the Julia set; indeed, all of these points are attracted to the fixed point that I have denoted by q_λ. No matter how large N is chosen, a similar picture results. The two different pictures for $\lambda \gtrsim 1/e$ (Fig. 2, center and right) are computed with different values of N and different values of λ. We have set N equal to 50 in Fig. 2 (left and center); choosing N larger will result in the disappearance of the black region as more points have a chance to escape. N was chosen to be 200 in Fig. 2 (right).

These results, together with many similar bursts, were suggested initially by mathematical experimentation. The idea of experimentation is becoming increasingly important in mathematics as the computer becomes the mathematician's laboratory. Experimentation has led to a number of significant new ideas, particularly in dynamical systems. As further examples of this, the above algorithm may be used with minor adjustments to find bursts in other families of complex entire functions. Figure 3 illustrates a burst in the family $i\lambda \cos(z)$ as λ is changed from ≈ 0.67 (left) to a value slightly larger (right). Figure 4 illustrates a burst in the family $(1 + \lambda i) \sin z$ for $\lambda = 0$ (left) and $\lambda \gtrsim 0$ (right).

Each of these bursts may be rigorously proven to occur. For the cosine family, $C_\lambda(z) = i\lambda \cos z$, the mechanism that produces the burst is analogous to that which occurs in the exponential family: an elementary saddle-node bifurcation occurs at the critical parameter value and allows the critical orbits to slip away to infinity. For the sine family, however, the mechanism is entirely different. The family $\lambda \sin z$ experiences an elementary bifurcation as λ increases through the value 1. This bifurcation is reminiscent of the period-doubling bifurcation as described in (*2, 13*), although it is technically somewhat different. It is known that such a bifurcation does not lead to a burst into chaos; rather, the states both before and after the bifurcation are quite stable. Nevertheless, if a different route in parameter space is chosen through the value $\lambda = 1$, then a burst is possible. Figure 4 (left) depicts the Julia set of $(1 + \lambda i) \sin z$; note the large black basin on either side of 0. For λ small and positive, the Julia set of $(1 + \lambda i) \sin z$ changes dramatically, as shown in Fig. 4 (right). As before, the computer screen fills with color, suggesting the explosion. Indeed, one may prove that there are parameter values arbitrarily close to 1 for which the corresponding Julia set is the whole plane (*19*). In fact, the above results suggest that any elementary bifurcation in complex dynamics (for entire transcendental functions) is accompanied by a direction in parameter space that leads to a similar burst.

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Forskolin and Phorbol Esters Reduce the Same Potassium Conductance of Mouse Neurons in Culture

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Second messenger systems may modulate neuronal activity through protein phosphorylation. However, interactions between two major second messenger pathways, the cyclic AMP and phosphatidylinositol systems, are not well understood. The effects of activators of cyclic AMP–dependent protein kinase and protein kinase C on resting membrane properties, action potentials, and currents recorded from mouse dorsal root ganglion neurons and cerebral hemisphere neurons grown in primary dissociated cell culture were investigated. Neither forskolin (FOR) nor phorbol 12,13-dibutyrate (PDBu) altered resting membrane properties but both increased the duration of calcium-dependent action potentials in both central and peripheral neurons. By means of the single-electrode voltage clamp technique, FOR and PDBu were shown to decrease the same voltage-dependent potassium conductance. This suggests that two independent second messenger systems may affect the same potassium conductance.

PHOSPHORYLATION OF INTRACELLULAR proteins may affect a variety of neurobiological control mechanisms (*1*). Second messenger systems such as adenosine 3′,5′-monophosphate (cAMP) and phosphatidylinositol (*2*) are important in regulating protein phosphorylation through protein kinases. Adenylate cyclase catalyzes the conversion of adenosine triphosphate to cAMP, which increases protein kinase A activity (*3*). Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol phosphates, which mobilize intracellular calcium, and to diacylglycerol, which activates protein kinase C (*4*). We have investigated the effects of activators of protein kinases A and C on resting membrane properties and action potentials recorded from mouse dorsal root ganglion (DRG) neurons and cerebral hemisphere neurons grown in primary dissociated cell culture. Forskolin (FOR) indirectly activates protein kinase A by activating adenylate cyclase (*5, 6*) whereas phorbol esters, which can substitute for diacylglycerol, directly activate protein kinase C. FOR and the phorbol ester phorbol 12,13-dibutyrate (PDBu) increased the duration of calcium-dependent action potentials in central and peripheral neurons without altering resting membrane properties. Data obtained by means of the single-electrode voltage clamp technique demonstrated that FOR and the PDBu decreased the same voltage-dependent potassium conductance.

