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MODULATION OF ACETYLCHOLINE-ACTIVATED K^+ CHANNEL FUNCTION IN RAT ATRIAL CELLS BY PHOSPHORYLATION

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SUMMARY

1. In voltage-clamped whole cells dialysed with GTP, extracellular application of ACh elicits an inwardly rectifying K^+ current which subsequently decreases to a steady-state level well below the maximally induced current (desensitization). The mechanism of desensitization of the acetylcholine(ACh)-activated K^+ channel current was studied in rat neonatal atrial cells at the single-channel level using the patch-clamp technique.

2. In cell-attached patches with ACh in the pipette, a similar pattern of K^+ channel current desensitization was present. Single-channel analyses revealed that the initial rapid decrease in channel activity was associated with progressive shortening of the mean open time (τ_o) and prolongation of the mean closed time (τ_c) of the K^+ channel.

3. In excised, inside-out patches with ACh in the pipette, GTP activated K^+ channels with a τ_o of $\sim$ 1·0 ms. Addition of ATP to the cytosolic surface resulted in progressive increases in τ_o (from 1 to 5 ms) and channel activity. These changes are similar but opposite in direction to those observed during the early phase of ACh-induced channel desensitization in cell-attached patches.

4. The effect of ATP on the channel kinetics was abolished in Mg^{2+}-free solution AMP-PNP (adenylyl-imidodiphosphate, a non-hydrolysable analogue of ATP), ADP, CTP (cytidine triphosphate), ITP (inosine triphosphate) or UTP (uridine triphosphate) did not alter the channel kinetics, suggesting that the ATP effect on channel gating probably occurs via phosphorylation by a membrane-bound kinase. H-8 (an isoquinolinesulphonamide derivative which inhibits protein kinases A and C) failed to prevent the action of ATP on the channel.

5. The increases in τ_o and channel activity produced by ATP could be completely reversed by an elevation of cytosolic $[Ca^{2+}]$ to 3×10^{-5} M or above.

6. The effect of Ca^{2+} on the ATP-induced changes in channel kinetics was blocked by sodium vanadate, a general phosphatase inhibitor. Okadaic acid, an inhibitor of protein phosphatase 1 and 2A, did not block the Ca^{2+} effect. Calmodulin antagonists, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W-7), trifluoroperazine, and calmidazolium, partially blocked the effect of Ca^{2+}.

7. Alkaline phosphatase (20 units/ml) reversed the ATP-induced increases in τ_o

and channel activity. These results suggest that the ACh-activated K^+ channel can be modulated by phosphorylation and dephosphorylation.

8. The early phase of desensitization of the K^+ current activated by ACh can largely be explained by time-dependent changes in the open- and closed-time durations of the channel, possibly by activation of a Ca^{2+}–calmodulin-dependent phosphatase. The secondary slower phase of desensitization was due to a progressive time-dependent decrease in the frequency of opening, and may involve effects on the receptor–G protein coupling.

INTRODUCTION

It is well documented that ACh binds to the muscarinic cholinergic receptor and activates an inwardly rectifying K^+ channel current in mammalian atrial cells via the inhibitory GTP-binding protein (Breitwieser & Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985; Kurachi, Nakajima & Sugimoto, 1986; Codina, Yatani, Grenet, Brown & Birnbaumer, 1987; Clapham & Kim, 1989). Earlier studies have shown that after the initial rapid onset, the ACh-evoked K^+ current or hyperpolarization in atrial preparations decreased quickly despite the continuous presence of the agonist (Jalife, Hamilton & Moe, 1980; Carmeliet & Mubagwa, 1986; Kurachi, Nakajima & Sugimoto, 1987). This type of response is typical of many hormone-stimulated biological effects observed during continuous stimulation by the agonist, and is generally referred to as 'desensitization'. Desensitization is an important physiological homeostatic mechanism that prevents the effector organs from being over-stimulated by hormones.

Although the existence of desensitization of the K^+ current response to ACh in mammalian atrial cells is clearly established, the cellular mechanism for this phenomenon is not known. Muscarinic receptor desensitization has previously been correlated with phosphorylation of the receptor in the chick and porcine heart, as well as in the brain (Ho, Ling, Dufield, Lam & Wang, 1987; Kwatra, Leung, Maan, McMahon, Ptasienski, Green & Hosey, 1987; Kwatra, Ptasienski & Hosey, 1989). The extent of phosphorylation of the nicotinic acetylcholine receptor has also been reported to be closely related to the rate of desensitization of the agonist-induced current (Hopfield, Tank, Greengard & Huganir, 1988). Similarly, in many other receptor systems, agonist-dependent phosphorylation of the receptor was found to be associated with changes in receptor function (Chad & Eckert, 1986; Sibley, Benovic, Caron & Lefkowitz, 1987; Kume, Takai, Tokuno & Tomita, 1989; Chen, Stelzer, Kay & Wong, 1990). Although the cardiac muscarinic receptor has been demonstrated to be a phosphoprotein, whether this is directly related to the observed desensitization of the muscarinic K^+ current response is not known. As this class of K^+ channels is activated by the inhibitory G proteins that are coupled to muscarinic receptors, direct modulation of the function of the G protein or the K^+ channel (or associated proteins) may also be involved in the gradual decrease in the physiological response to continued muscarinic receptor stimulation.

In earlier studies, the kinetic analysis of the muscarinic K^+ channel openings in several mammalian species showed mean open times of ~ 1 ms and the single-channel conductances between 35–40 pS at symmetrical 140 mM-K^+ (Sakmann, Noma & Trautwein, 1983; Soejima & Noma, 1984; Kurachi *et al.* 1986; Logothetis,

Kurachi, Galper, Neer & Clapham, 1987; Yatani, Codina, Brown & Birnbaumer, 1987; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989). However, these studies have not examined the early activation of the K^+ channel by ACh. In my preliminary studies, it was found that the pattern of K^+ channel opening during the first few seconds following seal formation with ACh in the pipette was markedly different from that observed after many seconds. Initial analyses revealed that channel gating kinetics were different.

In this paper, possible changes in K^+ channel kinetics following stimulation with ACh were investigated in detail at the single-channel level to determine whether such changes might account for the desensitization of the current response observed at the whole-cell level. The cellular processes by which the kinetics of the muscarinic K^+ channel are modulated were then studied using excised patches. In particular, the possibility that the observed channel modulation is caused by phosphorylation and dephosphorylation was investigated.

METHODS

Cell preparation

Hearts from 1- to 2-day-old newborn rats were dissociated with collagenase and trypsin. Right and left atrial tissues from whole hearts were cut out and placed in Ca^{2+}-free Hank's medium (Sigma). The tissues were then cut into small pieces (< 1 mm^3) with a sharp blade, and placed in Hank's balanced salt medium containing 0·05% collagenase type II and 0·03% trypsin (Worthington). Tissues were incubated at 37 °C for 10 min. Suspended cells were then removed and added to a same volume of 50% fetal calf serum to inhibit enzyme activities. Remaining tissues were incubated in new enzyme solution and allowed to dissociate for another 10 min. This procedure was repeated 5 times. Dissociated cells were collected by centrifugation (1000 r.p.m. for 10 min), and placed into culture medium which consists of Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, and 0·1% penicillin–streptomycin. Cells were plated on glass cover-slips and incubated at 37 °C in 5% CO_2 for 24 h before use.

Electrophysiology

Gigaseals were formed with Sylgard-coated pipettes with 1–3 MΩ (whole-cell) or 4–6 MΩ resistances (single channels) and channel currents were recorded using the method described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). For whole-cell recording, fast and slow capacitive transients were cancelled before adjusting the holding potential to desired levels. Channel currents were recorded with an Axopatch 1C patch-clamp amplifier, low-pass filtered at 5 kHz using a 8-pole Bessel filter (902-LPF; Frequency Devices, Inc., Haverhill, MA, USA) and stored on magnetic tape via digital data recorder (Instrutech Corp., Elmont, NY, USA). Later, digitized data were entered into an Atari computer and analysed to obtain duration and amplitude histograms and channel activity (averaged NP_o), using the analysis protocol described by Sigworth & Sine (1987). N is the number of channels, and P_o is the probability of channel opening. Note the logarithmic (abscissa) and square-root (ordinate) scales to represent dwell time distributions. Analysis of single-channel openings during 1 s periods represent averaged values over that period of time. The threshold amplitude for taking an opening event for analysis was set at 50% and the minimum duration was set at 100 μs. In general, the calculated mean open or closed times for a given record of channel openings varied by < 10% at minimum duration settings between 50 and 200 μs. All experiments were performed at 24–26 °C. All values are represented as the mean, or mean ± S.D.

