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ON THE ROLE OF G PROTEIN ACTIVATION AND PHOSPHORYLATION IN DESENSITIZATION TO ACETYLCHOLINE IN GUINEA-PIG ATRIAL CELLS

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SUMMARY

1. The ACh-activated K^+ current ($I_{K,ACh}$) has been investigated in guinea-pig atrial cells at 36 °C using the whole-cell patch-clamp technique.

2. During an exposure to ACh, $I_{K,ACh}$ faded as a result of desensitization. Throughout the fade of the current, the current reversed at E_K and showed inward-going rectification. The fade was, therefore, the result of a genuine decrease in $I_{K,ACh}$.

3. The onset of desensitization (as judged by the fade of $I_{K,ACh}$) was biphasic and the time constants of the fast and slow phases of desensitization were $1{\cdot}58 \pm 0{\cdot}14$ ($n = 16$) and $148{\cdot}2 \pm 12{\cdot}8$ s ($n = 18$) respectively. Recovery from the fast and slow phases of desensitization (after 30 s and 5 min exposures to ACh respectively) occurred with time constants of 52 and 222 s respectively. This suggests that two processes are involved in desensitization.

4. The Q_{10} of the rate constant of the fast phase of desensitization was $2{\cdot}2 \pm 0{\cdot}3$ ($n = 6$).

5. Intracellular perfusion with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) or extracellular perfusion with AlF_4^- were used to bypass the muscarinic receptor and trigger $I_{K,ACh}$ by directly activating the G protein, G_K, that links the muscarinic receptor to the K^+ channel. Both GTPγS and AlF_4^- activated a current with the same reversal potential and the same degree of inward-going rectification as the ACh-activated current.

6. Desensitization still occurred when the muscarinic receptor was bypassed and $I_{K,ACh}$ was triggered by direct activation of G_K with either GTPγS or AlF_4^-. This suggests that desensitization is, in part, the result of a modification of either G_K or the K^+ channel.

7. Activation of the muscarinic receptor by ACh resulted in greater desensitization than direct activation of G_K; at the end of a 5 min exposure to ACh, current was only 22 ± 1 % ($n = 19$) of its peak value, whereas, after direct activation of G_K by GTPγS for 5 min, current was 42 ± 6 % ($n = 5$) of its peak value. This suggests that desensitization also involves the muscarinic receptor.

8. When cells were perfused with GTPγS, the fast phase of desensitization could still occur, but the slow phase was reduced. This suggests that the fast phase involves G_K or the K^+ channel, whereas the slow phase involves the muscarinic receptor.

9. Intracellular perfusion with a stable analogue of ATP, 5′-adenylyl-imido-

phosphate (AMP-PNP), resulted in the loss of $I_{K,ACh}$ regardless of whether $I_{K,ACh}$ was activated by ACh or GTPγS. Phosphorylation of proteins by protein kinases cannot occur when AMP-PNP rather than ATP is available as a substrate and, therefore, the loss of $I_{K,ACh}$ could have been the result of dephosphorylation of either G_K or the K^+ channel.

10. 2,3-Butanedione monoxime, which has a phosphatase-like action, eliminated the fast phase of fade. This suggests that a phosphorylation/dephosphorylation reaction is responsible for the fast phase of desensitization during an exposure to ACh.

INTRODUCTION

Acetylcholine has negative chronotropic, inotropic and dromotropic effects on the heart and during either continuous stimulation of the vagal nerves *in vivo* or during continuous application of ACh *in vitro*, these effects fade as a result of desensitization (e.g. Martin, Levy & Matsuda, 1982; Martin, 1983; Boyett & Roberts, 1987; Boyett, Kirby, Orchard & Roberts, 1988). Because the vagal nerves to the heart are tonically active in the intact animal, desensitization is likely to occur under normal physiological conditions; desensitization is therefore important. The fade of the chronotropic and inotropic responses at least has been shown to be the result of the fade of the ACh-activated K^+ current ($I_{K,ACh}$); the effects of ACh on other currents (the hyperpolarization-activated current, I_f, and the Ca^{2+} current, I_{Ca}) do not fade (Honjo, Kodama & Boyett, 1991; Boyett *et al.* 1988). The aim of the present study was to investigate the fade of $I_{K,ACh}$ in guinea-pig atrial cells. $I_{K,ACh}$ is activated when ACh binds to the muscarinic receptor, which is coupled to the muscarinic K^+ channel via the G protein, G_K (made up of α-, β- and γ-subunits). On binding of ACh to the receptor, GDP is released from the α-subunit and GTP is bound instead. This causes the dissociation of the activated α-subunit (α-GTP) from the $\beta\gamma$-subunits. α-GTP interacts with a K^+ channel and causes it to open. The α-subunit has GTPase activity and the hydrolysis of GTP to GDP deactivates the α-subunit and the K^+ channel closes. The deactivated α-subunit (α-GDP) must bind to $\beta\gamma$-subunits before the cycle can begin again (Szabo & Otero, 1990). Two processes have been suggested to be responsible for desensitization: phosphorylation of the receptor and dephosphorylation of the K^+ channel (Kwatra & Hosey, 1986; Kwatra, Leung, Maan, McMahon, Ptasienski, Green & Hosey, 1987; Kwatra, Benovic, Caron, Lefkowitz & Hosey, 1989; Kim, 1991). The onset of fade of $I_{K,ACh}$ has been reported to be biphasic, which is consistent with two processes (with fast and slow time courses) being involved. If fast and slow processes are involved, recovery from the two processes is expected to occur along different time courses and this has been tested in the present study. The two processes suggested to be responsible for desensitization are postulated to act at separate sites: the receptor and the K^+ channel. To test this, the fade of $I_{K,ACh}$ was compared when $I_{K,ACh}$ was activated via the muscarinic receptor (as normal) or independently of the receptor. The two processes suggested to be responsible for desensitization involve phosphorylation/dephosphorylation reactions and to test this the effects of a variety of phosphorylating and dephosphorylating agents on $I_{K,ACh}$ were examined. A

preliminary report of some of the findings has been made to the Physiological Society (Zang & Boyett, 1991).

METHODS

Cell isolation

Guinea-pigs of either sex, weighing 300–500 g, were killed by a blow to the head or anaesthetized by intraperitoneal injection of sodium pentobarbitone (30 mg/kg). The chest was opened and the heart removed. The aorta was cannulated and the heart retrogradely perfused at constant pressure (40–47 cm H_2O) via the aorta and coronary arteries with normal Tyrode solution. After the blood had been washed out, the heart was perfused with nominally Ca^{2+}-free Tyrode solution for about 3–5 min. Next, the heart was perfused with Ca^{2+}-free Tyrode solution containing collagenase (Yakult, Japan), 0·1–0·15 mg/ml, and bovine serum albumin, 0·5–0·6 mg/ml, for 4–8 min. Finally, the heart was perfused with high-K^+, low-Cl^- solution (storage solution) for 5–6 min to wash out the collagenase-containing solution. During coronary perfusion all perfusates were maintained at 35–37 °C (with the exception of the storage solution, which was at room temperature) and equilibrated with O_2. At the end of the perfusion the atria were cut into pieces (about 2×2 mm) and placed in a beaker. The atrial cells were dispersed by gently shaking the beaker and the undigested tissue was removed by filtration through nylon mesh. The atrial cells were kept in the storage solution at 4 °C for at least 1 h before they were used experimentally.

Perfusion of the recording chamber

A drop of cell suspension was placed in a bath mounted on a Nikon Diaphot microscope. Cells were allowed to settle for about 10 min before perfusion of the bath was commenced. Up to four solutions were pumped by magnetic drive gear metering pumps (Micropump, Concord, CA, USA) to solenoid valves (The Lee Company, Westbrook, CT, USA) located next to the bath. The solenoid valves were used to direct one of the solutions to the bath and the remainder to waste. Before the solution entered the bath it was heated by a heating coil wrapped around the glass inflow tube. The bath had a volume of about 70 μl and it was perfused at a flow rate of 2·4 ml/min (thirty-five bath volumes per minute). The bath temperature was monitored using a miniature thermistor mounted in the bath wall. The bath temperature was maintained at 36 °C by manual or feedback control of current flow through the heating coil. The bath temperature did not vary more than $\pm 0{\cdot}5$ °C (manual control) or $\pm 0{\cdot}2$ °C (feedback control) during the course of an experiment. Bath level was kept constant using a feedback circuit (Cannell & Lederer, 1986). In order to change the solution in the bath rapidly, mixing of the two solutions had to be minimized. This was achieved by using solenoid valves to switch between solutions, a short length of small-bore tubing between the valves and the chamber, a small chamber, a fast flow rate and cells near the inlet tube to the chamber.

Recording and analysis

Spindle-shaped cells with clear striations were chosen for study. Membrane currents were recorded using the GΩ-seal patch-clamp technique in the whole-cell clamp configuration. An Axopatch-1C amplifier (Axon Instruments Inc., USA) and patch electrodes with resistances between 3 and 7 MΩ were used. At the start of an experiment the electrode and cell capacitance and series resistance were compensated. In all experiments the membrane potential was held at approximately −53 mV, which was corrected for junction potentials. The liquid junction potential for the normal patch electrode solution was +12 mV. It was checked that NaF and $AlCl_3$ at the concentrations used did not affect the junction potential of the bath earth electrode. During an experiment, membrane potential and current were recorded on a Tektronix 5000 series oscilloscope and a Gould 2400 series pen recorder. The signals were also recorded on video tape (a Neuro-corder, Neuro Data Instruments Corp., New York, NY, USA, and a JVC video recorder were used) for subsequent analysis. Data were captured using a CED 1401 analog-to-digital convertor (Cambridge Electronic Design Ltd, Cambridge, UK) and analysed using an IBM compatible computer. Time constants were calculated using Enzfitter (Biosoft, Cambridge, UK), a non-linear regression program.

Solutions

Normal Tyrode solution contained (mM): NaCl, 136·9; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·33; Hepes, 5·0; glucose, 5·5. The pH of the solution was adjusted to 7·4 by adding NaOH. The

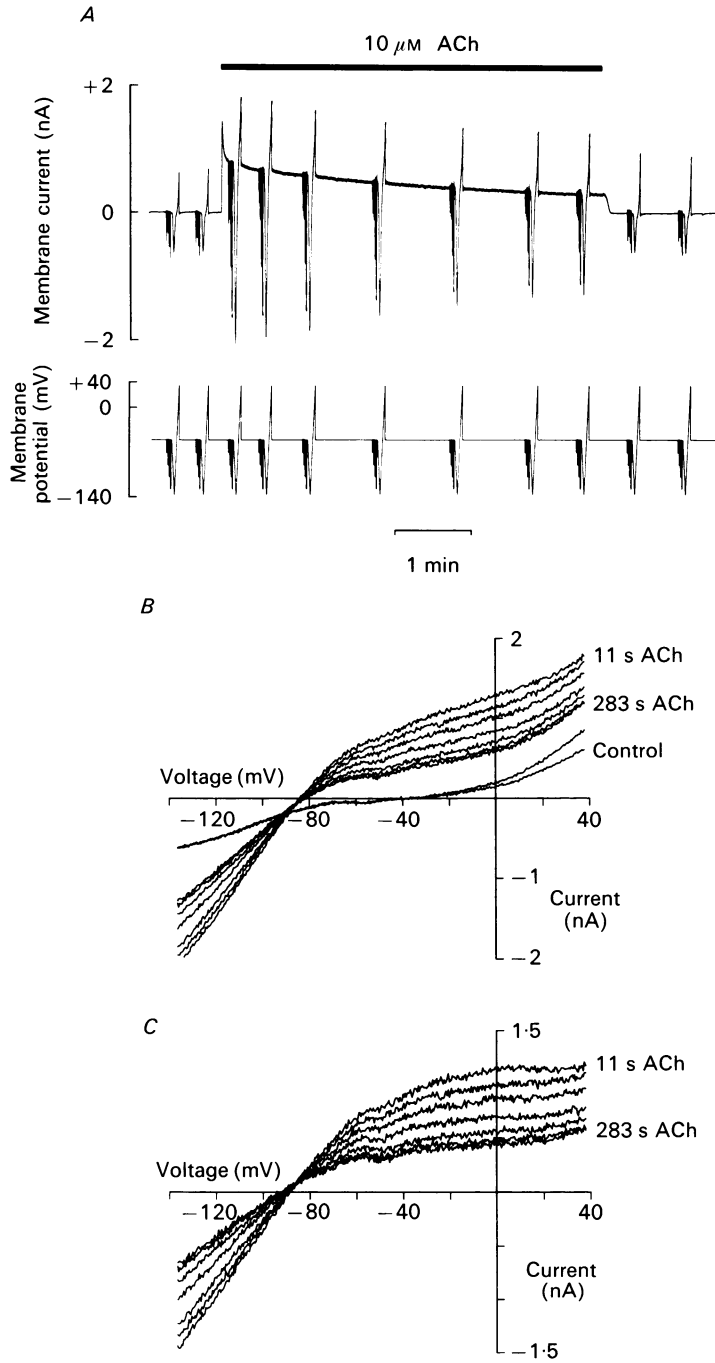

Fig. 1. Fade of $I_{K,ACh}$. *A*, activation and fade of $I_{K,ACh}$ during a 5 min exposure to ACh. In this and other figures the period of exposure to ACh (or other compound) is shown by the bar at the top of the figure. At regular intervals a series of 300 ms voltage-clamp pulses to −73, −93, −113, and −133 mV (pulse frequency, 1 Hz) and a ramp clamp from −143 to +37 mV at a speed of 45 mV/s were applied. *B*, current–voltage relationships, obtained from the ramp clamps, before, during and after the exposure to ACh. *C*, current–voltage relationships for the ACh-activated current at different times during the

