Receptor desensitization can determine the time course of transmitter action and profoundly alter sensitivity to drugs. Among P2X receptors, ion currents through homomeric P2X₄ receptors exhibit intermediate desensitization when compared with P2X₁ and P2X₃ (much faster) and P2X₇ and P2X₂ (slower). We recorded membrane currents in HEK293 cells transfected to express the human P2X₄ receptor. The decline in current during a 4-s application of ATP (100 μM) was about 30%; this was not different during whole-cell or perforated patch recording. Alanine-scanning mutagenesis of the intracellular C terminus identified two positions with much accelerated desensitization kinetics (Lys³⁷³: 92% and Tyr³⁷⁴: 74%). At position 373, substitution of Arg or Cys also strongly accelerated desensitization: however, in the case of K373C the wild-type phenotype was fully restored by adding ethylammonium methanethiosulfonate. At position 374, phenylalanine could replace tyrosine. These results indicate that wild-type desensitization properties require an aromatic moiety at position 374 and an amino rather than a guanidine group at position 373. These residues lie between previously identified motifs involved in membrane trafficking (XXKXK and YXXGL) and implicate the C-terminal also in rearrangements leading to channel closing during the presence of agonist.

P2X receptors belong to a family of ATP-gated ion channels, seven subunits of which have been currently identified and cloned (P2X₁₋₇) (1). Functional P2X receptors probably form as trimers, incorporating the same or different subunits (2, 3). The P2X subunits are considered to have two hydrophobic transmembrane domains with intracellular N and C termini and a large extracellular ectodomain containing conserved cysteines (1); the ectodomain also contains residues that appear to contribute to the binding site for ATP (4, 5).

In the central nervous system, P2X receptors have been implicated in mediating ATP-dependent fast excitatory neurotransmission (6–9). The P2X₄ subunit is highly abundant in the central nervous system, with expression in both spinal cord and brain, including dentate gyrus, CA1/CA3 pyramidal, and cerebellar Purkinje cells (10–12); they are particularly localized to glutamatergic synapses (12). Upon activation, P2X₄ can regulate cellular Ca²⁺ levels via direct permeation and by the activation of voltage-dependent Ca²⁺ channels. P2X receptor activity has therefore been proposed to be important in synaptic plasticity (13, 14).

The time course of the effect of ATP at P2X receptors is strongly influenced by receptor desensitization, a feature common to most other ligand-gated ion channels. Desensitization manifests itself as a decline in current amplitude during agonist occupancy of the receptor, and in the case of P2X₄, it can be modified pharmacologically (15). Recombinant P2X receptors display varying degrees of desensitization; membrane current recordings show that P2X₁ and P2X₃ undergo fast desensitization (tens of milliseconds), P2X₄ exhibits moderate desensitization (several seconds), and P2X₂, P2X₅, and P2X₇ show less desensitization (1, 16, 17). For various P2X receptors, desensitization can be regulated by cellular signaling events, which further suggests its importance in P2X receptor physiology. Several studies have demonstrated regulation of P2X desensitization by protein kinase activity, either via direct N-terminal phosphorylation (at a highly conserved protein kinase C site) or via phosphorylation of an associated protein (18, 19). P2X₄ desensitization can be decreased by the calcineurin-mediated dephosphorylation of the N terminus (20).

Experiments using chimeric constructs of P2X₁ and P2X₃ subunits identified desensitization critical domains as the transmembrane segments and the intracellular juxtamembrane regions (up to 15 amino acids in length). Other domains within the C terminus of P2X₂ have been identified by functional analysis of splice variants (21, 22). P2X receptors have very different C-terminals, and the regions important for P2X₄ subunits are not known. Given the likely importance of the P2X₄ receptor in central synaptic physiology, it seemed useful to understand further the molecular mechanisms that may contribute to P2X₄ desensitization.

**EXPERIMENTAL PROCEDURES**

Human P2X₄ subunit with a C-terminal EMYPME epitope tag subcloned into pcDNA3.1(+) was used as the template for all mutagenesis reactions. Mutagenesis was achieved using Pfu turbo polymerase and QuikChange methodology (Stratagene). Truncated receptors were generated by the introduction of a premature stop codon by mutagenesis. HEK293 cells were grown in 10% fetal calf serum in Dulbecco’s minimal essential medium at 37 °C with 5% CO₂ in a humidified incubator. Cells were transiently transfected with plasmids encoding wild-type or mutant P2X receptor (1 μg) and enhanced green fluorescent protein (0.1 μg) using Lipofectamine 2000 (Invitrogen).

