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Baculovirus Expression Provides Direct Evidence for Heteromeric Assembly of $P2X_2$ and $P2X_3$ Receptors

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$P2X_2$ and $P2X_3$ are subunits of P2X receptors, cation channels opened by binding extracellular ATP. cDNAs encoding $P2X_2$ and $P2X_3$ receptor subunits, each with one of two C-terminal epitope tags, were cloned into baculovirus. Virally infected insect cells (*Spodoptera frugiperda*) expressed moderate to high levels of the corresponding proteins, as detected by Western blotting, by the specific binding of [^{35}S]ATP and by whole-cell recordings of membrane current evoked by ATP or $\alpha\beta$methylene-ATP. In cells infected at the same time with two viruses encoding $P2X_2$ and $P2X_3$ receptors, the two proteins could be cross-immunoprecipitated with antibodies specific for either of the epitope tags. Whole-cell recordings from these cells showed that ATP and $\alpha\beta$methylene-ATP evoked currents with agonist sensitivity and desensitization quite distinct from those observed when $P2X_2$ or $P2X_3$ receptors were expressed alone. The results offer a method to express large amounts of P2X receptor protein, and they provide direct evidence that $P2X_2$ and $P2X_3$ subunits assemble to form heteromeric channels having distinct properties from those formed as homomers.

Key words: ATP; P2X receptors; baculovirus; cation channels; coimmunoprecipitation; subunit assembly

P2X receptors are membrane ion channels activated by extracellular ATP. Currently, seven subunits are known ($P2X_1$–$P2X_7$); each of these can form channels when expressed from the corresponding cDNA in cells such as *Xenopus* oocytes or human embryonic kidney (HEK) cells, although $P2X_5$ and $P2X_6$ do so only poorly (Collo et al., 1996; North, 1996). It is assumed that, when heterologously expressed singly, these subunits assemble into channels as homo-oligomers. On the other hand, two lines of evidence suggest that, as for ligand-gated channels of the nicotinic and glutamate superfamilies (Barnard, 1996), native P2X receptors in cells also might form by the hetero-oligomerization of different subunits. The first kind of evidence is that the same cells often express more than one subunit mRNA. For example, throughout the nervous system and in some epithelial cells, the mRNAs for the $P2X_4$ and $P2X_6$ subunits have a widely overlapping distribution (Collo et al., 1996). A second finding that may suggest heteromultimeric channels is that the properties of the currents evoked by ATP in native cells do not always correspond to those observed when subunits are expressed singly in heterologous systems. One example is that rat $P2X_4$ and $P2X_6$ receptors are relatively insensitive to the commonly used P2X receptor antagonists suramin and pyridoxal 5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), although responses to ATP recorded from central neurons that express this combination are blocked readily by these antagonists.

The further example in which the properties of native cells do not correspond well to those of any subunit expressed alone comes from sensory neurons. Many primary afferent neurons, including nodose ganglion cells (Khakh et al., 1995; Lewis et al., 1995) as well as some identified trigeminal ganglion nociceptors (Cook et al., 1997), respond both to ATP and its analog $\alpha\beta$methylene-ATP ($\alpha\beta$meATP) with an inward current that desensitizes only minimally during a few seconds (see Surprenant et al., 1995). In contrast, in cells expressing cloned P2X receptors, $\alpha\beta$meATP either elicits strongly desensitizing currents ($P2X_1$ or $P2X_3$) or has no effect ($P2X_2$, $P2X_4$, $P2X_5$, and $P2X_6$). The nondesensitizing response to $\alpha\beta$meATP observed in the sensory neurons can be reproduced in HEK 293 cells by coexpression of $P2X_2$ and $P2X_3$ receptor cDNAs; because this phenotype could not be accounted for readily by any simple mixing of channels with $P2X_2$ and $P2X_3$ properties, it was concluded that heteromeric channels must be formed (Lewis et al., 1995).

The main aim of the present experiments was to determine directly whether $P2X_2$ and $P2X_3$ receptor subunits could form a stable heteromeric complex. This was done by expressing the two cDNAs with distinct C-terminal epitope tags that could be used for immunoprecipitation and detection. Baculovirus was used as the vector, and membrane expression of the channels after infection of insect cells was shown by radioligand binding and whole-cell patch-clamp recording.