storage solution contained (mM): taurine, 20; glutamic acid, 70; KCl, 25; KH_2PO_4, 10; $MgCl_2$, 3; EGTA, 0·5; Hepes, 10; glucose, 10. The pH was adjusted to 7·4 by adding KOH. Aliquots of a 1 mM stock solution of ACh (acetylcholine chloride, Sigma, UK) were frozen. On the day of an experiment one was thawed and used and any stock solution remaining was discarded. An appropriate amount of the stock solution was added to the Tyrode solution to obtain a particular dose. To generate AlF_4^- ions, an appropriate amount of a 10 mM stock solution of $AlCl_3$ (Sigma) was added to the Tyrode solution to obtain a concentration of 10 μM $AlCl_3$. Sufficient solid NaF (Sigma) was then added to the Tyrode solution to obtain a concentration of 10 mM NaF. It was not possible to use higher concentrations of $AlCl_3$ and NaF, because a precipitate developed. Even with 10 μM $AlCl_3$ and 10 mM NaF a precipitate developed after a period of several hours, at which time fresh solutions were prepared. Electrodes were filled with the internal solution; this normally contained (mM): potassium aspartate, 130; KCl, 20; KH_2PO_4, 1; $MgCl_2$, 1; EGTA, 5·0; Na_2ATP (Sigma), 3; GTP (Na^+ salt, Sigma), 0·1; Hepes, 5·0. The pH was adjusted to 7·3 by adding KOH. In some experiments GTP was replaced by 0·1 mM GTPγS (guanosine 5′-*O*-(3-thiotriphosphate), tetralithium salt, Sigma) and ATP was replaced with 5 mM AMP-PNP (5′-adenylyl-imidodiphosphate, lithium salt, Sigma) or 5 mM ATPγ-S (adenosine-5′-(γ-thio)-triphosphate, tetralithium salt, Sigma).

Presentation of statistics

Data are presented as mean ± S.E.M. (number of cells). Comparisons were made using Student's *t* test or a paired *t* test as appropriate.

RESULTS

Fade of $I_{K,ACh}$

Figure 1*A* shows that the addition of 10 μM ACh resulted in the activation of an outward current, which then faded in the continued presence of ACh. This current was first recorded in guinea-pig atrial cells by Iijima, Irisawa & Kameyama (1985). The holding potential in this and all other experiments was close to −53 mV. To ensure that the fade was the result of a decrease in the ACh-activated outward current rather than a delayed activation of an inward current, current–voltage relationships were obtained. Voltage-clamp pulses to potentials between −73 and −133 mV and ramp clamps from −143 to +37 mV were applied before, during and after the application of ACh as shown in Fig. 1*A*. Current–voltage relationships obtained from the ramp clamps are shown in Fig. 1*B*. Current–voltage relationships obtained from the voltage-clamp pulses (not shown) were similar. To obtain current–voltage relationships for the ACh-activated current alone, the current under control conditions were subtracted from the current with ACh at each potential during the ramp clamp. Seven current–voltage relationships for the ACh-activated current at various times during the exposure to ACh are shown in Fig. 1*C*. The ACh-activated current early during the exposure to ACh showed inward-going rectification and reversed at −88 mV, which is close to −88·6 mV, the value of E_K calculated using the Nernst equation and the known intracellular and extracellular K^+ concentrations (see Methods). In thirteen cells the reversal potential of the ACh-activated current was −86·1 ± 1·6 mV, 7 ± 1 s after the start of an exposure to 10 μM ACh. The ACh-activated current late during the exposure to ACh was smaller than

exposure to ACh. The data were calculated from the data in *B*. At each voltage during the ramp clamp, the current under control conditions (before the exposure to ACh) was subtracted from the current in the presence of ACh to obtain the ACh-activated current alone.

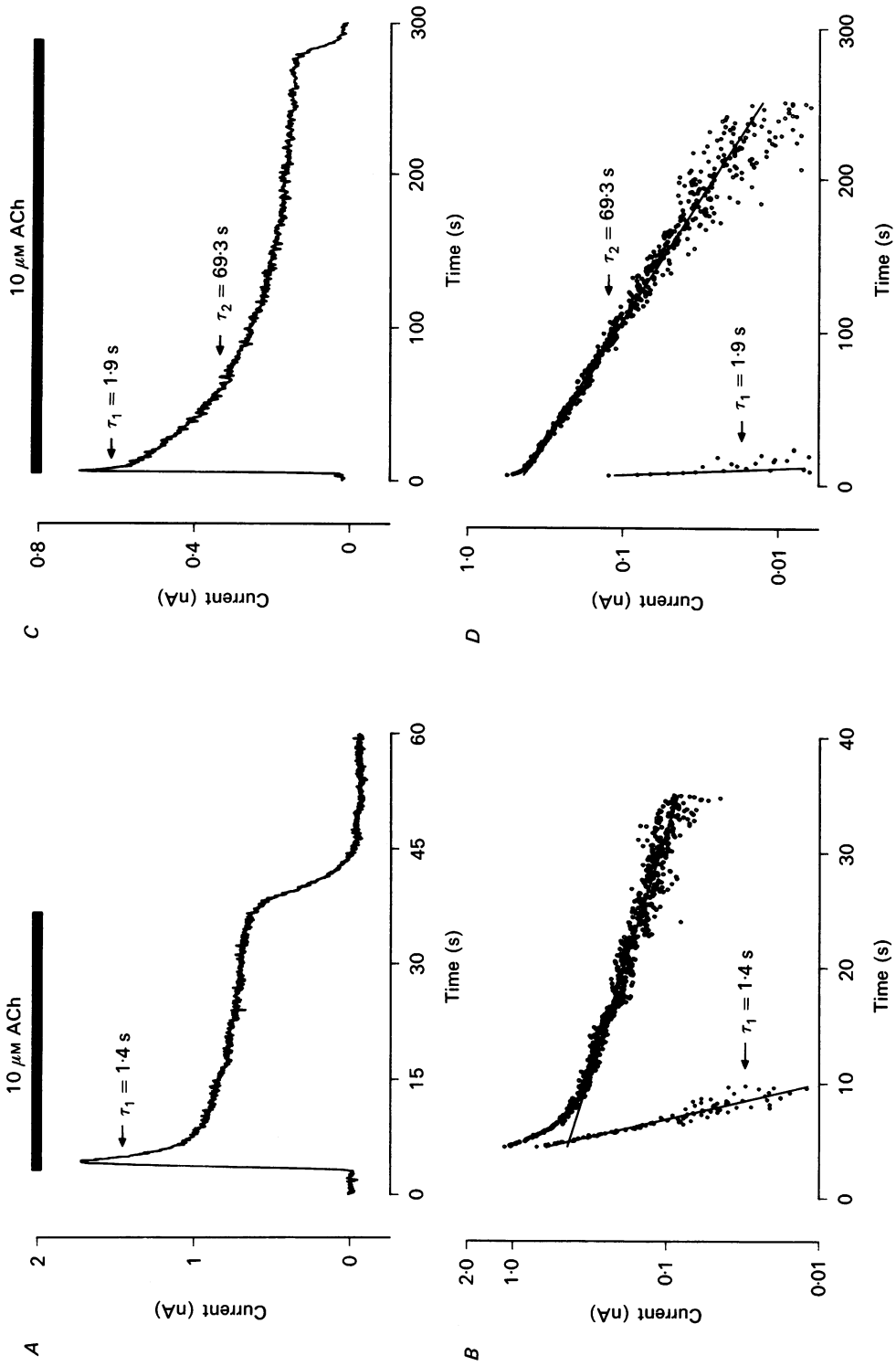

Fig. 2. For legend see facing page.

the current early during the exposure at all potentials, but it still showed inward-going rectification and reversed at −90 mV. In six cells the reversal potential of the ACh-activated current was −86·9 ± 2·3 mV, 113 ± 48 s after the start of an exposure to 10 μM ACh. It is concluded that on addition of ACh there was an activation of K^+ current ($I_{K,ACh}$) which then decayed in the continued presence of ACh.

Throughout this study 10 μM ACh was used, because desensitization is marked with high concentrations of ACh (Carmeliet & Mubagwa, 1986; Boyett & Roberts, 1987; Kurachi, Nakajima & Sugimoto, 1987). Furthermore, 10 μM ACh has been shown to activate $I_{K,ACh}$ near maximally (Carmeliet & Mubagwa, 1986). In twenty-three cells peak $I_{K,ACh}$ in response to 10 μM ACh was 19·9 ± 1·1 μA/μF and the time to peak $I_{K,ACh}$ was 887 ± 81 ms in eleven cells.

Kinetics of desensitization to ACh

Figure 2 shows that the onset of desensitization, as judged by the fade of $I_{K,ACh}$, was biphasic. Figure 2*A* shows $I_{K,ACh}$ during a short (30 s) exposure to ACh. During the exposure, $I_{K,ACh}$ declined rapdily at first and then more slowly. Figure 2*B* is a semilogarithmic plot of the fade of $I_{K,ACh}$ during the exposure and it shows that the decay can be fitted by two exponentials. The time constant of the fast phase of desensitization in this example was 1·4 s and in sixteen cells was 1·58 ± 0·14 s. The amplitude of the fast phase of desensitization in different cells was variable and perhaps, in part, depended on how abruptly ACh was applied to the cell (Boyett & Roberts, 1987). If there is mixing of the control and ACh-containing solutions, the ACh concentration next to the cell will increase gradually rather than in a step-like manner. As a result the fast phase of desensitization will develop at the same time as the activation of $I_{K,ACh}$, $I_{K,ACh}$ will be truncated and the fast phase of desensitization will not be manifest as a decrease in $I_{K,ACh}$. Mixing of the solutions was minimized as described in the Methods. At the end of a 30 s exposure to ACh, $I_{K,ACh}$ was 55 ± 2% of its peak value in sixteen cells.

The time constant of the slow phase of desensitization could not be measured accurately during short (30 s) exposures to ACh; the time constant measured in this way was found to be an underestimate. The time constant of the slow phase of desensitization was measured during long (5 min) exposures to ACh. Figure 2*C* shows an example of $I_{K,ACh}$ during a 5 min exposure to ACh and Fig. 2*D* is a semi-logarithmic plot of the fade of $I_{K,ACh}$. Once again the fade of $I_{K,ACh}$ can be fitted by two exponentials and the time constant of the slow phase of desensitization was

Fig. 2. Fade of $I_{K,ACh}$ is biphasic. *A*, $I_{K,ACh}$ during a 30 s exposure to ACh. *B*, semi-logarithmic plot of the fade of $I_{K,ACh}$ (same data as those in *A*). Current was assumed to be approaching asymptotically a value of 0·58 nA during the exposure to ACh. The upper set of data is the difference between current at any time and the asymptote, plotted on a logarithmic scale, versus time during the exposure to ACh. At times greater than about 10 s the data lie on a straight line and this represents the slow phase of desensitization. At times less than about 10 s the data diverge from the straight line. The difference between the data and the straight line has been plotted as the lower set of data. The lower set of data falls on a straight line and this represents the fast phase of desensitization. *C*, $I_{K,ACh}$ during a 5 min exposure to ACh. The results in *A* and *C* were obtained from different cells. *D*, semi-logarithmic plot of the fade of $I_{K,ACh}$ (same data as those in *C*). Data were plotted in a similar way to those in *B*.

