Substance P Abolishes the Facilitatory Effect of ATP on Spontaneous Glycine Release in Neurons of the Trigeminal Nucleus Pars Caudalis

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Glycine release was facilitated by the activation of presynaptic ATP receptors (P_{2X}-type) in a preparation of dissociated trigeminal nucleus pars caudalis neurons in which the native synaptic boutons were preserved. The action of ATP was completely blocked by substance P (SP) without alteration of the miniature IPSC (mIPSC) amplitude distribution. SP itself had no effect on mIPSC frequency or amplitude. The inhibitory effect of SP on ATP action was blocked by CP99994, indicating that the SP receptors are of the neurokinin-1 type. The ATP-induced facilitation of the mIPSC frequency was unaffected by Cd^{2+}. Moreover, SP did not inhibit the increase in mIPSC frequency induced high K^+ application, suggesting that SP did not modulate voltage-dependent calcium channels or subsequent steps in the release process.

KT5720 and phorbol 12-myristate 13-acetate did not block SP action, indicating that neither the cAMP–protein kinase A nor the protein kinase C pathway mediates the SP effects. However, in the presence of *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7), SP was no longer able to inhibit the ATP-induced stimulation of mIPSC frequency. 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine also suppressed the SP action, suggesting that SP modulates P_{2X} receptors via a Ca^{2+}/calmodulin-dependent protein kinase II-mediated pathway. In conventional whole-cell mode, the presence of W-7 in the patch pipette did not affect the SP inhibitory action. Thus, SP is not likely to be generating its modulation through the production of a retrograde signal (involving calmodulin) from the postsynaptic cell to the presynaptic boutons.

These results are the first demonstration of the modulation of one presynaptic receptor by another. Because SP inhibits the ATP stimulation of glycine release, SP may play a significant role in hyperalgesia or chronic pain.

Key words: dissociated neurons; substantia gelatinosa neuron; glycinergic nerve terminal; ATP and substance P receptors; calmodulin; CAM kinase II; glycinergic mIPSC; modulation; pain

Despite an intense effort to understand the neural circuitry underlying nociception in the spinal cord, there are still many uncertainties regarding the function of substance P (SP). Seemingly contradictory data from behavioral and electrophysiological experiments have been found regarding the role of SP. For example, neurokinin-1 (NK-1) receptor antagonists reduce the response to maintained inflammatory pain models but have no effect on brief noxious stimulation (Seguin et al., 1995). Electrophysiological experiments also indicate that NK-1 receptor inhibition decreases long-term noxious stimulation without a parallel effect on short-term C-fiber responses (Chapman and Dickenson, 1993). The destruction of dorsal horn neurons containing NK-1 receptors "left responses to mild noxious stimuli unchanged, but markedly attenuated responses to highly noxious stimuli and mechanical and thermal hyperalgesia" (Mantyh et al., 1997). Clearly the nature of the response, as well as the duration and magnitude of the stimuli must be considered. Moreover, parallel pathways mediating pain sensation or reflex activity complicate analysis.

SP mediates a bewildering variety of cellular responses. For example, certain subtypes of voltage-dependent calcium channels are inhibited by SP in rat nucleus basalis neurons. This inhibition appeared to be mediated by a pertussis toxin-independent G-protein (Margeta-Mitrovic et al., 1997). A variety of G-proteins are coupled to SP receptors (Nishimura et al., 1998). For submandibular neurons the decrease in calcium channel activity was reduced by protein kinase C (PKC) but not by protein kinase A (PKA) (Yamada et al., 1999). In contrast, locusttachykinins produced a depolarization mediated by cAMP and PKA (Lundquist and Nassel, 1997). In astrocytoma cells, SP and histamine appear to transiently raise the intracellular calcium levels through a mechanism involving phosphoinositide metabolism, which is then followed by a decrease mediated by a PKC stimulation of calcium pumps (Young et al., 1998). Moreover, inositol triphosphate levels increased within 30 sec of SP application to guinea pig urinary bladder (Martin et al., 1997). In adenoid tissue explants, the ciliostimulatory effect of SP appears to be mediated by nitric oxide (Runer and Lindberg, 1999). Last, SP activates mitogen-activated protein kinase with the expression of immediate-early genes in human astrocytoma cells (Luo et al., 1997).

Because of the complexity of the neural circuitry and the added complexity of the temporal relationships of the various inputs, it is difficult to resolve the cellular mechanisms underlying the response to sensory inputs. A preparation of acutely isolated

neurons from the substantia gelatinosa (SG) of the trigeminal nucleus pars caudalis retaining functional synaptic boutons (Rhee et al., 2000) provides a simplified system for studying the effects of modulators on synaptic transmission. The various nerve endings can be functionally isolated with appropriate inhibitors.

Here we demonstrate a role for SP in the modulation of the ATP-induced stimulation of glycine release. This effect of SP appears to be activated by the calmodulin–CaM kinase II system located in the presynaptic terminal. This finding represents the first demonstration to our knowledge of the presynaptic modulation of one receptor type by another.

MATERIALS AND METHODS

Preparation. Ten- to 14-d-old Wistar rats were decapitated under pento-barbital anesthesia. The spinal cord was quickly removed and was sliced at a thickness of 400 μm with a microslicer (DTK-1000; Dosaka, Kyoto, Japan). The spinal cord slices were kept in an incubation medium saturated with 95% O_2 and 5% CO_2 at room temperature (22–25°C) for at least 1 hr. Thereafter, the slices were transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Franklin Lakes, NJ), and the dorsal horn of the spinal cord was identified under a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). A fire-polished glass pipette was placed lightly onto the surface of the dorsal horn and was vibrated horizontally. We recorded the vibration movement with digital video camera and measured the speed and distance of movement. The speed and distance were regulated by an AC power supply. The vibration movement in our experiment was ~3–5 Hz for 2 min and over a 0.3–0.5 mm distance (Rhee et al., 1999). After the slices were removed, the mechanically dissociated SG neurons adhered to the bottom of the dish within 10 min. These neurons, which were dissociated without using any enzymes, retained some recognizable morphological features, including proximal dendritic processes. In addition some pinched-off synaptic boutons of a few micrometers in diameter remained attached and were capable of spontaneous release of neurotransmitter.

