cells were collected and immediately fixed by exposure to methanol and acetic acid (3:1, v/v). Slides containing fixed cells were air-dried and dipped in NTB2 Kodak emulsion as described by the manufacturer. Dipped slides were air-dried, stored at 4°C for 3 weeks, and then developed and stained with Giemsa. Cell cycle parameters were determined as follows. The time taken to first attain 50% of labeled metaphases is equivalent to G2 + M. The length of time from that point to the next point, where only 50% of metaphases are labeled, is equivalent to the duration of the S phase. The total length of the cell cycle is the interval between any two equivalent points on the curve. G_1 length is obtained by subtraction. [H. Quastler and F. G. Sherman, *Exp. Cell Res.* **17**, 420 (1959); A. Macieira-Coelho, in *Tissue Culture Methods and Applications*, P. F. Krause and M. K. Patterson, Jr., Eds. (Academic Press, New York, 1973), pp. 412–422].

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20. Empirical measurements with calibrated spheres of known dimensions showed that FSC as measured by flow cytometry on a fluorescence-activated cell sorter (FACScan, Becton Dickinson) was linearly related to the diameter of the spheres (J. Roberts, unpublished observations).
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22. Rat-1 fibroblasts were infected with retroviral expression vectors (LXSN) encoding cyclin E, c-Myc, and bcr-Abl and grown to confluence. No foci of proliferating cells were detected in cells infected with vectors encoding cyclin E alone or cyclin E in combination with either of the other two proteins. Also, none of these combinations was sufficient to allow growth in soft agar. The combination of c-Myc plus bcr-Abl, however, did induce the formation of foci and soft agar growth (E. Blackwood and M. Ohtsubo, unpublished data).
23. Late-passage primary human diploid fibroblasts were infected with LXSN or LXSN–cyclin E. Cells were selected for resistance to G418 and propagated. Control and cyclin E–overexpressing cells both senesced after 65 population doublings, with no alterations in proliferative potential (T. Norwood, M. Ohtsubo, J. Roberts, unpublished observations).
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31. We thank E. Firpo for contributing to our analysis of the expression of cyclin E in mammalian cells; A. Koff for affinity-purified antibodies to cyclin E; T. Norwood for low-passage primary human foreskin fibroblasts; D. Miller for help and advice in the construction of retroviral vectors expressing cyclins; and E. Blackwood for help in studying the effects of Myc and Abl on cells overexpressing cyclin E. M.O. is a recipient of fellowships from the Uehara memorial foundation (Japan) and the Leukemia Society of America. J.R. is a Lucille P. Markey scholar in the biomedical sciences and was supported in part by grants from the NIH and from the Lucille P. Markey Charitable Trust.

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NF-κB Controls Expression of Inhibitor IκBα: Evidence for an Inducible Autoregulatory Pathway

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The eukaryotic transcription factor nuclear factor–kappa B (NF-κB) participates in many parts of the genetic program mediating T lymphocyte activation and growth. Nuclear expression of NF-κB occurs after its induced dissociation from its cytoplasmic inhibitor IκBα. Phorbol ester and tumor necrosis factor–alpha induction of nuclear NF-κB is associated with both the degradation of preformed IκBα and the activation of IκBα gene expression. Transfection studies indicate that the IκBα gene is specifically induced by the 65-kilodalton transactivating subunit of NF-κB. Association of the newly synthesized IκBα with p65 restores intracellular inhibition of NF-κB DNA binding activity and prolongs the survival of this labile inhibitor. Together, these results show that NF-κB controls the expression of IκBα by means of an inducible autoregulatory pathway.

The NF-κB transcription factor participates in the regulation of multiple cellular genes involved in the immediate early processes of immune, acute phase, and inflammatory responses (*1*). NF-κB has also been implicated in the transcriptional activation of several viruses, most notably the type 1 human immunodeficiency virus (HIV-1) (*2*, *3*). In resting $CD4^+$ T lymphocytes, which serve as a primary cellular target for HIV-1, the heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, IκB (IκBα) (*4*, *5*). Expression of the active nuclear form of NF-κB, composed of 50- and 65-kD subunits, is post-translationally induced (*1*, *6*) by various stimuli including mitogens such as phorbol 12-myristate 13-acetate (PMA) (*2*, *6–8*) and cytokines including interleukin-1 (IL-1), IL-2, and tumor necrosis factor–alpha (TNF-α) (*9*). These stimulants appear to activate NF-κB by inducing the phosphorylation and release of IκB (*10*), thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus (*4*, *7*, *10*, *11*). However, the biochemical mechanism underlying the transient nature of NF-κB induction remains poorly understood. In the present study, an autoregulatory pathway involving NF-κB induction of its own inhibitor is identified.

