A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor

A study with forskolin and its analogs

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Received 14 February 1986

Forskolin, an activator of adenylate cyclase, and its analogs were studied on the nicotinic acetylcholine receptor-ion channel complex (AChR) of rat and frog skeletal muscles. At nanomolar concentrations, forskolin caused desensitization of the AChR located at the junctional region of innervated and the extrajunctional region of chronically denervated rat soleus muscles. The desensitization of the AChR occurred without alteration of the conducting state (channel lifetime, conductance or bursting) as shown by single channel currents. Accordingly, forskolin decreased the peak amplitude of the repetitive evoked endplate currents in frog sartorius muscles. These findings taken together with the good correlation found between the effects of forskolin and its analogs on the desensitization of the nicotinic AChR and their ability to activate adenylate cyclase suggested a possible involvement of phosphorylation of AChR via cyclic AMP on the desensitization process.

Forskolin *Nicotinic receptor* *cyclic AMP* *Desensitization* *Adenylate cyclase* *Acetylcholine sensitivity*

1. INTRODUCTION

The release of acetylcholine (ACh) from the presynaptic nerve terminal of nicotinic synapses and subsequent binding to recognition sites located on the subunits of the ACh receptor-ion channel complex (AChR) results in conformational changes of the AChR which yield channel opening. The AChR, upon binding of the agonist, can also undergo a slow transition to a refractory or desensitized state [1–5]. This condition, brought about by very high concentrations of the agonist, may not be evident under physiological conditions since the quantity of ACh released during repetitive nerve firing does not appear likely to be sufficient to induce desensitization [6]. However, desensitization of the AChR may serve as an autoregulatory function, protecting the junctional region against excessive depolarization. A major question is whether mechanisms other than repetitive ACh binding to the AChR complex may participate in the desensitization process.

An attractive possibility is phosphorylation of the AChR complex. Protein phosphorylation by specific protein kinases often has an autoregulatory role. Further, phosphorylation of the AChR complex has been demonstrated in electroplax membranes [7,8]. This phosphorylation appeared to involve a cyclic AMP (cAMP)-dependent protein kinase [9]. To investigate whether activation of cAMP-dependent protein kinases in situ could affect desensitization of the nicotinic AChR, the diterpene forskolin, a general activator of hormone-sensitive adenylate cyclases [10,11], was investigated. Forskolin has been shown to activate fully cAMP-dependent control

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00145793/86/$3.50

of physiological processes in nerve and muscle at 5 μM or less (for references see [11]). Also, forskolin has no effect on directly evoked contractures of soleus muscle [12]. In this study forskolin was used in low concentrations (up to 5 μM) along with two close structural analogs, one of which, namely 14,15-dihydroforskolin, is much less potent than forskolin in activating adenylate cyclase, while the other, 1,9-dideoxyforskolin, is inactive ([13]; see fig.2 for chemical structures). The actions of forskolin were investigated on the junctional region of innervated and extrajunctional region of the chronically denervated rat soleus muscles and on single channel currents in neonatal rat myoballs. We observed that at nanomolar concentrations, forskolin induced receptor desensitization but had no effect on the properties of ACh-activated single channel currents, thus leading to the suggestion that this effect could result from a mechanism involving phosphorylation of the AChR.

2. MATERIALS AND METHODS

2.1. *Preparations and recording techniques*

In vitro preparations of innervated and chronically denervated soleus muscles from female Wistar rats (180–200 g) were used in these studies. Denervation of the muscles 10 days prior to the day of experiment and measurement of junctional and extrajunctional ACh sensitivity to microiontophoretic application of ACh were performed according to [6,14,15]. Briefly, micropipettes filled with 3 M KCl with a resistance of 15–25 MΩ were used for recording ACh-induced potentials. The following procedure was observed for determination of junctional ACh sensitivity: only muscle fibers having a membrane potential between -70 and -80 mV were used. In a typical trial, the focal region of the endplate was located by the criterion of miniature endplate potentials (MEPPs) having a rise time of less than 0.8 ms. Once the focal region was found, without removing the recording microelectrode the tip of the ACh pipette was positioned as close as possible to the AChR-rich junctional region and brief 0.2 ms charges were applied to the pipette yielding a potential whose rising phase was <0.8 ms. In a typical trial of recording, the response to 1 or 2 single ACh-induced pulses was followed by a train of 100–200 pulses delivered at 8 Hz. At the end of the train, the response to single pulses was again determined. After 3–4 control steady responses the muscle was perfused with the drugs and the potentials recorded every 10 min up to 60 min. The data shown here are from recordings made 30–60 min after drug perfusion. After this period, the preparation was washed for up to 60 min with the same solution

