type Ca^{2+} channels.

In order to demonstrate the involvement of the intracellular Ca^{2+} store in the transient Ca^{2+} response induced by acetylcholine, complete depletion of the store was evoked by application of ryanodine and caffeine. Ryanodine has been shown to lock the Ca^{2+} release channels of the intracellular Ca^{2+} store in an open state so that the stored Ca^{2+} is progressively reduced (Meissner, 1986). Short (3 s) applications of 10 mM caffeine caused transient increases in $[Ca^{2+}]_i$ (Figure 2Aa), the amplitudes of which (190 ± 22 nM, $n = 17$) were not significantly different from those induced by acetylcholine applications (215 ± 26 nM, $n = 14$, $P > 0.05$). When the time interval between two successive applications was 3 min, the second transient increase in $[Ca^{2+}]_i$ induced by caffeine or acetylcholine was similar to the first Ca^{2+} response. When the cells were preincubated in the presence of 10 μM ryanodine for 60 min (Figure 2b), the first caffeine application induced a reduced Ca^{2+} response (166 ± 37 nM, $n = 10$) whereas the basal $[Ca^{2+}]_i$ level was progressively increased to 110 ± 5 nM ($n = 10$). A second application of caffeine, 3 min later, was ineffective ($n = 10$). Similarly, the acetylcholine-induced transient increase in $[Ca^{2+}]_i$ was not observed after a first application of caffeine ($n = 15$; Figure 2c), suggesting that acetylcholine released Ca^{2+} from the intracellular store which possesses ryanodine- and caffeine-sensitive channels. Finally, we used thapsigargin to induce Ca^{2+} store discharge (Thastrup *et al*., 1990) by inhibiting the Ca^{2+}-ATPases. Under these conditions, both acetylcholine- and caffeine-induced Ca^{2+} responses were abolished ($n = 8$; data not shown), indicating that thapsigargin prevented refilling of an internal Ca^{2+} store which can be mobilized by acetylcholine and caffeine. In the following experiments, acetylcholine-induced Ca^{2+} release was measured in Ca^{2+}-free, 0.5 mM EGTA-containing solution for 30 s.

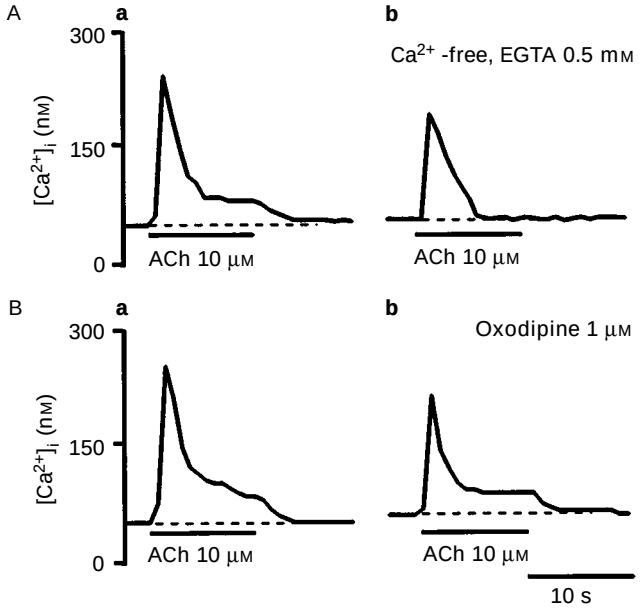

Figure 1 Effects of applications of 10 μM acetylcholine (ACh) in single mouse duodenal myocytes. The cells were loaded with fura-2AM and not patch-clamped. (A) In normal physiological solution with 2 mM Ca^{2+} (a), a long ejection of ACh (10 s) induced a transient increase in $[Ca^{2+}]_i$ followed by a sustained phase which decreased rapidly to baseline resting $[Ca^{2+}]_i$ upon removal of ACh. In Ca^{2+}-free, 0.5 mM EGTA-containing solution (b), a transient increase in $[Ca^{2+}]_i$ was still observed whereas the sustained phase was abolished. (B) Both transient and sustained Ca^{2+} responses induced by ACh in control conditions (a) were decreased by application of 1 μM oxodipine for 5 min (b).

