Cross-talk and Co-trafficking between ρ1/GABA Receptors and ATP-gated Channels*

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γ-Aminobutyric acid (GABA) and ATP ionotropic receptors represent two structurally and functionally different classes of neurotransmitter-gated channels involved in fast synaptic transmission. We demonstrate here that, when the inhibitory ρ1/GABA and the excitatory P2X2 receptor channels are co-expressed in Xenopus oocytes, activation of one channel reduces the currents mediated by the other. This reciprocal inhibitory cross-talk is a receptor-mediated phenomenon independent of agonist cross-modulation, membrane potential, direction of ionic flux, or channel densities. Functional interaction is disrupted when the cytoplasmic C-terminal domain of P2X2 is deleted or in competition with the main intracellular loop (of ρ1). This link between P2X2 and ρ1 receptors expressed in oocytes and the co-clustering of these receptors in transfected hippocampal neurons. Co-expression with P2X2 induces retargeting and recruitment of mainly intracellular ρ1/GABA receptors to surface clusters. Therefore, molecular and functional cross-talk between inhibitory and excitatory ligand-gated channels may regulate synaptic strength both by activity-dependent current occlusion and synaptic receptors co-trafficking.

Neuronal activity is regulated by a number of transmitters acting on different receptor types (1). Fast neurotransmission is achieved through different classes of transmitter-gated channels, including the P2X and nicotinic receptor superfamilies (1, 2). The family of P2X ATP-gated cation channels is composed of seven genes coding for subunits with two transmembrane domains, intracellular N and C termini, and a large extracellular loop (2). The nicotinic superfamily includes the GABA-gated1 receptors (5, 6). Recent work has demonstrated that a physical interaction between P2X and 5-HT3 receptors leads to an activity-dependent cross inhibition between the two cationic channels (26). We decided to explore the possibility that ligand-gated chloride channels may also interact with P2X ATP-gated channels through similar mechanisms.

Because P2X2 and ρ1/GABA receptors are co-expressed in the central nervous system (7–18), we investigated potential molecular and functional interactions between these two types of transmitter-gated channels in heterologous expression systems. We report here an intracellular inhibitory cross-talk between P2X2 and GABA receptors that can occur simultaneously on subunit level and which is dependent on the subunit choice.

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† The abbreviations used are: GABA, γ-aminobutyric acid; GABA, GABA subtype A; GABA, GABA subtype C; 5-HT, serotonin (5-hydroxytryptamine); DRG, dorsal root ganglia; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CT, carboxyl terminal; IL2, main intracellular loop (of ρ1); HIS, hexahistidine (His6); P2X2, TR, C-terminal truncated form of P2X2; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Wild-type rat P2X2, a C-terminal truncated form of P2X2 (P2X2,TR), and wild-type rat ρ1 clones were available from previous work (18, 27). Carboxyl-terminal epitope-tagged P2X2 subunits with either hexahistidine (His6)-tagged or enhanced YFP-tagged sequences and a C-terminal His6-tagged ρ1 subunit (ρ1-HIS) were obtained as described previously (28). N-terminal GFP-tagged ρ1 subunit (ρ1-GFP) was generated by insertion of two enhanced GFPs in tandem into a unique BamHI restriction site. This artificial BamHI site was created at amino acids positions Gly45-Ala46 by site-directed mutagenesis (QuickChange, Stratagene). The intracellular C-terminal domain of P2X2 (P2X2-CT) and the main intracellular loop (IL2) of the ρ1 subunit were amplified by PCR using 5′ and 3′-primers incorporating an initiation methionine and a stop codon, respectively. All constructs were
subcloned into pcDNA3 (Invitrogen) and verified by automatic dyeoxy DNA sequencing.

**Cell Culture—**Oocytes were prepared as described previously (26). Stage V and stage VI oocytes were manually defolliculated before the microinjection (Nanoject II, Drummond Scientific) of 0.2 ng of cRNA coding for P2X$_2$, YFP-tagged P2X$_2$ (P2X$_2$-YFP), or hexahistidine-tagged P2X$_2$ (P2X2-HIS) and 15–25 ng of RNA coding for P2X$_2$, TR, GABA, p1, the HIS-tagged p1 subunit (p1-HIS), or the GFP-tagged p1 subunit (p1-GFP) to reach similar levels of expression. Then the oocytes were incubated in Barth’s solution containing 1.8 mM CaCl$_2$ and gentamycin (10 µg/ml; Sigma) at 19 °C for 1–5 days prior to electrophysiological recordings. For competition experiments, RNAs coding for minigenes were injected (50–60 ng of each to reach a 2:1 minigene/neon receptor) independently, immediately after the injection of receptor RNA.