Materials and solutions

Acetylcholine, trifluoroperazine, calmidazolium and sodium vanadate were purchased from Sigma Chemical Co. ATP, ATPγS (adenosine-5′-*O*-3-thiotriphosphate), ITP (inosine triphosphate), CTP (cytidine triphosphate), UTP (uridine triphosphate), ADP, AMP, AMP-PNP (adenylyl

imidodiphosphate), GTP, GTPγS (guanosine-5′-*O*-3-thiotriphosphate) and alkaline phosphatase (porcine heart) were purchased from Boehringer Mannheim. W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) and W-13 were purchased from Seigaku America Inc. Okadaic acid was a generous gift from Dr Fujiki at the National Cancer Institute in Tokyo. Solutions with desired free Ca^{2+} concentrations were prepared using the programme of Fabiato & Fabiato (1979), modified to include in the solution only those ions and compounds that were used in this study. For whole-cell studies, cells were attached to the bottom glass cover-slip of the chamber (2 mm width and 2 cm in length) and perfused at 10 ml/min to produce fast exchange of solutions. With this set-up, complete exchange of solutions occurred within 500 ms. The pipette and bath solutions contained 140 mM-KCl, 5 mM-EGTA, 2 mM-$MgCl_2$, 10 mM-HEPES (pH 7·2), unless noted otherwise. For single-channel studies, cells or excised membrane patches were brought to the mouth of a polyethylene tubing through which test solutions flowed by gravity at approximately 2 ml/min. In experiments using the physiological solution, the bath solution contained 135 mM-NaCl, 5 mM-KCl, 1 mM-$CaCl_2$, 2 mM-$MgCl_2$, and 10 mM-HEPES (pH 7·2).

RESULTS

Whole-cell K^+ currents activated by ACh

Whole-cells were voltage clamped at −60 mV (60 mV negative to the resting potential) to record inward current. Both bath and pipette solutions contained 140 mM-K^+. Cells were dialysed with GTP (100 μM) and ATP (2 mM) by adding them to the pipette. Since the muscarinic receptor is coupled to a GTP-binding protein, addition of GTP was necessary. ATP was added to inhibit spontaneous opening of the ATP-sensitive K^+ channel. After stabilization of the holding current, ACh was applied extracellularly for approximately 3 min and then washed out. ACh evoked a rapid increase in current followed by a slow decline to a near-steady-state level after approximately 3 min (Fig. 1). At 1 or 10 μM-ACh, the decrease of the muscarinic K^+ current occurred in two phases; an initial rapid phase lasting approximately 30 s and a slower and longer-lasting second phase. Even at a lower concentration (0·1 μM), ACh produced a clearly detectable desensitization of the K^+ current but of a shorter duration. In all cells, the ACh-induced current returned to the control level when ACh was washed out. The maximum current elicited by ACh was concentration dependent as shown in Fig. 1. As reported previously (Kurachi *et al.* 1987), the current response to a second challenge of ACh after 30 s, but not after 5 min of wash-out, was greatly diminished, indicating that recovery from desensitisation requires several minutes. Qualitatively similar desensitizations were observed in the bath solution that included 5 mM-K^+ and 1 mM-Ca^{2+} when cells were held at −140 mV.

Desensitization of single-channel currents

Cells were perfused with bath solution containing 140 mM-K^+ and held at −80 mV to record inward single-channel currents. As soon as cell-attached patches were formed with ACh (10 μM) in the pipette, activation of the K^+ channels was observed (Fig. 2). These channels activated by ACh were very different in kinetics from that of the resting K^+ current i_{K1} which has a mean open time greater than 80 ms in these atrial patches. Since i_{K1} is present only in a small percentage of patches (< 10%), $i_{K,ACh}$ could be studied exclusively. Channel openings were recorded in the cell-attached configuration for at least 2 min to determine whether desensitization could be detected at the single-channel level. The earliest channel opening recorded was

delayed by ~ 1 s following formation of the seal, due to time taken to adjust the head stage gain and voltage of the amplifier. In nearly all of the patches (> 95%), channel activity was significantly greater during the initial few seconds than that after 1 or 2 min. In Fig. 2, three examples of channel currents obtained in cell-attached patches

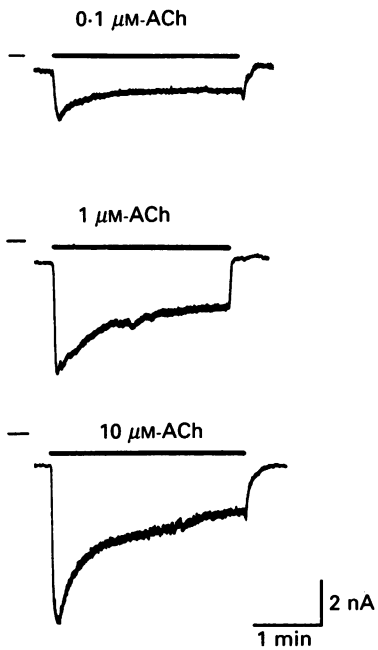


Fig. 1. Whole-cell currents evoked by ACh. Cells were dialysed with 100 μM-GTP and 2 mM-ATP first. At the holding potential of −60 mV, cell was perfused with ACh for ~ 3 min and washed at a rate of 10 ml/min. Zero current level is indicated by a horizontal bar on the left.

are shown. Single-channel openings during the first second and after 2 min of ACh exposure are shown on the right at higher time resolution. In Fig. 2*D* are histograms of channel openings recorded in Fig. 2*A*, and shows a typical distribution of open (on the left) and closed (on the right) times observed during the first second of ACh stimulation. Figure 2*E* shows open and closed time histograms of openings at 2 min of exposure to ACh. During the first second, the average mean open time was 5·2 ± 0·5 ms compared to 1·0 ± 0·1 ms at the end of 2 min (mean ± S.D., $n = 12$). The open time durations could be fitted with a single exponential. The closed time duration distributions could be fitted with two exponentials, indicating the presence of two closed states as reported previously (Sakmann *et al.* 1983). At 2 min, both the fast and slow mean closed times were increased compared to those at 0–1 s such that the net mean closed time was prolonged by 1·6-fold. Experiments done in physiological bath solution containing 5 mM-KCl, 135 mM-NaCl, 2 mM-$MgCl_2$, 10 mM-HEPES and 1 mM-Ca^{2+} also exhibited a similar type of desensitization of the single-channel currents (Fig. 2*C*).

To determine whether the kinetics of channel openings and closings change abruptly or progressively with time, more detailed kinetic analyses were performed on another current record (Fig. 3). Channel openings activated by 10 μM-ACh were analysed at several time points. For example, in Fig. 3*A*, channel openings at 0