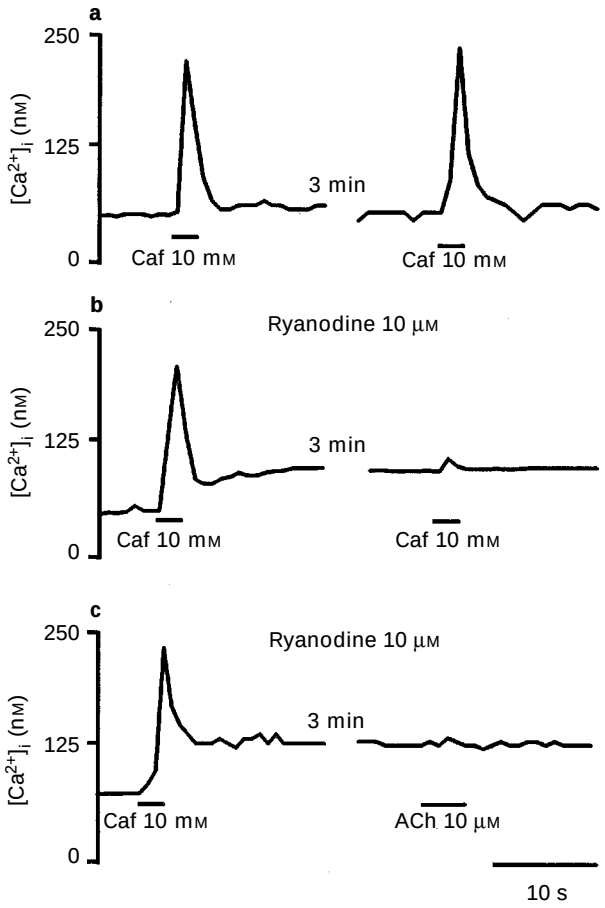

Figure 2 Effects of caffeine and ryanodine on the intracellular Ca^{2+} store. The cells were loaded with fura-2AM and not patch-clamped. (a) A short (3 s) application of 10 mM caffeine (Caf) caused only a transient increase in $[Ca^{2+}]_i$, the amplitude of which was similar to that induced by a second application of caffeine, separated by a 3 min interval. (b) When the cells were preincubated in the presence of 10 μM ryanodine for 60 min, the first 10 mM caffeine application induced a Ca^{2+} response, but the second one was ineffective. (c) Similarly, no response was evoked with a second application of 10 μM ACh.

Effects of muscarinic agonists and antagonists

The pharmacological profile of the acetylcholine-induced Ca^{2+} release was examined with compounds displaying selective affinity sequences for muscarinic receptor subtypes. In Figure 3a, the concentration-response curves for acetylcholine and carbachol indicate that maximal Ca^{2+} release was obtained at 100–300 μM for each compound. The concentrations producing half-maximal response (EC_{50}) were estimated to be 1.6 ± 0.4 μM for acetylcholine and 22.3 ± 1.5 μM for carbachol ($n=4$). The Hill coefficients obtained from Hill plots were close to unity. In contrast, oxotremorine produced a limited Ca^{2+} response which reached about 20–25% of the maximal response and pilocarpine was completely ineffective. A series of compounds antagonized the acetylcholine-induced Ca^{2+} release (Figure 3b) with the following concentrations producing half-inhibition of the control response (IC_{50}; $n=4-9$): 0.6 ± 0.2 nM for 4-DAMP, 0.8 ± 0.2 nM for atropine, 447 ± 78 nM for pirenzepine, 629 ± 67 nM for methoctramine and 50 ± 5 μM for gallamine. The rank order of potency was, therefore, 4-DAMP $\geqslant$ atropine > pirenzepine $\geqslant$ methoctramine > gallamine. α-Bungarotoxin (300 nM) had no effect on the acetylcholine-induced Ca^{2+} release ($n=9$).

Moreover, the Ca^{2+} responses induced by 100 μM oxotremorine were inhibited in a dose-dependent manner by low concentrations of 4-DAMP ($IC_{50} = 0.6 \pm 0.2$ nM, $n=6$) and high concentrations of methoctramine ($IC_{50} = 315 \pm 37$ nM, $n=6$), suggesting that the oxotremorine-induced Ca^{2+} response occurred through activation of muscarinic M_3 receptors.