Electrical measurements. Electrical measurements were performed in the nystatin-perforated patch recording mode at a holding potential of −60 mV under voltage-clamp conditions. Patch pipettes were made from borosilicate glass tubes (1.5 mm outer diameter, 0.9 mm inner diameter; G-1.5; Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PB-7; Narishige). The neurons were visualized with phase-contrast equipment on an inverted microscope (Diaphot; Nikon). The current and voltage were measured with a patch-clamp amplifier (CEZ-2300; Nihon Kohden, Tokyo, Japan), monitored on both an oscilloscope (Tektronix 5111A; Sony, Tokyo, Japan) and a pen recorder (Recti-Horiz 8K; Nippondenki San-ei, Tokyo, Japan), and stored on videotapes after digitization with a pulse-coded modulation processor (PCM-501 ES; Sony). The membrane currents were filtered at 1 kHz (E-3201A Dicade Filter; NF Electronic Instruments, Tokyo, Japan), and data were digitized at 4 kHz. The resistance between the recording electrode filled with the internal solution and the reference electrode in the external solution was 5–7 MΩ. All experiments were performed at room temperature (22–25°C).

Data analysis. Events were counted and analyzed using "Detectevent" (Ankri et al., 1994) and IgorPro software (Wavemetrics, Lake Oswego, OR). Analysis of miniature IPSCs (mIPSCs) was performed with cumulative probability plots. Cumulative amplitude histograms were compared using the Kolmogorov–Smirnov test for significant differences. Statistically significant differences were assumed for $p < 0.05$. Numerical values are provided as mean ± SEM. Differences in amplitude and frequency distribution were tested by a paired two-tailed t test.

Solutions. The ionic composition of the incubation medium was (in mM): 124 NaCl, 5 KCl, 1.2 KH$_2$PO$_4$, 24 NaHCO$_3$, 2.4 CaCl$_2$, 1.3 MgSO$_4$, and 10 glucose. The pH of the incubation medium was adjusted to 7.4 with 95% O_2 and 5% CO_2. The ionic composition of the external standard solution was (in mM): 150 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 10 HEPES. Ca^{2+}-free external solution contained (in mM): 150 NaCl, 5 KCl, 3 MgCl$_2$, 10 glucose, 10 HEPES, and 2 EGTA.

While recording mIPSCs, these external test solutions routinely contained 3×10^{-7} M tetrodotoxin (TTX) to block voltage-dependent Na$^+$ channels, 3×10^{-6} M 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), and 10^{-5} M DL-2-amino-5-phosphovaleric acid (DL-AP-5) to block glutamatergic responses, and 3×10^{-6} M bicuculline to block the GABA$_A$ responses. The ionic composition of the internal (patch pipette) solution

A
With TTX 3x10⁻⁷ M, CNQX 3x10⁻⁶ M, APV 10⁻⁵ M, Bic 3x10⁻⁶ M

Strychnine 10⁻⁶ M

B
a

SP 10⁻⁶ M
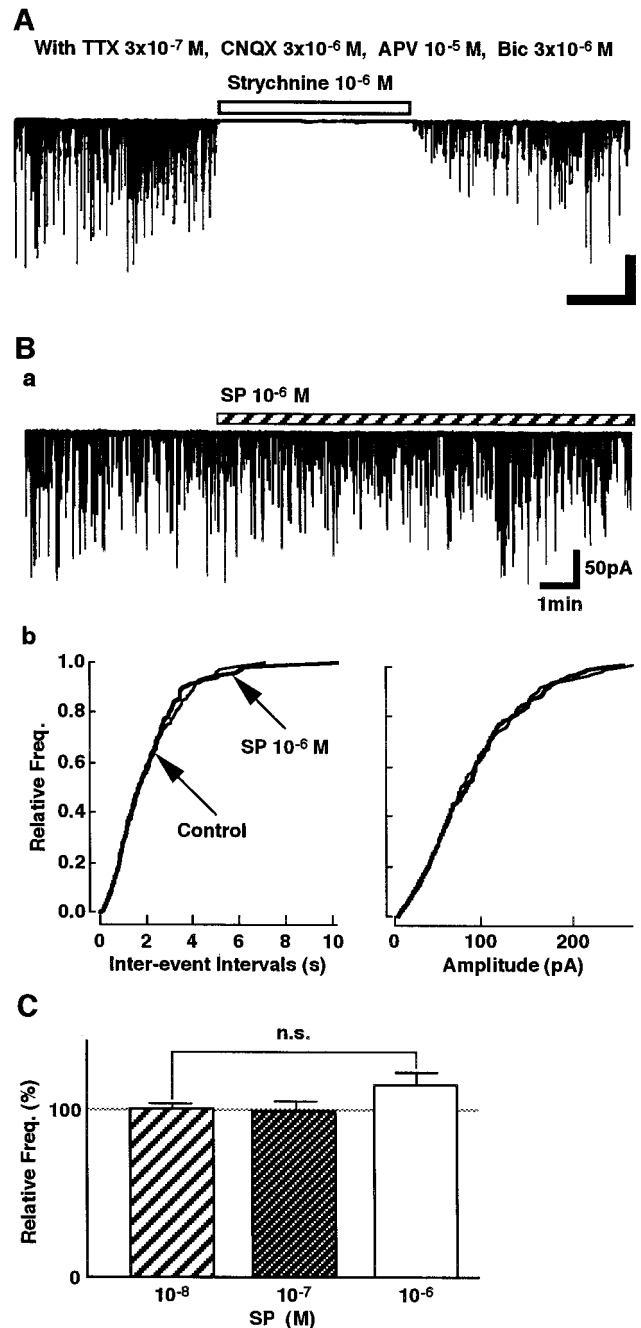
50pA
1min

b

c

Figure 1. Effects of SP on glycinergic mIPSCs. *A,* The spontaneous inhibitory currents recorded from the "synaptic bouton preparation" were completely blocked in a reversible manner by 10^{-6} M strychnine in the presence of 3×10^{-7}M TTX, 3×10^{-6} M CNQX, 10^{-5} M AP-5, and 3×10^{-6} M bicuculline, indicating that the spontaneous mIPSCs are glycinergic. *B,* The SP at 10^{-6} M itself facilitated the frequency of mIPSCs a little without altering the mIPSC amplitude distribution. *C,* The bar graph shows the frequency ratio of mIPSCs before and during application of SP. The vertical bar shows ±SEM. SP at a low concentration (10^{-8}–10^{-7} M) had no effect on mIPSC frequency and the current amplitude distribution. Pooled data at 10^{-6} M SP showed slightly increase of the mIPSC frequency but not significantly.