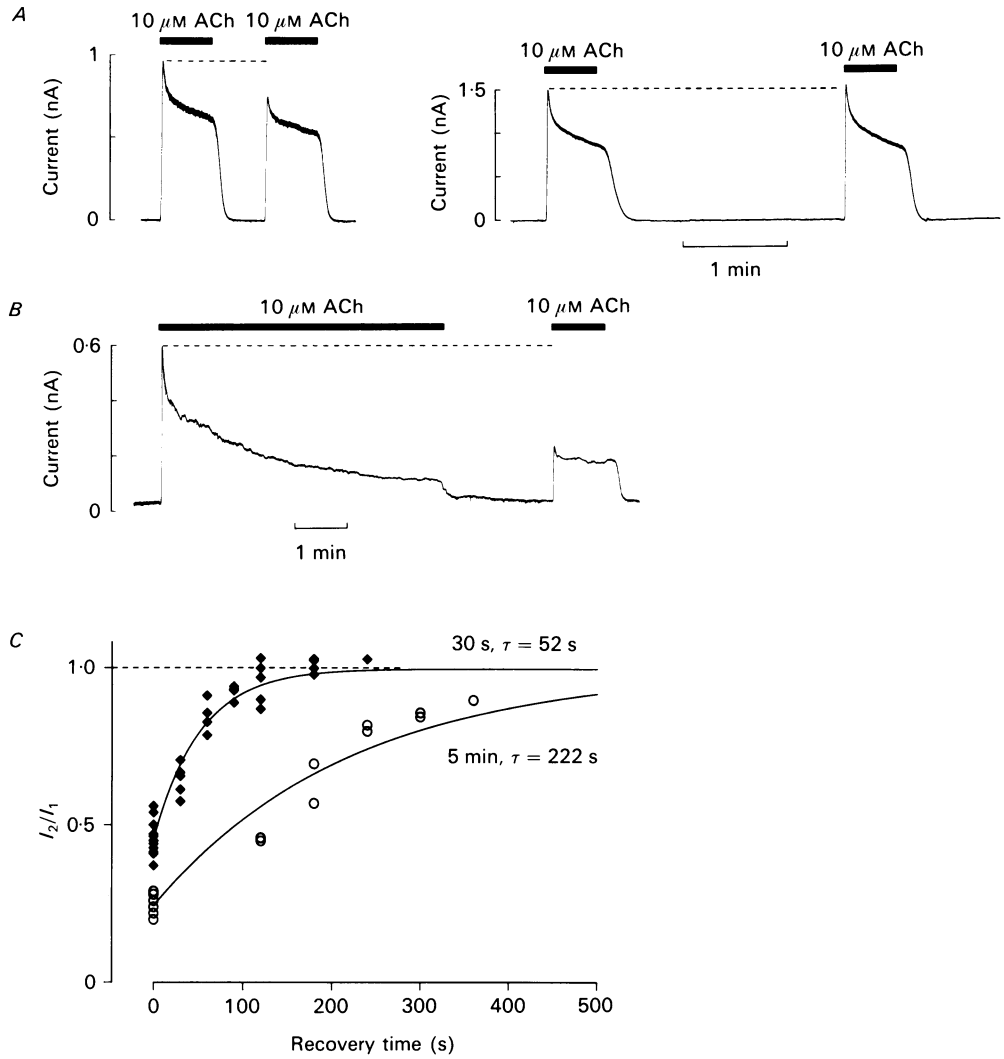

Fig. 3. Recovery of $I_{K,ACh}$ from desensitization. *A* and *B*, recovery from desensitization after 30 s (*A*) and 5 min (*B*) exposures to ACh. In each case a test exposure to ACh was applied after a control exposure. The dashed lines mark the peak current reached during the control exposures. Recovery times between the control and test exposures: 30 s (*A*, left trace) and 2 min (*A*, right trace and *B*). The three traces were obtained from different cells. *C*, the time course of recovery from desensitization after 30 s and 5 min exposures to ACh. Peak $I_{K,ACh}$ during the test exposure to ACh, I_2, as a fraction of peak $I_{K,ACh}$ during the control exposure, I_1, is plotted against the preceding recovery time. The dashed line shows a ratio of 1·0. ◆ show data obtained from sixteen cells after a 30 s exposure to ACh and ○ show data obtained from eight cells after a 5 min exposure. The data have been fitted with single exponential functions with the time constants shown. Note that the currents plotted for a recovery time of zero were the currents at the end of the control exposures to ACh immediately before the wash-out of ACh as a fraction of peak $I_{K,ACh}$ during the control exposures.

69·3 s in this example. In eighteen cells the time constant of the slow phase of desensitization was 148·2 ± 12·8 s. At the end of a 5 min exposure to ACh, $I_{K,ACh}$ was 22 ± 1 % of its peak value in nineteen cells.

Recovery from desensitization

As explained in the Introduction, recovery from the fast and slow phases of desensitization is expected to occur along different time courses. To investigate recovery from desensitization, a test exposure to ACh was applied after a control exposure and in different cells the recovery time between the two exposures was varied. Figure 3*A* shows two pairs of control and test exposures; each exposure was approximately 30 s in duration. A recovery time of 30 s (Fig. 3*A*, left trace) did not allow full recovery from desensitization and peak $I_{K,ACh}$ during the test exposure was less than that during the control exposure. However, a recovery time of 2 min (Fig. 3*A*, right trace) did allow full recovery from desensitization and $I_{K,ACh}$ during the test exposure was similar in amplitude to $I_{K,ACh}$ during the control exposure. In Fig. 3*C* peak $I_{K,ACh}$ during the test exposure as a fraction of peak $I_{K,ACh}$ during the control exposure is plotted against the recovery time. The filled symbols, obtained from sixteen cells, represent the time course of recovery from desensitization after a 30 s exposure to ACh. The data are fitted with a single exponential curve with a time constant of 52 s. During a 30 s exposure, only the fast phase of desensitization developed in full. The time constant, therefore, largely represents the time constant of recovery from the fast phase of desensitization. To study the recovery from the slow phase of desensitization, recovery was investigated after 5 min control exposures to ACh, during which the slow phase as well as the fast phase of desensitization developed. Figure 3*B* shows that $I_{K,ACh}$ during a test exposure to ACh, 2 min after a 5 min control exposure to ACh, was less than $I_{K,ACh}$ during the control exposure. This demonstrates that recovery from desensitization after a 5 min exposure to ACh was slower than recovery after a 30 s exposure, because a recovery time of 2 min was sufficient to allow full recovery after a 30 s exposure (Fig. 3*A*, right trace). Figure 3*C* also shows data obtained after 5 min exposures to ACh (open symbols); the open symbols represent the recovery from the slow phase of desensitization (data from eight cells shown). The data are fitted with a single exponential curve with a time constant of 222 s.

In all remaining experiments to be described (with one exception), ACh was applied for 30 s and if ACh was applied repeatedly there was an interval of 3 min or more between successive exposures to allow full recovery from the preceding exposure (Fig. 3*C*).

The temperature dependence of desensitization

The effect of temperature on $I_{K,ACh}$ is shown in Fig. 4. Figure 4*A* shows $I_{K,ACh}$ at 25 °C, 36 °C and on return to 25 °C and it shows that at the higher temperature $I_{K,ACh}$ was larger (1·7 × in this example). In Fig. 4*B* the first 2·5 s of responses to ACh at the two temperatures are superimposed. At the higher temperature, activation of $I_{K,ACh}$ was more rapid; in this example the maximum rate of activation of $I_{K,ACh}$ was 1·2 nA/s at 25 °C and 3·5 nA/s at 36 °C. Figure 4*A* shows that fade of $I_{K,ACh}$ was more prominent at the higher temperature. Fade of $I_{K,ACh}$ was also faster at the

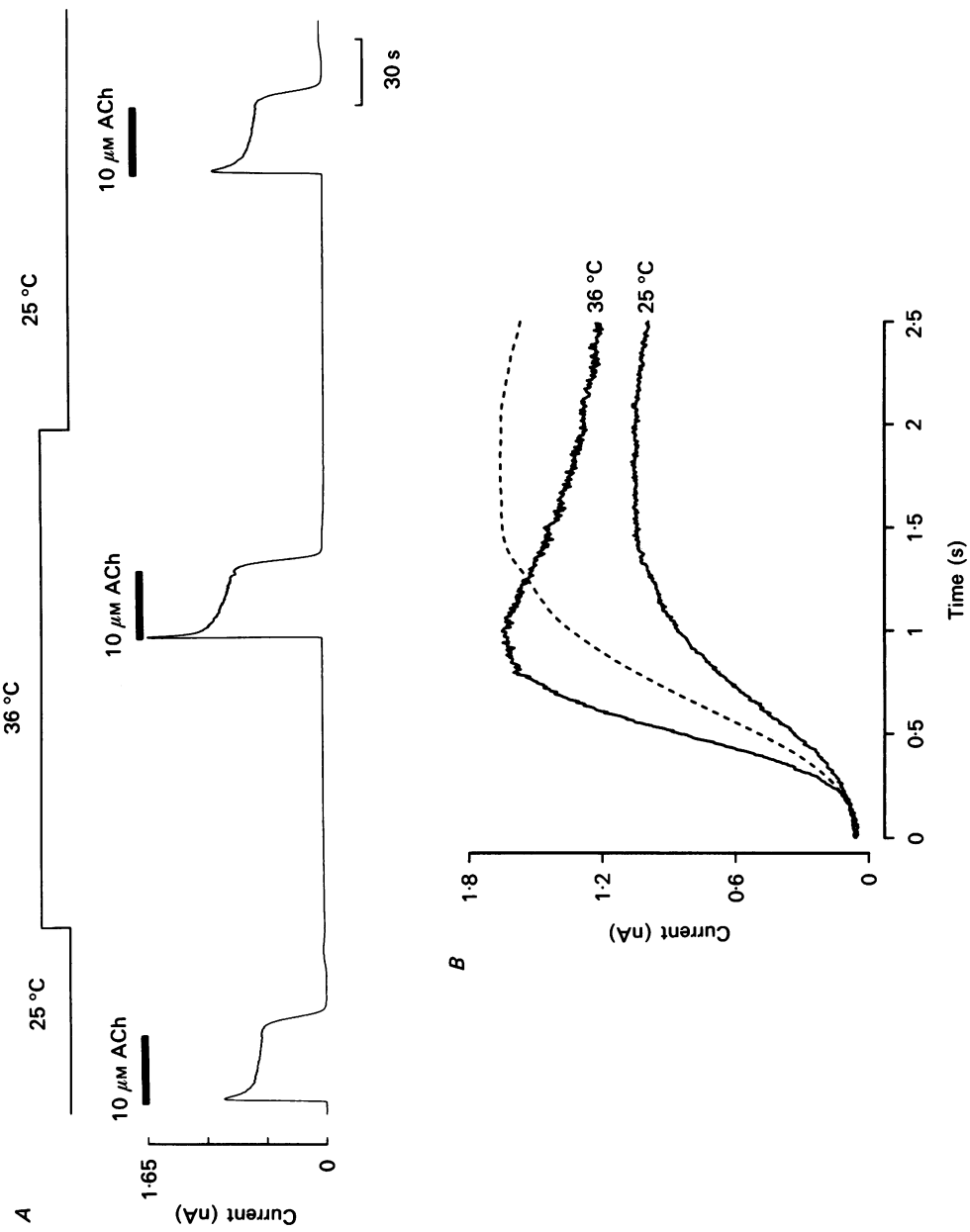

Fig. 4*A* and *B*. For legend see facing page.

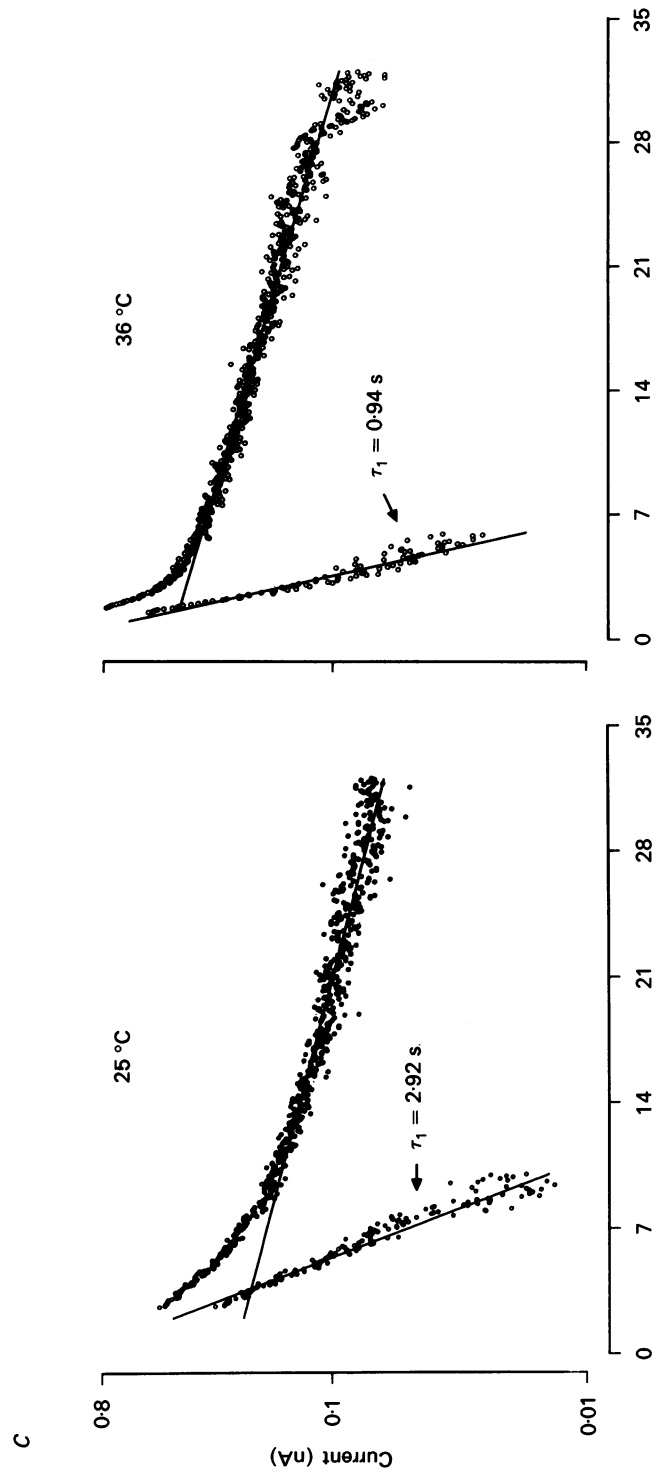

Fig. 4. The effect of temperature on $I_{K,ACh}$. *A*, $I_{K,ACh}$ at 25 °C, 36 °C and on return to 25 °C. *B*, fast time base records showing the activation of $I_{K,ACh}$ at 25 °C and 36 °C. ACh was assumed to reach the cell at time zero. The dashed line is $I_{K,ACh}$ at 25 °C scaled so that it is the same amplitude as $I_{K,ACh}$ at 36 °C. *C*, semilogarithmic plots of the fade of $I_{K,ACh}$ at 25 °C and 36 °C.

higher temperature; Fig. 4*C* shows semilogarithmic plots of the decline of $I_{K,ACh}$ during exposures to ACh at 25 and 36 °C and the time constant of the fast phase of desensitization was reduced from 2·92 to 0·94 s on increasing the temperature in this example. The time constant of the slow phase was not measured, because of the error in the measurement of this time constant during a 30 s exposure to ACh (see above). In six cells, the Q_{10} (the ratio of a variable at one temperature and a 10 °C lower temperature) was 1·9 ± 0·2 for the maximum rate of activation of $I_{K,ACh}$, 1·6 ± 0·1 for the amplitude of $I_{K,ACh}$ and 2·2 ± 0·3 for the rate constant of the fast phase of desensitization. The high Q_{10} for the rate constant of the fast phase of desensitization suggests that a chemical reaction may be involved in this process (see Discussion for further comment).