In order to ensure the absence of muscarinic M_2 receptor-induced Ca^{2+} response, we initially used 100 nM methoctramine (a concentration that completely but reversibly blocks muscarinic M_2 receptors, Doods *et al.*, 1993). Under these conditions, the acetylcholine-induced Ca^{2+} release reached 148 ± 23 nM ($n=8$) and was not significantly different from control values (155 ± 16 nM, $n=14$). Second, we pretreated myocytes with 10 nM 4-DAMP mustard and 100 nM methoctramine (in order to protect muscarinic M_2 receptors from 4-DAMP mustard alkylation) for 60 min at 37°C (Eglen & Harris, 1993). After washing (90 min at 37°C), Ca^{2+} release in response to applications of 5 or 10 μM acetylcholine was not detectable (data not shown; $n=38$), indicating that muscarinic M_2 receptor activation was not involved in intracellular Ca^{2+} mobilization in duodenal myocytes.

Effects of heparin, anti-PdtIns antibody and phospholipase C inhibitor

We used heparin to block inositol trisphosphate receptors and any further Ca^{2+} release via these receptors (Guillemette *et al.*,

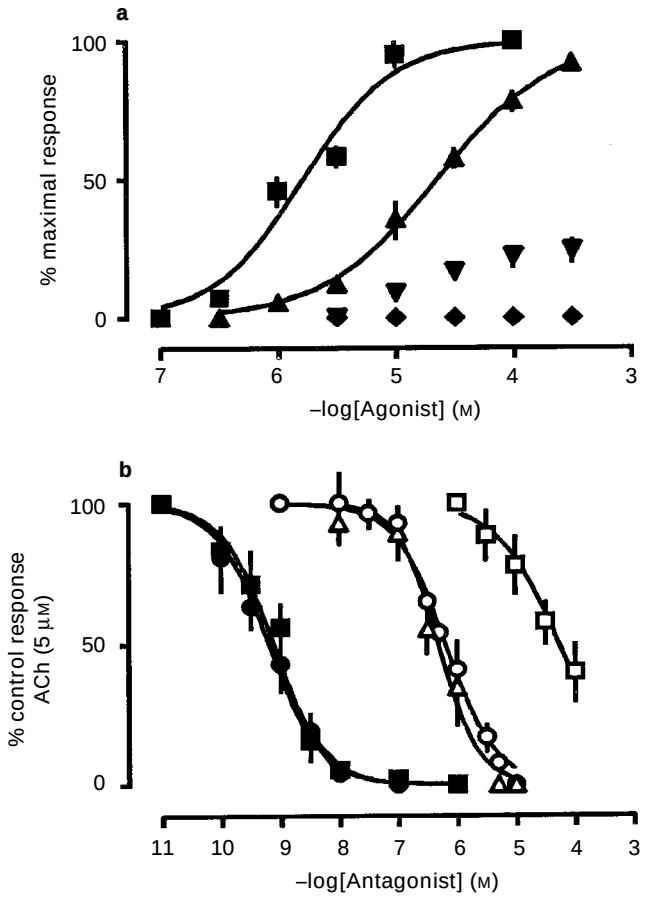

Figure 3 Effects of muscarinic agonists and antagonists on $[Ca^{2+}]_i$ in single cells loaded with fura-2 AM and not patch-clamped. (a) Concentration-response curves to (■) ACh, (▲) carbachol, (▼) oxotremorine and (◆) pilocarpine. $[Ca^{2+}]_i$ values were expressed as a percentage of the maximal response induced by ACh. (b) Inhibition of the ACh-stimulated $[Ca^{2+}]_i$ changes by (●) 4-DAMP, (■) atropine, (△) pirenzepine, (○) methoctramine and (□) gallamine. $[Ca^{2+}]_i$ values are expressed as a percentage of the response obtained with 5 μM ACh. Each point represents the mean for 5–9 cells; vertical lines show s.e.mean.

