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Src, a molecular switch governing gain control of synaptic transmission mediated by *N*-methyl-D-aspartate receptors

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ABSTRACT **The *N*-methyl-D-aspartate (NMDA) receptor is a principal subtype of glutamate receptor mediating fast excitatory transmission at synapses in the dorsal horn of the spinal cord and other regions of the central nervous system. NMDA receptors are crucial for the lasting enhancement of synaptic transmission that occurs both physiologically and in pathological conditions such as chronic pain. Over the past several years, evidence has accumulated indicating that the activity of NMDA receptors is regulated by the protein tyrosine kinase, Src. Recently it has been discovered that, by means of up-regulating NMDA receptor function, activation of Src mediates the induction of the lasting enhancement of excitatory transmission known as long-term potentiation in the CA1 region of the hippocampus. Also, Src has been found to amplify the up-regulation of NMDA receptor function that is produced by raising the intracellular concentration of sodium. Sodium concentration increases in neuronal dendrites during high levels of firing activity, which is precisely when Src becomes activated. Therefore, we propose that the boost in NMDA receptor function produced by the coincidence of activating Src and raising intracellular sodium may be important in physiological and pathophysiological enhancement of excitatory transmission in the dorsal horn of the spinal cord and elsewhere in the central nervous system.**

Appropriate modification of the transmission of information at synapses in the central nervous system (CNS) is essential for physiological processes such as development, learning, and memory. On the other hand, inappropriate alteration of synaptic transmission is a fundamental underpinning of various pathological conditions, including epilepsy and chronic pain. In the case of chronic pain, enhanced transmission in nociceptive pathways, i.e., pathways conveying pain-related information, is known from animal experiments to occur at various levels of the neuraxis including the dorsal horn of the spinal cord (1–3) and the trigeminal nucleus caudalis, the homologous region in the brainstem (4, 5). Synaptic transmission at fast excitatory synapses in the dorsal horn, as in most regions of the CNS, is mediated by glutamate receptors, and there is a growing body of evidence indicating that these receptors are crucial in conditions of enhanced nociceptive transmission (6–8). Activation of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor, in particular, appears critical for the initiation and maintenance of the enhanced responsiveness of dorsal horn nociceptive neurons that occurs in experimental pain models (4, 5, 9–11). The function of NMDA receptors, rather than being fixed at one level, is modulated over a wide range, and thus understanding the processes by which this modulation occurs has the potential to shed new light on our understanding of pathological alterations of synaptic transmission in chronic pain and other conditions in the CNS.

Over the past several years, it has become apparent that a fundamental process for regulating the function of NMDA receptors and other ion channels in neurons is tyrosine phosphorylation (12–16). The protein tyrosine kinase (PTK) Src has been identified as an endogenous PTK regulating NMDA receptor function (17). Src is one of the most well studied of the PTKs (for review, see ref. 18) and is highly expressed in the CNS (19, 20). Paradoxically, the functions of Src in the nervous system had been enigmatic. Recent observations indicate that the regulation of NMDA receptors by Src may mediate the induction of a form of synaptic plasticity known as long-term potentiation (LTP) in the hippocampus. Below, we outline the evidence for Src regulation of NMDA receptors (17) and the role of this kinase in LTP induction (21). We also describe novel observations showing that Src kinase governs the regulation of NMDA receptors by intracellular sodium (22).

NMDA Receptors and Regulation by Tyrosine Phosphorylation. NMDA receptors, as a main subtype of glutamate receptor, participate in rapid excitatory synaptic transmission in the spinal cord and throughout the CNS (23). NMDA receptors are members of the superfamily of ligand-gated ion channels, and a variety of NMDA receptor subunit proteins (NR1, NR2A-D, NR3) (24, 25) have been identified by using molecular cloning. Native NMDA receptors appear to be heterooligomeric complexes with the second membrane region of the subunits coming together to form a conductance pathway that is selectively permeable to cations (24, 26). NMDA receptors are activated by the binding of two molecules of glutamate (27) and two molecules of glycine, which acts as a coagonist at an extracellular site on the channel complex (28). Native NMDA receptors likely consist of one or more NR1 subunits, which may bind glycine (29, 30), a glutamate-binding subunit, NR2 (31), and possibly the more recently identified subunit, NR3 (32, 33).

Activated NMDA channels are permeable to monovalent cations, such as Na^+ and K^+, and also divalent cations, the most important of which is Ca^{2+} (34, 35). NMDA receptors are known to be regulated (23) at diverse extracellular (36–40) as well as intracellular sites (41), and the key intracellular process regulating NMDA receptor function is phosphorylation (42). Both serine/threonine (43–52) and tyrosine (12) phosphorylation have been shown to regulate NMDA receptor function.

In terms of NMDA receptor regulation by tyrosine phosphorylation, it has been found that when recombinant purified

Abbreviations: CNS, central nervous system; LTP, long-term potentiation; mEPSCs, miniature excitatory postsynaptic currents; NMDA, *N*-methyl-D-aspartate; NR, NMDA receptor; PTK, protein tyrosine kinase; SH2, Src-homology 2; SH3, Src-homology 3; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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protein tyrosine kinase (PTK) or protein tyrosine phosphatase (PTP) enzymes are applied into neurons, the whole-cell currents through native NMDA receptors are increased by PTK and are decreased by PTP (e.g., Fig. 1). Conversely, applying PTK inhibitors has been found to decrease NMDA currents, whereas PTP inhibitors potentiate these currents (12), indicating that native NMDA receptors are controlled by the balance of PTK and PTP activity. The increase in the ensemble NMDA currents that are measured by using the whole-cell recording method was found to be caused by increased activity of individual NMDA channels and there is no change in the single-channel conductance (53). This increase in NMDA channel activity is produced through enhancing the gating of already active receptors rather than through recruiting previously inactive NMDA receptors (54).