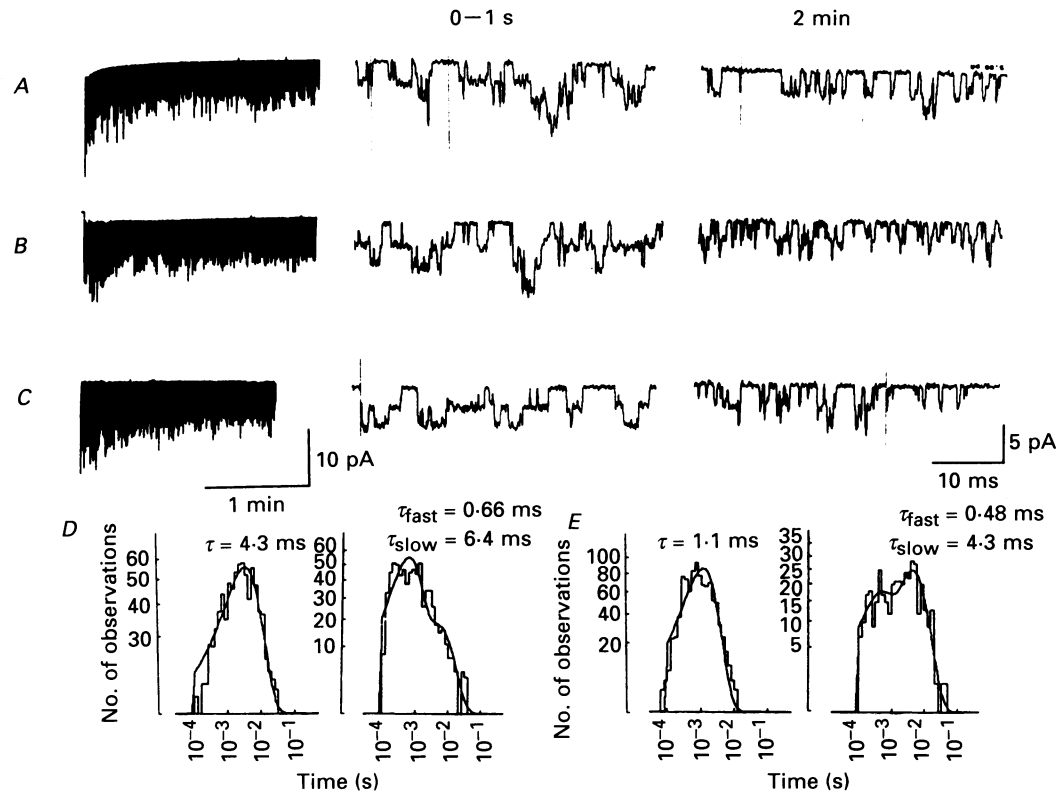


Fig. 2. Records of single K^+ channel current activated by ACh in cell-attached patches. Three examples are shown (*A*, *B* and *C*). On the right of each record are channel openings during the first, second and at 2 min at higher resolution. *D*, open- and closed-time duration histograms of channel openings during 0–1 s. *E*, histograms of openings during 120–122 s in record shown in *A*. The mean open or closed times are averaged values analysed over the entire 1 or 2 s periods.

(~ 1 s after seal formation), 2, 4, 6, 10, 16 and 120 s following stimulation with ACh are shown at fast speed. Kinetic analyses were done on channel openings observed during 0–1 s, 2–3 s, 4–5 s, and so on, and the results plotted in Fig. 3*C* and *D*. Clearly, there was a rapid but progressive decrease in the mean open time and the channel activity during the initial 20 s and a much more slower decrease during the next several minutes. The mean closed time (net sum of fast and slow closed times) increased progressively with time. These results indicate that ACh modulates the gating kinetics of the K^+ channel opening during current desensitization.

At 1 μM-ACh, the K^+ channel current also desensitized albeit at a rate slower than that produced by 10 μM-ACh (see Fig. 1). At the single-channel level, there was also a clear difference in the kinetics of channel opening during the first few seconds and at 60 s (Fig. 4). The average mean open time of channels active during 0–1 s and

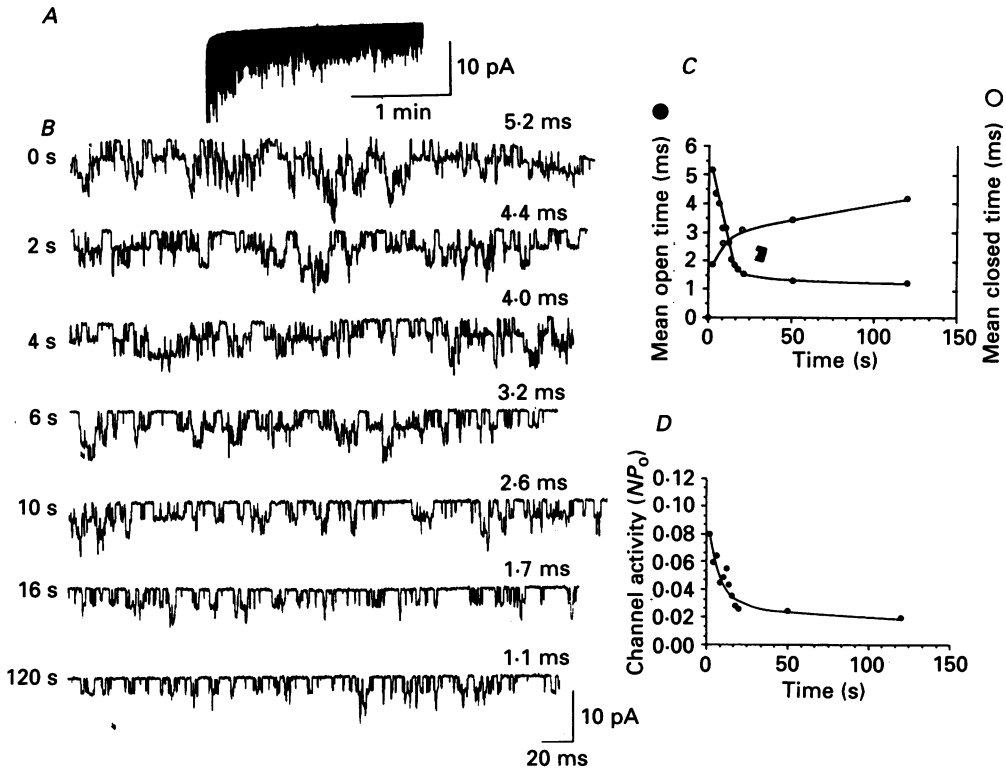


Fig. 3. A K^+ channel recording showing a progressive change in the channel kinetics following stimulation with ACh. *A*, a current record in a cell-attached patch with 10 μM-ACh in the pipette. There was a delay of ~ 1 s from the time of formation of gigaseal to actual first recording of channel opening (0 s). *B*, single-channel openings at various times after the recording of the channel opening. *C*, mean open and closed times during 1 s periods at various times. *D*, time-dependent change in the channel activity, calculated as mean NP_o during 1 s periods at indicated times.

60–62 s were 3·2 ± 0·4 and 0·9 ± 0·1 ms, respectively (mean ± S.D., $n = 6$). The channel activity also decreased significantly by ~ 40% during the first 1 min of channel recording. Thus, the initial phase of desensitization observed with 1 μM-ACh is also associated with changes in the kinetic behaviour of the K^+ channel. The open-time durations during early ACh stimulation were shorter than those seen with 10 μM-ACh, which may explain the smaller degree of desensitization with 1 μM-ACh compared to that with 10 μM-ACh. At 0·1 μM, although the whole-cell current exhibited desensitization, the onset of channel activity in cell-attached patches was

slow and the frequency of channel openings was very low compared to that at 1 or 10 μM-ACh. Therefore, appropriate kinetic analyses to yield interpretable results could not be done.

Test of the phosphorylation hypothesis

Phosphorylation of receptors and ion channels has been shown to modulate their physiological function (Sibley *et al.* 1987; Hopfield *et al.* 1988; Hartzell, 1989;

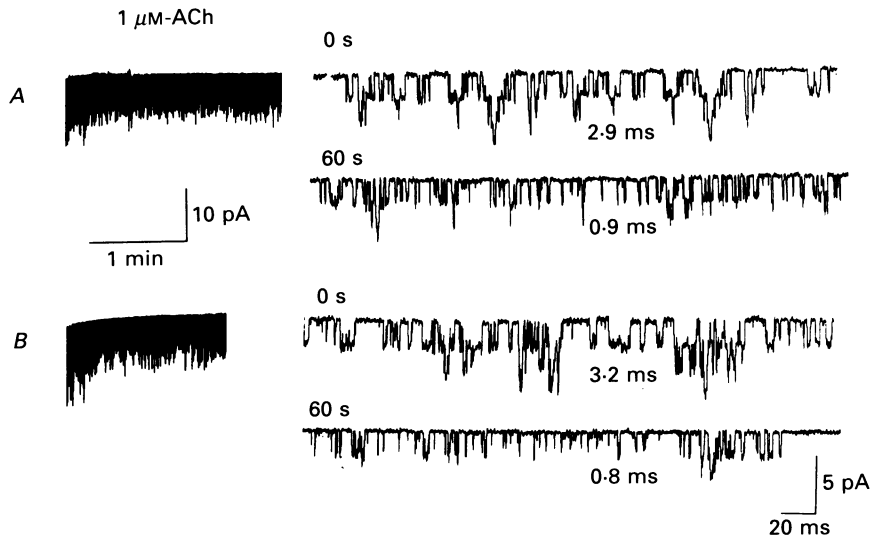


Fig. 4. Activation of K^+ channels by 1 μM-ACh in cell-attached patches. Two examples are shown (*A* and *B*). For each record, few hundreds milliseconds of channel activity at the beginning (0 s) and after 60 s of ACh exposure are shown at fast speed. The mean open time decreased from 3·2 to 0·9 ms, and channel activity decreased from 0·078 to 0·047 ($n = 6$).

