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16. The rate constant of FSK binding to the open state, k_{on} , may be calculated from the mean open durations obtained before and after application of FSK; $k_{on} = (1/\tau_f - 1/\tau_c)/[FSK]$, where τ_f is the mean open duration with FSK and τ_c is the mean open duration in the absence of FSK. Typically, k_{on} was 1×10^6 to 5×10^6 s⁻¹ M⁻¹, depending to some extent on the filter and sampling rates used to obtain the data. The infrequent openings of K_v channels with FSK present prevented a reliable determination of k_{off} from the mean-blocked time. It is possible, however, to roughly estimate the k_{off} to be on the order of 1 s at depolarized voltages (for example, +20 mV) as the channels were not observed to reopen frequently in the presence of FSK during 304-ms voltage pulses.
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22. We thank P. J. Casey for confirming the inability of our samples of 1,9-dideoxy-FSK to activate adenylate cyclase in plasma membranes from S49 cyc⁻ cells; and D. Baylor, M. P. Nusbaum, and H. Schulman for critical reading of this manuscript. Supported by NIH grants NS23294 and NS07158 and an NIH postdoctoral fellowship (T.H.).

10 February 1988; accepted 13 April 1988

Modulation of Acetylcholine Receptor Desensitization by Forskolin Is Independent of cAMP

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Biochemical and electrophysiological studies suggest that adenosine 3',5'-monophosphate (cAMP)-dependent phosphorylation of the nicotinic acetylcholine receptor channel is functionally significant because it modifies the receptor's rate of desensitization to acetylcholine. In studies that support this conclusion researchers have used forskolin to stimulate cAMP-dependent phosphorylation in intact muscle. It is now shown that although forskolin facilitated desensitization in voltage-clamped rat muscle, this effect was not correlated with the abilities of forskolin and forskolin analogs to activate adenylate cyclase or phosphorylate the receptor. Furthermore, elevation of intracellular cAMP or addition of the catalytic subunit of A-kinase failed to alter desensitization. Therefore, in intact skeletal muscle, cAMP-dependent phosphorylation does not modulate desensitization.

WHEN NICOTINIC ACETYLCHOLINE receptors (AChR) are exposed to cholinergic agonists, they show a decline in sensitivity (desensitization) that persists as long as the agonist is present (1, 2). Although the physiological role of desensitization is uncertain, it might be involved in neural plasticity (3) or in protecting the neuromuscular junction from prolonged depolarization (4). If desensitization is functionally significant, one might expect to find modulatory mechanisms (such as phosphorylation) that could cause relatively long-lasting changes in the process. A modulatory role for receptor phosphorylation is suggested by electrophysiological studies in which desensitization in rat muscle is accelerated by forskolin (FSK) (4, 5), an activator of adenylate cyclase (6) that

stimulates phosphorylation of the δ subunit of AChR in intact rat myotubes (7) or mouse myocytes (8). The apparent functional consequence of this phosphorylation is

suggested by flux measurements from reconstituted *Torpedo* AChR in which cAMP-dependent phosphorylation of the δ and γ subunits results in faster desensitization (9). We show here that FSK modulates desensitization in intact muscle by a mechanism that does not involve activation of adenylate cyclase or cAMP-dependent phosphorylation.

Sustained application of 10 μ M ACh to voltage-clamped rat myoballs evoked an inward current that peaked in 0.2 to 0.4 s and then declined (desensitized) (Fig. 1) with a time course described by the sum of two exponentials (Fig. 2A and Table 1) (2, 9, 10). This ACh-induced current was derived from openings of nicotinic channels, since the currents were abolished by overnight incubation with α -bungarotoxin (1 μ g/ml) (11). After bath application of 20 μ M (Table 1) or 50 μ M (Fig. 1) FSK for 3 min, ACh evoked an inward current that desensitized much more rapidly than control cur-

