Subunit-specific Coupling between \(\gamma\)-Aminobutyric Acid Type A and P2X\(_2\) Receptor Channels*

Received for publication, September 7, 2004
Published, JBC Papers in Press, September 29, 2004, DOI 10.1074/jbc.M410223200

Éric Boué-Grabot‡‡, Estelle Toumlé¶, Michel B. Émerit†, and Maurice Garret‡

From the ‡Laboratoire de Neurophysiologie, CNRS Unité Mixte de Recherche UMR 5543, Université Victor Segalen Bordeaux 2, 33076 Bordeaux cedex and ¶INSERM U288, Hopital de la Salpétrière, 75013 Paris, France

ATP and \(\gamma\)-aminobutyric acid (GABA) are two fast neurotransmitters co-released at central synapses, where they co-activate excitatory P2X and inhibitory GABA\(_A\) (GABA type A) receptors. We report here that co-activation of P2X\(_2\) and various GABA\(_A\) receptors, co-expressed in Xenopus oocytes, leads to a functional cross-inhibition dependent on GABA\(_A\) subunit composition. Sequential applications of GABA and ATP revealed that \(\alpha\beta\) or \(\alpha\beta\gamma\)-containing GABA\(_A\) receptors inhibited P2X\(_2\) channels, whereas P2X\(_2\) channels failed to inhibit \(\gamma\)-containing GABA\(_A\) receptors. This functional cross-talk is independent of membrane potential, changes in current direction, and calcium. Non-additive responses observed between cation-selective GABA\(_A\) and P2X\(_2\) receptors further indicate the chloride independence of this process. Overexpression of minigenes encoding either the C-terminal fragment of P2X\(_2\) or the intracellular loop of the \(\beta\) subunit disrupted the functional cross-inhibition. We previously demonstrated functional and physical cross-talk between \(\rho\)1 and P2X\(_2\) receptors, which induced a retargeting of \(\rho\)1 channels to surface clusters when co-expressed in hippocampal neurons (Boué-Grabot, E., Emerit, M. B., Toumlé, E., Seguela, P., and Garret, M. (2004) J. Biol. Chem. 279, 6967–6975). Co-expression of P2X\(_2\) and chimeric \(\rho\)1 receptors with the C-terminal sequences of \(\alpha2\), \(\beta3\), or \(\gamma2\) subunits indicated that only \(\alpha\beta\beta\) and P2X\(_2\) channels exhibit both functional cross-inhibition in Xenopus oocytes and co-clustering/retargeting in hippocampal neurons. Therefore, the C-terminal domain of P2X\(_2\) and the intracellular loop of \(\beta\) GABA\(_A\) subunits are required for the functional interaction between ATP- and GABA-gated channels. This \(\gamma\)-subunit-dependent cross-talk may contribute to the regulation of synaptic activity.

Synaptic transmission is achieved through the release of one or more neurotransmitters from the same presynaptic terminal, resulting in the activation of different classes of receptors co-localized at the same post-synaptic site. Recent reports have demonstrated that co-activation of distinct postsynaptic receptors by their respective transmitters induced cross-modulation of their functional properties (1). G-protein-coupled receptors and ligand-gated channels reciprocally affect their functions by direct interaction between intracellular domains, as illustrated for D5 and GABA\(_A\) (2) or D1 and N-methyl-D-aspartic acid (2, 3). Cross-talk between distinct ligand-gated channels has been described between ATP P2X receptors and either acetylcholine nicotinic receptors (4–7), 5-hydroxytryptamine 3 receptors (8, 9), or GABA\(_A\) receptors in dorsal root ganglia neurons (10), as well as between GABA and glycine receptors in spinal cord neurons (11). Cross-talk between ligand-gated channels is characterized by current occlusion during simultaneous agonist application, although the mechanisms remain unclear. Intracellular phosphorylation pathways underlie asymmetric cross-inhibition between GABA\(_A\) and glycine receptors (11). Cross-talk between GABA\(_A\) and P2X receptors expressed in dorsal root ganglia neurons has been described as a chloride- and calcium-dependent interaction (10), whereas we demonstrated that a physical interaction involving the intracellular domains of each receptor led to the functional cross-talk between 5-HT\(_3\) and P2X\(_2\) receptors (9), as well as between P2X\(_2\) and \(\rho\)1/GABA\(_A\) (12). Because GABA and ATP are synaptically co-released in spinal cord and hypothalamic neurons (13, 14), where they activate co-localized ATP- and GABA-gated channels, cross-talk between inhibitory GABA\(_A\) and excitatory P2X receptors may represent an important, fast process for controlling the signal transmission phenomenon. Therefore, our aim was to investigate a putative cross-modulation between GABA\(_A\) and P2X receptors and the mechanisms underlying this process.

GABA and P2X receptors have distinct structures. GABA\(_A\) receptors are pentameric structures formed by differential assembly of multiple subunits (15). Each of the five subunits contains four hydrophobic transmembrane domains and extracellular termini. GABA-gated chloride channels mediate fast inhibition and thus, play a fundamental role in the physiology and physiopathology of the nervous system (16). The diversity of their functional properties, pharmacology, and subcellular targeting depends to a large extent on subunit composition (16). P2X receptors are ATP-gated cation channels consisting of a family of seven subunits with two transmembrane domains, a large extracellular loop, and intracellular termini (17). P2X receptors are implicated in fast excitatory synaptic transmission (18). However, little is known about purinergic transmission in the brain, despite the wide distribution of P2X receptor subunits throughout the central nervous system (19).

* This work was supported in part by CNRS, Region Aquitaine, and Université Victor Segalen de Bordeaux2 (to E. B. G. and M. G.) and INSERM (to M. B. E.). 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.

‡ To whom correspondence should be addressed. Tel.: 33-(0)5-5767-4850; Fax: 33-(0)5-5690-1421; E-mail: eric.boue-grabo@umr5543.u-bordeaux2.fr.