Takano, Qin & Noma, 1990). In order to study the possible mechanisms by which ACh modulates the kinetic behaviour of the K^+ channel and thereby causes the early rapid desensitisation, the hypothesis that channel kinetics are modulated by phosphorylation was tested. This was done initially using inside-out patches.

With ACh (10 μM) in the pipette, application of GTP to the cytosolic surface of the membrane always activated channels with a mean open time of $\sim$ 1 ms (Fig. 5). When ATP (2 mM) and GTP (100 μM) were then applied together, there was a rapid change in the pattern of channel opening and the channel activity started to increase immediately. These results are different from our previous findings in which ATP did not always increase channel activity (see Discussion). At steady state, the mean open time of the channel openings recorded following addition of ATP was $5{\cdot}6 \pm 0{\cdot}4$ ms ($n = 14$, Fig. 5*C*). Washout of GTP, whether ATP was present or not, resulted in decrease of the channel activity to basal levels (one opening every few seconds), indicating that the K^+ channel activation is GTP dependent and thus G protein-

mediated (Fig. 5*A*). Readdition of GTP resulted in reactivation of the channels with kinetics identical to those observed in the presence of ATP and GTP. Thus, the effect of ATP was not reversible under these conditions. Since intracellular ATP itself did not activate a K^+ channel, it is unlikely that ATP has activated another population

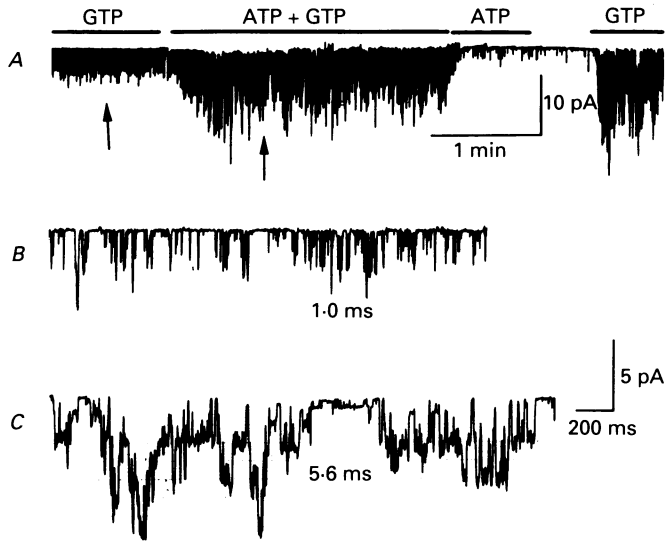


Fig. 5. Effect of ATP on the K^+ channel behaviour. *A*, K^+ channels in an inside-out patch activated with ACh in the pipette and GTP in the bath showed channel openings with a mean open time of 1·0 ms. Upon application of ATP, the pattern of channel opening changed, resulting in marked increases in mean open time and channel activity. Wash-out of ATP and GTP decreased the channel activity to basal levels. Reapplication of GTP activated channels with longer open time durations. *B*, channel openings at the arrow during GTP perfusion. *C*, channel openings at arrow during perfusion with GTP and ATP.

of K^+ channel in atrial cells. Instead, it is more likely that ATP has modified the kinetics of the existing, muscarinic K^+ channel, perhaps by phosphorylation of the channel or an associated regulatory protein. This is further supported by the nature of the progressive rather than an abrupt change in the channel kinetics (see below).

The kinetics of the K^+ channel openings and closings were analysed in more detail to test whether ATP produced a progressive change in channel kinetics similar to those observed during the fast phase of desensitization in cell-attached patches (see Fig. 3) or an abrupt change. Figure 6*A* is a recording from an inside-out patch with K^+ channels activated with ACh in the pipette and GTP in the bath. As soon as ATP was added to the perfusion solution, the channel behaviour began to change and the channel activity started to increase. Channel openings during 0–1, 5–6, 10–12 and 40–45 s after the addition of ATP were analysed, and the results from three experiments were averaged and plotted in Fig. 6*C* and *D*. Channel activity increased rapidly during the initial 10 s and reached a steady-state level in $\sim$ 20 s. The mean open time followed a similar time course and increased from 1·1 to 5·3 ms ($n = 3$). The

mean closed time decreased progressively such that by 10 s, it was reduced to 65% of the initial level. Thus, the large increase in the open-time duration and the reduction of the shut-time duration probably account for the major part of the increase in channel activity (Fig. 6*D*). Although the changes are in the opposite

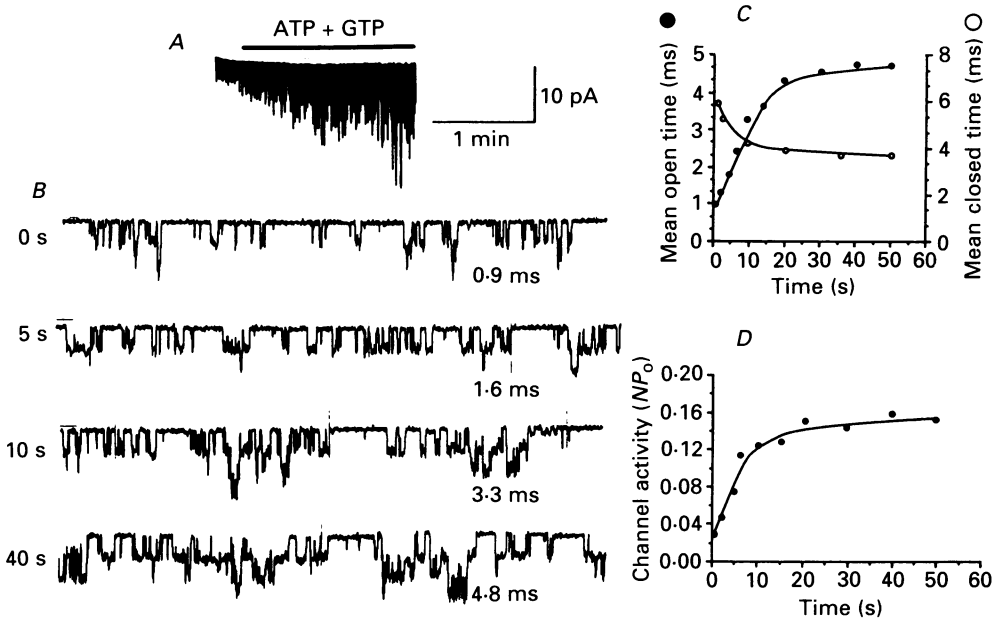


Fig. 6. ATP-induced increases in mean open time and channel activity showing progressive changes. *A*, K^+ channel in an inside-out patch activated with GTP was further perfused with GTP and ATP. *B*, channel openings shown at higher time resolution at various times after the perfusion with ATP. *C*, plot of mean open and closed times *versus* time of exposure to ATP. *D*, plot of the channel activity *versus* time.

direction, these ATP-mediated changes in the kinetics of the K^+ channel are reminiscent of those observed during the early phase of desensitization following stimulation with ACh.

Whether the ATP effect on the channel is the result of a kinase-mediated phosphorylation was examined next. Since kinases require Mg^{2+}–ATP for phosphorylation, experiments were repeated in inside-out patches perfused with Mg^{2+}-free solution containing ATP. Figure 7*A* shows K^+ channel activity initially in a cell-attached patch with ACh in the pipette. Upon formation of an inside-out patch, channel activity subsided to basal levels. Application of Mg^{2+}-free, GTP-containing solution resulted in a small activation of the K^+ channel (presumably due to some Mg^{2+} present initially) followed quickly by inhibition. The inhibition is expected as muscarinic-coupled G protein function requires Mg^{2+} for G protein subunit dissociation and subsequent activation of the K^+ channel (Gilman, 1987; Neer & Clapham, 1988). Further addition of 2 mM-ATP (Mg^{2+}-free) to the cytoplasmic face

of the patch did not produce any effect and only the background level of channel activity was present. After ~ 4 min of incubation with ATP, Mg^{2+} and GTP were applied together to the patch without ATP. This resulted in reactivation of the K^+ channel; however, the mean open time remained at ~ 1 ms, indicating that the ATP