Application of PDBu (1 μM) or FOR (10 or 100 μM) prolonged action potentials in a saturable and additive manner for DRG (Fig. 1; A1, A2, and B1) and cerebral hemisphere (*7*) neurons. Prolongation was maximal on the first action potential after application and action potential duration

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returned to control values in several minutes. The magnitude of the effect was dependent upon concentration, as reported (*6*) PDBu prolonged action potentials at concentrations above 1 n*M*, and FOR prolonged action potentials at concentrations above 1 μ*M*. Near maximal effects on action potential duration were produced by 1 μ*M* PDBu and 100 μ*M* FOR (Fig. 1, A1 and A2). Reduction of the action potential afterhyperpolarization, which is indicative of a decrease in potassium conductance, was concurrent with the increase in action potential duration produced by PDBu and FOR. These observations suggest that PDBu and FOR were prolonging action potentials by decreasing a voltage- and/or calcium-dependent potassium conductance.

While it was not demonstrated directly that FOR increased cAMP concentrations in these neurons, an effect of FOR on adenylate cyclase was suggested by a similar effect of cholera toxin and the partial blockade of the FOR effect by 2,5-dideoxyadenosine (*6*). Similarly, PDBu was not directly demonstrated to activate protein kinase C, but an action on protein kinase C was suggested since the inactive phorbol ester, 4-α-phorbol, did not modify action potential duration (*6*). Addition of 1 μ*M* PDBu to neurons exposed to 100 μ*M* FOR (Fig. 1A3) or addition of 100 μ*M* FOR to neurons exposed to 1 μ*M* PDBu (*7*) did not produce additive effects. On the contrary, the prolongation appeared to be maximal with either one or the combination. FOR at 10 μ*M* (Fig. 1B1) produced a significant but submaximal action potential prolongation alone; the prolongation was augmented by the subsequent (Fig. 1B2) or concurrent (Fig. 1B3) addition of 1 μ*M* PDBu. This additivity at submaximal concentrations in addition to the occlusion of the FOR and PDBu responses at maximal concentrations was suggestive of an effect of FOR and PDBu on a common site.

It has been suggested in mouse DRG neurons that FOR affected primarily a voltage- and/or calcium-dependent potassium conductance, while PDBu affected both voltage-dependent potassium and calcium conductances (*6*). The similarity of effects as well as their additivity and saturability (Fig. 1, A and B) raised the question of whether FOR and PDBu were affecting the same voltage-dependent potassium conductance (*6*). Further evidence for a common site of action of PDBu and FOR was obtained by repeating PDBu and FOR applications to neurons after membrane depolarization. When membrane potential was reduced by applying steady depolarizing current from −70 mV to less than −55 mV, the duration of the calcium-dependent action potential was increased and the afterhyperpolarization was reduced, suggesting that a voltage-dependent potassium conductance was inactivated at the reduced membrane potentials.

To compare the effect of PDBu and FOR on calcium-dependent action potential duration evoked from different membrane potentials, control PDBu and FOR responses were obtained when DRG (Fig. 1C) and cerebral hemisphere (*7*) neurons were held at −75 to −65 mV. Under these circumstances, both PDBu (Fig. 1C1) and FOR (Fig. 1C2) produced action potential prolongation. Neurons were then depolarized to less than −55 mV and action potential duration increased gradually with successive stimuli. After several minutes, a consistent, increased duration was obtained. PDBu and FOR were then reapplied, but neither drug increased action potential duration (Fig. 1C). When membrane potential was restored to −70 mV, the PDBu and FOR effects returned. These results suggest that PDBu and FOR both acted on a voltage-dependent channel that was inactivated by membrane depolarization.