¶ Recipient of a post graduate fellowship from the Ministère de l’Éducation Nationale et de la Recherche.

1 The abbreviations used are: GABA\(_A\), \(\gamma\)-aminobutyric acid type A; YFP, yellow fluorescent protein; P2X, TR, C-terminal truncated form of P2X\(_2\); CT, C-terminal; NT, N-terminal; NMDG, N-methyl-D-glutamate; PBS, phosphate-buffered saline; IL2, intracellular loop.
Cross-talk between GABA<sub>A</sub> and P2X<sub>2</sub> Receptors

**EXPERIMENTAL PROCEDURES**

**Molecular Biology**—Wild-type and YFP-tagged rat P2X<sub>2</sub>, P2X<sub>2</sub>TR, and P1 clones were available from previous work (9, 12). Human hβ1, hβ3, and mutated hβ3OSGE cDNAs were generously provided by Dr. Philip K. Ahring (Neurosearch, Copenhagen) (20). The cDNA fragments for rat hβ1, hβ3, hγ2, and hγ3 subunits were generated by replacing the pλ cDNA sequence from amino acid lys<sup>379</sup> to the stop codon by a homologous sequence of α2, β3, and γ2 subunits, respectively. The pλ cDNA fragment located between the natural HindIII restriction site and the 3′-end cloning site, XbaI, was replaced by a homologous α2, β3, or γ2 GABA<sub>A</sub> subunit fragment generated by PCR to create compatible HindIII and XbaI restriction enzyme sites. All constructs were subcloned into pcDNA3 (Invitrogen) and verified by automated dideoxy sequencing. Numerical values are expressed as mean ± S.E. Statistical comparisons were assessed using Student’s t test. The differences were considered significant if p < 0.05. For the experiments, currents were measured at the peak of the response obtained at several membrane potentials (∼60 to 30 mV). Reversal potentials (E<sub>rev</sub>) were estimated from the V-I relationship determined by linear regression analysis. All data were analyzed using Prism 4.0 (GraphPad, San Diego, CA).

**Cell Culture and Transfection**—Neurons were cultured as previously described (12). Briefly, hippocampi of rat embryos were dissected on day 18. Dissociation was achieved after trypsinization, with a Pasteur pipette (22). Hippocampi were transferred to a 90-mm dish containing medium containing B27 supplement (20). The cDNAs coding for minigenes were injected into the neuronal culture and incubated for 3 h at 37 °C. Expression was then conducted for 48 h in the original medium that was added back to the neurons. Under these conditions, the transfection rate reached 10–25%, and 60–70% of transfected neurons were co-transfected with two plasmids.

**Immunocytochemistry and Confocal Imaging**—Immunofluorescence was performed 48 h after transfection (9–10 days after plating). Coverslips were washed twice with PBS+ (phosphate-buffered saline containing 0.1 mM CaCl<sub>2</sub> and 0.1 mM MgCl<sub>2</sub>) at 37 °C, and fixed for 15 min with paraformaldehyde (4%) containing 4% sucrose, at 37 °C in PBS+. After three 10-min washes in PBS+, they were incubated for 30 min in antibody buffer (2% bovine serum albumin, 3% normal goat serum, 3% normal donkey serum, and 0.1% Triton X-100 in PBS+). P1 chimeras were labeled with affinity purified anti-p1 antibodies (1:200) directed against the extracellular N-terminal sequence (22) for 2 h at room temperature, and revealed with AlexaFluor<sup>488</sup>-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1:1000 dilution, for 1 h at room temperature. Immunofluorescence images were generated using a Leica TCS-400 laser scanning confocal microscope. Contrast and brightness were adjusted to ensure that all relevant pixels were within linear range. Images are the product of a 16-fold line average. For double labeling experiments, pictures were generated using Adobe Photoshop 5.0.

**Quantitative Measurements of Fluorescence Profiles**—Fluorescence profiles along labeled neurites were measured using Lucia 4.71 imaging software (Nikon). For each neuron, the contrast and brightness of confocal images were adjusted using identical parameters. The longest labeled neurite was then fitted with a polyline along which the fluorescence profile was plotted against the distance from the cell body. In all experiments, neurons overexpressing either receptor were excluded from the study. In co-transfection experiments, only neurons expressing similar number of both receptors were included in the study. The proximity and similarity of localization between individual neurons was overcome by using the cumulated fluorescence profiles obtained with 50 neurons of each group as a relevant parameter for quantifying the radial distribution of p1 chimeras.

**RESULTS**

**Reciprocal Cross-inhibition between αβ-containing GABA Receptors and P2X<sub>2</sub> Receptors**—To assess whether GABA<sub>A</sub> and P2X<sub>2</sub> receptors cross-modulated, we first co-expressed different combinations of GABA<sub>A</sub> subunits with P2X<sub>2</sub> channels in Xenopus oocytes and recorded responses to separate or combined application of ATP and GABA. In oocytes co-expressing αββ3 and P2X<sub>2</sub> channels, the application of saturating concentrations of either GABA (100 μM) or ATP (100 μM) evoked inward currents characteristic of the activation of GABA<sub>A</sub> and P2X<sub>2</sub> receptors, respectively (Fig. 1A and Table I summarizes data in Figs. 1 and 2). In co-transfection experiments, only neurons expressing similar number of both receptors were included in the study. The proximity and similarity of localization between individual neurons was overcome by using the cumulated fluorescence profiles obtained with 50 neurons of each group as a relevant parameter for quantifying the radial distribution of p1 chimeras.