MATERIALS AND METHODS

Baculovirus construction and amplification. $P2X_2$ and $P2X_3$ receptor coding regions were each fused at the C terminal via linker DPGLN to either of two epitope tags [EE tag: EYMPME, Grussenmeyer et al. (1985); 179 tag: CLEPYTACD, Whitehorn et al. (1995)]. The resulting four cDNAs were cloned into pFastBac1 and transformed into DH10Bac-competent cells (Life Technologies, Grand Island, NY). Recombinant baculovirus subsequently was generated by homologous recombination via transfection of 1×10^6 insect cells with 5 μg of each mini-prep recombinant bacmid DNA in the presence of 10 μg of Cellfectin (Life Technologies) in 35 mm tissue culture plates (Nunc, Roskilde, Denmark). Virus was harvested after 72 hr incubation at 27°C and amplified at a multiplicity of infection of 0.1 pfu/cell in suspension culture until virus titers approximating 10^8 pfu/ml were obtained. Large-scale virus stocks were harvested after 1 week by centrifugation at $1000 \times g$ for 45 min, and supernatants were stored at 4°C. Virus titers were estimated by plaque assay (Knudson and Tinsley, 1974) and confirmed by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) endpoint

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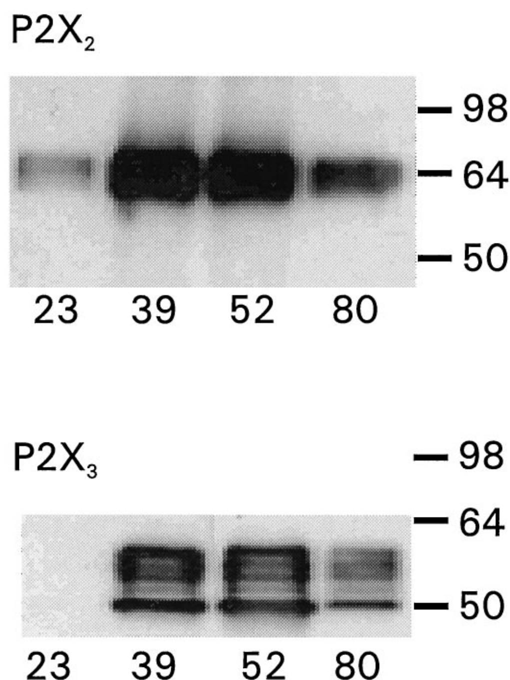

Figure 1. Time course of P2X receptor expression by sf9 cells infected with baculovirus. Immunoblots detected epitope-tagged $P2X_2$ (anti-179; *top*) and $P2X_3$ (anti-EE; *bottom*) receptors at four times (*23*, *39*, *52*, and *80* hr) after infection.

assay. For this assay, serial dilutions of virus (10^{-5} to 10^{-10}) were made directly into insect cells suspensions (8×10^5 cells/ml). Multiple repeats of 100 μl aliquots were incubated at 27°C for 72 hr. MTT (20 μl of 7.5 mg/ml) was added to each well, and the cleavage of tetrazolium salt to formazan by noninfected proliferating cells allowed spectrophotometric detection of the viral endpoint. Virus titer and associated error were calculated from the endpoint, as described by Nielsen et al. (1992).

Cell culture and infection. Spodoptera frugiperda (sf9) insect cells (CRL-1711, American Type Culture Collection, Rockville, MD) were maintained in SF900II serum-free medium (Life Technologies) as 100 ml suspension cultures in 250 ml Erlenmeyer flasks (Schott, Mainz, Germany) and shaken at 150 rpm on an orbital shaker at 27°C. Cultures were grown until 2–3 $\times 10^6$ cell/ml and subcultured at 3×10^5 cell/ml. Cell density was determined with a hemocytometer; viability was assessed by exclusion of 0.2% trypan blue (Sigma, St. Louis, MO). Reported cell viability estimations represent the mean of triplicate estimations ± 95% confidence interval calculated from variances estimated via the method by Nielsen et al. (1991).