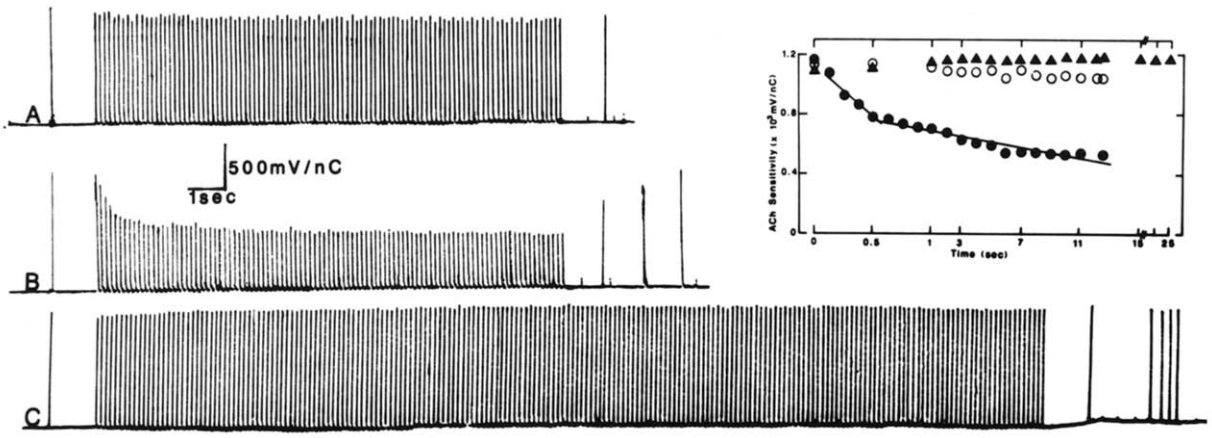

Fig.1. Effect of forskolin (1 μM) on the junctional ACh sensitivity of the endplate region of innervated rat soleus muscle. Potentials generated by microiontophoretic application of ACh in a train of 100 pulses at 8 Hz were recorded from the same cell under control conditions (A), 30 min after perfusion of forskolin (B) and 30 min after wash (C). Membrane potential, -70 mV. Vertical bar, ACh sensitivity (mV/nC). (Inset) The values of ACh sensitivity (mV/nC) shown in A (○), B (●) and C (▲) are plotted vs time (s). Initial fast phase of desensitization lasting approx. 0.6 s (slope $= -672$ mV·nC^{-1}·s^{-1}) was followed by a slow phase (slope $= -17.2$ mV·nC^{-1}·s^{-1}).

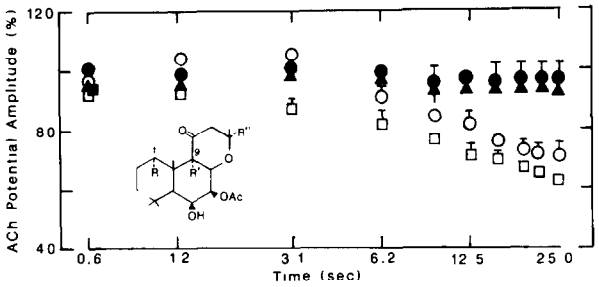

Fig.2. Effect of forskolin on the junctional sensitivity to microiontophoretic application of ACh at the endplate region of innervated rat soleus muscle. Each point, expressed in percent of the first response in a train of 200 responses evoked at 8 Hz, represents the mean ± SE of values recorded from at least 4 responses from 2 muscles. The recordings were made from the same cell under control conditions (●—●), 40–60 min after either perfusion of 0.2 μM (○—○) and 1.0 μM (□—□) forskolin or wash (▲—▲). (Inset) Chemical structures of forskolin (R = R′ = OH, R″ = -CH=CH_2); 14,15-dihydroforskolin (R = R′ = OH, R″ = -CH_2CH_3); 1,9-dideoxyforskolin (R = R′ = H, R″ = -CH=CH_2).

used for control recordings. Throughout these experiments the rate of perfusion was kept optimum so as not to disturb the recording conditions. The volume of the muscle chamber was 20 ml and with the solution supplied to the chamber at a rate of 25 drops/min a complete bath solution exchange was achieved in 9 min. The data presented for the junctional ACh sensitivity were obtained from the same fiber maintained throughout the control, drug-perfused, and recovery conditions.