for the nystatin-perforated patch recording was (in mM): 20 *N*-methyl-D-glucamine methanesulfonate, 20 Cs-methanesulfonate, 5 MgCl$_2$, 100 CsCl, and 10 HEPES. The pH of the internal solution was adjusted to 7.2 with Tris-OH. Nystatin was dissolved in acidified methanol at 10 mg/ml. The stock solution was added to the internal solution just before use at final concentration of 100–200 μg/ml.
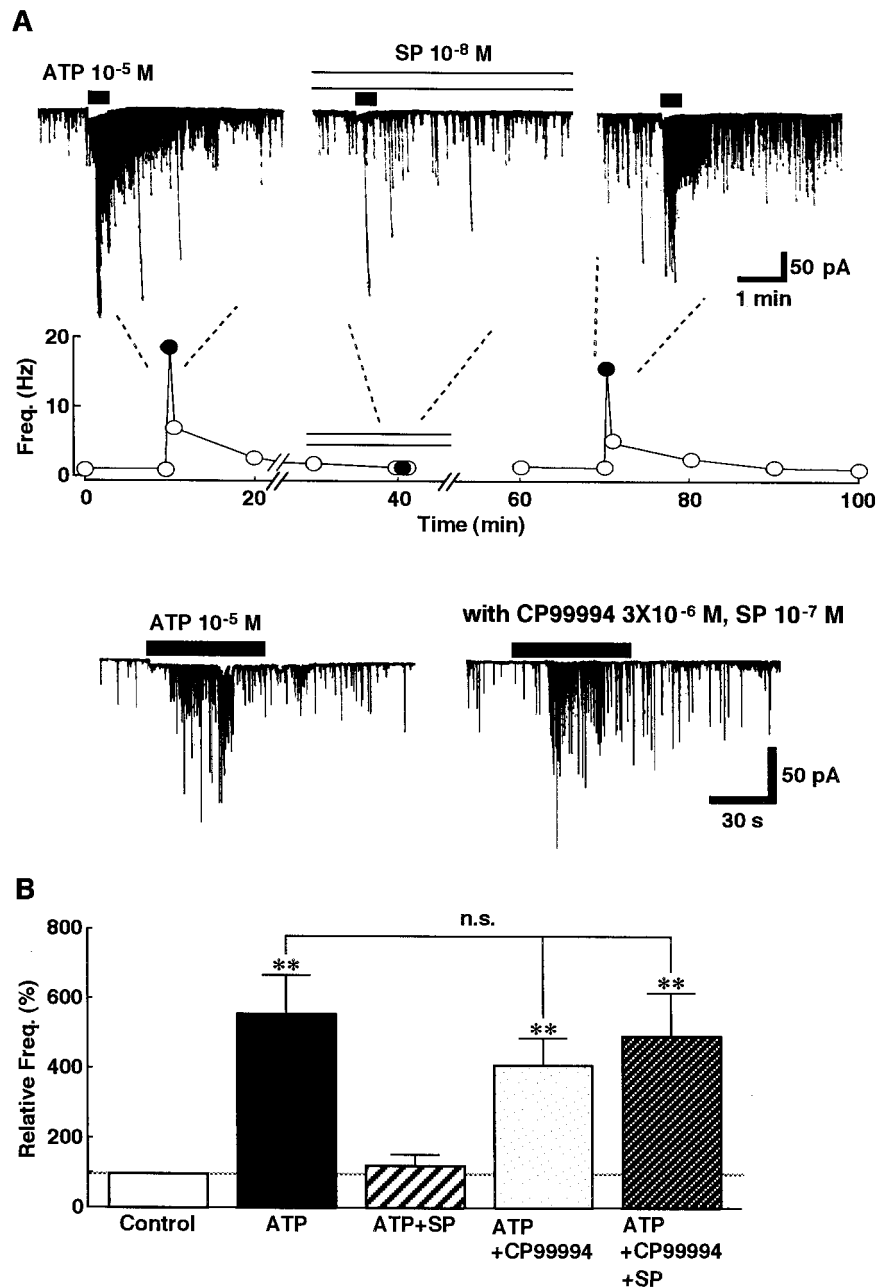
A

B

Figure 2. SP inhibits the facilitatory action of ATP on mIPSC frequency. *A,* Recordings of mIPSCs before, during, and after the application of ATP with or without SP. The SP was applied 15 min before the ATP application. *Bottom panel* shows the time courses of mIPSC frequency per 10 min during 30 sec applications of ATP with or without SP. *B,* CP99994, a selective NK-1 antagonist, blocked the inhibitory action of SP on the facilitatory effect of ATP on mIPSC frequency. The relative frequency shows the mIPSC frequency ratio of ATP in the normal external solution with or without CP99994 in the presence of SP. *Asterisks* represent statistically significant differences ($^{**}p < 0.01$; $n = 6$).

Drugs. Drugs used in the present study were 10^{-6} M strychnine, 10^{-5} M AP-5, 3×10^{-6} M bicuculline, 3×10^{-6} M CNQX, nystatin, and 10^{-6}-10^{-8} M substance P (SP) (Sigma, St. Louis, MO), 10^{-5} M ATP (Yamasa, Chiba, Japan), 3×10^{-7} M TTX (Wako Pure Chemicals, Osaka, Japan), 10^{-6} M 1-[*N,O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), 10^{-5} M PMA, 10^{-7} M *N*-(6-aminohexyl)-5-chloro-1-naphthalene (W-7), and 10^{-6} M KT5720 (Calbiochem, La Jolla, CA). CNQX was dissolved in dimethylsulfoxide (DMSO) at 10^{-2} M as a stock solution. A concentration of 3×10^{-6} M CP99994 was kindly provided by Dr. Yukio Takano (Fukuoka University, Fukuoka, Japan).

Drugs were applied by a "Y-tube system" (Akaike and Harata, 1994) that enables a solution exchange within 20 msec.

RESULTS

Spontaneous postsynaptic currents were recorded from the acutely dissociated SG neurons of the rat spinal trigeminal nucleus pars caudalis (layer II). Attached to these dissociated neurons were functional presynaptic nerve endings (Akaike et al.,

1992; Rhee et al., 1999). Electrical recordings were made using the nystatin perforated patch recording technique at a holding potential (V_H) of -60 mV. All external test solutions contained 3×10^{-7} M TTX, 3×10^{-6} M CNQX, 10^{-5} M AP-5, and 3×10^{-6} M bicuculline. As shown in Figure 1*A*, after a control period of spontaneous mIPSCs, the mIPSCs were blocked by 10^{-6} M strychnine in a reversible manner, indicating that the mIPSCs were glycinergic (Rhee et al., 2000).

SP itself did not alter the frequency or amplitude of glycinergic mIPSCs at the lower concentrations (10^{-8}-10^{-7} M). At a concentration of 10^{-6} M, SP slightly increased the mIPSCs frequency, although the increase was not significant (Fig. 1*B,C*). These data suggest that SP itself does not substantially modulate presynaptic glycine release or the postsynaptic glycine response.

