A Protein Kinase C Site Highly Conserved in P2X Subunits Controls the Desensitization Kinetics of P2X$_2$ ATP-gated Channels*

(Received for publication, December 30, 1999, and in revised form, January 24, 2000)

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P2X receptors are nonselective cation channels gated by extracellular ATP. Recombinant mammalian P2X subunits assemble in homomeric ionotropic ATP receptors that differ by their agonist sensitivity and desensitization rate in heterologous expression systems. Using site-directed mutagenesis and voltage clamp recording in Xenopus oocytes, we identified the highly conserved protein kinase C site TX(K/R) located in the intracellular N terminus of P2X2 subunits as a critical determinant of kinetics in slowly desensitizing (time constant, $>1$ min) rat P2X$_2$ receptors. Mutant receptors P2X$_2$T18A, T18N, and K20T devoid of this consensus site exhibited quickly desensitizing properties (time constant, $<1$ s). In contrast with wild-type receptors, mutant P2X$_2$ receptors with truncated C terminus exhibited variable cell-specific kinetics with quickly desensitizing currents converted to slowly desensitizing currents by phorbol ester-mediated stimulation of protein kinase C. Phosphorylation of Thr$^{18}$ was demonstrated directly by immunodetection using specific monoclonal antibodies directed against the phosphothreonine-proline motif. Our data indicate that both phosphorylation of the conserved threonine residue in the N-terminal domain by protein kinase C and interaction between the two cytoplasmic domains of P2X$_2$ subunits are necessary for the full expression of slowly desensitizing ATP-gated channels.

Fast ionotropic responses to extracellular ATP are mediated by the activation of ATP-gated channels or P2X receptors present on the surface of various cell types. A family of genes coding for seven P2X channel subunits has been identified in human and rodents (1, 2). The electrophysiological characterization of recombinant homomeric and heteromeric P2X receptors expressed in heterologous systems led to their grouping in three functional categories based on their sensitivity to ATP and on their desensitization properties: 1) P2X$_1$ (3) and P2X$_7$ (4, 5) assemble in quickly desensitizing homomeric receptors highly sensitive to ATP and $\alpha$-$\delta$-methylene ATP (EC$_{50}$ around 1 $\mu$M); 2) P2X$_2$ (6), P2X$_4$ (7, 8), P2X$_5$ (9), P2X$_{2,3}$ (5), P2X$_{2,5}$ (10, 11), and P2X$_{4,6}$ (12) receptors are less sensitive to ATP (EC$_{50}$ around 10 $\mu$M) and desensitize at slow to moderate rate; 3) P2X$_7$ receptors (13) show low sensitivity to ATP (EC$_{50}$ around 500 $\mu$M) and desensitize slowly. Modulation of the desensitization rate of neurotransmitter-gated channels is recognized as a potentially important mechanism for modulation of neuronal excitability (14). P2X receptors are nonselective cation channels with high permeability to calcium ions (15, 16), so the subtype-specific desensitization phenotype of ATP-mediated currents has a significant impact on the levels of intracellular calcium and subsequent activation of calcium-dependent effectors. Studies on the relationship between the slowly desensitization kinetics of P2X$_2$ receptor and its primary sequence have emphasized the requirement for several structural features including the transmembrane domains and their intracellular flanking regions (17), the negatively charged residue Asp$_{349}$ located in the second transmembrane domain (18), and the unique C-terminal tail of the P2X$_{2a}$ splicing variant (19–21). Chow and Wang (22) have reported that P2X$_2$ receptor kinetics can also be modulated by protein kinase A (PKA)$^1$-dependent phosphorylation of Ser$_{431}$ located in the C-terminal domain of the subunit. However, no structural motif distinctive of the P2X family has been linked to a specific functional property of these ionotropic ATP receptors so far.

Using site-directed mutagenesis and electrophysiological characterization of recombinant P2X receptors in Xenopus oocytes, we report here the identification of a phosphothreonine part of the highly conserved protein kinase C site located intracellularly in the N terminus of P2X$_2$ subunits as a critical determinant for the expression of slowly desensitizing P2X$_2$ ATP-gated channels.