Neuronal cultures were prepared according to a modification of the procedure described by Goslin et al. (29). Briefly, hippocampi of rat embryos were dissected at day 18. Dissociation was achieved with a Pasteur pipette after trypsinization. Cells were plated on poly-D-lysine-coated coverslips (Electron Microscopy Sciences) in complete Neurobasal medium supplemented with B27 (Invitrogen) containing 1 mM t-glutamine, penicillin G (10 units/ml), and streptomycin (10 mg/ml). Four hours after plating, the coverslips were transferred to dishes containing conditioned medium obtained by incubating the complete medium described above on glial cultures (70–80% confluence) for 24 h. The medium was partially changed every 3–4 days. Hippocampal neurons were transfected at 7–8 days in vitro as follows. For each coverslip, a total of 1.5 µg of plasmid DNA was mixed with 50 µl of Neurobasal medium without B27 supplement. For cotransfection experiments, the proportions of each plasmid were first adjusted to reach similar levels of expression, and the plasmids were thoroughly mixed prior to the addition of Plus™ packaging reagent (Invitrogen). After 15 min of incubation, 1.5 µl of LipofectAMINE 2000 (Invitrogen) in 50 µl of Neurobasal medium was added, and incubation was continued for another 30 min. After the addition of 150 µl of complete Neurobasal medium containing B27 supplement, the mix was applied on the neuronal culture for 3 h at 37 °C. Expression was then confirmed in the original medium every 3–4 days.

**Electrophysiology and Data Analysis—**Two-electrode voltage clamp recordings were performed using glass pipettes (1–2 megohms) filled with 3 mM KCl solution. Oocytes were perfused at a flow rate of 10–12 ml/min with Ringer’s solution (pH 7.4) containing 115 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, and 10 mM HEPES. Membrane currents were recorded through an OC-725B amplifier (W Barker Instruments) and digitized at 500 Hz. All drugs (purchased from Sigma) were dissolved in the perfusion solution and applied using a computer-driven valve system (BPPS, Alfa Scientific). We compared the actual responses to the predicted additive responses obtained with Axograph software (Axon Instruments). All recordings were made at room temperature. Statistical differences between means were assessed using Student’s t test. Dose-response curves were fitted to the Hill sigmoidal equation, and EC$_{50}$ values were determined by a non-linear regression analysis test using Prism 2.0 (Graphpad, San Diego, CA).

**Co-purification and Western Blotting—**Following the measurement of p1-GFP alone, P2X$_2$-YFP alone, P2X2-YFP + p1-HIS, or P2X2-HIS + P2X2-GFP expression levels by electrophysiological recordings, batches of 15 oocytes were homogenized in 10 mM HEPES and 0.3 M sucrose and solubilized in 0.8% Triton X-100 and protease inhibitors (Sigma) at 4 °C for 2 h. The oocytes were incubated overnight with equilibrated nickel resin (Qiagen) under agitation at 4 °C. After several washes with 20 mM imidazole, bound His$_6$-tagged proteins were eluted with 0.5 M imidazole solution and then loaded onto a 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunostaining of associated tagged receptors were performed with anti-GABA antibodies (1:10,000 dilutions), anti-P2X$_2$ antibodies (1:10,000 dilutions) for 2 h at room temperature. The plates were incubated in pH 9.5, 0.1 mM MgCl$_2$ and 0.1 mM CaCl$_2$ at 37 °C and then fixed with paraformaldehyde (4%) containing 4% sucrose at 37 °C in PBS+. Incubation of incubation with anti-rabbit peroxidase- and secondary antibodies (1:2000, Jackson Immunoresearch, West Groove, PA) for visualization by enhanced chemiluminescence (ECL, Amersham Biosciences).

**Immunocytochemistry and Confocal Microscopy—**Immunofluorescence was performed 9–10 days after plating and 2 days after transfection. Cells were fixed with phospho-buffered saline containing 0.1% paraformaldehyde (4%) containing 4% sucrose at 37 °C in PBS+. Incubation of incubation with anti-rabbit peroxidase-labeled secondary antibodies (1:2000, Jackson Immunoresearch, West Groove, PA) for visualization by enhanced chemiluminescence (ECL, Amersham Biosciences).