As it is known that NMDA receptor subunit proteins, in particular NR2A (55) and NR2B (56), are phosphorylated on tyrosine it is logical to ask whether the up-regulation of NMDA receptor function is due to phosphorylation of the subunit proteins themselves. Investigating this would appear to be technologically feasible, because in addition to increasing the function of native NMDA receptors, PTKs have been found to potentiate the function of recombinant NMDA receptors expressed heterologously (57, 58). The number of tyrosine residues on presumed intracellular domains of NR2A and -2B is large (54), but some residues are better candidates than others, depending on the sequence of the surrounding amino acids, which confers selectively for particular PTKs or groups of PTKs (59). Recently, through mutagenesis of residues in the C-terminal region of NR2A, three tyrosine residues in this region were found to be necessary for the up-regulation of recombinant NR1/NR2A receptors expressed in HEK293 cells (60). This combination of receptor subunits was known to be especially sensitive to inhibition by Zn^{2+} (61), and evidence was found indicating that the enhancement of currents by tyrosine phosphorylation was caused by removal of this inhibition for NR1/NR2A, and also for NR1/NR2B, receptors. These findings are surprising and intriguing (62) because the site for inhibition by Zn^{2+} is on the extracellular region of the receptor, whereas tyrosine phosphorylation is presumed to occur at an intracellular site. Thus, there must be an unknown

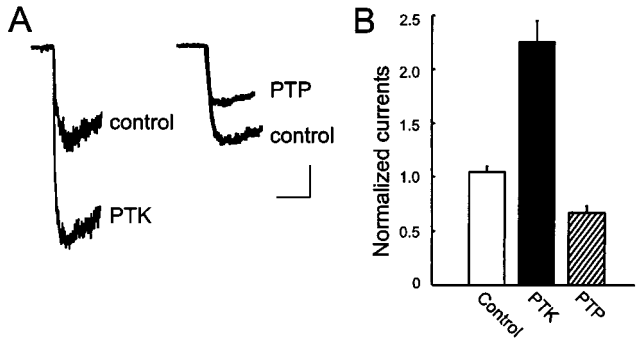

FIG. 1. NMDA currents are up-regulated by protein tyrosine kinase and down-regulated by protein tyrosine phosphatase. NMDA receptor-mediated currents recorded by using the whole-cell patch configuration were evoked by pressure application of L-aspartate (200 μM) at an interval of 1 min from a pressure pipette whose tip was positioned within 100 μm from the recorded neuron. *A* shows individual whole-cell current traces taken before (Control) or 20 min after the start of recording with PTK (src; 30 units/ml) or PTP (truncated T cell PTP; 100 μM) in the intracellular solution. *B* Normalized peak NMDA currents recorded with standard intracellular solution (Control; $n = 11$ neurons), or intracellular solution supplemented with PTK ($n = 8$) or PTP ($n = 7$). For each neuron peak, NMDA current is normalized by dividing the amplitude of current recorded at 20 min after the start of the recording (I_{20}) by that of the initial current (I_1). (Bar = 2 sec and 200 pA.)

mechanism for transmitting the effect of phosphorylation from the inside of the membrane to the outside.

These observations foreshadow a new mechanism that has potential relevance to the general issue of the regulation of ion channels. However, whether this mechanism applies to native NMDA receptors is doubtful, because it has been found that NMDA channel function is up-regulated by tyrosine kinase activity even when Zn^{2+} is chelated (63), and NMDA channels with low single-channel conductance, characteristic of NMDA channels that are insensitive to Zn^{2+} (64), are also up-regulated by PTKs (54). Therefore, it appears that removal of Zn^{2+} inhibition is not the means by which the function of native NMDA receptors is up-regulated by tyrosine phosphorylation. Thus, for native NMDA channels, the question of whether phosphorylation at the sites implicated by mutagenesis is the means for up-regulating NMDA channel function remains open. It is alternatively possible that this up-regulation is through phosphorylation of other tyrosine residues in the NMDA receptor subunit proteins or by phosphorylation of an associated protein, such as one of many proteins already known to bind to the receptors (65–68).

Src Is an Endogenous PTK That Up-Regulates NMDA Receptor Function. Once it had been determined that NMDA receptor function is regulated by PTKs and PTPs, a central question to be addressed was that of identifying the endogenous enzymes involved. Notionally, this is not a trivial task, because the mammalian genome is expected to encode more than a thousand PTKs (69) and nearly as many PTPs (70). Many of these enzymes are known to be expressed in the spinal cord and elsewhere in the CNS (20, 71), providing numerous potential candidates for the endogenous enzymes. Nevertheless, an endogenous PTK regulating NMDA receptors has been identified, as described below, and there is preliminary evidence for a possible PTP (72).