EXPERIMENTAL PROCEDURES

Mutagenesis—P2X$_2$ receptor mutants were generated by polymerase chain reaction with the original P2X$_2$ plasmid kindly provided by Dr D. Julius (University of California at San Francisco) (6) as DNA template using Pfu DNA polymerase (Stratagene) to minimize artifactual mutations. Point mutations were constructed using the QuickChange site-directed mutagenesis system (Stratagene) or the megaprimer mutagenic polymerase chain reaction. Briefly, primer containing one or several mismatch bases and a specific primer in the pcDNA3 vector (Invergent) sequence were used for the first amplification. The polymerase chain reaction product generated was used as megaprimer in a second round of amplification with an exact match oligonucleotide primers derived from P2X$_2$ sequence. The mutated fragment thus obtained was cut with unique restriction enzymes and subcloned into the original P2X$_2$ pcDNA3.

For construction of C-terminal truncated receptors, we used a primer derived from amino acid sequence $^{306}$DKVRPRK$^{314}$ of P2X$_2$ containing a single base mismatch to replace Thr$^{312}$ by an alanine and followed by a terminal XhoI site. The full-length and truncated P2X$_2$ (wild-type and mutant forms) were then ligated with an XhoI fragment containing in-frame His$_6$ or FLAG epitope followed by an artificial stop codon in pcDNA3 vector to generate tagged P2X$_2$ subunits as reported previously for other P2X subunits (11, 12). All the constructs were

* This work was supported by the Medical Research Council of Canada, the Heart and Stroke Foundation of Canada, and AstraZeneca Charnwood. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; TR, truncated.
subjected to automatic dideoxy sequencing (Sheldon Biotechnology Center, McGill University, Montreal, Canada).

Preparation and Injection of Oocytes—Ovary lobes were surgically retrieved from Xenopus laevis frogs under deep tricaine (Sigma) anesthesia. Oocyte-positive lobes were then treated for 0 h at room temperature with type I collagenase (Life Technologies, Inc.) in calcium-free Barth’s solution under vigorous agitation. Stage V–VI oocytes were then manually defolliculated before nuclear microinjections of 1–5 ng of cDNA. The cells were maintained in Barth’s solution containing 1.8 mM calcium chloride and 10 μg/ml gentamicin (Sigma) at 19 °C for up to 5 days.

Electrophysiology and Data Analysis—Two-electrode voltage clamp recordings were made 1–3 days after microinjection using an OC-725B amplifier (Warner Instruments). Signals were low pass filtered at 1 kHz, acquired at 500 Hz using a Macintosh IIci computer equipped with an NB-MIO-16XL analog-to-digital interface (National Instruments). Recorded traces were post-filtered at 20–50 Hz in Axograp (Axon Instruments). Ringer’s solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES buffered at pH 7.4 was perfused onto oocytes at a constant flow rate of 10–12 ml/min. Agonists and drugs were prepared in bath perfusion at their final concentration. ATP, PMA, 4aPMA, staurosporine, and chelerythrine were purchased from Sigma. Drugs were initially dissolved in dimethyl sulfoxide before being diluted at least 1000-fold in bath solution to give the final working solution. Dose response curves were fitted to the Hill sigmoidal equation and EC50 values were determined by nonlinear regression analysis using the Prism 2.0 software (Graphpad, San Diego, CA). Desensitization rates were measured during 10-s applications of 100 μM ATP. For the desensitization of truncated mutants, both the peak current and the current amplitude 5 s after the peak (sustained current phase) were measured, and desensitization is expressed as the peak/sustained current ratio. We used unpaired t test to compare desensitization rates, and statistical significance was set at p < 0.05.

