Desensitization of Acetylcholine Receptors in Rat Myotubes Is Enhanced by Agents That Elevate Intracellular cAMP

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Incubating skeletal muscle fibers with forskolin, an activator of adenylate cyclase, increases the rate at which nicotinic acetylcholine receptors (AChRs) desensitize when exposed to ACh. Several reports indicate that this is due to the phosphorylation of AChRs by cAMP-dependent protein kinase, but other studies suggest that forskolin interacts with AChRs directly and that second-messenger systems are not required. To help clarify this issue, we studied the effects of forskolin and several other drugs on AChR function in embryonic rat myotubes. AChR function was studied by recording ACh-induced membrane depolarizations and ACh-induced single-channel currents. Our results indicate that forskolin at low concentrations enhances AChR desensitization through the action of a second messenger, most likely cAMP. An analog of forskolin that is much less effective in activating adenylate cyclase (1,9-dideoxyforskolin) is also much less potent in enhancing desensitization. Forskolin at low concentrations does not alter single-channel conductance or mean channel open time. However, when used at concentrations above 20 μ M, forskolin may also exert direct drug effects on AChRs.

Protein phosphorylation appears to be a general mechanism for modulating the functional properties of ion channels (for review, see Kaczmarek and Levitan, 1987). Although many types of channels are modulated by agents that activate protein kinases, the underlying biochemical mechanisms are poorly understood. In most systems it is not known if kinases act by phosphorylating the channel itself or a separate regulatory protein.

Because it is by far the best characterized chemically-gated ion channel, the nicotinic acetylcholine receptor (AChR) should be useful in clarifying how channel function is modulated by protein phosphorylation. Abundant evidence indicates that the AChR is a phosphoprotein (Gordon et al., 1977; Teichberg et al., 1977; Haganir and Greengard, 1983; Haganir et al., 1984; Miles et al., 1987). AChRs from *Torpedo* electric organ are

substrates for cAMP-dependent protein kinase (A kinase), protein kinase C, and tyrosine-specific protein kinase. The likely phosphorylation sites have been identified in each case (Haganir et al., 1984). Although much remains to be learned about the functional significance of AChR phosphorylation, recent studies indicate that phosphorylation by A kinase increases the rate of AChR desensitization. Forskolin and other agents that stimulate cAMP-dependent pathways enhance AChR desensitization (Albuquerque et al., 1986; Middleton et al., 1986) and stimulate AChR phosphorylation (Miles et al., 1987) in rat muscle cells. Furthermore, rapid-flux studies of purified *Torpedo* AChRs have provided direct evidence linking AChR phosphorylation to an increased rate of desensitization (Haganir et al., 1986).

In this paper we extend our earlier physiological studies of AChR modulation to the single-channel level. The results indicate that forskolin, a powerful activator of adenylate cyclase, enhances agonist-induced desensitization without significantly changing the properties of individual single-channel currents. It seems likely that forskolin does so by promoting the phosphorylation of AChRs by A kinase. In contrast to results obtained in other systems (Akagi and Kudo, 1985; McHugh and McGee, 1986), we found no indication that forskolin at low concentrations affects AChR function directly.

Materials and Methods

Chemicals. Forskolin and 1,9-dideoxyforskolin were obtained from Calbiochem. Each was prepared as a 10 mM stock in 95% ethanol. L-15 and Dulbecco's Modified Eagle's Medium (DMEM) tissue culture media were obtained from GIBCO. The cAMP analogs 8-methylthio-cAMP and 8-(4-chlorophenylthio)-cAMP were obtained from ICN Biochemical and Boehringer Mannheim, respectively. The phosphodiesterase inhibitor RO 20-1724 was a generous gift from Hoffmann-LaRoche. All other chemicals were obtained from Sigma.

Cell cultures. Muscle cultures were prepared with conventional techniques (Rubin, 1985). Whole leg muscle was removed from rats ranging in age from embryonic day 19 to postnatal day 1. The muscle was minced and incubated for 20 min at 37°C in Ca^{2+} - and Mg^{2+} -free Puck's saline G with 0.05% trypsin. The tissue was pelleted, resuspended in DMEM supplemented with 20% fetal calf serum, and an additional 6 mg/ml glucose, triturated with a Pasteur pipette, and filtered to remove tissue fragments. The cells were preplated for 1 hr to remove fibroblasts, and those that remained in suspension were plated in 35 mm tissue culture dishes (400,000/dish) containing collagen-coated glass coverslips.

The cultures were fed every 3 d after plating with DMEM supplemented with 10% horse serum, 2% chick embryo extract, and an additional 6 mg/ml glucose. Sometimes 10^{-5} M cytosine arabinoside was added to 4-d-old cultures for 24–48 hr to slow the growth of fibroblasts, but in other cases this was unnecessary. Similar results were obtained in both cases.

Electrophysiology. For each experiment, a coverslip with myotubes was placed in a recording chamber on a Zeiss UEM microscope and viewed at $\times 500$ with a $\times 40$ water-immersion objective. The cells were

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We dedicate this paper to the memory of Dr. Stephen M. Schuetze.