To assess the dynamics of IκB protein expression occurring during T lymphocyte activation, subcellular fractions from human Jurkat T cells were treated with various inducing agents and then subjected to immunoblotting (Fig. 1A) and gel retardation (Fig. 1B) analyses. A 37-kD protein was readily detected in cytoplasmic extracts from unstimulated cells with peptide antiserum raised against the COOH-terminus (Fig. 1A) or NH_2-terminus (*12*) of IκBα (previously referred to as MAD-3) (*13*), a member of a growing family of proteins known to inhibit NF-κB DNA binding activity in vitro (*13*, *14*) and in vivo (*15*, *16*). This endogenous immunoreactive protein comigrated with authentic IκBα present in COS-7 cells transfected with an ex-

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ogenous IκBα cDNA expression vector (*12*).

In cells treated with either PMA or TNF-α, cytoplasmic IκBα completely disappeared within 40 min of stimulation (Fig. 1A, lane 3) without concomitant expression in the nucleus (*12*). During this time, the DNA binding activity of nuclear NF-κB reached peak levels (Fig. 1B, lane 3). IκBα protein reappeared in the cytoplasm after 2 hours of stimulation (Fig. 1A, lanes 4 to 6). Reexpression of IκBα required de novo protein synthesis because this response was completely blocked by several translation inhibitors, including cycloheximide (CHX; Fig. 1A), emetine, anisomycin, and puromycin (*12*). Parallel studies with cells treated with cycloheximide alone showed only a gradual decline in IκBα protein levels (CHX; Fig. 1A) associated with a slight increase in nuclear NF-κB activity (CHX; Fig. 1B). These findings presumably reflect the labile nature of IκBα and the requirement for its continuous basal production (*7*). Newly synthesized IκBα appeared fully functional because its reexpression was associated with a down-regulation of NF-κB DNA binding activity (Fig. 1B, lanes 4 to 6). These IκBα inhibitory effects occurred more rapidly when the induced cells were washed after 40 min of stimulation to partially deplete the agonists (*12*).

Induction of IκBα mRNA expression after PMA or TNF-α stimulation was also studied (Fig. 1C). Both PMA and TNF-α induced increases in IκBα mRNA amounts whereas sustained superinduction occurred when these agonists were added in the presence of cycloheximide. The time course of this response was similar to the induction kinetics for nuclear NF-κB expression (Fig. 1B). The effects of cycloheximide on IκBα mRNA expression suggest that this inhibitor is encoded by an immediate early gene whose expression in T cells is regulated by a preexisting factor.

To examine the possibility that the activation of NF-κB was a prerequisite for the subsequent induction of IκBα, parallel immunoblotting (Fig. 2A), gel retardation (Fig. 2B), and ribonuclease protection (Fig. 2C) studies were performed with Jurkat T cells stimulated with either PMA or TNF-α in the presence of pyrrolidinedithiocarbamate (PDTC), a potent free radical scavenger and metal chelator that inhibits NF-κB induction (*17*). PDTC efficiently blocked the induction of NF-κB DNA binding activity (Fig. 2B) mediated by either PMA or TNF-α. It also prevented the induced degradation of IκBα (Fig. 2A, lanes 3 and 7 and lanes 11 and 15). The induction of IκBα mRNA expression was also blocked by the addition of PDTC (Fig. 2C), thus suggesting that NF-κB might transcriptionally regulate the induction of IκBα gene expression.