For infections, cultures were pooled at 1.5×10^6 cell/ml, centrifuged at 1000 × *g* for 5 min, and resuspended in fresh medium. Resuspended cultures were inoculated at a multiplicity of 5 pfu/cell with baculovirus encoding PX_2179, $P2X_3EE$, or $P2X_2179$ and $P2X_3EE$. A further uninfected culture was used as a control. Cultures were shaken at 200 rpm at 27°C for 80 hr, and aliquots were removed at 0, 23, 39, 52, and 80 hr; these were were stored at −80°C.

Immunoprecipitation and Western blotting. Cells (2×10^7) were lysed on ice in 2 ml of Tris-buffered saline [TBS; (in mM): 20 Tris, 150 NaCl, 1 $CaCl_2$, and 1 $MgCl_2$) with 1% Triton X-100 and homogenized (Polytron; twice for 10 sec). Lysate was centrifuged at 12,000 × *g* for 10 min at 4°C, and the supernatant was transferred to 200 μl of a 1:1 mixture of washed protein A-agarose/protein G-agarose (Pharmacia, Uppsala, Sweden) for preadsorption of background proteins. Samples were rotated for 1 hr at 4°C, and beads were pelleted at 12,000 × *g* for 3 min. Supernatant (800 μl) was rotated at 1 hr at 4°C with either anti-EE or anti-179 antibodies (25 μg/ml final concentration) and rotated overnight after the addition of 100 μl of washed protein A-agarose/protein G. Beads were washed three times in lysis buffer by repeated centrifugation (12,000 × *g*

