Receptor kinase-dependent desensitization of the muscarinic K^+ current in rat atrial cells

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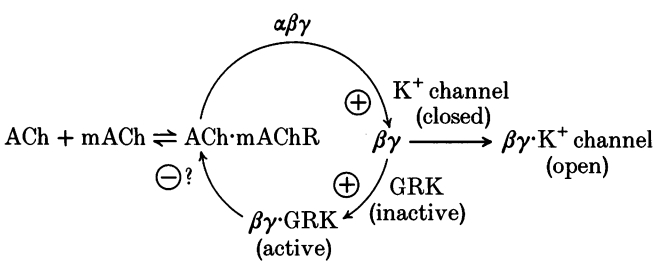
1. Activity of rat atrial muscarinic K^+ channels has been measured in five configurations of the patch clamp technique.

2. In configurations in which the normal intracellular solution was lost, the slow phase of desensitization (a slow decline of channel activity during an exposure to ACh) was much reduced (or absent) and deactivation (on wash-off of ACh) was slowed as compared with desensitization and deactivation in configurations in which normal intracellular solution was retained. This suggests that soluble intracellular regulators are involved in these processes.

3. When a G protein-coupled receptor kinase (GRK2) was applied to the cytoplasmic surface of conventional outside-out patches in the presence of ATP, the slow phase of desensitization was restored. In the absence of ATP, GRK2 failed to restore the slow phase.

4. It is concluded that (i) G protein-coupled receptor kinase dependent phosphorylation of the muscarinic receptor is responsible for the slow phase of desensitization and (ii) a soluble factor (such as a GTPase activating protein or 'GAP') is responsible for normal rapid deactivation.

The heart is under the control of ACh, which decreases the heart rate and the strength of contraction by activating the muscarinic K^+ channel. As well as activating the channel, ACh also initiates the process of desensitization, which causes the channel to 'inactivate' (e.g. Kurachi, Nakajima & Sugimoto, 1987). As a result, many of the actions of ACh on the heart, including those above, are transient and decline with time (e.g. Boyett, Kirby, Orchard & Roberts, 1988; Honjo, Kodama, Zang & Boyett, 1992). ACh activates the muscarinic K^+ channel by binding to the m2 muscarinic receptor (mAChR). The ACh-bound mAChR causes a G protein ($G_K \equiv G_i$) to dissociate into its constituent α- and $\beta\gamma$-subunits and the $\beta\gamma$-subunits then bind to and open the K^+ channel (Reuveny *et al.* 1994). The $\beta\gamma$-subunits also bind to and activate muscarinic receptor kinase, which is one of a family of G protein-coupled receptor kinases (GRK) (Haga, Haga & Kameyama, 1994). In the presence of ACh, the chick heart mAChR is phosphorylated over ~4–5 min (Kwatra *et al.* 1987), presumably by the activated kinase (Haga *et al.* 1994):



Phosphorylation of the mAChR by the kinase may be responsible for desensitization (e.g. by uncoupling the mAChR and G protein) (Haga *et al.* 1994). If it is responsible for desensitization of the muscarinic K^+ channel, wash-off of the kinase should stop desensitization and replacement should restore it.

METHODS

Rats were killed by cervical dislocation and atrial cells were prepared as described previously (Harrison, McCall & Boyett, 1992). Experiments were carried out with various configurations of the patch clamp technique. Sylgard-coated pipettes with resistances of 3 MΩ (perforated outside-out configuration) or 5 MΩ (other recording configurations) were used. Unless specified otherwise, intracellular solution contained (mM): potassium aspartate, 120; KCl, 20; KH_2PO_4, 1; $MgCl_2$, 5·5 (free Mg^{2+}, 1·8); EGTA, 5; Hepes, 5; Na_2ATP, 3; Na_3GTP, 0·02; pH 7·4 (titrated with KOH); extracellular solution contained (mM): KCl, 140; $MgCl_2$, 1·8; EGTA, 5; Hepes, 5; pH 7·4 (titrated with KOH). In the inside-out configuration, there was no GTP or ATP in the control intracellular solution and 0·1 mM GTP and 3 mM ATP in the test intracellular solution. In some conventional outside-out experiments in the absence of ATP, 20 μM glybenclamide was present in the extracellular solution to block ATP-sensitive K^+ channels. For the perforated outside-out patch clamp technique (Rae, Cooper, Gates & Watsky, 1991), a 200–400 μm column of intracellular solution without amphotericin B was drawn up into the tip of the pipette; the pipette was then back-filled with intracellular solution containing 0·25 mM amphotericin B. When the tip