It is well known that PTKs fall into two main categories: receptor and nonreceptor kinases (73, 74). Within each of these categories, there are numerous families with common features, in terms of primary sequence and domain structure. These common features have permitted the development of pharmacological tools, including peptides and antibodies with activity against particular families of enzymes, that have allowed the screening of broad groups of kinases. We took advantage of such reagents during our hunt to discover the PTK regulating NMDA receptors (17, 54). As a first step, we used a reagent that activates PTKs in the Src family: the phosphopeptide, EPQ(pY)EEIPIA (75), which was found to enhance NMDA channel function. Conversely, channel function is depressed by an antibody, anti-cst1, which inhibits Src-family PTKs (76). These results indicated that the endogenous PTK was a member of the Src family.

The family of Src kinases comprises a total of nine members, five of which—Src, Fyn, Lyn, Lck, and Yes—are known to be expressed in the CNS. All members of the Src family contain highly homologous regions—the C-terminal, catalytic, Src homology 2, and Src homology 3 domains (77). The various members do, however, have substantial differences in a region of low sequence conservation near the N terminus known as the unique domain. Therefore, reagents directed against this domain may distinguish between the various Src family members.

Src was identified as the specific member of the family that regulates NMDA channel function by means of testing one such reagent, the antibody, anti-src1, which selectively blocks the function of Src but not other members of the Src family (78). It was found that anti-src1 caused a decrease in NMDA channel activity when this antibody was applied to the cytoplasmic face of membrane patches containing NMDA channels (e.g., Fig. 2*A*). In contrast, a control IgG had no effect. Moreover, anti-src1 prevented the enhancement of NMDA channel activity by EPQ(pY)EEIPIA, indicating that Src is

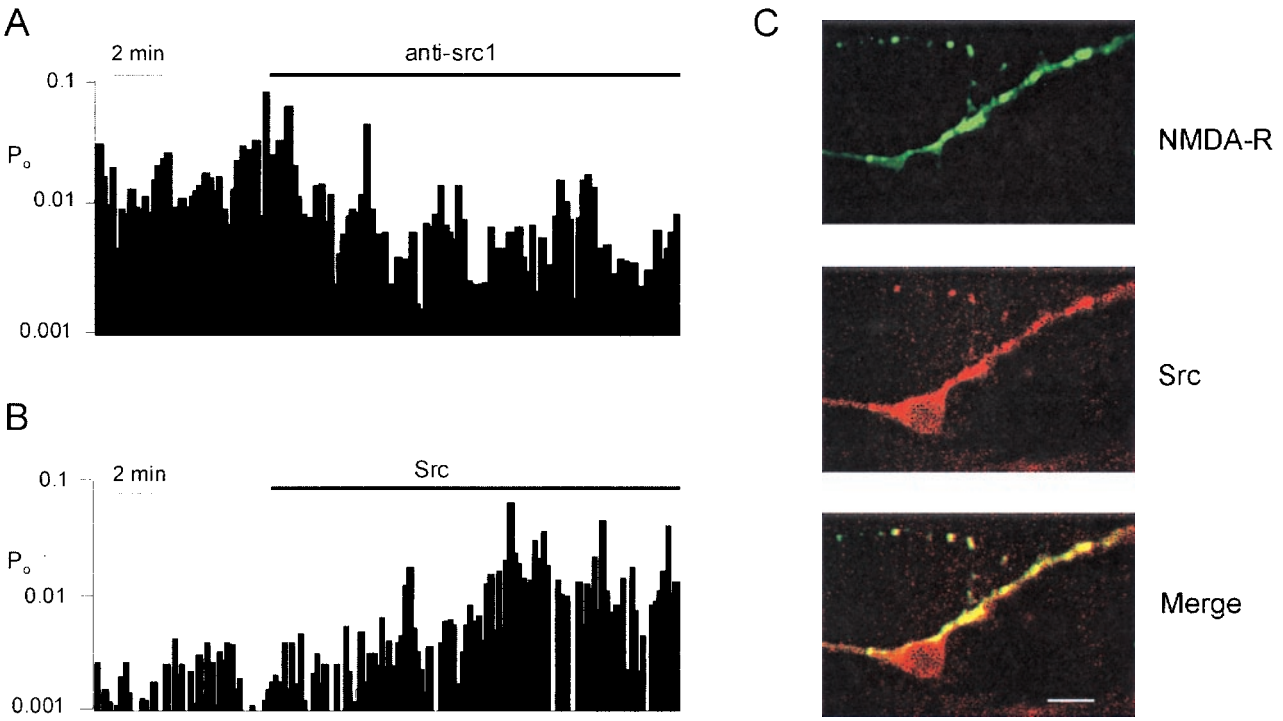

FIG. 2. Regulation of NMDA receptor single-channel activity in inside-out patches by Src and overlapping distribution of Src and NMDA receptor subunit proteins. (*A*)A continuous record of NMDA channel-open probability (P_o). Anti-src1 was applied to the cytoplasmic face of the patch during the period indicated. P_o was calculated in bins of 10 sec. (*B*) A record of NMDA channel P_o from a different inside-out patch with Src applied to the cytoplasmic side as indicated. (*C*) Confocal images show immunofluorescent labeling of a dorsal horn primary culture by antibodies recognizing NR2A/B subunit proteins (green; courtesy of R. Wenthold, National Institutes of Health, Bethesda, MD) or Src (red). *Bottom* shows the merged images; areas showing overlapping fluorescence are yellow. We found similar colocalization when anti-NR1 and anti-Src antibodies were used. Also, experiments without primary antibodies or with primary antibodies incubated with the respective immunogen peptides showed no labeling. (Bar = 10 μm.)

necessary for the effect of the activating peptide. As would be anticipated if Src indeed up-regulates NMDA receptors, we found that applying recombinant $pp60^{c\text{-}src}$ increased NMDA channel activity (Fig. 2*B*), an effect not produced by heat-inactivating the kinase just before use.