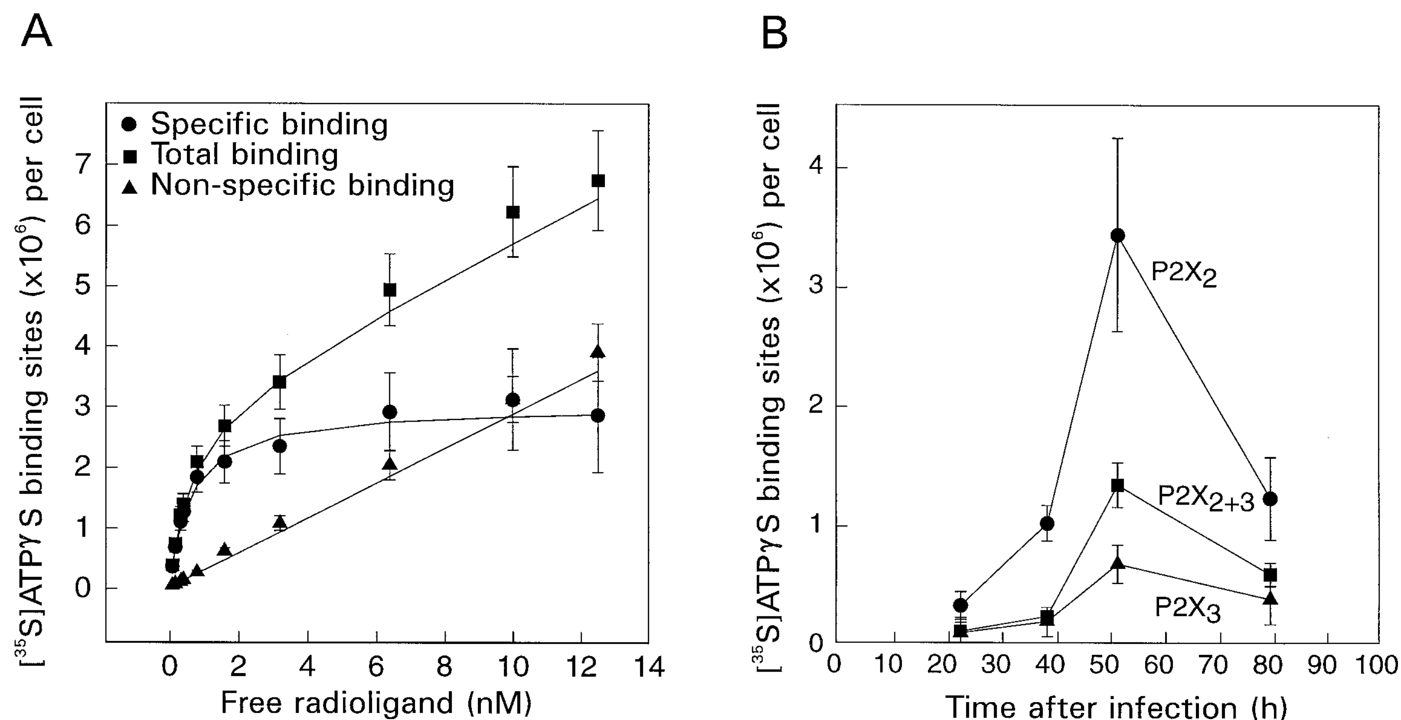

Figure 2. Binding of [^{35}S]ATPγS to sf9 cells infected at 48 hr (*A*) or at different times (*B*) after infection with baculovirus encoding the $P2X_2$ receptor. *A*, The points for specific binding are fit to B_{max} $[L]^n/([L]^n + K_D{}^n)$, where $[L]$ is the free [^{35}S]ATPγS concentration; they provide estimates for B_{max} of 2.8×10^6 binding sites per cell and K_D of 0.63 nM. The points are the means of triplicates from two experiments, and error bars indicate 95% confidence limits from Student's *t* distribution. *B*, The number of binding sites per cell was greater for $P2X_2$ than for $P2X_3$ or $P2X_2$ plus $P2X_3$. In each case this was maximal at 52 hr.

for 3 min) and rotation (4°C for 10 min). Each pellet was resuspended in 50 μl of sample buffer and boiled for 5 min; after 1 min of centrifugation 10 μl was run on 12% Tris-glycine gels (Novex, San Diego, CA). Samples immunoprecipitated with anti-EE or anti-179 antibody were detected by Western blot/ECL format (Amersham, Braunschweig, Germany), using the anti-179 or anti-EE antibody, respectively, as the primary antibody and anti-mouse IgG as the secondary antibody. In some experiments lysates of cells expressing $P2X_2$ receptors were treated with Endo H (Boehringer Mannheim, Mannheim, Germany) (50 mU/ml, overnight at 4°C).

[^{35}S]ATPγS binding. The ligand binding assay was similar to that described by Michel et al. (1996a,b), modified by the use of multiscreen membrane plates (0.65 μm pore size; Millipore, Bedford, MA) to separate bound from unbound radioligand. Intact insect cells infected with $P2X_2$ or $P2X_3$ receptor baculovirus (5×10^4/well) were incubated for 2–3 hr at 4°C with [^{35}S]ATPγS in 50 mM Tris-HCl, pH 7.1, plus 1 mM EDTA. This buffer, with 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 50 μg/ml N^α-tosyl-Lys chloromethyl ketone, also was used for sample and reagent dilution and plate washing. Unbound radioligand was washed from plates by vacuum filtration (3 × 250 μl/well), and plates were counted in a Wallac scintillator after the addition of 30 μl of scintillation fluid. For time course measurements, a single concentration of [^{35}S]ATPγS was used, which was close to the K_D (0.4 nM), and binding was measured at 23, 39, 52, and 80 hr postinfection. Nonspecific binding was defined by using 15 μM 2-methylthio-ATP; the total binding of [^{35}S]ATPγS observed in the presence of 15 μM 2-methylthio-ATP was the same for infected and noninfected cells.

Electrophysiology. At ~30 or 52 hr after infection, cells were transferred from the culture to coverslips and allowed to attach for 1–2 hr at 27°C. Whole-cell recordings were obtained with glass pipettes (4–6 MΩ) containing (in mM): 140 CsCl, 11 EGTA, and 10 HEPES. The external solution contained (in mM): 147 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 12 glucose, and 10 HEPES. Agonists were applied by U-tube delivery system (Evans et al., 1995). Responses were obtained by applying agonist for 1 sec at intervals of 2 min ($P2X_2$; $P2X_2$ and $P2X_3$) and 4 min ($P2X_3$).

