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4. Expression clones pαX1, pαX4, pαX7, pαX8, and pαX10 were derived from pMARα15 by subcloning appropriate restriction fragments into the β-galactosidase gene fragment of plasmid vector pUC8 [J. Viera and J. Messing, *Gene (Amsterdam)* **19**, 259 (1982)] in such a way as to put the mouse sequence in the correct reading frame from the *lac* promoter, resulting in constitutive high-level expression of mouse nAChR α-subunit/β-galactosidase hybrid proteins. Clone pαX11 was derived from pαX4 by in-frame deletion of the Bgl II–Bcl I fragment. pαX12 and pαX13 were obtained from pαX4 by digesting with Bgl II, treating the linear DNA with Bal 31 exonuclease to various extents, repairing the ends with the Klenow fragment of DNA polymerase I, cutting with Bal I, and recircularizing the plasmid DNA by blunt-end ligation. From among the resulting clones, we selected those in which a correct reading frame relationship was restored at the site of ligation by assaying αBTX binding on total protein from these cultures. We further characterized several such clones by sequencing the DNA using the Maxam-Gilbert procedure [A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499 (1980)]. The pαX1Ω and pαX4Ω were derived from their parent expression clones by insertional mutagenesis with the Ω DNA [P. Prentki and H. M. Krisch, *Gene (Amsterdam)* **29**, 303 (1984)]. This DNA fragment includes stop codons in all three frames and transcription-termination signals and was inserted downstream of the α-subunit sequence. Thus, the peptides encoded by these constructs have only a very short COOH-terminal extension following the α-subunit sequence (Ser-Gln-Ala-Gly-Asp-Pro-Val-Ile-Asp and Gly-Asp-Pro-Val-Ile-Asp, respectively, for X1Ω and X4Ω). For fusion protein production, the various plasmids were transformed into *E. coli* MC1061.
5. A 100-ml suspension of *E. coli* containing the expression plasmids was grown to an absorbance at 600 nm of 1. After centrifugation (5000*g* for 10 minutes), the bacteria were resuspended in 10 ml of a solution of lysozyme (1 mg/ml in 25 m*M* tris-HCl, 10 m*M* EDTA, *p*H 8.0) for 5 minutes at 4°C, then sonicated for three periods of 15 seconds. After centrifugation (30,000*g* for 40 minutes), the pellets were extracted sequentially for 1 hour at 4°C with 10 ml of 25 m*M* tris-HCl, 10 m*M* EDTA, containing 1% Triton X-100, *p*H 8.0; phosphate-buffered saline (PBS) containing 3*M* potassium thiocyanate (KSCN), *p*H 7.1; and 10 m*M* potassium phosphate buffer, 10 m*M* EDTA, containing 8*M* urea, *p*H 6.0, with centrifugation (13,000*g* for 30 minutes) between extractions. The final 8*M* urea extract was dialyzed against 10 m*M* tris-HCl, containing 2*M* urea, *p*H 8.0, then against 10 m*M* tris-HCl, *p*H 8.0. All buffers contained 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF) and 0.01% sodium azide. Owing to poor solubility in 8*M* urea, fusion proteins X12 and X13 were treated identically for the first two extractions (Triton X-100 and KSCN); the pellet was then dissolved directly in electrophoresis sample buffer.
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Increased Numbers of Ion Channels Promoted by an Intracellular Second Messenger

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The anomalous rectifier potassium current in *Aplysia* neurons was examined to determine the immediate cause of an increase in conductance induced by serotonin and mediated by adenosine 3′,5′-monophosphate. Voltage-dependent cesium ion block and steady-state current power spectral density were measured under voltage clamp before and after application of serotonin. The amplitude of the anomalous rectifier conductance was increased by adding serotonin, but the shapes of the conductance-voltage curve and the power spectrum were not altered. Calculation of the number of functional channels and of the single-channel conductance from the power spectra indicates that the serotonin-induced increase in conductance resulted from an increase in the number of functional channels, while the single-channel conductance and the open-channel probability were unchanged.