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Figure 1. Forskolin and a cAMP analog enhance AChR desensitization. Myotubes were stimulated with a train of 2 msec ACh pulses delivered at 7 Hz. In each panel the responses are shown superimposed, and the ionophoretic current is illustrated below. *A*, Ten consecutive responses recorded from a myotube in control medium. *B*, Twelve responses recorded from the same cell after 45 min in 10 μ M forskolin and 35 μ M RO 20-1724. *C*, Eleven responses recorded from the same cell 30 min after the drugs were removed. *D*, Eleven responses recorded from a different myotube after 4 min in 30 μ M 8-(4-chlorophenylthio)-cAMP. Resting membrane potentials in this and other experiments ranged from -50 to -70 mV and were unaffected by the drugs. Calibration bars, 4 mV (upper traces), 20 nA (lower traces), 20 msec.

bathed in L-15 medium or a saline solution containing (in mM) 150 NaCl, 3 KCl, 1 MgCl_2 , 1 CaCl_2 , 33 glucose, and 10 HEPES (titrated to pH 7.2 with NaOH). In some experiments, TTX (2 μ M) was added to stop spontaneous twitching. All experiments were performed at room temperature (21–23°C).

Ionophoretic assays of ACh sensitivity were made as described in Middleton et al. (1986). Myotubes were impaled with intracellular electrodes with resistances of ~ 80 M Ω when filled with 3 M KCl. Ionophoretic electrodes were similar but were filled with 2 M AChCl. Both electrodes were connected to an Axoclamp 2A amplifier. ACh leakage was minimized with a negative backing current of 1–2 nA. Ionophoretic pulses were 2 msec in duration, 5–15 nA in amplitude, and were delivered in 1–2 sec trains at 7 Hz. A laboratory computer (LSI 11/73; Digital Equipment Corporation) was used to deliver the pulses, record the responses, and calculate the ACh sensitivity in units of depolarization per unit charge delivered. AChR desensitization was characterized in terms of the decrease in response amplitude between the first and seventh pulses in a train (Fig. 1).

Single-channel recordings of AChRs were obtained with conventional techniques (Hamill et al., 1981) as described (Jaramillo and Schuetz, 1988). The patch pipette was filled with the bath saline solution (less glucose) supplemented with ACh (10 nM–1 mM). In some experiments, the filling solution also contained forskolin at a final concentration of 10–20 μ M. The pipette was connected to a List EPC-5 patch-clamp amplifier, and the current output signal was low-pass-filtered at 1–4 kHz (8-pole Bessel; Frequency Devices, Inc., Haverhill, MA) and digitized by a laboratory computer (LSI 11/73). The sampling frequency was usually 5 times the corner frequency of the analog filter.

Single-channel analysis. Measurements of single-channel amplitudes and distributions of open and closed times were made with a semiautomated procedure based on half-amplitude threshold analysis, as described in detail elsewhere (Jaramillo and Schuetz, 1988). Events shorter than twice the sampling interval were ignored.

In some experiments we studied the effects of forskolin on the gating properties of AChRs by comparing the open time distributions in the presence and absence of the drug. The interpretation of open times is generally unclear because of the presence of undetected brief closings, and it is therefore usually preferable to characterize events in terms of

their burst durations (Colquhoun and Sakmann, 1985). However, we were forced to use open times rather than burst durations because many of our experiments were done at high agonist concentrations, and under these conditions events are often so frequent that the definition of a burst becomes ambiguous.

Open time distributions were fitted with 1 or 2 exponentials by the method of maximum likelihood. At low agonist concentrations the open time distributions contained 2 exponential components. The amplitude of the faster component decreased with ACh concentration, and at high concentrations (typically ≥ 10 μ M) good fits were obtained with a single exponential (Jaramillo and Schuetz, 1988). In the experiments reported here, we focused on the slower of the 2 open time components.

The amount of AChR channel activity in a patch was expressed as Np_o , where N is the number of channels in the patch and p_o is the probability that a channel is open. Np_o was defined as I/i , where i is the single-channel current amplitude and I is the mean current during the entire record. I was measured by fitting a straight line to the baseline (correcting for drift as required) and integrating the area beyond the baseline. In some experiments this procedure was modified to express Np_o as a function of time. This was done by calculating a running average of I over a time window 5–60 sec long (see Logothetis et al., 1987).

Np_o varied widely between patches in control experiments, even though the recording conditions were apparently identical. Presumably, this was mostly due to variability in the number of AChRs in the patch. We controlled for this by averaging data from several patches for each experimental condition. A related problem was that the *mean* activity level varied between different platings of myotubes. For example, with 20 μ M ACh in the patch pipette Np_o varied from a low of 0.1 ± 0.1 in one group of cultures (mean $\pm$ SD, $n = 8$ patches) to an extreme value of 2.3 ± 1.8 in another ($n = 9$ patches). This problem was circumvented by obtaining the control and test patches for each experiment from the same cultures and often from the same myotubes.