To explore the correlation between the nuclear translocation of NF-κB and the induction of IκBα mRNA expression, the ability of NF-κB to activate IκBα mRNA and protein production was examined by performing transfection studies with COS cells (Fig. 3A). In this regard, NF-κB corresponds to a heterodimeric complex comprised of 65-kD (p65) and 50-kD (p50) DNA binding subunits, both of which are structurally related to the c-*rel* proto-oncogene (*18–21*). Although undetectable in cells transfected with a control expression vector lacking a cDNA insert (pCMV4), IκBα protein was readily observed on immunoblots of whole cell extracts from transfected COS-7 cells expressing p65, which contains a powerful transcriptional activation domain (*20–22*). In contrast, the p50 subunit of NF-κB, which lacks a potent transactivation domain (*20–22*), failed to augment IκBα protein expression. Consistent with its capacity to act only as a weak transcriptional activator relative to p65 (*21, 23*), c-Rel alone, or in combination with p50, only modestly induced endogenous IκBα protein expression. Combinations of either p65 and p50 or p65 and c-Rel produced effects that were similar to those obtained with p65 alone. These functional

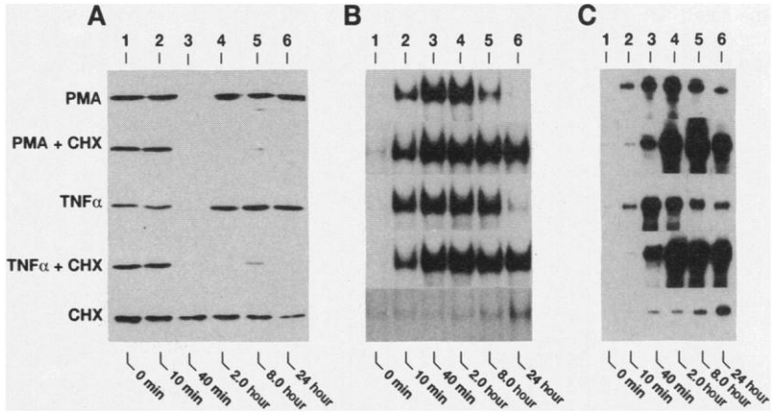

Fig. 1. Degradation and resynthesis of IκBα during T cell activation. Jurkat T cells were stimulated with either PMA (50 ng/ml) or TNF-α (140 U/ml) in the absence or presence of cycloheximide (CHX; 25 μg/ml). At the indicated times, cells were collected for the preparation of subcellular protein extracts (*24*) and total cellular RNA (*25*). (**A**) Immunoblotting analysis of IκBα. Cytoplasmic extracts (20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with peptide antiserum specific for the COOH-terminus (amino acids 289 to 317) of IκBα with the use of an enhanced chemiluminescence assay (Amersham). (**B**) Gel retardation assay of nuclear NF-κB. Nuclear extracts (10 μg) were added to DNA binding reactions (20 μl) (*26*) containing a ^{32}P-labeled palindromic κB probe (κB-pd) (*27*). Resultant nucleoprotein complexes were resolved on native 5% polyacrylamide gels and visualized by autoradiography. (**C**) Ribonuclease (RNase) protection analyses of IκBα mRNA expression. Total cellular RNA (10 μg) prepared in parallel from the cells was hybridized to a ^{32}P-radiolabeled RNA probe (225 bases) corresponding to nucleotides 824 to 1048 of the IκBα cDNA (*13*), then treated with RNase A and RNase T1, and subsequently fractionated on a denaturing urea 5% polyacrylamide gel (*28*).

