The P2X3 receptor is an ATP-gated ion channel predominantly expressed in nociceptive neurons from the dorsal root ganglion. P2X3 receptor channels are highly expressed in sensory neurons and probably contribute to the sensation of pain. Kinetics of P2X3 currents are characterized by rapid desensitization (<100 ms) and slow recovery (>20 s). Thus, any mechanism modulating rate of desensitization and/or recovery may have profound effect on susceptibility of nociceptive neurons expressing P2X3 to ATP. Here we show that currents mediated by P2X3 receptor channels and the heteromeric channel P2X2/3, composed of P2X2 and P2X3 subunits are potentiated by the neuropeptides substance P and bradykinin, which are known to modulate pain perception. The effect is mediated by the respective neuropeptide receptors, can be mimicked by phorbol ester and blocked by inhibitors of protein kinases. Together with data from site-directed mutagenesis our results suggest that inflammatory mediators sensitize nociceptors through phosphorylation of P2X3 and P2X2/3 ion channels or associated proteins.

P2X receptors mediate fast responses of excitable cells to application of ATP. So far, seven different P2X subunits have been cloned (P2X1–7), which form a gene family (1). They have two transmembrane domains with intracellular termini and a rather large extracellular loop (2, 3) and form non-selective cation channels with a high permeability to Ca2+. Various P2X receptors show differences both in the affinity to ATP and in the kinetics of activation and inactivation. P2X1 and P2X3 show high agonist affinity (EC50 ~ 1 μM) and rapid activation and desensitization with full activation in less than 10 ms and almost complete desensitization in less than 1 s (4, 5). Moreover, repeated application of the agonist leads to the disappearance of the ATP-gated currents (4, 5). In contrast, P2X2 and P2X4 through P2X6 have a low agonist affinity (EC50 ~ 10 μM), are slowly activating with time constants in the order of 100 ms, and show only partial desensitization (1, 6). Heterologous P2X2/3 channels show a mixed phenotype with high affinity to the agonist α,β-methylene ATP (α,β-meATP)1 like P2X3 receptors and incomplete desensitization like P2X2 receptors (5).

The desensitization properties of the rapidly activating and inactivating P2X2 and P2X3 are likely to be of physiological importance as synaptic transmission takes place in a few milliseconds. Modulation of the rate of desensitization and/or recovery of these receptors may contribute to synaptic efficacy. P2X4 is almost exclusively expressed in sensory neurons (7, 8), mostly in capsaicin-sensitive nociceptors (9–11). P2X7-mediated currents in sensory neurons are large (12), and P2X3 shows the strongest expression level in dorsal root ganglion (DRG) neurons compared with other P2X receptors (10). The P2X3 protein is found on the sensory endings as well as on the presynaptic membrane in inner lamina II of the spinal horn (9), and its activation by ATP might contribute to the sensation of pain. In the dorsal horn a presynaptic mechanism is involved, which leads to a potentiation of the excitatory postsynaptic potential (13).

Nociceptive neurons express homomeric P2X3 as well as heteromeric P2X2/3 receptors (5). Both types of channels can be expressed separately or together in individual neurons (14); recent data suggest that homomeric P2X3 is predominantly expressed on small diameter sensory neurons and heteromeric P2X2/3 on medium diameter sensory neurons (15–17). Application of α,β-meATP leads to nociceptive behavior in vivo (18), confirming a role for P2X3 and P2X2/3 receptors in nociception.

Here we show that the inflammatory mediators substance P (SP) and bradykinin (Bk) can potentiate currents through P2X3 and P2X2/3 expressed in Xenopus oocytes. Both induce an increase in peak current as well as in steady-state current with repeated application of ATP. Phorbol ester had a similar, non-additive effect, suggesting a pathway involving protein kinase C. Mutagenesis experiments suggest that a conserved threonine at the intracellular N terminus, which had been shown to be phosphorylated in P2X3 (19), is crucial for this effect. Together, our results suggest that inflammatory mediators potentiate ATP-gated currents in nociceptors through a mechanism involving either direct phosphorylation of P2X3 receptors or phosphorylation of a so far unidentified protein.