Whole-cell recordings were made from HEK293 cells at room temperature, 24–48 h after transfection. The extracellular solution contained (mM): 145 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, 13 d-glucose, and 10 HEPES. The intracellular pipette solution contained (mM): 145 NaCl, 10 HEPES, and 10 EGTA. Solutions had pH 7.3 after titration with 5 M NaOH. Chemicals were purchased from Sigma (Poole, UK). Pipettes had resistances of 3–5 MΩ. For perforated patch-clamp, perforation was achieved by the addition of 120 μg/ml amphotericin in the patch pipette solution. Perforation typically occurred 5–8 min after gigaseal formation. ATP was applied using an RSC 200 system (Biological Science Instruments, Grenoble, France). Ethyl ammonium methanethiosulfonate (MTSEA²). Toronto Research Chemicals) was prepared as a concentrated stock, stored at ~20 °C, and diluted in extracellular solution.

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² The abbreviation used is: MTSEA, ethyl ammonium methanethiosulfonate.
RESULTS

Decline in Response with Repeated ATP Application—We distinguish two forms of desensitization at the human P2X4 receptor. The first is the decline in the peak current with repeated brief applications of ATP. Thus, in whole-cell recording, the peak current amplitude progressively declined when ATP (100 μM, 2 s) was applied at 1-min intervals (Fig. 1a). This decline was well fit with a single exponential (τ = 93 ± 6.2 s, n = 25 cells), reaching a plateau level at ~20% of the initial peak amplitude. We observed no recovery from desensitization with repeated application when the application interval was increased to 5, 10, or 20 min (data not shown).

P2X4 currents recorded using the perforated patch configuration (amphotericin 120 μg/ml) did not show decline with repeated applications (Fig. 1a and b). However, we were unable to influence the decline in response by a range of other perturbations. Ivermectin (3 μM, 3 min) did not alter the kinetics of current decline (τ = 98 ± 5.4 s, n = 8, p > 0.05) (Fig. 1a), although it increased peak current amplitudes ~3-fold.

The rate of current decline was unchanged in external solutions containing 0 mM calcium and also not different when the recording pipette contained ATP (1 mM), GTP (1 mM), staurosporine (100 μM), genistein (100 μM), phosphatidylinositol 4,5-bisphosphate (50 μM), or U73122 (10 μM). P2X4ΔY378, which is truncated so as to lack the endocytic motif YEQL (Fig. 2), showed properties not different from wild type receptors (τ = 91 ± 5.2 s, n = 12 cells, p > 0.05).

Decline in Response during ATP Application—The second form of desensitization is the decline in the membrane current during the continued application of ATP, and this is the subject of the remainder of this paper. Currents evoked by ATP (100 μM) rose to their peak amplitude in 410 ± 8.2 ms, and the average peak amplitude was 151 ± 30 pA (n = 25 cells). During a 5-s application of ATP (100 μM), wild-type currents declined to a value that was 32 ± 7.9% (n = 25 cells) of their initial peak amplitude. These values were obtained in the whole-cell configuration, but the decline in current during the application was not different when recorded using the perforated patch configuration (27 ± 5.2%, n = 12 cells, p > 0.05) (Fig. 1c). However, the decline during the application was immediately prior to application. MTSEA was applied by superfusion at a rate of ~2 ml/min.

For immunohistochemistry, transfected cells were fixed with 4% paraformaldehyde for 5 min followed by permeabilization with 0.1% Triton X-100. Cells were blocked for 30 min (phosphate-buffered saline, 0.5% bovine serum albumin) and incubated with primary antibody (rabbit anti-EE, 1:500; Bethyl Laboratories) in blocking solution for 1–2 h at room temperature. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit, 1:200; Jackson ImmunoResearch) for 1–2 h at room temperature. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit, 1:200; Jackson ImmunoResearch) for 1–2 h at room temperature. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit, 1:200; Jackson ImmunoResearch) for 1–2 h at room temperature. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit, 1:200; Jackson ImmunoResearch) for 1–2 h at room temperature. After washing, cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit, 1:200; Jackson ImmunoResearch) for 1–2 h at room temperature.
P2X<sub>4</sub> Desensitization