THE MECHANISMS BY WHICH INTRACELLULAR second messengers effect long-lasting changes in neuronal activity are a focus of neurobiological research. Several instances of modulation of excitable cells by adenosine 3′,5′-monophosphate (cAMP) have been documented in which the cAMP mechanism is coupled to a K^+ conductance—calcium-dependent K^+ channel (*1*), serotonin-inactivated K^+ channel (*2*), A current (*3*) [for a review, see (*4*)]. In the identified *Aplysia* neuron R15, cAMP is coupled to the anomalous rectifier channel (*5*). In this last system, the causal relation of increased concentrations of intracellular cAMP to changes in the membrane conductance has been rigorously demonstrated by bath applications of membrane permeant, phosphodiesterase-resistant cAMP analogs and phosphodiesterase inhibitors, and intracellular injection of adenylate cyclase inhibitor and protein kinase inhibitor (*6*). However, the mechanism by which enhanced cAMP-dependent protein kinase activity results in anomalous rectifier conductance increases has not been identified. Because membrane conductance (G) is the product of three factors—$G = Np\gamma$, where N is the number of functioning channels, p is the open channel probability, and γ is the single-channel conductance—the conductance changes due to cAMP must result from changes in one or more of these three factors. Here we report results of noise analysis experiments on the anomalous rectifier in R15 that demonstrate that serotonin increases conductance by increasing the number of functioning channels in the membrane.

The serotonin-sensitive inward rectifying K^+ current in R15 has been identified with the anomalous rectifiers in muscle (*7*) and eggs (*8*) on the basis of blockage by Cs^+ and Ba^{2+} and by the distinguishing shift in the conductance-voltage curve with changes in external K^+ (*5*). Low concentrations of Cs^+ cause a characteristic time-dependent block of this current (*8*), becoming stronger with hyperpolarization (Fig. 1A). In the absence of Cs^+, the conductance approaches an asymptotic maximum. The conductance-voltage curve in the presence of Cs^+ retains its shape after the addition of serotonin, but is scaled upward in amplitude (Fig. 1A), indicating an essentially pure anomalous rectifier conductance. A Lorentzian-shaped power

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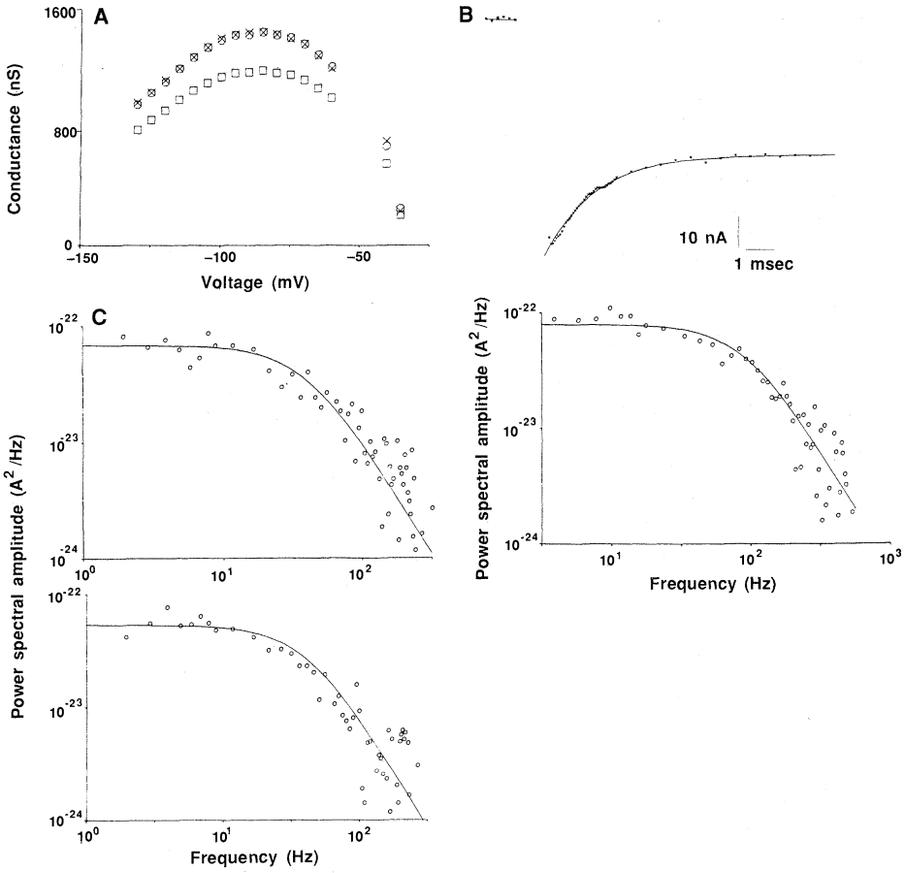