**Immunocytochemistry and Confocal Imaging**—Immunofluorescence was performed 48 h after transfection (9–10 days after plating). Coverslips were washed twice with PBS+ (phosphate-buffered saline containing 0.1 mM CaCl<sub>2</sub> and 0.1 mM MgCl<sub>2</sub>) at 37 °C, and fixed for 15 min with paraformaldehyde (4%) containing 4% sucrose, at 37 °C in PBS+. After three 10-min washes in PBS+, they were incubated for 30 min in antibody buffer (2% bovine serum albumin, 3% normal goat serum, 3% normal donkey serum, and 0.1% Triton X-100 in PBS+). P1 chimeras were labeled with affinity purified anti-p1 antibodies (1:200) directed against the extracellular N-terminal sequence (22) for 2 h at room temperature, and revealed with AlexaFluor<sup>488</sup>-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) at 1:1000 dilution, for 1 h at room temperature. Immunofluorescence images were generated using a Leica TCS-400 laser scanning confocal microscope. Contrast and brightness were adjusted to ensure that all relevant pixels were within linear range. Images are the product of a 16-fold line average. For double labeling experiments, pictures were generated using Adobe Photoshop 5.0.

**Quantitative Measurements of Fluorescence Profiles**—Fluorescence profiles along labeled neurites were measured using Lucia 4.71 imaging software (Nikon). For each neuron, the contrast and brightness of confocal images were adjusted using identical parameters. The longest labeled neurite was then fitted with a polyline along which the fluorescence profile was plotted against the distance from the cell body. In all experiments, neurons overexpressing either receptor were excluded from the study. In co-transfection experiments, only neurons expressing similar number of both receptors were included in the study. The proximity and similarity of localization between individual neurons was overcome by using the cumulated fluorescence profiles obtained with 50 neurons of each group as a relevant parameter for quantifying the radial distribution of p1 chimeras.
Cross-talk between GABA<sub>A</sub> and P2X Receptors

TABLE I

| P2X<sub>2</sub> + GABA<sub>A</sub> | I<sub>ATP</sub> | I<sub>GABA</sub> | I<sub>ATP+GABA</sub> | n | Inhibition |
|-----------------------------|-------------|-------------|----------------|---|-----------|
| αβ3                         | 0.23 ± 0.1  | 1.5 ± 0.88  | 1.48 ± 0.68    | 5 | ATP+GABA  |
| αβ2                         | 1.17 ± 0.21 | 1.11 ± 0.27 | 1.58 ± 0.25    | 15| ATP+GABA  |
| αβ3                         | 1.9 ± 0.65  | 1.3 ± 0.6   | 1.93 ± 0.5     | 5 | ATP+GABA  |
| αβ2β2                       | 0.66 ± 0.29 | 0.55 ± 0.32 | 0.82 ± 0.37    | 5 | ATP+GABA  |
| αβ2β2β3                    | 0.92 ± 0.6  | 1.2 ± 0.46  | 1.41 ± 0.67    | 6 | ATP+GABA  |
| αβ2β3γ3                    | 4.3 ± 1.04  | 2.9 ± 0.5   | 5.6 ± 0.89     | 22| ATP+GABA  |

These results showed that co-activation of P2X<sub>2</sub> and αβ-containing GABA<sub>A</sub> receptors induced an instantaneous, bidirectional current occlusion, demonstrating that GABA<sub>A</sub> and P2X<sub>2</sub> receptors are functionally non-independent.

Reciprocal Cross-inhibition Did Not Occur with γ-Containing GABA-gated Channels—Because most native GABA<sub>A</sub> receptors are composed of α, β, and γ subunits, we co-injected P2X<sub>2</sub> receptors with α2, β3, and γ2 or γ3 GABA<sub>A</sub> subunits (or cDNAs) at a ratio of 1:1:1.5 to ensure that a γ subunit would be associated with the αβ complex(es). In both cases, co-application of ATP plus GABA induced currents significantly smaller (p < 0.0005) than the sum of the individual I<sub>GABA</sub> and I<sub>ATP</sub> (Fig. 2, A and B, and Table I). The I<sub>GABA</sub> then ATP (100 µM GABA) in oocytes expressing α2β3γ3 (Fig. 2, A–C) and for P2X<sub>2</sub> plus α2β3γ3 (Fig. 2, B and C), respectively. When ATP was applied during GABA application, the amplitude of I<sub>GABA</sub> then ATP was also significantly smaller (p < 0.0005, n = 15) than the predicted current. I<sub>GABA</sub> then ATP represented 71.36 ± 3.08% (n = 15) and 66.62 ± 3.3% (n = 6) of the predicted current for P2X<sub>2</sub> plus α2β3γ3 and P2X<sub>2</sub> plus α2β3γ3, respectively (Fig. 2, A and C). Thus, the same degree of occlusion (~30%) was observed between P2X<sub>2</sub> and GABA<sub>A</sub> receptors formed by α, β, and γ subunits (Fig. 2) and between P2X<sub>2</sub> and GABA αβ receptors (Fig. 1 and Table I).

Interestingly, when GABA was applied during ATP application, the current amplitude was not significantly different (p > 0.5, n = 15) from the sum of I<sub>GABA</sub> and I<sub>ATP</sub> (100 µM) of the current evoked by 10 µM ATP (Fig. 2, A–C). Therefore, the γ subunit was not associated with the αβ subunits. As illustrated in Fig. 2D, the enhancement by flunitrazepam (1 µM) of the current evoked by 10 µM GABA (I<sub>GABA</sub> + flunitrazepam) = 152 ± 15% of the control, n = 6) indicated that γ subunits were associated with α and β subunit complexes. Taken together, these results show that ATP-gated channels interact functionally with GABA<sub>A</sub> receptors irrespective of their subunit composition and, more importantly, that the direction of current occlusion depends on the molecular composition of the GABA<sub>A</sub> receptors. GABA<sub>A</sub> receptors closed substantial proportions of P2X<sub>2</sub> receptors during co-activation (and vice versa), whereas ATP-gated channels failed to close GABA-gated channels associated with a γ subunit.
Cross-talk between GABA<sub>A</sub> and P2X Receptors