Given the similarity of the effects of FOR and PDBu on action potentials, voltage-clamp experiments were performed to assess whether a common conductance was being affected. DRG neurons were used in these experiments to increase the likelihood of an adequate space clamp. Membrane potentials were held at −70 mV and both hyperpolarizing (to −120 mV) and depolarizing (to 0 mV) steps of 140-msec duration were applied in 10-mV increments. Cadmium (400 μ*M*) was applied to block calcium current. PDBu and FOR each decreased the outward potassium current (Fig. 2, A1 and A2). Step depolarizations of +70 mV produced an outward current that gradually increased over the duration of the depolarization. This outward current was blocked by the application of tetraethylammonium chloride (TEA; 20 to 100 m*M*) (*7*). In some neurons, a smaller earlier transient current was evoked (Fig. 2, B1 and B2) that was blocked by the

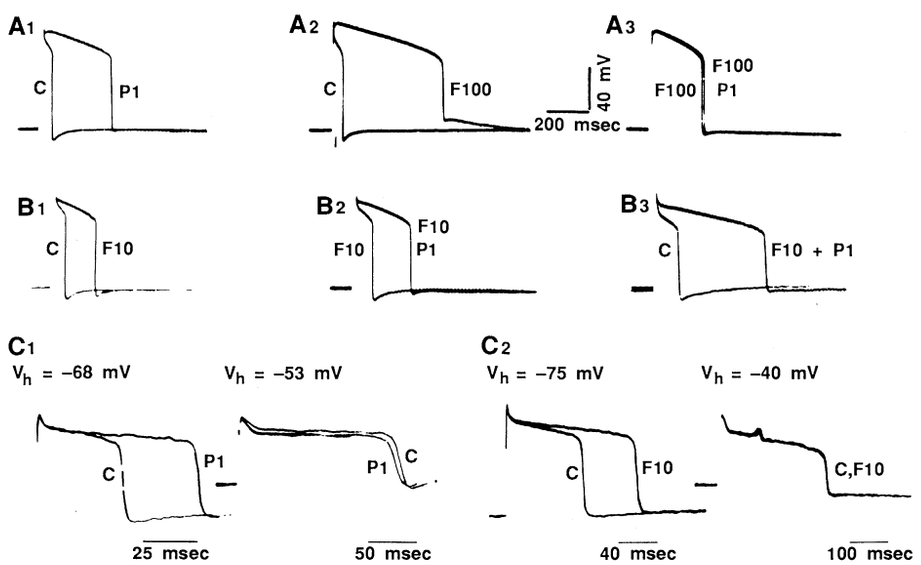

Fig. 1. PDBu and FOR increased action potential duration. Concentrations (μ*M*) of PDBu and FOR are shown after the single-letter abbreviations (P and F, respectively). Data are superimposed photographs of action potentials evoked at 15-second intervals prior to (C) and subsequent to application of either PDBu or FOR. In A3 and B2, FOR was present before and during the application of PDBu. FOR and PDBu increased action potential duration in DRG neurons in a saturable (**A**) and additive (**B**) manner; the PDBu and FOR effects were voltage-dependent (**C**). Data in (A) and (B) were obtained from one DRG neuron and in (C) from a different DRG neuron. Preparation of mouse spinal cord and DRG cocultures, cerebral hemisphere, neuron cultures (*23*), and electrophysiological techniques (*24*) were as described. Recording medium was a tris-HCl–buffered saline (*p*H 7.3, 320 mOsm) that contained (m*M*) NaCl (142.5); KCl (5.3); $CaCl_2$ (5.0); glucose (5.6); tris (13.0); TEA (5.0). For cerebral hemisphere neuron action potential experiments, the bathing medium contained 10 μ*M* tetrodotoxin. Neurons were impaled with micropipettes (20 to 45 Mohm) filled with either 4*M* potassium acetate (DRG) or 3*M* cesium acetate (cerebral hemisphere neurons) and action potentials were evoked by applying short (500 μsec) depolarizing stimuli every 15 seconds. FOR (Calbiochem, La Jolla, CA) and PDBu (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide as 10 m*M* stock solutions, aliquoted, and frozen. Drug dilutions in recording buffer were made immediately prior to use. PDBu was diluted in recording medium containing 0.1% bovine serum albumin. Application of dimethyl sulfoxide (1:100) as a control (C) produced less than a 4% change in action potential duration. FOR and PDBu were applied to single neurons by pressure ejection from micropipettes with tip diameters of 2 to 5 μm for 5 seconds prior to evoking an action potential. Under these conditions, DRG neuron action potentials had two components: an initial sodium-dependent portion that was partially sensitive to block by tetrodotoxin and a 2 to 25 msec broad plateau that was dependent upon calcium (*25*).

