Downregulation of muscarinic M_2 receptors linked to K^+ current in cultured guinea-pig atrial myocytes

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1. Desensitization of muscarinic K^+ current ($I_{K(ACh)}$) was studied in cultured atrial myocytes from guinea-pig hearts using whole-cell voltage clamp.

2. Three different types of desensitization could be identified. A fast component which upon rapid superfusion with ACh resulted in a partial relaxation of $I_{K(ACh)}$ within a few seconds to a plateau which was maintained in the presence of ACh. Recovery from this type of desensitization paralleled the decay of $I_{K(ACh)}$ after washout of the agonist. A second type of desensitization was observed within minutes. This was reversed around 10 min after washout of ACh. Both types were heterologous with regard to the A_1 receptor and the novel phospholipid (Pl) receptor, both of which activate $I_{K(ACh)}$ via the same signalling pathway.

3. A third type of desensitization (downregulation) occurred upon exposure of the cultures for 24–48 h to the muscarinic agonist carbachol (CCh). The level of downregulation depended on the concentration of CCh (0·1 μM $\leqslant$ [CCh] $\leqslant$ 10 μM). No recovery was observed within 5 h after washout of CCh. Thereafter sensitivity to ACh slowly returned (half-time ($t_{1/2}$), ~20 h).

4. Downregulation by CCh (0·1–5 μM) was characterized by an increase in EC_{50} for ACh with no reduction in maximum $I_{K(ACh)}$. With 5 μM CCh, EC_{50} was increased from 0·1 to 3·7 μM. At 10 μM CCh EC_{50} was increased to 15 μM and maximal current that could be evoked by ACh was reduced to 15%.

5. Downregulation by CCh was homologous with regard to A_1 and Pl receptors. Maximum $I_{K(ACh)}$, assayed by a saturating concentration of Pl, was not reduced in downregulated cells, suggesting a mechanism localized at the M_2 receptor.

6. The changes in the concentration–response curves can be accounted for by assuming an excess of M_2 receptors relative to the subsequent component of the signalling pathway.

7. As the intact heart is under tonic vagal control, downregulation is likely to contribute to controlling the sensitivity of the heart to vagal activity *in situ*.

Parasympathetic control of the heart is mediated by acetylcholine (ACh) released from vagal nerve endings. ACh binds to muscarinic M_2 receptors (M_2R), which belong to the superfamily of G protein-coupled receptors. Via activation of a pertussis toxin-sensitive G protein (G_i/G_K) a variety of cellular events is initiated. One major effector in supraventricular myocytes is an inward-rectifying K^+ (K_{ACh}) channel (Kurachi, 1994; Reuveny *et al.* 1994).

Long-term or repetitive exposure of a G protein-coupled receptor to an agonist results in events summarized under the term desensitization. Desensitization refers to different processes developing with different velocity upon stimulation by an agonist (Debburman & Hosey, 1995). These processes are either limited to the one type of receptor (homologous desensitization) or other receptors converging on the same signalling pathway (heterologous desensitization).

So far, the mechanisms underlying desensitization have been elucidated in only a few systems, originally for the β-adrenergic receptor–adenylate cyclase system (for review see Huganir & Greengard, 1990). Desensitization in that pathway involves receptor-dependent activation of a β-receptor kinase (β-ark) the substrate of which is the agonist-ligated receptor itself. The phosphorylated receptor represents one desensitized state. Internalization, degradation and inhibition of receptor synthesis provide additional mechanisms, summarized under the term 'downregulation', that contribute to long-term control of the sensitivity of a cell to a β-agonist (for review see Hausdorff, Caron & Lefkowitz, 1990). Analogous mechanisms have been shown to exist in muscarinic receptor (MR)-activated pathways (Debburman & Hosey, 1995). Phosphorylation of MRs concomitant with a loss of high-affinity muscarinic binding sites can be induced in embryonic chick heart by

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brief (~10 min) treatment with carbachol (Kwatra *et al.* 1987). There is evidence that a kinase belonging to the family of G protein-coupled receptor kinases (GRKs) is involved in M_2R phosphorylation (Kwatra, Benovic, Caron, Lefkowitz & Hosey, 1989).

One major mechanism by which vagal activity controls the heart is via the M_2R–G_i–$I_{K(ACh)}$ pathway (Hutter & Trautwein, 1955; Honjo, Komada, Zang & Boyett, 1992). $I_{K(ACh)}$, the major target of M_2R activation in cardiac supraventricular tissue shows partial desensitization upon application of agonist. In voltage clamp studies on isolated atrial myocytes at least two types of desensitization have been described, a fast decay with a half-time ($t_{½}$) of the order of a few seconds (Kurachi, Nakajima & Sugimoto, 1987; Kim, 1993) and a slower decay with a half-time of some tens of seconds (Honjo *et al.* 1992; Zang, Yu, Honjo, Kirby & Boyett, 1993; Wang & Lipsius, 1995). Evidence has been provided that the fast phase occurs at the level of the G protein although details remain to be elucidated; a contribution of $I_{K(ACh)}$ channels to rapid desensitization is also being discussed (Zang *et al.* 1993). The slow phase, which seems to be sensitive to a loss of an intracellular component in conventional patch clamp experiments, may reflect phosphorylation of the M_2R (Shui, Boyett & Zang, 1994).

Because of the tonic nature of vagal control in the intact organism, the M_2Rs are exposed to ACh on a time scale that is quite different from that achieved by superfusing a cell in an acute experiment. Little is known of the properties and consequences of desensitization related to the natural physiological situation. In the present study the effect of long-term ($\geqslant$24 h) exposure to carbachol (CCh) of cultured atrial myocytes on functional desensitization in the M_2R–G_i–$I_{K(ACh)}$ pathway was studied. A third novel type of desensitization (downregulation) with slow recovery over a period of days was identified and the underlying mechanism analysed.

This is the first study in a terminally differentiated cell, of desensitization caused by chronic treatment with an agonist, a situation which to an unknown extent is likely to prevail *in situ*. A prerequisite for this investigation is the availability of an *in vitro* system for adult myocytes which retain the quantitative properties of the relevant signalling pathway for several days. Preliminary data have been published in abstract form (Bünemann & Pott, 1994).

METHODS

Isolation and culture of atrial myocytes

Guinea-pigs of either sex (200–250 g) were killed by cervical dislocation. The method of enzymatic isolation of atrial myocytes has been described in detail previously (see Banach, Hüser, Lipp, Wellner & Pott 1993*b*). The 'standard' culture medium was bicarbonate-buffered M199 (Gibco, Dreieich, Germany) containing gentamicin (10 μg ml^{-1}) and kanamycin (10 μg ml^{-1}) (Sigma, Deisenhofen, Germany); culture medium was not supplemented with fetal calf serum (FCS). Cells were plated at several hundred cells per dish (36 mm diameter). Fifty to seventy per cent of the myocytes attached within 16–24 h. Cells were cultured and used experimentally for up to 8 days. The medium was changed every second day. If culture medium containing CCh was used this was changed every 24 h.