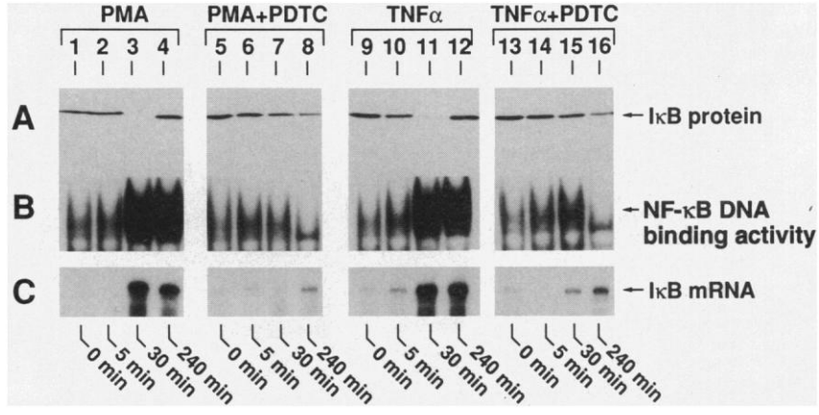

Fig. 2. Effect of PDTC on depletion and induction of IκBα (IκB). Jurkat cells were treated with 0.2 mM PDTC for 1 hour and then stimulated with either PMA (50 ng/ml) or TNF-α (140 U/ml). Subcellular protein extracts and RNA were prepared at the indicated times and subjected to immunoblotting (**A**), gel retardation (**B**), and RNase protection (**C**) analyses as described (Fig. 1).

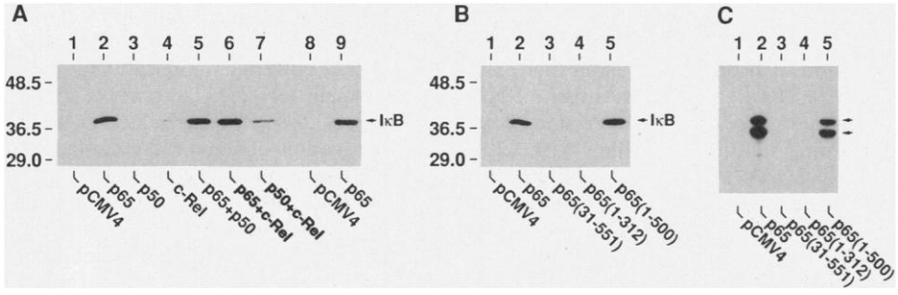

Fig. 3. Induction of endogenous IκBα by NF-κB p65. (**A**) Effect of Rel-related polypeptide expression on IκBα protein induction. COS-7 (lanes 1 to 7) and Jurkat cells (lanes 8 and 9) were transfected (*15*) with pCMV4-based cDNA expression vectors (2 μg/ml for COS and 40 μg/ml for Jurkat cells) encoding the indicated Rel polypeptides. Whole cell extracts were prepared (*15*) at 48 hours after transfection and subjected to SDS-PAGE (3.5 μg per lane) and immunoblotting analysis as described (Fig. 1A). Molecular size markers (in kilodaltons) are indicated. (**B**) Mutational analysis of p65-mediated IκBα induction. COS-7 cells were transfected with either wild-type (lane 2) or mutant (lanes 3 to 5) p65 expression vectors (*15*) and recipient cell extracts were assayed for IκBα protein expression as in (A). (**C**) Induction of IκBα mRNA by p65. Total cellular RNA isolated in parallel from COS transfectants in (B) were analyzed by RNase protection assay as described (Fig. 1C). The major protected IκBα RNA fragments are indicated by arrows.