ATP reversibly facilitated the frequency of mIPSCs without affecting the mean mIPSC amplitude in SG neurons. Indeed, the
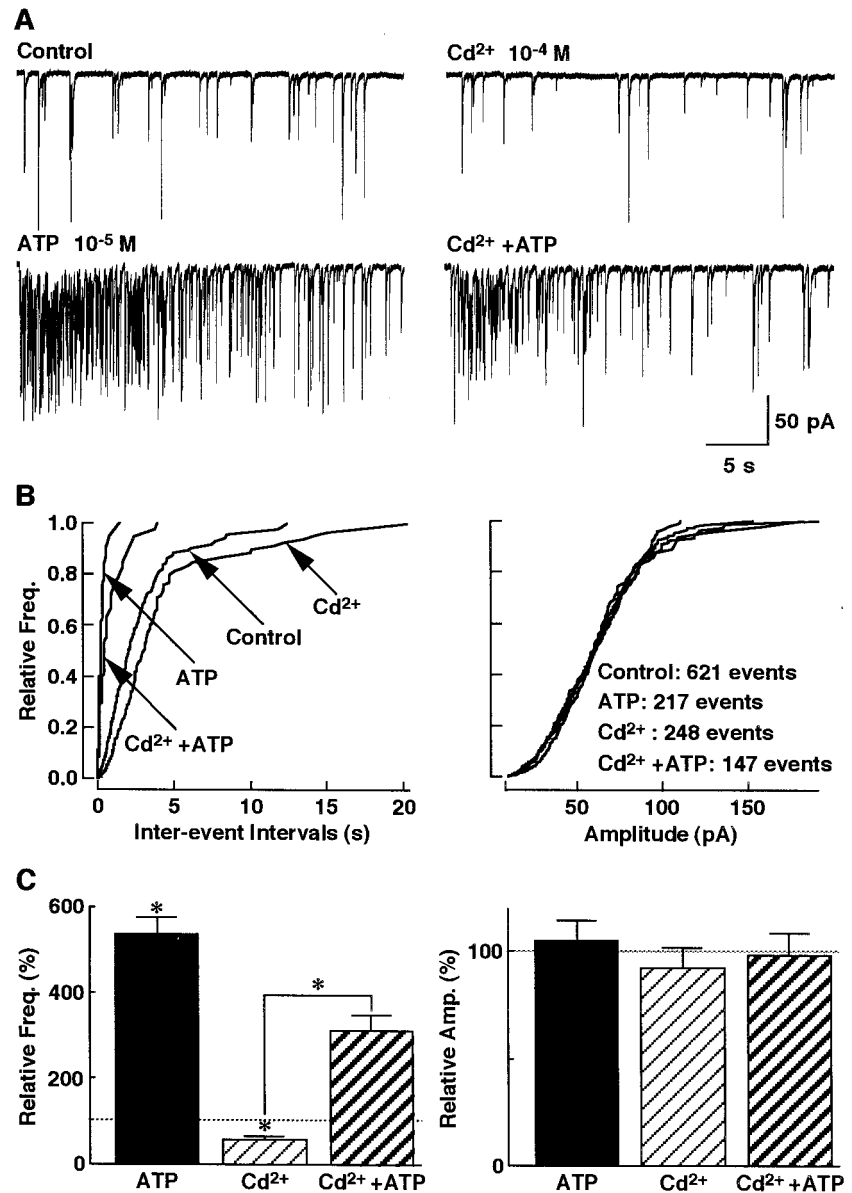
Figure 3. Cd^{2+} does not inhibit the ATP stimulation of the mIPSC frequency. *A,* Recordings of mIPSCs with and without Cd^{2+} (10^{-4}) in the *top panels*. The same experiment is shown in the presence of 10^{-5} M ATP in the *bottom panel*. *B,* Normalized cumulative curves of interevent interval show that Cd^{2+} only slightly inhibited mIPSC frequency from control values or from the ATP-stimulated state without altering the mIPSC amplitude. *Numbers* in the *inset* of the amplitude distribution indicate the numbers of samples used. *C,* Bar graph of the frequency ratio compared with control values of the ATP, Cd^{2+} alone, and Cd^{2+} in the presence of ATP. The vertical bars indicate ±SEM. The *asterisks* indicate significant differences (*$p < 0.05$) between the ATP and control state, between the Cd^{2+} and the control state, and between the Cd^{2+} and the Cd^{2+} + ATP. No significant differences ($p > 0.05$) were found for mIPSC amplitude.

facilitation was dependent on extracellular Ca^{2+}, indicating that the ATP action was mediated by P_{2X} receptors or by the activation of voltage-dependent calcium channels on the glycinergic presynaptic nerve terminals. This facilitation occurred in the presence of 2 mM EGTA in the patch pipette, suggesting that the ATP effect is, perhaps, not generated in the postsynaptic membrane which then, in turn, generates a retrograde signal modifying mIPSC frequency. Note this claim is weakened by the low buffer capacity provided by the 2 mM EGTA; a large local influx of Ca^{2+} could initially overwhelm the local buffer near the inside surface of the membrane (Rhee et al., 2000). The facilitatory effect of ATP on mIPSCs frequency was completely blocked by pretreatment with 10^{-8} M SP (105.11 ± 7.86% of control in standard external solution) without altering the mIPSC amplitude distribution (Fig. 2*A,B*). After washing out the SP, the facilitatory effect of ATP on glycine release gradually recovered over 30 min. However, the inhibitory effect of SP on ATP action was blocked by adding CP 99994, a selective NK-1 antagonist (Fig. 2*B*). The ATP action itself was not affected by adding CP

99994. The facilitatory ratios of mIPSC frequency after external addition of ATP were 491.21 ± 125.29 and 550.23 ± 115.29% ($n = 4$) (in the external solution) with or without CP 99994, respectively (Fig. 2*B*). There was no significant difference between the two values. These results indicate that activation of NK-1 receptor can modulate the P_{2X} receptors on glycinergic presynaptic nerve terminals.

