A Memory for Extracellular Ca^{2+} by Speeding Recovery of P2X Receptors from Desensitization

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Nerve endings of nociceptors (pain-sensing neurons) express an unusual subtype of ATP-gated ion channel, the P2X₃ receptor, that rapidly desensitizes (<100 msec) and slowly recovers (>20 min). Here we show that Ca^{2+} , or certain other polyvalent cations, binds to an extracellular site on rat sensory neurons and can increase current through P2X₃ channels more than 10-fold. Importantly, Ca^{2+} facilitates P2X₃ current to precisely the same level whether a transient Ca^{2+} change occurred just before or several minutes before activating the channels with ATP. This memory for past changes in Ca^{2+} is integrative in that a 90 sec Ca^{2+} stimulus delivered just before an ATP application

has the same effect as an earlier series of three, separated 30 sec Ca^{2+} stimuli. These diverse phenomena are explained by a single mechanism: Ca^{2+} speeds recovery of P2X channels from desensitization. Recovery follows an exponential growth curve that depends on the duration, but not the timing, of changes in recovery rate. Modulation of desensitization underlies a well described short-term memory in bacteria, and it might be similarly used in the nervous system.

Key words: P2X₃; purinergic; Ca^{2+} ; desensitization; recovery rate; rat; sensory neuron; nociceptor; learning; memory; short term memory; pain; hyperalgesia; dorsal root ganglia

ATP-gated ion channels (P2X receptors) are distributed throughout somatic and nervous tissues (Bean, 1992; Abbracchio et al., 1994; Surprenant et al., 1995). In response to micromolar extracellular ATP, P2X receptors pass cations nonselectively. The resulting depolarization triggers processes such as secretion, smooth muscle contraction, or neuronal excitation. Sensory neurons of rat dorsal root ganglia contain mRNA for six of the seven cloned members of the P2X family (Collo et al., 1996), and one, P2X₃, is found only in sensory neurons (Chen et al., 1995; Lewis et al., 1995). P2X₃ protein is localized both to nociceptive peripheral nerve endings (Cook et al., 1997), where it may mediate ATP-driven nociception (Bleehen and Keele, 1977; Burnstock and Wood, 1996), and to central presynaptic terminals (Vulchanova et al., 1997), where it facilitates neurotransmitter release (Gu and MacDermott, 1997).

ATP-activated current in nociceptors has transient (desensitizing) and persistent components. Channels made only of P2X₃ subunits account for transient current, and channels combining P2X₃ and P2X₂ subunits account for persistent current (Cook et al., 1997). Homomeric P2X₃ channels desensitize to sustained applications of ATP within 100 msec, yet they recover from desensitization in >20 min (Chen et al., 1995; Lewis et al., 1995; Robertson et al., 1996; Cook and McCleskey, 1997). These radically different time scales are unusual for ion channels (see Table

1). They suggest that cellular functions controlled by P2X₃ decrease after the channels are desensitized by recent exposure to ATP. However, P2X₃ current is so large in sensory neurons [Cook et al. (1997), and see below] that activation of a small fraction of the total P2X₃ channels should be sufficient to excite the cell. Therefore, P2X₃ may influence sensory physiology even when most of the channels are desensitized.

Here we show that elevated extracellular Ca^{2+} (or several other polyvalent cations) speeds the rate at which P2X₃ current recovers from desensitization. This explains a previously noted ability of Ca^{2+} to increase by many-fold the transient ATP-gated current of sensory neurons (Cook and McCleskey, 1997). This modulation has two surprising properties. First, extracellular, not intracellular, Ca^{2+} causes it. Second, a period in elevated Ca^{2+} increases P2X current to the same level regardless of when the period occurs in the interval between two ATP applications. Thus, a previous elevation of extracellular Ca^{2+} appears to lock P2X₃ channels into a more active state that does not diminish over time.

MATERIALS AND METHODS

Tissue culture and transfections. Dissociated sensory neurons were plated on polylysine/laminin-coated glass or plastic and maintained for ~3 hr at 37°C in 5% CO_2 in F12 media plus 50 ng/ml nerve growth factor (NGF). Media was then changed to L15 media plus 50 ng/ml NGF at 23°C in air until recording. Electrophysiological recordings occurred 24–48 hr after dissociation. Labeling, dissociation, and culturing of nociceptors and stretch receptors precisely followed Eckert et al. (1997). Dissociation of dorsal root ganglion neurons followed the protocol for nociceptors except that centrifugation through Percoll was not used.