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of the pipette touched a cell, negative pressure (20 cmH_2O) was applied to the pipette to produce a giga-seal. Once a giga-seal was formed the pressure was reduced to 10 cmH_2O for 10 s to produce a bigger vesicle within the tip of the pipette. The negative pressure was then released and the capacitive transients cancelled and the holding potential set. Over ~10 min the amphotericin B diffused into the pipette tip and permeabilized the membrane patch and membrane capacity transients in response to a rectangular command pulse appeared and became larger with time. When the amplitude of the capacity transients was stable, the pipette was withdrawn from the cell to form a perforated outside-out patch. Acetylcholine chloride (10 μM) was added to the extracellular solution when required. Currents were recorded with an Axopatch-1D amplifier and acquired with pCLAMP software (Axon Instruments Inc.). Whole-cell and single-channel currents were filtered at 2 and 5 kHz, respectively, with an 8-pole Bessel filter and sampled every 0·5 and 0·2 ms, respectively. Mean NP_o (the product of the number of channels in a patch and the open probability of a channel) was calculated for consecutive 200 ms episodes as the mean current during an episode divided by the unitary current. All experiments were carried out at room temperature and at a holding potential of −60 mV (inside with respect to outside). The bathing solution (outside a cell or patch) could be changed in ~200 ms using a rapid superfusion device consisting of six 0·5 mm stainless-steel tubes mounted in a pipette 2 mm from the tip (diameter, 0·5 mm). Solution flow was controlled using miniature solenoid valves and was directed onto the cell or patch under study. Sf9 cells were transfected with recombinant baculovirus containing cDNA encoding bovine GRK2 (Benovic, DeBlasi, Stone, Caron & Lefkowitz, 1989), and GRK2 expressed in Sf9 cells was purified as described previously (Kameyama, Haga, Haga, Moro & Sadée, 1994). A stock solution containing ~1 μM GRK2, 20 mM Hepes–KOH buffer (pH 7·5), 2 mM EDTA, ~300 mM NaCl, 0·07% CHAPS and 10% glycerol was stored at −80 °C until required. On the day of an experiment stock solution was diluted 100-fold using intracellular solution. In an assay, 10 nM GRK2 was sufficient to incorporate 5–10 mol of phosphate per mole of receptor, when 2–4 nM m2 mAChR was incubated with GRK2 for 60 min at 30 °C with 50 μM [^{32}P]ATP. Data are presented as means ± S.E.M. and statistical significance of differences was calculated using a one-way analysis of variance.

RESULTS

Responses to ACh in different patch clamp configurations

Figure 1*A* shows mean whole-cell current recorded from ten rat atrial cells with the whole-cell patch clamp technique. When 10 μM ACh was applied by a rapid superfusion device for 3 min, it resulted in the activation of muscarinic K^+ current ($I_{K,ACh}$). $I_{K,ACh}$ was an inward current under the experimental conditions used. On wash-off of ACh, $I_{K,ACh}$ was deactivated and membrane current returned to its control level. During the exposure to ACh, $I_{K,ACh}$ declined as a result of desensitization. There are known to be two phases of desensitization: a rapid phase in the first 20 s followed by a slow phase (Zang, Honjo, Kirby & Boyett, 1993). In the whole-cell configuration, although the fast phase could be seen, it was small. During the slow phase of desensitization (after the first 20 s of the exposure to ACh), the current declined by 41 ± 2% (of $I_{K,ACh}$ at 20 s; $n = 10$) with a time constant of 144 s. The decline of $I_{K,ACh}$ (or NP_o) during the slow phase in this and other configurations is shown in Fig. 2.