The site of desensitization as studied using GTPγS and AlF_4^-

As explained in the Introduction, desensitization has been suggested to involve the muscarinic receptor and the K^+ channel. It is also possible that it could involve G_K. To test which of these components is involved in desensitization, the muscarinic receptor has been bypassed and the K^+ channel has been activated directly. For example, if the muscarinic receptor is the only site of desensitization, desensitization should not occur under these conditions. GTPγS, acting via G_K, was used to directly activate the K^+ channel. It is well known that the hydrolysis-resistant GTP analogue, GTPγS, irreversibly activates $I_{K,ACh}$ by binding to the α-subunit of G_K to form activated α-subunit (α-GTPγS) (e.g. Breitwieser & Szabo, 1988). This irreversibly activates $I_{K,ACh}$, because the GTPγS cannot be hydrolysed.

Figure 5*A* shows the membrane current recorded from a cell dialysed with GTPγS (the patch electrode contained 100 μM GTPγS rather than 100 μM GTP). Immediately after the rupture of the membrane patch, the holding current slowly became more outward. Thirty seconds after the rupture of the patch ACh was applied for 30 s; $I_{K,ACh}$ activated and then faded as normal. The fact that $I_{K,ACh}$ faded is significant and will be considered in the Discussion. After the first exposure to ACh, the holding current remained outward. This is likely to have been the result of the persistent activation of $I_{K,ACh}$ by GTPγS. The large increase in GTPγS-activation of $I_{K,ACh}$ after the exposure to ACh is expected – agonist binding to the muscarinic receptor facilitates the binding of GTP or GTPγS to G_K (see Introduction; see also Breitwieser & Szabo, 1988). Figure 5*A* shows that $I_{K,ACh}$ activated by GTPγS was less than the peak $I_{K,ACh}$ at the start of the initial exposure to ACh. In seven cells the maximum $I_{K,ACh}$ activated by GTPγS was 52·9 ± 4·3 % of peak $I_{K,ACh}$ activated by ACh. There are three possible reasons for this: (i) the GTPγS concentration used was insufficient to activate G_K and $I_{K,ACh}$ maximally, (ii) the GTPγS concentration was sufficient to activate G_K and $I_{K,ACh}$ maximally, but GTPγS activated concurrently an inward current, or (iii) the GTPγS concentration was sufficient to activate G_K and $I_{K,ACh}$ maximally, but as a result of desensitization and an alteration in either G_K or the K^+ channel (brought about by the activation of G_K) $I_{K,ACh}$ activated by GTPγS was less than peak $I_{K,ACh}$ at the start of the exposure to ACh.

To test the first possibility, ACh was applied a second time, after the activation of $I_{K,ACh}$ by GTPγS (Fig. 5*A*). In the example shown and in three other cells there was no further activation of $I_{K,ACh}$ by ACh in the presence of GTPγS. This demonstrates

that the GTPγS concentration used was sufficient to activate G_K and $I_{K,ACh}$ fully in these cells. Although in four other cells ACh did activate further current (perhaps because dialysis with GTPγS was not continued for a sufficient length of time in these cells) the total current after the activation of the GTPγS-dependent current and the addition of ACh was still less than the peak $I_{K,ACh}$ during the first exposure to ACh. If data from all cells studied ($n = 8$) are grouped together, 6·9±0·7 min after the

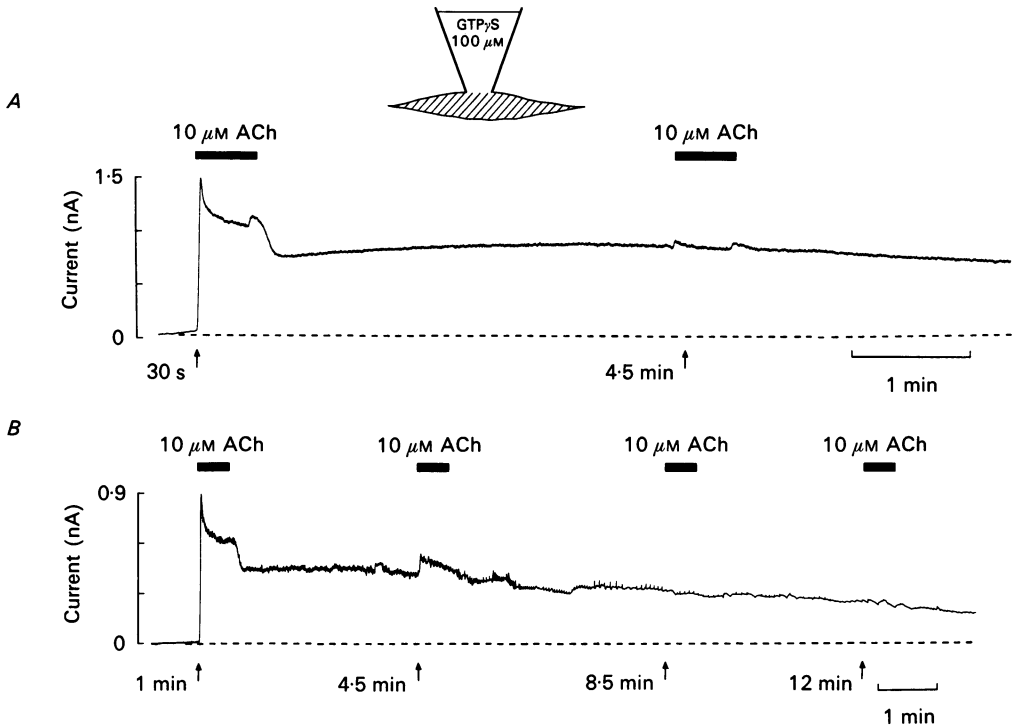

Fig. 5. Effect of intracellular perfusion with 100 μM GTPγS on $I_{K,ACh}$. ACh was applied at the times shown at the bottom of the traces. The dashed lines correspond to the level of current immediately after the rupture of the membrane patch. The results in *A* and *B* were obtained from different cells. The small outward shift in membrane current on wash-off of ACh in *A* was an artifact.

start of cell dialysis the GTPγS-activated current was 42·5±4·3%. At the same time, the ACh-activated current was 7·1±3·2% and the sum of the GTPγS- and ACh-activated currents was 49·8±6·0% (all currents expressed as a percentage of the peak $I_{K,ACh}$ during the first exposure to ACh). These data are shown graphically in Fig. 7*D*.

To test whether GTPγS concurrently activated an inward current, the reversal potentials of the GTPγS- and ACh-activated currents were measured. If GTPγS activated an inward current as well as $I_{K,ACh}$, the reversal potential of the GTPγS-activated current would be more positive than the reversal potential of the ACh-activated current. In five cells current–voltage relationships were obtained before and during the first exposure to ACh as well as after the exposure once GTPγS had activated an outward current. An example is shown in Fig. 6*A*. The changes in

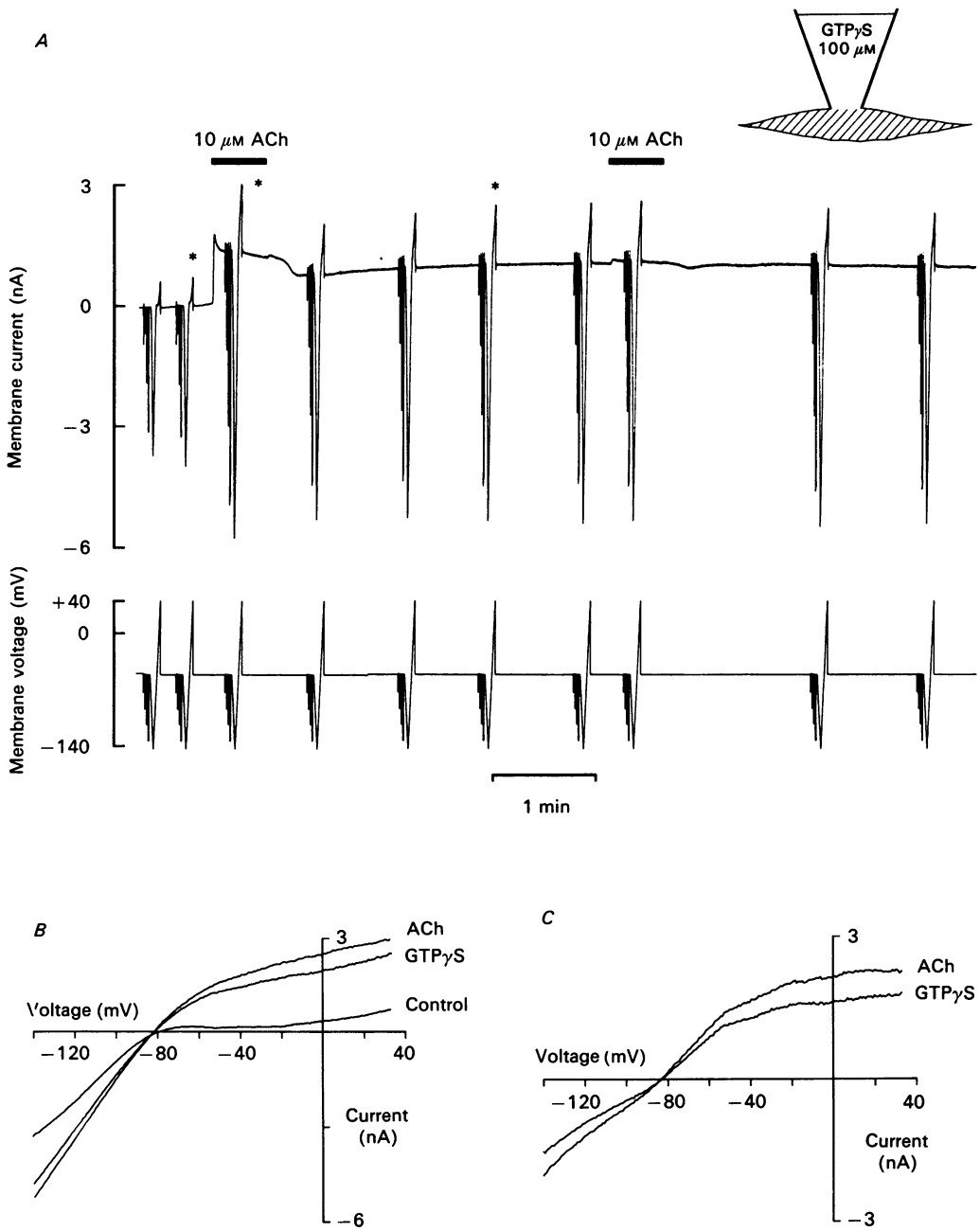

Fig. 6. Effect of intracellular perfusion with 100 μM GTPγS on the current–voltage relationship. *A*, the experimental protocol. At regular intervals voltage-clamp pulses and ramp clamps were applied (see Fig. 1*A* legend for details). ACh was applied twice as shown. *B*, current–voltage relationships, obtained from the ramp clamps marked with an asterisk in *A*, before the first exposure to ACh (the control), in the presence of ACh and after the activation of the GTPγS-dependent current. *C*, current–voltage relationships for the ACh- and GTPγS-activated currents. The data were calculated from the data in *B* (see Fig. 1*C* legend for details).

holding current are similar to those shown in Fig. 5. Square pulses and ramp clamps were applied at regular intervals. Selected current–voltage relationships obtained from the ramp clamps are shown in Fig. 6*B*. Difference current–voltage relationships (obtained from the data in Fig. 6*B*) are shown in Fig. 6*C*; these show the current–voltage relationships for the ACh- and GTPγS-activated currents alone. Both currents had a reversal potential of −84 mV and exhibited inward-going rectification. In five cells the reversal potential of the GTPγS-activated current was $-88{\cdot}2 \pm 2{\cdot}6$ mV. It is concluded that GTPγS did not activate an inward current concurrently with $I_{K,ACh}$. Therefore, the GTPγS-activated current was less than the peak ACh-activated current possibly as a result of desensitization brought about by the activation of G_K.