RESULTS

Time course of P2X receptor protein expression

P2X receptor subunit expression was monitored by antibody-specific protein production (Fig. 1). This was detectable but very weak at 23 hr after infection and much stronger at 39 and 52 hr. Both the $P2X_2$ and the $P2X_3$ receptors migrated as several closely spaced bands, with molecular weights of ~64 and 50 kDa, respectively (Fig. 1). We assume that these represent differently glycosylated forms: treatment of the $P2X_2$ receptor-infected cells with Endo H eliminated the higher molecular weight bands (data not shown). For both subtypes, some antibody-positive material disappeared between 52 and 80 hr after infection; bands at lower molecular weight that might result from degradation were not detected in either case. In cultures expressing the $P2X_2$ receptor, the fraction of cells excluding trypan blue, a measure of cell lysis, fell from 97.5 ± 9.5% at time 0, to 93.5 ± 9.7% at 23 hr, 83.5 ± 7.9% at 39 hr, 52.5 ± 5.4% at 52 hr, and 6 ± 0.9% at 80 hr (n = 6). The P2X receptor expression and lysis kinetics for the $P2X_3$ receptor-infected and coinfected cultures were not significantly different from those observed with $P2X_2$ alone.

Binding of [^{35}S]ATPγS

The specific binding of [^{35}S]ATPγS to cells infected with $P2X_2$ receptor constructs was well fit by a simple adsorption isotherm when it was measured 48 hr after infection (Fig. 2). From the direct fit of a hyperbola to the data, the K_D was 0.6 nM, and the B_{max} corresponded to 3×10^6 binding sites per cell. Saturation analysis also was performed for cells expressing $P2X_3$, and both receptors and the K_D estimates were not significantly different ($P2X_2$, 0.63 ± 0.08 nM; $P2X_3$, 0.54 ± 0.06 nM; $P2X_2$ and $P2X_3$, 0.57 ± 0.09 nM; n = 3). These values are similar to those described for $P2X_2$ and $P2X_3$ expressed in other systems (Michel et al., 1996a,b; Miller et al., 1996); in subsequent experiments a single concentration of [^{35}S]ATPγS was used (0.4 nM) with or without 15 μM 2MeSATP. For $P2X_2$ receptors, the number of specific binding sites was $3.4 \pm 0.8 \times 10^6$/cell at 52 hr and $1.2 \pm 0.3 \times 10^6$/cell at 80 hr; the corresponding values for cells infected with $P2X_3$ receptors were $6.6 \pm 1.6 \times 10^5$/cell at 52 hr and $3.7 \pm 2.1 \times 10^5$/cell at 80 hr; for cells infected with $P2X_2$ and $P2X_3$ receptors, they were $1.3 \pm 0.2 \times 10^6$/cell at 52 hr and $5.8 \pm 1.0 \times 10^5$/cell at 80 hr (n = 3 in each case). The decline between 52 and 80 hr correlates well with the results of immunoblotting (Fig. 1).