**Experimental Procedures**

cDNAs and Site-directed Mutagenesis—cDNAs coding for P2X3 and P2X2 are contained in the BamHI/NotI (P2X3) or the EcoRI/BssHII (P2X2) site of vector pCDNA3 (Invitrogen, Groningen, Netherlands). Splice variant P2X3β was described previously (20). cDNAs for SP and Bk receptors are contained in the HindIII (SP receptor) or the EcoRI (B2 receptor) site of vector pBluescript. Chimeric molecules between P2X2

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1 The abbreviations used are: α,β-meATP, α,β-methylene ATP; Bk, bradykinin; SP, substance P; PMA, phorbol 12-myristate 13-acetate; DRG, dorsal root ganglion.
Potentiation of P2X<sub>3</sub> by Inflammatory Mediators

**RESULTS**

Currents through ATP Receptor Channels Containing the P2X<sub>2</sub> Subunit Are Potentiated by the Inflammatory Substances P and Bradykinin—We used X. laevis oocytes as an expression system to investigate differential regulation of P2X receptor subtypes. SP or Bk application to oocytes expressing the SP receptor caused an immediate and transient (20 s) inward current (arrows in Figs. 2–4) resulting from activation of endogenous Ca<sup>2+</sup>-activated Cl<sup>−</sup> channels. A, transient potentiation of currents through P2X<sub>3</sub> homomeric channels showed a similar potentiation by SP (upper panel) and Bk (lower panel). B, P2X<sub>2</sub>/P2X<sub>3</sub> heteromeric channels showed no current potentiation. C, P2X<sub>2</sub>/P2X<sub>3</sub> receptors were activated by a pulse protocol as shown in Fig. 1. Once ATP-gated currents showed reproducible peak currents, inflammatory mediators were applied. Arrowheads indicate Cl<sup>−</sup> currents due to the activation of endogenous Ca<sup>2+</sup>-activated Cl<sup>−</sup> channels. A, transient potentiation of currents through P2X<sub>3</sub> homomeric channels following application of SP (upper panel) or Bk (lower panel). B, P2X<sub>2</sub>/P2X<sub>3</sub> heteromeric channels showed no similar potentiation by SP (upper panel) and Bk (lower panel). C, P2X<sub>2</sub>/P2X<sub>3</sub> receptors were activated by a pulse protocol as shown in Fig. 1. Once ATP-gated currents showed reproducible peak currents, inflammatory mediators were applied. Arrowheads indicate Cl<sup>−</sup> currents due to the activation of endogenous Ca<sup>2+</sup>-activated Cl<sup>−</sup> channels. A, transient potentiation of currents through P2X<sub>3</sub> homomeric channels following application of SP (upper panel) or Bk (lower panel). B, P2X<sub>2</sub>/P2X<sub>3</sub> heteromeric channels showed no similar potentiation by SP (upper panel) and Bk (lower panel). C, P2X<sub>2</sub>/P2X<sub>3</sub> receptors were activated by a pulse protocol as shown in Fig. 1. Once ATP-gated currents showed reproducible peak currents, inflammatory mediators were applied. Arrowheads indicate Cl<sup>−</sup> currents due to the activation of endogenous Ca<sup>2+</sup>-activated Cl<sup>−</sup> channels.

**Electrophysiology**—Experiments were done at room temperature 3–7 days after injection using 2-microelectrode voltage clamp. Currents were recorded with a TurboTac O1C amplifier (npi, Tamm, Germany), digitized at 30 Hz (ITC16, HEKA, Lamprecht, Germany) and stored on hard disk. The bath solution had the following composition (in mM): 115 NaCl, 2.5 KCl, 1.0 Na<sub>2</sub>HEPES, 1.0 MgCl<sub>2</sub>, 1.0 CaCl<sub>2</sub>, and 0.5 g/liter polyvinylpyrrolidon, pH 7.3. Bath solution contained Ca<sup>2+</sup> in order to avoid activation of endogenous Ca<sup>2+</sup>-activated Cl<sup>−</sup> channels. A, ATP, α,β-methylene ATP (α,β-meATP), SP, Bk, phorbol 12-myristate 13-acetate (PMA), and staurosporine were all purchased from Sigma-Aldrich (Deisenhofen, Germany).

P2X<sub>2</sub> receptors showed the pharmacological and kinetic characteristics reported in previous studies using heterologous expression systems and as known from native tissue (Fig. 1). We used 100 nm ATP for activation of P2X<sub>2</sub>, 300 μM for activation of P2X<sub>3</sub>, and 10 μM α,β-meATP or 300 μM ATP for activation of P2X<sub>2,3</sub>. In order to get sufficient current amplitude at low desensitization rate for all subunit combinations injected, we repeatedly applied the agonist for 50 s with 50-s intervals. During the first four to eight ATP pulses, current peak amplitudes decreased; thereafter, they remained constant (Fig. 1). We took three equal current peak amplitudes as the base line for judging regulatory effects on current amplitude.