Results

Forskolin enhances AChR desensitization in rat myotubes

We assayed AChR desensitization in rat myotubes by measuring the membrane depolarizations evoked by a train of ACh pulses. In untreated myotubes, each pulse in a 7 Hz train evoked about the same response amplitude, indicating little or no desensitization (Fig. 1*A*). After the myotubes were exposed to a combination of 10 μ M forskolin and the phosphodiesterase inhibitor RO 20-1724, the initial response amplitude was little changed, but successive responses fell by 65% within 1 sec (Fig. 1*B*). This indicates that forskolin enhanced the rate of agonist-induced AChR desensitization without affecting the ability of AChRs to be activated by ACh initially (but see below). ACh sensitivity recovered fully within less than a minute after the pulses were discontinued, and application of a second pulse train again evoked a rapid fall in response amplitude (not shown). The drug effects reversed fully after washout (Fig. 1*C*). These results are similar to those reported previously in studies of rat soleus muscles (Middleton et al., 1986).

Evidence that forskolin acts on AChRs via the cAMP pathway

If forskolin acts on AChRs via the cAMP pathway, the drug should enhance desensitization with a concentration dependence and time course consistent with its activation of adenylate cyclase. We tested this idea with the ionophoretic assay.

Seamon et al. (1981, 1984) reported that forskolin activates hormonally responsive adenylate cyclase with half-maximal effects at 5–10 μ M. We found that forskolin enhances AChR desensitization at concentrations as low as 1–2 μ M and with half-maximal effects at about 6 μ M (Fig. 2), in good agreement with the biochemical data. Concentrations higher than 30 μ M were not tested because at these drug levels the ACh sensitivity appeared to be lower than normal. That is, even the initial response to a train of ACh pulses was small, and it was impossible to

Figure 2. Dose-response curve for the forskolin-mediated increase in AChR desensitization. Forskolin was added at the indicated concentration with 35 μ M RO 20-1724 and 30–60 min later AChR desensitization was assayed in several myotubes as illustrated in Figure 1. Each symbol represents the mean amplitude ($\pm$ SEM) of the seventh response expressed as a percentage of the initial amplitude. Three to seven (mean = 4) cells were tested at each concentration.

quantify desensitization with this approach. The apparent decrease in ACh sensitivity may reflect an additional effect of forskolin at high concentrations. Alternatively, it is possible that the number of activable AChRs is unchanged, but desensitization is so rapid that many AChRs become inactive during the rising phase of the first response.

Forskolin activates adenylate cyclase rapidly and reversibly (Seamon et al., 1981, 1984). To determine if the drug's effects on AChRs are also rapid and reversible, we studied the same membrane sites before, during, and after treating myotubes with 10 μ M forskolin. We found that the level of desensitization reached a plateau within minutes of drug addition, and recovery took place within minutes after drug washout (Fig. 3). Again, the results are consistent with the biochemical data.

Several other observations support the idea that forskolin enhances desensitization by raising intracellular levels of cAMP. AChR desensitization was enhanced by the phosphodiesterase inhibitor RO 20-1724 used alone and by 2 analogs of cAMP (Fig. 1D and Table 1). The cAMP analog 8-bromo-cAMP proved ineffective (Table 1), but this has been observed in other systems thought to be regulated by cAMP (Dunlap, 1985). Furthermore, in a separate series of experiments using an ineffective concentration of RO 20-1724, the effects of 1 μ M forskolin were enhanced 4-fold. The percentage decrease in the response amplitude after 7 pulses of ACh with 1 μ M forskolin alone was 2 ± 4 (mean $\pm$ SD, $n = 6$), whereas in combination with RO 20-1724, it was 9 ± 7 ($n = 10$).

Possible direct effect of forskolin on AChR channels

In some systems forskolin apparently alters channel activity via a direct action, possibly open channel block, that is independent of its stimulation of adenylate cyclase (Akagi and Kudo, 1985; McHugh and McGee, 1986; Grassi et al., 1987). We tested for direct actions on myotube AChRs by comparing the effects of forskolin and 1,9-dideoxyforskolin, a forskolin analog that does not stimulate adenylate cyclase (Seamon et al., 1984). These

Figure 3. Time course of forskolin-stimulated increase in desensitization. AChR desensitization was measured at zero time from a myotube in control medium, and then the culture was bathed in 10 μ M forskolin and 35 μ M RO 20-1724. The same myotube was assayed repeatedly at the indicated times. After 1 hr the drugs were washed out (arrow) and the myotube was assayed again. The symbols indicate the mean $\pm$ SEM of 4 experiments.

experiments were done in the absence of the phosphodiesterase inhibitor RO 20-1724 because, as just noted, they partially mimic the effects of forskolin.

When used at 10 μ M, forskolin markedly enhanced AChR desensitization but 1,9-dideoxyforskolin did not (Fig. 4, A and B). This is consistent with the idea that forskolin acts via cAMP. However, both drugs enhanced desensitization when used at 20 μ M, although forskolin was clearly more potent (Fig. 4, C and D). In this experiment, 20 μ M forskolin also appeared to lower ACh sensitivity (Fig. 4D). As discussed above, this may reflect rapid desensitization rather than a true loss of sensitivity.