Figure 1 also shows recordings of muscarinic K^+ channel activity made with four other configurations of the patch clamp technique: Fig. 1*B* and *C* shows examples of currents from single patches (*B*, perforated outside-out patch; *C*, conventional outside-out patch), whereas Fig. 1*D*–*G* shows mean NP_o from seven to eight cell-attached (*D*), perforated outside-out (*E*), conventional outside-out (*F*) and inside-out (*G*) patches. After the application of ACh (or GTP; see below) the single-channel conductances were 37·6 ± 0·9, 37·5 ± 0·9, 36·9 ± 0·8 and 35·1 ± 1·2 pS (means ± S.E.M., $n = 5$) and the mean open times were 0·79 ± 0·17, 0·85 ± 0·24, 0·88 ± 0·18 and 0·91 ± 0·14 ms ($n = 5$) (measured at the end of the 3 min exposures to ACh or GTP) in the cell-attached, perforated outside-out, conventional outside-out and inside-out configurations, respectively. These values are typical of the muscarinic K^+ channel (e.g. Sakmann, Noma & Trautwein, 1983; Kaibara, Nakajima, Irisawa & Giles, 1991).

In cell-attached recordings (Fig. 1*D*), 10 μM ACh was included in the patch pipette. With this recording configuration it was not possible to observe the activation and deactivation of the muscarinic K^+ channel on application and wash-off of ACh. However, during the first 3 min of cell-attached recording there was a progressive decline in muscarinic K^+ channel activity. During the slow phase of desensitization, mean NP_o declined by 52 ± 7% ($n = 7$; Fig. 2) with a time constant of 51 s. There was no observable fast phase of desensitization, perhaps because it occurred when the patch pipette was first sealed onto the cell (channel activity was not recorded at this time). The extent and time course of desensitization in cell-attached recordings were similar to those in whole-cell recordings. This demonstrates that desensitization is a local response to ACh and does not require the participation of the whole cell.

Figure 1*F* shows mean NP_o during a 3 min application of 10 μM ACh to conventional outside-out patches. This is the first time that outside-out recordings of muscarinic K^+ channel activity have been reported. On application of ACh, the muscarinic K^+ channel was activated and, on wash-off of ACh, it was deactivated. However, during the 3 min exposure to ACh the pattern of desensitization was different from that observed with the whole-cell or cell-attached configurations: there was an initial fast phase of desensitization during the first 20 s of the exposure to ACh, but there was little or no subsequent slow phase (Fig. 2).

In the inside-out configuration it is not possible to apply and wash-off ACh rapidly. Therefore, ACh was present continuously in the patch pipette and we rapidly applied 0·1 mM GTP to the inside surface of the inside-out patches to activate the muscarinic K^+ channel (in the whole-cell and