Anti-src1 does not bind to the catalytic domain of Src, and we were therefore curious as to its mechanism of action. Along this line, it was determined that applying a peptide, Src(40–58), comprising the region in Src which the antibody recognizes, i.e., amino acids 40–58, to the cytoplasmic side of membrane patches reduced NMDA channel activity. A control peptide with the same amino acid composition but in random order, scrambled Src(40–58), had no effect on channel function. Because it was found that Src(40–58) did not block *in vitro* phosphorylation of a small substrate peptide by recombinant Src, we concluded that amino acids in the region 40–58 may interact with a component of the receptor complex and that this interaction is necessary for the effect of Src on NMDA channels. The region 40–58 is within the unique domain of Src, and these results implicated this domain as being functionally important in this well known enzyme.

Through kinetic analysis of the NMDA channel activity in the patches, it was determined that the effect of Src is due to an increase in channel gating during single activations of the receptor. This is relevant because synaptic responses mediated by NMDA receptors are caused by single receptor activations (79). Thus, if NMDA receptors that are synaptically stimulated are affected similarly to the receptors in the patches, which are by necessity extrasynaptic, it was predicted that Src should increase synaptic NMDA responses. This was confirmed in studies of spontaneously occurring miniature excitatory postsynaptic currents (mEPSCs) (17). Consistent with these electrophysiological results, it was found by using immunocytochemistry that the distribution of Src within neurons overlaps with that of NMDA receptors (Fig. 2*C*) and that Src is localized at sites where NMDA receptors are highly enriched, presumably at synapses.

Whether Src is physically associated with NMDA channels could not be determined from any of the previous experiments: membrane patches are large in comparison with the size of the proteins that comprise ion channels, and confocal microscopy does not have sufficient spatial resolution. While it was therefore possible that Src was separate from the NMDA receptor complex, we found that Src and NMDA receptor subunit proteins coprecipitate, demonstrating that Src is associated with the NMDA channel complex. The coprecipitation might be via a direct interaction between Src and an NMDA receptor subunit protein or, alternatively, it is possible that Src associates with NMDA channels by means of an intervening adaptor protein. Taking all of the information together, it was concluded that Src is physically associated with and up-regulates the function of NMDA receptors.

Src is expressed at high levels within the CNS with a number of neuron-specific isoforms being generated by alternative splicing of one or more cassettes (19, 80, 81) after amino acid 114, which is in the SH3 domain. In the nervous system, Src has been found to be localized both pre- and postsynaptically (20, 82). The postsynaptic localization is especially relevant to the modulation of NMDA receptor function because Src has been found in the postsynaptic density (82, 83), which is the main structural component of excitatory synapses and is where glutamate receptors are concentrated.

Src Up-Regulation of NMDA Receptors in Hippocampal LTP. The studies described above implicating Src in the up-regulation of NMDA receptor function were focused primarily on neurons from the spinal cord dorsal horn. Because NMDA receptors and Src are widely expressed in the nervous system, there is the possibility that by regulating the activity of

postsynaptic NMDA receptors, tyrosine phosphorylation/dephosphorylation might modulate the efficacy of synaptic transmission in many regions of the CNS. One region where Src is highly expressed is the CA1 region of the hippocampus (20). In this region, a lasting enhancement of the efficacy of synaptic transmission, LTP, is induced by tetanic stimulation of the Schaffer collateral inputs to CA1 neurons (84, 85). It has been established that LTP in the CA1 region is induced by a sequence of biochemical steps occurring in the pyramidal neurons (86, 87). Both PTK function (88) and NMDA receptor activation (89) have been found to be necessary for LTP induction in these neurons. Therefore, we used the reagents characterized previously to determine whether Src participates in LTP in CA1.

It was found that administering anti-src1 or Src(40–58) into CA1 neurons prevented the induction of LTP in an acute hippocampal slice preparation (21). On the other hand, administering recombinant Src or activating Src by means of the EPQ(pY)EEIPIA peptide induced a long-lasting enhancement of synaptic responses. This enhancement occluded the induction of LTP and *vice versa*, implying that Src activation is sufficient to cause LTP. In addition, by measuring Src activity biochemically *in vitro* by using an immune-complex kinase assay, it was found that tetanic stimulation, which produced LTP, caused an increase in the activity of Src within 1 min of the stimulation. Thus, Src is up-regulated very rapidly as a consequence of tetanic stimulation. Because inhibiting Src did not affect basal synaptic responses, it was concluded that rapid up-regulation of Src activity is necessary, as well as sufficient, for the induction of LTP.