These data indicate that forskolin may act on AChRs in 2 ways. At low concentrations (≤ 10 μ M), it apparently acts primarily by stimulating adenylate cyclase; at higher concentra-

Table 1. Effect of forskolin and other drugs on AChR desensitization

Drug	Desensitization (% decrease)
None	5 $\pm$ 1 (18)
0.095% EtOH	6 $\pm$ 3 (6)
10 μ M 1,9-dideoxyforskolin	10 $\pm$ 4 (3)
10 μ M forskolin	45 $\pm$ 6 (3)
10 μ M forskolin + PDI	65 $\pm$ 5 (4)
20 μ M forskolin	64 $\pm$ 4 (7)
20 μ M forskolin + PDI	83 $\pm$ 2 (4)
PDI alone	28 $\pm$ 4 (6)
30 μ M 8-(4-chlorophenylthio)-cAMP	23 $\pm$ 4 (5)
100 μ M 8-methylthio-cAMP	17 $\pm$ 2 (3)
100 μ M 8-bromo-cAMP	1 $\pm$ 1 (3)

Desensitization is expressed as the percentage decrease in response amplitude during a train of 7 ACh pulses as illustrated in Figure 1. Values are given as means $\pm$ SEM, and the number of myotubes tested is in parentheses. Forskolin and 1,9-dideoxyforskolin were added as 10 mM stock solutions in 95% ethanol to give the final concentrations shown (final ethanol concentration $\leq 0.19\%$). PDI indicates that the preparation was incubated with the phosphodiesterase inhibitor RO 20-1724 (35 μ M).

Figure 4. Relative potency of forskolin and 1,9-dideoxyforskolin in enhancing AChR desensitization. All 4 panels were obtained from the same myotube in the sequence shown. *A*, Nine consecutive responses recorded after 15 min in $10\ \mu\text{M}$ 1,9-dideoxyforskolin. *B*, Nine responses recorded after 26 min in $10\ \mu\text{M}$ forskolin. *C*, Nine responses recorded after 34 min in $20\ \mu\text{M}$ 1,9-dideoxyforskolin. *D*, Eight responses recorded after 16 min in $20\ \mu\text{M}$ forskolin. The culture was washed in control medium for 20 min between treatments. Calibration bars, 4 mV (upper traces), 20 nA (lower traces), 20 msec.

tions, the drug may also act on AChR channels directly. Consistent with this idea, previous work has indicated that $100\ \mu\text{M}$ forskolin appears to act as a modest open channel blocker of AChRs (Middleton et al., 1986). We turned to single-channel recordings to help distinguish direct and indirect actions of forskolin more cleanly.

Effects of forskolin on AChR activity at the single-channel level

AChR activity in cell-attached patches was quantified in terms of Np_o , where N is the number of AChR channels in the patch and p_o is the probability that any particular channel is open (see Materials and Methods). Potential direct effects were examined by adding forskolin to the patch pipette but omitting it from the bath. This exposes AChRs to forskolin but minimizes drug entry into the cell. We found no obvious effects of pipette-applied forskolin on AChR activity. Records obtained with the drug were indistinguishable from control records (Fig. 5, *A* and *B*).

A more rigorous, quantitative comparison was made using paired recordings from 8 different myotubes. In each case, one cell-attached patch was assayed with ACh alone (10 , 20 , or $100\ \mu\text{M}$) and another was studied with the same concentration of ACh in combination with forskolin (10 – $20\ \mu\text{M}$). Np_o averaged 0.11 ± 0.12 (mean $\pm$ SD) when forskolin was in the pipette and 0.11 ± 0.10 when it was not (Fig. 6). Forskolin also had no effect on the mean open time or unitary conductance of AChR channels (Table 1 and Middleton et al., 1986). These data indicate that the forskolin-enhanced desensitization in the ionophoretic experiments is not likely to be the result of a direct action on AChRs.

Indirect forskolin actions, possibly mediated by cAMP, were studied by adding the drug to the bath but omitting it from the pipette filling solution. This exposes virtually the entire cell to

Figure 5. Single-channel records from myotubes before and after exposure to forskolin. *A*, AChR channel openings recorded from a control myotube in the absence of forskolin. *B*, Record obtained from another myotube on the same coverslip with $10\ \mu\text{M}$ forskolin in the patch pipette. *C*, Record obtained from a third myotube on the same coverslip 20 min after forskolin was added to the bath (final concentration, $10\ \mu\text{M}$). All 3 records were obtained from cell-attached patches with $10\ \mu\text{M}$ ACh in the pipette at approximately the same time following the formation of each patch. The records were low-pass-filtered at 1 kHz, and the patch was hyperpolarized 20 mV from rest. Calibration bars, 500 msec, 3.5 pA.

forskolin, as in the ionophoretic experiments. However, the AChRs within the patch are not directly exposed to forskolin once the patch is formed since the pipette filling solution contains no drug. Inclusion of forskolin in the bath (but not in the pipette) resulted in a dramatic inhibition of AChR activity (Fig. 5C). In the experiment shown here, the patch was obtained 20 min after forskolin addition. Similar results were obtained after shorter exposures to forskolin, the shortest being 5 min (data not shown). Np_o averaged 0.27 ± 0.30 with myotubes in control medium ($n = 26$ patches, $20\ \mu\text{M}$ ACh). When the same cultures were studied in a bath containing 10 – $20\ \mu\text{M}$ forskolin, Np_o was only 0.0075 ± 0.0090 ($n = 12$ patches, $20\ \mu\text{M}$ ACh), a 36-fold decrease (Fig. 6).