Solutions

The culture medium was replaced 30 min prior to an experiment by a solution containing (mM): NaCl, 120; KCl, 20; $CaCl_2$, 2·0; $MgCl_2$, 1·0; Hepes–NaOH, 10·0, pH 7·4. The solution for filling the patch clamp pipettes for whole-cell voltage clamp experiments contained (mM): potassium aspartate, 100; KCl, 40; MgATP, 5·0; EGTA, 2·0; GTP, 0·01; Hepes–KOH, 10·0, pH 7·4. The intracellular concentration of Cl^- and other anions have been shown to affect sensitivity of $I_{K(ACh)}$ to both receptor agonists and intracellular GTP (Nakajima, Sugimoto & Kurachi, 1992). Dialysis of a cell with the above solution resulted in ACh-evoked currents, the amplitude of which remained fairly constant from starting the whole-cell mode throughout long-lasting recordings. Thus, sensitivity of the signalling pathway investigated was not measurably affected by the combination of anions in the dialysing solution.

Standard salts were obtained from Merck (Darmstadt, Germany). EGTA, Hepes, MgATP, GTP, ACh chloride, adenosine (Ado), CCh chloride, and serum lipids were obtained from Sigma.

Current measurement

Membrane currents were measured in the whole-cell mode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Pipettes were made from borosilicate glass with a filament (Clark; Pangbourne, Berks, UK) on a horizontal puller (DMZ; Munich, Germany) and were backfilled with pipette solution (see above). The DC resistance of the filled pipettes ranged from 2 to 6 MΩ. Current measurements were performed by means of a patch clamp amplifier (List LM/EPC 7; Darmstadt, Germany). Signals were passed through an analog filter (corner frequency, 1–3 kHz) and were digitally stored on the hard disk of an IBM-compatible AT-computer, equipped with a hardware–software package (ISO2; MFK, Frankfurt/Main, Germany) for voltage control, data acquisition, and data evaluation. Experiments were performed at ambient temperature (22–24 °C). If not otherwise stated, cells were voltage clamped at a holding potential of −90 mV, i.e. negative to the potassium reversal potential (E_K, −50 mV). K^+ channel currents under these conditions are in the inward direction. These experimental conditions were chosen because of the strong inward-rectifying properties of $I_{K(ACh)}$. Ramp-shaped changes in membrane potential (E_m) from −120 to +60 mV were used to measure current–voltage relations and to check constancy of electrical access to the cell. Rapid superfusion of the cells for application and withdrawal of different solutions was performed by means of a solenoid-operated flow system which permitted switching between up to six different solutions. The half-time of exchange of solution seen by the superfused cell was estimated as 200–400 ms using a change to a solution to which CsCl was added at 5 mM. This causes an instantaneous block of $I_{K(ACh)}$.

Whenever possible results are presented as means ± standard deviation (S.D.). Significance of differences was tested using Student's *t* test.

RESULTS

Two types of acute desensitization of $I_{K(ACh)}$

Superfusion of a myocyte with ACh at a concentration of $\geqslant$1 μM resulted in an inward current at holding potential of −90 mV. In individual cells this current either remained

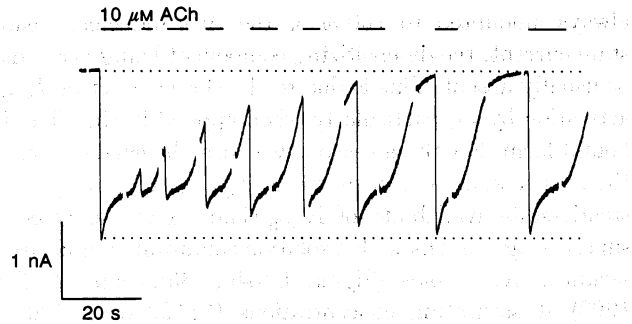

Figure 1. Rapid desensitization and recovery from desensitization of $I_{K(ACh)}$
Atrial myocyte cultured for 4 days in standard culture medium. ACh (10 μM) was superfused as indicated by horizontal bars. Dotted lines mark basal current level (upper line) and peak $I_{K(ACh)}$ (lower line). Gaps in the trace result from the fact that current deflections due to voltage ramps have been blanked out for clarity.

virtually constant in the presence of the agonist for periods up to 1 min, or partially desensitized with a single exponential time course ($t_{½}$, ~2 s) to a steady-state level which was maintained over several minutes. A representative example is illustrated in Fig. 1.

Superfusion of the cell with ACh-containing solution was interrupted by variable periods of ACh-free solution ranging from 2·2 to 17 s. Recovery from desensitization was directly related to the washout of ACh, i.e. as soon as the current had reached the baseline, a challenge by ACh produced the full response. This behaviour was found in all twelve myocytes tested in this way and was independent of the agonist concentration. A second, slower component of desensitization which has been reported to occur during acute application of an M-agonist (Honjo *et al.* 1992; Shui, Boyett, Zang, Haga & Kameyama, 1995; Wang & Lipsius, 1995) was not detected as a distinct kinetic relaxation phase during brief application of ACh but could be identified as a reduction in sensitivity to ACh (see below). The absence of a slow component to the relaxation of $I_{K(ACh)}$ in the present experiments may have been due to the lower temperature used here.

As shown previously, the concentration dependence of fast desensitization for this receptor–ion channel system is unusual. Increasing concentrations of ACh resulted in an increase in the desensitizing component of the current response; this was superimposed on a steady-state component which saturated at a lower concentration. A crossover of the two components because of 'stronger' desensitization to a lower steady-state current at large ACh concentrations, a situation which is typical of ionotropic receptors (e.g. Tang, Dichter & Morad, 1989), but which was also found in a study of $I_{K(ACh)}$ in intact Purkinje fibres (Carmeliet & Mubagwa, 1986), was never observed in the present investigation.

Fast desensitization was small or absent if the system was activated via A_1 receptors which, for a saturating concentration of Ado (≥0·1 mM), yielded less than 70% of the steady-state current available to ACh, presumably because of the lower density of A_1 compared with M_2 receptors (Bünemann & Pott, 1995). Activation of $I_{K(ACh)}$ by PI present in serum (Banach, Hüser, Lipp, Wellner & Pott, 1993*b*; Bünemann & Pott, 1993; Bünemann, Brandts, Meyer zu Heringdorf, van Koppen, Jakobs & Pott, 1995)