The principal means by which LTP is expressed in CA1 neurons is enhancement of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) component of synaptic responses and therefore, our conclusion seemed at odds with other findings (17) that the AMPA receptor-mediated synaptic response is not potentiated when Src is activated in dorsal horn neurons. However, in our studies on dorsal horn neurons, intracellular Ca^{2+} was highly buffered, whereas in the experiments in hippocampal slices low intracellular Ca^{2+} buffering was used, because this is required to induce LTP. When Ca^{2+} buffering was increased in the hippocampal neurons, Src no longer potentiated the synaptic AMPA responses, but synaptic NMDA responses were still potentiated by Src, as was the case in the dorsal horn neurons. Thus, enhancement of AMPA responses produced by activating Src depends on a rise in intracellular Ca^{2+}. Because Src is not a Ca^{2+}-dependent enzyme (90), these results indicated that Src does not up-regulate AMPA receptors directly but rather does so indirectly through one or more Ca^{2+}-dependent steps.

In other experiments, it was determined that blocking NMDA receptors prevents but does not reverse Src-induced potentiation of AMPA responses. Thus, like LTP induced by tetanus, NMDA receptors are necessary to produce, but not to maintain, the potentiation of AMPA responses induced by activating Src directly. Together, these findings required the development of a new model where activation of Src appears to be a biochemical mechanism gating the induction of LTP in CA1 neurons (54). It is hypothesized that during induction of LTP, Src is rapidly activated, leading to enhanced NMDA receptor function, which boosts the entry of Ca^{2+} sufficiently to trigger the downstream signaling cascade.

A key question opened up by this work is, what is the mechanism causing Src activation upon tetanic stimulation? Src has a number of regulatory sites (18), and there are numerous biochemical pathways that converge to activate (91, 92) or to inhibit (93, 94) this kinase. Determining whether the increase in Src activity is produced by stimulating an activating pathway or by blocking an inhibiting one and identifying the specific biochemical steps are central goals of future work in this area.

Because the role of Src in LTP induction appears to be to enhance NMDA receptor function, one potential mechanism is phosphorylation of one of the NMDA receptor subunit proteins, as discussed above. Indeed, it has been found that the level of tyrosine phosphorylation of the NR2B NMDA receptor subunit is increased after induction of LTP in the dentate gyrus in the hippocampus (95, 96). Like CA1, the dentate is a region where LTP induction is NMDA receptor-dependent and is blocked by inhibitors of PTKs (97). Another region where NMDA receptor-dependent synaptic plasticity has been associated with tyrosine phosphorylation of NMDA receptors is the insular cortex, where it has been found that taste aversion conditioning causes an increase in phosphorylation of NR2B (98). However, whether phosphorylation of NR2B mediates the plasticity in the dentate gyrus or in the insular cortex, and if so by what mechanism, remains to be determined (54).

Tyrosine kinases were first implicated in the induction of LTP from experiments showing that LTP is blocked by bath-applied tyrosine kinase inhibitors (88). It was found subsequently that mutant mice with targeted deletion of the *src* gene showed LTP in CA1, which is a genetic argument against the absolute requirement for Src in the induction of LTP. Also, it was reported that in mice lacking the Src-family kinase *fyn*, LTP is blunted but not abolished (99). The impairment in LTP is age-dependent in *fyn*$-/-$ mice, with young *fyn*$-/-$ animals showing LTP comparable to that in wild-type animals (100). The developmental time at which LTP becomes impaired in *fyn*$-/-$ mice correlates with a large decline in the level of Src expression. Src and Fyn are known to substitute for each other in various processes (101). Thus, from our evidence together with that from experiments using genetic manipulation, it appears that in wild-type animals, Src is a required mediator of LTP induction, whereas in animals that develop without *src*, another member of the *src* family, likely *fyn*, may substitute. It is possible that, for example, by being upstream of Src activation, Fyn might also be necessary for LTP induction in the wild-type animal, and this possibility needs to be examined in acute experiments by using Fyn-specific manipulations.

Intracellular Sodium Regulates NMDA Receptors. During high levels of neuronal discharge activity, such as occur during the induction of lasting changes in synaptic efficacy, there is a large influx of Na^+, leading to substantial increases in the intracellular concentration of Na^+ ($[Na^+]_i$) (102). It has been found that during such activity, the level of $[Na^+]_i$ may increase by 15–20 mM in the neuronal soma (103) and likely even more in the dendrites (102). While there can be no doubt that Na^+ is the major carrier of electrical charge responsible for producing action potentials and excitatory postsynaptic potentials, the possibility that raising $[Na^+]_i$ may act as a signaling factor in neurons had been virtually ignored. Thus, we recently set out to determine whether Na^+ might regulate synaptic function in postsynaptic neurons (22).

We first examined the effects of raising $[Na^+]_i$ on whole-cell NMDA currents evoked by exogenously applied NMDA. Perfusing the neurons with an elevated $[Na^+]_i$ solution produced an increase in the amplitude of the NMDA currents of nearly 40% (22). In this case, the intracellular perfusion contained $[Na^+]_i$ of 50 mM, but we only achieved an increase in $[Na^+]_i$ of about 30 mM, as determined by using the fluorescent Na^+-sensitive dye sodium-binding benzofuran isophthalate (SBFI), presumably because of the very active Na^+ pumping in the neurons. The increase in NMDA currents was not reproduced by perfusing Cs^+ at 50 mM, indicating that not all monovalent cations were able to cause the potentiation and, equally importantly, that the potentiation was not because of lowering intracellular $[K^+]$, which was required to maintain proper osmolarity.

