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Forskolin Acts as a Noncompetitive Inhibitor of Nicotinic Acetylcholine Receptors

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SUMMARY

Bath application of micromolar concentrations of forskolin to *Xenopus* oocytes that express either *Torpedo* electroplax or mouse muscle nicotinic acetylcholine (ACh) receptors leads to a reduction in the size of the ACh-elicited currents. This inhibition is concentration dependent and rapidly reversible, with full onset and recovery occurring within the exchange time of the recording chamber. *Torpedo* and mouse ACh receptors exhibit differential sensitivity to forskolin, with the *Torpedo* receptor showing higher affinity than the mouse receptor, with K_i values of 6.5 μM and 22 μM, respectively. The affinity for forskolin increases with ACh concentration, which rules out the possibility that forskolin acts as a competitive inhibitor. Single-channel analysis using excised patches shows that forskolin has no effect on either the single-channel amplitude or mean open time but, instead, reduces the number of channel openings per unit time, suggesting that forskolin either is a very slow channel blocker or alters receptor gating such that a fraction of the channels enter a state from which they are no longer available to open. Finally, through the use of a series of mouse-*Torpedo* hybrid ACh receptors, it is shown that the structural features responsible for the observed species difference in the affinity of ACh receptors for forskolin, and thus at least part of the binding site, are located on the γ subunit.

The diterpene forskolin activates adenylate cyclase in a receptor-independent fashion (1) and is commonly used to elevate intracellular cAMP levels in a wide variety of cells. It is generally assumed that any effects that are observed after forskolin treatment are due to this elevation in cAMP levels and the subsequent actions of cAMP itself. However, over the past few years a growing body of literature has demonstrated that forskolin itself can have direct effects that are independent of its activation of adenylate cyclase. For example, Sergeant and Kim (2) have shown that forskolin inhibits glucose transport in human erythrocytes in a cAMP-independent manner. In addition, forskolin produces a rapid decay in the normally noninactivating K_Z K^+ currents in PC12 cells, and this effect is due to channel blockade by forskolin (3, 4). Finally, several groups have reported that forskolin exerts cAMP-independent effects on the nicotinic AChR channel (5-7). In the case of the *Torpedo* AChR expressed in *Xenopus* oocytes, we showed that the acceleration in the decay of ACh-elicited currents by forskolin was independent of adenylate cyclase activation, and we suggested that forskolin acted like a local anesthetic, i.e., a channel blocker.

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In this report, we have further characterized the effects of forskolin on AChRs and suggest that forskolin does not act at the local anesthetic biding site but, rather, acts at some other site on the receptor. In addition, we provide some information concerning the site of interaction of this compound on the AChR complex.

Materials and Methods

Expression of AChRs in *Xenopus* oocytes. The mouse muscle AChR subunit cDNAs were obtained from J. P. Merlie (Washington University) (α subunit), N. Davidson (California Institute of Technology) (β and δ subunits), and S. Heinemann (Salk Institute) (γ subunit), and the *Torpedo* AChR subunit cDNAs were from T. Claudio (Yale University). The plasmids were linearized with the appropriate restriction enzyme and transcribed *in vitro* using SP6 RNA polymerase, as previously described (8). The bulk of the experiments were carried out using the mouse muscle instead of the *Torpedo* receptor, because the greater single-channel mean open time of the mouse receptor (approximately 10-20 times longer than for the *Torpedo* single channels) (9, 10) allows for better quality single-channel recordings. The appropriate mixture of AChR subunit mRNAs (10-15 ng, in a molar stoichiometry of $2\alpha:\beta:\gamma:\delta$, in 50 nl of water) was injected into the cytoplasm of immature, follicle cell-free, *Xenopus* oocytes. Injected oocytes were maintained at 19°, in SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1

ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NCI, noncompetitive inhibitor.

mM $MgCl_2$, 5 mM HEPES, pH 7.6) supplemented with 50 μg/ml gentamicin and 2.5 mM sodium pyruvate, for 18–72 hr before recording.