Western Blotting—DNA transfections of FLAG-tagged P2X2 subunits were performed in mammalian HEK cells using the calcium phosphate method as described previously (11, 12). Transfected HEK-293A cells or injected oocytes used for Western blots were collected in phosphate-buffered saline pelleted at 14,000 g for 5 min. Membrane proteins within supernatants were used for Western blots. Solubilized proteins were incubated with 100 μl of equilibrated anti-FLAG M2 affinity gel (Sigma) for 2 h at 4 °C under agitation. Then resin beads were washed three times in Tris-buffered saline containing 1% Triton X-100. Bound proteins were eluted from the anti-FLAG M2 affinity gel with 0.1 M glycine, pH 3.5. Samples were then loaded onto 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunostainings were performed first with an anti-phosphothreonine/serine/methionine monoclonal antibody (1:500) (New England Biolabs) followed by incubation with anti-mouse peroxidase-labeled secondary antibodies (1:2,000) for visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech). Membranes were then stripped by 30 min of incubation at 50 °C in a solution of 150 mM Tris, pH 7.5, 2% (w/v) sodium pyrophosphate. Membranes from cell lysates were solubilized with 1% Triton X-100 for 5 min. Membrane proteins within supernatants were used for Western blotting. Solubilized proteins were incubated with 100 μl of equilibrated anti-FLAG M2 affinity gel (Sigma) for 2 h at 4 °C under agitation. Then resin beads were washed three times in Tris-buffered saline containing 1% Triton X-100. Bound proteins were eluted from the anti-FLAG M2 affinity gel with 0.1 M glycine, pH 3.5. Samples were then loaded onto 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunostainings were performed first with an anti-phosphothreonine/serine/methionine monoclonal antibody (1:500) (New England Biolabs) followed by incubation with anti-mouse peroxidase-labeled secondary antibodies (1:2,000) for visualization by enhanced chemiluminescence (Amersham Pharmacia Biotech). Membranes were then stripped by 30 min of incubation at 50 °C in a solution of 150 mM Tris, pH 7.5, 2% (v/v) SDS, 0.8% (v/v) β-mercaptoethanol and washed four times in Tris-buffered saline before staining with anti-FLAG M2 murine monoclonal antibody (1:1000) (Sigma) followed by incubation with anti-mouse peroxidase-labeled secondary antibodies (1:5,000) for visualization by enhanced chemiluminescence.

RESULTS AND DISCUSSION

Members of an unique class of ligand-gated channels by their protein topology, P2X ATP receptors are produced by the omeromic assembly of two-transmembrane domain subunits with both their N and C termini located intracellularly (23). All known P2X ATP-gated channel subunits display a transmembrane residue in the highly conserved lysine N-terminal consensus site TX(K/R) in the intracellular N-terminal domain of the protein (Fig. 1A). To test the role of this conserved domain in the function of P2X2 ATP-gated channels, we first substituted Thr18 for Ala in the sequence of rat P2X2 subunit to eliminate this protein kinase C site. Following expression of this mutant P2X2T18A receptor in Xenopus oocytes, we observed a dramatic change of phenotype in response to application of extracellular ATP. In contrast with wild-type P2X2 receptors that do not desensitize significantly over the time of application of agonist, mutant P2X2T18A subunits assembled in functional ATP-gated channels that displayed fast kinetics with complete desensitization in less than 5 s (Fig. 1B).

Despite a short time of recovery (Fig. 2A), the rate of desensitization of P2X2T18A was faster than the rate of P2X2 receptors expressed in oocytes (Fig. 2B). We measured a time constant (50% of maximal response) of 0.36 ± 0.11 ms for P2X2T18A and 0.80 ± 0.11 ms for P2X2 receptors. Increase of desensitization rate could be linked to structural alterations that increase the agonist sensitivity of the mutant channels. Independently of the agonist concentration, an alteration in the structure of the subunits could also create an unexpected change of voltage sensitivity leading to abnormal kinetics at specific membrane potentials. So we tested the possible roles of the agonist concentration and of the membrane potential by recording the activity of P2X2T18A channels at increasing concentrations of ATP (from 1 μM to 1 mM) and at different voltages (from 0 mV to −100 mV) without detecting any significant changes in the desensitization rate (Fig. 2A). Confirming that an increase of sensitivity to agonist was not the mechanism causing the change, the EC50 of ATP for P2X2T18A was found to be 62 ± 2 μM, compared with an EC50 of 8.43 ± 1.4 μM for wild-type P2X2 receptors (Fig. 2C). For technical reasons, the EC50 measured for P2X2T18A could be slightly underestimated because of the fast kinetics of desensitization of these mutant receptors. However, in the range of agonist concentrations tested (1 μM to 1 mM), we did not observe a change of phenotype at high concentrations of ATP in the case of wild-type P2X2, receptors seven to eight times more sensitive than P2X2T18A mutant receptors.