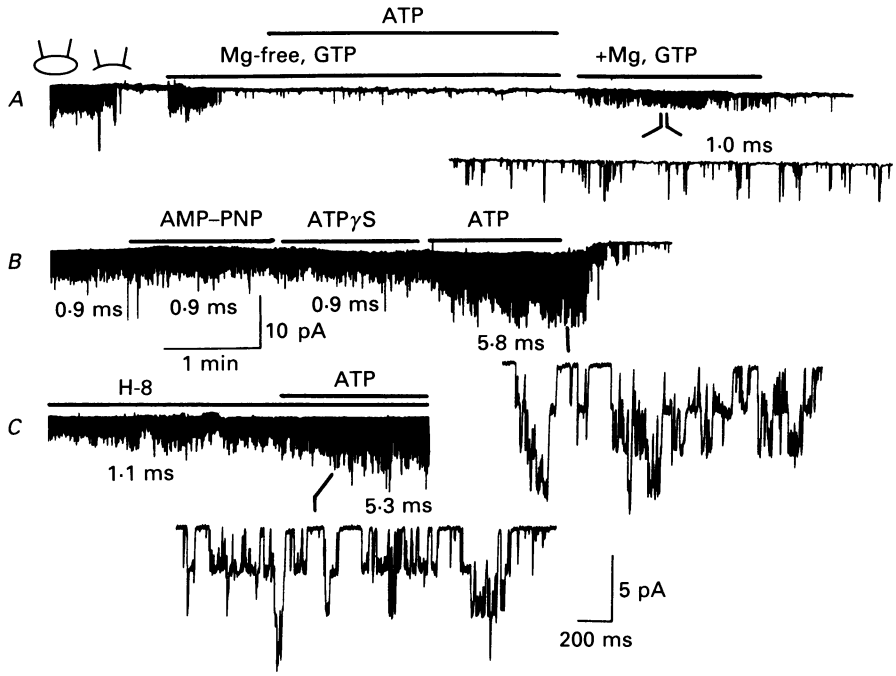


Fig. 7. Effects of Mg^{2+}-free solution, ATP analogues and a protein kinase inhibitor on ATP-induced changes in K^+ channel kinetics. *A*, in Mg^{2+}-free solution. GTP did not activate the K^+ channel, as expected for a G protein-coupled process. Under this condition, ATP failed to alter the channel kinetics when Mg^{2+} and GTP were added back to reactivate the channel. *B*, AMP-PNP and ATPγS both failed to produce any changes in channel kinetics. In the same patch, ATP rapidly increased the mean open time and channel activity. *C*, H-8, an inhibitor of protein kinases A and C, also failed to affect ATP-induced changes in channel kinetics.

effect on the channel kinetics was inhibited, presumably by failure of phosphorylation by a protein kinase in the absence of Mg^{2+}. Identical results were obtained in four additional cells. In another set of experiments, inside-out patches were exposed to GTPγS in normal bath solution to cause activation of $i_{K,ACh}$. After ~ 2 min, the cytosolic face of the patch was perfused with Mg^{2+}-free solution. Addition of ATP without Mg^{2+} had no effect on the mean open time of the channel, i.e. τ_0 remained at ~ 1 ms. When Mg^{2+} was then applied in the presence of ATP, the pattern of channel opening began to change, and this was accompanied by a gradual increase in the mean open time, as observed previously. These results confirm earlier results above that a protein kinase is mediating the effect of ATP on the channel kinetics.

The possible role of phosphorylation was tested further using adenylyl-imidodiphosphate (AMP-PNP) and adenosine-5′-*O*-3-thiotriphosphate (ATPγS) which are analogues of ATP. AMP-PNP, a non-hydrolysable analogue, failed to affect the channel kinetics (Fig. 7*B*). ATPγS, an analogue that can donate a

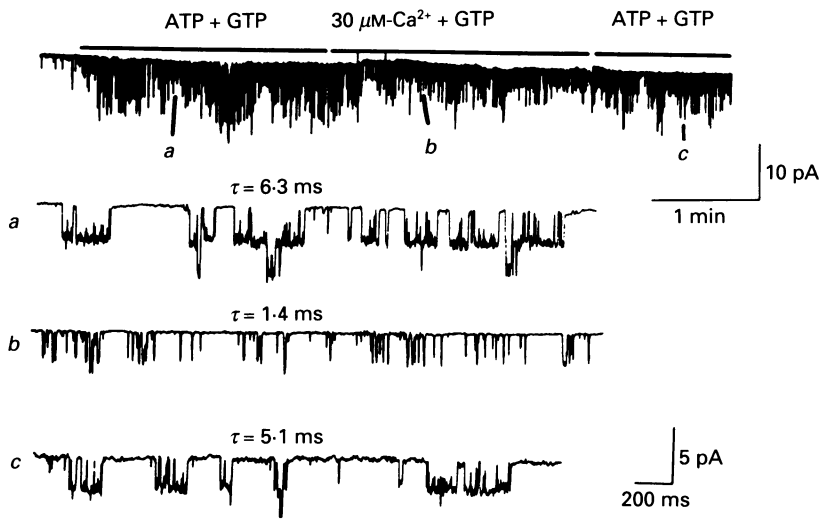


Fig. 8. Modulation of the K^+ channel by Ca^{2+}. In an inside-out patch, ATP and GTP activated channels with a τ_o of 6·3 ms (*a*). Further exposure of the patch to 30 μM-Ca^{2+} and GTP resulted in a gradual decrease in τ_o and channel activity. Wash-out of Ca^{2+} and addition of ATP prolonged the τ_o to 5·1 ms (*c*). In the presence of 100 μM-sodium vanadate, Ca^{2+} failed to reverse the effect of ATP.

thiophosphate group and form a product that is more resistant to hydrolysis, also did not affect the K^+ channel kinetics. In the same patch, however, application of ATP resulted in increases in channel activity and open-time durations as shown above. Although ATPγS is a potential donor of a phosphate group, several studies reported that it is not a suitable substrate for kinases (Palvimo, Linnala-Kankkunen & Maenpaa, 1985; Ohya & Sperelakis, 1989; Chen *et al.* 1990). This may explain the failure of ATPγS to produce effects similar to those produced by ATP, at least for the duration of the experiments in this study. It is also possible that the kinase involved in the regulation of the K^+ channel cannot use ATPγS as a substrate. Other compounds such as AMP, ADP, ITP, UTP, and CTP also did not alter ACh-activated K^+ channel activity or the kinetics. H-8 (30 μM), an isoquinoline-sulphonamide derivative that is a potent inhibitor of cyclic GMP- and cyclic AMP-dependent protein kinases and protein kinase C (Hidaka, Inagaki, Kawamoto & Sasaki, 1984), failed to block the effect of ATP (Fig. 7*C*), suggesting that a kinase other than those listed above may be involved.

In the absence of ACh stimulation, opening of the muscarine-like K^+ channels occurred at low frequency (one opening every few seconds). Perfusion of the cytosolic

surface of the inside-out patches with ATP also resulted in an increased duration of the K^+ channel from 0·9 ms to 4·6 ms ($n = 7$) but did not alter the frequency of opening. Patches with ACh in the pipette but no cytosolic GTP also exhibited a basal level of K^+ channel opening. A similar increase of the open-time duration was also observed in these patches in response to ATP. Intracellular application of GTPγS, a non-hydrolysable GTP analogue that causes gradual, irreversible release of endogenous G protein subunits (Gilman, 1987), resulted in activation of the K^+ channel with a τ_0 of 1·1 ms in patches not treated with ATP ($n = 3$), but a τ_0 of 4·9 ms in patches treated with ATP ($n = 3$). Therefore, under these conditions, the muscarinic receptor is unlikely to be involved in the ATP-mediated changes in channel kinetics. Rather, it is likely that direct phosphorylation of the channel or associated proteins alters the kinetic behaviour of the K^+ channel. Recently, it was shown that in guinea-pig atrial cells, ATP caused activation of the muscarine K^+ channels in the absence of agonist and GTP (Heidbuchel, Callewaert, Vereecke & Carmeliet, 1990). This was found to be due to transphosphorylation from ATP to G protein-bound GDP. No such activation of the K^+ channel by ATP was observed in rat atrial cells in the present study, at least for the duration of the experiments.