Electrophysiology. Macroscopic currents elicited by bath application of ACh were measured at a holding potential of −70 mV, using a standard two-electrode voltage-clamp [Axoclamp 2A (Axon Instruments, Foster City, CA) or OC-725 (Warner Instruments, Hamden, CT)]. Electrodes were filled with 3 M KCl and had resistances of 0.5–3 MΩ. The recording chamber was continuously perfused with saline with a low [Ca^{2+}] (SOS with 0.3 mM $CaCl_2$ and 2.5 mM $MgCl_2$), containing the desired concentrations of ACh and forskolin. Low [Ca^{2+}] solutions were used to reduce receptor desensitization, which requires extracellular Ca^{2+} (11). The data were recorded on a chart recorder and with a digitzer and a VCR (VR-10A; Instrutech Corp., Elmont, NY).

Single-channel records were obtained using an Axopatch 1B patch-clamp (Axon Instruments), and outside-out patches were obtained using the method described by Methfessel *et al.* (12). Patch pipettes were filled with 80 mM KF, 20 mM KCl, 10 mM K-EGTA, 10 mM HEPES, pH 7.6, and the bathing solution was 100 mM KCl, 10 mM K-EGTA, 10 mM HEPES, pH 7.6. The data were recorded on magnetic tape, using a digitizer-VCR combination, and were analyzed off-line, using an Atari-based data acquisition and analysis system (Instrutech). In general, the data were filtered at 1.5 or 2.5 kHz (−3-dB frequency) and sampled at 11.8 or 23.8 kHz, respectively.

Data analysis. The macroscopic inhibition curves were fit to a single-site inhibition curve by using a Levenberg-Marquart routine in a commercially available Apple Macintosh program (IGOR; WaveMetrics, Lake Oswego, OR):

$$\theta = (1 + ([F]/K_i))^{-1} \quad (1)$$

where θ is the fractional current remaining in the presence of forskolin, F, and K_i is the inhibition constant. Single-channel amplitude and dwell-time histograms were constructed and analyzed using a commercially available software package running on an Atari computer (TAC; Instrutech).

Results

Bath application of ACh to voltage-clamped *Xenopus* oocytes expressing mouse muscle ACh receptors elicits an inward current carried by monovalent cations. In low [Ca^{2+}] saline, low ACh concentrations give rise to maintained currents that show little, if any, desensitization. Under these conditions, if the perfusate is changed from saline containing 1 μM ACh to one containing the same concentration of ACh and 50 μM forskolin, the current is depressed and, if the perfusate is changed back to the forskolin-free ACh-containing solution, the current returns to its original level (Fig. 1A). The effect of forskolin is dose dependent and is well described by a single-site inhibition model (eq. 1), with a K_i of 22 ± 3.1 μM (Fig. 1B). As demonstrated previously with *Torpedo* AChRs (7), the time course of the depression and recovery is within the exchange time of the perfusion apparatus and reflects a direct effect of forskolin on the receptor.

Forskolin could cause a reduction in ACh-elicited currents by acting at one of several different sites. First, it could act at the ligand binding site and act as a competitive antagonist, such as *d*-tubocurarine. Second, it could act as a local anesthetic, such as lidocaine or QX-222, and directly block the channel. Third, it could alter the gating of the channel by altering the rates of one or more transitions between various states of the channel (i.e., acting as a nonblocking NCI). We can distinguish between the first and the other two mechanisms by examining the effect of forskolin at several different ACh concentrations. If forskolin were acting as a competitive antagonist, then its effect at a given concentration should be diminished as the ACh concentration is raised. The value of K_i determined at a given concentration of ACh will depend on both the intrinsic affinity of forskolin for the binding site ($K_{i_{true}}$) and the ratio of the concentration of ACh to its binding affinity, K_{ACh}:

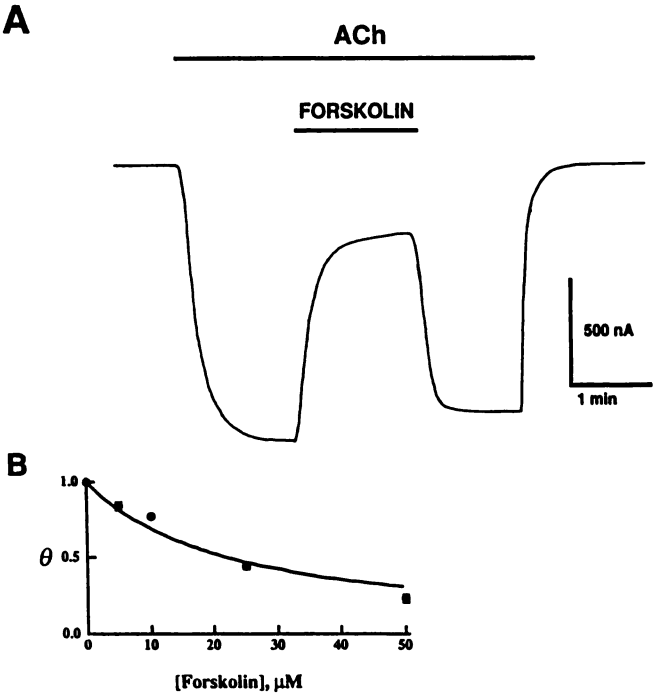

Fig. 1. Forskolin depresses ACh-elicited currents in a reversible, dose-dependent manner. A, AChR channels were opened by a 3.5-min bath application of 1 μM ACh to a voltage-clamped oocyte expressing mouse muscle AChRs. During the period of ACh application, the perfusate was changed to one containing 1 μM ACh plus 50 μM forskolin, for 1 min, and the fractional amount of current remaining, θ, was determined. In this particular case, $\theta = 0.31$. B, The concentration dependence of the current depression by forskolin is shown. Each *point* represents the mean ± standard error of five determinations, with all concentrations of forskolin being tested on each oocyte. The *solid curve* is a fit to the data using eq. 1, with K_i = 22 μM.

$$K_i = K_{i_{true}} (1 + ([ACh]/K_{ACh})) \quad (2)$$

In this case, K_i should increase (i.e., affinity decrease) as [ACh] is increased. In the case of the other two possibilities, one would expect either no change or a decrease in K_i as the ACh concentration is raised (13). Fig. 2 shows the effect of varying the ACh concentration on the measured value of θ at a fixed concentration (25 μM) of forskolin, which should be sensitive to changes in K_i. θ shows a dependence on ACh but, in contrast to the prediction of the competitive antagonist model, θ actually decreases as the ACh concentration is increased. This result strongly suggests that forskolin does not act as a competitive antagonist but, rather, acts at some other site on the receptor and interacts preferentially with the liganded, rather than unliganded, form of the receptor.

Aldrich and co-workers (3, 4) have shown that forskolin acts as a blocker of K_Z K^+ channels in PC12 cells. Although direct blockade is difficult to prove using macroscopic measurements, it should be readily detected at the single-channel level. Channel blockers can be considered to fall into three kinetic categories, fast, intermediate, and slow, each of which should give a distinctive behavior in single-channel recordings. If the bind-