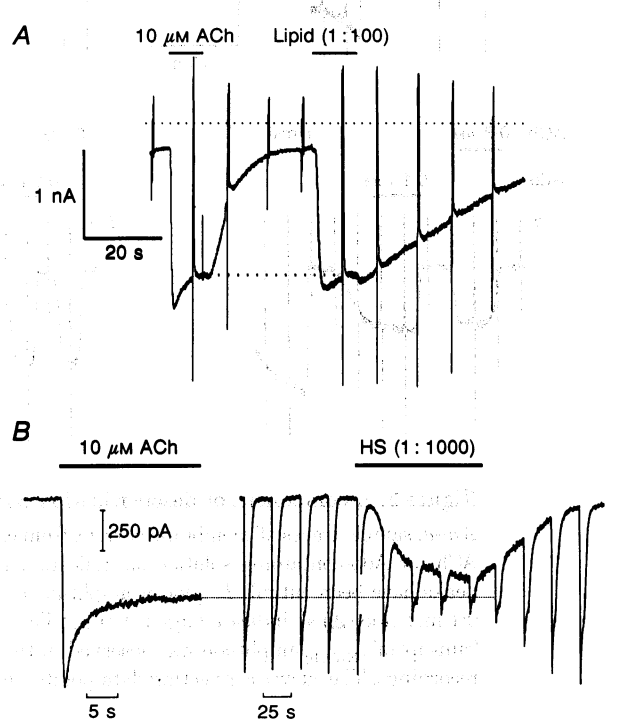

Figure 2. Activation and desensitization of $I_{K(ACh)}$ by a serum-derived phospholipid
A, identity of steady-state $I_{K(ACh)}$ levels due to saturating concentrations of ACh and active phospholipid (PI) factor. During the period of time marked 'Lipid (1 : 100)' the cell was superfused with solution containing serum-derived lipids (Sigma L4646, 1 : 100 dilution with standard solution). The upper and lower dotted lines indicate zero current and steady-state level of $I_{K(ACh)}$, respectively. *B*, desensitization of ACh-evoked $I_{K(ACh)}$ during slow activation with human serum (HS) diluted 1 : 1000. The right trace represents a low-speed recording of membrane current. Rapid inward current transients are due to periods (8 s in duration) of superfusion with ACh (10 μM). The left trace represents an expanded response caused by longer superfusion with ACh to reveal steady-state level of $I_{K(ACh)}$ (dotted line).

always amounted to 100% of the ACh-inducible steady-state current, the desensitizing component being very small or usually absent. This is due to the slower onset of $I_{K(ACh)}$ activation by PI, particularly when applied in the albumin-bound form. For these reasons we used the current level in the steady state as a measure of $I_{K(ACh)}$ availability. Total steady-state availability of K_{ACh} channels was assessed by superfusing the cells with a solution containing a mixture of serum-derived lipids (Sigma L4646, Bünemann & Pott, 1993) at saturating concentrations (1:100 or 1:500). A representative example is illustrated in Fig. 2*A* which compares $I_{K(ACh)}$ evoked by 10 μM ACh and the mixture of serum lipids.

Activation of $I_{K(ACh)}$ by PI causes cross-desensitization; the response to ACh is attenuated during partial activation of $I_{K(ACh)}$ by PI. This is illustrated in Fig. 2*B*. In this cell, $I_{K(ACh)}$ was repetitively activated by brief (8 s) pulses of ACh (10 μM). The desensitizing component was particularly prominent in this myocyte. During the period indicated, diluted (1:1000 with standard solution) human serum (HS), which contains PI in an albumin-bound but still active form, independent of its source (Banach *et al.* 1993*b*), was superfused. At this dilution, corresponding on average to the EC_{50} of HS, this particular cell responded with slow activation of $I_{K(ACh)}$ to about 80% of steady-state current. The identification of HS- or PI-induced current as $I_{K(ACh)}$ has been demonstrated previously (Bünemann & Pott, 1993; Banach *et al.* 1993*a*, *b*). Currents due to superimposed pulses of ACh are reduced in amplitude solely because of a reduction of the desensitizing component. Analogous to the observation illustrated in Fig. 1, the desensitizing effect of HS or PI is linked to the degree of activation of $I_{K(ACh)}$. Recovery of ACh-evoked current from desensitization strictly parallels the decay of the HS effect. This finding clearly demonstrates that fast (acute) desensitization is heterologous for ACh *vs.* PI. Heterologous (cross)-desensitization in the atrial muscarinic signalling pathway has been described previously also for ACh *vs.* Ado (Kurachi *et al.* 1987). The heterologous properties of the fast desensitizing component support the view of a target downstream from the receptors, such as a common pool of G proteins.

Using protocols similar to those illustrated in Figs 1 and 2 with various intervals between agonist applications, we saw desensitizing effects only. A potentiating effect of a pulse of ACh, or any other activator, on a subsequent response to ACh, as has been reported for perforated patch recordings from cat atrial myocytes (Wang & Lipsius, 1995), was not detected in the present conventional whole-cell recordings.