Voltage-, Calcium-, and Chloride-Independent Current Occlusion—Sokolova and coworkers (10) described a current occlusion between GABA<sub>A</sub>- and ATP-induced currents in dorsal root ganglia neurons and postulated that chloride ions were the main coupling agent. We therefore investigated a potential chloride or voltage dependence of the cross-talk by recording currents evoked by the activation of P2X<sub>2</sub> and GABA<sub>A</sub> receptors at different holding potentials. Currents induced by co-application of ATP plus GABA were significantly smaller (p < 0.0005, n = 7) than the sum of the individual I<sub>ATP</sub> and I<sub>GABA</sub> at holding potentials ranging from −60 to +30 mV (I<sub>ATP+GABA</sub> was between 64 and 76% of the predicted current, Fig. 3A). We also recorded cross-inhibition in the absence of CaCl<sub>2</sub> in the extracellular solution (Fig. 3A). Cross-inhibition was observed with outward GABA currents, i.e. chloride entry, suggesting that chloride was not essential to the occlusion process. However, ATP-induced outward currents are difficult to record due to a marked inward rectification of P2X<sub>2</sub> channels (23). We therefore used cationic GABA receptors to determine whether chloride was implicated in the cross-talk. Mutation of amino acids in the M1–M2 loop of the β3 subunit to the corresponding amino acids of the α<sup>7</sup> nicotinic acetylcholine subunit rendered the GABA<sub>A</sub> cation-selective, with no significant permeability to anions, on co-expression with wild type α and γ subunits (20). We co-injected oocytes with cDNAs encoding for P2X<sub>2</sub>, α<sup>7</sup> and human mutated β3 subunits (hβ3SGE) and recorded responses to individual and combined application of agonists at a holding potential of −60 mV. Application of GABA (100 μM) induced a slowly desensitizing inward current (I<sub>GABA</sub> = −0.71 ± 0.4 μA, n = 7), and ATP evoked non-desensitizing responses (I<sub>ATP</sub> = −2.8 ± 1.1 μA, n = 7). Co-activation of both receptors by simultaneous or sequential application evoked inward responses significantly smaller (p < 0.0005, n = 7) than the predicted sum of I<sub>ATP</sub> and I<sub>GABA</sub> (Fig. 3C). I<sub>ATP+GABA</sub>, I<sub>ATP</sub> then GABA, and I<sub>GABA</sub> then ATP represented 73.26 ± 4.7%, 73 ± 4.0%, and 65 ± 5.0% of the predicted current, respectively. Current-voltage relationships for α2β3γSGE receptors and wild-type α2β3 receptors were determined in normal Ringer solution Na<sup>+</sup>-R and in low Na<sup>+</sup> Ringer (NMDG-R). As depicted in Fig. 3D, the I-V curve for α2β3γSGE revealed a shift in reversal potential of −26 mV for NMDG-R (E<sub>rev</sub> = −5.17 ± 0.7 mV, n = 5) compared with Na<sup>+</sup>-R (E<sub>rev</sub> = −31.33 ± 2.0 mV, n = 5). In contrast, the I-V curve for wild type α2β3 receptors exhibited a minor shift of −4 mV for NMDG-R (E<sub>rev</sub> = −34.22 ± 2 mV, n = 4) compared with Na<sup>+</sup>-R (E<sub>rev</sub> = −30.28 ± 2 mV, n = 4). These data are in agreement with the cation-selective permeability of the α2β3γSGE receptor (20). Taken together, these experiments demonstrated that reciprocal cross-inhibition between GABA<sub>A</sub> and P2X<sub>2</sub> was a receptor-mediated, voltage-, chloride-, and calcium-independent phenomenon.

Interaction of Intracellular Domains of P2X<sub>2</sub> and GABA<sub>A</sub> Receptors Are Crucial for Cross-inhibition—Previous studies showed that intracellular domains of receptor channels were involved in cross-inhibition between P2X and 5-HT<sub>3</sub> or α1/β3 GABA<sub>A</sub> receptors (9, 12). We investigated whether the C-terminal domain of P2X<sub>2</sub> and the intracellular domains of GABA<sub>A</sub> subunits participated in the current occlusion (Fig. 4A). We first co-expressed a C-terminal truncated form of P2X<sub>2</sub> (P2X<sub>2</sub>TR) with α2β3 GABA<sub>A</sub> receptors. Co-activation of both receptors by co-application of ATP and GABA evoked inward currents (−2.06 ± 0.92 μA) not significantly different (p > 0.5, n = 5) from the sum of the individual I<sub>ATP</sub> (−0.54 ± 0.19 μA) and I<sub>GABA</sub> (−1.5 ± 0.73 μA). I<sub>ATP+GABA</sub> represented 100 ± 1.4% of the predicted value (Fig. 4, B and F) showing that P2X<sub>2</sub>TR and GABA<sub>A</sub> receptors are functionally independent. To further examine the role of the intracellular domains of each receptor in the cross-inhibition mechanism, we carried out competition experiments with minigenes encoding the N or C termini of P2X<sub>2</sub> receptors (X2-NT corresponding to amino acids 1–29 and X2-CT corresponding to amino acids 374–472) and a minigene coding for the intracellular loop between hydrophobic domains M3 and M4 of the β3 GABA<sub>A</sub> subunit (β3-IL2 corresponding to amino acids 327–450). Although overexpression of X2-NT had no effect (Fig. 4C), X2-CT (Fig. 4D) and β3-IL2 (Fig. 4E) significantly blocked the cross-inhibition between wild-type P2X<sub>2</sub> and α2β3 receptors. Co-application of ATP plus GABA elicited responses not significantly different (p > 0.5) from the sum of the individual responses of ATP and GABA. I<sub>ATP+GABA</sub> represented 103.1 ± 7.8% (n = 5) and 108.8 ± 11.17% (n = 5) of the predicted sum in presence of X2-CT and β3-IL2, respectively. On the contrary, I<sub>ATP+GABA</sub> was significantly smaller (I<sub>ATP+GABA</sub> = 72.03 ± 4.3% of the predicted current p < 0.0005, n = 5) than the sum of separate applications in the presence of X2-NT. These data demonstrate that the C-terminal domain of P2X<sub>2</sub> or the intracellular loop of the β3 subunits are required for functional interaction between P2X and GABA<sub>A</sub> receptors.
Cross-talk between GABA<sub>A</sub> and P2X Receptors