Reversal of the ATP effect by intracellular Ca^{2+}

In attempts to find ways to reverse the effect of ATP, i.e. shorten the time durations of channel opening and decrease the channel activity, it was discovered that exposure of the cytoplasmic surface of the membranes to Ca^{2+} at a concentration of 30 μM or above could reverse the ATP effect. Other ions such as H^+ (pH 5·0–8·0), Na^+ (0–135 mM) or Ba^{2+} (10–100 μM) had no effect on the open time durations. Figure 8 shows a typical experiment which illustrates the effect of Ca^{2+} on the muscarinic K^+ channel activity. After the kinetics of channel openings have been modified with ATP in an inside-out patch, 30 μM-Ca^{2+} and GTP were applied to the cytosolic surface for $\sim$ 1 min. This resulted in shortening of the open-time duration and concomitant decrease in channel activity. After the Ca^{2+} treatment, the averaged mean open time was shortened from $5·1 \pm 0·4$ to $1·4 \pm 0·1$ ms ($n = 11$), and the channel activity became comparable to that observed prior to ATP addition. These channels were again sensitive to further application of ATP which increased the channel activity and the open-time durations to levels similar to those recorded after the first ATP application. This indicates that non-specific effects due to Ca^{2+}-activated proteases are unlikely to be involved. At 10 μM-Ca^{2+}, either partial (from 5·2 ms to 2·6 ms; $n = 3$) or no reversal ($n = 3$) of the open-time duration occurred. The effect of Ca^{2+} on the K^+ channel kinetics did not require the presence of GTP, as shown in Fig. 9. Provided that ATP was not present, a brief exposure to Ca^{2+} completely reversed the ATP effect. The effect of Ca^{2+} on the channel kinetics was completely inhibited by simultaneous presence of 100 μM-sodium vanadate, a phosphatase inhibitor, in all five cells (τ_0: from 5·4 to 5·5 ms). These results indicate that the kinetics of the K^+ channel can be modulated by ATP- and Ca^{2+}-dependent pathways, involving phosphorylation and dephosphorylation, respectively.

Possible role of a Ca^{2+}-activated protein phosphatase

The hypothesis that the reversal of the ATP effect on the channel kinetics by Ca^{2+} may be mediated by a Ca^{2+}-dependent protein phosphatase was tested. Cohen (1987) has categorized protein phosphatases into several major forms: phosphatase 1, and

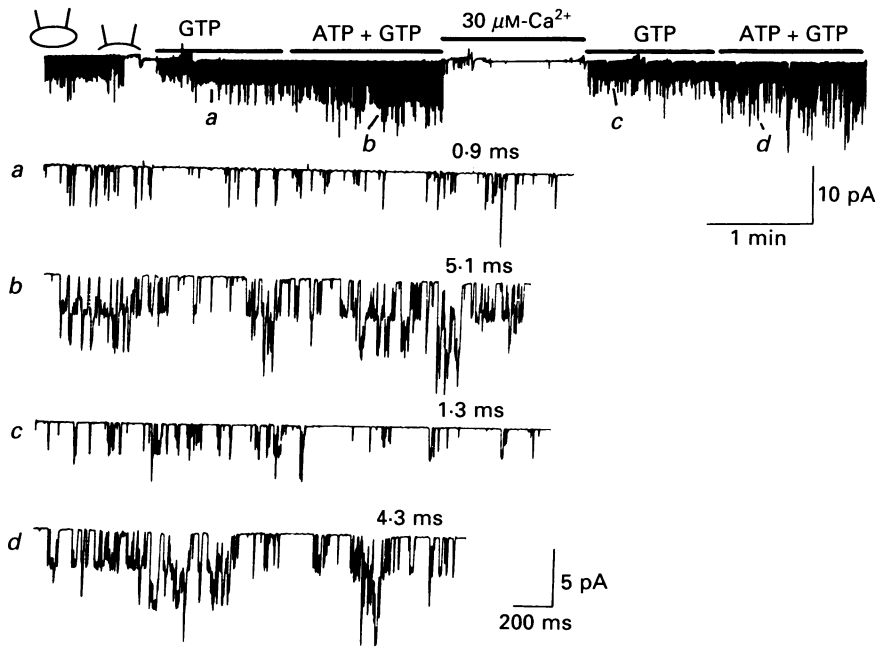


Fig. 9. Reversal of ATP effect by a brief Ca^{2+} exposure. In an inside-out patch with ACh in the pipette, bath application of GTP activated channels with a τ_0 of 0·9 ms (*a*). Addition of ATP prolonged the τ_0 to 5·1 ms and increased the channel activity (*b*). After a brief exposure of the patch to 30 μM-Ca^{2+}, GTP was applied. This resulted in the activation of the channels with τ_0 of 1·3 ms and in channel activity similar to that present before the addition of ATP (*c*). Further perfusion with GTP and ATP produced effects similar to those seen after the first challenge with ATP (*d*). Note that during Ca^{2+} exposure, the channel activity is decreased to basal levels due to absence of GTP.

phosphatases 2A, 2B and 2C. Of these, phosphatase 1 and 2A are potently inhibited by okadaic acid, a polyether derivative of a 38-carbon fatty acid isolated from a black sponge *Halichondria okadai* (Bialojan & Takai, 1988; Hescheler, Mieskes, Ruegg, Takai & Trautwein, 1988; Cohen, Holmes & Tsukitani, 1990). Figure 10*A* shows a typical experiment in which the effect of Ca^{2+} on the channel kinetics was tested in an inside-out patch in the presence of 5 μM-okadaic acid. In six of six patches, okadaic acid failed to inhibit the action of Ca^{2+}. Ca^{2+} treatment resulted in shortening of τ_0 from 5·4 to 1·3 ms. These results are in keeping with the properties of phosphatases 1 and 2A which do not require Ca^{2+} for their activity. The effect of Ca^{2+} was also not affected by 30 μM-H-8, suggesting that protein kinases A and C probably do not play a role in the reversal of ATP effect on the channel kinetics.

Phosphatase 2B has been shown to be Ca^{2+} dependent, stimulated by calmodulin and inhibited by calmodulin antagonists (Cohen, 1989). To further test the possibility that the Ca^{2+}-mediated reversal of the ATP effect is due to activation of phosphatase 2B, inside-out patches were incubated with 20 μM-W-7 (*N*-(6-aminohexyl)-5-chloro-1-

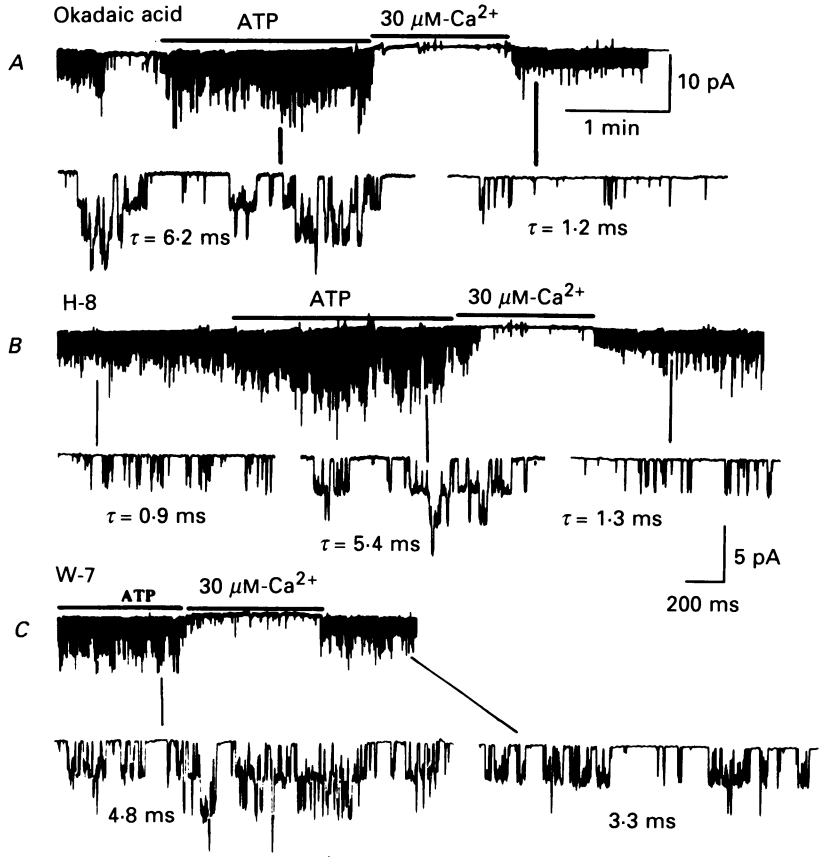


Fig. 10. Effect of a phosphatase inhibitor, a protein kinase inhibitor and a calmodulin inhibitor on Ca^{2+}-induced reversal of the ATP effect on K^+ channel kinetics. GTP was always present except during Ca^{2+} exposure. Thus no channel activity is present during Ca^{2+} exposure. *A*, okadaic acid, an inhibitor of phosphatases 1 and 2A, failed to block the effect of Ca^{2+}. *B*, H-8, an inhibitor of protein kinases A and C, also failed to block the effect of Ca^{2+}. *C*, W-7, a calmodulin antagonist, inhibited the effect of Ca^{2+} in four of six patches. The number below each tracing is mean open time.