Cell lines. Human embryonic kidney 293 (HEK293) cells (ATCC) were grown in a 1:1 ratio of F12 and DMEM (F12/DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals) at 37°C in 5% CO_2 /95% air. New thaws were started every 6 weeks. Stable HEK lines containing the human P2X₃ receptor (HEK-p271; generously provided by A. Surprenant, Glaxo Wellcome, Geneva, Switzerland) were maintained in 300 $\mu\text{g}/\text{ml}$ G418. HEK293 cells were subjected to transient transfection by electroporation as described by Bai et al. (1996). Cells

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were co-transfected with expression vectors for the rat P2X3 receptor [P2X3-p481 (Glaxo Wellcome) in the expression vector pCDNA3 (Invitrogen, San Diego, CA)] and for green fluorescent protein (pEGFP, Invitrogen) with or without the Ca^{2+} -sensing receptor. After electroporation, cells were allowed to recover in F12/DMEM for 24 hr and then cultured in serum-free F12/DMEM for 18 hr before electrical recordings. Electrical recordings were taken only from cells expressing intermediate levels of green fluorescent protein, as determined by fluorescence microscopy.

Electrical recording. Whole-cell currents were recorded with a patch-clamp amplifier. Unless indicated otherwise, holding and test potentials were -80 mV. To obtain a steady baseline of current despite the slow recovery from desensitization of P2X3 channels, a saturating concentration of ATP ($30 \mu\text{M}$) was always applied on a rigorous schedule (for example, at every 120 sec) so that the same fraction of channels was recovered from desensitization at each ATP application. Ca^{2+} facilitations of transient currents in nociceptors (see Fig. 1) and dorsal root ganglia neurons (see Figs. 2–6) were indistinguishable. Because the dorsal root ganglia neurons are more easily prepared, we used them for the bulk of the data gathered.

Normal internal solution consisted of (in mM): KCl 55, K_2SO_4 60, MgCl_2 7, EGTA 10, HEPES 10, pH 7.4 with KOH. BAPTA internal solution was (in mM): BAPTA (tetra-potassium salt) 40, HEPES 40, MgCl_2 7, K_2SO_4 10, pH 7.4 with KOH. Control extracellular solutions consisted of (in mM): NaCl 135, KCl 5, CaCl_2 1, MgCl_2 2, glucose 10, HEPES 10, pH 7.4 with NaOH. Divalent and trivalent cations were added to this solution as Cl^- salts. Control and test solutions perfused the vicinity of the cell through 1 or $10 \mu\text{l}$ pipettes with flow controlled by computer-operated solenoid valves. Solution exchange typically took 20 msec. Unless stated otherwise (e.g., see Fig. 2, top trace), ATP ($30 \mu\text{M}$) was applied in control solution (1 mM Ca^{2+}).

Analysis. The equation $I_t = I_{\text{max}}[1 - \exp(-t/\tau)]^2$ was fit to the recovery data in Figure 4C using the program NFIT (University of Texas Medical Branch, Galveston, TX), a least squares algorithm. I_{max} was set to 1.96 for all fits after finding that it was the best value for the Gd^{3+} curve. After determining recovery time constants in the 1 and 10 mM Ca^{2+} solutions, the curves (see Figs. 4A,B, 5B, 7) that described the effect of changes in Ca^{2+} concentration were calculated from: $I(t) = I_{\text{max}}[1 - \exp(-t_1/\tau_1)\exp(-t_2/\tau_2)]^2$, where $t = t_1 + t_2$, t_1 is the time spent at the first time constant, τ_1 , and t_2 is the time spent at the second time constant, τ_2 .

RESULTS

Extracellular Ca^{2+} increases transient P2X current

Three distinct types of P2X current were previously noted in retrogradely labeled tooth-pulp nociceptors and muscle stretch receptors (Cook et al., 1997). Increased extracellular Ca^{2+} only enhanced transient nociceptor P2X current that flows through homomeric P2X3 channels (Fig. 1). Persistent current, either from nociceptors or from stretch receptors, was unaffected. The enhanced current had the same kinetics as control (Fig. 2, bottom right; see also Fig. 6 legend) and appeared only in neurons with existing transient current, suggesting that the additional current does not result from promotion of a new type of P2X current.