**Fig. 3.** Current occlusion is independent from membrane potential or ion permeation. A, superimposed currents induced by application of ATP (100 μM), GABA (100 μM), or a mixture of both agonists recorded at different potentials (indicated on the left of each trace) in oocytes co-expressing P2X<sub>2</sub> and αβ3 GABA<sub>A</sub> receptors (n = 5). Non-additive ATP and GABA responses were observed between −60 to +30 mV. I<sub>ATP+GABA</sub> recorded at all tested potentials (black filled circles) and in calcium-free extracellular solution (black filled circles) was significantly smaller (p < 0.0005) than the predicted current (open circles). B and C, reciprocal current occlusion was also observed in oocytes co-expressing cationic α2β3SSGE GABA<sub>A</sub> receptors and P2X<sub>2</sub> channels. **D**, current (1.25 μA) from the third transmembrane domain of the α1 subunit is responsible for the cross-inhibition. The sequence downstream from the third transmembrane domain of the α1 subunit was swapped with the homologous domain of either α2, β3, or γ2 subunits of GABA<sub>A</sub> receptors (Fig. 5A). In Xenopus oocytes expressing either α1-α2, α1-β3, or α1-γ2 chimeric subunits, the application of GABA (10 μM) induced non-desensitizing inward currents similar to wild-type α1 channels, showing that the chimeric subunits formed functional homomeric GABA receptors. In addition, ATP (100 μM) did not gate or modulate GABA-evoked responses (Fig. 5A).

In oocytes co-expressing P2X<sub>2</sub> and α1-α2 channels, simultaneous application of GABA (10 μM) and ATP (100 μM) induced currents (−1.61 ± 0.34 μA, black squares) that were not significantly different (p > 0.5, n = 24) from the sum of responses to separate applications of ATP (−1.25 ± 0.34 μA) and GABA (−0.65 ± 0.13 μA). I<sub>ATP+GABA</sub> represented 95.47 ± 3.40% of the predicted current (Fig. 5A). When GABA was applied during ATP application or ATP during GABA responses, I<sub>ATP+GABA</sub> or I<sub>GABA+ATP</sub> represented 85.34 ± 1.8% and 87.10 ± 2.0% of the predicted current, respectively. These values are significantly different (p < 0.0005) from those previously recorded between wild-type α1 and P2X<sub>2</sub> channels (−75% of the predicted current), suggesting that α1-α2 channels exhibit a less marked functional interaction with P2X<sub>2</sub>. In oocytes co-expressing P2X<sub>2</sub> and α1-β3 chimeric receptors, significant inhibition (p < 0.0005, n = 10) was observed with both simultaneous and sequential application. I<sub>ATP+GABA</sub>, I<sub>ATP then GABA</sub>, or I<sub>GABA then ATP</sub> represented 66.54 ± 4.35%, 67.34 ± 1.80%, or 64.32 ± 2.50% of the predicted current, respectively (Fig. 5, C and E), showing that α1-β3 fully reconstituted the ability of α1 channels to interact functionally with P2X<sub>2</sub> channels. Finally, recordings of oocytes (n = 18) expressing α1-γ2 and P2X<sub>2</sub> channels showed that simultaneous application evoked responses representing 95.8 ± 6.4% of the predicted sum of individual ATP and GABA responses (Fig. 5, D and E). Additive responses were also recorded when GABA was applied during ATP application and vice versa (I<sub>ATP+GABA</sub> then GABA = 92.63 ± 5.57% and I<sub>GABA then ATP</sub> = 98.21 ± 5.5% of the predicted current) demonstrating that α1-γ2 and P2X<sub>2</sub> functioned independently in oocytes. These data suggest that the intracellular domain of β subunits is determinant in the functional cross-inhibition between GABA<sub>A</sub> and P2X<sub>2</sub> receptors.
"Inward currents evoked by ATP (100 μM) were predicted from the sum of individual ATP and GABA responses. Simultaneous application of ATP plus GABA (Actual) elicited responses that were not significantly different (ns, p > 0.5) from the sum of individual ATP and GABA responses (Predicted). C–E, inward currents evoked by ATP (100 μM), GABA (100 μM), and both agonists together (Actual) in oocytes co-expressing wild-type P2X2 and α2β3 GABA receptors. In the presence of a minigene encoding either X2-NT (C), X2-CT (D), or β3-IL2 (E), actual ATP plus GABA responses were not significantly different from the sum of individual ATP and GABA responses (Predicted ns, p > 0.5) in the presence of X2-CT or β3-IL2 minigenes. Non-additive responses were recorded in the presence of X2-N2.

When expressed alone in hippocampal neurons, p1 exhibited a punctate distribution restricted to the close vicinity of cell bodies and were scarcely found distally (Fig. 7A). Conversely, P2X2-YFP was uniformly distributed throughout the neurons with a predominantly surface topology (Fig. 7A). These data were consistent with previous reports (24, 25). A clear increase in the distance from cell bodies (radial topology) of p1-wt in the presence of P2X2-YFP subunits was revealed by cumulated fluorescence intensity profiles (Fig. 7, B and C), indicating that the presence of P2X2-YFP affected the routing of the p1-wt subunits. An increase in radial topology of p1-β3 was also observed in the presence of P2X2-YFP, whereas there was no difference between the cumulated fluorescence profiles obtained with neurons transfected with p1-α2 or p1-γ2, in the presence or absence of P2X2-YFP subunits (Fig. 7, B and C).