Fig. 1. (**A**) Effect of serotonin on Cs^+-blocked anomalous rectifier current showing steady-state conductance-voltage curves before (□) and after (○) the addition of 5 μM serotonin to the bath. The increased conductance equals the initial conductance scaled by a factor of 1.21 (×). The equilibrium potential of potassium (E_K) was −50 mV; Cs^+ = 25 μM. (**B**) Time dependence and frequency composition of Cs^+-blocked anomalous rectifier current. (Top) Current response to an 80-mV hyperpolarization. The exponential curve reflects a time constant of τ = 1.7 msec. (Bottom) Power spectrum of the steady-state current from the same cell. The Lorentzian curve is $S(f) = 8 \times 10^{-23}/[1 + (f/90)^2]$ A^2/Hz. The corner frequency of 90 Hz reflects a time constant of 1.8 msec. E_K = −40 mV; Cs^+ = 100 μM. (**C**) Effect of serotonin on steady-state current power spectra. Power spectra before (bottom) and after (top) addition of 5 μM serotonin to the bath. Both Lorentzian curves are drawn with f_c = 40 Hz and with zero-frequency asymptotes determined by a least-squares fit as $S(0) = 5.4 \times 10^{-23}$ A^2/Hz (bottom) and $S(0) = 7.0 \times 10^{-23}$ A^2/Hz (top)—a 30% increase. E_K = −41 mV; Cs^+ = 25 μM current was recorded at −120 mV. Experiments were performed principally on the identified neuron R15 from the abdominal ganglion of small *Aplysia* (0.5 to 5.0 g) (*21*). Experiments with R2 yielded similar data.

