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17. The *S. cerevisiae* strains used in all experiments were JK9-3d, a/α, a, or α *leu2-3,112 ura3-52 rme1 trp1 his4 HML*a (the diploid JK9-3da/α is homozygous for all markers except mating type) (*16*), or isogenic derivative of JK9-3d. Isogenic derivatives with only the changes indicated were as follows: JH1-2a, α *fpr1::URA3-1*; JH2-1b, α *ade2 fpr1::ADE2-2*; JH3-3b, α *fpr1::URA3-3* (*16*); JH6-1b, α *TRP1*; JH10, α *fpr1::URA3-3 TRP1*. The alleles *fpr1::URA3-1, fpr1::ADE2-2*, and *fpr1::URA3-3* are the same as *fpr1-1, fpr1-2*, and *fpr1-3* (*16*), respectively. Single and high copy number plasmids bearing *FPR1*, in both cases contained in a 2-kb Eco RI fragment, were YCp50::FPR1 and pYJH23, respectively. The plasmid YCp50::FPR1 is *amp cen ars URA3 FPR1*. The plasmid pYJH23 is *amp 2μ URA3 FPR1* (*16*).
18. Rapamycin was originally isolated as an antifungal compound and described to have potent activity against *Candida albicans* [H. Baker, A. Sidorowicz, S. N. Sehgal, C. Vezina, *J. Antibiot.* **31**, 539 (1978)].
19. FK506 inhibits transport of some amino acids in *S. cerevisiae*. Thus, auxotrophic strain JK9-3d (*trp1 his4*) is sensitive to FK506, and prototrophic derivatives (*TRP1* or *HIS4*) are resistant to FK506 (J. Heitman and M. N. Hall, unpublished data). For unknown reasons, a derivative that is prototrophic for only one amino acid (*TRP1*) becomes resistant although it still depends on import of another amino acid (*his4*). A possible explanation is that multiple auxotrophies are necessary to reach a threshold beyond which amino acids become limiting. Sensitivity of auxotrophic JK9-3d to FK506 appears not to be mediated through an FKBP-FK506 complex because FK506 still inhibit amino acid transport in an *fpr1* mutant.
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23. Antagonism between FK506 and rapamycin in T cells also requires an excess (100- to 500-fold of the limiting amount) of the competing compound (*4, 5*).
24. Oligonucleotides for polymerase chain reaction (PCR) were: 5′ oligo (P130), 5′-AAATTGCCGGAAGAGAGACG-3′; 3′ oligo (P132), 5′-ACGTATGCTTCTCCTTGTC-3′. Asymmetric PCR amplification reactions contained 50 pM P130 (330 ng), 1 pM P132 (6.6 ng), 50 μM dNTP (N = A, G, T, and C), and 10 μl of a 1:100 dilution of yeast genomic DNA (approximately 200 to 400 ng) in a final reaction volume of 100 μl. The reaction mixtures were overlaid with 10 μl of mineral oil and PCR-amplified with a Perkin-Elmers thermocycler for 30 cycles consisting of 95°C for 1 min, 45°C for 1 min, and 70°C for 2 min. Yeast genomic DNA was purified as described [C. S. Hoffman and F. Winston, *Gene* **57**, 267 (1987)]. Oligonucleotides for sequencing were P132 (above), P142 (5′-TAAGGCTCAGATACTTACC-3′), and P133 (5′-TTCACCAACAGACAACTT-3′).
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27. An *fpr1*×*tor1-1* diploid yielded 4 4R:0S, 1 2R:2S, and 14 3R:1S tetrads: *fpr1*×*tor2-1* diploid, 3 4R:0S, 6 2R:2S, and 22 3R:1S; *tor1-1*×*tor2-1* diploid, 6 4R:0S, 4 2R:2S, and 18 3R:1S.
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Factor XI Activation in a Revised Model of Blood Coagulation

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Coagulation factor XI is activated in vitro by factor XIIa in the presence of high molecular weight kininogen (HMWK) and a negatively charged surface. Factor XII deficiency is not associated with bleeding, which suggests that another mechanism for factor XI activation exists in vivo. A revised model of coagulation is proposed in which factor XI is activated by thrombin. In the absence of cofactors, thrombin is more effective (k_{cat}/K_m = 1.6 × 10^5) than factor XIIa (1.7 × 10^4) in activating factor XI. Dextran sulfate enhances activation of factor XI by thrombin 2000-fold; part of this effect is due to autoactivation of factor XI by activated factor XI.