Patch-clamp studies employing the cell-attached configuration were performed on both myoballs cultured from neonatal rat skeletal muscles and muscle fibers isolated from interosseal and lumbricalis muscles of adult frogs. The procedures for culture and isolation of the muscle fibers and the details of single channel current recordings were as described elsewhere [16–18]. The drug was applied as an admixture with ACh inside the patch pipette.

Endplate currents (EPCs) were recorded from the frog sartorius nerve-muscle preparation according to [19].

2.2. *Acetylcholinesterase (AChE) assay*

AChE from soleus muscles exposed to 1 μM forskolin and from muscle homogenates to which the drug was added (0.5–100 μM) was assayed according to a modification of the procedure by Ellman et al. [20]. The details of the procedure were as described [21].

2.3. *Solutions and drugs*

The physiological solution had the following composition (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 $NaHCO_3$, 1 Na_2HPO_4, 11 glucose; the pH was 7.2–7.3. ACh hydrochloride (Sigma) and tetrodotoxin (Calbiochem, TTX) solutions were freshly prepared from stock solutions stored at 4°C. Forskolin (Calbiochem), 1,9-dideoxyforskolin and 14,15-dihydroforskolin were dissolved in absolute ethanol to 1 mM and stored at 4°C. Forskolin analogs were kindly provided by Hoechst Pharmaceutical Ltd (Bombay, India). TTX (0.1–0.3 μM) was added to the bathing medium to avoid spontaneous twitching of muscle fibers and cell movement during single channel recordings.

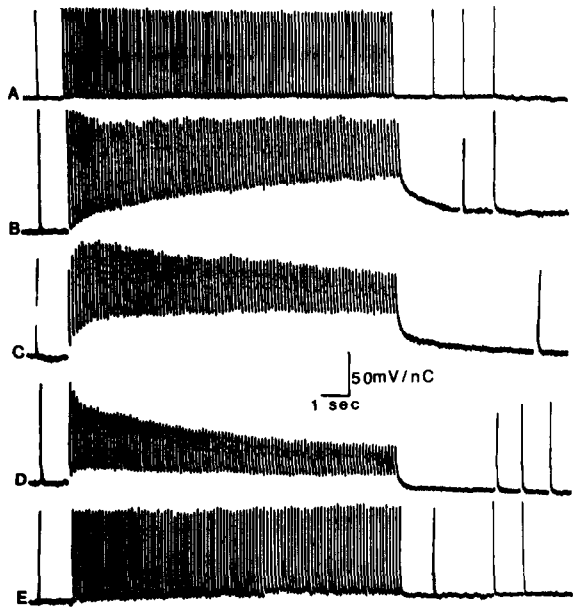

Fig.3. ACh potentials recorded at various concentrations of forskolin on the extrajunctional region of denervated rat soleus muscles. Responses were evoked at 8 Hz stimulation under control conditions (A) and 30 min after perfusion of 0.1 (B), 0.5 (C) and 1 (D) μM forskolin. Complete recovery from desensitization was seen after 60 min wash (E).

3. RESULTS

3.1. *Effect of forskolin on ACh sensitivity of the junctional region of innervated soleus muscle*

To study the effects of forskolin on the response of the junctional region to microiontophoretically applied ACh the following approach was taken: determinations of high values for ACh sensitivity at the junctional region were obtained in the presence of physiological salt solution plus 0.01–0.1% alcohol, since this was the vehicle for dissolving forskolin. The ACh sensitivity for the innervated junctional region varied from 1500 to 5000 mV/nC. To avoid desensitization during control conditions, the trains of 100–200 ACh potentials were evoked at frequencies of 1–8 Hz. Fig.1 shows experimental records of ACh sensitivity under control conditions, after 30 min exposure to forskolin (1 μM) and during washing depicting the recovery phase. After 30 min exposure to forskolin (0.2 and 1 μM) a significant depression of the amplitude of the ACh potentials was observed. While ACh potentials evoked at 1 Hz did not show any sign of desensitization, at 8 Hz a significant depression occurred such that at 1 μM forskolin by the 100th and 200th potentials the amplitudes of the ACh potentials had decreased by as much as 60% of the initial value (fig.2). The depression was often characterized by a fast phase followed by a slow steady decay (fig.1, inset). Upon cessation of the train, the amplitude of the ACh potentials returned to values identical to those generated at 1 Hz (see fig.1B). The desensitization induced by forskolin was reversible upon washing the muscles for 30 to 60 min (see figs 1 and 2).