**Data Analysis**—Data on graphs indicate the median of the maximal increase of ATP-elicited peak current after stimulation by SP or Bk. Values were normalized to the ATP stimulation just before application of SP or Bk. Error bars indicate the range of the results as these were not symmetrically distributed. Moreover, we compared the amplitude at the end of the 50-s ATP application (quasi-steady state) and report this as the I<sub>500</sub>.

Desensitization time constants were fitted to a single exponential function. They are reported as mean ± standard deviation. Statistical analysis was done with the unpaired Student’s t test.
It is known that the desensitization of the P2X receptor and the Bk B2 receptor (median increase: 74%, range: 38–175%, n = 12; Fig. 2 A and E; median increase of I_{50\%} = 89%, range: 33–140%, n = 9), showing that multiple neurotransmitters can modulate P2X receptors.

Using oocytes expressing the P2X\textsubscript{2,6} heteromer (obtained by coexpression of the P2X\textsubscript{2} and P2X\textsubscript{6} subunits) together with the SP receptor, application of SP yielded a similar transient and reversible increase of current (median increase of peak amplitude: 38%, range: 21–64%, n = 6, Fig. 2 B and E; median increase of I_{50\%} = 56%, range: 29–68%, n = 6). The effect was independent of the P2X receptor agonist; it could be elicited by 300 \mu M ATP as well as by 10 \mu M c3P-meATP. A similar effect was again seen with application of Bk when the Bk receptor was coexpressed (median increase of peak amplitude: 112%, range: 22–140%, n = 9, Fig. 2 B and E; median increase of I_{50\%} = 136%, range: 17–199%, n = 9). The desensitization rate could not be well fitted but showed an apparent decrease after application of inflammatory mediators (see Fig. 2B).

In contrast, the slowly desensitizing currents through P2X\textsubscript{2} receptors were not significantly increased by SP receptor activation (median increase of peak current amplitude: 3%, range: −9% to 15%, n = 13, p > 0.01, Fig. 2 C and E; median increase of I_{50\%} = 2%, range: −7% to 8%, n = 13, p > 0.05) nor by Bk receptor activation (median increase of peak current amplitude: 6%, range: −11% to 17%, n = 12, p > 0.05; Fig. 2 C and E; median increase of I_{50\%} = 1%, range: −25% to 17%, n = 12, p > 0.05). Thus, it seems that modulation by the neuropeptides is subunit-specific and that the P2X\textsubscript{2,6} subunit is responsible for modulation of the P2X\textsubscript{2,6} heteromer.

Potentiation by Inflammatory Mediators Is due to Activation of a Protein Kinase—It is known that the desensitization of nicotinic acetylcholine receptors is directly accelerated by SP without the need of the SP receptor (22). In oocytes expressing only P2X\textsubscript{2,6} receptors but no SP receptor, the neuropeptide was not able to sensitize currents through P2X\textsubscript{2,6} (median increase of peak amplitude: −2%, range: −5% to 10%, n = 6, Fig. 2 D and E; median increase of I_{50\%} = 6%, range: −3% to 17%, n = 6).
This shows that the effect is mediated by the SP receptor and suggests pathways downstream of the receptor to be responsible for sensitization of P2X receptors.

Since SP and Bk are supposed to act via the phospholipase C/protein kinase C pathway, we tested different pharmacological agents interacting with kinases for their effect on sensitization of P2X receptors. Oocytes expressing P2X3 and the SP receptor were incubated with the serine/threonine kinase inhibitor staurosporine (10 μM) for 10 to 30 min. After this incubation, application of SP no longer increased ATP-activated peak or steady state currents (median increase in peak amplitude: 6%, range: 16% to 9%, n = 10, Fig. 3 (A and D); median increase in I_{50,5}: 3%, range: 12% to 16%, n = 7), suggesting the involvement of protein kinase in the potentiating process. In addition, the effect of SP could be mimicked using the phorbol ester 4β-PMA (10 nM for 50 s, median increase of peak current: 66%, range: 7–337%, n = 22; Fig. 3 (B and D); median increase of I_{50,5}: 94%, range: 17–265%, n = 22) but not using the biologically inactive stereoisomer 4α-PMA (data not shown). Moreover, as shown in Fig. 3C, during PMA application SP was unable to further sensitize the current (median increase of peak current: 4%, range: 6% to 10%, n = 4; Fig. 3 (C and D); median increase of I_{50,5}: 8%, range: 9% to 42%, n = 4), suggesting that the same mechanism underlies both effects. Together, these experiments suggest that protein kinase C mediates the stimulation of ATP-gated currents through P2X3 and P2X2