application of 4-aminopyridine (5 mM). Since these currents were recorded in the presence of the calcium-channel blocker cadmium, it is likely that the TEA-sensitive current was the delayed rectifier current and the 4-aminopyridine–sensitive current was the A current. The slowly developing and persistent outward currents were reduced in the presence of 1 μM PDBu (Fig. 2A1) or 250 μM FOR (Fig. 2A2). The reduction in current observed with FOR was not enhanced by the addition of PDBu (Fig. 2A3).

The effects of PDBu and FOR were dependent on the holding potential. From a −70-mV holding potential, a step depolarization to 0 mV produced a large outward current that was reduced by the application of 1 μM PDBu (Fig. 2B1) or 100 μM FOR (Fig. 2B2). After membrane depolarization to −40 mV, a large outward current was evoked that inactivated over 10 to 20 seconds (*7*). After full inactivation of the current, step depolarizations from −40 to 0 mV evoked small, time-invariant outward currents. At this reduced holding potential, neither 1 μM PDBu (Fig. 2B3) nor 100 μM FOR (Fig. 2B4) altered the current. The larger amplitude current and the responsiveness to FOR and PDBu returned when the neurons were again held at −70 mV with steps of +70 mV (*7*).

Current-voltage relations for DRG neurons were obtained for five conditions: control, PDBu alone, FOR alone, PDBu plus FOR, and recovery (four neurons). Current measurements were made at 100 msec after the voltage steps. A representative plot is shown in Fig. 3. The current-voltage relation between −110 and −40 mV membrane potential was linear, representing leak current; this portion was not changed by application of FOR or PDBu. At more positive potentials, an outward current developed (Fig. 2A). PDBu (1 μM) and FOR (250 μM) each reduced the voltage-dependent outward current, measured 100 msec after the voltage step (Fig. 3). Addition of PDBu (1 μM) to FOR (250 μM) did not produce any more reduction in the outward current than application of FOR alone. Three minutes after removal of FOR and PDBu, the recovery current-voltage curve returned to control levels.

The application of FOR or PDBu to DRG and cerebral hemisphere neurons increased the duration of calcium-dependent action potentials in a voltage-dependent fashion while decreasing the afterhyperpolarization. In DRG neurons, single-electrode voltage clamp data suggested that both PDBu and FOR were decreasing the same voltage-dependent outward current, presumably the potassium delayed rectifier, which inactivates when the membrane potential is held at −40 mV (*8*). Protein kinases have been shown to alter ionic conductances in neurons. Protein kinase A (stimulated by increased cAMP levels) has been reported to (i) decrease potassium conductance in chick sensory (*9*) and gastropod (*10*) neurons; (ii) increase potassium conductance in gastropod neurons (*11*) as well as in amphibian oocytes and neurons (*12*); and (iii) increase sodium or sodium/calcium conductance in gastropod neurons (*13*) and vertebrate cardiac cells (*14*). Protein kinase C blocked a calcium-dependent potassium conductance in rat hippocampal neurons (*15*) and was reported to enhance calcium current in a gastropod (*16*). In dissociated mouse DRG and cerebral hemisphere neurons, FOR and phorbol esters decreased the same outward potassium current.