naphthalenesulphonamide), a calmodulin inhibitor (Fig. 10*C*). In experiments with W-7, results were somewhat variable. In one patch, W-7 completely blocked the effect of Ca^{2+} incubation and the channels still showed a τ_o of 5·5 ms following Ca^{2+} treatment. In three of six patches, W-7 partially prevented the effect of Ca^{2+} incubation, i.e. the open-time duration shortened from 5·1 to 3·1 ms ($n = 3$). In two patches, however, W-7 failed to block the Ca^{2+} effect. Experiments using other

calmodulin antagonists also produced variable results. For example, the Ca^{2+} effect was inhibited by trifluoroperazine (5 μM) in two of six patches, by calmidazolium (10 μM) in three of five patches, and by W-13 (200 μM) in two of five patches. Although the presence of inhibitory effects of calmodulin antagonists in about half

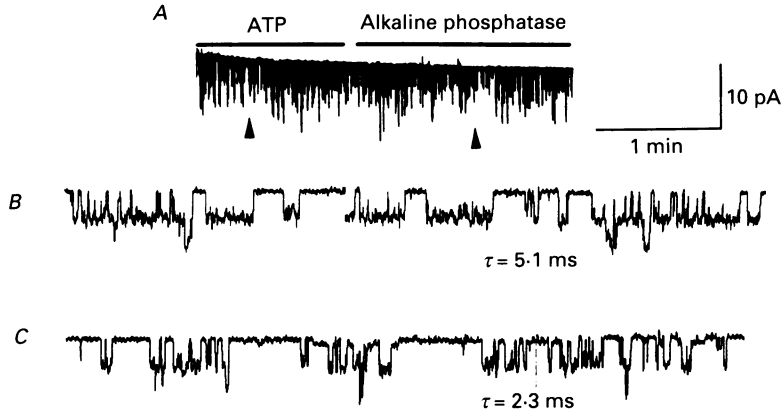


Fig. 11. Effect of alkaline phosphatase on the K^+ channel kinetics. *A*, following prolongation of the open-time durations of the channel by perfusing ATP to an inside-out patch, ATP was washed off and alkaline phosphatase (20 units/ml) was applied to the cytosolic surface of the membrane. This resulted in a gradual shortening of the open-time duration as illustrated in *B* (before the enzyme at the arrow) and *C* (after the enzyme at the arrow). Alkaline phosphatase was dialysed against the perfusion buffer solution for 8 h at 0 °C.

of the patches suggest that the phosphatase involved may be a Ca^{2+}- and calmodulin-dependent protein phosphatase, conclusive evidence is lacking.

Effect of alkaline phosphatase on the channel kinetics

Alkaline phosphatase hydrolyses many phosphocompounds non-specifically (McComb, Bowers & Posen, 1979). To test further whether the muscarinic K^+ channel is indeed modulated by phosphorylation and dephosphorylation, alkaline phosphatase was intracellularly applied to inside-out patches after the open-time durations of the K^+ channel have been increased by addition of ATP. Figure 11*A* shows an inside-out patch first perfused with ATP and GTP to cause prolongation of the open-time duration (Figure 11*B*). ATP was then washed out and alkaline phosphatase (20 units/ml) was applied to the perfusion solution with GTP. Alkaline phosphatase was able to reduce, although not completely, the open-time duration as well as the channel activity after approximately 20 s (Fig. 11*C*). The mean open time decreased from 5·3 to 2·4 ms ($n = 3$), and the channel activity (NP_o) decreased from 0·15 to 0·08 (mean values, $n = 3$). The effect of alkaline phosphatase was not due to wash-out of ATP, since the ATP alters the channel kinetics irreversibly in all patches. These results further support the view that phosphorylation prolongs and dephosphorylation shortens the durations of the K^+ channel opening.

Mechanism of the slow phase of desensitization

After the initial rapid phase of desensitization that lasts approximately 20 s, there is a slower decrease in channel activity that continues for several minutes. Since the mean open time does not change significantly after ~ 30 s, the slow decrease in

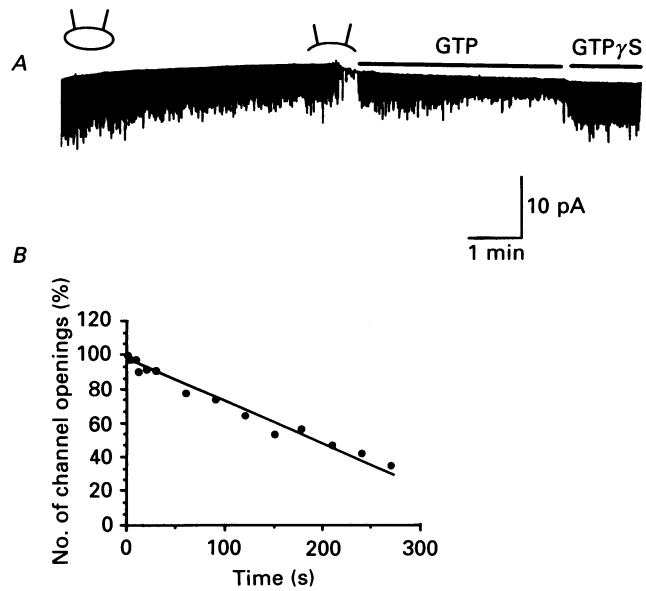


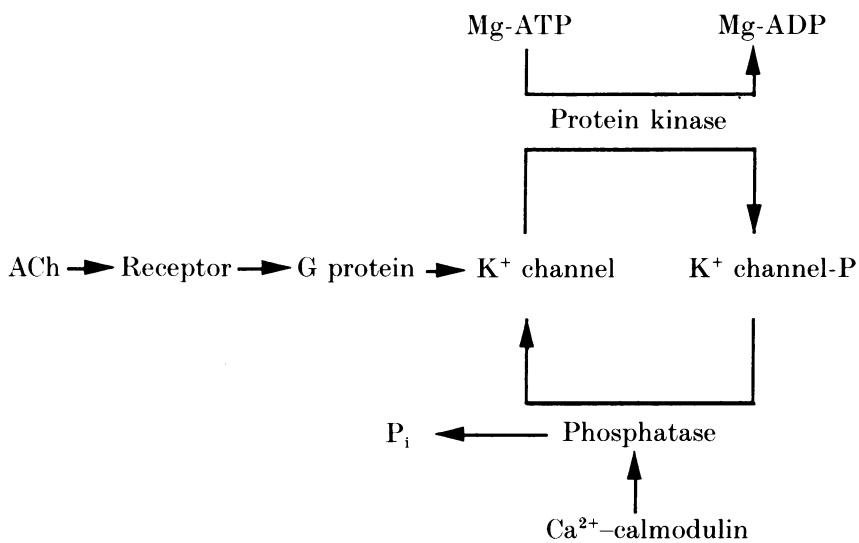
Fig. 12. The slow phase of desensitization in cell-attached and inside-out patches. *A*, this record shows a typical decrease in channel activity in a cell-attached patch with 10 μM-ACh in the pipette. After several minutes, an inside-out patch was formed and GTP added to reactivate the channels. The slow decrease in channel activity continued to occur. Application of GTPγS increased the channel activity to levels observed early (2–3 min) during the cell-attached configuration, but not completely to the maximal level present at the onset of ACh stimulation. The difference between the maximal activity and activity after GTPγS is probably due to channel run-down. *B*, a plot of the number of channel openings during 200 ms periods at various times from 0 to 270 s of channel activity. The mean open time at any point in time throughout the experiment remained between 1 and 1·2 ms.

channel activity must be due to mechanisms other than via modulation of the time duration of channel openings. Figure 12*A* shows a recording of K^+ channel openings beginning at 30 s after activation by 10 μM-ACh in a cell-attached patch, thus showing only the slow phase of desensitization. After several minutes, an inside-out patch was formed and perfused with GTP to reactivate the channels. The desensitization continued to occur for the next several minutes. The decrease in channel activity was not due to channel run-down, since GTPγS (a non-hydrolysable analogue of GTP that produces maximal and persistent activation of the K^+ channel) could cause an increase in channel activity in a desensitized patch. The mean open

time remained between 1·0 and 1·2 ms throughout, indicating that the desensitization was not due to shortening of the open-time duration. Analysis of the frequency of channel opening, i.e. measurement of the number of channel openings during a 200 ms period at several time points (Fig. 12*B*) illustrated rather that the decrease in channel activity was due to a progressive reduction in the number of channel openings with time of exposure to ACh. This is consistent with the slowly increasing mean closed times during ACh stimulation and is analogous to the mechanism of desensitization of the current response to nicotinic acetylcholine receptor stimulation (Hopfield *et al.* 1988).