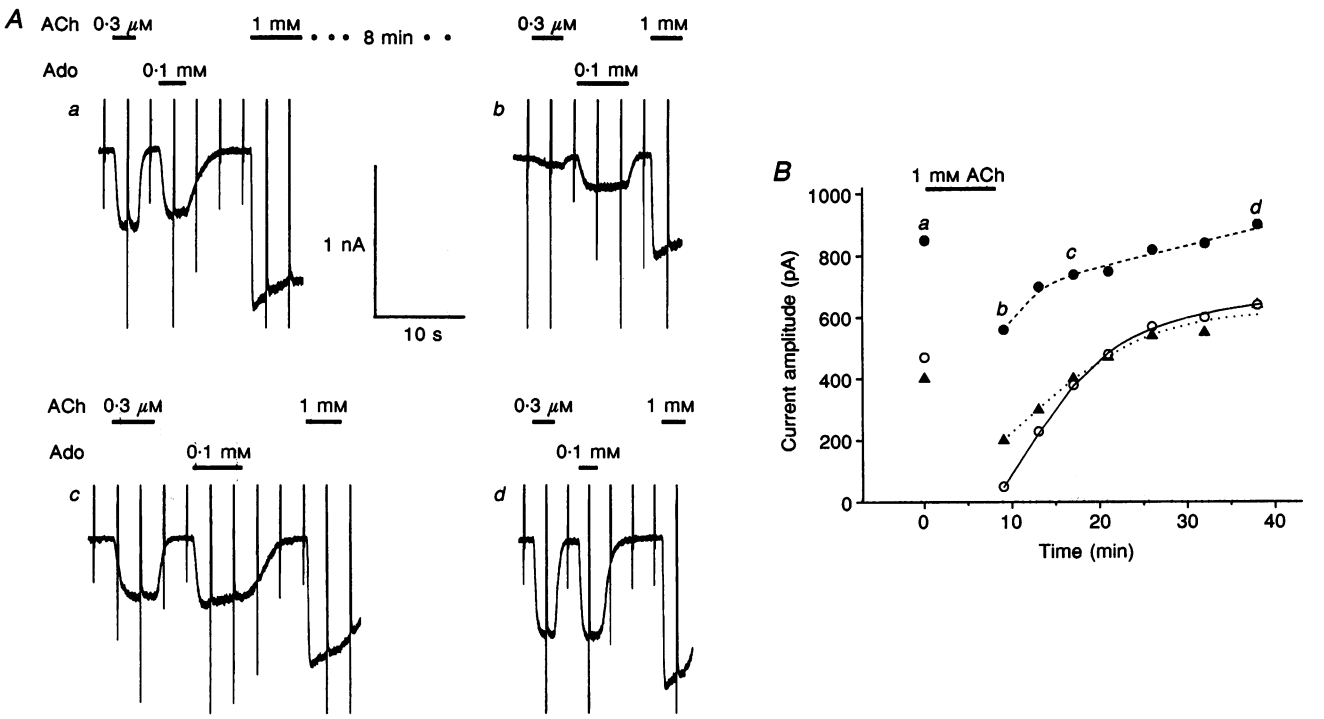

Figure 3. A component of desensitization with slow recovery

A a–d, sample traces of membrane current from corresponding times marked in *B*. Cell was superfused with ACh- or Ado-containing solution as indicated. Rapid current deflections were caused by voltage ramps; peaks have been cut off. *B*, plot of amplitude of inward $I_{K(ACh)}$ elicited by 0·3 (○) and 1 mM (●) ACh and 0·1 mM Ado (▲) as indicated against time. Time $t = 0$ corresponds to the beginning of trace *A a*. A slight 'run-up' of $I_{K(ACh)}$ amplitude was observed in this experiment which was sometimes seen during long-term recordings. The curves connecting data points were drawn by eye.

Although a slower component of desensitization as described previously (Kurachi *et al.* 1987; Shui *et al.* 1995) was not identified as a distinct relaxation phase, we did, however, identify a type of densensitization that was intermediate between the fast type described above and the stable long-lasting type which will be dealt with later.

In the experiment illustrated in Fig. 3 the cell was superfused either with ACh at two different concentrations (0·3 μM and 1 mM) or with Ado (0·1 mM). The low concentration of ACh and the challenge by Ado were used to test the sensitivity of the cell to either of the two agonists; the largely saturating concentration of 1 mM ACh which is more than 10^3-fold the published half-saturating concentration (e.g. Glitsch & Pott, 1978; Inomata, Ishihara & Akaike, 1989), served to desensitize the cell. At the beginning of the measurement (Fig. 3*A a*) the responses to 0·3 μM ACh and 0·1 mM Ado amounted to 54 and 48%, respectively, of the steady-state current elicited by 1 mM ACh. After 10 min superfusion with 1 mM ACh the subsequent responses were all reduced in amplitude (Fig. 3*A b*). Reduction of $I_{K(ACh)}$ caused by 1 mM ACh was 37%, whereas the response to the lower concentration of ACh was only 6·7% of the initial value. The response to Ado was reduced to 37% of control. Recovery of the reponses occurred with a half-time of around 10 min (Fig. 3*A b*, *A c*, and *B*), revealing a component of desensitization, which continues long after the desensitizing stimulus has been removed. The qualitatively parallel reduction and recovery of the currents evoked by 0·3 μM ACh and 0·1 mM Ado demonstrate that this slower type of 'acute' desensitization is also heterologous. Cross-desensitization was also observed if in a similar type of experiment sensitivity to Pl was tested (not shown).

The result illustrated in Fig. 3 is qualitatively representative of four other cells from which $I_{K(ACh)}$ was successsfully recorded for such a long period of time. It demonstrates that the major fraction of desensitization caused by exposure of a cell to a M-agonist for periods of tens of minutes is reversible with a similar time course but, in contrast to the fast phase of desensitization, outlasts the presence of the agonist. In six cells treated with 1 mM ACh in the incubator for 30 min, it was found that after a 60–90 min incubation with agonist-free solution sensitivity to ACh was indistinguishable from that of control cells from non-treated sister cultures. Thus, to investigate more stable components of desensitization or downregulation, the duration of agonist exposure has to be longer than in the above experiments, and after removal of the agonist cells have to be washed for periods of time long enough to ensure complete reversibility of the above type of desensitization.

A long-lasting component of desensitization

Long-term effects of muscarinic stimulation on sensitivity of atrial myocytes to ACh were examined using incubation for 24–48 h in the presence of the stable M-agonist carbachol (CCh). The long-lasting effects of such treatment on the sensitivity of a cell to ACh will be termed 'downregulation' in the following text. This does not imply any particular mechanism but distinguishes such effects from the acute types of desensitization described above. The time course of development of downregulation resulting from this treatment was not studied in detail. It was consistently found that after about 20 h a stable level of reduced ACh-sensitivity was reached which was dependent on the concentration of CCh in the culture medium. This degree of downregulation was maintained if incubation with the M-agonist was prolonged by another 24–28 h. Thus, the data presented below are based on measurements from myocytes which had been exposed to CCh for periods of between 24 and 48 h.

The concentration–response curve for acute activation of $I_{K(ACh)}$ by the stable agonist CCh was determined in a separate set of measurements in eight different cells (four cultures from two different animals; time in culture was 24–36 h). These measurements yielded a mean EC_{50} of 3·8 μM and a Hill coefficient (n_H) of 1·35. Maximum currents activated by ACh and CCh were identical (data not shown).

Cultures incubated with CCh were carefully washed 3 times with an excess of agonist-free medium, and were then equilibrated in culture medium at 37 °C for at least 60 min before being transferred to the voltage clamp setup. This

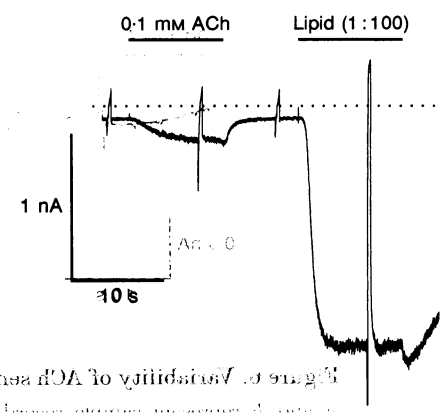

Figure 4. Reduction of ACh-evoked $I_{K(ACh)}$ in a CCh-treated cell
Typical response to ACh and serum lipid of a myocyte that was cultured for 2 days in the presence of 10 μM CCh. After treatment with CCh cultures were washed as described in Methods in order to avoid superposition with slow component of 'acute' desensitization.