Ca^{2+} might act at three different locations to cause the larger transient current in Figure 1: (1) it might be highly permeant through the pore of the channel; (2) it might enter the cell and bind to the channel or to a modulating molecule in the cytoplasm; or (3) it might bind to an extracellular site. Figure 2 distinguishes between these possibilities and demonstrates the general protocol used throughout the rest of the paper.

To obtain a consistent baseline of current despite the extremely slow rate of recovery from desensitization, we applied ATP on a rigorous schedule, precisely every 2 min in Figure 2. ATP ($30 \mu\text{M}$) was applied for 300 msec, sufficient to fully desensitize transient P2X current. We estimate (see below) that $\sim 6\%$ of the current recovers in the 2 min interval between ATP applications. When Ca^{2+} was elevated to 10 mM for 10 sec before and during the third ATP application at the top of Figure 2, there was no significant effect (traces are shown at an expanded time scale on

Figure 1. Elevated extracellular Ca^{2+} increases transient nociceptor current. A saturating concentration of ATP ($30 \mu\text{M}$) was applied for 300 msec at 2 min intervals in 1 mM Ca^{2+} solution. During the 2 min before ATP, extracellular Ca^{2+} was increased to 10 mM where indicated. Ca^{2+} returned to 1 mM 10 sec before test application of ATP. Means were (in nanoampere) -1.1 ± 0.3 , -2.0 ± 0.5 , and -0.32 ± 0.09 for transient nociceptor currents ($n = 9$), persistent nociceptor currents ($n = 11$), and stretch receptor currents ($n = 9$), respectively (1 mM Ca^{2+}). Percentage increase in current after 10 mM Ca^{2+} treatment was 260 ± 20 , 4 ± 4 , and 4 ± 4 for the three cell types.

the right). This demonstrates that the Ca^{2+} facilitation is caused neither by high Ca^{2+} permeability nor by Ca^{2+} entry through the P2X channel. In the bottom set of records, Ca^{2+} was elevated for most of the 2 min of the second interval, but then was dropped back to 1 mM before ATP was applied. Current increased 2.5-fold, demonstrating that it is the preincubation in elevated Ca^{2+} that causes facilitation. The last application of ATP produced an identical current to that produced before the Ca^{2+} increase, demonstrating that ATP reverses the facilitation. Such Ca^{2+} -induced facilitation could be repeated many times on a cell without attenuation (data not shown).

There is no evidence of Ca^{2+} -induced current during the 2 min incubation interval in Figure 2. Moreover, changes in holding potential (from -120 to -40 mV) that should alter Ca^{2+} entry during the interval neither caused facilitation nor altered Ca^{2+} -induced facilitation (data not shown). Chelation of intracellular Ca^{2+} by various compounds (10 mM EGTA , 40 mM BAPTA , 5 mM ATP) did not block facilitation (Fig. 2 shows 10 mM EGTA ; BAPTA and ATP are not shown). P2X current did not change

Figure 2. Prolonged pretreatment with elevated Ca^{2+} increases transient P2X current. The sequences of four ATP applications (arrows, 30 μM , 300 msec) occurred 2 min after P2X channels were desensitized by ATP. These partially recovered currents are $\sim 6\%$ of the maximal possible current (see Fig. 4C). Rightmost traces show currents elicited by the indicated ATP applications (asterisks) at an expanded time scale. In the top traces, extracellular Ca^{2+} was raised (filled bars) to 10 mM starting 10 sec before the third ATP and returned to 1 mM 10 sec after. Current did not increase. In bottom traces, Ca^{2+} was 10 mM for the duration of the interval between the ATP applications, but it was 1 mM when ATP was applied. Current increased 2.5-fold.

when we intentionally caused Ca^{2+} entry by opening voltage-gated Ca^{2+} channels just before ATP application (depolarizations to 0 mV for 0.1–20 sec; data not shown). Because increasing intracellular Ca^{2+} , buffering intracellular Ca^{2+} , or changing the transmembrane Ca^{2+} driving force caused no effect, we conclude that the Ca^{2+} binding site lies on the outside face of the cell.