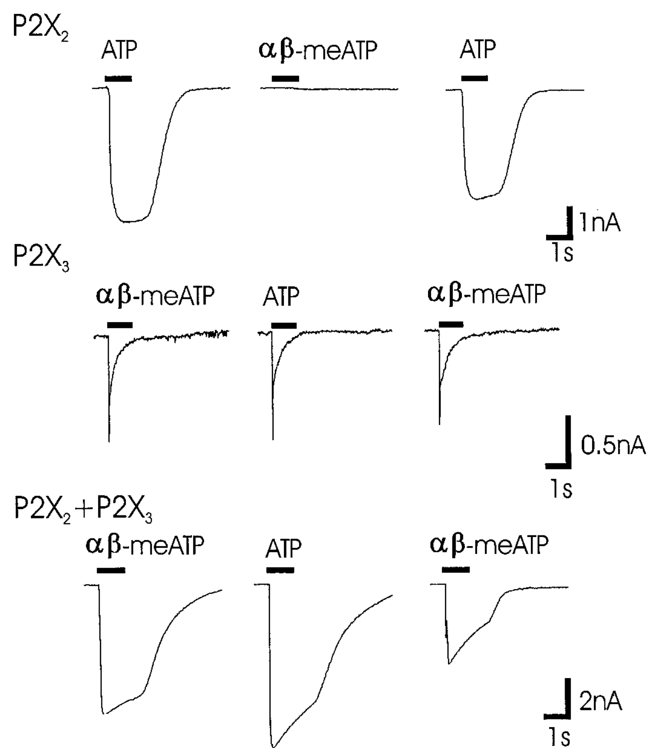

Figure 3. Membrane currents evoked by ATP and αβmeATP recorded in sf9 cells infected with baculovirus encoding $P2X_2$, $P2X_3$, or $P2X_2$ and $P2X_3$ receptors. *Solid lines above traces* indicate period of application of agonist. *Top,* Cells expressing $P2X_2$ receptors responded to ATP (30 μM), but not to αβmeATP (30 μM); the current showed little desensitization. All three traces were taken in order from the same cell; the second application of ATP was 8 min after the first. Recordings were made 30 hr after infection. *Middle,* Cells expressing $P2X_3$ receptors responded both to ATP (30 μM) and to αβmeATP (30 μM); the current desensitized almost fully within the period of application (1 sec). Recordings were made 50 hr after infection and 2 hr after adding apyrase (30 U/ml). *Bottom,* Cells coinfected with baculovirus encoding $P2X_2$ and $P2X_3$ receptors responded both to ATP (30 μM) and to αβmeATP (30 μM), but in this case the current desensitized much more slowly. Recordings were made 50 hr after infection.

ATP-induced currents in infected sf9 cells

ATP (30 μM) applied for 1–2 sec evoked a sustained current in cells infected with $P2X_2$ receptors, whereas αβmeATP (30 μM) had no effect (n = 2; Fig. 3). The current evoked by ATP (30 μM) was 4.5 ± 0.6 nA (n = 6) at 30 hr after infection, 1.3 ± 0.4 nA (n = 4) at 40 hr, and 1.5 ± 0.4 nA (n = 4) at 48 hr. In cells expressing $P2X_3$ receptors, αβmeATP (30 μM) evoked a rapidly desensitizing current in four of five cells tested (1.5 ± 0.3 nA, n = 4; 50 hr after infection; Fig. 3). However, this was seen only after pretreatment of cells with apyrase (30 U/ml for 2 hr); αβmeATP (30 μM) had no effect in five other cells infected with $P2X_3$