IN THE CASCADE (*1*) OR WATERFALL (*2*) hypothesis of hemostasis, blood coagulation proceeds through a series of reactions in which plasma zymogens of serine proteases are sequentially activated by limited proteolysis, culminating in the generation of thrombin, which then cleaves fibrinogen to produce fibrin. The putative "intrinsic" pathway of coagulation is initiated when blood is exposed to a negatively charged surface, which activates factor XII in a reaction involving high molecular weight kininogen (HMWK) and prekallikrein (*3*). Activated factor XII (factor XIIa) then activates factor XI (*4*) and factor XIa, in turn, is responsible for the activation of factor IX (*5*). It is apparent that factor XI is important in normal hemostasis because individuals deficient in factor XI suffer a variable bleeding diathesis that may be particularly severe after surgery (*6*). In contrast, deficiency of factor XII, HMWK, or prekallikrein is not associated with bleeding, suggesting that an alternative mechanism for the activation of factor XI exists in vivo. In a recent, revised model of coagulation, factor XI is not required for the initiation of coagulation, but instead, functions to sustain hemostasis after coagulation has been induced by the exposure of plasma to tissue factor at a site of injury (*7*). We therefore investigated the ability of thrombin, an enzyme generated late in the coagulation cascade, to activate factor XI.

Factor XI is a 160-kD homodimer composed of two identical 83-kD polypeptides connected by a disulfide bond. Each factor XI monomer is cleaved by factor XIIa at a single site, resulting in a 47-kD heavy chain and a 35-kD light chain, the latter of which contains the catalytic serine-protease site (*4*). Initial experiments demonstrated that thrombin cleaves factor XI to yield a pattern

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after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) identical to that produced by factor XIIa (Fig. 1A). No cleavage of factor XI occurs if thrombin is preincubated with the thrombin-specific inhibitor hirudin (*8*). Factor XIIa cleaves factor XI between amino acids Arg^{369} and Ile^{370} (*4*). The Lys-Pro-Arg sequence immediately preceding the cleavage site is a typical sequence for thrombin action (*9*).

Thrombin-mediated cleavage activates factor XI (*10*). The product hydrolyzes the chromogenic substrate (Glu-Pro-Arg-paranitroanalid, S-2366) (*11*) and activates factor IX in the presence of calcium ions (*12*). The kinetic constants for the activation of factor XI by thrombin and factor XIIa were determined (Fig. 1, B and C). The K_m, k_{cat}, and derived catalytic efficiency (k_{cat}/K_m) for the activation of factor XI by thrombin are 50 nM, 7.8×10^{-3}/min, and 1.6×10^5; the corresponding constants for factor XIIa activation of factor XI are ~2 μM, 3.4×10^{-2}/min, and 1.7×10^4, respectively. In the absence of additional cofactors, both thrombin and factor XIIa activated factor XI poorly, but the data predict that at concentrations of factor XI (30 to 60 nM) typically found in plasma thrombin would be the more potent activator.

