regularity.

Some amino acid residues, notably Ala, show relatively smooth and favorable distributions along the helix but are rarer at the caps. Ala has visible but slight preferences for outside over inside and middle over ends. Several of the amino acid residues that form side-chain H bonds (Ser, Thr, and Gln) show a smoothly increasing occurrence for C3, C2, and C1 but drop abruptly at the C-cap.

Preferences are relatively weak for the positions outside the caps, presumably because those residues have a variety of other roles and constraints. The charge asymmetry is essentially gone by then (Fig. 1, D and E). There is a significant but weak preference for H bonding residues (up by 2 sigma) over hydrophobic residues (down by 2.5 sigma) in positions N″, C′, and C″, while position N′ somewhat prefers the hydrophobics. The only individual preference in this region that is up by more than 3 standard deviations is for Thr in C″. Most of those C″ Thr's are on the solvent side of a β strand or at the beginning of the next α helix, but six of them show a specific interaction where they are in position 4 of a tight turn and both Oγ and NH of the Thr are hydrogen-bonded to the carbonyl O of residue C1.

It is interesting that a simple definition of helix ends in terms of Cα position gives stronger and more position-specific amino acid preferences than more precise definitions based on hydrogen bonding or φ, ψ angles. Some of this effect may come from local errors in the less well-determined crystal structures, but in looking at the structures it is hard to avoid the conviction that many of the local irregularities represent late folding adjustments of the protein to its detailed environment in the final tertiary structure. Interactions important for secondary-structure formation early in the folding process may persist only approximately in the native structure, as is also strongly suggested by the work of Presta and Rose (*22*). Presumably the definition that correlates most strongly with amino acid sequence will be best for use in structure predictions.

It remains to be seen whether these individual position amino acid preferences can be integrated into an improved helix prediction algorithm, and whether substitution of strongly preferred residues in appropriate positions can help to stabilize isolated helices or entire proteins.

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27. Supported by NIH grant GM-15000.

29 February 1988; accepted 28 April 1988

Effect of Forskolin on Voltage-Gated K^+ Channels Is Independent of Adenylate Cyclase Activation

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Forskolin is commonly used to stimulate adenylate cyclase in the study of modulation of ion channels and other proteins by adenosine 3′,5′-monophosphate (cAMP)–dependent second messenger systems. In addition to its action on adenylate cyclase, forskolin directly alters the gating of a single class of voltage-dependent potassium channels from a clonal pheochromocytoma (PC12) cell line. This alteration occurred in isolated cell-free patches independent of soluble cytoplasmic enzymes. The effect of forskolin was distinct from those of other agents that raise intracellular cAMP levels. The 1,9-dideoxy derivative of forskolin, which is unable to activate the cyclase, was also effective in altering the potassium channel activity. This direct action of forskolin can lead to misinterpretation of results in experiments in which forskolin is assumed to selectively activate adenylate cyclase.

NEURONAL EXCITABILITY CAN BE modulated by phosphorylation of ion channel proteins by protein kinases that are activated by specific second messengers, such as cAMP (*1*). Because of its high affinity for adenylate cyclase, forskolin (FSK) has been used to increase intracellular cAMP levels, leading to the presumed phosphorylation of ion channels by cAMP-dependent protein kinases (*1–9*). Recent reports, however, have suggested that some of the actions of FSK on ion channels and the glucose transporter may be inconsistent with a selective activation of adenylate cyclase and subsequent phosphorylation (*9–*

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14). Direct effects of FSK on ion channels, in addition to effects by means of the cyclase, could cause error in the interpretation of experiments designed to address the involvement of cAMP as a second messenger in neuronal modulation. By using the whole-cell and single-channel voltage-clamp techniques, we have found that FSK directly alters the gating of voltage-dependent K^+ channels in pheochromocytoma (PC12) cells.

In undifferentiated PC12 cells K^+ currents activate rapidly in response to depolarizing voltage pulses and then decline in amplitude slowly (Fig. 1). The bath application of FSK (1.2 to 120 μM) consistently reduced the peak current amplitude and accelerated the time course of the current decline. The reduction of current on application of FSK and the recovery on removal occurred within seconds and were probably limited by the diffusion time of the drug within the chamber. The fraction of the peak current reduced by FSK was not dependent on the test voltage. The actions of FSK did not require the presence of magnesium adenosine triphosphate (MgATP) in the pipette, suggesting that phosphorylation is not involved.