**Results**

**Reciprocal Cross-inhibition between GABA$_C$, and P2X$_2$ Receptors—**We co-expressed p1 and P2X$_2$ RNAs in Xenopus oocytes to investigate receptor function by two-electrode voltage clamp recordings (Vh = −60 mV; Fig. IA). Application of a saturating concentration of GABA (10 µM) induced non-desensitizing inward responses (IGABA = −1.8 ± 0.3 µA, n = 27). Similarly, application of a saturating concentration of ATP (100 µM) evoked slowly desensitizing responses (IATP = −2.7 ± 0.2 µA, n = 27). Kinetic profiles of individual responses are consistent with activation of GABA$_C$ and P2X$_2$ receptors, suggesting that activation of one receptor is not modified by the presence of the other (Fig. IA). If both receptors were functionally independent, we would expect that co-activation of both receptors evoked responses corresponding to the sum of individual responses. However, co-application of ATP and GABA (10 µM) evoked inward responses (denoted as Actual on Fig. 1A) significantly smaller (p < 0.0005) than the sum of individual responses (trace labeled Predicted on Fig. 1A is the arithmetic sum of I$_{GABA}$ and I$_{ATP}$ traces). Amplitude of I$_{ATP}$-GABA represents 73.4 ± 3% of the predicted current (Fig. IA; n = 27). When GABA was applied during application of ATP, and, conversely, when ATP was applied during GABA application, significant inhibition (p < 0.0005) of the responses was also observed (Fig. 1B). Amplitude of I$_{ATP}$ and GABA was 73 ± 4.5% and I$_{ATP}$ and GABA was 77 ± 4% of the predicted current (n = 16).

We showed previously, by recordings of oocytes expressing p1 GABA$_C$ receptors and 5-HT$_3A$ receptors, that co-activation by simultaneous application of GABA (10 µM) and 5-HT (100 µM) evoked responses (I$_{GABA} + 5-HT = −2.7 ± 0.6 µA$) corresponding to the sum of I$_{GABA} = −1.2 ± 0.4 µA$ and I$_{5-HT} = −1.5 ± 0.2 µA$, demonstrating that responses mediated by these two independent receptors are additive under similar experimental conditions (26). These results showed that p1 and P2X$_2$ channels do not function independently during co-activation and that the subunit-specific cross-talk induces rapid and reciprocal current occlusion.

**Current Occlusion Was Observed at Low Receptor Densities and Was Not Due to Cross-activation—**To check if the cross-inhibition was dependent on the density of receptors, the expression level of both receptors was decreased while the ratio of P2X$_2$/p1 subunits was kept constant. Co-application of ATP (100 µM) and GABA (10 µM) evoked currents with an amplitude (I$_{ATP} + GABA$ = −0.4 ± 0.1 µA) that was significantly lower than the expected sum of currents evoked separately by ATP (I$_{ATP}$ = −0.3 ± 0.1 µA) and by GABA (I$_{GABA}$ = −0.3 ± 0.1 µA; Fig. 2A). The amplitude of actual ATP + GABA responses represented 61.5 ± 2% of the predicted current (n = 8), indicating that current occlusion during co-activation of GABA$_C$ and P2X$_2$ receptors was not related to artificially high receptor densities.
Cross-talk and Co-trafficking between ρ1 GABAₐ and P2X₂ channels expressed in Xenopus oocytes. A, co-application of ATP + GABA induced currents (Actual) significantly smaller than the arithmetic sum (Predicted) of the individual ATP and GABA responses. ***, p < 0.0005, n = 27. B, non-additivity occurs whether ATP application begins before or after the start of GABA application, n = 16. Amplitudes of responses are normalized to the predicted response from each cell. Holding membrane potential (Vh = −60 mV) was monitored during recordings as illustrated in panel A.

Oocytes injected with P2X₂ RNA only did not respond to 10–100 μM application of GABA, and responses to 100 μM ATP (I_{ATP} = −4.6 ± 0.1 μA, n = 6) were not modulated by co-application of GABA (I_{ATP+GABA} = −4.65 ± 0.1 μA, n = 6) (Fig. 2B). Similarly, in oocytes expressing ρ1 GABAₐ receptors only, the application of saturating concentration of ATP did not activate GABAₐ channels, and co-application of ATP + GABA-induced currents (I_{ATP+GABA} = −1.9 ± 0.4 μA, n = 5) were identical in kinetics and amplitude to GABA responses (I_{GABA} = −1.9 ± 0.4 μA, n = 5; Fig. 2C). The ATP concentration-response curve of the P2X₂ channel was not modified by co-expression with ρ1 receptors (EC₅₀ for ATP was 2.6 ± 1.2 and 4.3 ± 1.3 μM, n = 7, respectively, for oocytes expressing P2X₂ alone and the P2X₂ + ρ1 subunit; Fig. 2D). Normalized ATP (100 μM) responses obtained from oocytes expressing P2X₂ alone or ρ1 + P2X₂ indicated that P2X₂ channel kinetics were not affected by the presence of ρ1 receptors (Fig. 2D). Conversely, GABA concentration-response curves determined from oocytes expressing either ρ1 receptor alone or ρ1 + P2X₂ (Fig. 2E) had similar EC₅₀ (2.5 ± 1.9 and 1.7 ± 1.5 μM, respectively, n = 5). Kinetics of GABAₐ receptors were identical in the absence or presence of P2X₂ receptors (Fig. 2F). These results showed that the cross-inhibition between GABAₐ and P2X₂ receptor is not mediated by agonist cross-modulation or by a shift in agonist sensitivity and further indicated that functional cross-talk occurs only when both channels are co-activated.