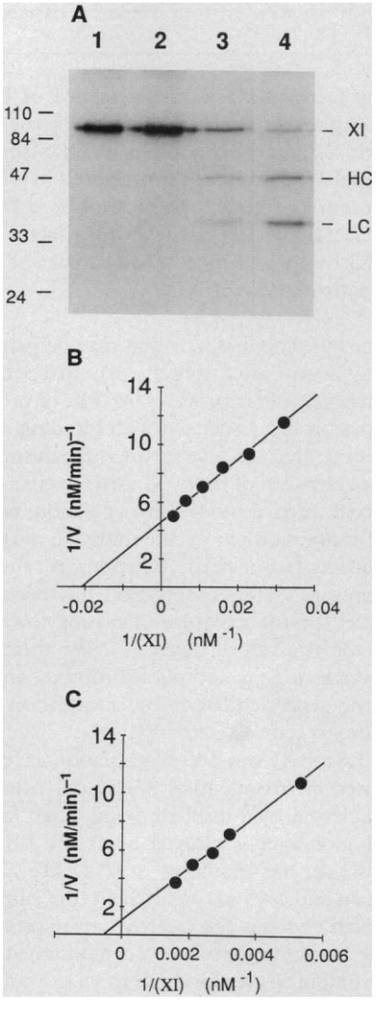

Fig. 1. Activation of factor XI by thrombin (*10*). (**A**) Cleavage of ^{125}I-labeled factor XI by thrombin and factor XIIa. ^{125}I-labeled factor XI (150 nM) was incubated for 60 min at 37°C with either thrombin (100 nM) or factor XIIa (100 nM) in tris-NaCl. Reduced samples were electrophoresed on a 15% SDS-polyacrylamide gel and examined by autoradiography. Lane 1, uncleaved factor XI; lane 2, factor XI with thrombin and hirudin, 25 units/ml; lane 3, factor XI with thrombin; lane 4, factor XI with factor XIIa. (XI) uncleaved factor XI, (HC) factor XIa heavy chain, (LC) factor XIa light chain. Relative molecular sizes are shown on the left in kilodaltons. (**B** and **C**) Lineweaver-Burke plots for activation of factor XI by thrombin (B) and factor XIIa (C). Factor XI was incubated with 25 nM thrombin or factor XIIa for 5 min and the production of factor XIa was determined by chromogenic assay (*18*). The K_m for activation by factor XIIa could not be determined with precision because it exceeded the achievable factor XI concentration.

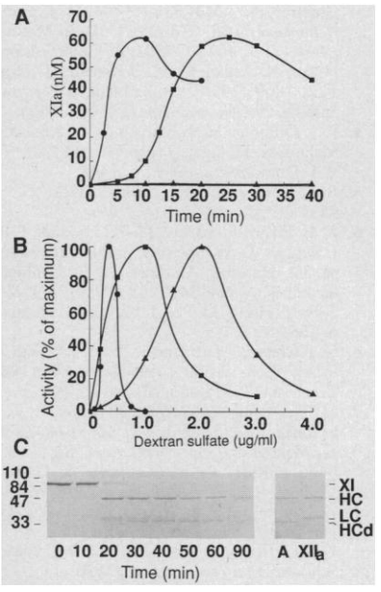

Fig. 2. Effect of dextran sulfate on factor XI activation. (**A**) Factor XI (60 nM) was incubated with (●) 1.25 nM thrombin and dextran sulfate (1 μg/ml) (average MW 500 kD), (■) dextran sulfate (1 μg/ml), or (▲) in the absence of thrombin and dextran sulfate. Samples obtained at the designated times were assayed for factor XIa production by chromogenic substrate assay (*18*). The inclusion of hirudin and CTI in reactions containing factor XI and dextran sulfate did not effect the apparent autoactivation. (**B**) Effect of dextran sulfate concentration on autoactivation of factor XI. Factor XI (●) 30 nM, (■) 60 nM, or (▲) 120 nM was incubated for 10 min at 37°C in the presence of dextran sulfate. Factor XIa production was determined by chromogenic assay (*18*) and plotted as the percent of maximum activation for each factor XI concentration. (**C**) Factor XI autoactivation in the presence of dextran sulfate. Factor XI (60 nM) was incubated at 37°C in tris-NaCl with dextran sulfate (1 μg/ml) and hirudin (25 units/ml). Reduced samples obtained at the indicated times were electrophoresed on a 15% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lane A, the final cleavage product of factor XI autoactivation; lane XIIa, factor XI cleaved by factor XIIa. (XI) uncleaved factor XI, (HC) factor XIa heavy chain, (LC) factor XIa light chain, and (HCd) a degradation fragment of factor XIa heavy chain. Relative molecular weights are shown on the left in kilodaltons.