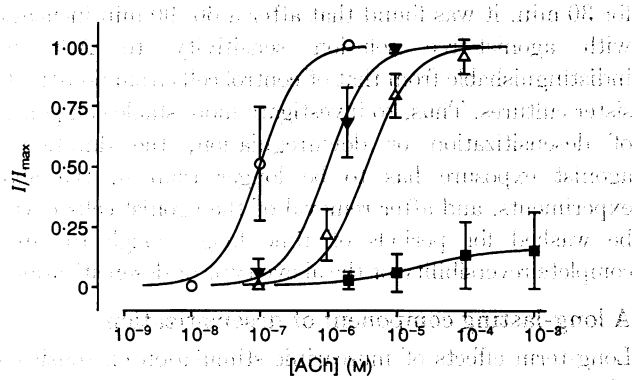

Figure 5. Effect of downregulation on concentration–response curves

$I_{K(ACh)}$ was activated by various concentrations of ACh and normalized to the current evoked by Pl (1 : 100) in the same cell. Effect of 0 (control, ○), 0·1 (▼), 5 (△), and 10 μM (■) CCh on the concentration–response curve. The parameters for the control, 0·1, 5 and 10 μM fitted curves are: EC_{50}: 0·14, 1·01, 3·7 and 13 μM, respectively; n_H: 1·47, 1·41, 1·32 and 1·2, respectively. Number of cells (n) was 6–8 for each data point. Control data were recorded from cells that were in culture for 16–50 h in standard culture medium.

protocol was applied so as to avoid contamination by the slow type of acute desensitization. As will be shown below, downregulation induced by incubation with the M-agonist was not measurably reversed over this period. By means of the Pl standard, the maximum $I_{K(ACh)}$ evoked by a saturating concentration of Pl, which yields M_2R-independent information on maximum steady-state $I_{K(ACh)}$ in each cell, responses to various desensitizing treatments could be normalized. A typical result recorded from a myocyte that had been treated for 48 h with a concentration of CCh (10 μM) close to saturation for activation of $I_{K(ACh)}$ is illustrated in Fig. 4. In cells treated in this way, a highly saturating concentration of ACh (100 μM) resulted in a slowly developing inward $I_{K(ACh)}$. The steady-state current, which was reached about 10 s after switching to ACh-containing solution – compared with typically less than 800 ms in an untreated cell (see Fig. 1) – amounted only to 9% of the Pl standard.

The mean value of the ACh-evoked current normalized to the Pl standard for these conditions (10 μM CCh for 24–48 h) was 0·15 ± 0·15 (n = 8). The typical behaviour of a control cell which, after isolation, was kept for ≥ 24 h in a CCh-free culture medium has been shown in Fig. 2; steady-state ACh-evoked current and lipid standard under those conditions are always identical. Densities of Pl-evoked currents of untreated and CCh-treated cells were not significantly different (not shown).

Effect of downregulation on the ACh concentration–response curve

In the example illustrated in Fig. 4 the maximum current that could be activated by ACh was reduced, a consistent finding, if a high concentration of CCh was used as a desensitizing treatment. The concentration–response curve for $I_{K(ACh)}$ activation by ACh obtained from cells treated with 0·1, 5 or 10 μM CCh (24–48 h), and the control curve representing cells that were cultured for the same period of time without CCh, are illustrated in Fig. 5 (for further details see legend). Formally downregulation by 10 μM ACh can be described by a 150-fold shift of EC_{50} (from 0·1 to 15 μM), and a reduction of the maximum current to a mean value of ~15%.

A shift in the concentration–response curve was observed at all CCh concentrations tested (0·1–10 μM). A concomitant reduction of maximal $I_{K(ACh)}$, however, was found only for concentrations > 5 μM. Incubation with CCh at 0·1 μM ≤ [CCh] ≤ 5 μM produced an increase in EC_{50} only; the magnitude of this increase was concentration dependent. Maximum current was not significantly reduced by such a moderate desensitizing treatment. In cells treated with 0·1 μM CCh EC_{50} was shifted from 0·1 μM (control) to 1·01 μM. Raising [CCh] to 5 μM resulted in an EC_{50} of 3·7 μM. The slope factors yielding the best fit to the experimental data were not significantly different: 1·47 (control); 1·41 (0·1 μM CCh) and 1·32 (5 μM CCh).

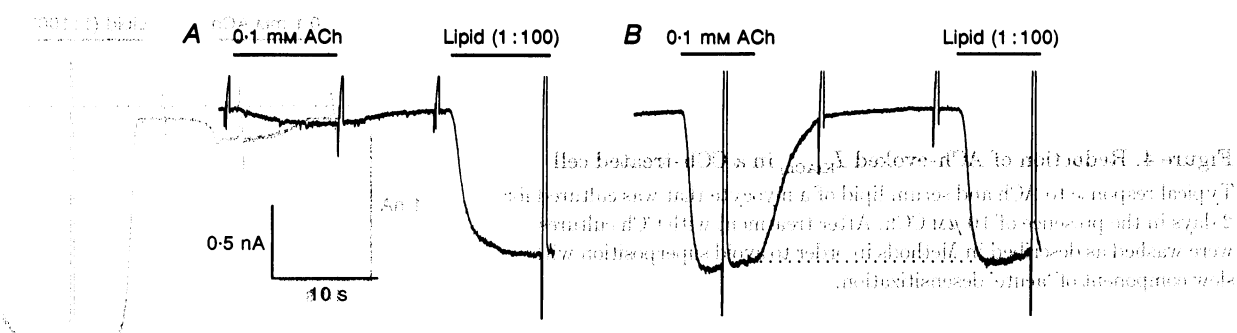

Figure 6. Variability of ACh sensitivity at an intermediate degree of downregulation

A and *B* represent sample recordings, showing exposure to ACh and serum lipids, from two different myocytes in the same culture incubated for 24 h with 7 μM CCh.

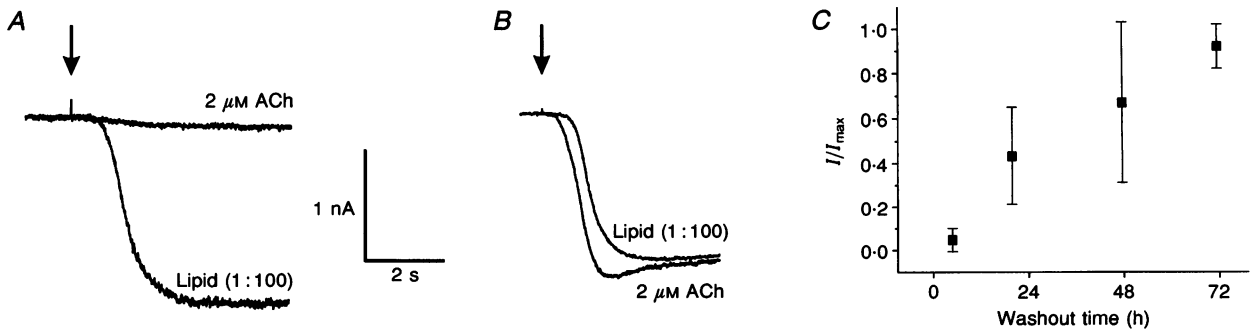

Figure 7. Recovery from downregulation

Cells were desensitized by incubation with CCh for 24–40 h and, thereafter, washed as described above. *A* and *B* are representative recordings from this series of measurements, upon exposure to ACh and serum lipids, from a myocyte that was studied within 2 h after the washing procedure (*A*) and after 3 days in CCh-free solution (*B*). *C*, plot of summarized data. Current evoked by ACh (2 μM) normalized to PI-induced current recorded from the same myocyte against time after withdrawal of CCh. ($t = 0$ corresponds to 90 min after the cell had been washed with CCh-free medium in order to avoid contamination by the intermediate type of desensitization.) The time values represent the time when the culture was removed from the incubator. Measurements were performed within 2 h thereafter; each data point represents 6 cells from 2 or 3 different cultures.