Although the experiment in Fig. 5 suggests that direct activation of G_K resulted in desensitization, it is possible that the desensitization was the result of another action of GTPγS, perhaps the formation of ATPγS from GTPγS by a trans-phosphorylation reaction; later it is shown that ATPγS eliminates $I_{K,ACh}$. Therefore, the effect of activation of G_K by AlF_4^- has also been investigated. It is well known that AlF_4^- activates G proteins, including G_i (G_K is possibly G_{i2} or G_{i3}, Robishaw & Foster, 1989) (Sternweis & Gilman, 1982; Gilman, 1984*a*, *b*; see also Blackmore & Exton, 1986; Magnússon, Halldórsson, Kjeld & Thorgeirsson, 1989; Woods, Dixon, Cuthbertson & Cobbold, 1990). AlF_4^- is able to activate G proteins by substituting for the γ-phosphate of GTP – there are structural similarities between AlF_4^- and phosphate (Bigay, Deterre, Pfister & Chabre, 1985). AlF_4^- could not be introduced into the cell via the patch electrode, because EGTA (present in the patch electrode) avidly binds Al^{3+}. Therefore, AlF_4^- was added to the bathing solution; extracellular AlF_4^- has been used by Woods *et al.* (1990) to activate G protein in hepatocytes.

The effect of AlF_4^- was investigated in eleven cells. A typical result is shown in Fig. 7*A*. Soon after the rupture of the patch (the standard patch electrode solution was used) ACh was applied for 30 s and $I_{K,ACh}$ was activated as usual. At the end of the exposure to ACh, the cell was superfused with bathing solution containing AlF_4^-. $I_{K,ACh}$ decayed on wash-out of ACh, but then the current became more outward as a result of AlF_4^-. In three of the cells current–voltage relationships were obtained before and during the first exposure to ACh as well as after the activation of the AlF_4^--dependent current. Current–voltage relationships from one cell are shown in Fig. 7*B* and the current–voltage relationships for the ACh- and AlF_4^--activated currents alone are shown in Fig. 7*C*. Both the ACh- and AlF_4^--activated currents had the same reversal potential and both showed inward-going rectification (Fig. 7*C*). In the three cells the reversal potential of the AlF_4^--activated current was $-89{\cdot}7 \pm 2{\cdot}1$ mV. It is concluded that AlF_4^- activated $I_{K,ACh}$. In one of the cells there was a further change in the current–voltage relationship later during the exposure to AlF_4^-: at potentials more negative than about −90 mV current became more outward as compared to current under control conditions. A similar, but less marked, tendency was observed in the other cells.

The effect of AlF_4^- was similar to the effect of GTPγS. In Fig. 7*A* the AlF_4^--activated current was less than the peak $I_{K,ACh}$ during the first exposure to ACh. In ten cells the peak AlF_4^--activated current was $43{\cdot}2 \pm 1{\cdot}9$ % of the peak $I_{K,ACh}$ during the first exposure to ACh. In Fig. 7*A* after the activation of the AlF_4^--dependent current ACh was applied a second time. The response to ACh was not eliminated and

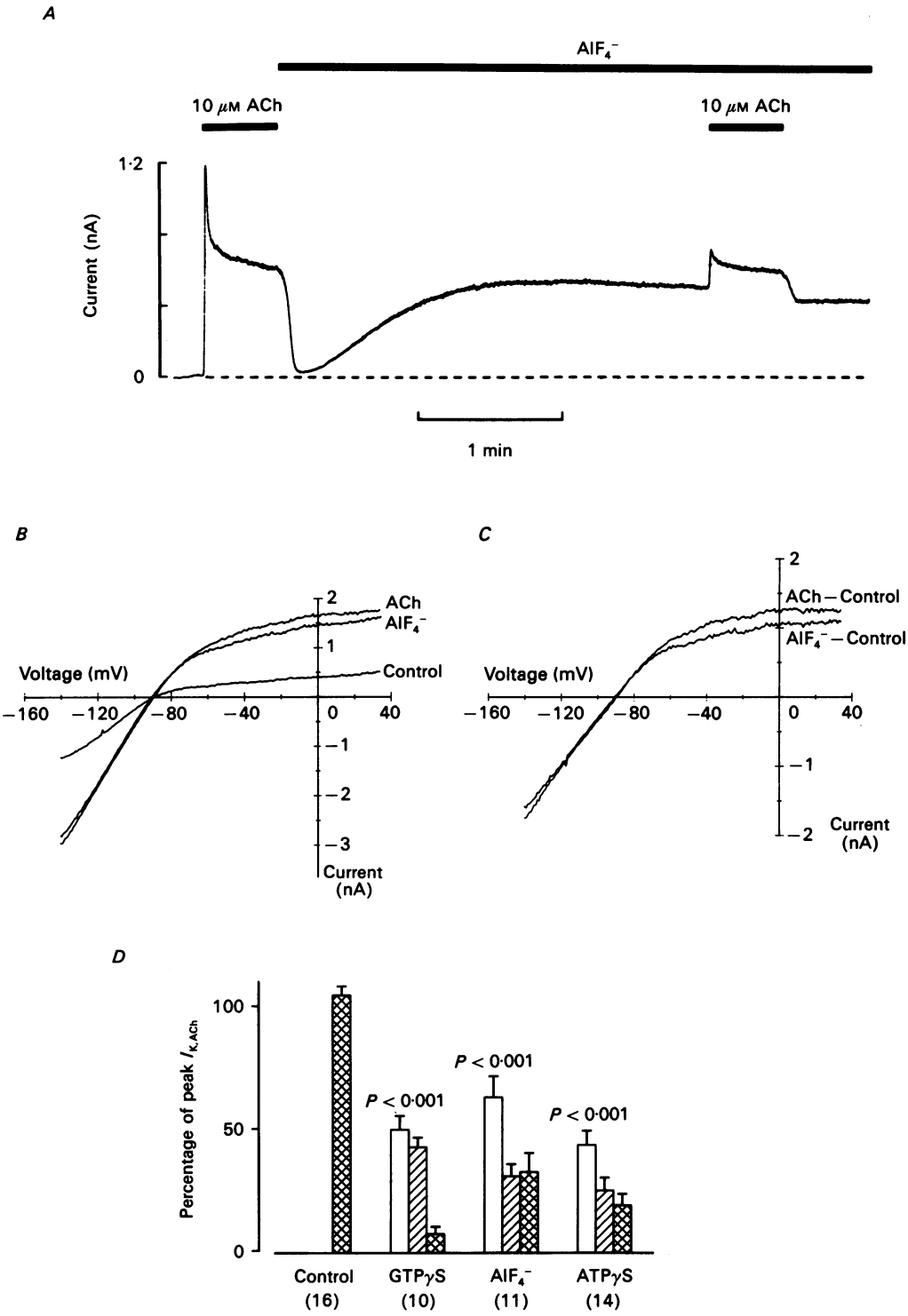

Fig. 7. For legend see facing page.

there was a further activation of $I_{K,ACh}$. Figure 7*D* summarizes the data obtained from eleven cells. It shows both the AlF_4^-- and ACh-activated currents as well as the sum of the two currents, 5·3 ± 0·9 min after the start of perfusion with AlF_4^-. In Fig. 7*D* and also the example shown in Fig. 7*A*, the sum of the AlF_4^-- and ACh-activated currents was less than the peak $I_{K,ACh}$ during the first exposure to ACh (taken to be 100 %). This is similar to the result obtained with GTPγS, which is also summarized in Fig. 7*D*. Figure 7*D* also shows the amplitude of the ACh-activated $I_{K,ACh}$ under normal conditions 5·6 ± 0·5 min after the start of cell dialysis. It is a control and it demonstrates that the response to ACh did not normally decline. It is concluded that activation of G_K by AlF_4^-, like activation by GTPγS, resulted in desensitization to ACh. The desensitization caused by GTPγS was, therefore, unlikely to have been the result of the formation of ATPγS.

In the experiment illustrated in Fig. 5*B* (similar to that in Fig. 5*A*). The GTPγS-activated current was monitored for 12·4 min and over this period the GTPγS-activated current gradually declined. A similar decline was observed in eight cells and perhaps was another result of desensitization brought about by activation of G_K. The AlF_4^--activated current also declined over a similar time course (not shown).

Figure 8*A* shows the record of $I_{K,ACh}$ activated first by ACh and then by GTPγS from Fig. 5*A* superimposed on the record of $I_{K,ACh}$ during a 5 min exposure to ACh from Fig. 2*C*. GTPγS will have acted via G_K, whereas ACh will have acted via the muscarinic ACh receptor (mAChR). The two records have been scaled so that their peak amplitudes are the same. It has already been argued that the GTPγS-activated current was less than the initial peak ACh-activated current as a result of desensitization presumably resulting from a change in G_K or the K^+ channel. However, it is clear from Fig. 8*A* that the decline of the current as a result of desensitization was greater when the muscarinic receptor was involved (i.e. during the 5 min exposure to ACh). This is confirmed by Fig. 8*B*, which summarizes results from many cells. It shows, on the same scale as Fig. 8*A*, the ACh-activated current at the end of a 5 min exposure to ACh and the GTPγS-activated current at the same time. This suggests that the muscarinic receptor is also involved in desensitization.

Fig. 7. Effect of extracellular perfusion with AlF_4^- on $I_{K,ACh}$. *A*, $I_{K,ACh}$ activated first by ACh and then by AlF_4^-. The dashed line corresponds to the level of current immediately after the rupture of the membrane patch. *B*, current–voltage relationships, obtained from the ramp clamps (see Fig. 1*A* legend for details), under control conditions, in the presence of ACh and after the activation of the AlF_4^--dependent current. The results in *A* and *B* were obtained from different cells. *C*, current–voltage relationships for the ACh- and AlF_4^--activated currents. The data were calculated from the data in *B* (see Fig. 1*C* legend for details). *D*, a summary of the effects of GTPγS, AlF_4^- and ATPγS on $I_{K,ACh}$. The bars show the mean amplitude of the ACh-activated current (⊠), the $GTP\gamma_S$-, AlF_4^-- or ATPγS-activated current (▨), and the sum of the two currents (□), 6·3 ± 0·6 (GTPγS), 5·3 ± 0·9 (AlF_4^-) and 8·9 ± 1·0 (ATPγS) min after the start of cell dialysis. The mean amplitude of the ACh-activated current under control conditions 5·6 ± 0·5 min after the start of cell dialysis is also shown. All current amplitudes are expressed as a percentage of the amplitude of the ACh-activated current at the start of cell dialysis. The *n* values are shown below the bars. The sums of the ACh- and GTPγS-, AlF_4^-- or ATPγS-activated currents were all significantly less than the amplitude of the ACh-activated current under control conditions.

The effect of dephosphorylating agents on $I_{K,ACh}$

AMP-PNP

As explained in the Introduction, phosphorylation/dephosphorylation reactions may be involved in desensitization to ACh and, therefore, the effects of phosphorylating and dephosphorylating agents on $I_{K,ACh}$ have been investigated. First, the effect of dephosphorylating agents will be considered. In the initial set of experiments the ATP in the patch electrode was replaced by a non-hydrolysable ATP analogue, AMP-PNP, which cannot be used as a substrate by protein kinases to phosphorylate proteins (Yount, 1975). The degree of phosphorylation of any protein depends on the balance of phosphorylation by protein kinases and dephosphorylation by protein phosphatases and, therefore, in the presence of AMP-PNP rather than ATP, phosphorylation will cease and there will be a net dephosphorylation of the protein.

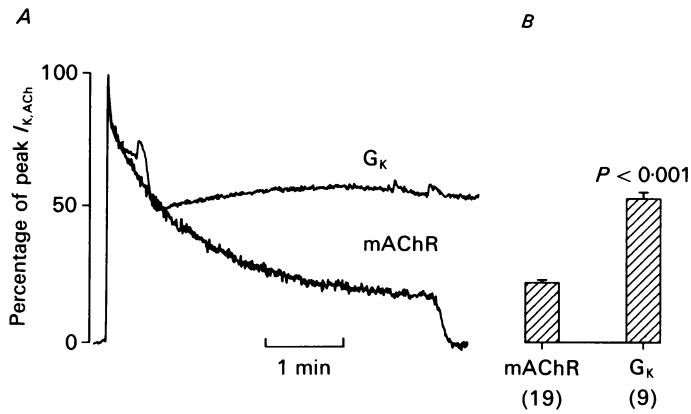

Fig. 8. Comparison of the fade of $I_{K,ACh}$ when $I_{K,ACh}$ was activated either by ACh acting on the muscarinic ACh receptor (mAChR) or by GTPγS acting on G_K. *A*, superimposed records of $I_{K,ACh}$ activated in one case by ACh (applied for 5 min) and in the second case first by ACh and then by GTPγS. The records (from different cells) are the same as those shown in Figs 2*C* and 5*A*; they have been scaled so that they have the same peak amplitude. *B*, the mean amplitude of $I_{K,ACh}$ at the end of a 5 min exposure to ACh (acting via the mAChR) and the mean amplitude of $I_{K,ACh}$ activated by GTPγS (acting via G_K) at the equivalent time. Measurements were made from experiments like those in *A*. The current amplitudes are expressed as a percentage of the peak amplitude of the ACh-activated current at the start of cell dialysis. Thus, the same scale applies to both *A* and *B*. The *n* values are shown below the bars.