In contrast, bath applied 1,9-dideoxyforskolin had little effect on AChR activity when the same experimental protocol was used. The average Np_o from 11 different myotubes was 0.18 ± 0.26 before exposure to the drug. After incubating the same cells in $10\ \mu\text{M}$ 1,9-dideoxyforskolin Np_o averaged 0.07 ± 0.09 (Fig. 6). This does not represent a significant decrease in AChR activity (Student's t test and the Wilcoxon signed-rank test; Snedecor and Cochran, 1967).

In the experiments illustrated in Figures 5 and 6, myotubes were incubated with the drugs for at least several minutes prior to seal formation, and therefore, different membrane patches were used for controls and experiments. We also tried recording from the same patch before and after adding forskolin to the bath. The results were unpredictable (Fig. 7). Sometimes forskolin addition was associated with a sharp drop in AChR activity, indicating an enhancement of desensitization, but at other times, the drug seemed to be without effect. We suspect that membrane distortions introduced by seal formation may interfere with second-messenger effects (see Discussion).

In summary, forskolin strongly decreased AChR activity when added to the bath but not when added to the patch pipette filling

Figure 6. Effects of forskolin and 1,9-dideoxyforskolin (analog) on AChR single-channel activity in cell-attached patches. The drugs were added at final concentrations of 10–20 μM to either the patch pipette filling solution or the bath. For each type of experiment, several control and experimental patches were obtained from the same cultures under similar conditions, and Np_o was measured in each patch. To control for variability in channel activity between platings of myotubes, all measurements of Np_o were divided by the mean control value of Np_o for that experiment. The control values illustrated were obtained from an experiment testing the effects of bath-applied forskolin; a similar range in relative activity in control patches was observed in all cases. The ACh concentration was 10–100 μM when forskolin was included in the filling solution and 20 μM otherwise.

solution. Bath-applied 1,9-dideoxyforskolin did not decrease AChR activity significantly. Taken together, these results strongly suggest that forskolin acts on AChRs via a second messenger, presumably cAMP, diffusing through the cytoplasm.

Forskolin appears to enhance AChR desensitization selectively

Records obtained in the presence of bath-applied forskolin seemed to differ from control records only in the frequency of channel openings and not in the characteristics of individual ACh-evoked currents. This was confirmed by more careful analysis. Bath-applied forskolin had virtually no effect on either single-channel conductance or mean channel open time of AChRs at any ACh concentration tested (Fig. 8, Table 2; also compare Fig. 9, *B* and *D*). These data argue further against a channel-blocking mechanism and are consistent with the idea that forskolin enhances AChR desensitization selectively.

One prediction of this hypothesis is that forskolin's effectiveness should depend on the concentration of ACh. The drug should strongly decrease AChR activity at high, desensitizing ACh concentrations but have little effect at low agonist levels. In contrast, if forskolin silences AChRs directly rather than enhances agonist-induced desensitization, then it should decrease AChR activity at all ACh concentrations. This was tested by studying forskolin's activity with various concentrations of ACh in the patch pipette.

In the single most complete experiment, 4 different cell-attached patches on the same myotube were studied under different conditions. The culture was first bathed in control medium and 2 patches were assayed at different ACh concentrations. Much more AChR activity was evoked by ACh at 100 μM ($Np_o = 0.58$; Fig. 9*B*) than at 0.1 μM ($Np_o = 0.06$; Fig. 9*A*). The culture was then bathed in 10 μM forskolin and 2 additional patches were obtained from the same cell. The activity level at 0.1 μM ACh was the same as control ($Np_o = 0.07$; Fig. 9*C*) but that at 100 μM ACh was much lower ($Np_o = 0.09$; Fig. 9*D*). In short, forskolin inhibited channel activity only at the higher, desensitizing ACh concentration.

Further studies confirmed and extended these results. Np_o values obtained in the absence or presence of forskolin with nondesensitizing ACh concentrations (0.01–0.1 μM) in the pipette were 0.02 ± 0.02 ($n = 11$) and 0.04 ± 0.06 ($n = 14$),

respectively. In contrast, when the pipette contained desensitizing concentrations of ACh (1–100 μM), the Np_o values before and after forskolin treatment were 1.2 ± 1.6 ($n = 36$) and 0.04 ± 0.06 ($n = 24$), respectively. This represents a 30-fold decrease in channel activity and supports the view that the drug does not silence AChRs directly but, rather, increases their susceptibility to agonist-induced desensitization.

Discussion

Forskolin enhances AChR desensitization

As reported previously for AChRs at rat soleus endplates (Mittleton et al., 1986), the major action of forskolin on myotube AChRs appears to be a marked enhancement of agonist-induced desensitization. Forskolin promoted a progressive, rapid decrease in the amplitude of responses evoked by a train of ACh pulses. However, except when the drug was used at concentrations higher than about 20 μM , forskolin did not reduce the amount of depolarization evoked by the first ACh pulse in a train. This indicates that forskolin did not desensitize AChRs directly nor decrease their initial response to agonist via some other mechanism. Rather, it apparently increased the rate at which they desensitized when exposed to ACh.