**FIGURE 2.** P2X<sub>4</sub> receptor C-terminal truncations. A, left, schematic representation of human P2X<sub>4</sub> receptor subunit showing topology and sites of C-terminal truncations used. Right, alignment of relevant parts of C terminus tail of P2X receptors. Boxed, YXX0K motif common to all receptors. Underlined, P2X<sub>2</sub> motif that binds to AP2 protein. Bold, Lys<sup>373</sup> and Tyr<sup>374</sup>. B, representative evoked currents (ATP; 100 μM, 5 s) for wild-type and three C-terminally truncated mutants (ΔY378, ΔK373, and ΔK362). Cells were voltage clamped at −60 mV. ΔK373 and ΔK362 were non-functional (n = 8 cells each). Currents shown were obtained during the first ATP application.

substantially slowed by ivermectin (3 μM, 3 min; 2.4 ± 0.8%, n = 12 cells, p < 0.01) (Fig. 1c) (15). The decline in response was independent of ATP concentration (1 μM, 30 ± 1.8%, 3 μM, 31 ± 1.4%, 30 μM, 31 ± 4.1%, 300 μM, 34 ± 2.0%; n = 6 cells). Further experiments were carried out with 100 μM ATP. The rate of current decline was not different when the extracellular calcium concentration was 0 mM as compared with 2 mM; it was also independent of holding potential (−60 mV versus +60 mV).

**Truncations and Alanine Scanning in the C Terminus**—The C terminus of the human P2X<sub>4</sub> receptor extends from residue Leu<sup>358</sup> at the end of the second transmembrane domain, to Glu<sup>386</sup>. A truncation that deleted the final eleven amino acids (ΔY378) had properties not different from wild type P2X<sub>4</sub> as it showed a similar desensitization during the first ATP application (Fig. 2) and a similar decline with repeated applications. This decline in the current was not different from that seen with P2X<sub>4</sub>[K373A], P2X<sub>4</sub>[Y374K], or P2X<sub>4</sub>[K373D,Y374K] receptors (92 ± 5.4%, 93 ± 3.2%, and 92 ± 4.8%, respectively, n = 8–12 cells, p > 0.05). Phenylalanine substituted fully for tyrosine at position 374: P2X<sub>4</sub>[Y374F] showed currents and desensitization not different from wild type (28 ± 6.2%, n = 8 cells, p > 0.05) (Fig. 3a).

**Further Studies at Lys<sup>373</sup>**—When arginine replaced lysine at position 373 (P2X<sub>4</sub>[K373R]) the receptors exhibited a current not significantly different from P2X<sub>4</sub>[K373A] (89 ± 3.4%, n = 8 cells, p > 0.05) (Fig. 3a). This indicates that the arginine side chain (–CH<sub>2</sub>–NH<sub>2</sub>) cannot substitute for that of lysine (–CH<sub>2</sub>–NH–) at position 373. We examined further the difference between the [ε-cystosin]-amino group and the guanidino moiety by expressing the P2X<sub>4</sub>[K373C] receptor. Current evoked by ATP at this receptor declined similarly to that seen with P2X<sub>4</sub>[K373A] (87 ± 3.4%, n = 8 cells, p > 0.05). However, MTSEA (1 mM; 10 min) restored the wild-type phenotype (38 ± 6.3%, n = 6 cells, p > 0.05) (Fig. 4a). This effect of MTSEA was prevented by intracellular cysteine (20 mM; in the recording pipette; Fig. 4b). MTSEA had no effect on wild type (33 ± 4.2%, n = 6–10) or P2X<sub>4</sub>[K373A] receptors (90 ± 4.2%, n = 8, p > 0.05), indicating that the effect of MTSEA was a specific modification of the cysteine at position 373 (Fig. 4).

These results indicate that maintained channel opening in the presence of ATP requires Lys<sup>373</sup> (or the equivalent side chain –S–S–(CH<sub>2</sub>)<sub>4</sub>–NH<sub>2</sub>). We asked therefore in how many subunits of a homotrimeric receptor was the lysine required? We co-expressed wild-type and P2X<sub>4</sub>[K373A] receptors in nominally equal amounts. Whole-cell recordings showed typical ATP-activated currents, but the decline during the application was intermediate between that seen for cells expressing either wild-type or P2X<sub>4</sub>[K373A] receptors alone (Fig. 4c). In fact, the current declined by 58 ± 8.4% over 4 s (n = 12 cells) during a 4-s application. This decline in the current was not different from that which would have been observed from an equal mixture of wild type and [K373A] substituted receptors. If one assumes that heterotrimer formation occurs readily between subunits containing alanine and those containing lysine and that only two (rather than four) phenotypes can occur, then this result indicates that a single alanine-containing subunit does not exert a dominant effect with respect to the desensitization kinetics (Fig. 4c).