As noted above, the ATP facilitation of mIPSC frequency depends on external calcium. To demonstrate that most of this effect was mediated by P_{2X} receptor rather than voltage-dependent calcium channels, we added 0.1 mM Cd^{2+} to the external solution. Figure 3 shows that the addition of Cd^{2+} to control case results in a modest decrease of the mIPSC frequency (Fig. 3*B*). In the presence of Cd^{2+}, ATP still had a significant facilitatory effect, although less than in the absence of the Cd^{2+} (Fig. 3*C*). These results are in substantial agreement with those found by Rhee et al. (2000) and suggest that the facilitatory effect of ATP is via activation of P_{2X} receptors. Terminal depolarization may secondarily activate voltage-dependent calcium chan-
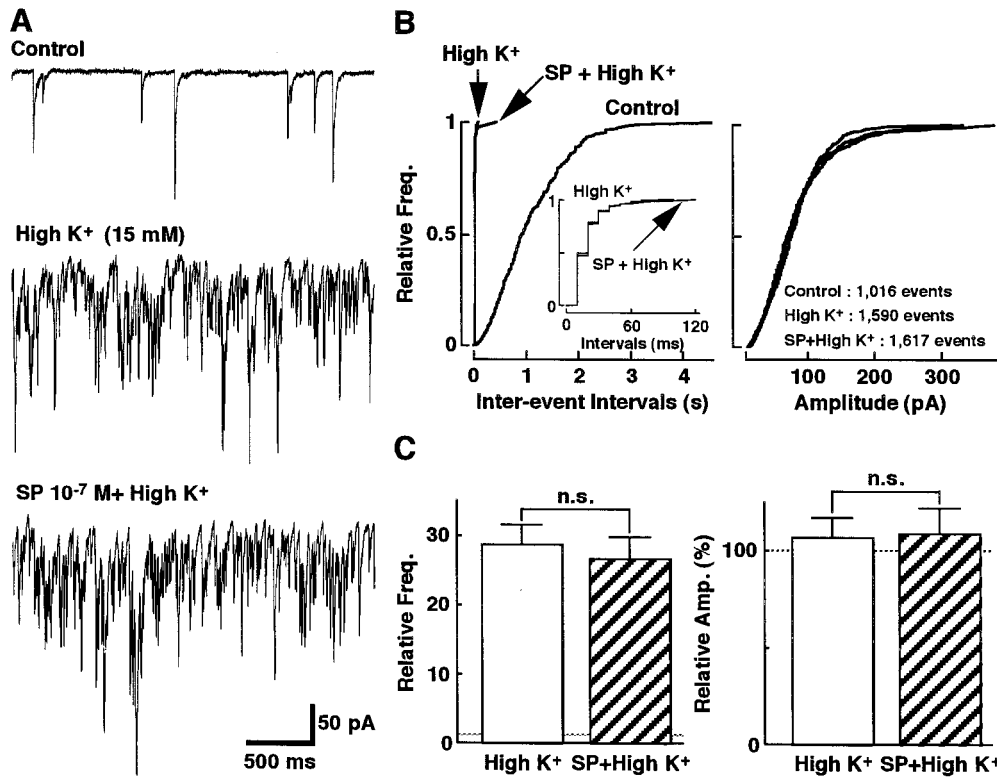
Figure 4. SP does not inhibit the facilitatory effect of elevated external potassium concentration on mIPSC frequency. *A,* mIPSC recordings for control, in elevated potassium (15 mM), and in elevated potassium + SP (10^{-6} M). *B,* Normalized cumulative interevent histograms show that SP does not reduce the frequency stimulation induced by the high potassium. The *inset* shows the same data set at an expanded time scale. The corresponding amplitude cumulative histogram also shows no differences. *C,* The bar graphs show that there are no significant differences between the high potassium with or without 10^{-6} M SP for mIPSC frequency or amplitude ($p > 0.05$).

nels, which then further contribute to the increase in mIPSC frequency.

The SP effect also does not appear to be mediated through the modulation of voltage-dependent calcium channels. As shown in Figure 4, the increase in mIPSC frequency was stimulated by depolarizing the terminal with high (15 mM) K^+ with no change of the relative mIPSC amplitude. Unlike the case for the ATP stimulation, SP (10^{-6} M) was unable to inhibit the stimulation caused by high K^+ (Fig. 4*B,C*). These results suggest that SP does not inhibit the release process or the voltage-dependent calcium channels.

To address the functional interaction between NK-1 and P_{2X} receptors on the glycinergic presynaptic nerve terminals, the intracellular mechanisms were investigated using pharmacological techniques. A previous report suggested that P_{2X} receptors could be modulated by PKA and PKC in Deiters cells (Chen and Bobbin, 1998). We therefore investigated whether the inhibitory effect of SP on ATP action is related to the PKA intracellular pathway. With or without externally applied KT5720, a PKA-selective antagonist, ATP almost equally facilitated the mIPSCs frequency (418.56 $\pm$ 43.9 and 502.86 $\pm$ 54.42% of control in standard external solution, respectively; $n = 4$) without altering the mIPSC amplitude distribution. In the presence of both KT5720 and SP, however, ATP failed to increase the mIPSC frequency (Fig. 5*A,B*), indicating that the effect of SP on P_{2X} receptors are not mediated through the PKA pathway in the glycinergic presynaptic nerve terminals.

Therefore, we examined the SP effect on ATP action in the presence of phorbol 12-myristate 13-acetate (PMA), a selective PKC activator. If PKC mediates the SP modulation of the ATP stimulation, then the application of PMA should inhibit the ATP response. Moreover, if PMA saturates the PKC binding sites, then additional activation through SP might be impossible. We found that regardless of the presence of PMA in the external
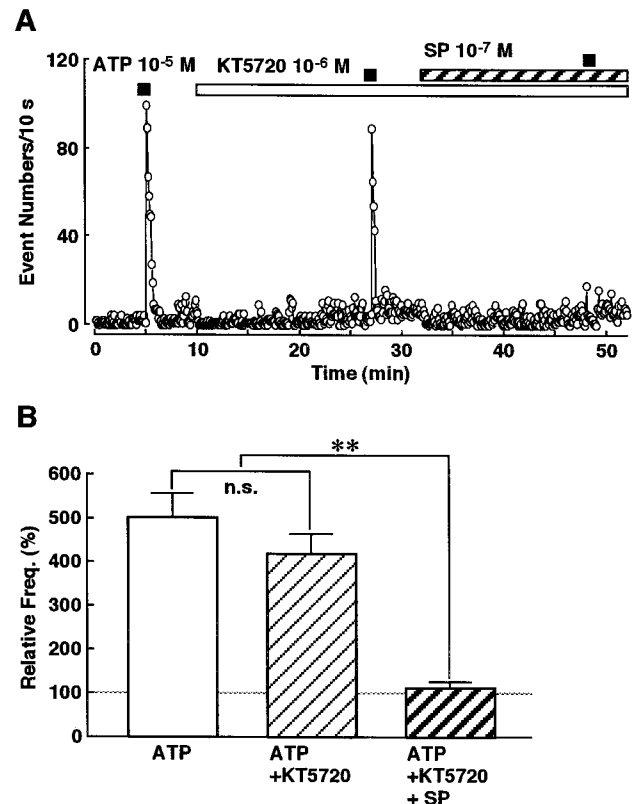