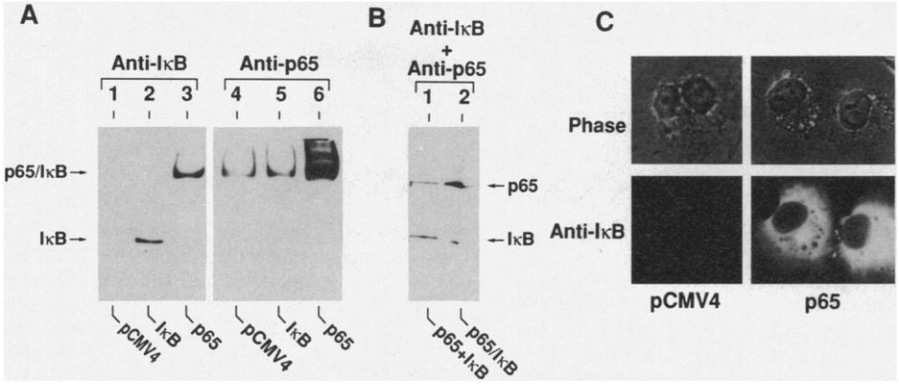

Fig. 4. Newly synthesized IκBα is present as a complex with p65 in the cytoplasm. (**A**) COS cells were transfected with either unmodified (lanes 1 and 4) or modified (lanes 2, 3, 5, and 6) cDNA expression vectors encoding the indicated proteins. Whole cell extracts were prepared at 48 hours after transfection (*15*), fractionated on a 5% native polyacrylamide gel, and subjected to immunoblotting analyses with either anti-IκBα (anti-IκB) (lanes 1 to 3) or p65 antiserum (lanes 4 to 6). (**B**) The major immunoreactive species in lane 3 of (A) was separated by SDS-PAGE and subjected to compositional analysis by immunoblotting with a mixture of IκBα- and p65-specific antisera (lane 2). Control extract from COS cells cotransfected with IκBα and p65 cDNA expression vectors is shown (lane 1). (**C**) Immunofluorescence analysis of COS cells transfected with parental expression vector (pCMV4; left panels) or p65 cDNA expression vector (p65; right panels). COS cells were grown on chamber slides (Nunc, Inc.), transfected with effector plasmids, and subjected to indirect immunofluorescence (*15*) with IκBα-specific antiserum.

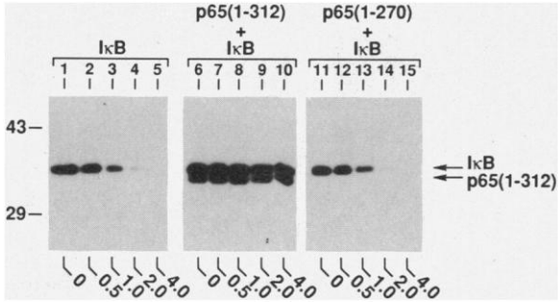

Fig. 5. Immunoprecipitation of ^{35}S–pulse-labeled COS cell extracts. COS cells were transfected with the IκBα (IκB) expression vector either alone (lanes 1 to 5) or in combination with cDNA expression vectors encoding p65(1–312) (lanes 6 to 10) or p65(1–270) (lanes 11 to 15). Forty-eight hours after transfection, cells were metabolically radiolabeled with [^{35}S]cysteine and [^{35}S]methionine and then immediately cultured in normal medium for the indicated times (in hours). Recipient cells were lysed and subjected to immunoprecipitation analysis with IκBα-specific antiserum.

results were also fully recapitulated in independent transfection studies performed with human Jurkat T cells. (Fig. 3A, lanes 8 and 9).

To investigate the biochemical basis for p65-mediated induction of IκBα synthesis, a panel of p65 deletion mutants was tested in functional transfection studies (Fig. 3B). The principal transactivation domain of p65 has been localized to its unique COOH-terminal region (*20–22*), whereas its DNA binding domain resides within its NH_2-terminal *rel* homology sequences (*15, 19, 20*). Deletion of either the NH_2-terminal 30 amino acids of p65 [p65(31–551)], which disrupts the DNA binding domain (*15*), or the COOH-terminal half of p65 [p65(1–312)], which deletes the transactivation function (*15*), abolished the ability of p65 to induce IκBα (Fig. 3B). In contrast, a functional mutant of p65 lacking only 51 COOH-terminal amino acids [p65(1–500)] retained the ability to induce IκBα. Parallel studies of RNA isolated from these transfectants (Fig. 3C) showed a marked increase in IκBα mRNA expression in cells transfected with functional forms of p65 but not with the transcriptionally inactive mutants of p65. Although effects of p65 on IκBα mRNA stability cannot be entirely excluded, the finding that IκBα induction requires fully functional p65 DNA binding and transactivation domains further strengthens the possibility that p65 activates expression of the IκBα gene at a transcriptional level. One potential explanation for these results is that the human IκBα gene contains a functional κB enhancer element or elements.

A possible function for NF-κB–mediated induction of IκBα would be to provide a tightly controlled mechanism of feedback inhibition in order to down-regulate the potent transcriptional activity of the p65 subunit. For this to be the case, newly synthesized IκBα must be capable of associating with NF-κB. To test this hypothesis, extracts from COS-7 cells transfected with either IκBα or p65 expression vectors were fractionated on a native polyacrylamide gel and then subjected to immunoblotting (Fig. 4A) with either IκBα-specific or p65-specific antiserum. IκBα was readily detected in cells transfected with the IκBα expression vector but not in mock-transfected cells. The predominant form of IκBα detected in cells transfected with the p65 expression vector exhibited retarded mobility relative to free IκBα and virtually comigrated with p65. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the low-mobility immunoreactive species detected in Fig 4A, lane 3, revealed the presence of both p65 and IκBα (Fig. 4B). A direct physical association of IκBα and p65 was further demonstrated in coimmunopre-