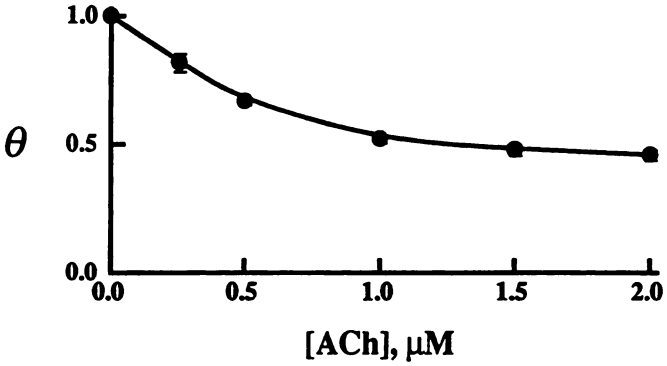

Fig. 2. Forskolin is not a competitive inhibitor. The effect of 25 μM forskolin on ACh-elicited currents was determined at several concentrations of ACh, and the fractional current θ is plotted as a function of the ACh concentration used. Each *point* represents the mean ± standard error of three to five determinations. Note that θ goes down as [ACh] goes up, in contrast to the expected behavior of a competitive antagonist.

ing/unbinding of the blocker is fast relative to the frequency response of the recording system, the observed single-channel current is the time average of the blocked and unblocked open channel, and the apparent single-channel current is reduced. If the kinetics of the blocker binding/unbinding reaction are on the same time scale as the channel opening kinetics, discrete interruptions ("flickers") of the current flowing through an open channel as the blocker resides in the channel are observed. Finally, if the blocking kinetics are extremely slow with respect to the channel gating kinetics, then flickering is not observed but long block durations are seen, and the mean open time may be either reduced or unchanged, depending on the values of the blocking rate constant relative to the gating rate constants. Fig. 3A shows single-channel records of mouse AChR channels recorded at −150 mV from an outside-out patch, in the presence of 1 μM ACh, before and after addition of 10 μM forskolin to the perfusate. The single-channel current in the presence and absence of forskolin is 14.1 and 14.0 pA, respectively, whereas the channel mean open time is 1.87 and 1.92 msec, respectively. Fig. 3B shows the ratios of single-channel current, mean open time, and Np_o, in the presence and absence of forskolin. The only parameter altered by forskolin is Np_o, which is reduced to about 45% of the control level. The lack of an effect on the single-channel amplitude eliminates the possibility that forskolin acts as a fast blocker, whereas the absence of flickering eliminates intermediate time scale blocking. What does appear to change, however, is the level of channel activity in the patch. This type of behavior would be expected if the rate constants for forskolin blockade were very slow with respect to channel opening. On the other hand, a similar result would be obtained if forskolin was not a channel blocker but, instead, either directly affected the gating of the channel or created a subpopulation of channels that could no longer be opened by ACh. "Classical" channel-blocking local anesthetics that contain a positive charge, such as procaine, exhibit voltage-dependent block (13), which is a reflection of the fact that the blocker must traverse a portion of the transmembrane field to reach its binding site in the channel. In the case of local anesthetics and the AChR, this binding site is 70–80% of the transmembrane field from the extracellular side. The tertiary amine-containing forskolin derivative 7β-desacetyl-7β-[γ-(*N*-methylpiperazino)-butyryl]forskolin (14) (which should exist predominantly in the charged form at pH 7.6), also inhibits AChRs expressed in