Factor XI activation through the intrinsic pathway is facilitated by a negatively charged surface, such as silica or dextran sulfate (*3, 13*). Although dextran sulfate enhances the activation of factor XI by thrombin at least 2000-fold, it appears that part of this effect is independent of thrombin because factor XI is activated in the presence of dextran sulfate alone (Fig. 2A). The sigmoidal shape of the activation curves is consistent with autoactivation of factor XI by factor XIa. In the absence of thrombin, this autoactivation is probably initiated by trace amounts of factor XIa present in the factor XI preparation (*10*). The optimal concentration of dextran sulfate for autoactivation (Fig. 2B) and thrombin-triggered activation of factor XI are the same, and vary with the concentration of factor XI, suggesting that a substantial portion of factor XI activation in the presence of thrombin and dextran sulfate is due to autoactivation. The pattern of factor XI cleavage during autoactivation appears similar to that induced by thrombin and factor XIIa; however, with time there is apparent degradation of both the heavy and light chains of factor XIa, corresponding to a loss in amidolytic activity (Figs. 2C and 3B).

The marked enhancement of factor XI activation by thrombin and factor XIa in the presence of dextran sulfate suggests that a polyanionic cofactor such as a proteoglycan or glycosaminoglycan may be required for optimal activation in vivo. Thrombomodulin (TM) is an integral membrane glycoprotein, found on the luminal surface of blood vessel endothelium, that enhances thrombin activation of the anticoagulant protein, protein C, (*14*). The soluble human recombinant TMs, rTM-105 that contains an endogenous galactosaminoglycan and rTM-75 that lacks the galactosaminoglycan (*15*), both enhance the activation of protein C by thrombin about 200-fold in the chromogenic substrate assay (*16*). In reactions with factor XI and thrombin, rTM-105 enhances factor XI activation 10- to 20-fold, whereas rTM-75 is associated with a 0- to 2-fold increase (*16*). Neither of the recombinant TMs promote autoactivation of factor XI. The modest enhancement by TM of factor