Treatment with an intermediate concentration of 7 μM CCh resulted in extremely variable responses. This variability in sample traces recorded from two cells of the same culture is illustrated in Fig. 6.

Recovery from downregulation

To determine whether long-term downregulation can be reversed, cultures were first treated with CCh (10 μM) for 24–30 h, subsequently washed with CCh-free medium and placed in the incubator for various periods of time. $I_{K(ACh)}$ evoked by 2 μM ACh, normalized to the PI standard, was again used to assess the degree of downregulation. The summarized results are illustrated in Fig. 7. No significant recovery was observed within the first 5 h after withdrawal of CCh. This is further evidence that the process(es) underlying downregulation is different from the desensitization caused by short-term exposure to agonist (e.g. Fig. 3).

After a lag of several hours, $I_{K(ACh)}$ recovered with a half-time of around 20 h. Full recovery was reached after about 50 h. As indicated by the size of the error bars in the steep part of the recovery curve in Fig. 7 the data are extremely variable, analogous to the observation illustrated in Fig. 6. Comparison of the concentration–response curves at various stages of recovery (Fig. 8), shows a time-dependent increase in maximum current (I_{max}) and a decrease in EC_{50}. In this series of measurements, EC_{50} ranged from 30 μM (no recovery) to 0·3 μM after 3 days, which is close to the EC_{50} determined in the series of control experiments shown in Fig. 5. No significant difference was found in this series of measurements between concentration–response curves after 3 and 6 days.

Downregulation is homologous

Evidence has been presented that the rapid desensitization shown in Fig. 1 proceeds at the level of the G protein, although the final nature of this process so far is not completely understood. One observation in support of this view is that rapid desensitization is heterologous for ACh–Ado (Kurachi *et al.* 1987) and, as shown above (Fig. 2), also for ACh–PI. Downregulation of the putative PI receptor by chronic exposure to an M-agonist can be excluded. In cells in which the response to an intermediate concentration of ACh is downregulated to less than 10% the density of the current available to activation by PI is not

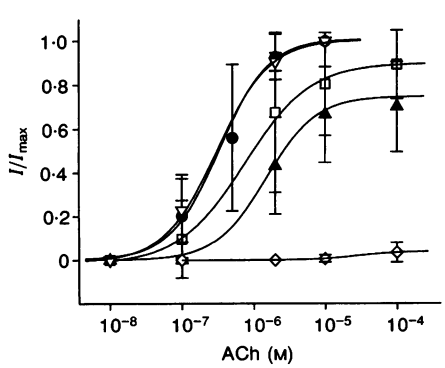

Figure 8. Recovery from downregulation reverses shift of concentration–response curves

ACh-evoked membrane currents normalized to PI-induced current in the same cell. Cultures were incubated for 24 h with 100 μM CCh and thereafter washed as described. Concentration–response curves were determined within 2 h of the times indicated (◇, 0 h; ▲, 20 h; □, 48 h; ●, 72 h; ▽, 6 days).

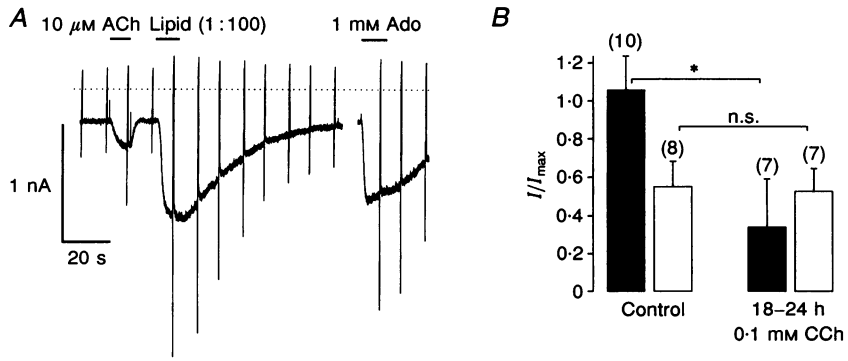

Figure 9. Downregulation is homologous

A, sample recording of membrane current from a cell that was cultured in the presence of CCh (1 mM) for 18 h and washed using the standard procedure. *B*, comparison of normalized (to Pl) responses to 10 μM ACh (■) and 1 mM Ado (□) in control cells and cells treated with CCh for 18–24 h. n.s., not significantly different; * $P < 0{\cdot}01$. Values in parentheses are numbers of cells.

different from untreated cells. As shown recently, treatment of the cells with Pl (or sphingosine-1-phosphate (SPh-1-P), an agonist at the putative Pl receptor) also causes desensitization which is strictly homologous, i.e. it does not affect sensitivity to ACh (Bünemann *et al.* 1995).

To study the specificity of long-term downregulation of the response to the M_2 receptor, $I_{K(ACh)}$ activated by Ado was measured. We have previously shown that in the same preparation maximal activation of $I_{K(ACh)}$ by Ado on average was about 60% of steady-state $I_{K(ACh)}$ activated either by ACh or Pl (Bünemann & Pott, 1995).

Figure 9*A* illustrates a representative current recording from a cell that had been exposed to 10 μM CCh for 24 h. The response to 10 μM ACh was slow (cf. Fig. 1) and only reached 25% of the response to Pl. Superfusion with a solution containing 1 mM Ado resulted in a current which was 3-fold larger than that elicited by ACh. On the other hand, the ratio of Ado- to Pl-induced current did not differ from that in control cells. The summarized data from seven CCh-treated cells are illustrated in Fig. 9*B* and demonstrate that downregulation of the pathway under study is homologous, i.e. it does not seem to affect other seven-helix receptors converging on the same signalling pathway.

A simple model to account for the increase in EC_{50}

As shown in Figs 5 and 8, various degrees of downregulation can formally be described by a shift in EC_{50} and, for strong downregulation, an additional reduction of maximum current. It is evident that the shift in EC_{50} is unlikely to reflect a corresponding reduction of affinity of the functional M_2 receptors, since this would require that the affinity changes over more than two orders of magnitude. Within that range, receptors with different affinities should exist. The resulting concentration–response curves in the range of 'mixed K_Ds' should display reduced slopes compared with the control situation.