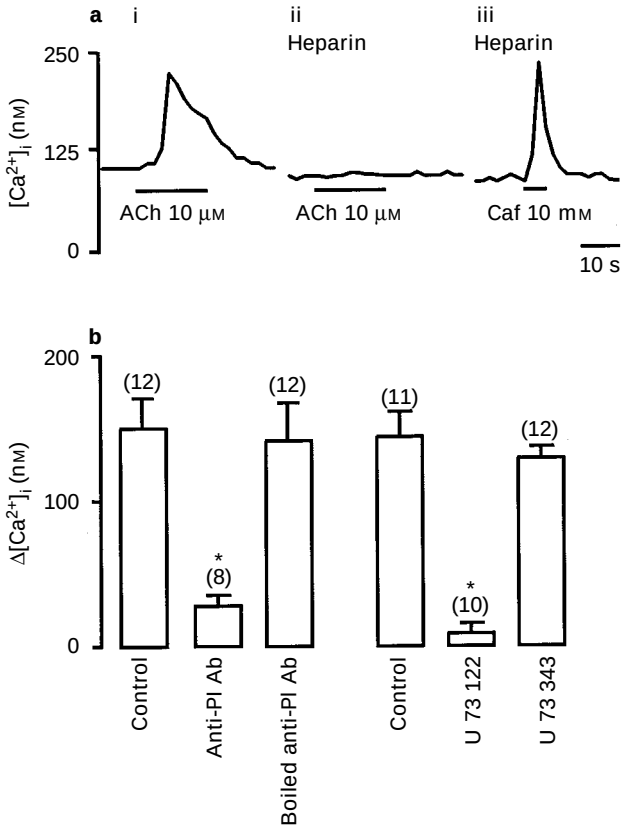

Figure 4 Effects of heparin anti-PdtIns antibody, U73122 and U73343 on the increase in $[Ca^{2+}]_i$ induced by ACh or caffeine. In voltage-clamped cells at a holding potential of -70 mV, fura-2 (100 μM) was added to the pipette solution. (a)(i) Ca^{2+} responses were induced in control conditions by 10 μM ACh. In cells dialysed with a pipette solution containing 5 mg ml^{-1} heparin for 5 min, Ca^{2+} responses were induced by ACh (ii) or caffeine (iii). (b) Peak increases in $[Ca^{2+}]_i$ evoked by 10 μM ACh in control conditions, in a cell dialysed with 15 μg ml^{-1} anti-PdtIns antibody or boiled anti-PdtIns antibody (95°C for 30 min) for 7 min through the patch clamp pipette and in cells superfused with 100 μM U73122 or U73343 for 5 min. Data are given as means ± s.e.mean with number of experiments in parentheses. External solution was a Ca^{2+}-free, 0.5 mM EGTA-containing solution. *Values significantly different from those obtained under control conditions, $P<0.01$.

1989). The effects of acetylcholine and caffeine were studied with heparin in the pipette solution (Figure 4a). In cells held at −70 mV, the basal $[Ca^{2+}]_i$ (119±8.5 nM, $n=20$) was larger than that measured in non patch-clamped cells loaded with fura-2AM. In the presence of 5 mg ml^{-1} heparin for 5 min, the basal $[Ca^{2+}]_i$ was not different from that observed under control conditions (120±20 nM, $n=7$). Application of 10 μM acetylcholine was unable to evoke a noticeable transient Ca^{2+} release (Figure 4a, $n=5$) whereas application of 10 mM caffeine induced a Ca^{2+} release (135±25 nM, $n=5$) similar to control values (130±21 nM, $n=9$). This effect was specific to heparin, as, when chondroitin sulphate (5 mg ml^{-1}) was added to the pipette solution instead of heparin, the amplitude of the acetylcholine-induced Ca^{2+} release was not significantly affected (control: 145±12 nM; in the presence of chondroitin sulphate: 147±18 nM; $n=12$). To determine whether phosphatidylinositol hydrolysis is involved in the generation of second messengers in response to muscarinic receptor activation, we tested the effects of an anti-PtdIns antibody on the acetylcholine-induced Ca^{2+} release. As shown in Figure 4b, intracellular application of 15 μg ml^{-1} anti-PtdIns antibody for 7 min inhibited the acetylcholine-induced Ca^{2+} release ($n=8$). When anti-PtdIns antibody (15 μg ml^{-1}) was inactivated by heating at 95°C for 30 min, the acetylcholine-induced Ca^{2+} release was unaffected (control: 150±21 nM, $n=12$; in the presence of inactivated anti-PtdIns antibody: 141±26 nM, $n=12$). We also tested the effects of U73122 which has been proposed as a phospholipase C inhibitor (Sohn *et al*., 1993; Macrez-Leprêtre *et al*., 1996). Pretreatment of the cells with 100 μM U73122 for 5 min largely inhibited the acetylcholine-induced Ca^{2+} release (Figure 4b; $n=10$) while it did not significantly affect the caffeine-induced Ca^{2+} response ($n=14$; data not shown). In contrast, pretreatment of the cells with 100 μM U73343 (the inactive analogue of U73122) did not significantly affect acetylcholine-induced Ca^{2+} release (Figure 4b).