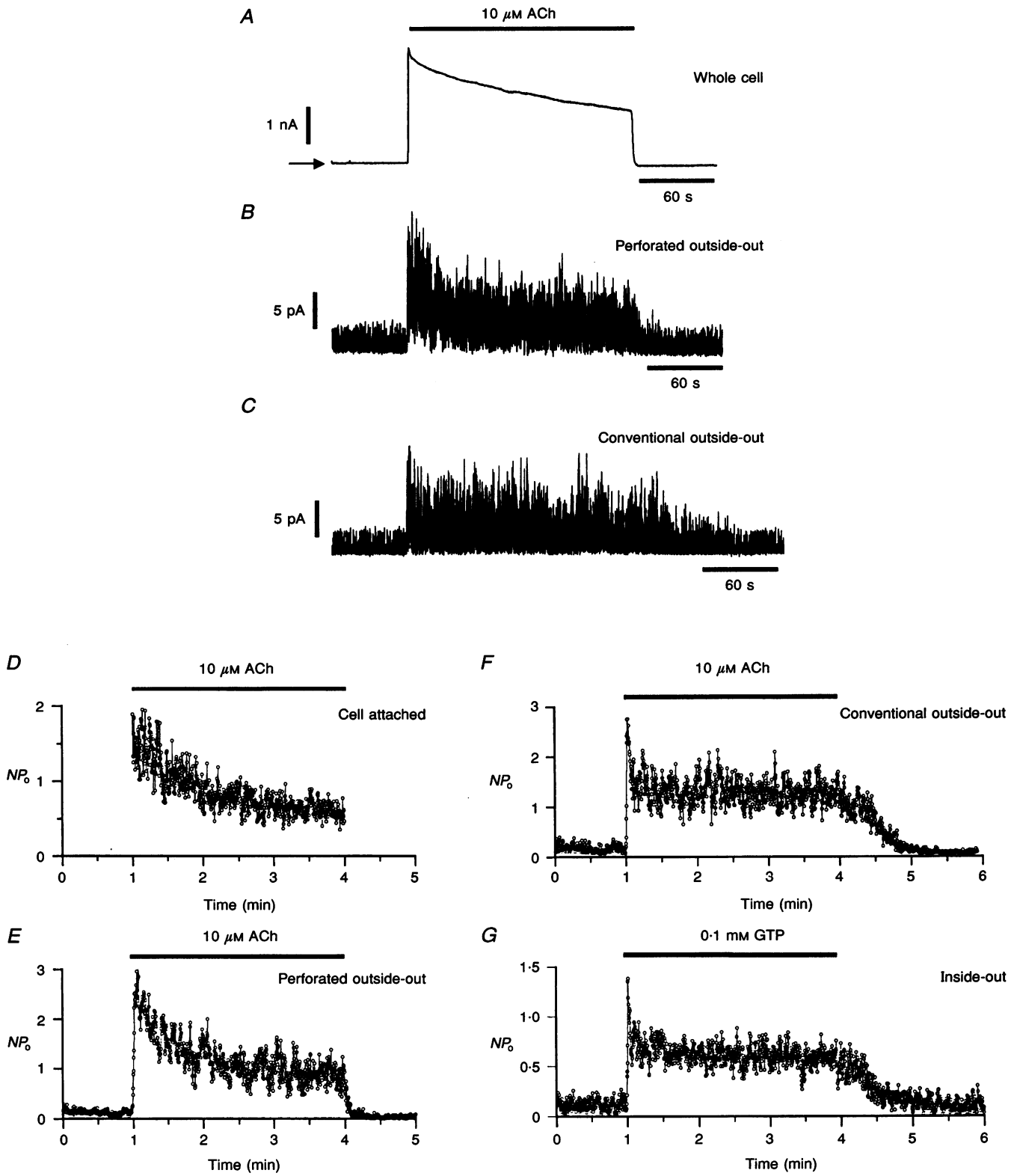

Figure 1. Muscarinic K^+ channel activity in different recording configurations

Muscarinic K^+ channel activity recorded with the whole-cell (*A*), cell-attached (*D*), perforated outside-out (*B* and *E*), conventional outside-out (*C* and *F*) and inside-out (*G*) configurations of the patch clamp technique is shown. 10 μM ACh or 0·1 mM GTP was applied during the period shown by the bar. Mean whole-cell current (from 10 cells) is shown in *A*, current from single patches is shown in *B* and *C* and mean NP_o (from 7–8 patches) is shown in *D*–*G*. Inward current is shown upwards in *A*–*C*. The arrow in *A* shows zero current.

conventional outside-out configurations, 0·1 mM GTP was present continuously in the patch pipette and, in the cell-attached and perforated outside-out configurations, natural GTP was present on the inside surface of the membrane). Figure 1*G* shows mean NP_o during a 3 min exposure of inside-out patches to 0·1 mM GTP. Application of GTP resulted in the activation of the muscarinic K^+ channel. During the 3 min exposure to GTP, the pattern of desensitization was similar to that observed with conventional outside-out patches: there was an initial fast phase of desensitization during the first 20 s of the exposure to ACh, but little slow phase (Fig. 2).

Figure 1*E* shows mean NP_o during a 3 min application of 10 μM ACh to perforated outside-out patches. Application of ACh resulted in the activation of the muscarinic K^+ channel and during the exposure to ACh the pattern of desensitization was similar to that observed with the whole-cell and cell-attached configurations: during the slow phase of desensitization NP_o declined by $55 \pm 7\%$ ($n = 7$; Fig. 2) with a time constant of 38 s.

The time course of activation and deactivation of muscarinic K^+ channel activity on application and wash-off of ACh or GTP in the different recording configurations is shown in Fig. 3. Activation of muscarinic channel activity on application of ACh or GTP was fast in all configurations and took ~1–2 s (time to peak). Activation was fastest in the whole-cell configuration, but we do not know whether this is significant. In whole cells it is well known that deactivation of the muscarinic K^+ current on wash-off of ACh is fast and this was also true in the present study. Deactivation was also fast in the perforated outside-out configuration, but it was about an order of magnitude slower in the conventional outside-out and inside-out configurations. In the conventional outside-out and inside-out configurations, there was a delay before deactivation occurred after the wash-off of ACh or GTP. In Fig. 3, deactivation was fitted by the following descriptive equation:

$$y = \frac{y_{max} - y_{min}}{1 + (t/t'_{½})^s} + y_{min}, \quad (1)$$

where y is $I_{K,ACh}$ or NP_o, y_{max} is the value of $I_{K,ACh}$ or NP_o 10 s prior to the wash-off of ACh or GTP, y_{min} is the value of $I_{K,ACh}$ or NP_o after deactivation, t is time, $t'_{½}$ is the time at which $y = (y_{max} - y_{min})/2$, and s is a slope factor. The half-time for deactivation, $t_{½}$, is $t'_{½} - 240$ (wash-off occurred at $t = 240$ s). In the whole-cell, perforated outside-out, conventional outside-out and inside-out configurations, $t_{½}$ values were $0·8 \pm 0·005$, $3·2 \pm 0·1$, $32·9 \pm 0·9$ and $23·0 \pm 0·9$ s, respectively, and s values were $-567·9 \pm 6·1$, $206·0 \pm 21·3$, $26·7 \pm 2·5$ and $41·9 \pm 5·1$, respectively.

In summary, the fast phase of desensitization was present and activation was fast in all recording configurations (with the exception of the cell-attached configuration, in which there was no fast phase of desensitization, perhaps as a

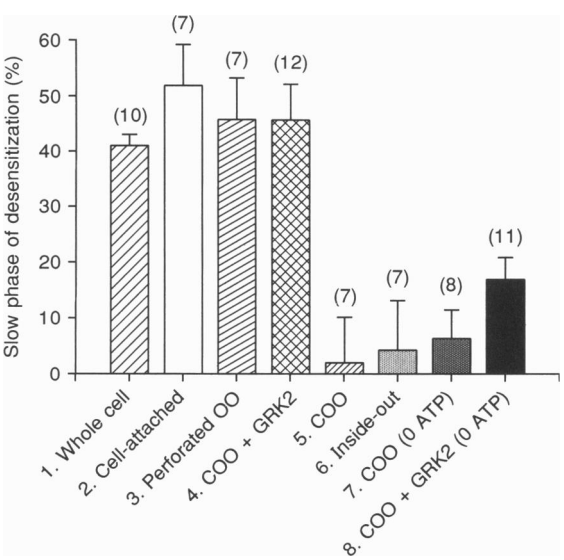

Figure 2. Slow phase of desensitization in different recording configurations

(1) whole-cell, (2) cell-attached, (3) perforated outside-out, (4) conventional outside-out with GRK2, (5) conventional outside-out, (6) inside-out, (7) conventional outside-out (0 ATP) and (8) conventional outside-out with GRK2 (0 ATP). After the first 20 s of an exposure to ACh or GTP (to exclude the fast phase of desensitization) the decline of $I_{K,ACh}$ or NP_o during the remainder of the 3 min exposure to ACh or GTP is expressed as a percentage of $I_{K,ACh}$ or NP_o at 20 s. The calculations were based on mean values of $I_{K,ACh}$ or NP_o over a 2 s period at 20 s after the start of the exposure and a 10 s period at the end of the exposure. Means ± S.E.M.s are shown (n in parentheses). 1, 2, 3 and 4 are all significantly greater than 5, 6, 7 or 8. No significant differences among 1–4 or 5–8.

result of a technical limitation). However, the slow phase of desensitization was present and deactivation was fast only in those configurations in which normal intracellular fluid was retained (whole-cell, cell-attached and perforated outside-out configurations). The slow phase was small or absent and deactivation was slow in those configurations in which the normal intracellular fluid was lost (conventional outside-out and inside-out configurations). In the conventional outside-out and inside-out configurations the slow phase was significantly less ($P < 0{\cdot}01$) than that in the whole-cell, cell-attached and perforated outside-out configurations. These results suggest that soluble intracellular regulators are involved in the slow phase of desensitization and normal rapid deactivation.

Effect of GRK2 on conventional outside-out patches

We have shown previously that when the mAChR is bypassed and the muscarinic K^+ current is activated by GTPγS, there is no slow phase of desensitization (Zang *et al.* 1993). The slow phase of desensitization could, therefore, be receptor dependent and the soluble intracellular regulator lost in the conventional outside-out patches could be muscarinic receptor kinase (the kinase is a soluble

