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ACCELERATED COMMUNICATION

Forskolin Alters Acetylcholine Receptor Gating by a Mechanism Independent of Adenylate Cyclase Activation

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SUMMARY

The diterpene forskolin activates adenylate cyclase in a receptor-independent fashion and is commonly used to obtain a rapid elevation of intracellular cAMP levels. Application of 10–20 μM forskolin to *Xenopus* oocytes that express *Torpedo* nicotinic acetylcholine (ACh) receptors leads to an acceleration in the decay of ACh-elicited currents, which could be taken as evidence for modulation of ACh receptor gating by cAMP-dependent protein kinase. However, the effect is not mimicked by phosphodiesterase inhibitors or intracellular injection of a cAMP analog. In addition, 1,9-dideoxyforskolin, which is unable to activate adenylate cyclase, has a similar effect. Finally, the action of forskolin is rapidly reversible, with full onset and recovery occurring within the exchange time of the recording chamber. These results suggest that forskolin is a potent local anesthetic and that this property of this widely used compound must be taken into account when using it to study ion channel modulation.

Second messenger-mediated protein phosphorylation is a common mechanism for short term modification of many cellular processes (1). In addition to the alteration of the activity of many enzymes, a variety of ion channels are thought to be modulated by phosphorylation/dephosphorylation (2), among them cardiac Ca^{2+} channels (3, 4) and a K^+ channel from *Aplysia* sensory neurons (5). In all but one case, it has been inferred, but not proven, that the channel itself is phosphorylated, rather than some associated protein. Recently, Huganir *et al.* (6) demonstrated that nicotinic AChRs isolated from *Torpedo* electroplax and phosphorylated *in vitro* by cAMP-dependent protein kinase desensitize more rapidly in a reconstituted ion flux assay than do nonphosphorylated receptors.

Forskolin, a diterpene isolated from the roots of *Coleus forskohii*, activates adenylate cyclase in a receptor-independent fashion (7) and has become a common tool for rapidly elevating intracellular cAMP levels in a variety of cell types. It is generally assumed that alterations in cellular functions caused by micromolar concentrations of forskolin are due to the elevation of cAMP levels and the subsequent activation of cAMP-dependent protein kinase. Recently, Albuquerque *et al.* (8) and Middleton *et al.* (9) reported that forskolin increases the rate of AChR desensitization in rat soleus and frog sartorious muscle, and both groups concluded that an elevation in intracellular cAMP was responsible for this phenomenon, as expected from the work of Huganir *et al.* (6). In this report, I show that micromolar concentrations of forskolin alter the gating properties of *Torpedo* AChRs expressed in *Xenopus* oocytes by a mechanism independent of activation of adenylate cyclase activity, most likely by acting as a local anesthetic.

Materials and Methods

Torpedo californica AChRs were expressed in *Xenopus* oocytes by microinjection of mRNAs for all four receptor subunits, prepared by *in vitro* transcription as described (10). Currents elicited by bath application of 5 μM ACh were measured using a standard two-microelectrode voltage clamp (Axoclamp 2A, Axon Instruments, Burlingame, CA). Electrodes were filled with 3 M KCl and had resistances of 0.5–2 MΩ. The recording chamber was continually perfused with saline (96 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/ 1 mM $MgCl_2$/ 5 mM HEPES, pH 7.6/ 0.3 μM atropine) containing the appropriate drugs. The exchange time of the 0.5-ml chamber was 5 sec. Cells were held at −25 mV, the Cl^- equilibrium potential of oocytes, in order to avoid any contamination by small currents from the endogenous Ca^{2+}-activated Cl^- channels (11). A recovery period of 3 min of perfusion with drug-free saline elapsed between ACh applications, which was sufficient to allow the receptors to recover from desensitization and channel blockade. Currents were recorded on tape using a modified digital audio processor/ videocassette recorder unit (Unitrade, Philadelphia, PA) and then analyzed off-line on a laboratory computer system (Indec Systems, Sunnyvale, CA).