Table 1. Desensitization kinetics of ACh-evoked currents from control and treated cells. Kinetic constants were derived from exponential fits to the decay phase of the currents. Currents obtained from cells with elevated cAMP were typically unsteady, and satisfactory quantitative fits could not always be obtained. These currents, which included three cells in which cAMP and 90 μ M IBMX were included in the patch electrode and five that had been treated with dibutyryl cAMP, were at least as slow as controls but are excluded from the means below. Abbreviations: τ_f , time constant of the fast component; τ_s , time constant of the slow component; and %A_f, percentage of the extrapolated peak current accounted for by the fast component. Results are presented as means $\pm$ SEM.

Treatment	n	τ_f (s)	τ_s (s)	%A _f
Control	11	2.66 $\pm$ 0.45	23.94 $\pm$ 1.88	56.0 $\pm$ 5.0
FSK				
20 μ M	2	0.67 $\pm$ 0.25	9.97 $\pm$ 3.23	94.8 $\pm$ 1.0
50 μ M	2	0.22 $\pm$ 0.06	1.87 $\pm$ 0.21	91.2 $\pm$ 1.1
1,9-Dideoxy-FSK				
20 μ M	3	0.61 $\pm$ 0.08	19.60 $\pm$ 1.39	94.5 $\pm$ 0.2
50 μ M	2	0.53 $\pm$ 0.02	7.64 $\pm$ 3.30	89.8 $\pm$ 3.1
L858051 (50 μ M)	3	4.32 $\pm$ 1.33	24.04 $\pm$ 5.82	64.5 $\pm$ 11.3
cAMP _i (1 to 4 mM)	3	1.24 $\pm$ 0.32	17.13 $\pm$ 1.40	70.5 $\pm$ 4.7
8-btcAMP (100 μ M)	6	2.92 $\pm$ 1.57	25.66 $\pm$ 14.16	58.2 $\pm$ 9.0
PT (300 ng/ml)	3	2.84 $\pm$ 0.25	19.77 $\pm$ 0.58	44.5 $\pm$ 2.2
CS (100 μ g/ml)	3	1.89 $\pm$ 0.43	17.50 $\pm$ 3.36	63.0 $\pm$ 12.6

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rents (Fig. 1) (12). This effect was characterized by an increase in the rates of decay of both components and an increase in the relative magnitude of the fast component (Fig. 2B and Table 1).

The effects of FSK and its analogs were not coupled to their abilities to activate adenylate cyclase because external application of the membrane-permeable inactive analog 1,9-dideoxy-FSK (13) greatly accel-

erated desensitization (Fig. 1 and Table 1). In contrast, the water-soluble active analog 7-deacetyl-7-(4-methylpiperazino)butyryloxy-FSK (L858051) (14) (included in the patch electrode) had no statistically significant effect on the time course of the ACh-induced currents (Fig. 1 and Table 1).

We tested the effect of cAMP on desensitization by directly manipulating its intracellular concentration. To avoid possible disruption of phosphorylating mechanisms that could occur during whole-cell voltage clamp, intact myoballs were treated with 100 μ M solutions of the membrane-permeable cAMP analog 8-benzylthioadenosine 3',5'-monophosphate (8-btcAMP) for 0.5 to 2 hours (37°C and 5% CO₂) before application of ACh; 8-btcAMP failed to influence the rate of desensitization (Fig. 3 and Table 1). In five additional experiments, treatment with another cAMP analog, dibutyryl cAMP (300 μ M), was also without effect. Middleton *et al.* (5) also failed to obtain an FSK-like effect on desensitization after treatment of rat endplates with dibutyryl cAMP. Similarly, when cAMP [with or without the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX)] was introduced directly into the cell through the patch electrode, the desensitization rates and magnitudes remained unchanged from control (Fig. 3 and Table 1). Attempts to directly affect receptor phosphorylation with the catalytic subunit of cAMP-dependent protein kinase (CS) (in the patch electrode for 10 min before ACh application), to alter the resting level of receptor phosphorylation [alkaline phosphatase (100 μ g/ml) in the patch electrode; $n = 3$], or to disinhibit adenylate cyclase with 24-hour incubations with pertussis toxin (PT) were also ineffective (Table 1).