Figure 5. PKA does not affect SP action. *A.* Time course of event numbers per 10 sec. ATP increased the mIPSC frequency in the presence of 10^{-6} M KT5720, a PKA-selective antagonist. The ATP action was still completely blocked by adding 10^{-7} M SP. *B,* Histogram of the mIPSC frequency during the application of ATP with or without KT5720 and in the presence of both ATP and KT5720 with SP. *Asterisks* represent statistically significant differences (**p < 0.01; $n = 4$).
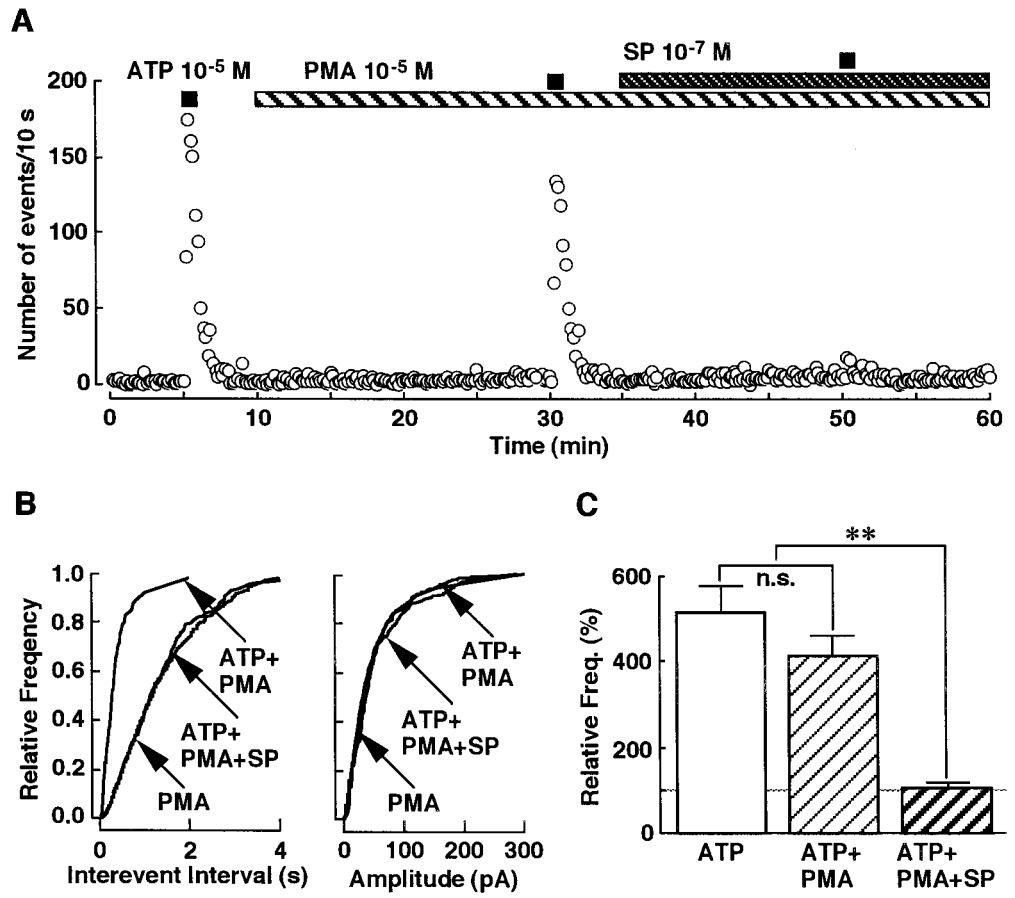
Figure 6. PKC does not affect SP action. *A,* Time course of the number of events per 10 sec. The activation of PKC by PMA, a selective PKC activator, did not affect the facilitatory action of ATP on mIPSC frequency. Successive applications of SP completely blocked the facilitatory effect of mIPSC frequency by ATP in the presence of PMA. *B,* Normalized cumulative frequency distribution (*left*) and current amplitude distribution (*right*) of ATP with or without SP in the presence of PMA. *C,* Effect of ATP on mIPSC frequency in the standard external solution with or without SP in the presence of PMA. Histogram of the mIPSC frequency during the application of ATP with or without PMA and in the presence of PMA with or without SP. *Asterisks* represent statistically significant difference (**$p < 0.01$; $n = 4$).

solution, ATP equally potentiated the mIPSCs frequency (525.87 ± 59.617 and $413.82 \pm 46.23\%$ of control in standard external solution, respectively; $n = 4$) without altering the mIPSC amplitude distribution (Fig. 6). The facilitatory action of ATP on mIPSC frequency disappeared in the presence of both SP and PMA without changing the mIPSC amplitude, indicating that PKC does not mediate the inhibitory effect of SP on ATP action.

The increase of mIPSC frequency by P_{2X} receptor activation is induced by Ca^{2+} entry into the terminal (Rhee et al., 2000). We, therefore, wished to investigate the possible role of the calcium–calmodulin system (Ca–CAM system) in the mediation of the SP response. Figure 7 shows that ATP stimulation of mIPSC frequency was unaffected by W-7, a Ca–CAM antagonist, and persisted in the presence of SP with W-7 (Fig. 7*A*). ATP potentiated the mIPSCs frequency in the external solution with or without SP in the presence of 10^{-7} M W-7 ($677.85 \pm 104.67\%$ with ATP, $449.28 \pm 35.55\%$ with ATP + W-7, and $446.69 \pm 46.34\%$ ATP + W-7 + SP of control in external solution; $n = 4$) without altering the mIPSC amplitude (Fig. 7*C*). We therefore investigated whether the calmodulin-activated kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), mediated the SP action. In the presence of KN-62, a CaMKII antagonist, SP once again failed to block the facilitatory effect of ATP as shown in Figure 8. In standard W-7 and W-7 + SP external solutions, ATP potentiated the mIPSCs frequency ($457.8 \pm 41.42\%$ in standard external solution, 429.59 ± 30.03 and $341.51 \pm 22.97\%$ in external solution with W-7 and W-7 + SP, respectively; $n = 4$) without altering the mIPSC amplitude distribution. These results suggest that SP modulates P_{2X} receptors through the Ca–CAM and CaMKII pathways in the glycinergic presynaptic nerve terminals or retro-

gradely by activation of postsynaptic receptors that activate some subsequent signal that affects the nerve terminals.