In the experiment shown in Fig. 9*A* the patch electrode contained 5 mM ANP-PNP rather than ATP as normal. One minute after the start of cell dialysis application of 10 μM ACh resulted in the activation and fade of $I_{K,ACh}$ as usual. When ACh was reapplied at later times $I_{K,ACh}$ was reduced and 8 min after the start of cell dialysis the response to ACh was eliminated. In seven cells dialysed with AMP-PNP, $6{\cdot}5 \pm 0{\cdot}8$ min after the start of cell dialysis, $I_{K,ACh}$ was $6{\cdot}7 \pm 1{\cdot}6$ % of $I_{K,ACh}$ during the first exposure to ACh. In contrast, when the patch electrode contained 3 mM ATP as usual, $5{\cdot}6 \pm 0{\cdot}5$ min after the start of cell dialysis, $I_{K,ACh}$ was $104{\cdot}4 \pm 3{\cdot}6$ % ($n = 16$) of $I_{K,ACh}$ during the first exposure to ACh (not illustrated). In addition, when it

contained 5 mM ATP, 11·0±1·3 min after the start of cell dialysis, $I_{K,ACh}$ was 103·0±8·6% ($n = 8$) of $I_{K,ACh}$ during the first exposure to ACh (not illustrated).

Figure 9*A* shows that AMP-PNP also reduced the fast phase of desensitization. In seven cells, which had been dialysed with AMP-PNP for 5·0±0·6 min, the fade of $I_{K,ACh}$ during the first 5 s of an exposure to ACh was significantly ($P < 0·01$) reduced to 32·2±12·3% of that seen during the first exposure to ACh.

AMP-PNP could eliminate $I_{K,ACh}$ by an action on the muscarinic receptor, G_K or

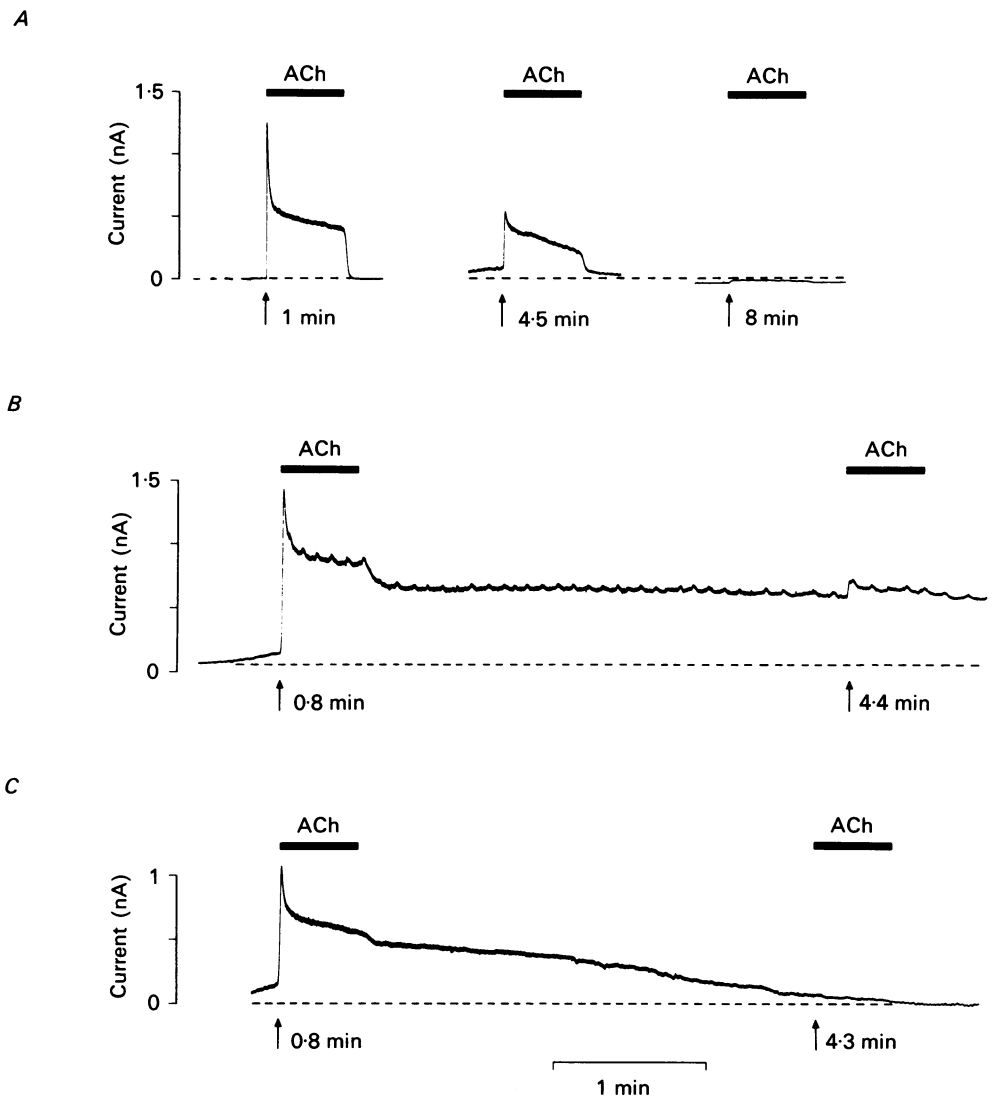

Fig. 9. The effect of AMP-PNP on $I_{K,ACh}$. *A*, effect of dialysis with 5 mM AMP-PNP (and 0·1 mM GTP) on ACh-activated current. *B*, GTPγS-activated current under control conditions (the cell was dialysed with 0·1 mM GTPγS and 3 mM ATP). *C*, effect of dialysis with 5 mM AMP-PNP (and 0·1 mM GTPγS) on GTPγS-activated current. The traces shown in *A*, *B* and *C* were obtained from different cells. ACh was applied at the times (after the start of cell dialysis) shown below the traces. The dashed lines show the membrane current at the start of cell dialysis (*A* and *B*) or the end of the experiment (*C*).

the K^+ channel. To investigate which of the three components was affected, cells were dialysed with AMP-PNP and GTPγS simultaneously. For comparison, the effect of GTPγS alone is shown in Fig. 9*B*; the patch electrode contained 0·1 mM GTPγS rather than GTP. The result is similar to that in Fig. 5*A*. Soon after the start of cell dialysis 10 μM ACh was applied and it resulted in the normal activation of $I_{K,ACh}$. On wash-off of ACh, $I_{K,ACh}$ remained as a result of the activation of G_K by GTPγS. The GTPγS-activated current persisted and only slowly declined with time: in ten cells, 6·3 ± 0·6 min after the start of cell dialysis the GTPγS-activated current was 42·6 ± 3·9 % of the current activated by ACh soon after the start. The effect of GTPγS and AMP-PNP together is shown in Fig. 9*C*; the patch electrode contained 0·1 mM

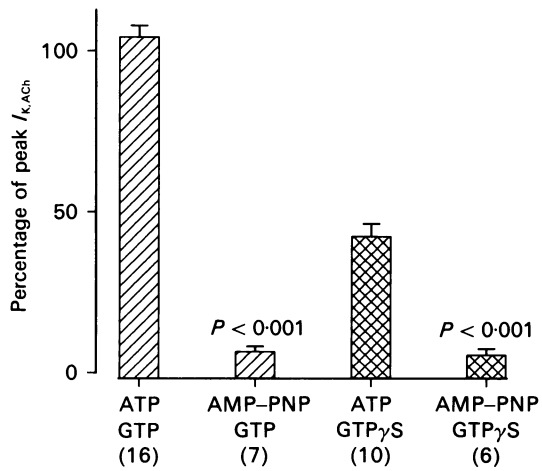

Fig. 10. The amplitude of the ACh- and GTPγS-activated currents in the absence and presence of AMP-PNP. The key component of the electrode solution is shown below the bars. The bars show the mean amplitude of the ACh- (first two bars) or GTPγS- (final two bars) activated current 5·6 ± 0·5 min (ATP + GTP), 6·5 ± 0·8 min (AMP-PNP + GTP), 6·3 ± 0·6 min (ATP + GTPγS) or 5·7 ± 0·6 min (AMP-PNP + GTPγS) after the start of cell dialysis. All current amplitudes are expressed as a percentage of the amplitude of the ACh-activated current at the start of cell dialysis. The *n* values are shown below the bars. The currents recorded in the presence of AMP-PNP were significantly less than their respective controls.

GTPγS instead of GTP and 5 mM AMP-PNP instead of ATP. Once again 10 μM ACh at the start of cell dialysis resulted in the activation of $I_{K,ACh}$ and on wash-off $I_{K,ACh}$ remained as a result of the activation of G_K by GTPγS. However, the GTPγS-activated current declined with time: in six cells, 5·7 ± 0·6 min after the start of cell dialysis the GTPγS-activated current was 5·6 ± 2·9 % of the current activated by ACh soon after the start.

The results obtained from the four experimental protocols are summarized in Fig. 10, which shows the amplitude of $I_{K,ACh}$ 6–7 min after the start of cell dialysis. The first two bars show the amplitude of the ACh-activated current in the absence and presence of AMP-PNP, whereas the final two bars show the amplitude of the GTPγS-activated current in the absence and presence of AMP-PNP. Both the ACh- and GTPγS-activated currents were significantly smaller in the presence of AMP-PNP.

It is possible that the effect of AMP-PNP on $I_{K,ACh}$ is the consequence of net

dephosphorylation brought about by the cessation of phosphorylation and the continuation of dephosphorylation by a phosphatase (see above). Inhibition of the phosphatase, therefore, should inhibit the action of AMP-PNP. Okadaic acid is a specific inhibitor of phosphatases 1 and 2A (Cohen, 1989), but in five cells, which were incubated in 5 μM okadaic acid-containing solution for $\sim$ 2 h and dialysed with 50 μM okadaic acid via the patch electrode, AMP-PNP still caused a decrease in the GTPγS-activated $I_{K,ACh}$. Okadaic acid at 50 μM in the patch electrode increased the L-type Ca^{2+} current in four guinea-pig ventricular myocytes, which proves that the okadaic acid was active. In five cells 100 μM sodium vanadate, a general inhibitor of protein phosphatases (Seargeant & Stinson, 1979), was applied via the patch electrode, but it was without effect on the AMP-PNP-dependent decrease in the ACh-activated $I_{K,ACh}$. Therefore, if a phosphatase was involved in the response to AMP-PNP, it was not one inhibited by either okadaic acid or 100 μM sodium vanadate.

BDM

The effects of 2,3-butanedione monoxime (BDM), an antidote for nerve gas poisoning, have been attributed to its ability to remove phosphorus moieties from proteins, i.e. a phosphatase-like activity for which it was synthesized (Wilson & Ginsburg, 1955). In neuromuscular poisoning it reactivates acetylcholinesterase by dephosphorylation (Askew, 1956) and it inhibits cardiac and neuronal L-type Ca^{2+} currents with ID_{50}s (the dose required to inhibit by 50%) of 5·8 and $\sim$ 20 mM respectively, an effect again attributed to dephosphorylation (Chapman, 1992; Huang & McArdle, 1992). Figure 11*A* shows $I_{K,ACh}$ recorded from two cells under control conditions and in the presence of 32 mM BDM in the bathing medium. Under control conditions, $I_{K,ACh}$ faded as usual during an exposure to ACh, but in the presence of BDM the fade was much reduced. The results from ten cells are summarized in Fig. 11*B*, which shows peak $I_{K,ACh}$ and $I_{K,ACh}$ at 5, 10 and 30 s during a 30 s exposure to ACh both under control conditions and in the presence of 32 mM BDM. The peak $I_{K,ACh}$ was taken to be 100% in both cases. The difference between peak $I_{K,ACh}$ and $I_{K,ACh}$ at the end of an exposure is a measure of the extent of fade during the exposure and this was significantly less in the presence of BDM. Fade of $I_{K,ACh}$ during the first 5 s of an exposure to ACh is plotted against the BDM concentration in Fig. 12*A*. About 5·3 mM BDM reduced fade by 50% and this is comparable to the ID_{50} of 5·8 mM for BDM to inhibit the cardiac L-type Ca^{2+} current by dephosphorylation (Chapman, 1992).

BDM also tended to reduce the peak $I_{K,ACh}$ during an exposure to ACh. An example of this effect is shown in the lower trace of Fig. 11*A*. The open bars in Fig. 11*C* show the peak $I_{K,ACh}$ on exposure of a group of cells to 32 mM BDM. Peak $I_{K,ACh}$ was reduced when the cells had been exposed to BDM for 3·5 min and it was further reduced once the cells had been exposed to BDM for 9 min. Peak $I_{K,ACh}$ is plotted as a function of the BDM concentration in Fig. 12*B*. The concentration of BDM required to inhibit peak $I_{K,ACh}$ by 50% was in excess of 32 mM.