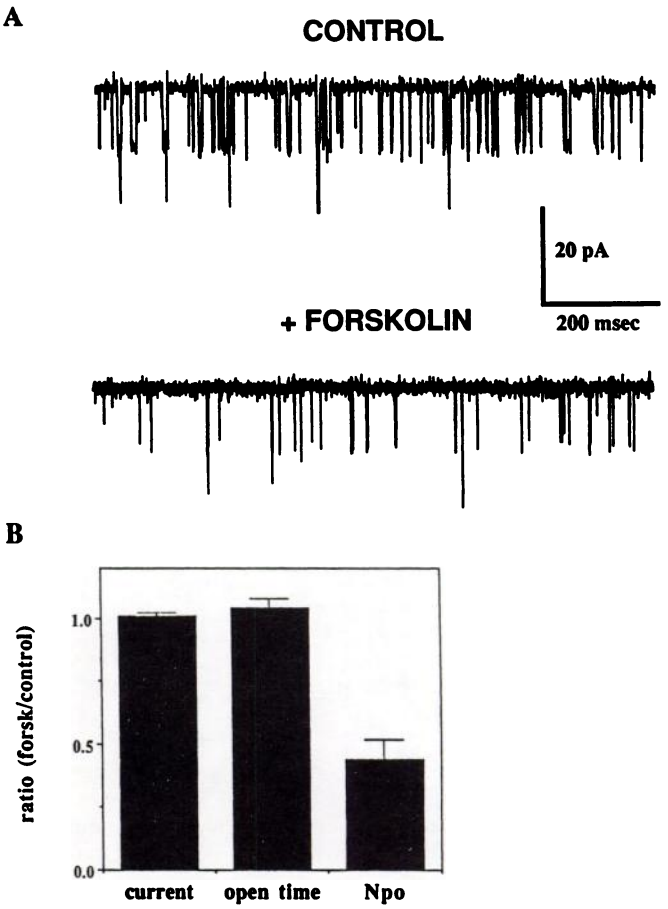

Fig. 3. Forskolin does not act as a channel blocker. A, Representative single-channel recordings from an outside-out patch at −150 mV in the presence of 1 μM ACh (*CONTROL*) and after switching to a perfusate containing 1 μM ACh and 10 μM forskolin (*+FORSKOLIN*). Note that, although the channel activity seems to be decreased by forskolin, the openings themselves in the presence of forskolin do not appear to be markedly different from those in the absence of forskolin. B, Single-channel conductance, mean open time, and Np_o in the absence and presence of forskolin, were determined for four different patches, from at least 1000 transitions, patch, and the ratio of the value of each parameter in the presence of forskolin to that in its absence is shown. Note that the only property affected by forskolin is Np_o.

Xenopus oocytes; however, we have been unable to detect any voltage dependence to this inhibition (data not shown). Therefore, if forskolin is acting as a channel blocker, its binding site is different from the normal local anesthetic binding site.

The data presented in Figs. 2 and 3 strongly suggest that forskolin exerts its effect on AChRs at a site other than the ligand binding site or the local anesthetic binding site in the ion channel. As a first step towards the localization of this site, we have taken advantage of the fact that forskolin has different affinities for the mouse and *Torpedo* AChRs. Fig. 4 shows the concentration dependence of the inhibition by forskolin for both mouse and *Torpedo* AChRs. The *Torpedo* receptor is clearly more sensitive to forskolin (K_i = 6.5 μM versus 22 μM for the mouse receptor). If the difference in forskolin affinities is due to a discrete structural difference between the subunits that contain the binding sites of the two receptors, then the possibility exists that most, if not all, of the "species identity" could be assigned to a particular subunit. Hybrid receptors, in which a particular subunit from one species is replaced by the

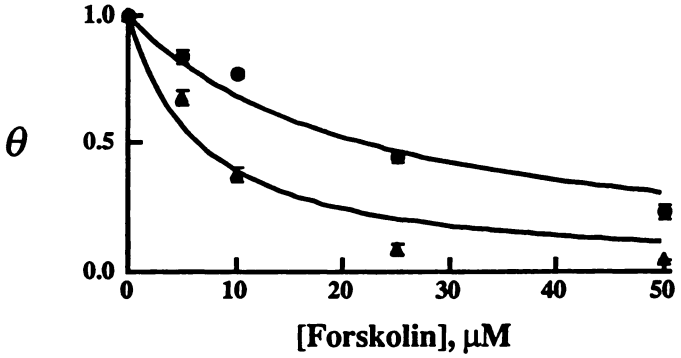

Fig. 4. Mouse and *Torpedo* ACh receptors exhibit differential sensitivity to forskolin. The concentration dependence of the effect of forskolin on either mouse (●) or *Torpedo* (▲) ACh receptors is shown. Each *point* represents the mean ± standard error of three to seven determinations. The *solid curves* are fits to the data using eq. 1, with K_i values of 22 μM and 6.5 μM for the mouse and *Torpedo* receptors, respectively.