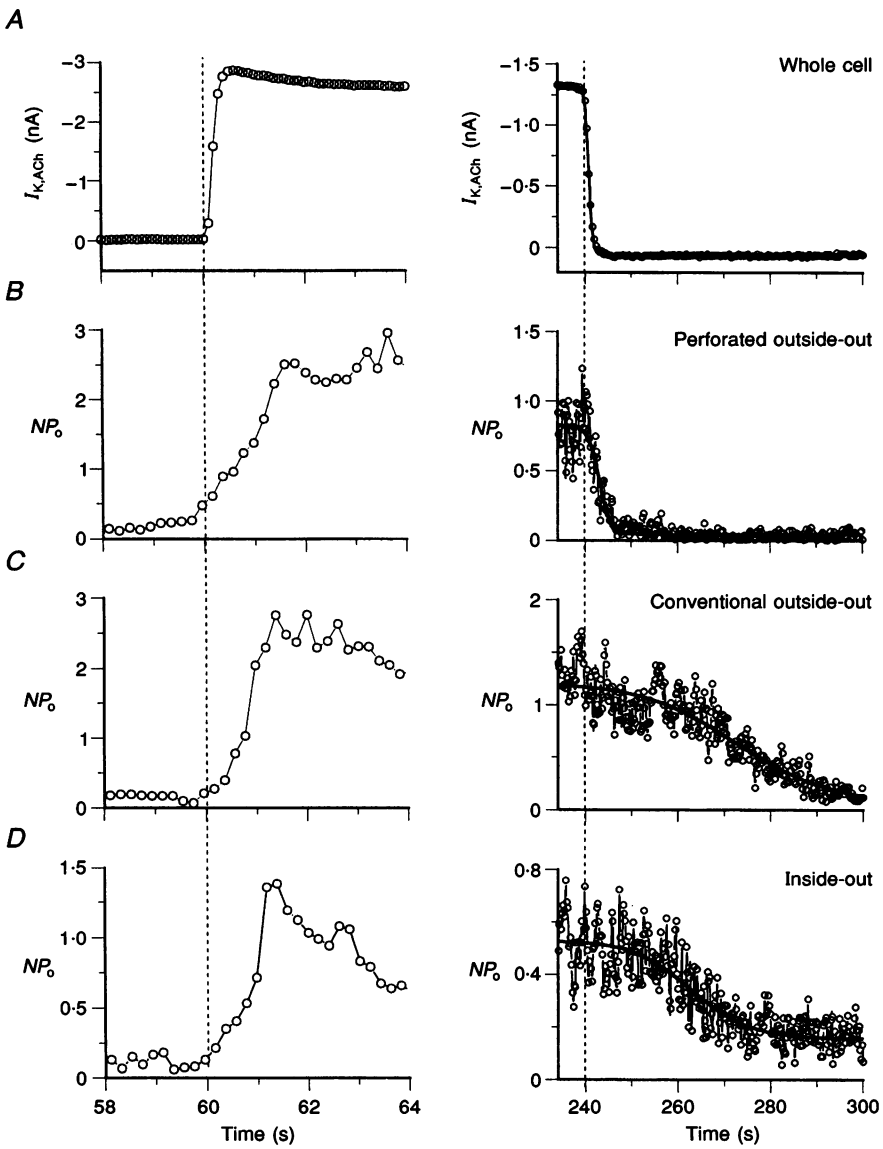

Figure 3. Activation and deactivation of the muscarinic K^+ channel in different recording configurations

Time course of activation (left) and deactivation (right) of the muscarinic K^+ channel is shown in the whole-cell (*A*), perforated outside-out (*B*), conventional outside-out (*C*) and inside-out (*D*) configurations of the patch clamp technique. ACh or GTP was applied or washed off at the times shown by the dashed lines. The decline of $I_{K,ACh}$ or NP_o on wash off of ACh or GTP has been fitted with eqn (1) with $t_{½} = 0{\cdot}8$ s and $s = -567{\cdot}9$ (whole cell), $t_{½} = 3{\cdot}2$ s and $s = 206{\cdot}0$ (perforated outside-out), $t_{½} = 32{\cdot}9$ s and $s = 26{\cdot}7$ (conventional outside-out) and $t_{½} = 23{\cdot}0$ s and $s = 41{\cdot}9$ (inside-out). Same data as shown in Fig. 1.