Prolonged facilitation and its mechanism

Figure 2 shows that prolonged (2 min) but not brief (10 sec) elevation of Ca^{2+} facilitated P2X current. Figure 3 further describes the time dependence of facilitation and shows that it does not decay as expected. Two minutes separated each ATP application (arrows) in Figure 3A, and Ca^{2+} changed between 1 and 10 mM when indicated. Current increased twofold if Ca^{2+} increased for 1 min and 3.6-fold if Ca^{2+} increased for 2 min. Thus, facilitation increases with increasing time in elevated Ca^{2+} . However, facilitation did not diminish after Ca^{2+} was lowered. Current increased to the same level whether a 1 min Ca^{2+} elevation had just completed (“late” application) or had completed 1 min earlier (“early” application). All cells showed such behavior (Fig. 3B). This contrasts with the expectation that modulation should diminish after the modulating compound is removed.

To determine why facilitation fails to decay, we replotted the early and late data in Figure 3B as a pair of points (boxes) on the graph in Figure 4A. The horizontal axis gives the time since the last application of ATP, 2 min; the various curves are explained below. Figure 4B shows results from analogous experiments in which 6 min separated ATP applications. Ca^{2+} was elevated for 2 min either at the beginning or the end of the 6 min interval. Regardless of the time elapsed since Ca^{2+} was elevated, the current increased to the same level (50% above that seen with 1 mM Ca^{2+} throughout the 6 min interval; asterisk). Because facilitation was identical although 4 min separated the two Ca^{2+} elevations, the “memory” for Ca^{2+} persisted for those 4 min, as it had for the 1 min in Figure 4A.

Figure 3. Transient P2X receptors remember previous Ca^{2+} exposure. *A*, ATP-evoked currents (right) followed a previous ATP application (arrows) by 2 min. The time scale changes at break in current baseline. Extracellular Ca^{2+} was increased to 10 mM for length of time indicated (filled bars) during the 2 min between ATP applications (30 μM ATP, 1 mM Ca^{2+}). Relative magnitudes of current are indicated in parentheses. *B*, The summary of data for six such experiments shows that Ca^{2+} modulation increased with increased duration of Ca^{2+} elevation. However, a 1 min increase has the same effect whether it is applied early or late in the interval between ATP applications. This memory for elevated Ca^{2+} persists for at least 4 min (see Fig. 4B).

Figure 4C shows that Ca^{2+} speeds the recovery of P2X channels from desensitization, the only effect on kinetics that we have found. At time 0 in the graph, an ATP application fully desensitized the channels. Recovery from desensitization was examined with subsequent applications at the times indicated on the axis. Recovery was approximately twofold faster in 10 mM Ca^{2+} than in 1 mM. Another multivalent cation, Gd^{3+} , sped recovery fourfold at a 1000-fold lower concentration than Ca^{2+} . The sigmoidal data in Figure 4C is fit with the equation $I_t = I_{\text{max}} \times [1 - \exp(-t/\tau)]^2$ (solid curves).

The solid curves in Figures 4, *A* and *B*, are the first 2 and 6 min, respectively, of the recovery equations from Figure 4C for 1 mM ($\tau = 7.1$ min) and 10 mM Ca^{2+} ($\tau = 3.5$ min). The dashed curves show what should happen if recovery time constants toggle between 7.1 and 3.5 min when Ca^{2+} concentration changes between

Figure 4. Increased rate of recovery from desensitization creates the Ca^{2+} memory. Facilitation is undiminished for 1 min (*A*) or 4 min (*B*) after removal of Ca^{2+} . Data points (boxes $\pm$ SEM) were obtained using a protocol similar to that of the *middle traces* in Figure 3*A*. ATP was applied at either 2 min (*A*) or 6 min (*B*) intervals. Ca^{2+} was increased from 1 to 10 mM at either the beginning of the interval (■) or the end (□) for either 1 min (*A*) or 2 min (*B*). Early and late Ca^{2+} treatments gave identical currents in each case. *Solid lines* are the recovery curves from Figure 4*C* (see below) for 1 mM Ca^{2+} ($\tau = 7.1$ min) or 10 mM Ca^{2+} ($\tau = 3.5$ min). *Dashed curves* are the theoretical predictions (see Materials and Methods for calculation), assuming that the recovery rate changes immediately after changes in Ca^{2+} concentration (*heavy arrows*). This mechanism predicts the perfect persistence of facilitation between the Ca^{2+} applications. Data from each cell were normalized to the value obtained with 1 mM Ca^{2+} (*asterisks*); vertical scales are identical to that in Figure 1*C*. *C*, Ca^{2+} and Gd^{3+} speed recovery from desensitization. ATP (30 μM) was applied at the indicated times after current was fully desensitized at time 0. During the interval between ATP applications, extracellular solution contained either 1 mM Ca^{2+} (○, $n = 8$), 10 mM Ca^{2+} (●, $n = 8$), or 10 μM Gd^{3+} and 1 mM Ca^{2+} (■, $n = 6$). In all cases, the solution was changed to 1 mM Ca^{2+} 10 sec before the ATP application. Peak currents from each cell were normalized relative to that recorded with a 4 min interval in 10 mM Ca^{2+} (*asterisk*). Error is expressed as SEM. The non-normalized currents ranged from -1.5 to -9.8 nA; mean = -4.6 ± 0.6 nA (4 min interval and 10 mM extracellular Ca^{2+}). Curves represent