Finally, BDM could increase the amplitude of $I_{K,ACh}$ at the end of an exposure to ACh. An example of this effect is shown in the upper trace of Fig. 11*A*. In Fig. 11*C* the hatched bars represent the amplitude of $I_{K,ACh}$ at the end of an exposure to ACh and when the cells had been exposed to 32 mM BDM for 3·5 min this was significantly increased, although the current at the end of an exposure was no longer increased

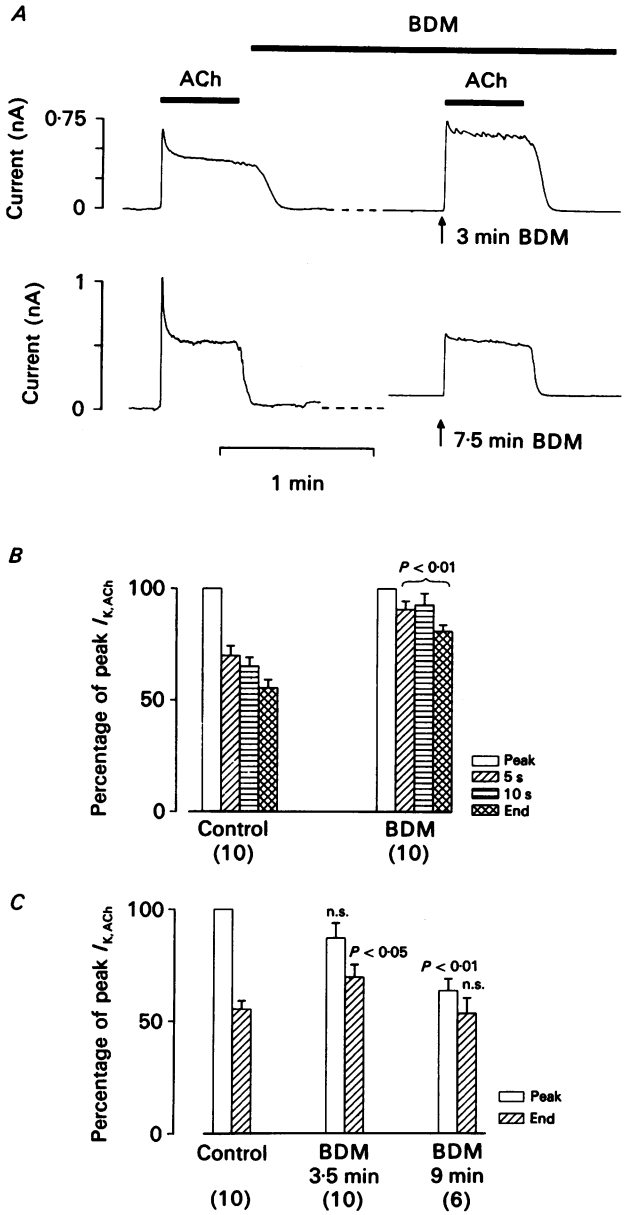

Fig. 11. The effect of 32 mM 2,3-butanedione monoxime (BDM) on $I_{K,ACh}$. *A*, effect of BDM on $I_{K,ACh}$ in two cells. ACh was applied at the times shown by the bars. When ACh was applied a second time the cells had been exposed to BDM for the times shown below the traces. *B*, effect of BDM on the fade of $I_{K,ACh}$ during an exposure to ACh. The bars show the mean amplitude of $I_{K,ACh}$ at the time of the peak current and 5, 10 and 30 (end) s after the start of an exposure to ACh under control conditions and after the cells had been exposed to BDM for 8·8 ± 0·8 min. All currents are expressed as a percentage of the peak $I_{K,ACh}$ at the start of the exposure to ACh. In the presence of BDM the currents shown are significantly greater than the respective currents under control conditions. *C*, effect of BDM on the amplitude of $I_{K,ACh}$. The bars show the mean amplitude of $I_{K,ACh}$ at the time of the peak current and at the end of an exposure to ACh under control conditions and after the cells had been exposed to BDM for the times shown below the

once the cells had been exposed to BDM for 9 min. There were *maintained* increases in $I_{K,ACh}$ at the end of an exposure to ACh at lower concentrations of BDM, although only the increase with 8 mM BDM was significant ($P < 0.05$). The possible significance of this increase will be considered in the Discussion.

The effect of phosphorylating agents on $I_{K,ACh}$

ATPγS

In the first set of experiments to investigate the effect of phosphorylating agents, cells were dialysed with another ATP analogue, ATPγS. ATPγS can be used as a substrate by protein kinases to thiophosphorylate proteins, but, because thiophosphate is a poor substrate for protein phosphatases, in general thiophosphorylated proteins are more slowly dephosphorylated by protein phosphatases than normal phosphorylated proteins (Yount, 1975; Trautwein & Hescheler, 1990). The result is an increase in the degree of phosphorylation of proteins. Fourteen cells were perfused with ATPγS via the patch electrode, which contained 5 mM ATPγS rather than 3 mM ATP. In the majority of cells the response was similar to the response to GTPγS and AlF_4^-: the holding current became more outward in the majority of cells and the ACh-activated $I_{K,ACh}$ decreased in amplitude. The results are summarized in Fig. 7*D* and can be compared to results obtained with GTPγS and AlF_4^-. Although the decrease in ACh-activated $I_{K,ACh}$ could have been the result of phosphorylation, it also could have been the result of the formation of GTPγS from ATPγS by a transphosphorylation reaction (see Discussion).

Isoprenaline

Isoprenaline, a β-agonist, ultimately activates protein kinase A, a kinase responsible for the phosphorylation of a variety of proteins. It had no effect on $I_{K,ACh}$. In seven cells 6·5±0·9 min after the application of 1 μM isoprenaline via the bathing solution, $I_{K,ACh}$ was 100·6±8·7% of its value under control conditions. Isoprenaline had no effect on the fade of $I_{K,ACh}$ during a 30 s exposure to ACh.

Phosphatase inhibitors

Inhibition of the phosphatase responsible for the dephosphorylation of a protein is expected to result in an increase in the degree of phosphorylation of the protein. The effects of the phosphatase inhibitors, okadaic acid and sodium vanadate, on $I_{K,ACh}$ have been investigated. Nine cells were perfused with 50 μM okadaic acid via the patch electrode and ACh was applied for 30 s every 3·5 min: 6·1±0·6 min after the start of cell dialysis in eight of the cells, the amplitude of $I_{K,ACh}$ was significantly ($P < 0.05$) reduced to 82·1±6·7% of that during the first exposure to ACh (in the ninth cell there was an apparent large increase in $I_{K,ACh}$ and this cell was excluded from the analysis). Okadaic acid also significantly ($P < 0.01$) reduced the fast phase of desensitization in the eight cells: after dialysis with okadaic acid, the fade of $I_{K,ACh}$ during the first 5 s of an exposure to ACh was reduced to 58·7±9·6% of that during

bars. All currents are expressed as a percentage of the peak $I_{K,ACh}$ at the start of the exposure to ACh *under control conditions*. In the presence of BDM some currents were significantly different but others were not (n.s.) as compared to the respective currents under control conditions. In *B* and *C*, *n* values are given below the bars.

the first exposure to ACh. The effect of 100 μM sodium vanadate, which was applied via the patch electrode, was investigated in a similar way in eight cells and it had no significant effect on $I_{K,ACh}$: 5·4 ± 0·6 min after the start of cell dialysis, the amplitude of $I_{K,ACh}$ was 102·2 ± 9·8 % and the fade of $I_{K,ACh}$ was 95·1 ± 4·1 % of those during the first exposure to ACh. Because vanadate and phosphate compete for the same

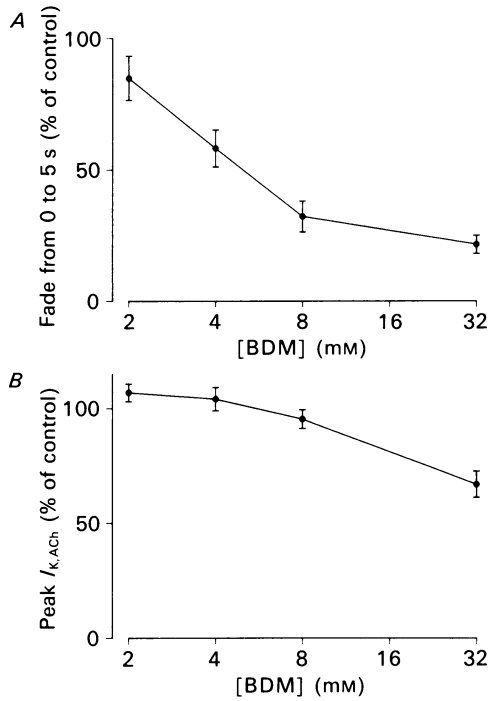

Fig. 12. The effect of BDM at concentrations ranging from 2 to 32 mM on the fade of $I_{K,ACh}$ during the first 5 s of an exposure to ACh (*A*) and the peak amplitude of $I_{K,ACh}$ early during an exposure to ACh (*B*). Both measurements are expressed as a percentage of the respective measurements under control conditions. The cells had been exposed to BDM for 6–9 min when measurements were made. *n* values range from eight to ten cells.

binding site on the phosphatase (Seargeant & Stinson, 1979) and the intracellular phosphate concentration is about 6 mM (Allen, Morris, Orchard & Pirolo, 1985), we also investigated the effect of 5 mM sodium vanadate in seven cells. In six of the cells it resulted in significant ($P < 0·02$) decreases in both the amplitude and the fade of $I_{K,ACh}$: 5·3 ± 0·8 min after the start of cell dialysis, the amplitude of $I_{K,ACh}$ was 71·9 ± 7·1 % and the fade of $I_{K,ACh}$ was 52·8 ± 7·1 % of those during the first exposure to ACh. In the seventh cell it resulted in an apparent large increase in fade and this cell was excluded from the analysis above.

DISCUSSION

Activation of $I_{K,ACh}$ by ACh, GTPγS and AlF_4^-

ACh, GTPγS and AlF_4^- activated a current which had a reversal potential of −87·2 ± 1·2, −88·2 ± 2·6 and −89·7 ± 2·1 mV respectively (close to the calculated value of E_K of −88·6 mV) and showed inward-going rectification (Figs 1, 6 and 7).

This suggests that ACh, GTPγS and AlF_4^- activated the same current, $I_{K,ACh}$. Although GTPγS has previously been shown to activate $I_{K,ACh}$, this is the first study (to our knowledge) to demonstrate that AlF_4^- can also activate $I_{K,ACh}$.

Kinetics of the onset of desensitization to ACh

Fade of $I_{K,ACh}$ activated by 10 μM ACh was biphasic with mean time constants of $1{\cdot}58 \pm 0{\cdot}14$ and $148{\cdot}2 \pm 12{\cdot}8$ s. Carmeliet & Mubagwa (1986) have reported that fade of $I_{K,ACh}$ in rabbit Purkinje fibres is also biphasic with time constants of 12·5 and 76·9 s during an exposure to 10 μM ACh. However, these time constants can be expected to be in error, because multicellular preparations were used and application of ACh to individual cells within the preparation would have been slow and non-uniform (for example, in one case $I_{K,ACh}$ reached a peak in ~ 18 rather than ~ 1 s on application of 10 μM ACh) (Carmeliet & Mubagwa, 1986). Kim (1991) has reported that fade of $I_{K,ACh}$ in rat atrial cells is biphasic, although time constants were not given. The presence of two phases of desensitization suggests that at least two mechanisms may be involved in desensitization to ACh. Honjo *et al.* (1991) have suggested that the fade of $I_{K,ACh}$ is responsible for the fade of the chronotropic response to ACh in rabbit sino-atrial node cells. Fade of the chronotropic response of multicellular preparations of the rabbit sino-atrial node to 0·01–10 μM ACh is biphasic with time constants of 14 and 348 s (Boyett & Roberts, 1987), which are roughly similar to the time constants of fade of $I_{K,ACh}$ in guinea-pig atrial cells. This supports the hypothesis that the fade of $I_{K,ACh}$ is responsible for the fade of the chronotropic response.

Kinetics of recovery from desensitization to ACh

As predicted, recovery of $I_{K,ACh}$ from the fast and slow phases of desensitization (measured after 30 s and 5 min exposures to ACh respectively) occurred along different time courses with time constants of 52 and 222 s respectively (Fig. 3). This supports the conclusion that at least two processes are responsible for desensitization. In the rabbit sino-atrial node, recovery of the chronotropic response after a 30 s exposure to ACh, during which only the fast phase of desensitization develops, is fast with a time constant of 58 s, whereas recovery after a 5 min exposure, during which the slow phase of desensitization also develops, is slow with a time constant of 362 s (Boyett & Roberts, 1987). The time constants of the recovery of $I_{K,ACh}$ (52 and 222 s) and the chronotropic response (58 and 362 s) from the fast and slow phases of desensitization are therefore similar and this further supports the hypothesis that fade of $I_{K,ACh}$ is responsible for fade of the chronotropic response.

Although the activation of a cation channel by the nicotinic ACh receptor, at the neuromuscular junction for example, is different from the activation of the K^+ channel by the muscarinic ACh receptor in the heart (the former does not involve a G protein), both ACh-activated currents fade with time as a result of desensitization. Furthermore, the fade of the nicotinic ACh receptor-activated current is biphasic and the recovery from the two phases of desensitization once again occurs along different time courses, recovery from the slow phase being slower (Feltz & Trautmann, 1982; Chesnut, 1983; Boyd, 1987). As in the case of $I_{K,ACh}$ (Fig. 3), recovery is slower after long exposures to the agonist (Feltz & Trautmann, 1982; Boyd, 1987).