component of intracellular fluid). To test this hypothesis, a second series of experiments was carried out on conventional outside-out patches with ~10 nM GRK2 (also known as βARK1 and the same as or closely related to muscarinic receptor kinase) included in the patch pipette. Figure 4*A* shows an example of current from a conventional outside-out patch in the presence of GRK2 and Fig. 4*B–D* shows mean NP_o ($n = 11$) from this second series of experiments. In this series of experiments ATP was present as usual. The presence of GRK2 had no effect on activation and deactivation on application and wash-off of ACh: activation was still fast (complete in ~1–2 s) and deactivation was still slow ($t_{½} = 39{\cdot}7 \pm 0{\cdot}7$ s, $s = 24{\cdot}8 \pm 1{\cdot}7$) (Fig. 4*C* and *D*). However, in the presence of GRK2, the slow phase of desensitization was restored (compare Fig. 4*B* with Fig. 1*F*). During the slow phase of desensitization NP_o declined by $46 \pm 6\%$ ($n = 12$; Fig. 2) with a time constant of 46 s. In conventional outside-out patches in the presence of GRK2, the slow phase was significantly greater ($P < 0{\cdot}01$) than that in conventional outside-out patches in the absence of GRK2, but it was not significantly different from that observed in the whole-cell, cell-attached and perforated outside-out configurations (Fig. 2). To test whether the effect of GRK2 was the result of phosphorylation, the effect of GRK2 on conventional outside-out patches in the absence of ATP was investigated. There was little slow phase of desensitization in conventional outside-out patches in the absence of both

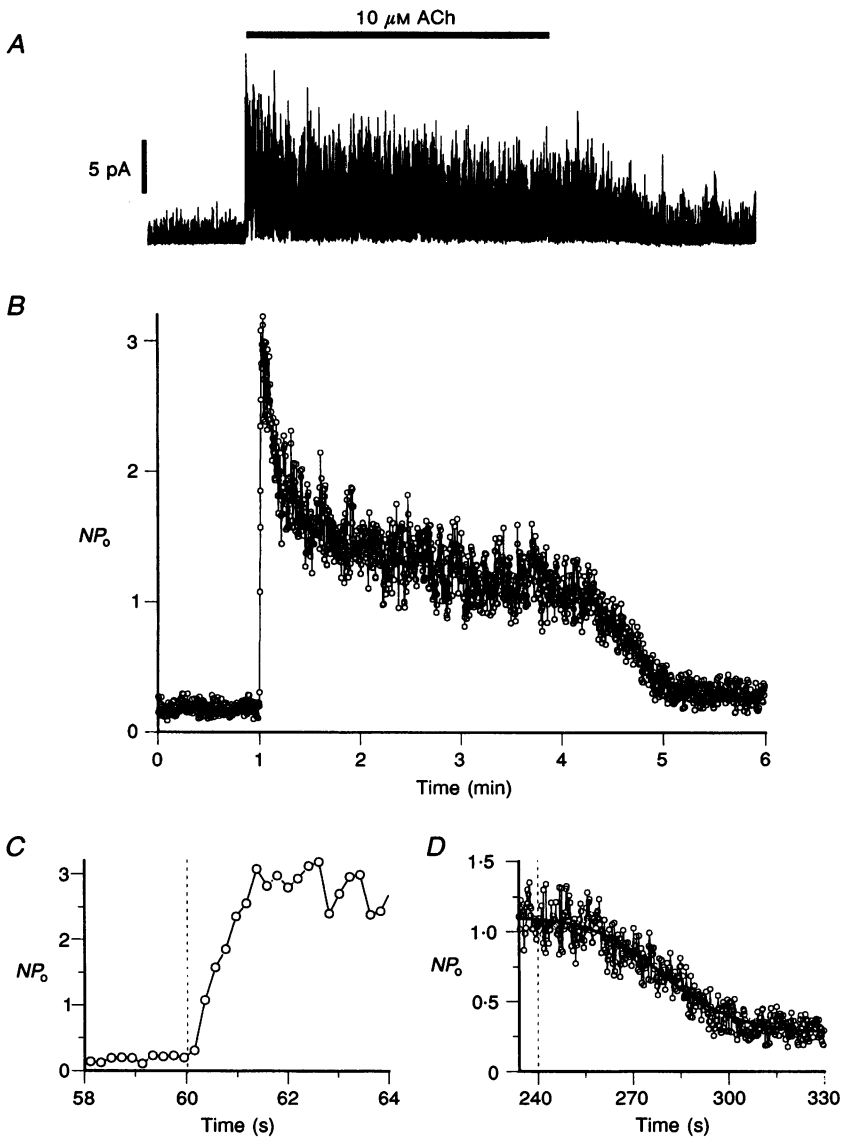

Figure 4. GRK2 restores the slow phase of desensitization in conventional outside-out patches
A, current from a single conventional outside-out patch (inward current is shown upwards). *B*, mean NP_o from 11 conventional outside-out patches. 10 μM ACh was applied during the period shown. *C* and *D*, activation (*C*) and deactivation (*D*). ACh was applied or washed off at the dashed lines. In *D* the decline of NP_o on wash-off of ACh has been fitted with eqn (1) with $t_{½} = 39{\cdot}7$ s and $s = 24{\cdot}8$. Same data as *B*. The patch pipette contained intracellular solution plus 10 nM GRK2.