The single-channel data support this interpretation. Forskolin treatment effected a striking decrease in the amount of channel activity in cell-attached patches, but the openings that did occur

Table 2. Effect of forskolin on AChR single-channel currents

Drug added	Bath/ pipette	[ACh] (μM)	γ (pS)	τ (msec)
None		0.01–0.1	32 ± 2 (11)	6.3 ± 2.3 (11)
Forskolin	Bath	0.01–0.1	35 ± 2 (4)	5.2 ± 1.0 (6)
None		10–100	35 ± 2 (9)	7.8 ± 1.9 (9)
Forskolin	Bath	10–100	41 ± 6 (4)	8.1 ± 2.5 (5)
Forskolin	Pipette	10–100	40 (1)	8.1 (2)

Single-channel conductances (γ) and open times (τ) were measured from AChRs in cell-attached patches exposed to various concentrations of ACh. Values are expressed as means $\pm$ SD whenever the number of measurements (given in parentheses) was greater than 2. Each conductance measurement represents the slope conductance determined from at least 3 different holding potentials. Forskolin was added to the bath at 5–20 μM or to the patch pipette filling solution at 20 μM .

Figure 7. Variable effect of forskolin on AChR channel activity when added to the bath after seal formation. *A* and *B*, AChR activity plotted against time for a different cell-attached patch. Activity is expressed as a running average of Np_o (50 sec averaging window) versus time. At the times indicated by the arrows, forskolin was added to the bath to give a final concentration of 10 μ M. The ACh concentration was 20 μ M. *A*, Np_o averaged 0.26 before forskolin was added and 0.07 afterwards. *B*, Corresponding values were 0.37 and 0.28. See text for details.

were unchanged in their visual appearance, unitary conductance, and mean open time. Furthermore, forskolin decreased channel activity only at ACh concentrations of 1 μ M and above; at lower ACh concentrations, experimental and control records were indistinguishable. This supports the idea that forskolin promotes the susceptibility of AChRs to ACh-induced desensitization.

Desensitization of nicotinic AChRs occurs in 2 distinct phases: fast and slow (Sakmann et al., 1980; Feltz and Trautmann, 1982). When exposed to high concentrations of ACh, AChRs desensitize on a time scale of tenths of seconds to seconds. If ACh is removed quickly, recovery from desensitization is also rapid. Prolonged application of ACh, even at low doses, pushes AChRs into a second desensitized state characterized by on and off rates roughly 10-fold slower. We have not characterized which of these 2 processes is modulated by forskolin, but the

Figure 8. Distributions of channel open times with (*B*) and without (*A*) bath-applied forskolin. Both cell-attached patches were on the same myotube and were hyperpolarized 40 mV from rest and exposed to 100 μ M ACh. The records were low-pass-filtered at 1 kHz and fitted with simple exponential curves. The best-fit time constants were 6.8 msec (*A*) and 7.1 msec (*B*). The experimental record was obtained 30 min after 10 μ M forskolin and 35 μ M RO 20-1724 were added to the bath.

rapid rates of onset and offset suggest that it is the faster one. In principle, a definitive answer could be obtained by analyzing the kinetics of clusters of openings in single-channel records, but our attempts to do so were unsuccessful. Even with high concentrations of ACh (100 μ M), clusters of AChR channel openings were not as distinct as described in frog muscle (Sakmann et al., 1980).

Mechanism of forskolin's action

An alternative explanation for many of our results is that forskolin treatment does not modulate AChR desensitization via a second messenger but, instead, blocks open channels directly so that they become nonconducting. If the blocked state is long-lived, responses to a train of ACh pulses would become progressively smaller as more and more channels become nonconducting, as we observed. In addition, blocking would explain the decreased activity in our single-channel records.

Figure 9. Effects of forskolin on AChR activity at different ACh concentrations. All 4 recordings were obtained from different patches on the same myotube. *A*, Consecutive traces recorded in control medium with $0.1 \mu\text{M}$ ACh in the patch pipette. Np_o for the entire record was 0.06. *B*, Traces recorded in control medium with $100 \mu\text{M}$ ACh ($Np_o = 0.58$). *C*, Traces recorded with $0.1 \mu\text{M}$ ACh after $10 \mu\text{M}$ forskolin and $35 \mu\text{M}$ RO 20-1724 were added to the bath ($Np_o = 0.07$). *D*, Traces recorded with $100 \mu\text{M}$ ACh in the presence of the same drugs as in *C* ($Np_o = 0.09$). The records were low-pass-filtered at 1 kHz, and the patch was hyperpolarized 20 mV from rest. Calibration bars, 25 msec, 3 pA.

A key feature of this model is that forskolin is postulated to act by interacting with AChRs directly. This has been reported to occur in other systems (Akagi and Kudo, 1985; McHugh and McGee, 1986; Grassi et al., 1987). To some extent, forskolin may also affect myotube AChRs directly. In the ionophoretic assay, $20 \mu\text{M}$ 1,9-dideoxyforskolin partially mimicked the action of forskolin, even though it does not activate adenylate cyclase. Furthermore, previous results showed that forskolin at $100 \mu\text{M}$ decreases channel open time slightly, consistent with a moderate channel-blocking activity (Middleton et al., 1986).