Inhibition of G protein function and expression

Pertussis toxin (PTX) ADP ribosylates both G_i and G_o proteins, thus preventing their activity. Cells were incubated in a culture medium containing 0.5 μg ml^{-1} PTX for 20 h. This pretreatment suppressed α_2-adrenoceptor-induced stimulation of Ca^{2+} channels, which has been demonstrated to depend on activation of a G_i protein (Macrez-Leprêtre *et al*., 1995). Acetylcholine-induced Ca^{2+} release was not affected by the PTX pretreatment (control: 147±20 nM, $n=12$; PTX-pretreated cells: 144±17 nM, $n=12$) while intracellular application of 1 mM GDP-β-S for 5–6 min completely abolished acetylcholine-induced Ca^{2+} release ($n=7$), suggesting the involvement of PTX-insensitive G proteins. Antibodies directed against the carboxyl terminus of the α subunits of G proteins have been shown to be useful tools for identifying transduction pathways. When an anti-α_q/α_{11} antibody was added to the basic pipette solution for 6 min, the acetylcholine-induced Ca^{2+} release was concentration-dependently inhibited, as illustrated in Figure 5a. Maximal inhibition was obtained with a concentration of 20 μg ml^{-1} anti-α_q/α_{11} antibody. Intracellular application of the anti-α_q/α_{11} antibody (20 μg ml^{-1}) inactivated by heating at 95°C for 30 min did not significantly affect the acetylcholine-induced Ca^{2+} release (control: 135±25 nM, $n=9$; in the presence of inactivated anti-α_q/α_{11} antibody: 125±21 nM, $n=15$; Figure 5a).

In order to discriminate between G_q and G_{11} protein, we used antisense oligonucleotides designed to inhibit selectively the expression of G_q or G_{11} protein. For each experiment, we compared the Ca^{2+} responses of antisense oligonucleotide-injected cells located within a marked area on the glass slide to sense or scrambled oligonucleotide-injected cells or non-injected cells outside this marked area. This procedure ensures that antisense oligonucleotides-injected cells were always compared to control cells that were otherwise grown, treated and analysed under identical conditions, i.e. culture, incubation, microinjection and loading with fura-2 AM. The increases in $[Ca^{2+}]_i$ evoked by 10 μM acetylcholine and 10 mM caffeine, in Ca^{2+}-free, 0.5 mM EGTA-containing solution for 30 s, were measured for each cell, and mean values were calculated from all the cells of each experiment. As illustrated in Figure 5b, the acetylcholine-induced Ca^{2+} release was similarly inhibited in cells injected with 10 μM anti-$\alpha_{q/11com}$ and anti-α_q oligonucleotides while the caffeine response was not significantly affected. In contrast, cells injected with 10 μM anti-α_{11} oligonucleotide showed no inhibition of either acetylcholine- or caffeine-induced Ca^{2+} responses. Increasing the concentration of injected anti-α_{11} oligonucleotide to 20 μM did not induce a further inhibition ($n=12$). Furthermore, we used sense $\alpha_{q/11com}$ and scrambled anti-$\alpha_{q/11com}$ oligonucleotides which did not significantly anneal to the target sequence of $G\alpha_{q/11}$ subunits. Acetylcholine-induced Ca^{2+} release was not significantly affected by injection of these oligonucleotides (non-injected cells: 148±14 nM, $n=15$; sense $\alpha_{q/11com}$-injected cells: 155±18 nM, $n=12$ and scrambled anti-$\alpha_{q/11com}$-injected cells: 142±11 nM, $n=12$). These results suggest different tasks for G_q and G_{11} proteins since G_q protein transduces the muscarinic signal for Ca^{2+} release while G_{11} protein does not.

Effects of anti-β_{com} antibody and βARK_1 peptides

The anti-α_q/α_{11} antibody and antisense oligonucleotide block of the acetylcholine-induced Ca^{2+} response cannot distinguish whether α or $\beta\gamma$ subunits are transducing the signal that activates Ca^{2+} release from the intracellular stores. Therefore, we