Voltage, Ionic Flux Direction, and Ca²⁺ Independence—To investigate a potential voltage-dependence of the cross-talk, we recorded responses obtained from the same oocyte expressing ρ1 and P2X₂ channels after successive applications of ATP (100 μM), GABA (10 μM), and a mixture of both agonists at different holding potentials. Currents induced by co-application of ATP + GABA (actual) were significantly lower than the expected sum of individual ATP and GABA responses (prediction) at holding potentials ranging from −80 to 20 mV (Fig. 3, A and B). Amplitude of I_{ATP+GABA} was 65 ± 6% at −80 mV, 70 ± 5% at −60 mV, 75 ± 4% at −40 and −20 mV, 60 ± 3% at 0 mV, and 52 ± 10% at 20 mV of the corresponding predicted current (n = 7).

P2X₂ channels are non-selective cationic channels and display a current-voltage relationship with marked inward rectification and a reversal potential close to 0 mV (30), whereas ρ1 channels are mainly permeable to Cl⁻ and display a linear I-V relationship with a reversal potential close to −20 mV from oocytes recorded in normal ringer solution (31). In oocytes co-expressing GABAₐ and P2X₂ channels, the experimental current-voltage relationship for P2X₂ was inwardly rectifying, whereas that for ρ1 channels was linear with reversal potential of +3.4 ± 1.3 mV for ATP and −19 ± 2 mV for GABA respectively (Fig. 3, A and C). Current-voltage relationship for actual and predicted ATP + GABA currents were linear with a slight inward rectification and displayed no difference in reversal potential (−6.7 ± 6 mV for actual and −7.6 ± 4 mV for predicted). These data indicated that both channels participated to...
current represents only 51 ± 4% of the prediction, showing that current occlusion occurred during Cl− entry. P2X2 and ρ1 GABA receptors also elicited non-additive responses when calcium ions (Ca2+) were removed (or substituted with Ba2+, not shown) from extracellular Ringer solution (Fig. 3E). ATP + GABA responses represent 56.7 ± 7% of the prediction (n = 6). Taken together, these results indicated that cross-inhibition is voltage-independent and did not arise from Cl− efflux via ρ1 channels or Ca2+ entry via P2X2 channels.

**Intracellular Domains of Both Receptors Are Involved in Non-additivity**—To determine whether intracellular domains of receptors were involved in cross-inhibition between P2X2 and GABA2 receptors, we first co-expressed ρ1 subunits with P2X2-TR, a C-terminal truncated form of P2X2 (Fig. 4A). Activation of P2X2-TR with 100 μM ATP evoked inward responses with slow to fast desensitizing kinetics, depending on oocytes as shown previously (27). As for wild-type P2X2, P2X2-TR receptors expressed alone in oocytes were not gated or modulated by the application of GABA (Fig. 4B). Co-activation of P2X2-TR and ρ1 channels evoked currents (IATP + GABA = −0.8 ± 0.2 μA), not significantly different (p > 0.5, n = 11) from the sum of the individual responses (IATP = −0.3 ± 0.1 μA and IGABA = −0.4 ± 0.1 μA). IATP + GABA represented 107 ± 3.5% of the prediction (Fig. 4C). Similarly, additive responses were recorded when GABA was applied during application of ATP and when ATP was applied during application of GABA (Fig. 4D), accounting for 97.2 ± 1 and 98.5 ± 0.6% of the predicted currents respectively (n = 5). These results showed that P2X2-TR and ρ1 receptors were independent, indicating a prominent role of the cytoplasmic domain of these channels in the cross-talk.

To determine whether both intracellular domains were involved in cross-inhibition between P2X2 and ρ1 channels, we examined the ability of minigenes coding for P2X2-CT, the C-terminal domain of P2X2 receptors (amino acids 374–472; Fig. 5A), and minigenes encoding the intracellular loop between the M3 and M4 transmembrane domains of ρ1 subunits (p1-IL2, amino acids 366–457; Fig. 5A) to disrupt the functional cross-talk by competition. As illustrated in Fig. 5, B and C, over-expression of P2X2-CT (Fig. 5B) or p1-IL2 (Fig. 5C) significantly blocked the cross-inhibition observed between P2X2 and GABA2 receptors. Co-application of ATP + GABA elicited currents that were not significantly different (p > 0.5) from the arithmetic sum of individual ATP and GABA applications. IATP + GABA recorded in the presence of either P2X2-CT or p1-IL2, represented 101.4 ± 6% (n = 10) and 100.4 ± 3% (n = 7), respectively, of the predicted sum. These data confirm that interactions between cytoplasmic domains are critical for the reciprocal current occlusion between P2X2 and GABA2 receptors.