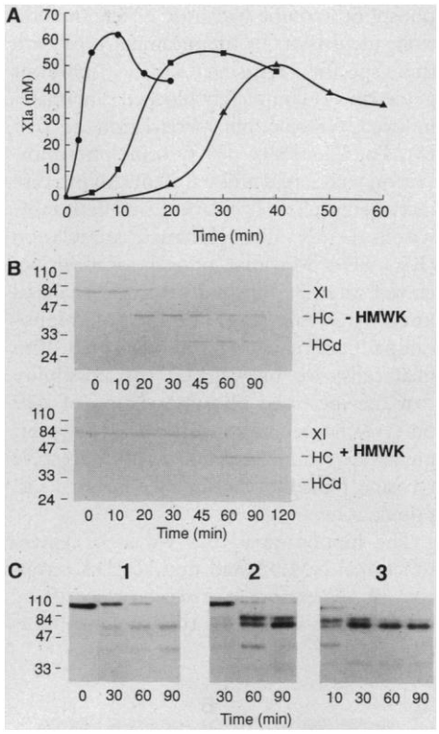

Fig. 3. HMWK and factor XI activation (*10*). (**A**) Effect of HMWK on thrombin activation of factor XI. Factor XI (60 nM) was preincubated for 10 min with (●) 0, (■) 225 nM, or (▲) 450 nM HMWK prior to the addition of 1 μg/ml dextran sulfate, 1.25 nM thrombin, and further incubation at 37°C. Factor XIa production was determined by chromogenic assay (*18*). (**B**) Western immunoblot of factor XI autoactivation. Factor XI (60 nM) was preincubated with or without 500 nM HMWK for 10 min prior to the addition of dextran sulfate (1 μg/ml) and further incubation at 37°C. Western blotting of samples obtained at the indicated times were performed as described (*24*) with the use of a mouse monoclonal antibody that recognizes the factor XIa heavy chain. (XI) uncleaved factor XI, (HC) factor XIa heavy chain, and (HCd) a degradation product of the factor XIa heavy chain. (**C**) Cleavage of HMWK during factor XI activation. ^{125}I-labeled HMWK (500 nM) was incubated in the presence of dextran sulfate (1 μg/ml) at 37°C in tris-NaCl and (1) 5 nM thrombin, (2) 60 nM factor XI, or (3) 5 nM thrombin and 60 nM factor XI. Reduced samples obtained at the indicated times were electrophoresed on a 15% SDS–polyacrylamide gel and autoradiographed. Relative molecular weights are shown on the left in kilodaltons.

XI activation by thrombin is unlikely to be physiologically relevant, but supports the notion that a glycosaminoglycan or proteoglycan may serve as a cofactor for factor XI activation.

Factor XI circulates in plasma as a complex with HMWK, a 110-kD glycoprotein containing the vasoactive peptide bradykinin (*17*). HMWK, at physiologic concentrations (500 nM), depresses thrombin activation of factor XI by 40 to 50% (*18*). In the presence of dextran sulfate, HMWK inhibits activation of factor XI by thrombin (Fig. 3A) and by autoactivation (Fig. 3B) in a time-dependent fashion. Both factor XIa and thrombin cleave HMWK, although different patterns after SDS-PAGE are generated (Fig. 3C). Thrombin-mediated cleavage of HMWK is dependent on the presence of dextran sulfate, whereas factor XIa–mediated cleavage of HMWK occurs with or without dextran sulfate. The pattern of HMWK proteolysis seen in the reaction including thrombin, factor XI, and dextran sulfate is similar to the pattern produced during factor XI autoactivation.

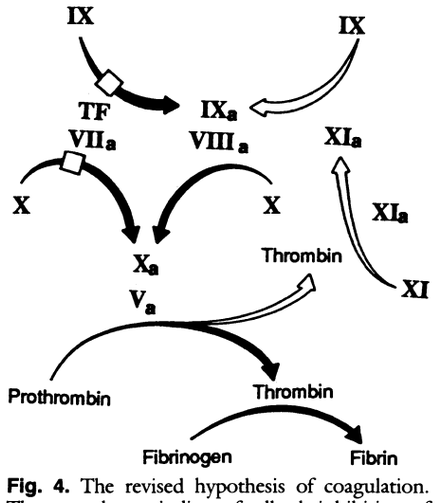

Fig. 4. The revised hypothesis of coagulation. The open boxes indicate feedback inhibition of the factor VIIa–tissue factor complex by LACI (*7*). The open arrows indicate the thrombin-initiated pathway for factor XI activation. The requirement for phospholipids and calcium in certain reactions is not included.

The observation that patients deficient in factor XII, HMWK, or prekallikrein do not bleed, despite in vitro clotting abnormalities (*19*), suggests that a factor XIIa–independent mechanism for the activation of factor XI exists in vivo. In a current model of hemostasis, coagulation is initiated when factor VIIa in plasma comes in contact with its cofactor, tissue factor (TF), which is produced constitutively by cells underlying the blood vessel endothelium (*7*). The factor VIIa–TF complex catalyzes the formation of a small amount of activated factor X (factor Xa) (*20*) and factor IX (factor IXa) (*21*) before it is rapidly inactivated by lipoprotein-associated coagulation inhibitor (LACI) (*7*) (Fig. 4). Additional factor Xa is produced by the action of factor IXa in the presence of its cofactor, factor VIII (*22*). The severe bleeding diathesis seen in patients with hemophilia who lack either factor IX or factor VIII indicates that this formation of additional factor Xa is critical for normal hemostasis. Incorporation of the current data into this scheme (Fig. 4) suggests that thrombin produced through the initial action of factor VIIa–TF could activate factor XI. The factor XIa thus generated, and possibly added to by autoactivation of additional factor XI, would sustain coagulation by activating more factor IX (*23*). These steps may require the presence of a cofactor, possibly a glycosaminoglycan or proteoglycan.