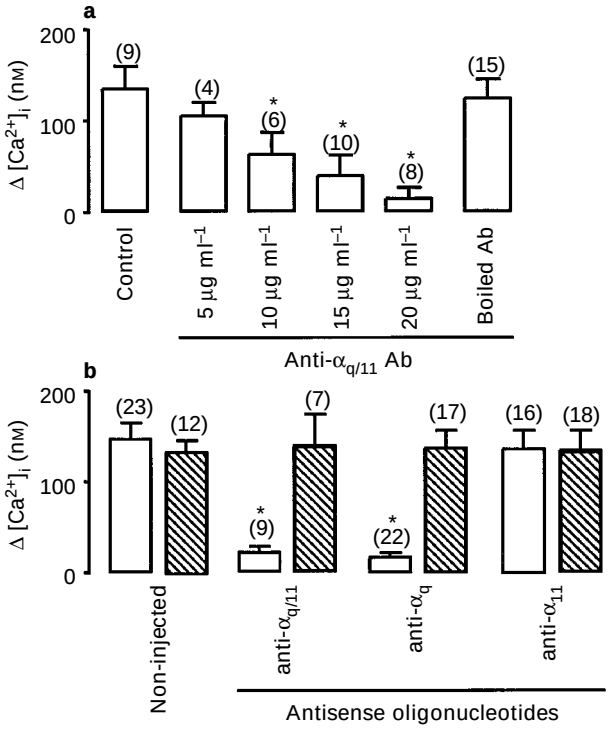

Figure 5 Effects of anti $\alpha_{q/11}$ antibody and antisense oligonucleotides on the increase in $[Ca^{2+}]_i$ induced by 10 μM ACh. (a) In other voltage-clamped cells (at a holding potential of −70 mV) fura-2 (100 μM) was added to the pipette solution. Shown are ACh-induced Ca^{2+} responses in control conditions, in cells dialysed with a pipette solution contained various concentrations of anti-$\alpha_{q/11}$ antibody or 20 μg ml^{-1} boiled anti-$\alpha_{q/11}$ antibody (95°C for 30 min) for 6 min. (b) Peak increases in $[Ca^{2+}]_i$ evoked by 10 μM ACh (open columns) or 10 mM caffeine (hatched columns) in non-injected cells and in cells injected with 10 μM anti-$\alpha_{q/11}$, anti-α_q or anti-α_{11} antisense oligonucleotides. Myocytes were used 3 days after injection. *Values significantly different from those obtained in non-injected cells, $P<0.01$. External solution was a Ca^{2+}-free, 0.5 mM EGTA-containing solution.

dialysed an anti-β_{com} antibody into the cell by the patch pipette for 8 min. Anti-β_{com} antibody (10 μg ml^{-1}) induced a slight increase of acetylcholine-induced Ca^{2+} release (Figure 6). In a second set of experiments, we dialysed peptides corresponding to fragments of βARK_1 (Nair *et al*., 1995) into the cell by the patch pipette for 5 min. Carboxyl-terminal fragments of βARK_1 have been used to bind G$\beta\gamma$ subunits and to block activation of effectors (Stehno-Bittel *et al*., 1995; Nair *et al*., 1995). Intracellular applications of 100 μM peptide G (corresponding to the G$\beta\gamma$ binding region of βARK_1) or peptide A (corresponding to a domain of βARK_1 not involved in G$\beta\gamma$ binding) had no significant effects on acetylcholine-induced Ca^{2+} release (Figure 6). Taken together, these results suggest that G$\beta\gamma$ subunits are not involved in the muscarinic M_3 receptor-activated transduction coupling leading to Ca^{2+} release from intracellular stores.

Discussion

Our results show that, in mouse duodenal myocytes, the muscarinic M_3 receptor is specifically coupled to the G_q protein and activates a phosphatidylinositol-specific phospholipase C leading to intracellular Ca^{2+} release. This conclusion is based on experiments with antibodies raised against the carboxyl terminus of Gα subunits to block interactions of G proteins with muscarinic receptors, antisense oligonucleotides to block expression of G protein subunits and synthetic peptides acting as $G_{\beta\gamma}$ subunit inhibitors.

In duodenal myocytes, our results are consistent with the idea that a single intracellular Ca^{2+} store is mobilized by acetylcholine as well as by caffeine and ryanodine. This conclusion is supported by the following observations: (1) when Ca^{2+}-ATPases were blocked with thapsigargin, which induces Ca^{2+} leaks from the intracellular Ca^{2+} store, acetylcholine- and caffeine-induced Ca^{2+} responses were abolished; (2) intracellular application of heparin (5 mg ml^{-1}), which completely inhibits $InsP_3$ binding at sites responsible for the Ca^{2+} mobilizing effect of $InsP_3$, suppressed the acetylcholine-induced Ca^{2+} response; (3) pretreatment with ryanodine and caffeine abolished the intracellular Ca^{2+} store sensitive to acetylcholine; (4) the basal $[Ca^{2+}]_i$ was increased (from 50 to 110 nM) in the presence of ryanodine and caffeine suggesting that depletion of the Ca^{2+} store leads also to increased Ca^{2+} entry into the cell (Missiaen *et al*., 1990). These results suggest that the $InsP_3$- and caffeine-sensitive Ca^{2+} store may represent, at least functionally, a single releasable Ca^{2+} pool, in agreement with previous observations in smooth muscle cells (Leprêtre & Mironneau, 1994; Zholos *et al*., 1994).