The action of SP could be either on presynaptic NK-1 receptors, which directly modulate presynaptic P_{2X} receptors, or postsynaptic NK-1 receptors, which activate pathways that produce a retrograde message that modulates the presynaptic P_{2X} receptors. In the hippocampus, nitric oxide produced in the postsynaptic element modulates transmitter release from the presynaptic element (Arancio et al., 1996). To eliminate the possibility of a postsynaptically mediated retrograde mechanism, we voltage-clamped the SG neurons in the whole-cell recording mode with the pipette solution containing 10^{-7} M W-7. Soluble second messenger components should diffuse up into the pipette. Moreover, the W-7 should block the action of postsynaptic calmodulin. The results indicate that the relative mIPSC frequency was enhanced ~10-fold (10.7 ± 2.87 times) by ATP (Fig. 9*A*). This enhancement was abolished in the presence of SP (1.26 ± 0.36-fold increase, not significantly different from the control frequency). The amplitude distribution was not significantly different from control (Fig. 9*B*). These results strongly argue that the SP effects are not mediated by postsynaptic SP receptors that, in turn, generate a retrograde signal. Indeed, SG neurons are thought not to have NK-1 receptors (Brown et al., 1995).

DISCUSSION

The use of the "dispersed neuron with attached synaptic bouton preparation" (Akaike et al., 1992; Rhee et al., 1999) has allowed unambiguous demonstration that SP directly modulates the stimulatory effect of ATP on the release of glycine. SP has postsynaptic as well as presynaptic actions (Shapiro and Hille, 1993),
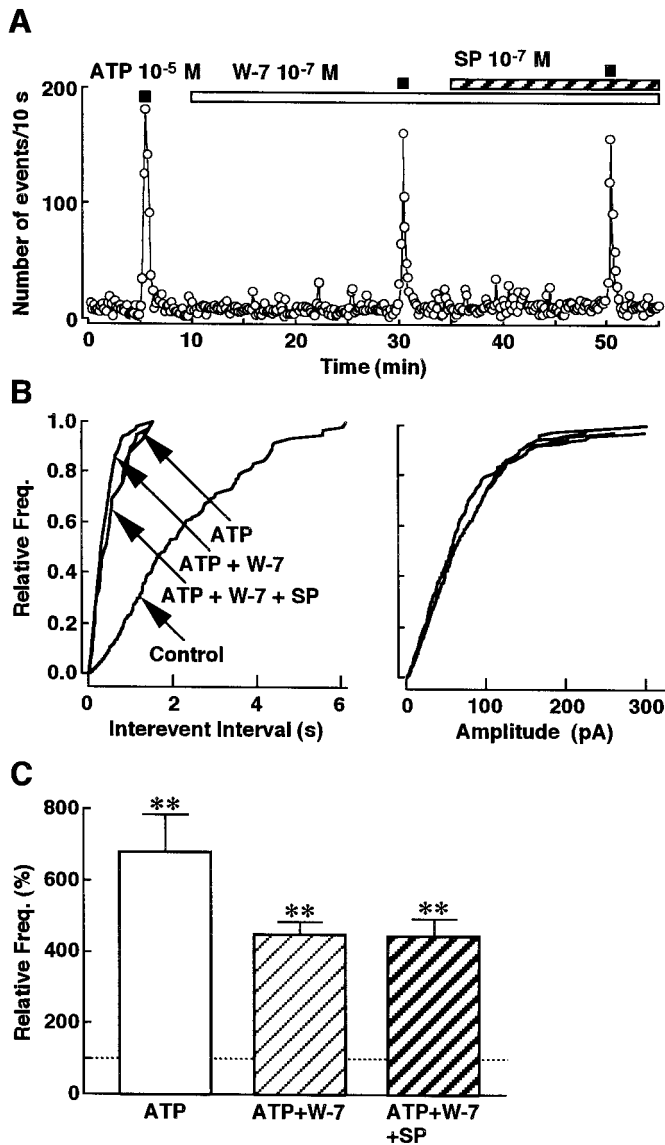
Figure 7. SP action is mediated by Ca–CaM. *A,* Time course of the number of events per 10 sec. SP did not block the ATP action in the presence of W-7, a selective Ca–CAM antagonist. *B,* Normalized cumulative mIPSC frequency of ATP action in the normal external solution, in the presence of W-7, and in the presence of both W-7 and SP (*left*), and cumulative mIPSCs amplitude curves (*right*). *C,* Histogram of the mIPSCs frequency during the application of ATP with or without W-7 and in the presence of W-7 with or without SP.

Figure 8. CaMKII participates in SP action. *A,* Time course of number of events per 10 sec is plotted. SP did not block the ATP action in the presence of KN-62, a selective CAMII antagonist. *Ba,* Normalized cumulative mIPSC frequency of ATP action in the normal external solution, in the presence of KN-62, and in the presence of both KN-62 and SP (*left*). *Bb,* Effects of ATP action on mIPSC frequency in the standard external solution and with or without SP in the presence of KN-62.

Figure 9. SP action is not mediated by a retrograde messenger from the postsynaptic element. *A,* Histogram of the mIPSC frequency in SG cells perfused with a pipette solution containing 10^{-7} M W-7 in the whole-cell recording mode. The graphs show the control, the effect of ATP stimulation, and the inhibition of the ATP stimulation by SP. *B,* Histograms showing a lack of effect on mIPSC magnitude of W-7 dialyzed in the postsynaptic cell body.