To characterize the effects of intracellular Na^+ on NMDA channel gating, single-channel currents were recorded by using cell-attached patches, and $[Na^+]_i$ was varied by application of

the Na^+-ionophore monensin (104). Ratiometric measurement of $[Na^+]_i$ was done under similar conditions so that single-channel activity could be correlated to the actual change in $[Na^+]_i$. It was found that NMDA channel activity followed the level of $[Na^+]_i$—increasing when Na^+ was raised and falling when $[Na^+]_i$ was reduced (Fig. 3). We found no change in the single-channel conductance of the NMDA channels, indicating that the increase in whole-cell current when Na^+ was raised could be accounted for by increased NMDA channel gating.

Neurons express a diversity of Na^+-permeable channels, e.g., ionotropic glutamate receptors and voltage-gated Na^+ channels, and therefore we wondered whether Na^+ influx through these various channels could affect NMDA channel function. This was investigated by examining single-channel activity by enclosing single channels within a patch pipette attached to the cell and stimulating surrounding channels in the cell by bath-applying activators. An important consideration in these experiments was to avoid the known voltage dependence of NMDA channel gating (105), and this was done by adjusting the transmembrane potential of the patch so that it was at a constant level with respect to the channel reversal potential. We thus found that bath-applying agonists to activate NMDA or non-NMDA receptors outside the patch led to an increase in activity of the NMDA channels within the patch. This increase in activity was prevented when Na^+ was removed from the bath solution. Importantly, removal of Ca^{2+} from the bath did not alter the effect of stimulating the extra-patch NMDA receptors. Also, depolarizing the cells by bath-applying a high-K^+ solution to mimic the depolarization caused by applying the agonists did not affect NMDA channel function. Thus, we concluded that NMDA receptor function may be up-regulated by Na^+ influx through neighboring glutamate receptors.

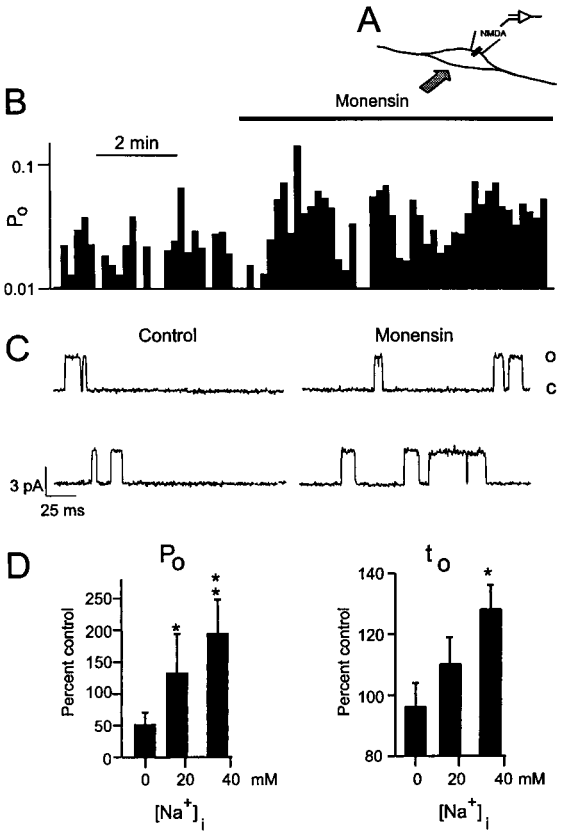

FIG. 3. Increases in $[Na^+]_i$ by application of monensin potentiate single NMDA channel activity recorded in the cell-attached configuration. *A* shows the recording configuration. (*B*) A continuous record of NMDA channel P_o from neurons bathed with extracellular solution containing 50 mM Na^+. (*C*) shows representative single-channel currents before and during monensin application. *D* Changes in P_o and mean open time (t_o) versus $[Na^+]_i$ during monensin application. For each Na^+ concentration, six patches were tested. *, $P < 0.05$; **, $P < 0.01$, Mann–Whitney *U* test when compared with the channel activity at 0 mM Na^+.

Another main route for Na^+ entry into neurons is via voltage-gated channels permeable to Na^+, such as those responsible for the generation of action potentials. To produce a consistent activity of voltage-gated Na^+ channels and thereby attain a stable membrane potential of the cell, as required to accurately record channel function in the cell-attached patches, we bath-applied the alkaloid veratridine (106). Veratridine caused a significant increase in NMDA channel activity, and the effects of veratridine were prevented when it was applied in the presence of tetrodotoxin. Thus, by using veratridine as a surrogate for evoking action potentials, we concluded that influx of Na^+ through tetrodotoxin-sensitive voltage-gated Na^+ channels is sufficient to increase NMDA channel activity.

The NMDA receptors studied by using the cell-attached recordings are of necessity localized extrasynaptically. To determine whether synaptic NMDA receptors are affected by changing $[Na^+]_i$, we studied spontaneous mEPSCs. We found that applying Na^+ into neurons via the patch pipette significantly increased the NMDA receptor-mediated component of the mEPSCs. By contrast, the non-NMDA receptor component of the mEPSCs was not altered by raising $[Na^+]_i$. Applying Cs^+ into the cell did not affect either the NMDA or the non-NMDA receptor-mediated components of the mEPSCs. Thus, increasing $[Na^+]_i$ selectively amplifies synaptic responses mediated by NMDA but not non-NMDA receptors. Taking into account the effects of varying Na^+ levels on NMDA channel activity in cell-attached patches described above, it appears that the efficacy of synaptic transmission through NMDA receptors tracks the level of Na^+ in the postsynaptic neuron.