3.2. *Effect of forskolin and its analogs on the ACh sensitivity of chronically denervated rat soleus muscles*

Similar results to that observed on the junctional region of the innervated muscles were obtained on the chronically denervated soleus muscle in the presence of various concentrations (0.1–5 μM) of forskolin (figs 3 and 4). Under control conditions, no desensitization was observed (fig.3A); however, at concentrations as low as 0.1 μM, forskolin produced depression of responses in a train which usually revealed one phase. ACh potential amplitude was fully recovered upon cessation of repetitive stimulation (fig.3). In addition, as seen with innervated muscles, no desensitization was observed after washing muscles with drug-free solution (fig.3E).

To investigate whether the effects of forskolin were correlated with its known stimulatory effects on adenylate cyclase, two close structural analogs were tested. 1,9-Dideoxyforskolin, which is inactive with respect to activation of adenylate cyclase

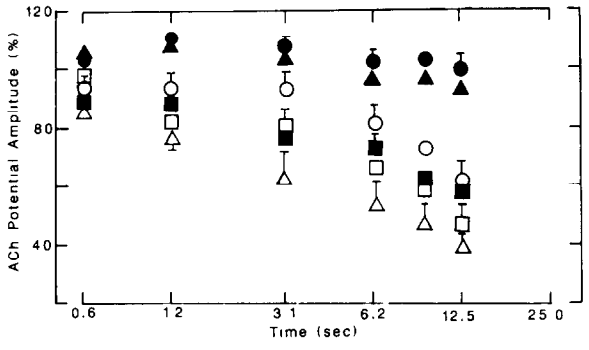

Fig.4. Effect of forskolin on the extrajunctional ACh sensitivity of the chronically denervated rat soleus muscle. ACh potentials (100) evoked at 8 Hz were recorded under control conditions (●), 40–60 min after perfusion of 0.1 (○), 0.5 (■), 1.0 (□), or 5.0 (△) μM forskolin and 45–60 min after wash (▲). Each point represents the mean ± SE of values from at least 4–5 fibers in 3 muscles, expressed as percent of the first potential in a train.

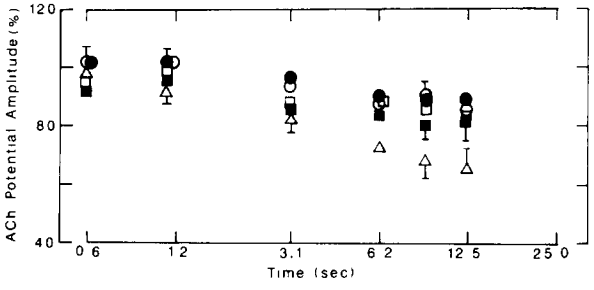

Fig.5. Effect of 1,9-dideoxyforskolin and 14,15-dihydroforskolin on the extrajunctional sensitivity to microiontophoretic application of ACh in the denervated soleus muscle of the rat. ACh potentials were evoked at 8 Hz under control conditions (●) and after perfusion with 1,9-dideoxyforskolin (○, 0.5 μM; □, 1.0 μM) or 14,15-dihydroforskolin (■, 0.4 μM; △, 1.2 μM). Each point, expressed as percent of the first potential in a train of 100 potentials, represents the mean ± SE of values obtained after 45–60 min drug perfusion from at least 3 fibers in 2 muscles.