If the alteration of K^+ channel gating by FSK is mediated by the adenylate cyclase and the increase in intracellular cAMP, other agents that increase the cAMP level should produce the same effect. In fact, they did not. Several minutes after bath application of membrane-permeable phosphodiesterase inhibitors (PDEIs) [3-isobutyl-1-methyl xanthine (IBMX) or theophylline], whole-cell K^+ currents increased slightly in amplitude at lower voltages (≤ 0 mV) and decreased at higher voltages (Fig. 1B). This effect was not immediately reversible. In contrast to the effect of FSK, PDEIs did not markedly alter the time course of the K^+ currents. The concurrent application of both FSK (24 to 60 μM) and IBMX (120 to 240 μM) or theophylline (5 mM), however, consistently resulted in a decrease of whole-cell K^+ currents ($n = 7$) similar to that obtained in the presence of FSK alone (Fig. 1C). Dibutyryl cAMP (1 mM) in the bath did not alter the effect of FSK.

The reduction of peak K^+ current and the accelerated time course of the K^+ current decline was dependent on the FSK concentration (Fig. 2). The concentration required for half-maximal reduction in current was approximately 19 μM. This value is comparable to the dissociation constant of adenylate cyclase for FSK determined in enzymatic assays (*2*). Application of 1,9-dideoxy-FSK, an FSK derivative incapable of stimulating the adenylate cyclase (*2*), resulted in a decrease of whole-cell K^+ currents that was indistinguishable from that caused by FSK. This result indicates that the effect of FSK on K^+ current does not involve the cyclase. Succinyl-FSK, a more hydrophilic derivative of FSK, which is approximately 1/10 as effective in stimulating the cyclase (*2*), was similarly less effective in reducing K^+ currents (Fig. 2). This result suggests that the hydrophobic properties of FSK are important in its ability to alter K^+ currents and to activate the adenylate cyclase. The organic solvents used to solubilize FSK, either ethanol (1%) or dimethyl sulfoxide (DMSO) (1%), had no effect when applied to the bath alone.

The mechanisms of the FSK action on voltage-dependent K^+ channels were further analyzed with single-channel currents in

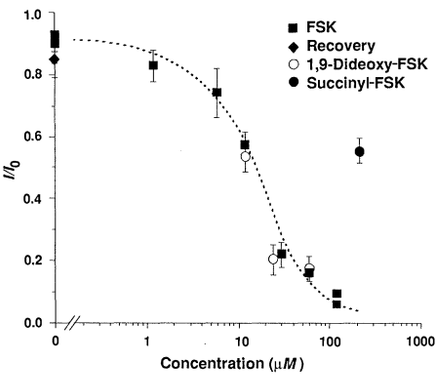

Fig. 2. The progressive decrease of the K^+ current amplitude with increasing concentrations of FSK and derivatives. The current amplitude at the end of a 225-ms voltage pulse to +60 mV was used as a measure of the efficacy of FSK. The current was normalized to control traces taken from the same cell, before perfusion (three to five times the chamber volume) of FSK, using 1% ethanol or DMSO as vehicle. All points are the averages of at least three separate determinations. Standard errors are shown for each point or are smaller than symbols. The effect of FSK was independent of the vehicle used, as indicated by multiple symbols at indicated values of concentration. Recovery was measured after FSK was washed out (three to five times the chamber volume). The outward current measured in the presence of vehicle only (ethanol or DMSO) is given as points at 0 μM FSK. The decrease in the current at these control points and after recovery is in part due to run-down. The dashed line was fitted to the data by eye according to the following relation: $I/I_0 = C - (n[\text{FSK}])/(K_{App} + [\text{FSK}])$ where the slope, n, was equal to 1, and the half-maximal reduction in current in the presence of FSK, K_{App}, was equal to 19 μM. The constant C was equal to 0.92 instead of 1.0 to account for a small amount of rundown in current amplitude during the experiment; 1,9-dideoxy-FSK was equivalent in potency to FSK but succinyl-FSK was 1/10 as effective in reducing K^+ current. Because the current at the end of the 225-ms pulse had not reached a steady-state value at low concentrations of FSK, the steepness of the curve is somewhat overestimated.