Compounds were injected into oocytes using the positive displacement pipettor used for RNA injections, which allows one to reproduc-

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ABBREVIATIONS: AChR, acetylcholine receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ibly and precisely inject volumes in the 20–200 nl range. In all cases described here, 50 nl of a 10 nM solution of the indicated compound in water were injected into each oocyte. This is equal to 5% of the total volume of the cell, and one can observe a slight swelling of the cell as it is injected. Injection of water alone has no effect on the cell.

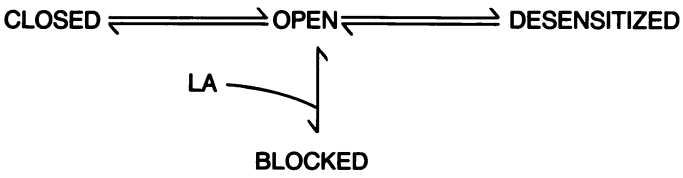
Forskolin was obtained from Calbiochem (La Jolla, CA), and 1,9-dideoxyforskolin was the generous gift of Dr. K. Seamon, National Institutes of Health (this compound is now available from Calbiochem). Both drugs were prepared as 20 mM stock solutions in absolute ethanol. Ethanol at a concentration of 0.1% had no effect on ACh-elicited currents. Synthetic protein kinase inhibitor (12) was the gift of Dr. T. Reisine, University of Pennsylvania (Philadelphia, PA).

Results and Discussion

Injection of *Torpedo* AChR subunit-specific mRNAs into the cytoplasm of immature *Xenopus* oocytes results in the appearance of functional AChRs on the oocyte surface (10, 13). Bath application of 5 μM ACh to a previously injected oocytes voltage-clamped at −25 mV elicits an inward current, due to activation of the *Torpedo* AChRs (Fig. 1A). In the prolonged presence of agonist, the initial increase in current is followed by a biphasic decay characteristic of receptor desensitization. When 10 μM forskolin is present in the perfusion solution, the peak current amplitude is decreased to 57.4 ± 4.1% of the control value (mean ± standard error of eight determinations), the rate of current decay is increased, and the final current level before ACh washout is decreased (Fig. 1B). Raising the forskolin concentration to 20 μM increases all three of these effects (Fig. 1C). Application of 0.1% ethanol has no effect on the ACh-elicited currents (data not shown).

The apparent increase in both the rate and extent of desensitization after forskolin treatment might be taken as evidence for cAMP-dependent protein kinase-mediated modulation of AChR gating kinetics. However, there are several aspects of this effect that suggest that this may not be the case. First, the effect is not mimicked or potentiated by a 1-hr preincubation with phosphodiesterase inhibitors [10 mM theophylline (four trials) or 1 mM isobutylmethylxanthine (three trials)] or injection of 500 pmol of 8-(4-chlorophenylthio)-cAMP, a phosphodiesterase-resistant cAMP analog that is a better activator of cAMP-dependent protein kinase than is cAMP (14) (four trials; data not shown). Second, preincubation with forskolin is not necessary to observe the effect; the full effect is observed immediately after the addition of forskolin. Finally, the effect reverses rapidly, with full recovery seen immediately after washout of the compound (six trials). This rapid reversibility is demonstrated in Fig. 2, which shows ACh elicited by a 2-min application of 5 μM ACh, with a 30-sec pulse of a solution containing 5 μM ACh and 10 μM forskolin, 15 sec after ACh addition. The effect of forskolin appears and disappears with the exchange time of the perfusion chamber (5 sec). This time course is not consistent with the complex biochemical cycle of adenylate cyclase activation, cAMP level elevation, and receptor phosphorylation and dephosphorylation. In addition, 1,9-dideoxyforskolin, which does not activate adenylate cyclase (15), also reduces and alters the time course of the ACh-elicited currents (five trials; Fig. 3). Note that although the acceleration of the current decay in the presence of 1,9-dideoxyforskolin is less than that caused by an equivalent concentration of forskolin, the current at the end of the ACh application is actually lower than in the presence of forskolin. This presumably reflects a difference in the kinetics of the blocking reaction of the two drugs. Finally, the effect of forskolin is not blocked by injection of 500 pmol of synthetic protein kinase inhibitor (12) (three experiments; data not shown).