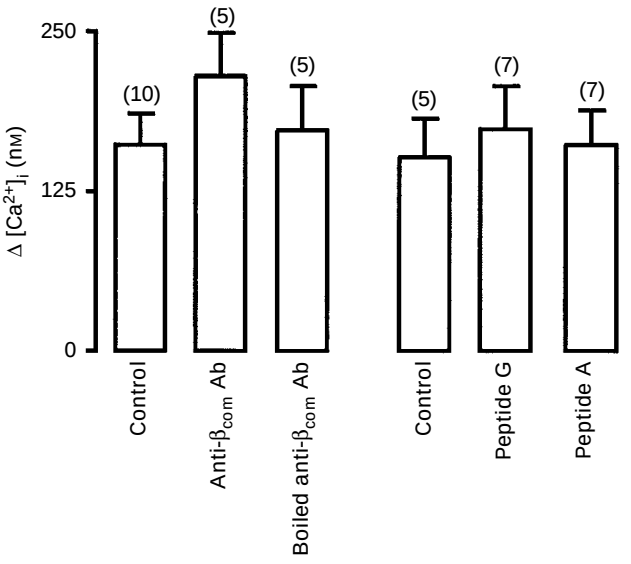

Figure 6 Effects of anti β_{com} antibody and βARK_1 peptides on the increase in $[Ca^{2+}]_i$ induced by 10 μM ACh. In voltage-clamped cells (held at -70 mV), antibodies (10 μg ml^{-1}) and peptides (100 μM) were dialysed intracellularly for 5–8 min with the pipette solution containing 100 μM fura-2. Data are given as means$\pm$s.e.mean with number of experiments in parentheses. External solution was a Ca^{2+}-free, 0.5 mM EGTA-containing solution.

Using affinity sequences for agonists and antagonists and the method of irreversible alkylation of muscarinic M_3 receptors with 4-DAMP mustard in conjunction with protection of muscarinic M_2 receptor by methoctramine (Eglen & Harris, 1993), we showed that only the muscarinic M_3 receptor subtype is involved in intracellular Ca^{2+} release in duodenal myocytes. Evidence supporting this conclusion was obtained from the following results: (1) acetylcholine and carbachol were equally effective at inducing maximal $[Ca^{2+}]_i$ increases, while oxotremorine showed partial agonist properties that were antagonized by low concentrations (nM) of 4-DAMP. Pilocarpine (a muscarinic M_1 receptor agonist, Baumgold *et al*., 1995) had no effect at all. The effect of acetylcholine on $[Ca^{2+}]_i$ was not affected by α-bungarotoxin indicating that functional nicotinic receptors are not present in duodenal myocytes; (2) 4-DAMP and atropine showed a high affinity (around 1 nM) for inhibiting the acetylcholine-induced Ca^{2+} release, whereas pirenzepine, methoctramine and gallamine show a low affinity. This affinity sequence is typical of muscarinic M_3 receptors (Doods *et al*., 1994): (3) after alkylation of the muscarinic M_3 receptor population by 4-DAMP mustard and protection of muscarinic M_2 receptors, application of acetylcholine did not initiate a transient increase in $[Ca^{2+}]_i$. These results are consistent with previous data on the pharmacological profile of muscarinic receptors in smooth muscles (Doods *et al*., 1993; Eglen & Harris, 1993).