On the other hand, at lower drug concentrations, actions mediated by a second messenger appear to be much more important than direct drug effects. Forskolin at $10 \mu\text{M}$ effected a striking decrease in the response of myotubes to a train of ACh pulses, whereas $10 \mu\text{M}$ 1,9-dideoxyforskolin did not. Albuquerque et al. (1986) have reported similar results in a different system using a lower concentration of forskolin and the same inactive analog. Stronger evidence for a second-messenger mechanism comes from single-channel experiments. Placing 10 – $20 \mu\text{M}$ forskolin in the patch pipette, which should permit direct effects, had no detectable effect on the level of AChR channel activity, open time, or conductance. In contrast, placing $10 \mu\text{M}$ forskolin in the bath (and omitting it from the recording pipette) effected a 36-fold decrease in the amount of AChR channel activity in cell-attached patches. This argues strongly for the involvement of a second messenger in forskolin's actions.

The most likely second messenger is cAMP. Forskolin is known to elevate intracellular levels of cAMP by activating adenylate cyclase, and it does so over the same concentration range with which it enhances AChR desensitization (Seamon et al., 1981, 1984). Other observations further support this view. The drug was more effective when combined with a phosphodiesterase inhibitor than when used alone, and the phosphodiesterase in-

hibitor RO 20-1724 and 2 analogs of cAMP mimicked the effects of forskolin.

An attractive mechanism for forskolin's actions is that it enhances desensitization by stimulating the phosphorylation of myotube AChRs by cAMP-dependent protein kinase (A kinase). Recently, Miles et al. (1987) studied the effects of forskolin on the phosphorylation of AChRs in rat myotubes *in vitro* under conditions similar to those used here. Incubating the cultures in a combination of $20 \mu\text{M}$ forskolin and $35 \mu\text{M}$ RO 20-1724 stimulated the phosphorylation of the AChR δ -subunit 20-fold over basal levels. The effect was rapid and dose dependent. At $20 \mu\text{M}$ forskolin, a half-maximal response was observed within 5 min. When assayed under steady-state conditions, the cultures exhibited half-maximal phosphorylation at $8 \mu\text{M}$ forskolin. Thus, forskolin stimulates AChR phosphorylation and enhances desensitization with a similar time course and concentration dependence. This interpretation agrees with the conclusions of Haganir et al. (1986), who found a direct link between phosphorylation and desensitization rate in *Torpedo* AChRs.

Forskolin also stimulates phosphorylation of the AChR α -subunit in rat myotubes. This effect occurs more slowly—half-maximal phosphorylation after 30 min (Miles et al., 1987)—than the enhanced desensitization reported here. However, it does correlate temporally with a forskolin-stimulated increase in the decay rate of miniature endplate currents in rat soleus muscles (Middleton et al., 1986). Because this decay rate is directly related to channel lifetime, this suggests that phosphorylation of the AChR α -subunit may decrease the mean channel open time. However, in the current experiments we found no evidence for a change in AChR channel open time even after prolonged (>1 hr) exposure to forskolin.

One possibility is that phosphorylation of the AChR α -subunit causes a further increase in the desensitization rate, not

resolved here, so that AChRs may go directly from the open state to the desensitized state at high ACh concentrations (Middleton et al., 1986). In this view, at the high ACh concentration (ca. 1 mM) present during synaptic transmission, AChRs desensitize so fast that endplate currents decay more rapidly. In principle, this hypothesis could be tested in single-channel recordings by using an ACh concentration as high as that found in the synaptic cleft. We attempted to do this, but our results were confounded by the channel-blocking activity of high concentrations of ACh (Ogden and Colquhoun, 1985). Channel block would not be a problem during synaptic transmission because the ACh pulse is so brief (Magleby and Stevens, 1972; Land et al., 1984).

Effect of seal formation on forskolin treatment

Although bath-applied forskolin was very effective in decreasing AChR channel activity in pretreated cultures, it was much less effective when added to the bath after cell-attached patches were formed. One could argue that the crucial feature of pretreatment is some type of direct interaction between forskolin and AChRs that remains stable after patch formation. This seems unlikely. The rapid reversibility of forskolin's effects (Fig. 3) suggests that any drug associated with AChRs in the patch should wash out quickly after seal formation. Furthermore, forskolin was ineffective when added to the patch pipette filling solution.

It seems more likely that seal formation prevented the phosphorylation of AChRs within the patch by A kinase. One possibility is that the Ω -like shape of the patch hindered access of kinase to the AChRs. Alternatively, seal formation may have inhibited phosphorylation by disrupting either the structure of the patch membrane or its association with the underlying cytoskeleton. A similar effect has been noted by others (Strong et al., 1987). After treatment with agents that activate protein kinase C (phorbol esters), *Aplysia* bag cell neurons express a type of calcium channel that is not seen in control cells. These channels are not detected when the activators are added to the bath after seal formation. Furthermore, phorbol esters are ineffective when added to the pipette filling solution but omitted from the bath. Taken together with our data, these results suggest that seal formation may interfere with the modulation of ion channels by second-messenger systems.