**Physical Coupling and Co-trafficking of ρ1 and P2X2 Receptors**—To assess the existence of complexes containing P2X2 and GABA2 channels, we first verified cross-inhibition in oocytes co-expressing P2X2 and GABA2 channels. A, co-application of ATP + GABA induced currents (Actual) significantly smaller than the arithmetic sum (Predicted) of the individual ATP (100 μM) and GABA (10 μM) responses in oocytes expressing low levels of each receptor. B and C, superimposed current traces obtained with 10 μM of GABA, 100 μM of ATP, or a mixture of ATP and GABA from oocytes expressing P2X2 receptors alone (B) and homomeric ρ1 receptors (C). D, ATP dose-response curves from oocytes expressing P2X2 alone (filled squares) or with ρ1 (filled circles). Normalized ATP responses obtained from oocytes expressing P2X2 alone and P2X2 + ρ1 are presented in the inset. Duration of application was 5 s. E, GABA dose-response curves from oocytes expressing ρ1 alone (filled squares) and with P2X2 (filled circles). Normalized GABA (10 μM) responses obtained from oocytes expressing ρ1 only and P2X2 + ρ1 are presented in the inset. Duration of application was 5 s. In panels D and E, mean peak currents were normalized to the maximal response (mean ± S.E.) from 5–7 oocytes. Vh = −60 mV.

inhibited ATP + GABA currents induced by co-application of ATP and GABA.

It has been proposed that cross-inhibition between P2X2 and GABA2 receptors expressed in DRG neurons was mainly due to action of Cl− efflux (generated by GABA inward currents) on P2X2 channels and by Ca2+ entry on GABA receptors (25). We expressed P2X2 and ρ1 in order to obtain outward currents with similar amplitudes. In these conditions, at 30-mV holding potential, we observed similar responses to each agonist (IATP = 0.3 ± 0.1 μA and IGABA = 0.4 ± 0.1 μA, n = 6). ATP + GABA induced responses with amplitudes (IATP + GABA = 0.4 ± 0.1, n = 6) significantly smaller (p < 0.0005) than the arithmetic sum of IATP and IGABA (Fig. 3D). The ATP + GABA
ATP (100 M) induced currents elicited by application of tagged tors in transfected hippocampal neurons. Wild-type and GABA (10 M) and ATP + GABA at different holding potentials ranging from −80 to 20 mV in 20-mV steps. Prediction (Predicted) represents the arithmetic sum of individual ATP and GABA responses at each potential. B, co-application of ATP + GABA induced currents smaller than the sum of IATP and IGABA, *p < 0.05; **p < 0.005; ***p < 0.0005; n = 7. C, current-voltage relationship of P2X receptors from co-activation of both channels (gray filled circle). The predicted current-voltage relationship of p1 + P2X2 channels is represented in black filled circles. Each point corresponds to the current amplitude mean ± S.E. obtained from 7 oocytes. Reversal potentials are indicated by arrows. D and E, superimposed currents induced by application of ATP (100 M), GABA (10 M), or a mixture of both agonists recorded from oocytes co-expressing P2X and GABAC receptors (n = 6). Non-additivity of ATP and GABA responses were observed at 30 mV holding membrane potential (D) and in calcium-free extracellular solution (E); ***p < 0.0005.

At 7 days in vitro, uniform immunoreactivity were detected on cell bodies and proximal dendrites in agreement with an earlier report (32). Confocal microscopy, associated with Alexa Fluor 488-phalloidin staining to label the F-actin cytoskeleton and reveal the outline of the cell, showed that most puncta were intracellular (Fig. 7A). This observation was confirmed by surface labeling of the extracellular p1 epitope. At lower concentrations of Triton X-100 (0.002%), the extracellular component of the p1 immunolabeling could be visualized (Fig. 7B). In this condition, very low levels of uniform immunoreactivity were detected on cell bodies and proximal dendrites, suggesting that only a minor fraction of p1 subunits were located at the cell surface. Thus, GABAC receptors expressed in hippocampal neurons exhibit a distribution compatible with a predominant intracellular localization.

In contrast to GABAC receptors, we found P2X2-YFP receptors uniformly distributed throughout the neurons, with a predominant surface localization consistent with previous reports (33). YFP fluorescence was not restricted to proximal dendrites but extended to the very extremities of neuronal processes (Fig. 7C, middle). This localization was divided into diffuse and clustered distribution, with a typical topology suggestive of P2X receptors at the plasma membrane, occasionally associated in dendritic clusters (Fig. 7D, middle).