Our results are consistent with the view that the effects of FSK on receptor desensitization were not the result of a cAMP-dependent phosphorylation. As we obtained no effects with a water-soluble FSK analog, we suggest that FSK (and similarly lipophilic, dideoxy-FSK) interacts with the nicotinic receptor by a mechanism involving hydrophobic moieties on or near the receptor. McHugh and McGee (15) have suggested that FSK inhibits receptor activation in neuronal pheochromocytoma (PC12) cells by altering lipid structure. Direct effects of FSK are also seen on ACh-induced depolarization in rat sympathetic ganglia (16) and on K⁺ currents in neurons (17) and squid axon (18).

Both FSK and 8-btcAMP stimulate phosphorylation of δ and α subunits of nicotinic receptors from rat myotubes (7). Although we performed no measurements of receptor phosphorylation, the conditions of our ex-

Fig. 1. The effects of forskolin and forskolin analogs on ACh-induced inward currents in rat skeletal muscle. Continuous application of 10 μ M ACh (horizontal bar) was by means of a U-tube system (21). The U-tube was placed approximately 200 μ m from the cell. For each experiment a different cell (and cover slip) was used because repeated applications of ACh to the same cell, even 10 to 15 min apart, resulted in progressively faster desensitization kinetics (22). Currents have been scaled to superimpose their peak amplitudes, allowing comparison of desensitization kinetics. Actual peak currents: control, 0.87 nA; L858051, 0.97 nA; FSK, 0.9 nA; and 1,9-dideoxy-FSK, 0.96 nA. All currents were obtained from cells voltage-clamped to -30 mV with the whole-cell configuration of the patch-clamp technique (23). To eliminate any contribution of K⁺ currents to the net current, the reversal potential for K⁺ currents was set to approximately -30 mV by using a 1:3 (external:internal) ratio of K⁺ concentrations. ACh-induced inward currents were initially recorded on FM tape and later digitized (100 ms per point). Rat skeletal muscle was grown on plastic cover slips (24). Although culture conditions produced primarily myotubes of skeletal muscle, spontaneously occurring round myoballs (8 to 15 μ m in diameter), which have electrical properties similar to those of myotubes (24), were present and were used exclusively for this study. Electrode resistances were 2 to 5 megohms, seal resistances 5 to 50 gigohms, and series resistance compensation typically 40 to 50%. All experiments were performed at room temperature. Preparation and use of FSK and FSK analogs are described in (26) and patch-clamp solutions in (25).

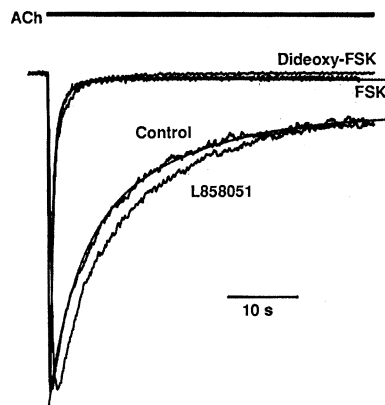


Fig. 2. Semilogarithmic plots of the time course of the decay phase (desensitization) of the ACh-evoked inward currents in the control (A) and FSK-treated (B) cells shown in Fig. 1. The time scales are the same in (A) and (B). The time course of the current decay phase was fitted by the sum of two exponentials with a nonlinear least squares routine. The sum of the two exponentials (solid lines) from this figure are shown superimposed on the actual currents in Fig. 1. The lines drawn through the current samples here and in Fig. 1 demonstrate the goodness of fit that was typically obtained. The time constants in (A) were 2.9 and 17.3 s, with the fast component extrapolating to 49% of the peak current. In (B), the time constants were 0.3 and 1.6 s, with the fast component representing 87% of the extrapolated peak current. The cell in (B) was treated with 50 μ M FSK for 4 min before ACh application, throughout which time the cell was maintained under voltage clamp.