[10], did not produce any effect on the ACh sensitivity at a concentration up to 1 μM (fig.5). 14,15-Dihydroforskolin, which is about 8-fold less potent than forskolin as an adenylate cyclase activator [10], at 0.4 and 1.2 μM induced much less desensitization compared to forskolin. As shown in fig.5, 1.2 μM dihydroforskolin caused only 25% depression of the 100th ACh potential, thus reflecting a much weaker activity than the parent compound forskolin. The latter depressed the ACh potential by nearly 40% even at the concentration of 0.1 μM (see figs 3 and 4). Similar to forskolin, the effect of 14,15-dihydroforskolin was completely reversible upon washing.

3.3. *Effect of forskolin on the ACh-activated single channel currents*

Single channel currents were recorded from cultured rat myoballs (6-day-old culture) under cell-attached patch configuration using a micropipette filled with either ACh (0.1 μM) alone or together with 0.1–1.0 μM forskolin. ACh, as has been reported [18,22], activated predominantly channel openings with conductance of 30 pS at 20°C. The excessive number of fast events contributed to a departure from the single-exponential distribution. The best fit to a double-exponential function obtained by nonlinear regression provided τ values of 0.7 and 17.8 ms for the fast and slow phases, respectively. Addition of forskolin to the patch pipette solution did not cause significant change in either channel conductance, duration or distribution of the open times or in the frequency of channel openings. The bursting-type activity similar to that reported for high agonist concentration [23] or for open channel blockers [25] was not observed. The effects of high concentrations of forskolin (up to 100 μM) were also tested on the isolated frog muscle fibers. As in the rat myoballs, no significant changes in channel lifetime or conductance were observed.

3.4. *Effect of forskolin on endplate currents of frog sartorius muscles*

Preliminary EPC experiments on the neuromuscular junction of the frog sartorius muscles showed that only high concentrations (1–100 μM) of forskolin decreased the EPC peak

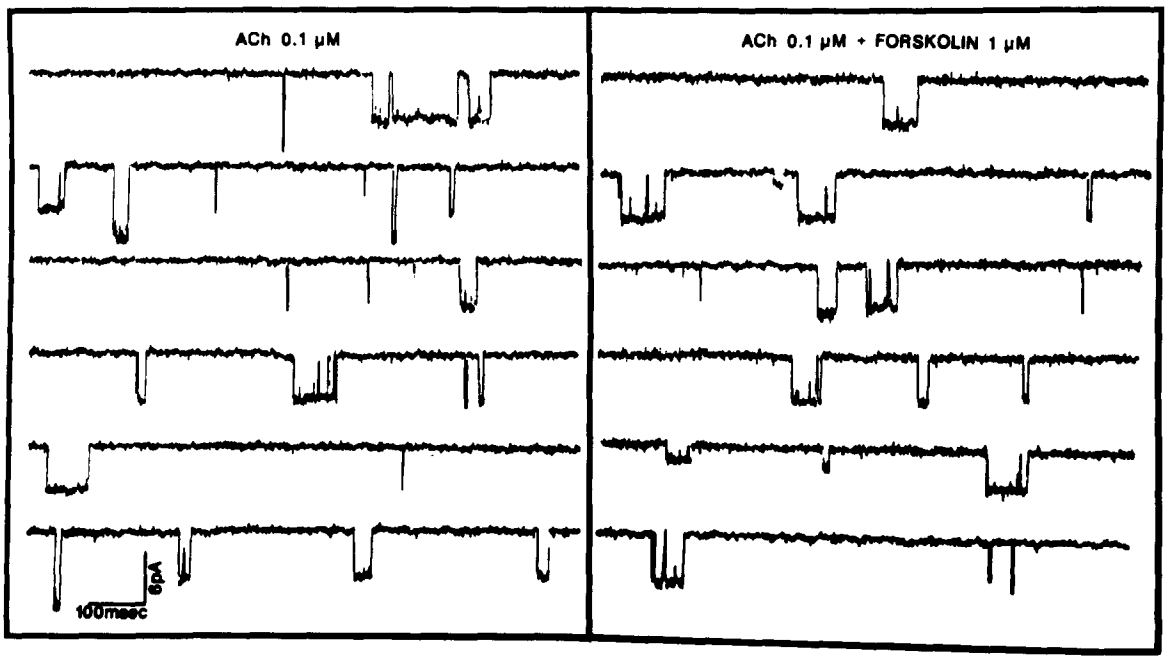

Fig.6. Samples of single channel currents recorded from rat myoballs. The recordings were performed under the cell-attached patch configuration with a micropipette filled with ACh either alone or in the presence of forskolin. Temperature, 20°C; holding potential, −180 mV.