Both the shift of apparent affinity and the reduction of I_{max} can be accounted for by assuming an excess of receptor molecules (spare receptors) converging on the subsequent signalling component, whereas the binding affinity of functional receptors remains unaffected in treated cells. For a minimal model of this behaviour one can suppose that downregulation does not affect the number of available channels. This supposition is supported by the finding that maximal $I_{K(ACh)}$ density, assessed by means of the Pl standard, remains unchanged in CCh-treated cells. For the same reason it is unlikely that the number of functional

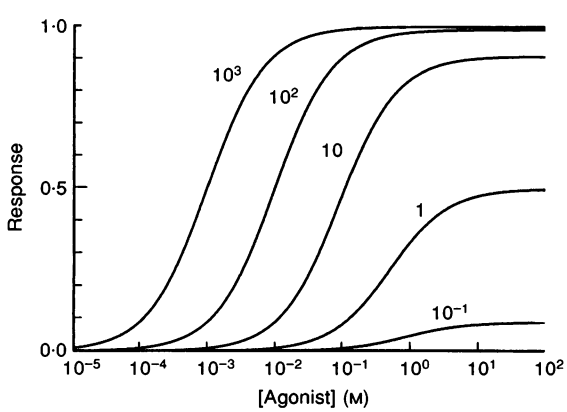

Figure 10. Simulation of concentration–response curves

Concentration–response curves have been calculated using eqn (2), for different receptor densities ranging from 10^3 to 10^{-1}. All other variables have been set to 1.

G protein molecules is affected since the response to neither Pl nor Ado differs from controls in CCh-treated cells. Both receptors are assumed to be linked to $I_{K(ACh)}$ by the same population of G_i/G_K as M_2R. In that case the response in the steady state depends on the fraction of active G protein subunits, which is assumed to be under the control of the steady-state number of ligated receptors.

Steady-state receptor occupancy is given by simple saturation kinetics (for simplicity a Hill coefficient of 1 is used):

$$[AR] = \frac{R_T[A]}{[A]K_A}, \quad (1)$$

where AR is the agonist (A)–receptor (R) complex, R_T is the total number of receptor molecules, and K_A is the equilibrium constant for agonist–receptor interaction. Inserting eqn (1) into a binding equation for steady-state (one-to-one) interaction of AR with a subsequent component of the signalling pathway, such as the G protein (G), yields:

$$[AR - G] = \frac{CG_T}{1 + (K_G/R_T)(1 + K_A/[A])}, \quad (2)$$

with G_T and R_T denoting the total numbers of G protein and receptor molecules respectively, and K_G denoting the equilibrium constant for the G_i–channel interaction. C is a constant to account for the lifetimes of activated G_i, kinetics of $G_{\beta\gamma}$–channel interaction, lifetime of the activated state of the channel, etc. Assuming that downregulation affects receptor density (R_T) only, C, G_T, K_G, and K_A have been set to unity. A simulation of concentration–response curves for different receptor densities over a range of four orders of magnitude is illustrated in Fig. 10. In this simple simulation a reduction of receptor density by a factor of ten, from 10^3 to 10^2 times G_T, results in a rightward shift of the concentration–response curve by a factor of ten.

A further 10-fold reduction has the same effect plus an additional decrease of maximum current by ~10%. With R_T set to 0·1, EC_{50} is shifted further, and the maximal response is reduced to 9%. Thus, the effects of different concentrations of the downregulating agonist on the concentration–response curve (Fig. 5), as well as the behaviour during recovery from downregulation (Fig. 7) can be accounted for by the assumption that there is: (i) an excess of receptors with regard to the downstream signalling element in the control situation, and (ii) a reduction of the density of functional receptors by long-term agonist treatment.

For the above formula EC_{50} at large R_T is proportional to $1/R_T$, whereas at low R_T it converges to K_D, the true dissociation constant for agonist–receptor interaction. The set of concentration–response curves presented in Fig. 9 suggests the true K_D for ACh–M_2R binding to be >30 μM.

A reduction in density of functional receptors by several orders of magnitude – depending on the assumption of the initial density – can be without effect on maximum steady-state $I_{K(ACh)}$ but strongly affects the horizontal position of the concentration–response curve, manifesting itself as a reduction in receptor affinity.

DISCUSSION

Cardiac muscarinic K^+ channel expressed primarily in supraventricular myocytes represents the prototype of a directly G protein-gated channel (Clapham, 1994; Kurachi, 1995). Current evidence indicates that the signalling pathway consists of three components. (i) The M_2R, or other receptors, converging on G_i-type G proteins, such as the A_1 adenosine receptor (Kurachi, Nakajima & Sugimoto, 1986) or the Pl receptor (Bünemann *et al.* 1995). (ii) The G protein G_i. Whether or not there is a specific 'G_K', different from the species that inhibits adenylyl cyclase (Kirsch, Yatani, Codina, Birnbaumer & Brown, 1988) has not been settled. (iii) The channel. This was originally described as one gene product (GIRK1: Kubo, Reuveny, Slesinger, Jan & Jan, 1993), but more recent data suggest, that the functional channel represents a heteromultimeric complex of the GIRK1 protein and a second type of inward-rectifying channel subunit (CIR: Kofuji, Davidson & Lester, 1995; Krapivinsky, Gordon, Wickmann, Velimirovic, Krapivinski & Clapham, 1995).

Agonist-induced desensitization is a universal feature of different groups of membrane receptors. The term desensitization of a G protein-linked signalling pathway does not denote a single type of mechanism but comprises a number of different reactions leading to an adaptation to prolonged or repetitive exposure to an agonist (Debburman & Hosey, 1995). The mechanisms underlying agonist-induced desensitization have so far been studied in greatest detail for the β_2-adrenergic (cyclic AMP-dependent) pathway (for recent reviews see Haga, Haga & Kameyama, 1994).

It is conceivable that downregulation or desensitization, respectively, could occur at any level of the M_2R–G_i–$I_{K(ACh)}$ signalling pathway. Evidence has been provided that fast desensitization within this system takes place at the level of the G protein, although other targets such as the receptor have also been suggested (Kurachi *et al.* 1987; Kim, 1993; Zang *et al.* 1993). Our observation that recovery from rapid desensitization strictly parallels decay of $I_{K(ACh)}$ during washout of the M-agonist confirms the view of close coupling of receptor occupancy and the fast type of desensitization. In previous studies it was found that acute desensitization also following brief exposure to ACh, outlasts the presence of agonist (e.g. Zang *et al.* 1993; Wang & Lipsius, 1995). This apparently contradictory observation possibly reflects the fact that in those studies performed at higher temperatures, the intermediate component of desensitization was much more pronounced. This might have prevented seeing the fast component in isolation. The final nature of acute desensitization and its localization at present are not fully understood. Since the G protein links

various types of receptors to the channel, desensitization localized at this level should be heterologous, as observed here. The same would apply to the channel itself. Activation of $I_{K(ACh)}$ by PI or SPh-1-P cross-desensitizes the response to ACh. Acute heterologous desensitizing interactions in line with the common G protein as the molecular site have previously been described also between M_2 and A_1 receptors in atrial cells (Kurachi *et al.* 1987; Bünemann & Pott, 1995).

A second type of desensitization which develops on a time scale of minutes in the continuous presence of ACh was found in the present study. This type of desensitization outlasts the complete decay of the current due to the ACh-challenge by some tens of minutes and is comparable to the slow component of acute desensitization described by Zang *et al.* (1993) and Wang & Lipsius, (1995). The slow development and recovery of this component of desensitization are compatible with a stable intermediate such as a phosphorylated receptor. A likely candidate to catalyse this phosphorylation is a GRK (Debburman & Hosey, 1995). The substrate of this class of kinases is a ligated receptor, which implies that desensitization processes involving a GRK should be homologous. As illustrated in Fig. 3, however, exposure to ACh (1 mM, 10 min) results in depression of subsequent responses to both ACh and Ado, i.e. the slow component of acute desensitization is heterologous.