The Intracellular C Terminus of P2X Receptors Controls Potentiation by Inflammatory Mediators—Intracellular signaling cascades most likely act on intracellular parts of the P2X receptors. To identify these regions, we made use of the differential effect of SP on P2X3 and P2X2. Designing chimeras between P2X3 and P2X2. Exchanging the intracellular C termini yielded active channels. The P2X2 receptor with P2X3 C terminus (P2X2-C-X3) formed rather slowly desensitizing ion channels, which were, however, faster than P2X3 wild type channels (mean of desensitization rates ± S.D. 16.7 ± 0.7 s, n = 6, in comparison to 95.6 ± 32.4 s with P2X2 wild type, n = 8). As shown in Fig. 4A, the P2X2-C-X3 receptor gained potentiation by SP. It showed an increase in the steady state current that lasted longer than the effect on P2X3 but was still transient (median increase of peak current: 62%, range: 48–92%, n = 10, Fig. 4 (A and E); median increase of I_{50,5}: 71%, range: 48–101%, n = 10). P2X2 receptors with P2X3 C terminus (P2X2-C-X3) showed faster desensitization than the P2X3 receptor and high agonist affinity as the P2X3 receptor. Because of the fast desensitization of the P2X2-C-X3 chimera, we investigated this chimera as heteromer with P2X2. The P2X2-C-X3/P2X2 receptor activated either with αβ-meATP or with ATP lost SP regulation (median increase of peak current: 7%, range: 21% to –3%, n = 4; Fig. 4 (B and E)), implicating the cytoplasmic C terminus as an important region for regulation by SP.

To further investigate the role of the P2X2 C terminus we tested the P2X2Δ3 receptor, a P2X2 splice variant in which 69 amino acids of the cytoplasmic C terminus are missing (20). Fig. 4C shows that this splice variant was also sensitized by SP (median increase of peak current: 36%, range: 18–49%, n = 4; Fig. 4E; median increase of I_{50,5}: 33%, range: 23–50%, n = 4). Thus, the P2X2 C terminus is not necessary for SP modulation. Rather, it seems that the wild type P2X2 C terminus inhibits the modulation by SP and that this inhibition is relieved in the splice variant. This hypothesis is further supported by a gain-of-regulation with a P2X2 receptor truncated at position 401 (P2X2Δ401) deleting 72 of the about 116 cytoplasmic amino acids at the C terminus. This mutant showed kinetics similar to that of the P2X2 wild type but gained SP regulation (median increase of peak current: 21%, range: 16–23%, n = 3, Fig. 4 (D and E); median increase of I_{50,5}: 21%, range: 16–24%, n = 3).

N-terminal chimeras were also functional. A P2X2 receptor with P2X3 N terminus (P2X2-N-X3) was not regulated by SP, whereas a P2X3 receptor with P2X2 N terminus (P2X3-N-X2) was regulated by SP (data not shown), confirming that the C terminus controls regulation of P2X receptors by SP.

A Conserved N-terminal Consensus Site Is the Most Likely Target for Phosphorylation—Regulation by SP might be due to direct phosphorylation of the channel protein. We, therefore, constructed a series of point mutations, either single or in combination, of cytoplasmic serine or threonine residues in the P2X3 receptor. These mutations include all intracellular serines or threonines that are in a consensus sequence for phosphorylation by protein kinase A or C (R/K(X)0–3)R/K). The effect of these mutations is summarized in Fig. 5B. Combined mutation of all serines or threonines that are in a consensus sequence for phosphorylation at the cytoplasmic C terminal (P2X3T364A/T365A/T369A/S371A/T382A/S387A/S388A) leads to a functional, SP-regulated channel, rendering it rather unlikely that direct phosphorylation of the C terminus mediates the regulation. Moreover, there is no C-terminal consensus site conserved between P2X3 and P2X2

Single or combined amino acid substitutions of the serines or threonines at the N terminus lead in all cases to functional, regulated channels with the exception of P2X3, T12A, which was not measurable. This threonine residue is conserved in all known P2X receptors, contained in a conserved consensus sequence for phosphorylation (TXKK/K/R) and has recently been shown to be phosphorylated in the P2X3 receptor (19). Mutation of lysine 14 in P2X5 contained in the consensus sequence (P2X3, K14Q) similarly leads to not measurable channels, whereas mutation of threonine 13 leads to functional, regulated channels (Fig. 5), identifying the consensus motif TXKK/K/R as crucial for normal channel function. A T12E mutant mimicking constitutive phosphorylation was also not measurable, demonstrating that it is not just the presence of a negative charge at this position, which is important. Mutation of the corresponding threonine in either the P2X3-C-X3 or the P2X3-N-X3 chimera, or the P2X2Δ3 splice variant leads always to not measurable channels, rendering it impossible to directly assess functional consequences of this mutation. P2X2Δ3 possesses, apart from this completely conserved threonine, only one other serine/threonine at its cytoplasmic N terminus. Mutation of
this second serine (serine 11) leads to functional, regulated channels.