The effect of forskolin on AChR gating is suggestive of the action of local anesthetics, such as lidocaine, which block the open channel (16). A simplified scheme for local anesthetic action is shown below:



where all of the closed states of the receptor are lumped into a single *CLOSED* state, and *LA* is the local anesthetic. If *LA* is present, once a channel opens it can be blocked by the *LA*, and the rate of blockade increases as the *LA* concentration increases, as is seen in Fig. 1. Because the *LA* binds to the *OPEN* state of the channel and drives it into the *BLOCKED* state, the channels are prevented from going into the *DESENSITIZED* state. This is seen in Fig. 2, where the current immediately after the forskolin washout are larger than those elicited without the forskolin pulse, because the blocked channels were prevented from desensitizing by forskolin.

Several other groups have reported that forskolin has effects on channel gating that are unrelated to adenylate cyclase activation. Coombs and Thompson (17) showed that the effect of forskolin on transient K^+ currents in nudibranch neurons was not reproduced by intracellular injection of cAMP and suggested that forskolin directly blocked the channel. However, they were not able to detect either voltage-dependent or use-dependent block by forskolin. In addition, it took up to 30 min for the forskolin effect to appear. Krause *et al.* (18) demonstrated that the effects of forskolin on voltage-dependent K^+ currents in lymphocytes were mimicked by 1,9-dideoxyforskolin. McHugh and McGee (19) studied the inhibition of car-

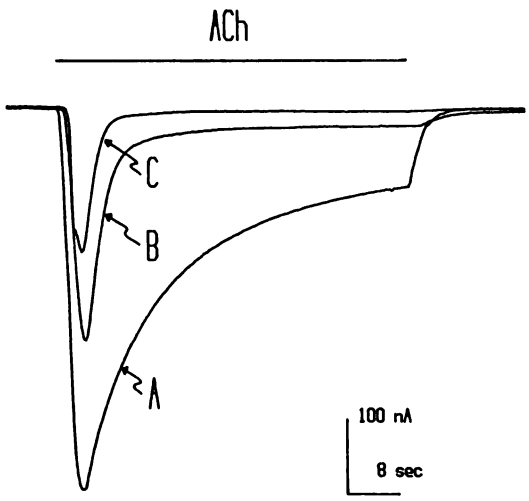


Fig. 1. Forskolin accelerates the decay of ACh-elicited currents. Oocytes were voltage clamped 48 hr after microinjection with *Torpedo* AChR mRNAs as described in the text. The response to a 1-min bath application of 5 μM ACh was measured in the absence of forskolin (A) or in the continued presence of either 10 μM (B) or 20 μM (C) forskolin. Forskolin was added to the bathing solution 10 sec before the application of ACh, and the ACh solutions contained the indicated concentration of forskolin. Note the decrease in the evoked current and the increase in the rate of decay in the presence of forskolin.

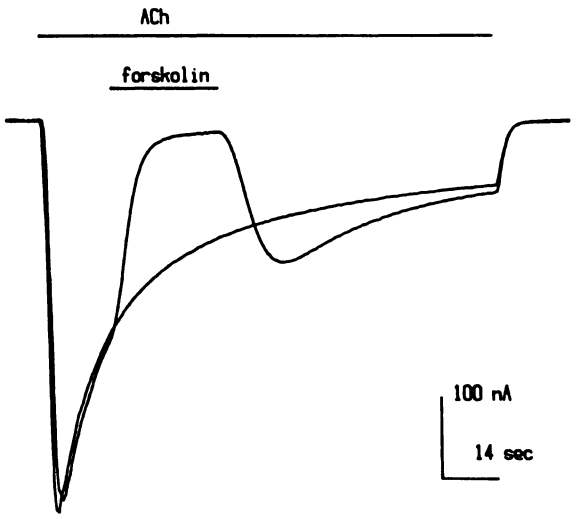


Fig. 2. Forskolin blocks AChR channels rapidly and reversibly. A control current was elicited by a 2-min application of 5 μM ACh (the *trace* with the smooth decay). After the 3-min recovery period, ACh was applied again, and 15 sec after the initiation of the ACh perfusion, a solution containing 5 μM ACh and 10 μM forskolin was applied for 30 sec, after which the chamber was perfused with forskolin-free 5 μM ACh for the remaining 75 sec. Note the rapid alteration in current level as forskolin washes in and out of the chamber and note that the current after forskolin washout is greater than that in the absence of forskolin treatment at the equivalent time.