We decided to eliminate the protein kinase C site by replacing the conserved Thr18 by Asp instead of Ala to check whether the observed effects on the kinetics could be due to a structural change unrelated to the presence of a phosphorylation site. The
Desensitization Determinant of P2X Channels

Fig. 2. Characterization of mutant P2X$_{2}$T18A receptor desensitization and recovery. A, ATP-induced currents in oocytes expressing P2X$_{2}$T18A fully recovered after repeated applications at 2-min intervals. The rate of desensitization did not change significantly with increasing concentration of ATP or with the membrane potential. B, comparison of the rate of desensitization of P2X$_{2}$ (filled squares), P2X$_{2}$T18A (open circles), and P2X$_{2}$ (filled circles) receptors. P2X$_{2}$T18A currents evoked by a 10-s application of 100 μM ATP had a time constant of desensitization <1 s similar to the currents recorded in oocytes expressing P2X$_{2}$ receptor. C, activation dose response curves of P2X$_{2}$ wild-type (filled squares) and P2X$_{2}$T18A mutants (open squares) for ATP. Mean peak currents were normalized to the maximal response (means ± S.E. from 8–10 oocytes). Holding membrane potential = −60 mV.

Fig. 3. Mutation of the conserved N-terminal PKC consensus site dramatically increased the rate of desensitization. Representative currents evoked by application of 100 μM of ATP to oocytes expressing wild-type and several PKC site mutant receptors. Current amplitudes recorded in oocytes expressing different PKC site mutants were decreased in comparison with wild-type receptors. The mean current amplitudes were 16 ± 2.8 μA for P2X$_{2}$, 4.53 ± 1.4 μA for P2X$_{2}$T18A, 1.3 ± 0.4 μA for P2X$_{2}$T18N, 11.2 ± 1.95 μA for P2X$_{2}$K20T, and 8.7 ± 1.39 μA for P2X$_{2}$P19A. All mutant receptors lacking the PKC consensus site TXK showed fast and complete desensitization.

| PKC site | P2X$_{2}$ wt | P2X$_{2}$ T18A | P2X$_{2}$ T18N | P2X$_{2}$ K20T | P2X$_{2}$ P19A |
|----------|-------------|----------------|----------------|----------------|----------------|
| N-terminal | [MVRLA]CGCWSAFWDY[VPK]IVVRNR | [MVRLA]CGCWSAFWDY[VPK]IVVRNR | [MVRLA]CGCWSAFWDY[VPK]IVVRNR | [MVRLA]CGCWSAFWDY[VPK]IVVRNR | [MVRLA]CGCWSAFWDY[VPK]IVVRNR |

Desensitization Determinant of P2X Channels

Mutant receptor P2X$_{2}$T18N had the same quickly desensitizing kinetics than P2X$_{2}$T18A (Fig. 3). It could happen also that this threonine residue plays an unique structural role independently of being a phosphate acceptor, so we eliminated the protein kinase C site by substituting the positively charged Lys$^{260}$ that is a second critical part of the kinase recognition site by PKC while preserving the conserved Thr$^{18}$. The mutant receptor P2X$_{2}$K20T displayed the same fast phenotype than P2X$_{2}$T18A and P2X$_{2}$T18N receptors (Fig. 3). To test the importance of the structural integrity of the protein kinase C motif TPK in P2X$_{2}$ kinetics, we replaced Pro$^{19}$ by Ala. P2X$_{2}$P19A receptors generated slowly desensitizing currents in response to ATP, similar to those observed with wild-type channels (Fig. 3). The results of these experiments suggest strongly that the presence of the protein kinase C recognition motif TXK is a critical determinant of the kinetics of wild-type P2X$_{2}$ receptors, without excluding the possibility that Thr$^{18}$ plays also a structural role.