TABLE 1

Effect of subunit composition on forskolin affinities

K_i values for forskolin were determined for the various subunit combinations shown here and were normalized to the K_i value for the all-*Torpedo* receptor (TTTT) at the same ACh concentration used to elicit the currents. Each value represents the mean ± standard error of the number of determinations shown in parentheses. The "species" were assigned on the basis of these normalized values; receptors with values of ≤1 were considered to be *Torpedo*-like, whereas those with normalized values of >1 were considered to be mouse-like.

Receptor ($\alpha\beta\gamma\delta$)	Normalized K_i	"Species"
MMMM	4.2 ± 0.1 (9)	Mouse
TMMM	7.0 ± 0.1 (3)	Mouse
MTMM	1.9 ± 0.1 (3)	Mouse
MMTM	1.0 ± 0.1 (4)	*Torpedo*
MMMT	7.5 ± 1.3 (4)	Mouse
TTTT	1.0 (10)	*Torpedo*
MTTT	0.3 ± 0.1 (4)	*Torpedo*
TMTT	0.7 ± 0.1 (2)	*Torpedo*
TTMT	8.4 ± 0.4 (2)	Mouse
TTTM	0.5 ± 0.1 (2)	*Torpedo*

analogous subunit from another species, can be formed in *Xenopus* oocytes by the injection of the appropriate combinations of RNAs (9, 15, 16). We have measured the K_i values for forskolin for a series of receptors in which one subunit has been replaced by the appropriate subunit from the other species. Not all combinations are expressed to the same extent in oocytes (14), so different concentrations of ACh were necessary to obtain currents large enough to carry out the titrations with some of the hybrids. Because K_i shows some dependence on [ACh] (Fig. 2), all values were normalized to that for *Torpedo* receptors at the same ACh concentration used for that particular hybrid.

Table 1 shows the normalized K_i values for the 10 receptors used in this study (all mouse, all *Torpedo*, and the eight single-subunit switches). All of the hybrid receptors are inhibited by forskolin; however, the normalized K_i values range from 0.4 ± 0.2 to 8.4 ± 0.2. If we consider those receptors with normalized K_i of >1 to be "mouse-like" and those with normalized K_i of ≤1 to be "*Torpedo*-like," then it becomes apparent that the major determinant of the "species" (as far as forskolin is concerned) is the γ subunit. For example, the MMMM and TTMT receptors have a low mouse-like affinity for forskolin, whereas TTTT and MMTM receptors have high *Torpedo*-like affinity for forskolin. The reciprocal nature of the γ subunit effect (i.e., the affinity of the MMTM receptor goes up while that of the TTMT receptor goes down) suggests strongly that the a region of the γ subunit forms part of the forskolin binding site.

Discussion

Second messenger-mediated protein phosphorylation is a common mechanism for short term modification of many cellular processes, including electrical excitability (17). In the case of cAMP-mediated modulation, both increases and decreases in channel activity have been observed. For example, treatment of cardiac cells with β-adrenergic agonists leads to an enhancement in voltage-activated Ca^{2+} currents (18, 19), whereas serotonin treatment of *Aplysia* sensory neurons leads to a reduction in K^+ currents (20). Forskolin is commonly used to bypass the receptor step in the process, by directly activating adenylate cyclase. It is generally assumed, in this case, that alterations in channel properties are due to cyclase activation and the subsequent biochemical events. However, there are now many examples of cAMP-independent effects of forskolin on ion channels (3–7, 21–24). This direct effect is usually demonstrated by showing that 1,9-dideoxyforskolin, which is unable to activate adenylate cyclase (25), produces a similar effect, eliminating the possibility that the observed effect is due to an elevation in cAMP levels. In all of these cases, the effect of forskolin is to lead to either a reduction in current or an acceleration of current decay and, in one case, the K_Z K^+ channel from PC12 cells, it has been shown that forskolin acts as a channel blocker (4).