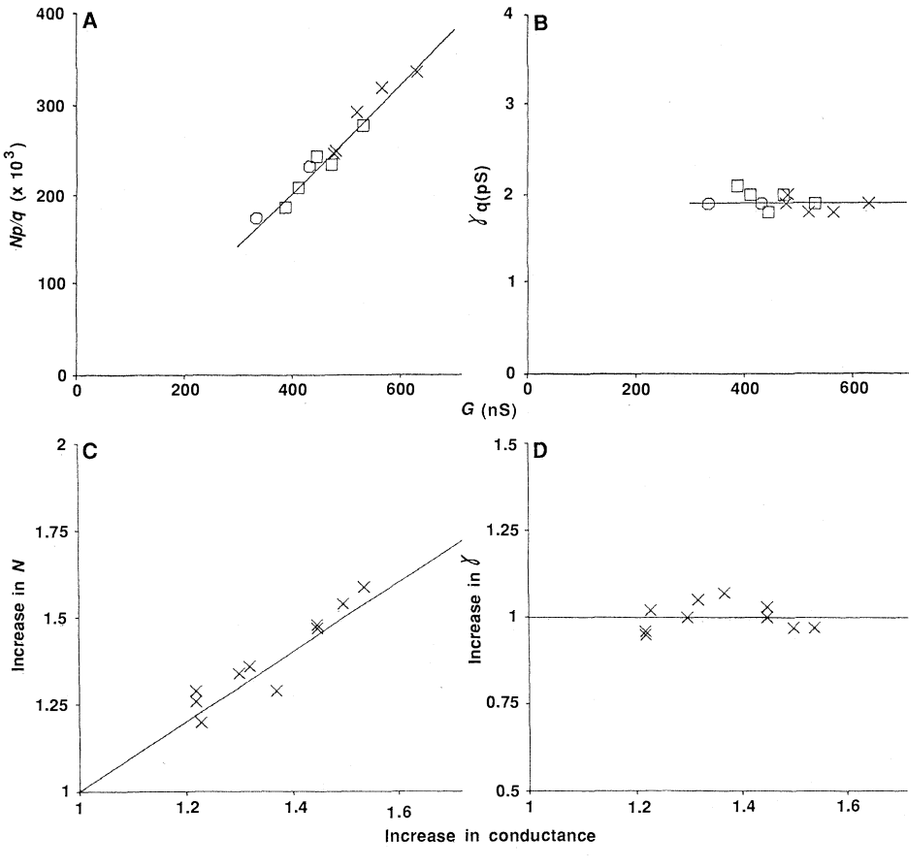

Fig. 2. Effect of serotonin on the number of channels (N) and the single-channel conductance (γ). (**A**) Change in $\bar{I}^2$/Var = Np/q as conductance increased after application of serotonin (5 μM). The straight line is drawn to $0.6G - 38$. E_K = −39 mV; Cs^+ = 25 μM; $V - E_K$ = −70 mV (×), −80 mV (□), and −90 mV (○). The standard error of the fit of the pooled data to the straight line is 10.5 units. (**B**) Change in Var/$[I(V - E_K)] = \gamma q$ as conductance increased as a result of application of serotonin (5 μM). The line is drawn at 1.9 pS (SE = 0.13 pS). Data are from the same experiment as in (A). The response to serotonin was generally maximal in 15 minutes. In this experiment, however, the conductance continued to increase for 1 hour, which allowed several power spectra to be calculated in the interim. (**C** and **D**) Percentage increase in N and γ as conductance was increased as a result of application of serotonin (5 μM). The straight lines are drawn with slopes 1 and 0 in (C and D), respectively. Linear regression in (C) yields a slope of 1.02 (SE = 0.02); that in (D), a slope of 0.02 (SE = 0.01). By the binomial distribution assumption, the variance of the current is Var = $Npq\gamma^2$ $(V - E_K)^2$ and the mean current is $\bar{I} = Np\gamma(V - E_K)$. By Parseval's theorem, the variance can be calculated from the power spectrum as Var = $S(0)f_c\pi/2$.

spectrum is also obtained in the presence of Cs^+, and the corner frequency of this Lorentzian (f_c) is related to the time constant (τ) ofthe current relaxation by the formula $f_c = 1/(2\pi\tau)$ (Fig. 1B), as expected for a spectrum reflecting fluctuations in the current due to Cs^+ block. Neither the Lorentzian spectrum nor the exponential current relaxation is seen in the absence of Cs^+, and moderate concentrations of Cs^+ eliminate both the anomalous rectifier conductance and the Lorentzian power spectrum. As with the conductance-voltage curve, serotonin increases the amplitude of the power spectrum without changing its shape (Fig. 1C).

Taken together, the data in Fig. 1 indicate a well-isolated anomalous rectifier current that responds to serotonin and yields an identifiable power spectrum when blocked by Cs^+. These results can be used to factor the total conductance into N and γ (*9*) under the assumption that the blocked and unblocked channels constitute a binomial distribution. Np/q and γq ($q = 1 - p$) are plotted parametrically against conductance in Fig. 2, A and B, with time since the addition of serotonin as the independent variable. [The choice of these ordinates in preference to N and γ is determined by the relatively large uncertainties resulting from small errors in estimating a q of approximately 0.1; the small value of q is necessary in these experiments for a sufficiently slow Cs^+ block (*10*).] Here p is the proportion of channels left unblocked by Cs^+. Since p and q are unchanged during the experiment (Fig. 1, A and C), Np/q and γq are directly proportional to N and γ, respectively. The value of N increases linearly with G as the conductance is increased by the action of serotonin (Fig. 2A). At the same time, γ is essentially unchanged (Fig. 2B). In Fig. 2, C and D, serotonin-induced increases in N and γ are plotted against increases in G, for nine experiments. Again the increase in conductance is due to an increase in the number of functional channels, while the single-channel conductance remains essentially unchanged.

The possibility exists of a fast, intrinsic gating process responding to serotonin. Such a process, coupled with the slower Cs^+ block, would be reflected in the power spectrum as the sum of two Lorentzians, the high-frequency one being outside the range of resolution. However, the apparent number of functioning channels calculated with only the visible portion of the power spectra and the observed Cs^+ block would still be equal to the true number of functioning channels N (*11*). So the existence of a hidden gating process would underestimate the single-channel conductance, but would not invalidate the result that serotonin increases the conductance only by increasing the number of functional channels.

Whether the new channels prompted by the serotonin are inserted from a cytoplasmic source or already reside in the plasma membrane in a nonfunctional state cannot be determined from these data (*12*). Channels continuously present in the membrane and occasionally converted between the functional and nonfunctional states by even basal concentrations of cAMP would, in the absence of some physical modification such as phosphorylation, blur the distinction between gating and recruitment of new channels. In our results, a voltage-independent process operating at a rate slower than 1 Hz would not appear in the records and would be considered an exchange between functional and nonfunctional pools.