DISCUSSION

ACh binds to the muscarinic receptors in atrial cells to cause activation of an inwardly rectifying K^+ current which contributes to the hyperpolarization and the slowing of the heart rate. Earlier studies have shown that the ACh-induced increase in K^+ conductance progressively fades or desensitizes even with maintained vagal or ACh stimulation (Jalife *et al.* 1980; Salata & Jalife, 1985; Carmeliet & Mubagwa, 1986; Kurachi *et al.* 1987). The desensitization of the K^+ current response of ACh occurs in two phases; a rapid initial phase lasting approximately 20–30 s and a subsequent slower phase that continues for many minutes. The results presented here indicate that the rapid phase of desensitization is primarily due to a change in the kinetics of the K^+ channel opening and closing, whereas the secondary slower phase is primarily due to decreased frequency of channel opening. The experiments to examine the cellular mechanisms of the early rapid desensitization suggest that phosphorylation and dephosphorylation of the channel or associated regulatory proteins may be the underlying processes that determine the kinetics of the muscarinic K^+ channel. Based on the results presented here, the following scheme for ACh-induced early desensitization of the K^+ channel activity is proposed.



It is proposed that the ACh-activated K^+ channel or an associated regulatory protein can exist either in a phosphorylated or dephosphorylated (or less

phosphorylated) state. Depending on the state of the channel, ACh (via the G protein) will activate the channel which opens with a τ_o of $\sim$ 1 ms or longer (4–6 ms). When ACh binds the receptor, a rapid activation of a kinase occurs to cause phosphorylation and thereby prolongation of the open-time duration. A slower activation of a phosphatase (possibly a Ca^{2+}–calmodulin-dependent phosphatase) then gradually causes dephosphorylation of the protein involved, leading to shortening of the mean open time and associated desensitization. Since this state of the channel is then maintained in the presence of ACh despite millimolar amounts of ATP in the cell, the phosphatase presumably remains in an active state. In excised outside-out patches with GTP in the pipette, ACh was also clearly capable of increasing the mean open time of the channel but only in the presence of cytosolic Mg-ATP, supporting the involvement of a kinase. In excised patches, however, the machinery that causes dephosphorylation must have been impaired due to wash-out of certain cytosolic factor(s), since the kinetics of the channel modified by ATP did not spontaneously reverse as those seen in cell-attached patches.

The kinase involved in modulating the channel kinetics was not identified in this study. The failure of H-8, a potent inhibitor of protein kinase A and C, to block the effect of ATP suggests the existence of another type of kinase that mediates the ATP effect. Many kinases require second messengers such as Ca^{2+}, cyclic AMP or diacylglyerol to become active (Nestler & Greengard, 1984). Therefore, it is surprising that addition of ATP only was sufficient to alter the K^+ channel kinetics. However, it is possible that the unidentified kinase may only require ATP and not other co-factors, the co-factors may be associated with the membrane and not washed off, or the kinase may already be in an active state. Other studies have also shown that addition of ATP only caused changes in ion conductances via phosphorylation, although the kinases involved were not identified (Augustine & Bezanilla, 1990; Chen *et al.* 1990). In another study, the ATP-induced phosphorylation could be attributed to a second messenger-independent kinase that was different from any of the known kinases (Sweetnam, Lloyd, Gallombardo, Malisan, Gallagher, Tallman & Nestler, 1988). In an earlier study using rat atrial cells, ATP itself was unable to affect the K^+ channel kinetics in $\sim$ 40% of the patches (Kim & Duff, 1990). In these patches not responsive to ATP, the channel open-time duration increased upon further addition of the cyclic AMP-dependent protein kinase (Kim, 1990). Although the reasons for different effects of ATP are not clear at this time, the findings suggest that there may be more than one type of kinase that is involved in the modulation of the muscarinic-gated K^+ channel.

The ability of Ca^{2+} to cause reversal of the ATP effect on the K^+ channel kinetics, the inhibitory effects of vanadate and calmodulin antagonists, and the effect of alkaline phosphatase all indicate involvement of a protein phosphatase. Due to variable effects of calmodulin antagonists, whether a Ca^{2+}–calmodulin-dependent phosphatase is indeed involved remains speculative. Nevertheless, since the phosphatase that is dependent on Ca^{2+} and inhibited by calmodulin antagonists but not by okadaic acid belongs to the phosphatase 2B class (Cohen, 1989), perhaps such a phosphatase is present in the rat cardiac membrane. A class 2B phosphatase has previously been purified from bovine cardiac muscle (Wolf & Hofmann, 1980; Cohen, 1989), and has also been identified in the brain (Klee, Crouch & Krinks, 1979). A high-Ca^{2+} solution (5×10^{-4} M) has been reported to reverse the ATP effect in GABA

receptor-mediated current via activation of such a phosphatase (Chen *et al.* 1990). The gradual shortening of the open-time duration and the progressive decrease in the K^+ channel activity during the rapid phase of desensitization may therefore be related to a slow activation of the Ca^{2+}- and calmodulin-dependent protein phosphatase. It is not clear why such a high concentration of Ca^{2+} is required to activate the phosphatase. Perhaps there is an intracellular co-factor that lowers the Ca^{2+} requirement and is lost during patch excision. Exactly how ACh regulates such a phosphatase is unknown at the present time.

During the slow phase of desensitization, i.e. decrease of channel activity from $\sim$ 30 s after the exposure to ACh during which there is a minimal change in the mean open time, it appears to be the slow decrease in the frequency of channel opening which accounts for the slow decline in the channel activity. During this process, it is likely that the muscarinic receptor–G protein function is modulated to reduce the amount of activation of the K^+ channel. Possible changes include gradual uncoupling of the receptor from its associated G protein, modification of the receptor at the cell surface to reduce binding, modification of the G protein, and slow endocytosis of the receptor. Kwatra & Hosey (1986) reported that the level of phosphorylation of the muscarinic receptor from desensitized heart was 10- to 12-fold greater than that from control hearts. Thus, phosphorylation of the receptor may play an important role in the slow desensitization phase. Phosphorylation of the G proteins has been reported in hepatocytes (Pyne, Murphy, Milligan & Houslay, 1989), platelets (Carlson, Brass & Manning, 1989) and dictyostelium (Gundersen & Devreotes, 1990). Thus, direct phosphorylation of G proteins could be involved in the rapid and/or slow desensitization processes observed in this study.

It was reported recently that chick cardiac muscarinic receptors can be phosphorylated by the β-adrenergic receptor kinase (Kwatra, Benovic, Caron, Lefkowitz & Hosey, 1989). This kinase has been shown to phosphorylate the β-adrenergic receptor and its activity to correlate with the desensitization of the receptor-coupled adenylate cyclase activity (Benovic, Stone, Caron & Lefkowitz, 1989). In my initial experiments, heparin, a potent inhibitor of the kinase (Benovic *et al.* 1989), was used to determine whether the kinase was involved in the slow desensitization of the muscarinic K^+ current response. Heparin at concentrations ranging from 0·1 to 10 μM, however, failed to affect the rate of time-dependent decrease in K^+ current in five cells, suggesting that phosphorylation by the β-adrenergic receptor kinase does not alter ACh-induced K^+ current. More recently, we and others have found that arachidonic acid metabolites could modulate $i_{K,ACh}$ in atrial cells (Kim *et al.* 1989; Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989). Arachidonic acid metabolites may thus be involved in the desensitization process. This requires further investigation.

In summary, data are presented to suggest that the rapid phase of desensitization of the ACh-activated K^+ current is mainly due to modulation of the duration of the K^+ channel opening and closing via phosphorylation by a second messenger-independent kinase, and via dephosphorylation possibly by a Ca^{2+}–calmodulin-dependent phosphatase. The slow, monophasic decrease in the frequency of channel opening that occurs following ACh application probably contributes to a small degree to the initial rapid desensitization. The slower phase of desensitization is

mainly due to a time-dependent decrease in the frequency of channel opening. The cellular processes by which stimulation of the muscarinic receptor by ACh leads to activation of a kinase and a phosphatase remain to be studied.

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