**DISCUSSION**

Our results distinguish two modes of P2X<sub>4</sub> receptor desensitization. In our whole-cell experimental conditions, P2X<sub>4</sub> responses declined both during ATP applications lasting a few seconds and with applications repeated at 1-min intervals (Figs. 1a and 2a). With perforated patch recording to minimize dialysis of intracellular contents, the decline with repeated application (“run-down”) was completely prevented, whereas the decline during ATP applications was the same as in whole-cell recordings (Fig. 1). The prevention of P2X<sub>4</sub> rundown by per-
forated patch recording is comparable with the work by Lewis and Evans (25), who showed that the run-down of a P2X1-like current in smooth muscle cells did not occur with amphotericin-permeabilized recordings. The phenomena of run-down has been observed for P2X1, P2X3, P2X4, and P2X7; other P2X receptors give robust steady-state currents with repeated ATP applications. A likely explanation for P2X4 run-down in whole-cell recording is that a diffusible cytosolic factor that tonically regulates the receptor is lost during intracellular dialysis. The identity of such a factor is not obvious, but from our experiments it seems not to be ATP, GTP, or phosphatidylinositol 4,5-bisphosphate.

The C terminus of P2X4 is short (30 residues) relative to that of other P2X receptors: 62% of these residues are either charged or aromatic (tyrosine) (Fig. 2). By alanine scanning we identified two residues (Lys373 and Tyr374) as being important for the time course of desensitization during ATP application. Both K373A and Y374A mutations resulted in receptors with much accelerated decline in response during ATP application as compared with wild-type receptor currents (Fig. 3a).

Ivermectin increases the currents evoked by ATP at P2X4 receptors (26). A recent careful study by Priel and Silberberg (15) distinguished two effects: the first is an increase in the maximum current that is seen at concentration around 0.3 μM, and the second is a slowing of deacti-
hydrocarbon chain). We tested this by expressing the P2X4[K373C]
receptor and using MTSEA to add a side chain of similar length (–S–
S–CH2–CH2–NH3+ to that of lysine (–CH2–CH2–CH2–CH2–NH3+). This
completely restored the wild-type phenotype. Control experi-
ments showing that MTSEA did not modify the wild-type channel or
the fast desensitizing mutant P2X4[K373A], and that the effect of
MTSEA was prevented by intracellular cysteine (29–31), strongly sup-
port the interpretation that MTSEA is acting by modification of
the cysteine side chain at position 373. This indicates that lysine rather
than arginine is required for structural reasons (e.g. the arginine side
chain –CH2–CH2–CH2–NH–(C(NH2) = NH3+ is too long to be accommo-
dated) or perhaps that the lysine is subject to posttranslational modi-
fication. For example, post-translational modification of lysine has been
shown to be important for the function of other ion channels (32).
However, there are no consensus motifs for lysine modification appar-
ent at position 373 and the relatively fast time course (5–10 min: Fig. 4b)
with which MTSEA restored the wild type phenotype effect also argues
against such an interpretation. The simplest explanation of why K373A
mutation accelerates the decline in response during ATP application is
that the critical positioning of this positive charge is required for chan-
el opening to be maintained when ATP is bound. This implies that
gating transitions involve not only conformational changes in the
ectodomain and transmembrane hydrophobic regions but also the first
part of the C terminus (33–36). It is difficult to make further mechanis-
tic interpretation, given that the residue at this position is lysine in one
other P2X subunit (P2X7, which shows rapid desensitization) and neg-
atively charged (Glu or Asp) in all others.