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In 40–50% of neurons that were cotransfected with p1 and P2X2-YFP, significant differences in GABAC receptor distribution were noticed (Fig. 7C) when compared with neurons transfected with p1 alone (Fig. 7A). In conditions of co-expression, whereas P2X2-YFP receptor topology seemed unaffected by the presence of the p1-subunit, GABAC receptors were translocated to the cell surface and colocalized with P2X2-YFP clusters (Fig. 7D). Co-clustering of p1 and P2X2-YFP subunits was particularly obvious on proximal dendrites (Fig. 7D). The external immunolabeling of p1 subunits appeared to overlap with P2X2-YFP fluorescence, indicating that the addressing of GABAC receptors was changed by the presence of P2X2-YFP receptors and that the two receptors were largely colocalized in surface clusters (Fig. 7E). Furthermore, p1 immunofluorescence was found more distally to the cell body, indicating that the targeting of GABAC receptors was also following the one exhibited by P2X2-YFP subunits. The specificity of this effect was verified by transfecting neurons with p1 GABAC and 5-HT3A receptors; no changes in subcellular labeling were ob-
served following co-transfection (not shown). It can be concluded from these results that, in neurons expressing both receptors, P2X2 receptors regulate the addressing of GABAC receptors in a dominant way by inducing their translocation from an internal vesicular compartment to surface clusters common to both receptors.

**DISCUSSION**

No functional or biochemical interaction was previously described between identified subtypes of excitatory and inhibitory ligand-gated channels. We demonstrate here a functional cross-talk and a physical coupling between P2X2 and GABAC receptors. A reciprocal current inhibition (25–50%) was recorded from oocytes co-expressing homomeric P2X2 and GABAC channels when they were simultaneously activated, showing that these two unrelated subtypes of receptor channels are not functionally independent. The percentage of current occlusion is identical whatever the sequence of agonist application (co-application, GABA during application of ATP, or vice versa), indicating that each type of receptor has an equal propensity to inhibit the other one. Similarities in kinetics (i.e. activation and desensitization) between recorded and predicted currents (see Fig. 1A) induced by the co-application ATP + GABA suggested that the cross-talk closed an important proportion of both receptors. Application of ATP did not gate or modulate GABAC receptors, and GABA did not activate or modulate P2X2 receptors, showing that nonspecific cross-activation or cross-desensitization between one type of transmitter and the other type of receptor could not explain the cross-inhibition observed during co-application. A similar reciprocity of cross-inhibition was observed between P2X2 and nicotinic or 5-HT3 receptors (24, 26).

Interestingly, an inhibitory cross-talk between P2X and GABAA channels reported in dorsal root ganglion neurons indicates a more pronounced inhibitory effect of GABA receptors on P2X channels than the reverse (25). It was proposed that the chloride efflux generated by GABA-gated channels could inhibit P2X receptors and, inversely, that the Ca2+ influx mediated by P2X channels could inhibit the GABA-gated channels (25). It is unlikely that these mechanisms play a major role in the functional interaction between the P2X2 and GABAC receptors studied here, because we observed a reciprocal current occlusion when the oocytes co-expressing both channels are clamped between −80 to 30 mV (above the reversal potential of...
chloride ions) and in the absence of extracellular calcium ions. The cross-talk was also recorded at low channel densities, giving rise to small current amplitudes at saturating concentrations of agonists and suggesting a significant affinity of coupling between P2X2 and GABA\(_C\) receptors.

Co-expression of \(\beta 1\) subunits with a functional truncated version of the P2X2 subunit lacking most of its C-terminal domain resulted in receptor independence, as indicated by current additivity. Moreover, an excess of minigene coding for the C-terminal domain of P2X2 or for the main intracellular loop (IL2) of \(\beta 1\), co-expressed with P2X2 and GABA\(_C\) receptors, resulted also in the loss of functional cross-talk despite normal individual current phenotypes. The results of these mutagenesis and competition experiments underlined the importance of the intracellular domains of both receptor subunits in this cross-inhibition. The crucial role of cytoplasmic domains was also demonstrated in the functional coupling between P2X and 5-HT\(_3\) receptor channels. However, in competition experiments these cytoplasmic domains were not able to disrupt physical association between 5-HT\(_3\) and P2X receptors (26). We also observed co-purification of GABA\(_C\) and P2X2 receptors expressed in oocytes. No clear sequence homology exists between the sequences of the large intracellular loops of members of the nicotinic receptor superfamily and between the C-terminal domains of the different P2X family members. Taken together, this led us to formulate the hypothesis of the existence of two distinct mechanisms involved in the physical and functional coupling between these two families of ligand-gated channels. To identify putative sequences required in both mechanisms, the propensity of other members of P2X family to interact with GABA\(_C\) or different subunit combinations of GABA\(_A\) receptors should now be investigated.