1 and 10 mM. Our equation assumes that the recovery time constant changes immediately after change of extracellular Ca^{2+} . These curves predict the equivalence of the data points and closely approximate their absolute magnitude. This indicates that the Ca^{2+} dependence of recovery from desensitization fully explains the memory for Ca^{2+} . As described further in Discussion, the equivalence of the data points relies on an unintuitive property of exponential growth: regardless of when a transient change in growth rate occurs, the end effect is always the same.

The Ca^{2+} memory is integrative

The above mechanism predicts that the memory for Ca^{2+} should be integrative. If several Ca^{2+} elevations are applied in succession, their effect should persist and should be identical to that of a single stimulus having the same total time in elevated Ca^{2+} . Figure 5 tests this prediction by comparing three 30 sec Ca^{2+} elevations, each separated by 30 sec, with a single 90 sec elevation. Because the 90 sec elevation occurs just before an ATP application whereas the last of the 30 sec elevations occurs well before, the experiment also tests for the persistence of integration. The *dashed lines* on the traces in Figure 5*A* indicate the amplitudes that the model predicted for Ca^{2+} facilitation; they closely approximate the observed facilitated currents, which are almost identical to each other. Figure 5*B* shows the average results from five experiments (*boxes*) along with calculated time courses (*dashed curves*) used to predict the amplitudes of facilitation.

Agonists of the Ca^{2+} binding site

Some multivalent cations substitute for Ca^{2+} and others do not. At 1000-fold lower concentration, Gd^{3+} sped recovery faster than 10 mM Ca^{2+} (Fig. 4*C*). The actions of Gd^{3+} and Ca^{2+} were dose-dependent and increased current ~ 13 -fold at 30 μM and 100 mM, respectively (Fig. 6*A*). Ba^{2+} and spermine were as potent as Ca^{2+} , whereas Mg^{2+} , Ni^{2+} , Cd^{2+} , and Zn^{2+} were relatively ineffective (Fig. 6*B*). The variety of effective agonists raises the possibility that the putative physiological ligand is not Ca^{2+} or another metal. More modest changes in Ca^{2+} also caused significant increases in current. When Ca^{2+} was increased from 1 to 3 mM, P2X current increased in 5 of 10 cells (average increase, 4.4-fold; range, 1.7–6.7-fold). We found no changes in recovery when Ca^{2+} was dropped below 1 mM.

This unusual agonist profile resembles that of the Ca^{2+} -sensing receptor (CaSR), a G-protein-coupled receptor that detects plasma Ca^{2+} concentration and alters homeostatic responses accordingly (Brown et al., 1993; Ray et al., 1997). We considered whether the CaSR might mediate facilitation of P2X channels and ruled out this hypothesis with the following experiments. Using an antibody to the CaSR, we detected a protein of appropriate M_r in Western blots from sensory ganglia preparations and saw immunocytochemical staining in cultured sensory neurons [data not shown; also see Bukoski et al. (1997)]. However, intracellular perfusion of 300 μM GTP γ S did not modify facilitation; it should if a G-protein is involved. Moreover, transiently transfected rat P2X3 receptors exhibited Ca^{2+} and Gd^{3+} facilitation, although the parent cell line (HEK293 cells) exhibited no immunostaining for the CaSR. Co-transfection of the

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a fit to the equation $I_t = I_{\max} \times [1 - \exp(-t/\tau)]^2$. Recovery time constant $\tau = 7.1$, 3.5, and 1.7 min for 1 mM Ca^{2+} , 10 mM Ca^{2+} , and 10 μM Gd^{3+} , respectively. *Dashed line* represents predicted 100% recovery ($I_{\max} = 1.96$ corresponding to a mean maximum current = -9.0 nA).