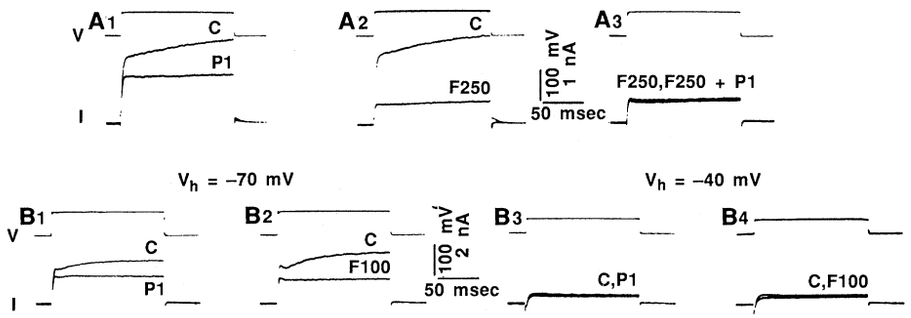

Fig. 2. PDBu and FOR decreased a voltage-dependent outward current in DRG neurons. Abbreviations are as described in Fig. 1. Data are superimposed traces of voltage step commands and the currents recorded with single-electrode voltage clamps evoked prior to (C) and subsequent to application of either PDBu or FOR. In (A) the holding potential (V_h) was −72 mV. In B1 and B2, V_h was −70 mV and in B3 and B4, V_h was −40 mV. Step depolarizations were made to 0 mV while PDBu and FOR were applied. For DRG voltage clamp experiments, the medium contained 10 μM tetrodotoxin. Micropipettes (18 to 25 Mohms) containing 3M potassium chloride were used. DRG neurons were voltage-clamped with an Axoclamp −2 preamplifier (Axon Instruments, Burlingame, CA) that switched between voltage recording (70% of each cycle) and current passing (30% of each cycle) at 6 kHz. Step voltage commands were maintained for 140 msec and measurements were made at 100 msec.

It is likely that both FOR and PDBu reduce more than one outward potassium current. In these studies, we have shown that FOR and PDBu reduced a late outward sustained current in the presence of a calcium-channel blocker. We have not studied FOR and PDBu effects on A current or calcium-activated potassium current. It remains to be determined whether protein kinases A and C act on the same substrate, in this case perhaps some portion of the ion channel or a common phosphorylating enzyme, or whether there is a convergence in the two pathways perhaps at the level of GTP-binding regulatory proteins. Protein kinases A and C (*17*) have a number of common substrate proteins in *Hermissenda* neurons (*18*) and a mouse anterior pituitary tumor cell line (*19*). Protein kinase C has

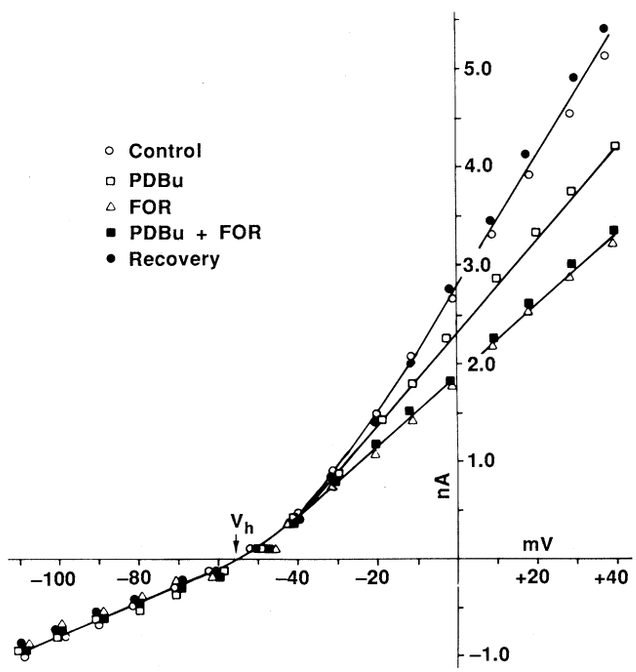

Fig. 3. Steady-state current-voltage relation for a DRG neuron after FOR and/or PDBu application. V_h was −55 mV.

been shown to modify the regulatory and/or catalytic subunits of the adenylate cyclase system in rat cortical slices (*20*) and anterior pituitary cells (*21*). In fibroblasts, phospholipids associated with the phosphatidylinositol system act via the inhibitory regulatory protein of adenylate cyclase (*22*). We suggest that two distinct second messenger systems may converge at some level to decrease the same potassium conductance.

