for the M-V analysis. If the change in mean and/or variance is sufficiently small, or the event duration sufficiently brief, then cross-bridge attachments become difficult to resolve from periods when no actomyosin interaction occurs (this limitation applies equally well to other methods that have been used to distinguish single events in the actomyosin system). Under such conditions, regions of the M-V plot in which no cross-bridge is attached overlap with the "attached" region, and so some form of background subtraction is required. Another problem with M-V analysis, as it is presented, is that it is not clear which particular sequences of the time-series data are included in the attached pool, and which in the detached pool (that is, all time information for points is discarded). This makes some of the potentially exciting analysis of the records difficult, and does not readily allow the method to be cross-checked easily against the raw data to see if sensible results are being obtained. Notwithstanding these restrictions, the method is a real step forward in the automation of analysis.

One significant difference between this report and earlier work concerns the proportion of displacements that occur within the level of the baseline noise. Whereas we reported a majority of such events (Molloy et al., 1995), for Guilford et al. these represent the minority. The distributions of the positions of displacements reported in their paper are generally biphasic, displaying rather tight distributions (SEM $\pm$ 0.7 nm) around displacements of +10 nm and −10 nm (their Table 1). This difference in observations has led to important differences of interpretation. First, to explain the tight distributions, Guilford et al. propose that each attachment occurs very close to the mid-position of the thermal noise (their Fig. 3). Second, the observation of both positive and negative events, occurring at nearly equidistant positions from the baseline, requires that the cross-bridge working stroke be able to go both backward as well as forward. That is, a cross-bridge may push as well as pull, although with a statistical bias for the forward direction. The implication is that Molloy et al. were not able to observe this because, in their work, the two distributions were smeared into one much broader peak. Other groups, however, have also reported seeing only single-peaked distributions (on the positive side), but with a low proportion of displacements occurring within the baseline noise, and consequently computed a much greater working stroke (Finer et al., 1994, ~10 nm; Ishijima et al., 1996, ~23 nm,) than that found by Molloy et al. (1995, ~4 nm).

These relatively new single-molecule studies permit one to ask and answer any number of important questions relating to the detailed mechanism of the cross-bridge cycle. Guilford et al. introduce a new way of analyzing the data, and thereby raise the intriguing possibility that myosin pushes, as well as pulls, and weigh in with their own answer to the controversial question, "Exactly how big is the myosin work stroke?" This line of research seems certain to remain a lively area, at least in the near term.

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Why Do Cyclic Nucleotide-Gated Channels Have the Jitters?

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Cyclic nucleotide-gated (CNG) channels were discovered in retinal rod photoreceptors, where they generate the electrical response to light. Similar channels were found subsequently in cone photoreceptors and in olfactory receptor neurons, where they serve the analogous purpose of generating electrical signals in response to the binding of odorants. CNG channels have recently been identified in a variety of other tissues, both neural and nonneural, but the physiological roles of these channels are uncertain (reviewed in Finn et al., 1996). The retinal rod channel is a heteromultimer consisting of α- and β-subunits (Kaupp et al., 1989; Chen et al., 1993; Körschen et al., 1995), both of which bind cGMP (Brown et al., 1995). A number of functional properties of both native and expressed channels have been elucidated in excised membrane patches containing hundreds to thousands of channels. In terms of gating, for example, channels are known to be activated rapidly by the binding of at least three molecules of cGMP. The channels are therefore exquisitely sensitive to changes in cGMP concentration. In terms of permeation, the pore is similar in several respects to that of voltage-gated Ca^{2+} channels. There is a high-affinity binding site (or sites) for divalent cations formed by a set of pore region glutamate residues. The binding of Ca^{2+} or Mg^{2+} to these residues

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reduces the effective single-channel conductance and allows a small trickle of Ca^{2+} into the cell, which plays a key role in the recovery of the light response and light adaptation.

Despite this information, several fundamental properties that are best studied at the single-channel level remain controversial and poorly understood. The native channel flickers very rapidly between closed and open states (in the total absence of divalent cations). In fact, the flicker is generally more rapid than the usable bandwidth of the recording system. Whether this extremely rapid flickering has any purpose other than to make life difficult for biophysicists trying to make an honest living is unclear. It has made it difficult to accurately measure the conductance of open channels, the probability that a fully liganded channel is open, and the kinetics of opening and closing. Furthermore, although it is known that α-subunit homomultimers exhibit stable openings (Kaupp et al., 1989), and that coexpression of the β-subunit confers the property of rapid flickering (Chen et al., 1993; Körschen et al., 1995), little is known about the molecular basis of the flickering behavior. In this issue of *Biophysical Journal*, Bucossi et al. (1997) present the first detailed study of the single-channel properties of expressed heteromultimeric channels. They also demonstrate that mutations in the pore region of α-subunit homomultimers can modify the probability of opening, indicating that the pore region is an integral component of the gating apparatus.

There are two important general findings about heteromultimeric channels. The first is that at least three types of channels with different properties were observed. Type 1 (4 of 29 patches) exhibited a single stable opening at positive potentials and multiple openings at negative potentials. The openings were somewhat noisier than homomultimeric channels, but not as flickery as native channels studied to date. Type 2 channels (14 patches) and type 3 channels (11 patches) both exhibited very rapid flickering reminiscent of the native channel, with type 2 having a low apparent conductance (5–25 pS) and type 3 having a large apparent conductance (25–45 pS). The different properties may arise from different subunit stoichiometries, different spatial arrangement of subunits, or posttranslational modifications. Some controversy has existed about the conductance of native channels. Sesti et al. (1994) reported values of about 50 pS from both salamander rod inner-segment patches and outer-segment patches containing multiple channels. Taylor and Baylor (1995) reported a value of 25 pS from three salamander outer-segment patches. Interestingly, the latter channels resemble type 2 channels in the current study by Bucossi et al. Although bovine sequences were used in the expression studies, and the cellular conditions were different, it is possible that rods contain channels with the different properties observed in the current study. The second important finding is that the rapid flicker in type 2 and type 3 channels appears to be an intrinsic gating property and is not caused by rapid proton block. The power spectra of current fluctuations were indistinguishable at extracellular pH values of 7.6 and 9.1.