Figure 5. P2X channels integrate and retain facilitations caused by multiple Ca^{2+} stimuli. *A*, The first ATP (30 μM) application (smaller expanded current, *right*) followed 4 min in 1 mM Ca^{2+} ; the second application (larger current) followed the indicated Ca^{2+} elevations. *Dashed lines* show the predicted value of facilitation (see Materials and Methods for description of the calculation). Facilitation was about equal to the predicted values and was identical whether Ca^{2+} was elevated for one 90 sec period just before evoking current (*bottom protocol*) or three 30 sec periods completed 1.5 min before evoking current (*top protocol*). *B*, Average facilitation ($n = 5$) relative to 1 mM Ca^{2+} (asterisk, mean current = 4.8 ± 0.9 nA) after the one 90 sec ($\square$, mean increase = 1.61 ± 0.10) or three 30 sec Ca^{2+} elevations ($\blacksquare$, mean increase = 1.62 ± 0.07). *Solid curves* show 4 min of the recovery curves for 1 and 10 mM Ca^{2+} from Figure 4C, and *dashed curves* show the calculated recovery in response to the indicated changes in Ca^{2+} concentration.

CaSR with P2X3 receptors made no discernible difference. Another molecule proposed to sense extracellular Ca^{2+} , the metabotropic glutamate receptor (Kubo et al., 1998), was also ruled out as a mediator because of its absence in HEK293 cells and the failure of receptor agonists to facilitate P2X current in sensory neurons. Thus, we found no molecule that conferred Ca^{2+} sensitivity on P2X channels. Interestingly, Ca^{2+} and Gd^{3+} only weakly facilitated human P2X3 channels in HEK293 cells (data not shown). These results suggest that the Ca^{2+} binding site is on the channel and that Ca^{2+} facilitates rat more effectively than human P2X3 receptors.

DISCUSSION

The simplest interpretation of our results is that Ca^{2+} binds directly to an extracellular site on P2X3 receptors of rats, and this

Figure 6. Gd^{3+} potently increases transient nociceptor current. *A*, ATP (30 μM) was applied at 1 min intervals in 1 mM Ca^{2+} ; the indicated ion was present (in addition to 1 mM Ca^{2+}) during the entire 1 min interval, but not during the ATP application. Gd^{3+} ($\blacksquare$) increased P2X current at almost 10,000-fold lower concentration than Ca^{2+} ($\square$). Mg^{2+} ($\bullet$) was ineffective. Data are expressed as means of P2X current $\pm$ SEM. For Gd^{3+} , Ca^{2+} , and Mg^{2+} , $n = 8, 10$, and 5, respectively. Although elevation of Ca^{2+} from 3 to 100 mM increased current 9 ± 2 -fold (Fig. 6A), desensitization rate did not decrease ($\tau_{\text{decay}} = 45 \pm 9$ msec at 3 mM Ca^{2+} ; $\tau_{\text{decay}} = 29 \pm 4$ msec at 100 mM Ca^{2+}). *B*, Various multivalent cations increased P2X current. At 10 mM, barium (Ba) and spermine (Spm) were as effective as Ca^{2+} . In contrast, nickel (Ni), cadmium (Cd), and zinc (Zn) were ineffective. Data are expressed as means of peak currents, normalized to current with 1 min pretreatment in 10 mM Ca^{2+} , $\pm$ SEM for $n = 5$ –11 neurons.

Ca^{2+} -bound receptor recovers more quickly from desensitization. Although we did not see an effect of Ca^{2+} on persistent P2X current in sensory neurons, Ca^{2+} may speed recovery of other desensitizing P2X receptors (e.g., P2X1). By modulating recovery, a transient change in extracellular Ca^{2+} facilitates P2X3 current to precisely the same degree, no matter when the change occurs. This endows the channel with a memory for transient changes in extracellular Ca^{2+} . Facilitation should persist no longer than the time it takes to complete recovery from desensitization, approximately 20 min. Because many channels, receptors, and enzymes involved in neural signaling undergo desensitization, modulation of recovery is worth considering as a mechanism for certain short-term memories. Below we discuss general properties of this mechanism and speculate about its possible role in P2X-mediated sensation.