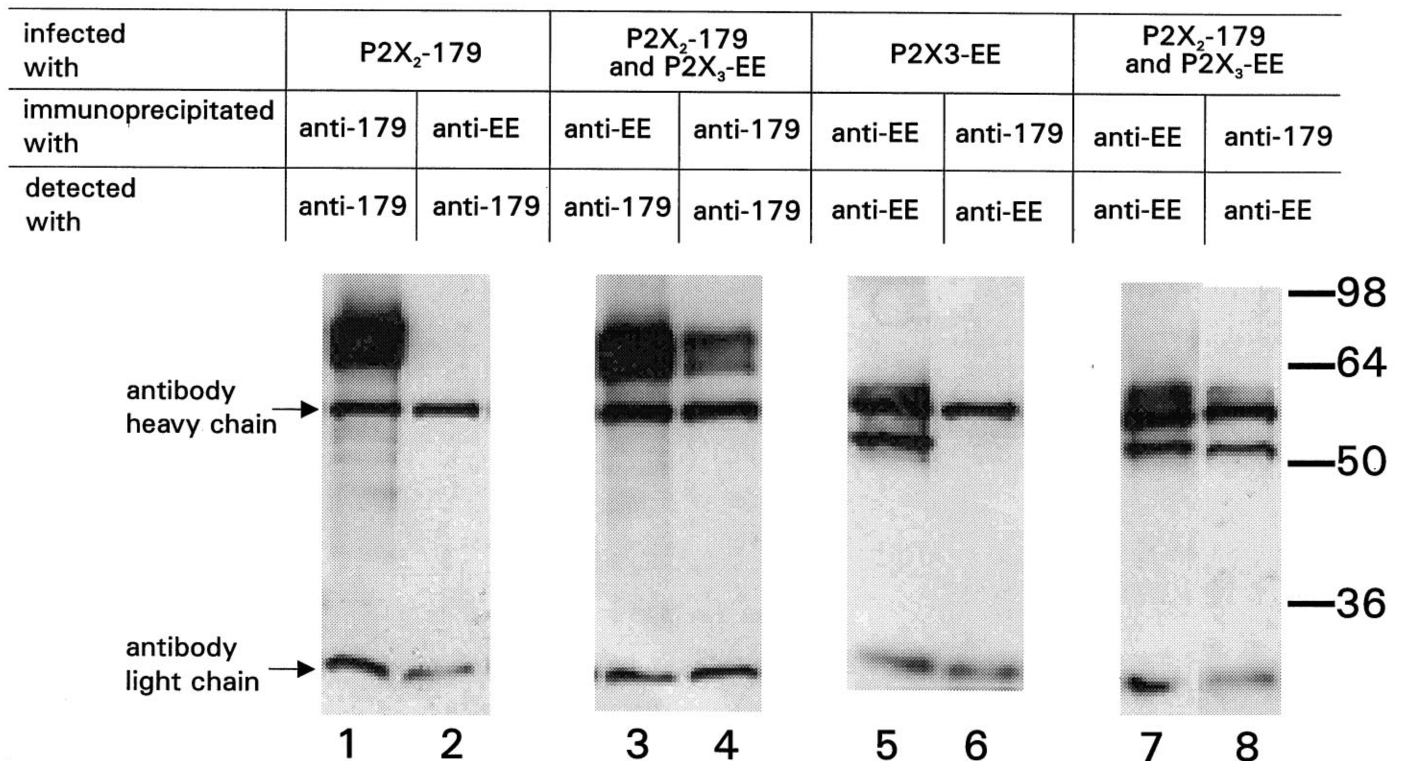

Figure 4. Coimmunoprecipitation of $P2X_2$ and $P2X_3$ subunits. Cells were infected with baculovirus encoding epitope-tagged $P2X_2$ receptors (*$P2X_2$179*), $P2X_3$ receptors (*$P2X_3$EE*), or both receptors. Cells were harvested at 52 hr and solubilized with 1% Triton X-100; the supernatant was immunoprecipitated with either antibody, as indicated. Then either antibody was used to identify the proteins in the immunoprecipitate. The antibody heavy chain lies within the $P2X_3$ bands.

receptor baculovirus, which were not treated with apyrase. This result is similar to that observed for HL60 cells (which express $P2X_1$ receptors), in which chronic desensitization by ATP released from the cells prevents any current being elicited by ATP unless apyrase is applied first (Buell et al., 1996). Cells infected with both the $P2X_2$ and $P2X_3$ baculovirus responded to ATP (30 μM; 4.7 ± 2.7 nA; $n = 2$) and $\alpha\beta$meATP (30 μM; 5.9 ± 2.9 nA; $n = 2$) with a sustained current (Fig. 3; tested 30 hr after infection); this was similar to that previously observed for HEK cells transfected with both receptor cDNAs (Lewis et al., 1995).

Coimmunoprecipitation of $P2X_2$ and $P2X_3$ subunits

Insect cells infected with $P2X_2$179, $P2X_3$EE, or both receptors were harvested after 52 hr and solubilized with Triton X-100; then the supernatant was immunoprecipitated with anti-179 or anti-EE antibodies. $P2X_2$179 receptors could be immunoprecipitated with anti-179, but not with anti-EE (Fig. 4, *lanes 1* and *2*). $P2X_3$EE receptors could be immunoprecipitated with anti-EE, but not with anti-179 (Fig. 4, *lanes 5* and *6*). In cells coinfected with both $P2X_2$179 and $P2X_3$EE, the material immunoprecipitated by anti-179 or anti-EE could be detected with either anti-179 (Fig. 4, *lanes 3* and *4*) or anti-EE (Fig. 4, *lanes 7* and *8*). In each case the immunoprecipitated material corresponded in size to the cognate receptor; $P2X_2$179 ran as a broad smear of ~64 kDa, and $P2X_3$EE ran as a series of bands, of which the smallest was ~50 kDa (Fig. 4).