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10. All coagulation factors were isolated from human plasma. Factor XI was prepared by a modification of the method of B. N. Bouma and J. H. Griffin, *J. Biol. Chem.* **252**, 6432 (1977), with the use of an anti–factor XI monoclonal immunoglobulin G affinity column. The preparation appeared homogeneous by SDS-PAGE under reducing and non-reducing conditions and had an activity of 160 units/mg. Approximately 0.2% of the preparation was factor XIa as determined by chromogenic assay. Thrombin was prepared from prothrombin [J. Miletich, C. Jackson, P. W. Majerus, *J. Biol. Chem.* **253**, 6908 (1978)]. A single major band at 35 kD was seen on SDS-PAGE. The preparation did not activate prekallikrein, indicating it was not contaminated with factor XIIa. Its specific activity was 2700 units/mg. Factor XIIa and HMWK were purchased from Enzyme Research Laboratories (Indianapolis, IN). Factor XII was activated by incubation with dextran sulfate (50 μg/ml) and separated from dextran sulfate by Amberlite CG-50 chromatography. Factor XIIa appeared homogeneous by SDS-PAGE. After treatment with corn trypsin inhibitor, the isolated factor XIIa did not induce factor XI autoactivation, indicating that essentially all of the dextran sulfate had been removed.
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16. Human protein C (500 nM) or factor XI (60 nM) was incubated with recombinant thrombomodulin (15 nM) in 200 μl of tris-NaCl, 25 μM ZnCl for 60 min at 37°C in the presence or absence of thrombin (2 nM). Calcium, at previously determined optimal concentrations for each type of rTM, was included in samples containing protein C (*15*). Reactions were terminated by addition of hirudin to a final concentration of 25 units/ml and factor XIa production was determined by chromogenic assay (*18*). A higher concentration of thrombomodulin did not increase the production of factor XIa.
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Stimulation of Protein Tyrosine Phosphorylation by NMDA Receptor Activation

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The *N*-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptors, plays a key role in synaptic plasticity in the nervous system. After NMDA receptor activation, calcium entry into the postsynaptic neuron is a critical initial event. However, the subsequent mechanisms by which the NMDA receptor signal is processed are incompletely understood. Stimulation of cultured rat hippocampal cells with glutamate resulted in the rapid and transient tyrosine phosphorylation of a 39-kilodalton protein (p39). Tyrosine phosphorylation of p39 was triggered by the NMDA receptor and required an influx of Ca^{2+} from the extracellular medium. Because p39 was found to be highly related or identical to the microtubule-associated protein 2 kinase, the NMDA receptor signal may be processed by a sequential activation of protein kinases.

GLUTAMATE IS THE PRINCIPAL EXCITATORY neurotransmitter in the mammalian central nervous system and acts on postsynaptic neurons through glutamate receptors that fall into two main categories, the NMDA and non-NMDA subtypes (*1*). NMDA receptors are critical for long-lasting physiological modifications of neurons (*2*) and neuronal degeneration (*3*). However, the intracellular mechanisms of NMDA receptor signaling and the biochemical processes through which these receptors modify neuronal properties and connections during development and in the mature nervous system are unclear. Alterations in neuronal phenotype have been suggested to involve protein phosphorylation (*4*). Because tyrosine kinases are particularly abundant in neurons (*5*), we tested the hypothesis that protein tyrosine phosphorylation plays a role in propagating the NMDA receptor signal.