Together, these results suggest that the completely conserved threonine at the cytoplasmic N terminus of P2X receptors is the best candidate for an amino acid directly phosphorylated after stimulation by SP. Alternatively, protein kinases might phosphorylate an unrelated protein, which controls activity of P2X receptors.

**DISCUSSION**

Both P2X3 and P2X2/3 receptors are expressed in sensory neurons, and there is accumulating evidence that they have a specific role in nociception (17, 23–26). Moreover, activation of P2X3 leads to a much stronger nociceptive effect in inflamed compared with normal tissue (27, 28) and the P2Xδ2 δ heteromer might mediate mechanical allodynia (11). Thus, it seems that sensitization of nociceptors leads to a bigger response to ATP. Our study addressed the underlying mechanism by reconstituting the relevant signaling cascades in *Xenopus* oocytes.

Sensitization of nociceptors is due to the release of inflammatory mediators such as the neuropeptides SP (29) and Bk (30). SP is expressed in small diameter afferent neurons (31, 32) and is released upon peripheral nociceptive stimulation in the periphery as well as in the superficial dorsal horn (33). It binds with high affinity to the tachykinin receptor (NK-1), which is expressed in neurons of the spinal cord (34, 35). There are conflicting data, however, on expression of the SP (NK-1) receptor in primary afferent neurons from the DRG (34, 36–38). Although the receptor has not been identified by immunocytochemistry (34) SP can elicit an inward current in DRG neurons, which is mediated by a non-selective cation channel (37). Moreover, it had been shown that application of SP to sensory neurons can potentiate currents, which are gated by ATP (39). This suggests a modulation of P2X receptors by SP also in sensory cells. So far, neither the P2X subunit involved nor the intracellular signaling cascade was known.

The main receptor for Bk in primary afferent neurons is the B2 receptor, which couples similar to the SP receptor to a signaling pathway using Gs and phospholipase C and leading to a rise in intracellular Ca2+. Our study demonstrates that the inflammatory mediators SP and Bk can potentiate currents through P2X3 and P2X2/3 receptors in *Xenopus* oocytes.

Heteromeric P2X2/3 receptor channels showed a decrease of the desensitization rate after treatment with inflammatory mediators, which could explain the potentiation. As the potentiation was only transient, we were not able to measure the time constant for recovery. Rate of desensitization of P2X3 was not significantly changed, but the fast desensitization rate of P2X3 was decreased desensitization rate as the underlying mechanism of current potentiation. This would imply that the C terminus interacts with the N terminus to control phosphorylation. In this model the C terminus of P2X3 would stabilize phosphorylation at the N-terminal TXK site leading to constitutive phosphorylation, whereas the C terminus of P2X3 and the splice variant P2X2–2 would destabilize phosphorylation, allowing phosphorylation to be regulated. This would also imply that splicing at the C terminus of P2X3 would be a means to control modulation of receptor activity by phosphorylation at the N terminus. Interaction of cytoplasmic termini has already been shown for other structurally related channels (40). As we cannot directly prove the implication of the N-terminal phosphorylation site in the regulation of P2X receptor activity, we still must consider, however, the possibility that a different, so far unidentified protein may be phosphorylated and control activity of P2X receptors.

Hu et al. (39) reported that application of 100 nM SP leads to a transient potentiation by 127.2 ± 6.7% of ATP-gated currents in sensory neurons. This potentiation was blocked by the protein kinase inhibitor H7 and the SP (NK1) receptor antagonist spantide, resembling our own results and suggesting that the underlying mechanism is active in sensory neurons.

For Bk it is well established that it acts directly on primary sensory neurons, and several studies have suggested that PKC mediates this effect (41–43). Moreover, Bk sensitizes heat-activated currents in isolated nociceptors, and this sensitization can be mimicked by phorbol ester and blocked by staurosporine (44). Our results now suggest that another target for PKC in nociceptors may be P2X3 and that Bk may sensitize different pain-related ion channels using similar mechanisms. At the same time, we show that different inflammatory mediators may converge on the same ion channel.

Together, we show that modulation of P2X3 and P2X2/3 activity by the inflammatory mediators SP and Bk may account for sensitization of nociceptors to the action of ATP.

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Inflammatory Mediators Potentiate ATP-gated Channels through the P2X<sub>3</sub> Subunit

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