although apparently not in SG neurons (Brown et al., 1995). In an intact spinal cord, many interneurons could participate in mediating the final effect on glycine release. In the synaptic bouton, the entire interneuron circuitry has been removed so that only the presynaptic terminals remain. Under such simplified conditions, we were able to demonstrate a novel mechanism of the modulation of the stimulatory action of ATP. This is, as far as we have been able to determine, the first demonstration of the presynaptic modulation of one presynaptic receptor by another. Thus, this particular action of SP requires the concomitant activation of presynaptic P_{2X} receptors. The inhibitory action of SP in the presence of ATP acts like a logical conditional-negation operation: SP alone does nothing, ATP alone stimulates, and the combination inhibits the ATP stimulation.
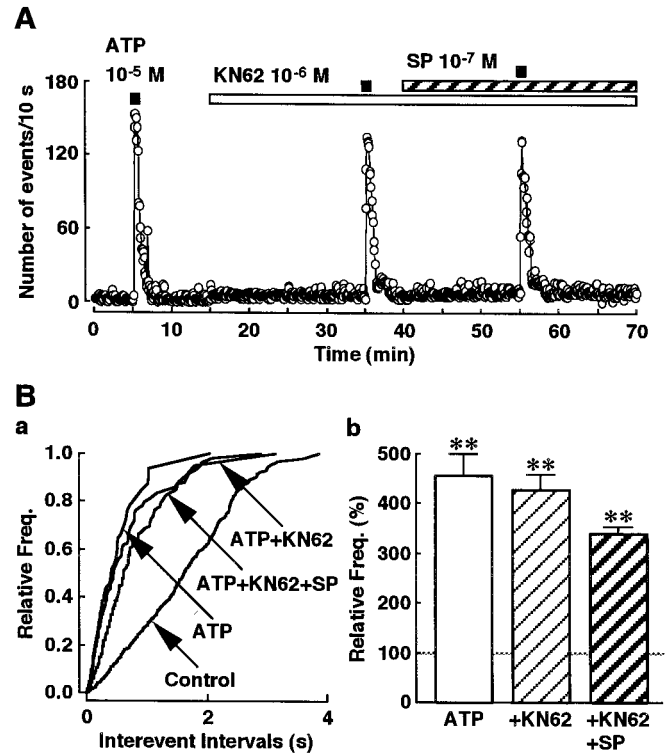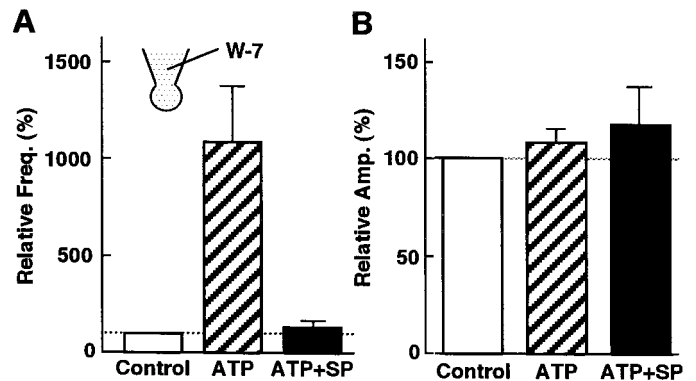
Using pharmacological agents, we have been able to demonstrate that the SP modulation of P_{2X} receptors involves the CAM kinase II pathway. Inhibition of either calmodulin by W-7 or of the CaMKII by KN-62 suppressed the inhibitory action of SP on the ATP stimulation of mIPSC frequency. Because the P_{2X} receptor acts to promote Ca^{2+} permeability, the activation of the calmodulin pathway by SP may be enhanced by preactivation of

the P_{2X} pathway. Our data do not rule out the possibility of another intervening step between the kinase activity and the final reduction of the ATP effect. Our data also does not unambiguously demonstrate that the P_{2X} receptor has been modulated. SP could, for example, block some distal step in the release process and leave the P_{2X} receptor unaffected. Indeed a role for CAM kinase in the process of vesicle docking has been proposed (Llinas et al., 1991). The observation that SP has little effect on spontaneous release does argue against a direct effect of SP on the release process itself. Moreover, SP had no effect on the increase in mIPSC frequency induced by elevated external potassium that presumably depolarized the terminal. This result argues against an inhibitory effect of SP on either the release process or the voltage-dependent calcium channels. It may be, however, that there are, in fact, partial inhibitory reactions that are overwhelmed and saturated by the high (15 mM) K^+ used in this experiment. Furthermore, because the ATP stimulation itself was only slightly effected by Cd^{2+}, the ATP-induced response was probably not mediated through voltage-dependent calcium channels (Rhee et al., 2000). Hence, a modulatory effect of SP on ATP-stimulated calcium channels is unlikely. A direct effect of the CaMKII on the P_{2X} receptor activation could be determined by imaging calcium entry into the terminal in the presence and absence of SP.

It may seem surprising that SP activates the calmodulin–CaMKII pathway at all. Calmodulin is activated by calcium ions that presumably increase in intracellular concentration after P_{2X} activation. SP could perhaps release local calcium stores to further activate the calmodulin. Indeed, SP applied locally to cultured dorsal root neurons caused the intracellular calcium levels to rise briefly. The response appeared to desensitize after repeated applications (Marvizon et al., 1998). Similarly Li and Zhao (1998) have shown that small rat dorsal root ganglion cells express NK-1 receptors that produce long-lasting inward currents presumably through a nonselective, calcium-permeable cation channel. They also suggest that autoreceptors on the primary afferent terminals could be responsible for hyperalgesia. Another interesting possibility is that the calmodulin is activated by P_{2X} receptor activation but cannot activate CaMKII that is in an inactive form. SP, in this model, would then function to activate the CaMKII (by some unknown pathway), which could then be activated by Ca-calmodulin triggered by the calcium influx through the P_{2X} receptor. Thus, not only would the SP inactivate the ATP response, but the SP effect would require an elevated intracellular calcium concentration from either the P_{2X} receptor or some other source. This suggestion that the CAM kinase requires activation by SP implies that merely raising intracellular Ca^{2+} by through the voltage- or ligand-dependent channels would be incapable of activating the kinase.

The role of CAM kinase II in the mediation of the ATP receptor modulation is reminiscent of the mechanisms underlying LTP. Activation of postsynaptic receptors on the SG cells could activate the CAM kinase pathway (via Ca^{2+} and calmodulin), in turn activating nitric oxide synthase. The nitric oxide could then diffuse retrogradely back to the presynaptic terminals and alter release (Arancio et al., 1996). Our experiments in which the SG cells were patch-clamped in whole-cell mode with W-7 included in the pipette strongly argue against such a model. Mobile components such as calmodulin, CAM kinase, ATP, L-arginine should all diffuse up the tip of the pipette. Moreover, W-7 should block any residual calmodulin effect.

The synaptic bouton preparation cannot, of course, demon-strate how SP functions physiologically. Behavioral and psychophysical experiments are necessary to discriminate reflexive, sensory, and affective dimensions. Indeed, the role of SP appears to reside partly in its site of action. Lamina I neurons bearing NK-1 receptors appear to be involved in intensity coding of pain, whereas the deep neurokinin-1 cells seem to be involved in spatial localization or submodality discrimination (Polgar et al., 1999). Our results are consistent with the suggestion that in severe pain the SP modulation of the P_{2X} receptor results in a decreased inhibitory input to the SG cells. The exact consequences of such modulation of SG excitability depend, of course, on whether the SG neurons are themselves excitatory or inhibitory with respect to the pain pathway. In view of the autophosphorylating property of CAM kinase II, the modulating effect of SP could be greatly prolonged and constitute a mechanism for promoting allodynia and hyperalgesia. Future experiments will be required to determine the time course of the modulatory effects of SP and the role of specific phosphatases.

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