The specific rerouting of both wild-type p1 and p1-β3 receptors triggered by P2X2 receptors suggested that the intracellular domain of β subunits was involved in molecular interaction between GABA<sub>A</sub> and P2X<sub>2</sub> receptors. However, significant differences were also noticed when we compared p1-β3 to p1-wt. First, although p1-β3 and P2X2-YFP were co-localized in co-transfected neurons, these areas were typically intracellular (Fig. 6), consistent with vesicular co-targeting. Second, the change in radial topology in the presence of P2X2-YFP was less..."
pronounced when compared with p1-wt (Fig. 7C). This suggests that chimera modified the anchoring properties of the complex within neuron membranes. Data in Figs. 4–7 demonstrate that the intracellular domain of the β subunit is necessary for molecular and functional interaction between GABA<sub>α</sub> receptors and P2X channels.

**DISCUSSION**

In this study, we provide evidence in both *Xenopus* oocytes and transfected hippocampal neurons that molecular and functional interaction between P2X<sub>2</sub> and GABA<sub>α</sub> receptors is a receptor-mediated phenomenon, dependent on GABA<sub>α</sub> subunit composition.

Simultaneous application of ATP and GABA triggered an instantaneous current occlusion in oocytes co-expressing P2X<sub>2</sub> and GABA<sub>α</sub> receptors containing various α and β subunits, with or without γ subunits (see Table I). These results showed that P2X<sub>2</sub> channels interacted functionally with all major types of GABA<sub>α</sub> receptors. However, sequential applications of agonists revealed that the relative contribution of ATP or GABA receptors to current occlusion was dependent on the composition of the GABA receptors. Although αβ or αβγ GABA<sub>α</sub> receptors inhibited P2X<sub>2</sub> channels, P2X<sub>2</sub> channel activation failed to inhibit GABA<sub>α</sub> receptors containing γ2 or γ3 subunits. Cross-inhibition between P2X<sub>2</sub> receptors and either nicotinic, 5-HT<sub>3</sub>, or p1/GABA<sub>α</sub> receptors was reciprocal (4–7, 9, 12), whereas asymmetrical current occlusion was observed between GABA<sub>α</sub> and P2X or GABA and glycine receptors (10, 11). We demonstrated that the occlusion direction was regulated by the subunit composition of the GABA<sub>α</sub> receptors, giving the first evidence of a specific cross-talk mechanism between two unrelated ligand-gated channels. The γ2 subunit has been shown to modify the functional properties of GABA<sub>α</sub> receptors (26, 27).

The open state of GABA<sub>α</sub> receptors promoted by a γ subunit may decrease the ability of P2X receptors to occlude GABA-gated channels. It is noteworthy that the percentage of current occlusion when both agonists were applied together was maintained when the GABA<sub>α</sub> complex contained a γ subunit. These data suggest that the cross-inhibition between these channels is the result of an equilibrium between their open and closed states. Because γ subunits in GABA<sub>α</sub> also play a central role in receptor localization and clustering by interacting with associated proteins, such as GABARAP or GODZ (28, 29), another possibility is that protein interactions promoted by this subunit stabilize the receptor complex or modify the coupling with P2X<sub>2</sub> and, consequently, the bidirectional cross-talk.

Current occlusion cross-talk observed between GABA<sub>α</sub> and P2X in dorsal root ganglia neurons was described as being chloride- and calcium-dependent (10). Our data showed that cross-talk between P2X<sub>2</sub> and GABA<sub>α</sub> could occur in the absence of extracellular calcium and at a range of holding potentials from −60 to +30 mV, i.e. irrespective of the direction of chloride or calcium fluxes. Similar calcium and voltage independence was demonstrated for the cross-talk between P2X<sub>2</sub> and either nicotinic, 5-HT<sub>3</sub>, or GABA<sub>α</sub> receptors (4, 7, 9, 12). Moreover, the use of cation-selective GABA<sub>α</sub> receptor-channels, generated by mutation of β3 subunits (20), did not prevent the functional cross-inhibition with P2X<sub>2</sub> receptors, clearly demonstrating that chloride is not involved in this coupling. However, cross-talk between other P2X subtypes and GABA<sub>α</sub> receptors has yet to be investigated.

Modulation of the state of receptor phosphorylation is responsible for the asymmetrical cross-talk between GABA<sub>α</sub> and glycine observed in spinal neurons (11). Although GABA<sub>α</sub>- and P2X-receptor functions are modulated by phosphorylation mechanisms (21, 30), the unchanged current kinetics and im-

**FIG. 6.** Differential co-localization of chimeric p1/GABA<sub>α</sub> receptors and P2X<sub>2</sub> channels in transfected hippocampal neurons. Hippocampal neurons were co-transfected with wild-type p1 (p1-wt) or each chimera plus P2X<sub>2</sub>-YFP subunits. p1-wt and p1-β3 co-localized with P2X<sub>2</sub>-YFP, whereas p1-α2 and p1-γ2 did not. Anti-p1/AlexaFluor<sup>647</sup> and YFP fluorescence profiles were measured on polylines drawn along the neurites. Note the overlap for p1-wt and p1-β3 but not p1-α2 or p1-γ2 with P2X<sub>2</sub> (bottom panels). Co-localization of p1-wt and P2X<sub>2</sub>-YFP occurred partially on surface clusters, whereas co-localization of p1-β3 and P2X<sub>2</sub>-YFP was essentially intracellular. Representative images are shown. Scale bars: 10 μm.
mediate recovery of both currents after co-activation are incompatible with such mechanisms.