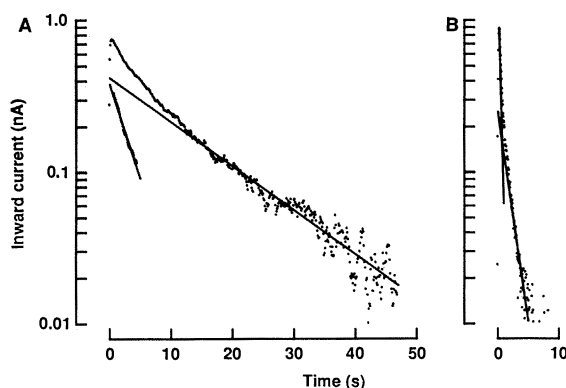
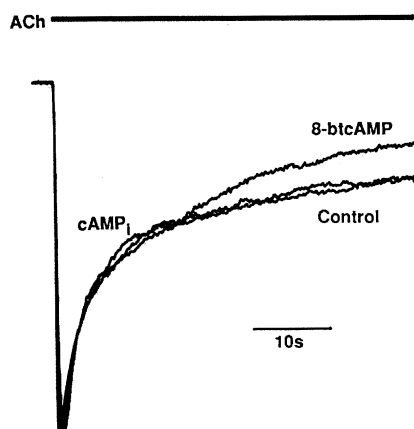


Fig. 3. Effects of elevating internal cAMP on ACh-evoked currents; cAMP_i was elevated by introducing 4 mM cAMP through the patch electrode for 4 min before ACh application, or by treatment for 2 hours with 100 μ M 8-btcAMP. All currents are scaled to the same peak. Actual peak currents: control, 2.9 nA; cAMP_i, 1.5 nA; and 8-btcAMP, 1.3 nA. IBMX, cAMP, dibutyryl cAMP, CS, and alkaline phosphatase were obtained from Sigma; 8-btcAMP was obtained from ICN, and PT from List Biological Laboratories.



periments (8-btcAMP, in particular) were similar or identical to those in which phosphorylation in rat myotubes is demonstrated (7). Our results would therefore imply that in contrast to nicotinic receptors from *Torpedo* (9), the extent of receptor phosphorylation in rat myoballs did not influence the time course of desensitization. One explanation for this difference might be that cAMP-dependent phosphorylation of the γ subunit, which occurs in *Torpedo* but not in rat myotubes, accounts for the acceleration of desensitization observed with *Torpedo* receptors. Because the proposed site of γ subunit phosphorylation by cAMP-dependent protein kinase is missing in most other species (19), our results may not be surprising (20).

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12. The concentrations of FSK used here are similar to those that accelerate desensitization at rat soleus endplates (5) or cause substantial phosphorylation in rat myotubes (7). In contrast, FSK (but not dideoxy-FSK) has also been reported to have similar desensitization effects (also at rat soleus endplates) after a 30-min exposure to concentrations $\leq 1 \mu M$ (4). In myotubes, 1-hour treatments with such low concentrations of FSK do not effect appreciable phosphorylation (7).
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25. The intracellular (pipette) solution contained 127 mM sodium aspartate, 30 mM KCl, 1 mM MgCl₂, 2 mM magnesium adenosine triphosphate, 10 mM Hepes, and 10 mM glucose; pH 7.4; osmolality, 300 to 308 mosM. The extracellular solution contained 127 mM NaCl, 10 mM KCl, 500 nM tetrodotoxin, 10 mM Hepes, and 10 mM glucose; pH 7.4; osmolality 285 to 300 mosM. The extracellular solution also contained 0 to 5 mM CaCl₂ and 1 to 4 mM MgCl₂. In control experiments, varying the external CaCl₂ and MgCl₂ concentrations had no effect on the rate of ACh current desensitization.
26. Forskolin and 1,9-dideoxy-FSK (Calbiochem) were maintained as stock solutions in dimethylsulfoxide