Figure 7. Calculated effects of 2 min Ca^{2+} increases occurring at various times during recovery from desensitization. Twenty minutes of the calculated recovery from desensitization in 1 mM Ca^{2+} (bottom solid curve, $\tau = 7.1$ min), 10 mM Ca^{2+} (top solid curve, $\tau = 3.5$ min), or 18 min in 1 Ca^{2+} and 2 min in 10 Ca^{2+} (middle dashed curves). The six dashed curves correspond to six different starting times for the 2 min interval in 10 Ca^{2+} . All six curves superimpose after completion of the Ca^{2+} changes. Curves are solutions of $\text{Frac}(t) = [1 - \exp(-t_1/7.1)\exp(-t_2/3.5)]^2$, where Frac is the fraction of channels available to be activated, $t = t_1 + t_2$, t_1 and t_2 are the number of minutes spent in 1 and 10 mM Ca^{2+} , respectively. The same equation fit the 2, 6, and 4 min of data in Figures 4A,B and 5B.

Memory through desensitization

The ability of P2X3 receptors to encode a transient change in Ca^{2+} as a perfectly persisting change in activity relies on a basic property of exponential growth: no matter when it occurs, a transient change in growth rate has precisely the same outcome on a later value of the exponential. As an example, consider 5% interest on \$1000 compounded yearly for 20 years. The profit will be increased by \$258.71 if the interest increases to 10% for the final 2 years. If the interest increases to 10% for the initial 2 years, the extra profit is only \$107.50 at first. However, 5% interest accrues on this until the final extra profit is precisely the same: \$258.71 at the end of the 20 year interval. Therefore, the value of the final profit provides a record of an interest rate change regardless of when the change occurs. [In general, when interest compounds either at rate b or at rate c , the value after t intervals is $F(t) = F_0(1 + b)^{t_1}(1 + c)^{t_2}$, where F_0 is the starting value, $t = t_1 + t_2$, t_1 is the number of intervals at rate b , and t_2 is the number of intervals at rate c . The final value depends on the number of intervals spent at each rate, but not on the timing of the intervals.] The same holds true for any continuously changing exponential function. For example, if exponential decay occurs with a time constant τ_1 for a time t_1 and with a time constant τ_2 for a time t_2 , the value is $F(t_1 + t_2) = \exp(-t_1/\tau_1)\exp(-t_2/\tau_2)$, regardless of whether the interval at τ_1 occurs first or second.

During recovery from desensitization, the number of activatable P2X channels increases along an exponential curve that is subject to the above principle. The calculation in Figure 7 shows 20 min of the recovery of P2X receptors exposed to Ca^{2+} concentrations (1 and 10 mM) that altered recovery rate twofold. The middle curves show the recovery if channels were exposed to 10 mM Ca^{2+} for 2 min at various starting times. All of these curves are identical after their respective Ca^{2+} elevations have occurred. This creates a memory of a previous Ca^{2+} elevation because one that occurred many minutes before has the same effect on the number of available channels as one that just occurred.

Table 1. Desensitization and recovery of ligand-gated ion channels

Channel	Desensitization (sec)	Recovery (sec)	Ratio
P2X3 (transient nociceptor)	0.03	460	15,000
P2X2/3 (persistent nociceptor)	4	60	15
AMPA	0.004	0.03	8
Kainate	1	60	60
NMDA	0.05	1	20
GABA	0.02	0.09	4.5
Glycine	1	20	20
Nicotinic ACh	0.05	2	40
5-HT3	0.4	30	80
H ⁺	0.5	20	40

Comparison of recovery and desensitization rates of various ligand-gated ion channels illustrates the unique nature of P2X3 current. Desensitization and recovery time constants are given in the first two columns and their ratio is in the third. For P2X3 current, this ratio is hundreds-fold larger than those of other ligand-gated ion channels. P2X3 may be especially sensitive therefore to modulation of recovery rate. (Data from Konnerth et al., 1987; Bufler et al., 1993; Jones and Westbrook, 1996; Barann et al., 1997; Cook and McClesky, 1997a; Wilding and Huettner, 1997).