Changes in conductance due to phosphorylation have been investigated in several other systems. Siegelbaum *et al.* (*13*) found that serotonin changes the number of functional (S) channels in *Aplysia* sensory neurons. In that preparation, application of serotonin decreased N. In contrast, Reuter *et al.* (*14*) found that β-adrenergic agonists stimulated a cAMP-modulated increase in the open-channel probability of Ca^{2+} channels from heart myocytes, and Ewald *et al.* (*15*) observed an increase in Np upon addition of the catalytic subunit of cAMP-dependent protein kinase to Ca^{2+}-dependent K^+ channels derived from snail neurons. Phosphorylation processes thus seem to affect ion channels through multiple modes of action (*16*).

Estimates of γ from these data are subject to inflated scatter because of the small value of the closed-channel probability q. If the calculations are made nonetheless, the result is an estimate of $\gamma = 7 \pm 4$ pS ($\bar{X} \pm$ SD) in a 40 mM K^+ external solution. This value is similar to that of the anomalous rectifier in rat myotubes (*17*) and in eggs from the tunicate (*18*) and the marine polychaete *Neanthes* (*19*), and it is about half that in the guinea pig heart (*20*). The number of anomalous rectifier channels in these R15 cells was approximately 50,000 to 100,000 and increased by an average of 38% when the cells were exposed to 5 μM serotonin.

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11. A channel with two independent gates will have three time constants in the correlation function:

$$C(t) = Ni^2 p_S p_F \{[p_F + q_F \exp(-t/\tau_F)] \times [p_S + q_S \exp(-t/\tau_S)] - p_S p_F\}$$

where t is time, N is the number of channels, i is the single-channel current, p is probability of an open gate, q equals $1 - p$, and S and F refer to slow and fast gates. However, if one gate is much faster than the other, the two fastest time constants nearly coincide:

$$C(t) = Ni^2 p_S p_F [q_F \exp(-t/\tau_F) + p_F q_S \exp(-t/\tau_S)]$$

In this case the power spectrum is divided into two Lorentzians. The areas subtended by them are $\mathrm{Var}_F = Np_S p_F q_F \gamma^2 (V - E_\kappa)^2$ and $\mathrm{Var}_S = Np_S p_F^2 q_S \gamma^2 (V - E_\kappa)^2$, and the mean current is $\bar{I} = Np_S p_F \gamma (V - E_\kappa)$. It then follows that $\gamma' = \mathrm{Var}_S / [\bar{I} q_S (V - E_\kappa)] = \gamma p_F$ and $N' = \bar{I}/[\gamma' p_S (V - E_\kappa)] = N$.
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21. A second inward rectifying current, apparently also a K^+ current, is often seen in R15 from the small animals. This current is distinguished from the anomalous rectifier by being impervious to Ba^{2+} and Cs^+ and by displaying a Lorentzian power spectrum with voltage-independent corner frequency of 25 Hz. This current may be the same as that reported by H. Hayashi and H. M. Fishman [*Biophys. J.* **49**, 165 (abstr.) (1986)]. Cells containing a major component of this current were not used in this study. The isolated ganglia were washed with a weak trypsin solution (0.4 mg/ml) for 0.5 hour. The bathing solution contained 420 mM NaCl, 40 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM Hepes-NaOH buffered to pH 7.6. The cells were voltage-clamped with two microelectrodes (3M KCl, 5 megohm) and held at E_κ between pulses. The temperature was kept at 17°C. Power spectra were calculated from steady-state current recorded under voltage clamp. The current was passed through an anti-aliasing filter with a corner frequency of 800 Hz and a passive high-pass filter with a corner frequency of 2 Hz to remove the mean current before being amplified and sampled by the analog-to-digital converter (250 μsec per point, 2048 points per record). A cosine taper window was applied to each record before calculation of the power spectrum by a fast Fourier transform program. Twenty-four power spectra at each test potential were averaged to reduce scatter. The background noise, due mostly to membrane capacitive current induced by thermal noise, was removed by subtracting control spectra taken at E_κ. The variance of the background noise from 0 Hz to the corner frequency averaged 27% of the total variance (channel gating plus background).

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