DISCUSSION

The main result of the present work is that $P2X_2$ and $P2X_3$ receptors express robustly in sf9 cells infected with baculovirus constructs, which encode them, and that they can be coimmunoprecipitated. Receptor expression was determined in three ways. Epitope tags presumably would detect individual subunits whether or not in the plasma membrane. Binding of [^{35}S]ATPγS would detect subunits or, presumably, multimeric forms in the plasma membrane; however, at later times postinfection this would include intracellular membranes from cells undergoing lysis. Electrophysiological recording detects functioning multimeric receptors in the membrane of intact cells.

The time course of appearance of P2X receptor protein was consistent with what is known about the progress of infection of baculovirus in insect cells (O'Reilly et al., 1994). Minimal protein was detectable at 23 hr, but there was strong expression at 39 hr. By the time of maximal expression (52 hr), whether measured by epitope tags or by ligand binding, >40% of the cells were permeable to trypan blue. There were no obvious differences in these kinetics between $P2X_2$ and $P2X_3$ receptors. However, there was three- to fourfold less protein expressed by the $P2X_3$-infected cells than the $P2X_2$-infected cells. Coinfection with $P2X_2$ and $P2X_3$ baculovirus also resulted in fewer [^{35}S]ATPγS binding sites per cell than expression of $P2X_2$ receptors alone. These differences were also qualitatively apparent in the immunoblots (see Fig. 1).

The binding sites for [^{35}S]ATPγS on baculovirus-infected sf9 cells have the same K_D (~0.5 nM) as those reported in more complete studies by Michel et al. (1996a,b) for $P2X_2$ receptors expressed by Semliki forest virus in Chinese hamster ovary cells. We observed maximal expression of ~3.3 × 10^6 binding sites per cell; this corresponds to ~50 pmol/mg protein (assuming that one

cell has 1.1×10^{-7} mg of protein), which is approximately twofold larger than the values reported for Semliki forest virus infection by Michel et al. (1996a). [^{35}S]ATPγS also has been used previously to label $P2X_1$ receptors (Michel et al., 1996b) and $P2X_3$ receptors (Miller et al., 1996) expressed by Semliki forest virus. The relatively high levels of expression and the ability to follow the receptor by ligand binding may be useful in efforts to purify significant amounts of protein.

The electrophysiological studies on infected insect cells were more difficult on cultures 50 hr after infection because cells were poorly adherent and fragile to gigaseal formation. However, satisfactory recordings were readily made 30 hr after infection, and large (up to 9 nA) ATP-induced currents were observed. With respect to desensitization of the current during the application and also with respect to the relative effects of ATP and $\alpha\beta$meATP, these currents closely resembled those observed from other cells infected (Semliki forest virus) or transfected with $P2X_2$ and $P2X_3$ receptors (Evans et al., 1995, 1996). Most strikingly, cells infected with both $P2X_2$ and $P2X_3$ receptor baculovirus showed the phenotype of a slowly desensitizing response to $\alpha\beta$meATP, which is not readily accounted for by the independent expression of the two subunits (Lewis et al., 1995).

These electrophysiological results and the more extensive previous work (Lewis et al., 1995) strongly suggest that $P2X_2$ and $P2X_3$ subunits can form a new phenotype by heteropolymerization. The immunoprecipitation now demonstrates this directly and shows that the association in the membrane between $P2X_2$ and $P2X_3$ receptor subunits is sufficiently strong to withstand solubilization with 1% Triton X-100. The method now can be extended to the other members of the P2X receptor family in an effort to determine which subunits can copolymerize when heterologously expressed. Such a direct approach will be particularly useful because many of the possible combinations are not expected to provide any unique phenotype, given the fact that their properties are similar when expressed as single subunits (Collo et al., 1996). On the other hand, they do not address the as yet unsolved question of the actual receptor stoichiometry. Similar experiments on native cells and tissues, using coimmunoprecipitation with antibodies raised against the naturally occurring receptors (Vulchanova et al., 1996; Cook et al., 1997), also will be important in view of the recent evidence that specific combinations may underlie the responses of neurons in defined functional pathways (Cook et al., 1997).

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