A Src Kinase Controls the Enhancement of NMDA Channel Function by $[Na^+]_i$. To lay the foundation for understanding the mechanisms and molecules by which intracellular Na^+ alters NMDA channel function, we examined the effects of Na^+ applied directly to the cytoplasmic side of the membrane in inside-out patches. In contrast to the effects found in cell-attached recordings from intact neurons, applying 50 mM Na^+ to the cytoplasmic face of inside-out patches did not change NMDA channel activity. We reasoned that the action of Na^+ on NMDA receptors may depend on a molecule(s) lost from the excised patches or a biochemical process that had been disrupted. Because regulation of NMDA receptors by protein phosphorylation is well established, we considered the possibility that phosphorylation may be involved in the effects of intracellular Na^+. To examine this, we used a broad-spectrum inhibitor of protein kinases, staurosporine, which was found to abolish the increase in NMDA channel activity caused by raising Na^+ through bath-applying monensin during cell-attached patch experiments. Importantly, staurosporine did not prevent the rise in $[Na^+]_i$ produced by the application of monensin. Thus, it was concluded that protein kinase activity may be required for the up-regulation of NMDA receptor activity by raising intracellular Na^+.

From these results, two mechanistic possibilities emerged: that the effect of Na^+ is mediated by activating a kinase (or inhibiting a phosphatase) that is present in the cell but is lost from the patches, or alternatively, but not mutually exclusively, that the activity of a kinase is required for the effect of Na^+ but is not directly a mediator. Because it had been established that endogenous Src could be up-regulated in the excised patches, we wondered whether this up-regulation would permit an effect of Na^+. Indeed, this was found to be the case, because

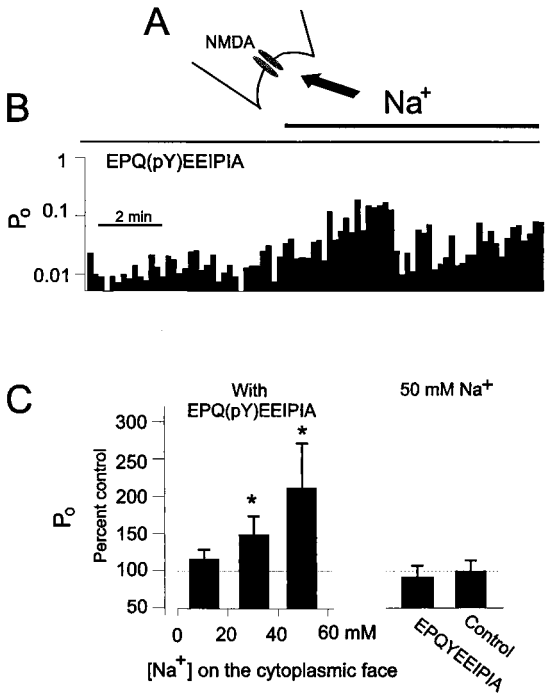

FIG. 4. NMDA channel activity is increased by raising the Na^+ concentration on the cytoplasmic side of the membrane in inside-out patches during activation of Src-family kinases. *A* shows the inside-out recording configuration. (*B*) A continuous record of NMDA channel P_o. The peptide EPQ(pY)EEIPIA was applied 3–5 min before the recording and throughout the recording period. Na^+ (50 mM) was applied as indicated. (*C*) *Left*, changes in P_o versus $[Na^+]$ on the cytoplasmic side in the presence of the peptide EPQ(pY)EEIPIA; *Right*, effects of 50 mM Na^+ on NMDA channels in control experiments and in the presence of the nonphosphorylated peptide EPQY-EEIPIA. *, $P < 0.05$, Wilcoxon test or paired *t* test.

applying Na^+ to the cytoplasmic face increased NMDA channel activity in patches treated with the Src family-activating phosphopeptide EPQ(pY)EEIPIA (Fig. 4). In contrast, the nonphosphorylated form of this peptide, which does not activate Src kinases, did not permit the effect of Na^+. We also found that at concentrations well above those expected under physiological conditions, Na^+ increased NMDA channel activity in the untreated inside-out patches (22). Thus, the Src-activating peptide shifted the concentration–response curve for Na^+ to the left.

Therefore, from the convergence of evidence described above, we have developed a working model that the sensitivity of NMDA channels to intracellular Na^+ is controlled by a channel-associated Src kinase. This model is represented diagrammatically in Fig. 5. For simplicity of presentation, we represent the entire complex of NMDA channel subunits and associated proteins together as one pair of ovals forming the ion channel. This is not to imply that either the target of phosphorylation or the site of action of Na^+ is necessarily one of the NMDA channel subunit proteins. Rather, as alluded to above, the target of phosphorylation remains to be determined, as does the site of action of Na^+. Also, because the Src-activating peptide stimulates all Src-family kinases, it needs to be established whether the enhanced sensitivity to Na^+ is caused by Src itself or another member of the family.