Full-length rat P2X$_{2}$ subunits have a large intracellular C-terminal domain that contains two other potential protein kinase C sites on Thr$^{172}$ and Thr$^{184}$ and one protein kinase A site on Ser$^{431}$ (22). Shorter splicing variants of P2X$_{2}$ subunits display different sets of these protein kinase sites, and several studies have shown that the structure of the C-terminal domain in these variants plays a modulatory role on the desensitization kinetics of P2X$_{2}$ channels (19–21). To eliminate these additional potential phosphorylation sites, we produced truncated (TR) forms of wild-type and mutant P2X$_{2}$ subunits lacking the native C-terminal sequence from His$^{375}$ to the Stop codon and mutated also on Thr$^{172}$ to eliminate the remaining proximal protein kinase C site. Larger deletions of the C-terminal domain that contains two other potential protein kinase A sites on Ser$^{431}$ (22). These deletions are consistent with previous reports of variability in the levels of basal protein kinase activity in oocytes (24). The functional importance of the C-terminal domain is also demonstrated by the differences of current amplitudes recorded between the full-length and the truncated versions of P2X$_{2}$T18A and P2X$_{2}$K20T mutant channels. The small amplitudes of peak currents (less than 500 nA at 100 μM ATP) obtained with truncated P2X$_{2}$T18A or K20T mutants (Fig. 4) indicates that a specific intracellular protein-protein interaction could underlie the efficient assembly and surface expression of ATP-gated channels.

If the variable phenotype observed with truncated P2X$_{2}$ receptors is linked to variable basal PKC activities in oocytes by stimulating pharmacologically the PKC with phorbol esters, we
should be able to observe a double effect on the mutant phenotype: a decrease of the desensitization rate and less variability in the kinetics of ATP-induced currents. To test this hypothesis, we preincubated the oocytes expressing wild-type P2X2, truncated P2X2, or P2X2K20T (Fig. 5) with the phorbol ester PMA or with its inactive enantiomer 4αPMA. As expected if Thr18 is a phosphate acceptor in a protein kinase C site, all hybrid currents generated by truncated P2X2 channels were converted into slowly desensitizing currents of the same peak amplitude in presence of PMA but not in a presence of the control phorbol ester 4αPMA. Slowly desensitizing wild-type P2X2 receptors or mutant P2X2K20T devoid of N-terminal PKC site were found insensitive to phorbol esters (Fig. 5) and to inhibitors of PKC (10–50 μM staurosporine and 10–100 μM chelerythrine; data not shown). Both the low variability of wild-type P2X2 phenotype and the lack of sensitivity to PKC stimulation or inhibition would be consistent with the early constitutive phosphorylation of Thr18 by PKC in oocytes as well as in transfected mammalian cells (21, 22). The fact that wild-type P2X2 receptors do not display such a variability in their phenotype in heterologous systems confirms that the C-terminal domain plays an important role in the stabilization of the slowly desensitization kinetics. In our hypothesis C terminus domain would promote phosphorylation of the subunit and/or

**Fig. 4.** Functional expression of P2X2 channels with truncated C-terminal domain. A, schematic diagrams showing the primary structure and location of mutations on full-length P2X2 and C-terminal truncated P2X2 channel subunits. PKC and PKA consensus sites are indicated by closed circles and open circles, respectively. Hatched boxes represent epitope tags. B, deletion of the C-terminal domain of P2X2, wild-type and mutants led to functional channels as shown here by representative currents evoked by 100 μM of ATP. The truncated P2X2 channels exhibited two phenotypes when expressed in oocytes. ATP responses were either slowly desensitizing (s) or had a biphasic phenotype (b) with a quickly desensitizing current followed by a sustained phase. The quickly desensitization phenotype of P2X2T18A and K20T mutants was not altered by the C-terminal deletion.