Conclusions

The primary action of forskolin on rat myotube AChRs is a strong enhancement of their rate of agonist-induced desensitization. This effect is probably mediated by cAMP and may involve the phosphorylation of the AChR's δ -subunit by A kinase. Forskolin at concentrations above 20 μ M may have additional, direct effects on muscle AChRs. In the future it will be important to identify the endogenous activators of A kinase at the neuromuscular junction and to clarify the physiological role of AChR modulation.

References

- Akagi, H., and Y. Kudo (1985) Opposite actions of forskolin at pre- and post-synaptic sites in rat sympathetic ganglia. *Brain Res.* 343: 346–350.
- Albuquerque, E. X., S. S. Deshpande, Y. Aracava, M. Alkondon, and J. W. Daly (1986) A possible involvement of cyclic AMP in the expression of desensitization of the nicotinic acetylcholine receptor. A study with forskolin and its analogs. *FEBS Lett.* 199: 113–120.
- Colquhoun, D., and B. Sakmann (1985) Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end-plate. *J. Physiol. (Lond.)* 369: 501–557.
- Dunlap, K. (1985) Forskolin prolongs action potential duration and blocks potassium current in embryonic chick sensory neurons. *Pfluegers Arch.* 403: 170–174.
- Feltz, A., and A. Trautmann (1982) Desensitization at the frog neuromuscular junction: A biphasic process. *J. Physiol. (Lond.)* 322: 257–272.
- Gordon, A. S., C. G. Davis, D. Milfay, and I. Diamond (1977) Phosphorylation of acetylcholine receptor by endogenous membrane protein kinase in receptor-enriched membranes of *Torpedo californica*. *Nature* 267: 539–540.
- Grassi, F., L. Monaco, and F. Eusebi (1987) Acetylcholine receptor channel properties in rat myotubes exposed to forskolin. *Biochem. Biophys. Res. Commun.* 147: 1000–1007.
- Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* 391: 85–100.
- Huganir, R. L., and P. Greengard (1983) cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* 80: 1130–1134.
- Huganir, R. L., K. Miles, and P. Greengard (1984) Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. *Proc. Natl. Acad. Sci. USA* 81: 6968–6972.
- Huganir, R. L., A. H. Delcour, P. Greengard, and G. P. Hess (1986) Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. *Nature* 321: 774–776.
- Jaramillo, F., and S. M. Schuetze (1988) Kinetic differences between embryonic- and adult-type acetylcholine receptors in rat myotubes. *J. Physiol. (Lond.)* 396: 267–296.
- Kaczmarek, L. K., and I. B. Levitan (1987) *Neuromodulation. The Biochemical Control of Neuronal Excitability*, Oxford U.P., New York.
- Land, B. R., W. V. Harris, E. E. Salpeter, and M. M. Salpeter (1984) Diffusion and binding constants for acetylcholine derived from the falling phase of miniature endplate currents. *Proc. Natl. Acad. Sci. USA* 81: 1594–1598.
- Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neher, and D. E. Clapham (1987) The $\beta\gamma$ subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature* 325: 321–326.
- Magleby, K. L., and C. F. Stevens (1972) A quantitative description of end-plate currents. *J. Physiol. (Lond.)* 223: 173–197.
- McHugh, E. M., and R. McGee, Jr. (1986) Direct anesthetic-like effects of forskolin on the nicotinic acetylcholine receptors of PC12 cells. *J. Biol. Chem.* 261: 3103–3106.
- Middleton, P., F. Jaramillo, and S. M. Schuetze (1986) Forskolin increases the rate of acetylcholine receptor desensitization at rat soleus endplates. *Proc. Natl. Acad. Sci. USA* 83: 4967–4971.
- Miles, K., D. T. Anthony, L. L. Rubin, P. Greengard, and R. L. Huganir (1987) Regulation of nicotinic acetylcholine receptor phosphorylation in rat myotubes by forskolin and cAMP. *Proc. Natl. Acad. Sci. USA* 84: 6591–6595.
- Ogden, D. C., and D. Colquhoun (1985) Ion channel block by acetylcholine, carbachol and suberyldicholine at the frog neuromuscular junction. *Proc. R. Soc. London [Biol.]* 225: 329–355.
- Rubin, L. L. (1985) Increases in Ca^{2+} mediate changes in acetylcholinesterase and acetylcholine receptors caused by muscle contraction. *Proc. Natl. Acad. Sci. USA* 82: 7121–7125.
- Sakmann, B., J. Patlak, and E. Neher (1980) Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature* 286: 71–73.
- Seamon, K. B., W. Padgett, and J. W. Daly (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. USA* 78: 3363–3367.
- Seamon, K. B., R. Vaillancourt, M. Edwards, and J. W. Daly (1984) Binding of [3 H]forskolin to rat brain membranes. *Proc. Natl. Acad. Sci. USA* 81: 5081–5085.
- Snedecor, G. W., and W. G. Cochran (1967) *Statistical Methods*, Iowa State University Press, Ames, Iowa.
- Strong, J. A., A. P. Fox, R. W. Tsien, and L. K. Kaczmarek (1987) Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* 325: 714–717.
- Teichberg, V. I., A. Sobel, and J.-P. Changeux (1977) In vitro phosphorylation of the acetylcholine receptor. *Nature* 267: 540–542.