cipitation assays (*12*). Thus, in keeping with the late inhibition of DNA binding by NF-κB (Fig. 1), newly synthesized IκBα can physically associate with p65. Furthermore, immunofluorescent staining of COS-7 cells transfected with the p65 expression vector confirmed that the newly synthesized IκBα was primarily localized in the cytoplasm (Fig. 4C), a finding that is fully consistent with the known subcellular location of inactive NF-κB complexes in resting human T cells (*4*, *11*).

Because IκBα rapidly disappears after its release from NF-κB (Fig. 1), another potential mechanism of IκBα regulation could involve the stabilization of this labile molecule when complexed with p65 in the cytoplasm (*7*). To compare the relative half-life ($T_{1/2}$) of survival of free and p65-complexed IκBα, pulse-chase experiments (Fig. 5) were performed in COS-7 cells transfected with an IκBα expression vector in the absence or presence of vectors encoding p65 deletion mutants differing in their capacity to complex with IκBα. These COOH-terminal deletion mutants lacking a transactivation domain were selected to preclude the induction of endogenous IκBα expression. Mutant p65(1–312) possesses a fully functional IκBα binding domain whereas mutant p65(1–270) fails to bind IκBα (*15*). In the absence of either of these truncated p65 proteins, IκBα was rapidly degraded ($T_{1/2} \approx 40$ min). In contrast, in the presence of p65(1–312), the intracellular half-life of IκBα was extended to more than 4 hours. However, with p65(1–270), IκBα exhibited a $T_{1/2}$ indistinguishable from that observed in cells transfected with IκBα alone. These findings suggest that the intracellular survival of IκBα is prolonged when this cytoplasmic inhibitor associates with p65.

In summary, these findings indicate that IκBα is a physiological inhibitor of the heterodimeric NF-κB complex whose expression is induced by NF-κB in activated T cells. Specifically, this particular inhibitor is rapidly degraded in PMA- or TNF-α–stimulated human T cells in concert with the liberation and nuclear import of NF-κB. After degradation of released IκBα, the cytoplasmic reservoir of this inhibitor is completely replenished by NF-κB– induced de novo synthesis of IκBα protein, which is preceded by a marked increase in IκBα mRNA expression. Because IκBα mRNA induction occurs in the presence of translation inhibitors and requires functional coexpression of the transactivation and DNA binding domains of p65, it seems likely that p65 mediates the activation of IκBα gene expression by a direct mechanism. This novel autoregulatory loop provides a dynamic mechanism of feedback control for transcriptional induction mediated by NF-κB p65, thus ensuring its rapid but transient pattern of biological action.

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Activity-Dependent Regulation of Conductances in Model Neurons

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Neurons maintain their electrical activity patterns despite channel turnover, cell growth, and variable extracellular conditions. A model is presented in which maximal conductances of ionic currents depend on the intracellular concentration of calcium ions and so, indirectly, on activity. Model neurons with activity-dependent maximal conductances modify their conductances to maintain a given behavior when perturbed. Moreover, neurons that are described by identical sets of equations can develop different properties in response to different patterns of presynaptic activity.

Most neurons survive for almost as long as the animal in which they are found and can retain stable electrical properties for much of the animal's lifetime. This stability results from a dynamic equilibrium because the ion channels that control the electrical activity of each neuron are replaced by protein turnover and because the neuron may change size or shape. Realistic models of neurons, with multiple active currents, are sensitive to small changes in parameters (*1*, *2*). How then do neurons maintain stable electrical activity?

We suggest a model in which the intrinsic properties of a neuron are regulated by its activity. This model uses the intracellular Ca^{2+} concentration as an indicator of activity, although other intracellular correlates of electrical activity might be involved. In the model, the maximal conductance of each ionic current is a dynamical variable rather than a fixed parameter.

We modified the model of Buchholtz and co-workers (*3*) that was derived from voltage-clamp data from the lateral pyloric

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