amplitude, but they had little or no effect on the decay time constant. Forskolin (100 μM) did not produce a marked departure from linearity of the current-voltage relationship of the EPCs. In addition, the influence of the frequency of nerve stimulation on EPC amplitude was analyzed. Under control conditions, trains of EPCs evoked up to 50 Hz did not show any depression of the peak amplitude. However, in the presence of forskolin (40–100 μM), trains of EPCs evoked at membrane potentials varying from -50 to -150 mV at 50 Hz disclosed a significant depression. The latter reached an apparent steady state by the 40th to 50th EPC (not shown).

4. DISCUSSION

This study demonstrated that forskolin, an activator of adenylate cyclase, induces a reversible AChR desensitization at the junctional and extrajunctional regions of rat soleus muscles. Such an action occurred without changing the kinetics of open ion channels associated with the AChR such that neither single channel conductance nor lifetime was affected.

Forskolin was used at concentrations at which it is effective in altering physiological responses mediated through cAMP in a variety of systems [11]. Physiological responses are usually fully altered by concentrations of forskolin less than 10 μM and in many cases with smooth muscle, cardiac preparations, or epithelial cells, the effects of forskolin on relaxation, contraction or ion transport occur with ED_{50} of 200 nM or less. Concentrations >5–10 μM were avoided since other 'nonspecific' effects of forskolin, viz. direct interactions with AChR through allosteric or noncompetitive mechanisms, may occur at such high concentrations as described for a variety of drugs [5].

The sequence of receptor desensitization by forskolin appears to involve an initial fast phase followed by a slow almost steady-state second phase (see fig.1). However, further quantitative analysis is now in progress to clarify such an observation. A good correlation between the elec-