Phosphotyrosine-containing proteins in primary rat hippocampal cells were examined before and after glutamate stimulation (*6*). Immunoblot analyses with two distinct antibodies to phosphotyrosine demonstrated that stimulation of hippocampal cells with 10 μM glutamate rapidly induced an increase in tyrosine phosphorylation of a 39-kD protein (p39) (Fig. 1A). Several other phosphotyrosine-containing polypeptides were also detected, but these did not undergo any apparent change in the amount of tyrosine phosphorylation upon glutamate stimulation. The specificity of the polyclonal antibodies for phosphotyrosine was demonstrated by incubation of the antibody with 5 mM phosphotyrosine, which prevented subsequent binding to p39. In contrast, treatment with 5 mM phosphoserine or 5 mM phosphothreonine had little effect. In addition, incubation of hippocampal cells with the specific tyrosine kinase inhibitor genistein (*7*) completely blocked glutamate-induced tyrosine phosphorylation of p39 (*8*). The kinetics of p39 tyrosine phosphorylation were rapid and transient. An increase in tyrosine phosphorylation was detectable within 1 min after glutamate stimulation (Fig. 1D). Maximal phosphorylation occurred after 3 min and returned to basal levels by 60 min (Fig. 1D). Because hippocampal cultures contain about 10 to 20% glial cells, we investigated the possibility that the increased phosphorylation of p39 on tyrosine occurs in glial cells. However, glutamate stimulation failed to induce p39 tyrosine phosphorylation in pure glial cell populations.

The hippocampal cells we used express functional NMDA and non-NMDA receptors (*9*). Therefore, the receptor specificity of glutamate-stimulated tyrosine phosphor-

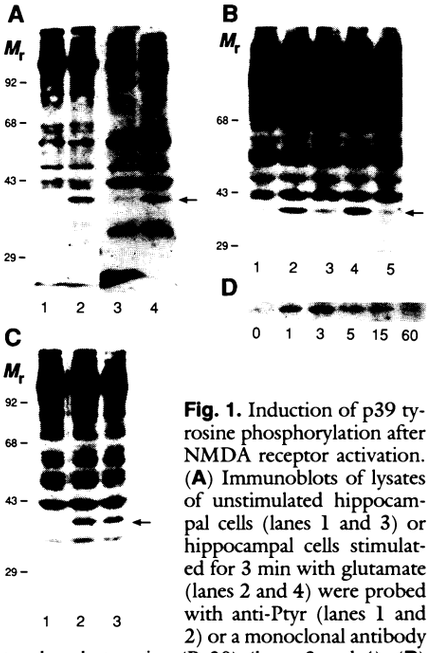

Fig. 1. Induction of p39 tyrosine phosphorylation after NMDA receptor activation. (**A**) Immunoblots of lysates of unstimulated hippocampal cells (lanes 1 and 3) or hippocampal cells stimulated for 3 min with glutamate (lanes 2 and 4) were probed with anti-Ptyr (lanes 1 and 2) or a monoclonal antibody to phosphotyrosine (Py20) (lanes 3 and 4). (**B**) Anti-Ptyr immunoblot analyses of unstimulated hippocampal cells (lane 1), cells stimulated for 3 min with glutamate (lane 2), or cells stimulated for 3 min with glutamate in the presence of 100 μM APV (lane 3), 40 μM CNQX (lane 4), or 1 mM sodium kynurenate and 11.3 mM $MgCl_2$ (lane 5). Cells were pretreated with APV, CNQX, or sodium kynurenate and 11.3 mM $MgCl_2$ for 10 min before glutamate stimulation. (**C**) Anti-Ptyr immunoblot analyses of unstimulated hippocampal cells (lane 1) and cells stimulated for 3 min (lane 2) and 5 min (lane 3) with 50 μM NMDA. The arrows in (A), (B), and (C) indicate p39. M_r, ^{14}C-labeled protein molecular weight standards ($\times 10^{-3}$). (**D**) Time course (in minutes) of the glutamate-induced increase in p39 tyrosine phosphorylation. Only part of the immunoblot is shown.

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