Agonist-dependent M_2R phosphorylation in the chick heart occurring on a time scale of several minutes has been shown to result in a reduction of affinity (Kwatra *et al.* 1987), measured by radioligand binding. Apart from affecting affinity, phosphorylation is assumed to result in functional uncoupling of the ligated receptor and its G protein (Haga *et al.* 1994). Thus, in a functional type of measurement – as in the present study – the fraction of phosphorylated receptors should be silent.

At present the only direct experimental evidence for a contribution of a GRK to desensitization and/or downregulation of M_2R in native cardiac cells comes from the observation that a slow component of acute desensitization seen in perforated patch measurements is lost in conventional patch clamp recordings. This component can be recovered by an exogenous GRK (Shui *et al.* 1995). Downregulation upon chronic muscarinic stimulation has not so far been investigated functionally in this system. A number of investigations, however, have been performed previously on desensitization/downregulation of MRs in different expression systems.

M_2 receptor phosphorylation by two isoforms of human β-ark (GRK2, GRK3) has been shown to occur in transfected insect SF-9 cells concomitant with a perturbation of receptor–G protein coupling (Richardson, Kim, Benovic & Hosey, 1993). Transfected M_2 receptors have been studied recently in a fibroblast cell line where they were found to be efficiently downregulated by internalization (Wei, Yamamura & Roeske, 1994), whereas M_1 receptors in the same system underwent downregulation without internalization upon long-term exposure to an agonist.

GRKs phosphorylate a number of G protein-coupled receptors in an agonist-dependent fashion, and might represent the most common first step involved in desensitization amongst this family of receptors. Phosphorylation is supposed to result in rapid decoupling, but also renders the receptor susceptible to slower processes such as internalization and degradation (e.g. Moro, Lameh & Sadee, 1993).

Evidence has been provided that the receptor is not the only site at which desensitization may occur. For different receptors, including human M_1, downregulation of the α-subunit of the coupling G protein occurs under desensitizing conditions (Mullaney, Dodd, Buckley & Milligan, 1993). In PC12 cells one component of desensitization of the signalling pathway linked to the A_{2a} adenosine receptor has been identified as inhibition of adenylyl cyclase, i.e. the target of a G protein-coupled signalling cascade (Chern, Lai, Fong & Liang, 1993).

Thus, apart from the β-ark (GRK) type of desensitizing regulatory mechanism, the target of which is the agonist-ligated receptor, there are examples of regulatory effects of long-term agonist treatment at various levels of signalling pathways.

Here we have used a 'physiological assay' for maximum $I_{K(ACh)}$ density. We have shown previously (Bünemann & Pott, 1993; Banach *et al.* 1993*b*) that $I_{K(ACh)}$ in atrial myocytes can be activated by a lipid factor (PI) the identity of which is still under investigation (Bünemann, Ferrebee, Tigyi & Pott, 1994). PI from any source such as sera from different animal species, crude, i.e. non-delipidated albumin, acid methanol extracts of crude albumin, or – as in the present investigation – a commercially available mixture of serum lipids, causes activation of $I_{K(ACh)}$ in each myocyte tested. A saturating concentration of PI obtained from either of the sources listed above results in activation of $I_{K(ACh)}$ corresponding to the maximal steady-state level observed in the presence of a saturating concentration of ACh (>2 μM). This means that in untreated cells ACh and PI activate the same population of $I_{K(ACh)}$ channels, and this is additionally supported by the finding that the effects of ACh and PI on $I_{K(ACh)}$ are additive, but never exceed the maximal $I_{K(ACh)}$ activated by a saturating concentration of either of the two activators alone (Banach *et al.* 1993*b*).

Even under almost complete desensitization of the response to ACh, sensitivity to PI is not different from that in untreated cells. A corresponding result was obtained in a CCh-treated cell, whose sensitivity to Ado was determined. Thus, in contrast to the acute components of desensitization, the stable component described in the present study is

strictly homologous. As the M_2, the A_1, and the putative PI receptor converge on the same class of G protein, these results clearly imply that desensitization due to long-term treatment with a M-agonist can be localized to the M_2 receptor. As illustrated in Fig. 5, the major effect of downregulation formally is a reduction of the apparent affinity for ligand. With higher concentrations an additional reduction in the maximum current occurs. If the effect of a desensitizing treatment on a single receptor molecule was to convert it from a high-affinity to a low-affinity state, both representing functional receptors, the effect on the concentration–response curve would be different. In an intermediate range of concentrations, where both high- and low-affinity receptors should co-exist, one would expect to see a mixed concentration–response curve. The resulting curve would have some intermediate K_D and, more importantly, would be flattened, resulting in a reduction of the formal Hill coefficient. The slope of the concentration–response curve in the present study, however remained unaffected. This behaviour can be satisfactorily simulated by assuming that an excess number of receptors converges on a smaller number of G protein molecules. Additional assumptions for this model are: (i) no downregulation on the level of the K_{ACh} channel, which is supported by the finding that maximum K_{ACh} channel density is the same in control- and downregulated cells, and (ii) no effect at the G protein. This assumption is less straightforward, since at present it is not known whether different species of receptors interact with a common pool of G protein molecules.

In combination with the findings that the responses mediated via A_1 and PI receptors, both converging on the same class, and probably the same population, of G proteins, are not affected by treatment of a cell with an M-agonist the simulation provides additional support for the interpretation that the downregulation described here is limited to the M_2 receptor. The shift of EC_{50} from 0·1 to 3·7 μM in moderately desensitized cells, without a decrease in I_{max}, suggests that the density of functional receptors can be reduced by almost a factor of forty without becoming limiting for the number of K_{ACh} channels active in the steady state.

The slow reversibility of the loss of functional receptors, with no recovery at all within the first 5 h after omission of the agonist, suggests the mechanism(s) to be on the transcriptional level (Debburman & Hosey, 1995). Attempts to study this question using the protein synthesis inhibitor cytochalasin B have so far failed in our hands due to the cytotoxic effects of this substance. A reduction of M_2R has been shown to occur upon incubation of embryonic chick heart myocytes on a time scale of several hours. In contrast to the present findings, however, this downregulation on the transcriptional level had heterologous properties with regard to A_1 and angiotensin receptors (Habecker & Nathanson, 1992).

The present study was performed on adult, i.e. terminally differentiated, atrial myocytes. In long-term culture these cells do not proliferate. With regard to different aspects of cellular signal transduction mechanisms, they retain their tissue-specific properties, thus providing a preparation that is highly suitable for studying long-term regulation of signalling pathways under controlled *in vitro* conditions in a highly differentiated cell with defined physiological function without superimposed developmental changes that occur in cells of embryonic or neonatal origin.

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