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Yeast KEX2 Protease Has the Properties of a Human Proalbumin Converting Enzyme

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Several classes of proteolytic enzymes have been proposed to have a role in the processing of precursor forms of proproteins at paired basic amino acid residues. In higher eukaryotes, a single endopeptidase has yet to fulfill the necessary criteria as the physiologically relevant convertase. The observation of proalbumin circulating in a child with a bleeding disorder caused by an unusual α_1-antitrypsin mutation led to speculation that the presence of this α_1-antitrypsin mutant was inhibitory to the convertase. This provided an additional means of characterizing the processing enzyme. In this study the yeast KEX2 enzyme, a calcium-dependent thiol protease, was found to have all the properties expected for this processing enzyme. KEX2 correctly recognized and cleaved the prosequence in proalbumin. In addition, KEX2 was specifically inhibited by the mutant α_1-antitrypsin but not by other serine protease inhibitors.

PROPROTEIN CLEAVAGE OCCURS AT pairs of basic residues, and cleavage at such sites is an essential feature in the processing of peptide hormones, neuropeptides, and some of the plasma proteins (*1, 2*). A clue to the enzyme involved is provided by the yeast *Saccharomyces cerevisiae*, in which mutations of the *KEX2* gene, coding for a proposed Golgi enzyme, block the processing at Lys-Arg sequences of the secreted yeast α-factor and killer toxin peptides (*3*). Membrane preparations from these yeast mutants have also lost the ability to cleave the dibasic peptide substrate *t*-butyloxycarbonyl -Gln -Arg -Arg -amino -4-methylcoumarin (BOC-Gln-Arg-Arg-MCA). However, reintroduction of the normal *KEX2* gene to the yeast, either on a multicopy plasmid or by integration into the genome, restores the missing proteolytic processing activities (*4*) and the killer expression [Kex^+ (*5*)] phenotype. No such mutation of a converting enzyme has been observed in higher eukaryotes, but several instances of a failure in proprotein cleavage have been recorded in humans. In particular, one unusual and unexplained instance of a failure of proalbumin cleavage occurred in a child who had a reactive center variant of the plasma serine protease inhibitor α_1-antitrypsin (*6, 7*). We report here experiments with the KEX2 enzyme that precisely duplicate the findings in this case. We conclude that the convertase that processes proalbumin to albumin in the liver is likely to be closely related to the KEX2 protease of yeast.

The production of albumin requires, as a final step in the Golgi vesicles (*8*), the cleavage at an Arg-Arg sequence of a six-residue propeptide from proalbumin (*9, 10*). A complete failure of proalbumin processing has been observed in two individuals with variations in this Arg-Arg cleavage site: proalbumin Christchurch (*11*) in which there is Arg-Gln, and proalbumin Lille (*12*) in which there is His-Arg. A third, more unusual, instance of a failure in propeptide cleavage occurred in a child with an abnormal α_1-antitrypsin (*6*), whose plasma contained uncleaved normal proalbumin (*13*) (Fig. 1A). The new variant inhibitor, α_1-antitrypsin Pittsburgh (*7*), had a replacement of its reactive center (358 methionine by arginine), which changed its activity from a general inhibitor of serine proteases to a relatively specific inhibitor of serine proteases that cleave at arginine or lysine residues (*14*). Albumin and α_1-antitrypsin are co-processed in the liver (*15*), and the reasonable conclusion from the observations in the child from Pittsburgh was that the mutant α_1-antitrypsin was acting as an inhibitor of the convertase that cleaves proalbumin (*13*). However, the inference from this, that the proalbumin convertase was a serine protease, ran counter to some evidence that suggested the mammalian propolypeptide or prohormone processing enzymes are thiol enzymes and perhaps cathepsin B–like (*10, 16*).

Together, these experiments of nature provide an exacting set of conditions to be met by a prospective mammalian cell con-

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