Our immunolocalization experiments in the primary culture of neurons revealed that expression of the \(\beta 1\) subunits was mainly intracellular and compatible with a prominent vesicular localization. Whether these vesicular compartments are part of the routing mechanism involved in \(\beta 1\)
subunit trafficking, an early endosomal compartment involved in subunit recycling, or a lysosomal network remains to be determined. Co-expression with P2X2 resulted in a dramatic change in the distribution of GABAC receptors that were translocated to the cell surface and colocalized with P2X2 clusters. When co-expressed with P2X2, the ρ1 receptor was also found more distally to the cell body, indicating that the radial topology of GABAC was driven by P2X2 receptors. The co-targeting in transfected neurons and the co-purification of P2X2 and ρ1 receptor channels expressed in oocytes demonstrate the existence of physical complexes formed by the constitutive association of P2X2 and GABAC receptor channels in a different expression system.

Our findings suggest also that, besides the regulation of neuronal activity by modulation of current amplitude during co-activation, physical association between receptors could regulate the location and the number of surface receptors, which is an important means for synaptic neuronal regulation. Recent studies showed that P2X2 and P2X3 receptors undergo rapid agonist-dependent internalization and recycling, contrary to P2X2 which appeared to be more stable at the cell surface (33–36). The molecular mechanism of P2X receptor trafficking has yet to be elucidated despite identification of some associated intracellular proteins (37, 38). Dynamic regulation of GABAC and GABAR receptor numbers at the membrane is regulated by interactions with several proteins. For example, gephyrin and dystrophin have been shown to play a role in the anchoring of GABAC receptors, although no physical interaction with gephyrin has been demonstrated in vitro (39, 40). GABARAP, a microtubule-associated protein, and Plic-1, a ubiquitin-related protein, interact with GABAC receptor subunits and are involved in receptor trafficking (41, 42). MAP1B is a large protein that binds actin and tubulin and is the only GABAC receptor demonstrating the existence of physical complexes formed by