(DMSO). At the concentrations that resulted after final dilution (0.25% or less), the solvent had essentially no effect on the time course of desensitization ($n = 2$). Because of its lipophilicity, 1,9-dideoxy-FSK showed a marked tendency to crystallize out of DMSO upon dilution. To maintain 1,9-dideoxy-FSK in solution, heated extracellular solution was added to the stock dropwise while vortexing. L858051 (Calbiochem) was added directly to the intracellular solution (25).

27. We thank R. Dingledine, G. Oxford, and T. K. Harden for comments on the manuscript, and G. Scarborough for helpful discussions. Supported by NIH grant GM32211 and the University of North Carolina Research Council.

10 February 1988; accepted 14 April 1988

Probing the Mechanisms of Macromolecular Recognition: The Cytochrome b₅-Cytochrome c Complex

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The specificity of complex formation between cytochrome b₅ (cyt b₅) and cytochrome c (cyt c) is believed to involve the formation of salt linkages between specific carboxylic acid residues of cyt b₅ with lysine residues on cyt c. Site-directed mutagenesis was used to alter the specified acidic residues of cyt b₅ to the corresponding amide analogues, which resulted in a lower affinity for complex formation with cyt c. The dissociation of the complex under high pressure resulted in specific volume changes, the magnitude of which reflected the degree of solvation of the acidic residues in the proposed protein-protein interface.

ALTHOUGH MUCH EFFORT HAS BEEN expended on the mechanisms of protein-nucleic acid recognition and multisubunit interactions (1), considerably less insight is available into the details surrounding the establishment of specific heterologous protein-protein complexes. Perhaps the most widely studied model system is a complex between mammalian cyt b₅ and cyt c (2-7). Cytochrome b₅ is a 17,000-dalton heme protein involved in the transfer of reducing equivalents to a variety of physiological acceptors. In the hepatic endoplasmic reticulum, cyt b₅ serves as the electron donor to the fatty acid desaturase complex and to cytochrome P-450 (8-14). A similar cyt b₅ lacking a 35-amino acid membrane-anchor domain acts as a soluble reductant of

hemoglobin in erythrocytes (15, 16). Cytochrome c serves as a carrier in the mitochondrial electron transfer system and, although it does not normally meet cyt b₅ physiologically, it can serve as a facile in vivo acceptor. Direct evidence for the formation of a stoichiometric complex between these two proteins was obtained by Mauk et al. (3) and by La Mar (17). Kinetic documentation of electron transfer from the heme of cyt b₅ ($E_0 = +6$ mV) to that of cyt c ($E_0 = +260$ mV) in a diprotein complex was observed by McLendon et al. (4).

With the use of a high-resolution three-dimensional structure of both cyt b₅ and cyt

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Table 1. Proposed surface charge interactions in cyt b₅ with several redox proteins at pH 7 and pH 8. Abbreviations: cyt c, horse heart cyt c; Hb, β_2 subunit of horse methemoglobin; and Myo, bovine heart myoglobin.

pH 7				pH 8	
Cyt b ₅	Cyt c	Hb*	Myo†	Cyt b ₅	Cyt c
E48	K13	K59		E48	K72
E44	K27	K61	K50	E13‡	K79
E43	K25	K65	K47		
D60	K72	K95	K98	D60	K87
Heme-COO-	K79	K66		E56	K86

*Proposed docking based on computer simulation (19). †Docking based on computer simulation and ¹H nuclear magnetic resonance studies (20). ‡In the modeling studies bovine cyt b₅ was used. All of the residues referred to above in the bovine cyt b₅ are conserved in our rat liver sequence (with the exception of Glu¹³, which is Gln¹³ in the rat liver sequence).