ATP and GRK2 (Fig. 2). The slow phase was not significantly greater in the presence of GRK2 (ATP absent), and the slow phase was significantly less ($P < 0.01$) than that in conventional-outside patches in the presence of both ATP and GRK2 (Fig. 2).

DISCUSSION

The slow phase of desensitization in conventional outside-out patches was restored by GRK2. A high concentration of the $\beta\gamma$-binding domain of GRK2 (10–20 μM) has been shown to compete with the muscarinic K^+ channel for G protein $\beta\gamma$-subunits (Reuveny *et al.* 1994) and this could explain the decline in muscarinic K^+ channel activity attributed to desensitization. However, the requirement for ATP rules out this possibility, because the interaction of GRK2 with $\beta\gamma$-subunits occurs in the absence of ATP (Pitcher *et al.* 1992). The requirement for ATP confirms that GRK2 affects the system via phosphorylation. The substrate of GRK2 is most likely to be the m2 mAChR, because the m2 mAChR has been shown to be phosphorylated by GRK2 *in vitro* and *in vivo* (Kameyama, Haga, Haga, Kontani, Katada & Fukada, 1993; Tsuga, Kameyama, Haga, Kurose & Nagao, 1994). GRK2 does not phosphorylate G_i *in vitro* under the same conditions that the m2 mAChR is phosphorylated, and GRK2 is known to phosphorylate the agonist-bound mAChR only except for a weak autophosphorylation (Haga *et al.* 1994). It is concluded that receptor kinase-dependent phosphorylation of the mAChR is responsible for the slow phase of desensitization of the muscarinic K^+ channel. In the present study the fast phase of desensitization was not affected by procedures that affected the slow phase and, therefore, the fast phase must be the result of another mechanism (Kim, 1991; Zang *et al.* 1993).

What is the nature of the intracellular regulator responsible for normal rapid deactivation? Deactivation is the result of the hydrolysis of GTP by the G protein α-subunit (a GTPase). The rate constant of GTP hydrolysis (k_{cat}) by G_i, G_s, G_o and transducin in isolated membranes or solubilized preparations of G proteins ranges between 1 and 4 min^{-1}, equivalent to half-times ranging between 10 and 42 s (Higashijama, Ferguson, Smigel & Gilman, 1987). These half-times are comparable to the half-times of deactivation of the muscarinic K^+ channel in conventional outside-out and inside-out patches (32·9, 23·0 and 39·7 s; Figs 3 and 4), but are about an order of magnitude greater than the half-times of deactivation of the muscarinic K^+ channel in whole cells and perforated outside-out patches (0·8 and 3·2 s; Fig. 3). The muscarinic K^+ channel is one of a family of effectors activated via G proteins and it is well known that *in vivo* the rate of deactivation of many of these systems is substantially faster than k_{cat} of G proteins *in vitro* (Bourne & Stryer, 1992). This realization led to the discovery of GAPs (GTPase activating proteins) (Bourne & Stryer, 1992). The intracellular regulator in the present study could, therefore, be a GAP. In many cases it is the α-subunit that activates the effector and the effector is the GAP (Bourne & Stryer, 1992). However, the muscarinic K^+ channel is activated by the $\beta\gamma$-subunits of the G protein (Reuveny *et al.* 1994) and, therefore, it is unlikely that the channel is a GAP (furthermore, this would not explain the difference in the rate of deactivation among the different patch clamp recording configurations).

It is concluded that the slow phase of desensitization of the muscarinic K^+ channel during an exposure to ACh is the result of phosphorylation of the mAChR by a G protein-coupled receptor kinase (such as GRK2), and the normal rapid deactivation of the muscarinic K^+ channel on wash-off of ACh is the result of a soluble intracellular factor such as a GAP.

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