Changing Src activity from the lowest to the highest level produces an ≈3- to 4-fold increase in NMDA channel function. Raising Na^+ from 0 to 40 mM produces an ≈1.5- to 2-fold change in NMDA channel activity. The change produced by Na^+ is over and above that produced by Src and therefore, we estimate that the total range over which NMDA channel activity is regulated by Src and Na^+ is about 5- to 8-fold. This degree of change would represent a dramatic alteration in synaptic efficacy and would be expected to have sizable effects on synaptic integration of individual neurons and on the behavior of neural networks. Moreover, because the influx of Ca^{2+} through NMDA receptors follows the amplitude of the currents (e.g., ref. 12), then a 5-fold increase in current would result in a 5-fold increase in Ca^{2+} influx. This boost in Ca^{2+} influx may then allow the resultant rise in the level of intracellular Ca^{2+} to be large enough to engage Ca^{2+}-activated signaling pathways in the cell.

We expect that the boost in NMDA channel function by coincidence of Src activation and raising $[Na^+]_i$ may be relevant to the induction of lasting enhancement of synaptic efficacy in phenomena such as LTP. The role of Src in this process has already been established, but what about a role of Na^+? High levels of action potential discharge, similar to those that have been shown to cause rises in $[Na^+]_i$, occur as a result of tetanic stimulation. Action potentials initiated in the soma are known to propagate into the dendrites (107–109), and this action potential backpropagation may participate in the induction of LTP (110, 111). It is thought that the backpropagation of action potentials works by promoting Ca^{2+} entry

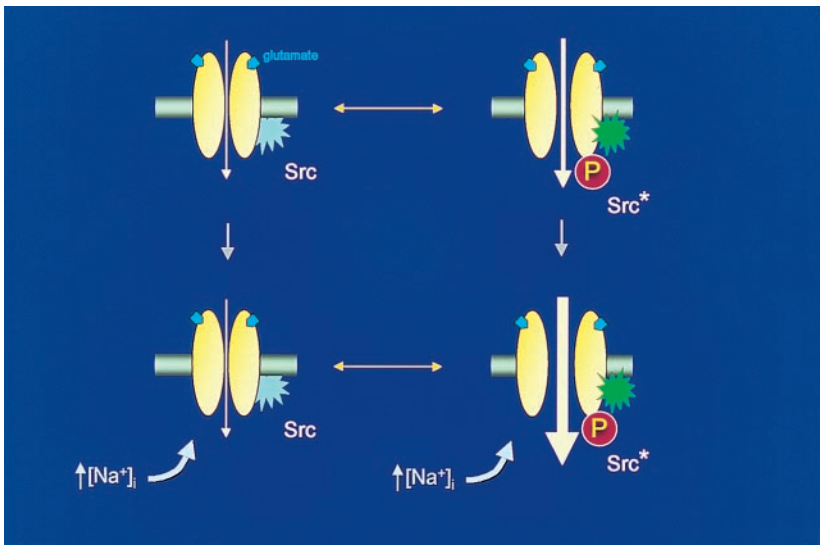

FIG. 5. Diagram illustrating a working model for the regulation of NMDA channel gating by Src and Na^+. See text for details.

through voltage-gated Ca^{2+} channels and by producing depolarization that relieves the Mg^{2+} block of NMDA channels. Our recent results, however, open up a new possibility, i.e., that it is the rise in intracellular Na^+ that is the important event. This rise in Na^+, when coincident with postsynaptic activation of Src, may lead to a large amplification of NMDA receptor function sufficiently large to set off the rest of the intracellular signaling cascade.

What about implications for pain? Excitatory synaptic transmission in nociceptive pathways in the spinal cord is facilitated by stimulation of C fiber nociceptors (112–114), apparently as a result of increased responsiveness of NMDA receptors (6, 11). It is not yet known whether Src is activated under such circumstances, but it seems likely given that Src can be activated through a number of signaling pathways, such as stimulating G protein-coupled receptors and receptor tyrosine kinases (91, 92), and these types of pathways have been implicated in enhancement of nociceptive transmission (115, 116). Moreover, the discharge rate of central nociceptive neurons responding to noxious inputs is sufficiently high as to be expected to produce dramatic rises in $[Na^+]_i$. Therefore, it is possible that the up-regulation of NMDA receptors by Src and sodium is involved in the central up-regulation of transmission in nociceptive pathways. This may be relevant to human pain states because this central up-regulation appears to be sufficient to produce hyperalgesia and allodynia (e.g., ref. 117).

CONCLUSIONS

The gene encoding Src was found over 20 years ago as the first protooncogene, with particular Src mutations causing cancer (118). Since then, this enzyme has been found to have many roles in cell signaling in a variety of cell types. In neurons, Src up-regulates the function of NMDA receptors and thereby gates the induction of a lasting enhancement of synaptic transmission, LTP in the CA1 region of the hippocampus. Src also sensitizes NMDA channels to up-regulation by intracellular Na^+. Thus, the coincidence of Src activation and a rise in $[Na^+]_i$ may be important for boosting synaptic NMDA receptor function and initiating the intracellular signaling cascades that produce persistent alterations in synaptic function. Because NMDA receptors are implicated in a variety of pathophysiological conditions in the CNS, the regulation by Src and Na^+ represent potential targets for developing new types of therapeutic intervention in a variety of CNS disorders.

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