In a previous study by Koshimizu et al. (22), the region 376–381
(EDYEQQ) was identified as being important in controlling P2X4
desensitization during sustained applications of ATP. They measured
the decline in intracellular calcium concentration over periods of sev-
eral hundred seconds, so it is difficult to interpret those results with
respect to the present studies in which ionic current was measured over
a period of 5 s. Under our experimental conditions, these residues do
not participate in receptor desensitization, as determined by alanine
substitution or truncation. Previous work by Royle et al. (27) has shown
that this region contains the first four amino acids of the motif (YEQQ)
that interacts with the μ2 subunit of the adaptor protein AP2 and is thus
involved in receptor internalization. In general terms, this would be
consistent with our interpretation that the decline in the current during
the time frame of a few seconds that we have studied is unrelated to
receptor internalization.

P2X receptors are generally considered to operate as trimers (1). Our
final question was whether the rapid desensitization was observed when
only one of the three subunits carried alanine at position 373 (i.e.
P2X4[K373A]:P2X4:P2X4) (Fig. 4); in other words, did this mutation confor
a dominant phenotype. The result of the co-expression of wild-type
and mutant subunits in equal amounts (Fig. 4c) strongly indicates that
accelerated desensitization is seen only in channels with two or more sub-
units carrying the alanine (i.e. P2X4[K373A]:P2X4[P2X4] (Fig. 4)). This
would imply that normal channel function can occur even though one of
the three subunits is mutated to alanine at this position. We cannot exclude
the alternative explanation for the results of the co-expression, which is that
the lysine to alanine mutation prevents the formation of heteromeric chan-
nels, and that the currents observed flow through approximately equal
parts of the homomeric wild type P2X4 and mutant P2X4[K373A] channels.
This seems unlikely given that the P2X4[K373A] has properties similar in
many respects to wild type channels, which shows that alanines in this
position (at least with three of them) do not themselves prevent formation
of functional homomers.

In summary, we have identified Lys373 and Tyr374 in the C terminus of

FIGURE 4. Reconstitution of wild-type properties by MTSEA-induced cysteine modi-
fication of P2X4[K373C]. a, representative superimposed normalized traces showing
currents at P2X4[K373C] and effect of extracellular perfusion with MTSEA (1 μm). The
right panel illustrates the absence of effect of MTSEA on wild-type receptors. b, summary
data showing the time-dependent effect of MTSEA on current decline during ATP appli-
cation (100 μμ, 4 s). There is no effect of MTSEA on wild-type receptor (open squares).
P2X4[K373C] receptor currents show almost 100% decline, in the absence of MTSEA
(filled circles). P2X4[K373C] receptor currents show wild-type decline after 10 min in
MTSEA (closed triangles). MTSEA effect on P2X4[K373C] current was prevented by intra-
cellular cysteine (20 μμ, open diamond). n = 6–10 cells each; p < 0.01 Student’s t test,
compared with K373C in the absence of MTSEA. c, averaged and superimposed macro-
scopic currents recorded from cells expressing either wild-type or P2X4[K373A] receptor
alone or co-expressing wild-type and P2X4[K373A] receptors; standard error is shown at
200-ms intervals; n = 8–15 cells each. The gray line is the macroscopic current predicted
to be observed if two independent populations of wild-type and P2X4[K373A] receptors
exist and assuming equal contribution. Currents shown were obtained during the first
ATP application. d, schematic showing predicted receptor stoichiometry and ratios
assuming equal mixing between wild-type (white) and P2X4[K373A] (black) receptor
subunits following co-expression.

heart of the YKYV motif that is not normally used for clathrin-mediated
desensitization of P2X4 receptors (24, 27).

We asked whether critical was the requirement for tyrosine at position
374 and found that P2X4[Y374F] functioned essentially as the wild-type
receptor with respect to the decline in current during the ATP applica-
tion (Fig. 3a). This indicates that the requirement for aromaticity at this
position for the channel to remain open during agonist occupancy. It
further indicates that Tyr374 is unlikely to be phosphorylated: for exam-
ple, previous work (28) on P2X7 receptors has indicated that repeated
activation leads to receptor dephosphorylation and that this underlies
the decline in the responses in that case.

On the other hand, arginine could not substitute for lysine at position
373 (Fig. 3a). This suggests that the critical moiety is not simply the
positive charge but other features of the lysine side chain (such as the
hydrocarbon chain). We tested this by expressing the P2X4[K373C]
the P2X4 receptor as being key determinants of P2X4 receptor desensitization on the time scale of seconds. This implicates the juxtamembrane C terminus in playing an important role in determining the duration of the physiological action of ATP at the P2X4 receptors.

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