The functional independence between the C-terminal truncated form of P2X₃ receptors and α₂β₃ GABAA receptors, as well as the suppression of cross-talk between wild-type P2X₃ and α₂β₃ GABAA receptors in competition experiments with either the C-terminal domain (but not with the N-terminal) of P2X₃ or the intracellular loop (IL2) of β₃ GABAA subunits demonstrated the involvement of intracellular domains in the functional interaction between these channels. Because α1/1 GABAC receptors are homomeric complexes interacting functionally and physically with P2X₃ channels (12), we co-expressed P2X₃ and chimeric α1-GABAA channels with α₂, β₃, or γ₂ sequences downstream from TM3. This demonstrated that the cytoplasmic domain of the β₃ subunit was indispensable for functional cross-talk, whereas that of α₂ and γ₂ GABAA was unnecessary. Similarly, we observed a co-clustering of α1-β₃ chimeras (but not α1-α₂ or α1-γ₂ chimeras) with P2X₃ in co-transfected hippocampal neurons and a modification of the α1-β₃ radial topology in the presence of P2X₃ subunits, indicating a physical association between the receptors. Thus, we propose that the physical and functional interactions between GABAA and P2X receptors specifically involve the large intracellular loop (IL2) of GABAA β subunits and the C-terminal domain of P2X subunits.

These results and previous data suggest that a generic molecular mechanism underlies the functional coupling between ATP-gated channels and members of the nicotinic superfamily. Because the IL2 loop is not conserved among the nicotinic superfamily, the C-terminal domain of P2X subunits are of varying lengths and sequences, and the cytoplasmic domains of ligand-gated channels are involved in interactions with several binding partners, it is likely that specific protein complexes promote associations between these ligand-gated channels. Interacting complexes may propagate conformational changes induced by activation of one type of receptor to neighboring receptors of different types. This type of conformational spread has been proposed as a universal mechanism for signal integration (31). Molecular complexes implicated in the cell surface localization or anchoring of GABAA receptors remain unclear, even though the involvement of proteins such as gephyrin, tubulin, dystrophyn, and GABA receptor-associated protein has been demonstrated (28, 32–34). Recently, it has been suggested that a conserved cytoplasmic motif stabilizes surface

**Fig. 7.** Modification of radial topology of homomeric ρ1 chimeras in the presence of P2X₃-YFP receptors in transfected hippocampal neurons. A, determination of radial topology for P2X₃-YFP and ρ1-wt subunits. Fluorescence profiles for YFP and anti-ρ1/AlexaFluor648 were measured along polylines drawn on the longest neurites. Note that P2X₃-YFP subunits were distributed distally to the cell body and found along the entire length of the neurites, whereas ρ1-wt subunits were usually found proximal to the cell body. B, determination of radial topology for ρ1 chimeras in the absence (top row) or presence (bottom row) of P2X₃-YFP subunits. The presence of P2X₃-YFP subunits changed the radial topology of ρ1-wt and ρ1-β3, but not ρ1-α₂ and ρ1-γ₂. C, cumulated fluorescence profiles measured as above, for 50 neurons in each group. From left to right: ρ1-wt, ρ1-α₂, ρ1-β3, ρ1-γ₂. Gray curves: without P2X₃-YFP, black curves: with P2X₃-YFP. The average radial trafficking was increased by the presence of P2X₃-YFP for ρ1-wt and ρ1-β3 but not for ρ1-α₂ and ρ1-γ₂.
P2X receptors (35), but protein complexes associated with P2X receptors require further investigation.

GABA and ATP are co-released in the spinal cord and brain, and their associated receptors are co-localized in the postsynaptic density (13, 14), thus providing the physiological conditions for cross-talk between GABA_\text{A} and P2X receptors. Furthermore, postsynaptic ATP/GABA transmission examined at a potential between the reversal potentials of both receptors revealed a lack of mixed excitatory and inhibitory currents in lateral hypothalamus synapses (14). Non-additive ATP and GABA currents observed in these neurons is consistent with negative cross-talk between GABA_\text{A} and P2X receptors described in this work.

Synaptic GABA_\text{A} receptors are composed of αβγ subunits, whereas neuronal ATP-gated channels are thought to be homomeric or heteromeric associations of P2X_2, P2X_3, and P2X_6 subunits (18). The finding that a γ subunit within GABA_\text{A} receptors prevents their inhibition by the activation of P2X receptors indicates that cross-talk may have a stronger effect on the ATP component of mixed ATP/GABA synapses in the dorsal horn of the spinal cord, as well as in the lateral hypothalamus. Reciprocal cross-talk may also regulate the functioning of extrasynaptic P2X_2 and GABA_\text{A} receptors thought to be composed of αβ or αββ subunits (36, 37), which may be co-activated by ATP and GABA released from neighboring astrocytes (38–40). Both GABA_\text{A} and P2X receptors have also presynaptic roles, as shown in afferent terminals of dorsal root ganglia neurons (41).

ATP facilitates glutamate release (13, 42), whereas GABA produces a presynaptic inhibition (43). Cross-inhibition between ATP- and GABA-gated channels may modify the balance between excitation and inhibition and, consequently, contribute to sensory information processing. Taken together with other published data, our findings showed that cross-talk between separate ligand-gated channels is a widespread mechanism, regulated by their molecular complexity, that profoundly influences receptor functions.

Acknowledgments—We thank Dr. Philip K. Ahring (NeuroSearch A/S, Denmark) for providing mutated GABA cDNA clones and Dr. Philippe Séguela (Montreal Neurological Institute, McGill University, Montreal, Canada) for his helpful comments during preparation of the manuscript.