REFERENCES

1. Barnard, E. A. (1996) Trends Pharmacol. Sci. 17, 305–309
2. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
3. Bornmann, J. (2000) Trends Pharmacol. Sci. 21, 16–19
4. Bonnert, T. P., McKernan, R. M., Farrar, S., Lebourdelle, B., Heavens, R. P., Smith, D. W., Heswion, L., Rigby, M. R., Sirnathsinghji, J. S., Brown, N., Wafford, K. A., and Whiting, P. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9891–9896
5. Pan, Z. H., Zhang, D., Zhang, X., and Lipton, S. A. (2000) J. Neurosci. 16, 3137–3145
6. Qian, H., and Ripp, H. (1999) Proc. Roy. Soc. Lond. Ser. B Biol. Sci. 266, 2149–2155
7. Vuchelen, L., Riedl, M. S., Schuster, J., Bueli, G., Surprenant, A., North, R. A., and Elde, R. (1997) Neurropharmacology 36, 1229–1242
8. Zheng, X., Wu, X., Zhang, A., Strong, J. A., Wang, L., Xu, M., and Lu, L. (2003) J. Biol. Chem. 278, 48321–48329
9. Wheeler-Schilling, T. H., Margiotta, K., Kohler, K., Jabs, R., and Guenther, E. (2002) Brain Res. Mol. Brain Res. 76, 415–418
10. Brandle, U., Guenther, E., Irie, C., and Wheeler-Schilling, T. H. (1998) Brain Res. Mol. Brain Res. 59, 269–272
11. Koenen, P., Brandstatter, J. H., Kroger, S., Enz, R., Bornmann, J., and Wasielewski, H. (1997) J. Comp. Neurol. 386, 520–532
12. Enz, R., Brandstatter, J. H., Wasielewski, H., and Bornmann, J. (1998) J. Neurosci. 18, 4479–4480
13. Koenen, P., Brandstatter, J. H., Enz, R., Bornmann, J., and Wasielewski, H. (1998) J. Neurosci. 18, 115–127
14. Kanghan, R., Housley, G. D., Burton, L. D., Christie, D. L., Kippenberger, A., Throndt, P., Luo, L., and North, R. A. (1999) J. Comp. Neurol. 407, 11–32
15. Wegelius, K., Pastorneck, M., Hiltnoen, J. O., Rivera, C., Kaila, K., Saarma, M., and Rechen, M. (1998) Eur. J. Neurosci. 10, 350–357
16. Boue-Grabot, E., Bouhobba, M., Baezales, L., Tranu, G., Bloch, B., and Garret, M. (1998) J. Neurochem. 70, 899–907
17. Stojilkovic, S. S., Tomic, M., Van Goor, F., and Koschmider, T. (2000) Biochem. Cell Biol. 78, 393–404
18. Boue-Grabot, E., Taupignon, A., Tramu, G., and Garret, M. (2000) Endocorpus 114, 1627–1632
19. Jo, Y. H., and Schlichter, R. (1999) Nat. Neurosci. 2, 241–245
20. Jo, Y. H., and Role, L. W. (2000) J. Physiol. 521, 4784–4804
21. Barajas-Lopez, C., Espinosa-Luna, R., and Zha, Y. (1998) J. Physiol. 513, 671–683
22. Zhou, X., and Galligan, J. J. (1998) J. Physiol. 513, 685–697
23. Searf, T. J., Redman, R. S., and Silinsky, E. M. (1998) J. Physiol. 510, 783–791
24. Khakh, B. S., Zhou, X., Sydes, J., Galligan, J. J., and Lester, H. A. (2000) Nature 406, 405–410
25. Sokolova, E., Nistri, A., and Giniatullin, R. (2001) J. Neurosci. 21, 4958–4968
26. Boue-Grabot, E., Barajas-Lopez, C., Chakfe, Y., Blais, D., Belanger, D., Emerit, M. B., and Seguela, P. (2003) J. Neurosci. 23, 1246–1253
27. Boue-Grabot, E., Archambault, V., and Seguela, P. (2002) J. Biol. Chem. 275, 10190–10195
28. Lee, K. R., Boue-Grabot, E., Archambault, V., and Seguela, P. (1999) J. Biol. Chem. 274, 15415–15419
29. Geslin, K., Asmusson, H., and Banker, G. (1998) in Culturing Nerve Cells (Banker, G., and Geslin, K., eds) pp. 339–370, MIT Press, Cambridge, MA
30. Brake, A. J., Wagenseil, M. J., and Julus, D. (1994) Nature 371, 519–523
31. Wotring, V. E., Chang, Y., and Weiss, D. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9891–9896
32. Cheng, Q., Burkut, P. M., Kulli, J. C., and Yang, J. (2001) J. Neurophysiol. 86, 2605–2615
33. Bobanovic, L. K., Royle, S. J., and Murrell-Lagnado, R. D. (2002) J. Neurosci. 22, 4814–4824
34. Li, G. H., Lee, E. L., Blair, D., Holding, C., Ponnukk, P., Cook, D. I., Barden, J. A., and Bennett, M. R. (2000) J. Biol. Chem. 275, 29107–29112
35. Dutton, J. L., Ponnukk, P., Li, G. H., Holding, C. A., Worthington, R. A., Vandenberg, R. J., Cook, D. I., Barden, J. A., and Bennett, M. R. (2000) Neuronapharmacology 39, 2054–2066
36. Royle, S. J., Bobanovic, L. K., and Murrell-Lagnado, R. D. (2002) J. Biol. Chem. 277, 35378–35386
37. Kim, M., Jiang, L. H., Wilson, H. L., North, R. A., and Surprenant, A. (2001) EMBO J. 20, 6347–6358
38. Gendreau, S., Schirmier, J., and Schmalzing, G. (2003) J. Chromatogr. 786, 311–318
39. Giustetto, M., Kirsch, J., Fritschy, J. M., Coronado, D., and Sasso, F. (1998) J. Comp. Neurol. 395, 231–244
40. Kruessel, I., Mastrocola, M., Zarell, R. A., Baurhauer, B., Schaub, M. C., and Fritschy, J. M. (1999) Eur. J. Neurosci. 12, 4457–4462
41. Bedford, F., Kitterl, J., Muller, E., Thomas, P., Uren, J., Merlo, D., Wiesen, W., Triller, A., Smart, T., and Moss, S. (2001) Nat. Neurosci. 4, 908–916
42. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999) *Nature* **397**, 69–72
43. Hanley, J. G., Koulen, P., Bedford, F., Gordon-Weeks, P. R., and Moss, S. J. (1999) *Nature* **397**, 66–68
44. Dunn, P. M., Zhong, Y, and Burnstock, G (2001) *Prog. Neurobiol.* **65**, 107–134
45. Bouvier, M. (2001) *Nat. Rev. Neurosci.* **2**, 274–286
46. Liu, F., Wan, Q., Pristupa, Z. B., Yu, X. M., Wang, Y. T., and Niznik, H. B. (2000) *Nature* **403**, 274–280
47. Lee, F. J., Xue, S., Pei, L., Vukusic, B., Chery, N., Wang, Y., Wang, Y. T., Niznik, H. B., Yu, X. M., and Liu, F. (2002) *Cell* **111**, 219–230
48. Li, Y., Wu, L. J., Legendre, P., and Xu, T. L. (2003) *J. Biol. Chem.* **278**, 38637–38645
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