**Fig. 5.** Activation of protein kinase C by phorbol ester converts the quickly desensitization kinetics of truncated P2X2 into a slowly desensitization phenotype. A, C, and E show the typical currents recorded in oocytes expressing P2X2TR (A), wild-type P2X2 (C), and P2X2 K20T (E) recorded during two successive applications of 100 μM ATP before and after a 5-min pretreatment with 1 μM PMA. B, negative control: P2X2TR was treated in the same way with the inactive phorbol ester 4αPMA. D shows the rate of desensitization (ratio of the maximal amplitude over the amplitude at 5 s after the peak, mean ± S.E.) measured during the first ATP application (control) and after treatment with phorbol esters PMA and 4αPMA. n = 6 for P2X2; n = 7–9 for P2X2TR. Asterisks, p < 0.001.
mass markers are indicated in kDa.

with anti-FLAG M2 monoclonal antibody after stripping. Molecular 
antagonists for FLAG epitope-tagged P2X2 constructs were purified on an anti-

from HEK-293A cells transiently transfected with wild-type and mu-

is active and that the subunits are constitutively phosphoryl-

ated on Thr18 in oocytes and transfected HEK293 cells hetero-

heterologous expression systems. The absence of Thr(P)18 in 

protect phosphorylated subunits from phosphatase activity. Indeed, in absence of the C terminus domain, the expression of a stable slowly desensitizing phenotype required the exogenous stimulation of PKC, likely to fully phosphorylate the P2X2 subunits.

To directly assess the phosphorylation status of Thr18 in Western blot from oocytes and transfected HEK293 cells expressing P2X2 receptors, we used specific monoclonal antibodies directed against the phosphothreonine-proline motif that would be present only if the conserved PKC site of the receptor subunit is active. After affinity purification of FLAG epitope-tagged P2X2 receptors, we detected a strong positive signal corresponding to wild-type P2X2 receptors, but no signal was observed in the case of mutant P2X2K20T receptors lacking the N-terminal PKC site in oocytes (data not shown) as well as in transfected HEK293 cells (Fig. 6A). We controlled for similar levels of receptor expression in the same samples with anti-FLAG epitope antibodies (Fig. 6B). We chose the P2X2K20T mutant as negative control in this experiment because in previous recordings we noticed robust currents related to its high level of expression (Fig. 3). The detection of a single phosphothreonine convincingly demonstrate that the conserved PKC site present in the N-terminal domain of P2X2 channel subunit is active and that the subunits are constitutively phosphorylated on Thr18 in oocytes and transfected HEK293 cells heterologous systems. The absence of Thr(P)-Pro-positive signal in the mutant P2X2K20T indicates also that the putative PKC site Thr369KVRTPK374 present in the C-terminal domain of the subunit is not constitutively used.

We conclude from these converging data that Thr(P)18 located in the N-terminal PKC site of the P2X2 subunit is a critical determinant for the characteristic slow rate of desensitization of homomeric P2X2 ATP-gated channels (Fig. 7). This intracellular post-translational modification appears to be constitutive in oocytes as well as in transfected HEK293A cells heterologous expression systems. The absence of Thr(P)18 in mutants devoid of the conserved PKC site consistently pro-
eicosanoids receptors coupled to phospholipase C activation would sensitizate the sensory neurons to extracellular ATP via the desensitization rate of calcium-permeable P2X channels. The N-terminal PKC site TX(K/R) is conserved in all known P2X subunits, but whether all P2X subunits assembled in ATP receptors with slowly desensitizing or quickly desensitizing phenotypes are constitutively phospho-rylated and subject to PKC-dependent heterologous functional regulation remains to be investigated.

Acknowledgments—We thank David Reese and Audrey Speelman for their expert technical assistance as well as Dr. Khanh-Tuoc Lê for sharing initial observations on P2X_{18A} mutants.

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