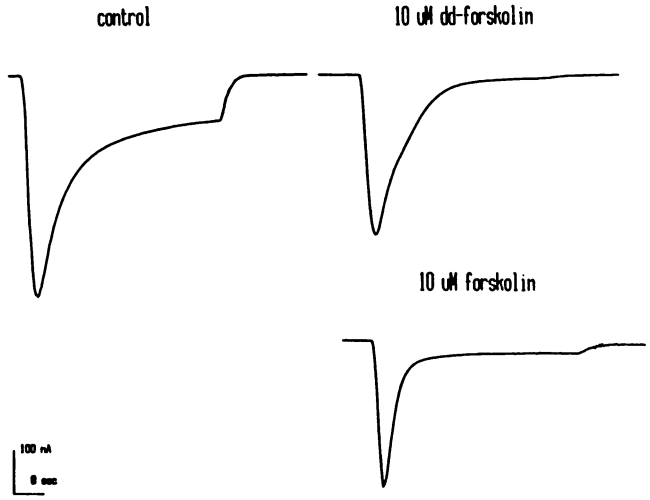


Fig. 3. The effect of forskolin is mimicked by 1,9-dideoxyforskolin. Currents were elicited by a 1-min application of 5 μM ACh in the absence of forskolin (*control*) or in the presence of 10 μM forskolin or 10 μM 1,9-dideoxyforskolin. Note that the initial current is depressed by the presence of the two compounds and that the currents decay to a final level much lower than that of the control. The difference in current waveform in the presence of the two diterpenes most likely reflects a difference in blocking kinetics.

bachol-stimulated uptake of $^{86}Rb^+$ through neuronal AChRs in PC12 cells (which are biochemically different from the muscle-type AChRs studied here) by forskolin and demonstrated that the effect was unrelated to adenylate cyclase activation. These authors suggested that forskolin exhibited "anesthetic-like" effects on the neuronal AChR.

What of the reports by Albuquerque *et al.* (8) and Middleton *et al.* (9) concerning the effect of forskolin on endplate AChRs, which are biochemically, pharmacologically, and electrophysiologically similar to the *Torpedo* receptor studied here? Middleton *et al.* (9) did not use dideoxyforskolin as a control and mentioned that dibutyryl cAMP was without effect, which would suggest that the effect of forskolin was unrelated to cAMP accumulation. However, they did show that the effect was potentiated by phosphodiesterase inhibitors, which does indicate that at least some of the effect was due to activation of adenylate cyclase. The authors did detect a slow open-channel block by forskolin, but this effect was only detectable at 100 μM forskolin, whereas the acceleration of endplate current decay was seen at 10–20 μM forskolin. Albuquerque *et al.* (8) used 1 μM forskolin in their study and did not find any effect of 1,9-dideoxyforskolin at this concentration. The data presented in Fig. 3 suggest that this compound shows slower blocking kinetics than forskolin, which may explain their finding. These authors reported that forskolin up to 100 μM had no effect on single-channel conductance or kinetics, which is curious, because one would expect that a compound that increased the rate of desensitization should alter the gating at the single-channel level as well. One might reconcile the clear local anesthetic actions of forskolin on the *Torpedo* receptor with the findings of these two groups by assuming that, although the receptors are very similar to each other, the channel pores are different enough to make the *Torpedo* receptor very sensitive to blockade by forskolin, whereas the others are not as sensitive.

The results presented here demonstrate quite clearly that forskolin can act as a local anesthetic in at least one type of modulatable ion channel. In the absence of any supporting evidence implicating elevation of cAMP levels, such as potentiation by phosphodiesterase inhibitors or mimickry by membrane-permeant derivatives of cAMP, the possibility that any alteration in channel gating kinetics, such that the currents are reduced and/or decay more quickly in the presence of forskolin, is due to the local anesthetic activity of this compound must be seriously considered.

Acknowledgments

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