The temperature dependence of $I_{K,ACh}$

The temperature coefficient (Q_{10}) of the amplitude of $I_{K,ACh}$ was low ($1{\cdot}6 \pm 0{\cdot}1$). This low temperature sensitivity is typical of the conductance of many channels and is like that for that aqueous diffusion of ions (Hille, 1984). The Q_{10} of the rate of activation of $I_{K,ACh}$ was higher ($1{\cdot}9 \pm 0{\cdot}2$) and is a value like that of many enzyme reactions and conformational changes of proteins (the activation of $I_{K,ACh}$, of course, depends on a cascade of reactions). The Q_{10} of the rate constant of the fast phase of fade ($2{\cdot}2 \pm 0{\cdot}3$) was also high and this suggests that an enzyme reaction or a conformational change of a protein is involved in the fast phase. The gating of voltage-dependent channels is known to involve conformational changes of channel proteins and the Q_{10} of channel gating is typically $\geqslant 2$ (Hille, 1984).

The site of desensitization

Both GTPγS and AlF_4^- produced desensitization to ACh and, because they bypass the muscarinic receptor and activate $I_{K,ACh}$ via G_K, this suggests that desensitization does not exclusively involve the receptor. Several lines of evidence show that GTPγS and AlF_4^- caused desensitization to ACh: first, the sum of the GTPγS- or AlF_4^- and ACh-activated currents (after the development of the GTPγS- or AlF_4^--activated current) was less than the peak amplitude of the control ACh-activated current (in the absence of GTPγS or AlF_4^-) (Fig. 7*D*). Secondly, the GTPγS- and AlF_4^--activated currents declined over a period of minutes (Fig. 5*B*). Finally, even when GTPγS rather than GTP was available for G_K activation, $I_{K,ACh}$ still rapidly faded on application of ACh: during the first exposure to ACh in Figs 5, 6 and 9*B*, GTPγS was available (as indicated by the rise in outward current before and after the exposure) and yet the current still faded. If desensitization involved the receptor only, fade of $I_{K,ACh}$ would not be expected when GTPγS was available, because the receptors would only have to be momentarily rather than continuously available to facilitate a once-only binding of GTPγS to G_K (it is assumed that normally there is a continuous cycle of GTP binding and GTP hydrolysis and therefore the receptor has to be available continuously throughout an exposure to ACh). The conclusion that desensitization does not exclusively involve the muscarinic receptor is supported by the finding reported by Kurachi *et al.* (1987) that adenosine, which acts via purinergic receptors (thus bypassing the muscarinic receptor) to activate *muscarinic* K^+ channels, causes desensitization to ACh. When the muscarinic receptor is bypassed, the desensitization must result from either a modification of G_K or the muscarinic K^+ channel.

The extent of desensitization was greater when the muscarinic receptor was involved, i.e. when ACh rather than GTPγS was used to activate $I_{K,ACh}$ (Fig. 8). This finding is consistent with the observation of Kim (1991) that after the decrease in muscarinic K^+ channel activity in inside-out patches from rat atrial cells as a result of desensitization to ACh, GTPγS partially, but not wholly, restored channel activity. This suggests that the muscarinic receptor is also involved in desensitization. The desensitization during an exposure to ACh is presumably the result of a modification of both the muscarinic receptor and G_K or the K^+ channel. Figure 8 suggests that of the desensitization taking place during a 5 min exposure to ACh $\sim 60\%$ (that seen when the muscarinic receptor was bypassed) was the result of

modification of either G_K or the K^+ channel and $\sim 40\%$ (the extra seen when $I_{K,ACh}$ was activated via the muscarinic receptor) was the result of a modification of the muscarinic receptor. In the presence of GTPγS the fast phase of desensitization still occurred as discussed above, whereas the slow phase was reduced or stopped (although the GTPγS-activated current did decline, the decline of the ACh-activated current during the slow phase of desensitization was faster – compare Figs 2*C* and 3*B* with Figs 5, 6 and 9). This suggests that the fast phase of desensitization results from a change in G_K or the K^+ channel, whereas the slow phase results from a change in the receptor.

The muscarinic receptor not only activates a K^+ channel, it also inhibits adenylate cyclase. We have observed that effects of ACh mediated by adenylate cyclase do not fade: in rabbits sinoatrial node cells the ACh-induced decreases in both i_f and β-agonist potentiated I_{Ca} (effects mediated by adenylate cyclase) do not fade, whereas $I_{K,ACh}$ does fade during an exposure to ACh (Honjo *et al.* 1991; H. Honjo & M. R. Boyett, unpublished observations); the inotropic effect of ACh on β-agonist potentiated guinea-pig ventricular cells (presumably mediated by adenylate cyclase) does not fade, whereas the inotropic effect of ACh on guinea-pig atrial cells (presumably mediated by $I_{K,ACh}$) does fade (M. R. Boyett, unpublished observations). If it is assumed that the same muscarinic receptor is responsible for the two actions, these results suggest that the muscarinic receptor cannot be the site of desensitization. This, however, is difficult to reconcile with the findings from this paper and, therefore, either different receptors are responsible for activating the K^+ channel and inhibiting adenylate cyclase and only the receptor coupled to the K^+ channel is affected by desensitization, or desensitization decreases the coupling of the receptor to G_K but not to the G protein linking the receptor to adenylate cyclase (this assumes that the G proteins are different).

AMP-PNP

It is possible that desensitization is the result of dephosphorylation or phosphorylation (of the muscarinic receptor, G_K or the K^+ channel). Kim (1991) suggested that desensitization is, in part, the result of dephosphorylation. Kim (1991) showed that ATP increased ACh- and GTPγS-activated K^+ channel activity in isolated patches from rat atrial cells, whereas AMP-PNP (which cannot be used to phosphorylate proteins) failed to do so. Such effects could explain the decrease in the ACh- or GTPγS-activated $I_{K,ACh}$ when guinea-pig atrial cells were perfused with AMP-PNP (Fig. 9). Therefore, the effect of ATP seen in isolated patches is possibly seen in whole cells. In isolated patches the effect of ATP did not spontaneously reverse on wash-out of ATP (Kim, 1991) and only reversed on addition of 30 μM Ca^{2+} (presumably as a result of the activation of a Ca^{2+}-dependent phosphatase). The reversal induced by Ca^{2+} was unaffected by the inhibitor of phosphatases PP1 and PP2A, okadaic acid, (as was the AMP-PNP-induced decrease in $I_{K,ACh}$ in guinea-pig atrial cells in the present study), but was inhibited by another phosphatase inhibitor, 100 μM vanadate. In contrast, 100 μM vanadate failed to prevent the AMP-PNP-induced decrease in $I_{K,ACh}$ in guinea-pig atrial cells in the present study (however, it is possible that 100 μM vanadate is ineffective in blocking phosphatases in the intact cell).

If desensitization is the result of *phosphorylation*, the effect of AMP-PNP on the

amplitude of $I_{K,ACh}$ cannot be explained. AMP-PNP also reduced the fast phase of desensitization (Fig. 9*A*), an effect expected regardless of whether desensitization is the result of dephosphorylation or phosphorylation: in the case of the dephosphorylation hypothesis, the dephosphorylation (and hence desensitization) will be irreversible, whereas in the case of the phosphorylation hypothesis, phosphorylation (and hence desensitization) will not occur.

It is interesting that Kaibara, Nakajima, Irisawa & Giles (1991) found that AMP-PNP abolished the spontaneous activity (i.e. activity in the absence of agonist) of the muscarinic K^+ channel. They conclude from this that 'phosphorylation of the channel itself or associated proteins is involved in their activation'. Our data are consistent with this conclusion. Regardless of the underlying mechanism, the effect of replacement of ATP by AMP-PNP raises the possibility that during myocardial ischaemia, when intracellular ATP is depleted, there may be a loss of sensitivity to ACh.

BDM

BDM has a phosphatase-like activity. If desensitization is the result of dephosphorylation, a phosphatase (if active at the site responsible for desensitization) is expected to reduce the peak amplitude of $I_{K,ACh}$ and abolish the fade of $I_{K,ACh}$ by dephosphorylating the site which is normally dephosphorylated (thus bringing about desensitization) during an exposure to ACh. If desensitization is the result of phosphorylation, a phosphatase (again if active at the site responsible for desensitization) is expected to either not affect or increase the peak amplitude of $I_{K,ACh}$ and abolish the fade of $I_{K,ACh}$ by antagonising the phosphorylation of the site responsible for desensitization during an exposure to ACh. BDM greatly reduced the fast phase of desensitization and the concentration of BDM required to decrease the fade of $I_{K,ACh}$ by 50% was approximately the same as that required to reduce the cardiac L-type Ca^{2+} current by 50%, an effect which has been attributed to dephosphorylation. This is consistent with either hypothesis. BDM did decrease the peak amplitude of $I_{K,ACh}$ (consistent with the dephosphorylation hypothesis), but only at higher concentrations and not in parallel with the decrease in fade, as expected from the dephosphorylation hypothesis. BDM often increased $I_{K,ACh}$ at the end of exposure to ACh (Fig. 11*A*, top trace; this is why lower concentrations inhibited fade and yet the peak amplitude of $I_{K,ACh}$ was little affected) and this is consistent with the phosphorylation hypothesis, although the decrease in the peak amplitude of $I_{K,ACh}$ at higher concentrations cannot be explained by this hypothesis.

Protein kinases

Regardless of whether desensitization is the result of dephosphorylation or phosphorylation, phosphorylation by a protein kinase will be involved. Although no systematic attempt was made to identify the protein kinase possibly involved in desensitization, it was observed that isoprenaline was without effect on $I_{K,ACh}$ and this suggests that protein kinase A is not involved in the regulation of $I_{K,ACh}$. In addition, in the present study the intracellular Ca^{2+} concentration was $\sim 10^{-10}$ M and under these conditions protein kinase C and other Ca^{2+}-dependent protein kinases

will be inactive (Morgan, 1989) and, therefore, they cannot be involved. Kwatra *et al.* (1989) have suggested that desensitization to ACh is the result of phosphorylation caused by β-adrenergic receptor kinase (β-ARK). However, in twelve cells 10 or 100 μM heparin (a blocker of β-ARK; Benovic, Stone, Caron & Lefkowitz, 1989) had no effect on $I_{K,ACh}$ (W.-J. Zang & M. R. Boyett, unpublished observations).

Protein phosphatases

If fade of $I_{K,ACh}$ during an exposure to ACh is the result of dephosphorylation by a phosphatase, block of the phosphatase should eliminate fade and increase or leave unchanged the amplitude of $I_{K,ACh}$. Alternatively, if fade of $I_{K,ACh}$ is the result of phosphorylation, block of the phosphatase involved should eliminate fade and decrease $I_{K,ACh}$ (because the phosphorylation would be irreversible). There are four types of protein phosphatase according to the classification scheme of Cohen (1989): PP1, PP2A, PP2B and PP2C. Both okadaic acid (an inhibitor of phosphatases PP1 and PP2A) and 5 mM vanadate (a general phosphatase inhibitor) reduced both the amplitude of $I_{K,ACh}$ as well as the fast phase of desensitization. Although this can be explained by the phosphorylation hypothesis, it can also be explained by the dephosphorylation hypothesis, if the decrease in the amplitude of $I_{K,ACh}$ is the result of another mechanism. Kim (1991) has suggested that fade of $I_{K,ACh}$ is the result of dephosphorylation caused by PP2B (Ca^{2+}–calmodulin dependent protein phosphatase). However, PP2B is inactive at Ca^{2+} concentrations below about 10^{-7} M (Klee, Draetta & Hubbard, 1988) and in the present study the intracellular Ca^{2+} concentration was $\sim 10^{-10}$ M and, therefore, this phosphatase cannot have been responsible for the fade of $I_{K,ACh}$ in the present study.

ATPγS

Dialysis of cells with ATPγS resulted in desensitization to ACh (Fig. 7*D*). Although this could have been the result of irreversible phosphorylation by ATPγS, there is an alternative explanation. Nucleoside diphosphate kinase (NDPK) can transfer the thiophosphate from ATPγS to GDP bound on G_K. This transphosphorylation results in the formation of activated α-subunit (α-GTPγS), which of course irreversibly activates $I_{K,ACh}$ (Otero, Breitwieser & Szabo, 1988; Otero, 1990; Heidbüchel, Vereecke & Carmeliet, 1991). In the present study ATPγS, like GTPγS, activated an outward current and, therefore, it is possible that the desensitization to ACh shown in Fig. 7*D* was the result of the formation of GTPγS and the activation of G_K rather than phosphorylation.

The nicotinic ACh receptor-activated current is also regulated by phosphorylation (Huganir, Delcour, Greengard & Hess, 1986; Huganir & Greengard, 1987). The receptor is phosphorylated by cAMP-dependent protein kinase, protein kinase C and tyrosine kinase. Although ACh-induced desensitization of the nicotinic ACh receptor is not the result of phosphorylation (desensitization can be demonstrated in purified preparations of the receptor and does not require the presence of protein kinases or ATP), phosphorylation controls (accelerates) the rate of desensitization.

In summary, we conclude that desensitization involves at least two mechanisms and the fast phase of desensitization involves G_K or the muscarinic K^+ channel,

whereas the slow phase involves the muscarinic receptor. Because the fast phase of desensitization has a high Q_{10} and is reduced by AMP-PNP, BDM, okadaic acid and vanadate, it may involve a phosphorylation/dephosphorylation reaction.

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