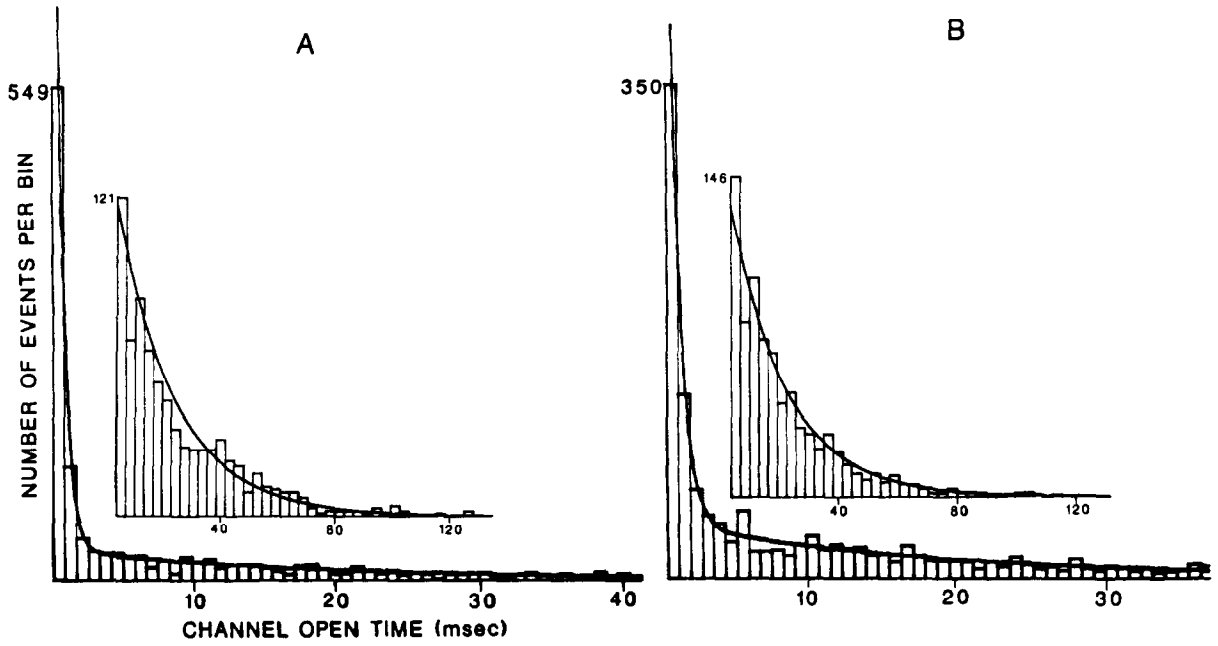

Fig.7. Open time histograms of the channels activated by ACh (0.1 μM) either alone (left) or together with forskolin (1 μM) from rat myoballs. τ values for the fast (τ_f) and slow (τ_s) phases obtained from the best fit of the distribution to a double-exponential function (nonlinear regression) were: left: τ_f = 0.7 ms, τ_s = 17.8 ms; right: τ_f = 0.5 ms, τ_s = 17.8 ms. (Inset) Histograms of the slow phase on an expanded scale.

trophysiological effect described here and the ability of forskolin and some of its analogs to activate adenylate cyclase was observed. 1,9-Dideoxyforskolin, an analog that is inactive on adenylate cyclase [13], was unable to induce any sign of desensitization even at high concentrations. In addition, compared to forskolin, 14,15-dihydroforskolin, a less potent activator of adenylate cyclase [13], was less potent in inducing desensitization. Such structure-activity correlations strongly suggested a possible involvement of cAMP in the process of desensitization by forskolin. However, alternative mechanisms for induction of desensitization by forskolin can be considered. First, a blockade of AChR in the open state. This possibility appears unlikely since no alterations in either channel lifetime or conductance were observed. However, a recent study [26] on effects of forskolin (10–30 μM) on synaptic transmission in sympathetic ganglia showed a decrease in the nicotinic receptor activity, which was postulated as perhaps due to a blockade of the open channel. Such a hypothesis was not confirmed here. Even at concentrations from 40 to 100 μM forskolin, as revealed both directly by single channel recording and indirectly by the analysis of the decay time constant of the EPC, did not have any effects typical of open channel blockade [5,25]. Both presynaptic facilitation and 'anticholinergic' postsynaptic effects were reported in the study with sympathetic ganglia [26], and from our study it appears likely that the latter effects are related to the facilitatory effects of forskolin in desensitization. Second, forskolin might enhance desensitization through mechanisms that do not involve activation, bursting and marked decrease in frequency of ion channel opening of the AChR as observed with noncompetitive blockers such as meproadifen [27,28] or high concentrations of agonist [23,24]. Bursting and decrease in frequency were not observed at any concentrations of forskolin tested, 0.1–1 μM in myoballs and 0.1–100 μM in isolated frog muscle fibers. Although we have not observed a decrease in channel opening frequency, one possible explanation is that it has been missed due to the limitations inherent in the cell-attached patch technique and the variation in density of AChRs in the myoballs. It is also conceivable that a short bath incubation of the myoballs with forskolin prevents the accumulation of cAMP to a level which would phosphorylate the AChR leading to the appearance of signs characteristic of desensitization. Third, since forskolin (1–100 μM) did not induce voltage- and time-dependent effects on the EPCs as seen with many other noncompetitive antagonists of the AChR such as phencyclidine, phenothiazines, meproadifen and histrionicotoxin [5,6,27–29], the possibility of a blockade of AChR in the resting state seems unlikely. The possibility that the desensitization observed with forskolin could be due to continuous and excessive exposure to ACh because of a blockade of AChE by forskolin was also considered. However, concentrations of 1–100 μM of the drug did not block AChE assayed in homogenates of soleus muscle. Under the present circumstances, taken together, the evidence suggests that the desensitization observed with forskolin may be mediated via a mechanism involving an activation of adenylate cyclase by forskolin, resulting in cAMP formation, activation of cAMP-dependent protein kinase and phosphorylation of the AChR. Phosphorylation of the AChR could occur either in the resting state or in states with bound ACh. Such phosphorylation would then enhance pathways leading to possible desensitized, nonconducting states.

In conclusion it is suggested that forskolin, a general activator of hormone-sensitive adenylate cyclase, enhances desensitization via a process in which one or more of the subunits comprising the AChR macromolecule is phosphorylated by a cAMP-dependent protein kinase.

ACKNOWLEDGEMENTS

We are most grateful to Dr Frederick C. Kauffman for having made the determinations of AChE for us. We would like to thank Ms Mabel A. Zelle for computer programming and assistance in the analysis of the data. This study was supported by US Army Research and Development Command Contract DAMD 17-84-C-4219.

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