An important difference between the two subunits is that β contains a glycine at the equivalent position in the pore region in which α contains the critical glutamate important for divalent cation block. Bucossi et al. have studied the mutant subunit E363G expressed alone or in combination with the w.t. α-subunit, to determine whether this residue is responsible for the flickery behavior of heteromultimeric channels. Channels formed from E363G alone exhibited a very low probability of opening at saturating cGMP (0.007 versus 0.8 for w.t. channels), as well as rapid flickering. However, coexpression of E363G with w.t. subunits resulted in a power spectrum that was more similar to w.t. than to E363G, suggesting that the presence of glycine in the β-subunit is not the major cause of rapid flickering in heteromultimeric channels. The low open probability of E363G channels is a dramatic demonstration, however, of the coupling between gating and residues in the pore. Perhaps this residue in conjunction with others is responsible for flickering.

Bucossi et al. also demonstrate that mutations E363D and T364M give rise to multiple apparent open states at negative potentials. The probability of these states was independent of cGMP concentration but dependent on the external pH. Lower pH was found to favor the smallest conducting level. A similar behavior has been observed and studied carefully in expressed catfish olfactory CNG channels (Root and MacKinnon, 1994), which contain a methionine at the position equivalent to T364 in the retinal channel. Bucossi et al. suggest that the different open states observed in the α-subunit variants may be related to the flickering behavior of heteromultimeric channels, in that the flickering may be caused by very rapid transitions between distinct open states with different conductance. However, the flicker is independent of external pH, and it is not clear that the "multiple open states" do not simply reflect different degrees of occupancy of pore residues by protons, as suggested by Root and MacKinnon (1994). In any case, the present results do suggest that the pore of these channels is a dynamic structure that participates in channel gating and may be responsible in some way for the rapid flickering.

This study raises some interesting questions and puzzles for the future that are accessible with current methods. First, why have cGMP-dependent subconductance states been observed in some studies (e.g., Taylor and Baylor, 1995) and not others (e.g., this one)? Second, what role do pore residues contributed by the β-subunit play in both permeation and gating? Are these residues, in particular, responsible for rapid flickering? Finally, are native rod channels a mixture of all of the different heteromultimeric species identified here, or do they assemble in more fixed stoichiometries or patterns?

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A Closer Look at a Molecular Motor by Atomic Force Microscopy

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Myosin, the prototype chemomechanic energy converter in biology, has been subject to numerous functional and structural analyses. A breakthrough in understanding the mechanics of this amazing machine came by the atomic model of the actin-myosin complex derived from x-ray and electron microscopy data (Rayment et al., 1993). More recently, imaging intermediate states in the energy conversion cycle has provided support for the lever model developed from this structure (Whittaker et al., 1995). In spite of this progress, many open questions have remained and call for further studies. The S1 fragment and S1-decorated actin filaments, but not the entire myosin molecule, have been analyzed to atomic detail. Because both heads and a segment of the tail are required for full biological activity of the smooth muscle myosin under physiological conditions, a closer analysis of the entire myosin dimer is of acute interest.

Electron microscopy of negatively stained (Walker and Trinick, 1989) or heavy-metal-shadowed myosin molecules (Winkelmann et al., 1984) has provided detailed insight into the architecture of this biomolecule, although the heavy-metal deposits are likely to obscure the finer features of the native structure. Scanning transmission electron microscopy (STEM) of unstained molecules, which does not rely on metal coats, has made it possible to determine mass maps (Walzthöny et al., 1984). However, the signal of unstained biomolecules is small, even in the STEM dark-field mode, thus limiting the resolution of single particle images. Therefore, the images acquired by Zhifeng Shao and collaborators (this issue) of dehydrated myosin molecules with a cryo-atomic force microscope (cryo-AFM) represent a breakthrough, as they show the molecules with an unprecedented clarity. Accordingly, myosin heads were observed not only to assume a compact or an extended conformation, but to exhibit substructure that confirms the projections recorded by electron microscopy and which can be interpreted in terms of the heavy chain and regulatory domains. Moreover, the tails, which are often kinked, show distinct elevations repeated every 7 nm, consistent with the cross-over distance of coiled-coils, 14 nm.

The superior quality of these topographs called for a detailed comparison of nonphosphorylated and thiophosphorylated myosins. Amazingly, the tails turned out to be about 5 nm shorter in the phosphorylated state, with the foreshortening identified to occur between the two major bends. This led to the interesting hypothesis that phosphorylation of the regulatory chain might induce an α-helix–random coil transition of a tail segment between the major bends. This is indeed a new finding that may explain an induced flexibility of the tail required for docking of the phosphorylated head domain to the actin filament. In addition, a distinct difference in the population of extended and close-packed heads upon thiophosphorylation was found, suggesting an effect of phosphorylation on the flexibility of the head-tail junction.

Cryo-AFM has thus demonstrated its power in providing new and exciting data. The stability of individual biomolecules at low temperature and the preservation of their structure during the rather simple preparation steps allow high-resolution images of superb clarity to be recorded. This advantage is paid for with the requirement to dehydrate the sample, which prevents the observation of biomolecules in buffer solution. However, myosin molecules have been imaged at room temperature in liquid with the AFM (Hallett et al., 1995), suggesting that dynamic measurement under physiological conditions may become feasible.

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