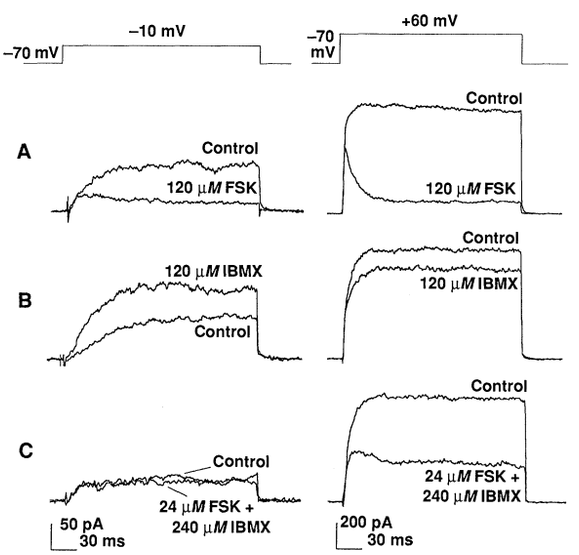

Fig. 1. The effect of FSK on K^+ currents is distinct from that of PDEIs. Whole-cell recordings (*20*) of K^+ currents were elicited in response to 225-ms depolarizing pulses to a test voltage of −10 or +60 mV from a holding potential of −70 mV. Linear leak and capacitative currents have been subtracted. (**A**) The decrease in K^+ current compared to control records from the same cell after the application of 120 μM FSK alone. (**B**) K^+ current elicited from a cell 15 min after the application of 120 μM IBMX alone did not show the distinct decrease in current and kinetic changes observed in the presence of FSK. (**C**) Concurrent application of 24 μM FSK and 240 μM IBMX to a cell on a fresh cover slip resulted in a decrease of K^+ current and an increase in decay rate similar to that seen with FSK alone. The differences between the FSK plus IBMX traces and the 120 μM FSK traces in (A) illustrate the slower decay of current with lower concentrations of FSK. MgATP (5 mM) was added to the pipette solution in this experiment. The bath solution contained 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, pH adjusted to 7.2 with N-methylglucamine. The pipette contained 140 mM KCl, 11 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 nM free Ca^{2+}, pH adjusted to 7.2 with N-methylglucamine. Working solutions were made up immediately before use from stock solutions of FSK (Sigma or Calbiochem), 1,9-dideoxy-FSK, and succinyl-FSK (7-*O*-hemisuccinyl-7-deacetyl-FSK) (purity >99%; Calbiochem) in 95% ethanol or 100% DMSO stored at −20°C. IBMX (Sigma or Calbiochem) was made up fresh from stock solutions kept in 50% ethanol at −20°C. Solutions containing theophylline (Sigma), dibutyryl cAMP (Sigma), or MgATP (Sigma) were also made up immediately before use. PC12 cells were obtained from the laboratory of E. M. Shooter. The cells were maintained in the absence of nerve growth factor in Dulbecco's modified minimum essential medium (Gibco) with 5% supplemental calf and 5% horse sera (Hyclone Laboratories) (*21*). The cells were plated on glass cover slips 1 to 14 days before use.

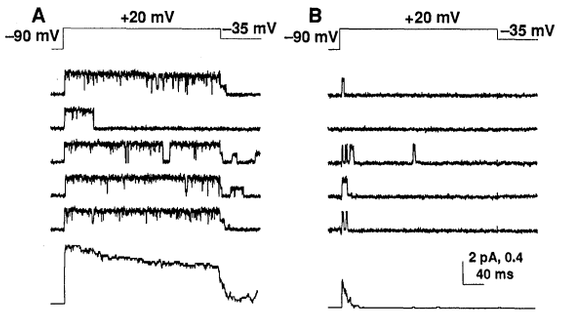

Fig. 3. Effect of FSK on the activity of single K_z channel in an "outside-out" cell-free patch. Representative openings of a K_z channel recorded before (**A**) and after (**B**) addition of 120 μM FSK to the bath. The patch was depolarized to +20 mV for 304 ms from the holding voltage of −90 mV and then repolarized to −35 mV for 40 ms. The pulses were applied every 6 s. The data were filtered through an eight-pole Bessel filter at 1.2 kHz and digitized at 10 kHz. The bottom trace in each column is an ensemble average of the openings, showing the time course of the probability of the channel being open. The scale bar for the ensemble averages is expressed in terms of probability (calibration equals 0.4).

cell-free patches. Under these conditions, any changes in the cAMP concentration caused by the activity of the adenylate cyclase in a membrane patch should have been negligible. Furthermore, the concentrations of diffusible cytoplasmic enzymes, such as protein kinases, were also unlikely to be significant. A single class of K^+ channels, the K_z channel, is the dominant channel contributing to the whole-cell K^+ current in PC12 cells (*15*). The channel opened rapidly on depolarization and generally exhibited bursting activity until it was repolarized (Fig. 3). On repolarization, the channel often remained open for a few milliseconds, resulting in single-channel "tail" openings. An ensemble average of many such traces shows that the probability of the channel being open declines very slowly during the voltage pulse (Fig. 3).