REFERENCES
1. Salter, M. W. (2003) Trends Neurosci. 26, 235–237
2. Liu, F., Wan, Q., Pristupa, Z. B., Yu, X. M., Wang, Y. T., and Niznik, H. B. (2000) Nature 403, 274–280
3. Lee, F. S. (2003) Exp. Neurol. 183, 269–272
4. Barajas-Lopez, C., Espinosa-Luna, R., and Zhu, Y. (1998) J. Physiol. 513, 671–683
5. Searl, T. J., Redman, R. S., and Silinsky, E. M. (1998) J. Physiol. 510, 783–791
6. Zhu, X., and Galligan, J. J. (1998) J. Physiol. 513, 685–697
7. Khalik, B. S., Zhou, X., Sydes, J., Galligan, J. J., and Lester, H. A. (2000) Nature 406, 405–410
8. Barajas-Lopez, C., Montano, L. M., and Espinosa-Luna, R. (2002) Am. J. Physiol. 283, G1238–G1248
9. Boue-Grabot, E., Barajas-Lopez, C., Chakfe, Y., Blais, D., Belanger, D., Emerit, M. B., and Seguela, P. (2003) J. Neurosci. 23, 1246–1253
10. Sokolova, E., Nistri, A., and Ginatellin, R. (2001) J. Neurosci. 21, 4985–4988
11. Li, Y., Wu, L. J., Legrandre, P., and Xu, T. L. (2003) J. Biol. Chem. 278, 38637–38645
12. Boue-Grabot, E., Emerit, M. B., Touline, E., Seguela, P., and Garret, M. (2004) J. Biol. Chem. 279, 6967–6975
13. Jo, Y. H., and Schlchter, R. (1999) Nat. Neurosci. 2, 241–245
14. Jo, Y. H., and Role, L. W. (2002) J. Neurosci. 22, 4784–4804
15. Barnard, E. A., Skolnick, P., Olsen, R. W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Batesen, A. N., and Langer, S. Z. (1998) Pharmacol. Rev. 50, 291–313
16. Fritschi, J. M., and Brunig, I. (2003) Pharmacol. Ther. 98, 299–323
17. Khakh, B. S., Burnstock, G., Kennedy, C., King, B. F., North, R. A., Seguela, P., Voigt, M., and Humphrey, P. (2001) Pharmacol. Rev. 53, 197–118
18. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
19. Illes, P., and Ribeiro, J. A. (2004) Curr. Top Med. Chem. 4, 831–838
20. Jensen, M. L., Timmermann, D. B., Johansen, T. H., Schousboe, A., Varming, T., and Ahring, P. K. (2000) J. Biol. Chem. 275, 41448–41447
21. Boue-Grabot, E., Archambault, V., and Seguela, P. (2000) J. Biol. Chem. 275, 10190–10195
22. Boue-Grabot, E., Roubaraki, M., Bascles, L., Tramu, G., Bloch, B., and Garret, M. (1998) J. Neurochem. 70, 899–907
23. Brake, A. J., Wagenbach, M. J., and Julius, D. (1994) Nature 371, 519–523
24. Cheng, Q., Burkat, P. M., Kulli, J. C., and Yang, J. (2001) J. Neurophysiol. 86, 2605–2615
25. Bobanovic, L. K., Royle, S. J., and Murrell-Lagard, R. D. (2002) J. Neurosci. 22, 4814–4824
26. Fisher, J. L., and Macdonald, R. L. (1997) J. Physiol. 505, 283–297
27. Lorez, M., Benke, D., Luscher, B., Mohler, H., and Bensen, J. A. (2000) J. Physiol. 527, 11–31
28. Wang, H., Bedford, F. K., Brandon, N. J., Moss, S. J., and Olsen, R. W. (1999) Nature 397, 69–72
29. Keller, C. A., Yuan, X., Panzanelli, P., Martin, M. L., Aldred, M., Sassoe-Pognetto, M., and Luscher, B. (2004) J. Neurosci. 24, 5881–5891
30. Kitter, J. T., and Moss, S. J. (2003) Curr. Opin. Neurobiol. 13, 347–347
31. Bray, D., and Duke, T. (2004) Annu. Rev. Biophys. Biomol. Struct. 33, 53–73
32. Essrich, C., Lorez, M., Benson, J. A., Fritschi, J. M., and Luscher, B. (1998) Nat. Neurosci. 1, 563–571
33. Kneusel, M., Brandstatter, J. H., Laube, B., Stahl, S., Muller, U., and Betz, H. (1999) J. Neurosci. 19, 9289–9297
34. Brunig, I., Suter, A., Kneusel, I., Luscher, B., and Fritschi, J. M. (2002) J. Neurosci. 22, 4805–4813
35. Chaumont, S., Jiang, L. H., Penna, A., North, R. A., and Bassendine, F. (2004) J. Biol. Chem. 279, 29626–29638
36. Nusser, Z., Sieghart, W., and Somogyi, P. (1996) J. Neurosci. 16, 1693–1703
37. Rubino, M. E., and Soto, F. (2001) J. Neurosci. 21, 641–653
38. Guthrie, P. B., Knuppenberger, J., Segal, M., Bennett, M. V., Charles, A. C., and Kater, S. B. (1999) J. Neurosci. 19, 520–528
39. Cotrina, M. L., Lin, J. H., Lopez-Garcia, J. C., Naus, C. C., and Nedergaard, M. (2000) J. Neurosci. 20, 2835–2844
40. Nedergaard, M., Takane, T., and Hansen, A. J. (2002) Nat. Rev Neurosci. 3, 748–755
41. MacDermott, A. R., Role, L. W., and Siegelbaum, S. A. (1999) Annu. Rev. Neurosci. 22, 443–485
42. Bardoni, R., Golstein, P. A., Lee, C. J., Gu, J. G., and MacDermott, A. B. (1997) J. Neurosci. 17, 5287–5304
43. Graham, B., and Redman, S. (1994) J. Neurophysiol. 71, 538–549
Subunit-specific Coupling between \( \gamma \)-Aminobutyric Acid Type A and P2X\(_2\) Receptor Channels

Éric Boué-Grabort, Estelle Toulmé, Michel B. Émerit and Maurice Garret

*J. Biol. Chem.* 2004, 279:52517-52525.  
doi: 10.1074/jbc.M410223200 originally published online September 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410223200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 43 references, 19 of which can be accessed free at http://www.jbc.org/content/279/50/52517.full.html#ref-list-1