Finally, we showed that the transduction pathway activated by muscarinic M_3 receptors leads to stimulation of a G_q protein and hydrolysis of phosphatidylinositols by a phospholipase C. Hydrolysis of phosphatidylinositols by phospholipase C in response to activation of muscarinic M_3 receptors was supported by the observations that intracellular applications of heparin or anti-PtdIns antibody inhibited the acetylcholine-induced Ca^{2+} release. In addition, external application of U73122 (a phospholipase C inhibitor) also blocked the acetylcholine-induced Ca^{2+} release. Identification of the G_q protein which couples muscarinic M_3 receptor to phospholipase C was obtained by using antibodies directed against different subtypes of G proteins and antisense oligonucleotides designed to block synthesis of G protein subunits. Our results indicate that the acetylcholine-induced Ca^{2+} release is selectively inhibited by an anti-$\alpha_{q/11}$ antibody. As this antibody is directed against the carboxyl terminus of the Gα subunit, it cannot discriminate between α_q and α_{11} subunits. Therefore, we performed selective inhibitions of α_q or α_{11} subunit by nuclear injection of antisense oligonucleotides. Only inhibition of the α_q subunit expression was able to suppress the acetylcholine-induced Ca^{2+} release in duodenal myocytes. This is in contrast with a previous study on muscarinic m_1 receptor-induced Ca^{2+} responses by Dippel *et al*. (1996); they proposed a similar role for α_q and α_{11} subunits in inducing release of stored Ca^{2+}. There are at least two explanations for these discrepancies. First, as the latter experiment was performed in Ca^{2+}-containing solution, acetylcholine-induced increases in $[Ca^{2+}]_i$ may depend on both Ca^{2+} release from intracellular stores and Ca^{2+} influx from the external medium. It is possible that α_q and α_{11} subunits may have distinct functions and couple to Ca^{2+} release and Ca^{2+} entry, respectively. It has been recently shown that a nonselective cation channel, the *Drosophila* trpl channel, may be stimulated in a membrane confined way by the Gα_{11} subunit (Obukhov *et al*., 1996). As our experiments were performed in Ca^{2+}-free EGTA-containing solution, we measured only the acetylcholine-induced Ca^{2+} release from the intracellular store. Second, the receptor-G protein interaction may depend on both the membrane environment and structural constraints achieved by the receptor/G protein/effector

complex (Sato *et al*., 1995). These possibilities could also explain why, depending on the cell type or *in vitro* assays used, it has been proposed that phospholipase C-β can be activated by several different Gα proteins including Gα_0 (Blitzer *et al*., 1993), Gα_{i1}, Gα_{i2}, Gα_{i3} (Kaneko *et al*., 1992; Dell' Acqua *et al*., 1993), Gα_q (Aragay *et al*., 1992; Wu *et al*., 1992), Gα_{11} (Aragay *et al*., 1992) and Gα_s (De la Pena *et al*., 1995). Although phospholipase C-β activation through the α subunit of G_q and G_{11} proteins has been largely described, it has been recently shown that, in *Xenopus* oocytes, activation of overexpressed muscarinic m_3 receptors leads to Ca^{2+} release via the $\beta\gamma$ subunits of G_q/G_{11} proteins (Stehno-Bittel *et al*., 1995). In contrast, in duodenal myocytes, the muscarinic M_3 receptor-activated Ca^{2+} release was not affected by an anti-β_{com} antibody or by synthetic peptides derived from βARK_1, which selectively bind to the $G_{\beta\gamma}$ subunits. It is likely that $G_{\beta\gamma}$ subunits are bound to βARK_1 peptides and anti-β_{com} antibody as these substances, used at similar concentrations, inhibit the angiotensin II-induced stimulation of Ca^{2+} channels in vascular myocytes (N. Macrez, J.L. Morel, F. Kalkbrenner, P. Viard, G. Schultz & J. Mironneau, unpublished data). Our results suggest that the stimulation of phospholipase C by activation of muscarinic M_3 receptors in duodenal myocytes occurs via the α subunit of the G_q protein. As the PLC-β isoforms mediating Ca^{2+} release from the intracellular store have not been identified in either cell, it can be postulated that in duodenal myocytes, the Gα_q subunit predominantly activates PLC-β_1 (Jhon *et al*., 1993) whereas in *Xenopus* oocytes, the G$\beta\gamma$ subunit predominantly activates PLC-β_2 (Camps *et al*., 1992).

In conclusion, in mouse duodenal myocytes, the release of Ca^{2+} from the intracellular store is mediated through activation of M_3 muscarinic receptors which couple with the G_q protein and activate a phosphatidylinositol-specific phospholipase C-β.

This work was supported by grants from Centre National de la Recherche Scientifique et Centre National des Etudes Spatiales, France. We are indebted to Dr G. Guillon (INSERM 469, Montpellier) for giving us anti-α_q/α_{11} antibodies and to Pr G. Schultz and Dr F. Kalkbrenner (Institut für Pharmakologie, Berlin) for giving us oligonucleotides.

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(Received January 8, 1997
Revised February 24, 1997
Accepted February 27, 1997)