Receptor-based desensitization governs a well described short-term memory in bacteria (Morimoto and Koshland, 1991). To swim up a gradient of amino acid nutrients, bacteria sample the medium, compare the present to a past sample, and adjust swimming behavior accordingly. Increasing or decreasing gradients shift the balance between a fast, positive regulatory response (receptor activation by attractant) and a slower, negative regulatory response (receptor desensitization). The memory of past samples occurs because of the comparatively slow time course of a covalent modification that controls the level of receptor desensitization.

Molecular mechanisms proposed for short-term memory in neurobiology include ion channel modulation (Shuster et al., 1985), ion channel desensitization (Heckmann and Dudel, 1997), and presynaptic Ca^{2+} dynamics (Tank et al., 1995). Modulation of recovery from desensitization provides yet another possible mechanism. Any memory based on it would have three properties that are evident in the Ca^{2+} facilitation of P2X receptors. First, although the Ca^{2+} facilitation of P2X receptors can last many minutes in the absence of extracellular ATP, it can vanish in <1 sec when ATP is applied. The ATP desensitizes the channels and thereby erases the Ca^{2+} facilitation. Second, Ca^{2+} facilitation can last no longer than the time to complete the recovery from desensitization. This can range from tens of milliseconds in some channels to the 20 min for P2X3 receptors, a range that encompasses the durations (seconds to minutes) of behavioral short-term memory. Third, facilitations caused by a series of Ca^{2+} stimuli sum to the same value as that of a single stimulus of the same total duration. Such additivity is like short-term memory, which is strengthened by rehearsal, and unlike long-term potentiation, which is evoked by a single tetanus that renders subsequent tetani ineffective.

Does Ca^{2+} concentration change outside sensory neurons?

Ca^{2+} modulation of recovery from desensitization can be relevant only if extracellular Ca^{2+} changes in physiological conditions. Because of the minute dimensions of interstitial spaces, the evidence regarding such changes is equivocal. However, a Ca^{2+} precipitation method provided a clear demonstration of a standing gradient of extracellular Ca^{2+} in skin (Menon et al., 1985).

The inner epidermis has lower Ca^{2+} than the outer epidermis, and this gradient plays a crucial role in keratinocyte development (Hennings et al., 1980; Bikle et al., 1996). Injury to skin might disrupt these outer epidermal Ca^{2+} reservoirs as well as release ATP from damaged cells. Spread of high Ca^{2+} to inner layers would increase the ATP sensitivity of sensory nerve endings.

Ca^{2+} might also change in the synaptic cleft. Central terminals of sensory neurons express P2X₃ receptors (Vulchanova et al., 1997), and activation of presynaptic P2X receptors enhances glutamate release (Gu and MacDermott, 1997). Changes in cleft Ca^{2+} concentration with synaptic activity have been proposed (Benninger et al., 1980; Pumain et al., 1987; Stabel et al., 1990). P2X receptors are the third class of synaptic receptors (metabotropic glutamate receptors and nicotinic acetylcholine receptors are the other two) that are modulated by extracellular Ca^{2+} (Mulle et al., 1992; Kubo et al., 1998). These findings suggest a role for extracellular Ca^{2+} in synaptic function.

Chronic desensitization

Ca^{2+} modulation of recovery from desensitization impacts only desensitized P2X receptors. Two properties suggest the possibility of chronic desensitization. First, the ratio of rapid desensitization and slow recovery rates for P2X₃ current is at least 100-fold greater than that reported for other ligand-gated channels (Table 1). Release of ATP more frequently than once every 20 min would cause chronic desensitization of P2X₃. Second, the channels are grossly overexpressed. The amplitude of nondesensitized transient P2X current (mean maximum current = -9 nA, $n = 14$) (legend to Fig. 4C) compares with that through voltage-gated Na^+ channels in the same cells. The Na^+ current drives 100 mV depolarizations, yet sensory potentials need to be only 10–20 mV. Overexpression would allow P2X₃ channels to generate sufficient current for sensory potentials even if the majority of channels were desensitized. If chronic desensitization limits P2X₃ current in sensory neurons, promotion of recovery rate (by extracellular Ca^{2+} or other multivalent cations) would provide a powerful mechanism for controlling ATP sensitivity of sensory nerve endings and presynaptic terminals.

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