Middleton *et al.* (26, 27) have provided rather convincing evidence that forskolin can increase the rate of AChR desensitization via a cAMP-mediated pathway in rat myotubes, consistent with the finding that the rate of *Torpedo* AChR desensitization is governed by protein phosphorylation (28, 29). In the case of the myotube AChR, the authors reported that, in addition to the cAMP-mediated actions, which showed half-maximal effects at a concentration of 6 μM, a direct effect of forskolin was observed at high concentrations (50–100 μM). We have never observed cAMP-mediated effects of forskolin on AChRs expressed in *Xenopus* oocytes; however, this is not surprising, because in our hands forskolin, even at a concentration of 100 μM, has little effect on cAMP levels in intact *Xenopus* oocytes (30). Therefore, unlike Middleton *et al.*, we are able to study the direct effect of forskolin without interference from any cAMP-mediated effects.

The data presented in this study further characterize the effects of forskolin on AChRs expressed in *Xenopus* oocytes. In a previous report (7), we suggested that effects of forskolin on macroscopic currents were consistent with the notion that forskolin acted as a channel blocker. The data presented here, on the other hand, suggest that forskolin may not be acting as a channel blocker but, rather, alters the gating of the channel so as to put the channels into a state in which they cannot open. These two contradictory conclusions can be reconciled by noting that the channel block hypothesis was suggested on the basis of the observation that a pulse of forskolin prevented channels from going into the desensitized state. As long as forskolin moves the channels into a new kinetic state different from those into which it would normally go during the course of activation and desensitization, whether it be a blocked state or some other state, the same effect should be observed.

Forskolin, therefore, falls into the broad class of agents

known as NCIs, which bind at sites distinct from the ligand binding site. NCIs are thought to act via several different mechanisms, i.e., 1) channel blockade (13, 31), 2) promotion of desensitization (32), and 3) prevention of channel opening (33); a given NCI can exhibit different actions at low and high concentrations (34, 35). In the case of forskolin, our data are consistent with the first and third possibilities. If forskolin acts as a channel blocker, our data suggest that forskolin does not act at the local anesthetic binding site but, rather, acts at some other site in the channel. In the case of the third possibility, forskolin would affect the transition from closed to open channels. Similar behavior has been observed for alkanols (33) and micromolar concentrations of chlopromazine (34). The fact that the apparent affinity of forskolin for the receptor increases as the ACh concentration increases suggests that forskolin preferentially interacts with a liganded form of the receptor. The possibility that forskolin acts by promoting desensitization is unlikely, because the effect both appears and disappears within the time course of the perfusion system (5 sec), even under conditions where desensitization takes many minutes (see Fig. 1A). If this were the underlying mechanism, then it would require forskolin to increase the rates of both entering and leaving the desensitized state manyfold, in order to account for the observed behavior.

We have taken advantage of the fact that the mouse and *Torpedo* receptors show differential sensitivity to forskolin to try to localize the forskolin binding site. The fact that, as far as forskolin is concerned, MMTM receptors behave as *Torpedo* receptors and TTMT receptors behave as mouse receptors, coupled with the finding that none of the other tested combinations showed this single subunit "species definition," suggests that a major part of the forskolin binding site is contained on the γ subunit. Several studies have suggested that the two ligand binding sites are formed at the α-γ and α-δ interfaces (36, 37). When ACh binds to these two sites, a conformational change that results in channel opening takes place. It is conceivable that forskolin bound to the γ subunit could interfere with this change. Further localization of the forskolin binding site should be possible through the use of species-chimeric subunits, as was initially done to localize the ion conduction pathway to the M2 region (38).

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