When FSK (120 μM) was applied directly to the extracellular face of the patches, it decreased the overall probability of the K_z channel being open during a voltage pulse (Fig. 3). It also decreased the mean open duration in a concentration-dependent manner (to approximately 1/5 as long at 120 μM), suggesting that FSK binds to a conductive conformation of the channel (*16*). However, FSK did not noticeably change the amplitude of unitary channel openings. A comparison of the ensemble averages before and after applying FSK showed the peak probability of being open typically decreased by approximately 1/2 (Fig. 3). With FSK present, the decline of the probability of the channel being open during depolarizing pulses was greatly accelerated, and openings near the end of the pulse were absent. Additionally, the tail openings were no longer observed on repolarization (−35 to −50 mV). However, K_z channels could reopen in the presence of FSK after a few seconds of hyperpolarization (−70 to −100 mV), indicating that the effect was not an irreversible decrease in opening probability. The probability that a K_z channel failed to open during a depolarizing pulse did not show a marked increase in the presence of FSK at pulse intervals of 6 s.

The observations of the single-channel activity agree well with those made on whole-cell K^+ currents. These effects occurred in the absence of MgATP in the pipette and were readily reversible with washout of FSK, suggesting that phosphorylation was not involved. Additional experiments on cell-free patches support the conclusion that the action of FSK was not mediated by cAMP-dependent phosphorylation. Application of millimolar concentrations of cyclic nucleotides (8-bromo–cAMP, $n = 6$; 8-bromo-guanosine 3′,5′-monophosphate, $n = 2$) to inside-out patches containing K_z channels had no effect. Bath application of FSK alone in the cell-attached configuration also had essentially no effect ($n = 3$). In contrast, preliminary experiments indicated that applying PDEIs to the bath increased K_z channel activity recorded in the cell-attached patches; this finding is consistent with the potentiation of current with PDEIs at lower voltages in whole-cell experiments (Fig. 1). The results confirm that diffusible cytoplasmic agents are not involved in the FSK action on the K_z channels.

When FSK was applied to the intracellular side of an inside-out patch, it did not alter K_z channel activity within 5 min ($n = 6$). Thus, FSK must act preferentially from the extracellular side of the channel. Other classes of voltage-dependent K^+ channels and voltage-dependent Na^+ channels were not noticeably affected by FSK, except for a large conductance Ca^{2+}-dependent K^+ channel in which the probability of opening appears to increase slightly, similar to an effect seen with cell-attached patches from cultured kidney cells (*17*).

The effectiveness of FSK in altering the K_z channel activity in cell-free patches, the ability of 1,9-dideoxy-FSK to duplicate the effect, and the inability of PDEIs to reproduce the effects of FSK on whole-cell K^+ current argue that FSK acts independently of adenylate cyclase (*18*). It is most likely that FSK acts by directly binding at a hydrophobic site at or near the extracellular face of the K_z channel protein. Other reports suggesting direct effects of FSK not mediated by increases in intracellular cAMP have appeared. McHugh and McGee (*10*) have proposed that FSK decreases Rb^+ flux through nicotinic acetylcholine receptors (AChR) in a manner similar to the effect of local anesthetics. Middleton *et al.* (*9*) have reported that FSK alters the activity of single AChR channels but only at high concentrations (100 μM) of FSK. Coombs and Thompson (*12*) and Watanabe and Gola (*13*) have also suggested that FSK directly decreases K^+ currents in molluskan neurons, although these effects were slow to develop and reverse (>30 min).

In addition to its effects on ion channels and adenylate cyclase, FSK has also been shown to bind to the glucose transporter and disrupt transport (*14*). Although the underlying mechanisms of the effects of FSK on K^+ channels, the glucose transporter, and the adenylate cyclase are probably different, the concentration of FSK needed to induce half-maximal effect is similar (1 to 20 μM). This value may be limited by the physical ability of the drug to bind to a hydrophobic site. It is possible that these membrane-bound proteins, in addition to nicotinic AChRs, have a binding site for FSK at a membrane-protein interface.

The direct effect of FSK on ion channels limits the usefulness of this drug in the study of the modulation of excitability and suggests that caution be applied in its use. Decreases in peak current and accelerated time course of decay of FSK-treated K^+ currents have been reported in a number of different cell types (*4–6*) where they have been attributed to phosphorylation. Our results suggest that the direct effect of FSK on K^+ channels may be responsible in part for these previous results. Increases in action potential duration or amplitude that are induced by FSK are also consistent with direct actions on K^+ channels (*6–8*). Experiments that do not directly control the membrane voltage, such as flux and binding assays on populations of cells, are particularly vulnerable to FSK-induced alterations of excitability that are independent of the cAMP cascade.

Note added in proof: Similar direct effects of FSK have been recently found on K^+ channels of human T lymphocytes (*19*).

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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 μg/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 ± SEM.

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

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