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Cloning and Pharmacological Characterization of a Fourth P2X Receptor Subtype Widely Expressed in Brain and Peripheral Tissues Including Various Endocrine Tissues

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We have isolated cDNA encoding a fourth member (P_{2X-4}) of the ATP receptor P_{2X} receptor family from a rat pancreatic islet cDNA library. Rat P_{2X-4} is a protein of 388 amino acids which shares 50%, 49%, and 47% identity with P_{2X-1}, P_{2X-2}, and P_{2X-3}, respectively, and has two putative transmembrane segments. Rat P_{2X-4} mRNA is widely expressed in brain and peripheral tissues, including various endocrine tissues and it is also expressed in various hormone-secreting cell lines. We have heterologously expressed the cloned P_{2X-4} in *Xenopus laevis* oocytes and have characterized its pharmacological properties. ATP, its analogs and ADP activate cation-selective ion channels. The order of agonist potency is ATP ADP 2-methyl-thioATP(2MeSATP) $\gg$ $\alpha\beta$-methelene-ATP ($\alpha\beta$meATP). ATP-evoked currents are only partially blocked by suramin, reactive blue-2, or H_2DIDS. The present study suggests that P_{2X-4}, with pharmacological properties distinct from those of P_{2X-1}, P_{2X-2}, and P_{2X-3} mediates extracellular ATP-induced biological effects in non-neuronal cells, including endocrine cells, as well as in neuronal cells.

ATP functions as extra- and intra-cellular signals as well as the fundamental carrier of metabolic energy in cells. ATP is co-stored with neurotransmitters in the synaptic vesicles of neuronal cells and with hormones in the secretory vesicles of endocrine cells (1). Extracellular ATP co-released with neurotransmitters or with hormones from these cells induces a variety of biological responses in many cells by binding to P_2 purinoreceptors on the cell surface (2). In pancreatic β-cells, ATP is co-stored with insulin in secretory vesicles (3). We have recently shown by calcium imaging analysis that upon stimulation of pancreatic β-cells, ATP is released from them and that the β-cells then respond to it (4), suggesting that ATP may act on pancreatic β-cells to stimulate insulin secretion through P_2 purinoreceptors.

The P_2 purinoreceptors are classified into ATP-gated ion channels (P_{2X}) (5, 6) and G-protein-coupled receptors (7, 8). Recently, three members of the P_{2X} receptor family have been identified (9–12). They have distinct tissue distributions, unique pharmacological properties, and potentially different functions. However, the molecular basis of the ATP receptors in pancreatic β-cells is unknown.

To search for a novel P_{2X} receptor subtype expressed in pancreatic β-cells, we screened a rat pancreatic islet cDNA library using a partial P_{2X-2} cDNA as a probe under low stringency conditions and isolated cDNA encoding a fourth member of the P_{2X} family.

MATERIALS AND METHODS

General methods. Standard methods were carried out as described (13). Total cellular RNA was prepared from various rat tissues and clonal endocrine cells by the guanidinum isothiocyanate/CsCl procedure. DNA sequencing was done by the dideoxynucleotide chain termination procedure after subcloning appropriate DNA fragments into M13mp18, mp19 (Takara), and pBluescript SK (Stratagene). Both strands were sequenced.

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cDNA cloning of P_{2X-4}. A partial cDNA fragment (nucleotide +164 to +1129) encoding P_{2X-2} (10) was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) (14) using total RNA (20 μg) isolated from the rat pheochromacytoma cell line PC12. Approximately 7 $\times 10^5$ plaques of a rat islet cDNA library (15) were screened using a ^{32}P nick-translated partial P_{2X-2} cDNA fragment as a probe under the low stringency hybridization conditions previously described (16). Positive clones were isolated and sequenced.

RNA blot analysis. For RNA transfer blots, 20 μg of total RNA from the various tissues and cells except for pituitary and thyroid (10 μg each) were denatured with formaldehyde, electrophoresed on 1% agarose gel, and transferred to a nylon membrane. The blot was probed with a ^{32}P nick-translated P_{2X-4} cDNA. Hybridization was carried out under the standard conditions previously described (17). The nylon membranes were washed in 0.1 × SSC and 0.1% SDS at room temperature for 1 h and then for 1 h at 50°C before autoradiography.

Expression and electrophysiological analysis of the cloned P_{2X-4} in Xenopus laevis oocytes. Ten μg of pGEM11Zf(+)(Promega) containing a full length cDNA encoding P_{2X-4} were linearized with Hind III and transcribed *in vitro* with T7 RNA polymerase as described previously (14). *Xenopus* oocytes were injected with 60 ng of transcribed cRNA. After 2–4 days, electrophysiological measurements were performed using a two-electrode voltage clamp (14), and the oocytes were voltage-clamped at a holding potential of −80 mV. Recordings were performed in a continuous-flow chamber (volume, 0.2 ml; perfusion rate, 6 ml/min). Microelectrodes were filled with 3 M KCl. The bathing solution contained (in mM) NaCl 88.0, KCl 1.0, $NaHCO_3$ 2.4, $MgSO_4$ 0.82, Ca $(NO_3)_2$ 0.33, $CaCl_2$ 0.41, and Tris 10.0, at pH 7.6. Agonists and antagonists were applied for the periods indicated in Fig. 3. Recordings were made at 20–22°C. The drugs used were ATP, ADP, 2-methyl-thio ATP (2MeSATP), $\alpha\beta$-methelene-ATP ($\alpha\beta$meATP), suramin, reactive blue-2 (RB-2), and 4,4′-diisothiocyanatodihydrostilbene-2,2′-disulfonic acid (H_2DIDS).

RESULTS

Eight positive λ clones were obtained by screening a rat pancreatic islet cDNA library using P_{2X-2} as a probe. DNA fragments isolated from a λ clone carrying the longest insert, designated λP2W, were subcloned and sequenced. λP2W contains a 1,164-nucleotide open reading frame encoding a 388-amino acid protein with a predicted Mr of 43.5 K (Fig.1). The predicted amino acid sequence of λP2W shows 50%, 49%, and 47% identity with P_{2X-1} cloned from rat vas deferens (9), P_{2X-2} from PC12 cells (10), and P_{2X-3} from rat dorsal root ganglia (11), respectively, suggesting that it represents a new member of the P_{2X} family, designated, therefore, P_{2X-4}. Hydropathic analysis of this protein predicts two transmembrane segments, a feature characteristic of the P_{2X} receptor family. There are ten cysteine residues in the putative extracellular region, the amino acid residue conserved among the four P_{2X} receptor subtypes identified to date. There is no potential cAMP-dependent protein kinase phosphorylation site. There are two potential protein kinase C-dependent phosphorylation sites in the intracellular amino-terminal region (Thr-17 and Ser-25). There are seven potential N-linked glycosylation sites in the extracellular region (Asn-75, Asn-110, Asn-153, Asn-184, Asn-199, Asn-208, and Asn-287).

RNA blot analysis (Fig. 2) reveals that a 2.0-kilobase transcript is widely expressed in rat tissues, including cerebrum, cerebellum, lung, heart, stomach, and colon. Interestingly, P_{2X-4} mRNA is expressed in all of the endocrine tissues examined, as well as in the various hormone-secreting cell lines: the insulin-secreting cell lines RINm5F and HIT-T15, the growth hormone-secreting cell line GH3 and the catecholamine-secreting cell line PC12.

We also examined the pharmacological properties of the cloned P_{2X-4} expressed in *Xenopus laevis* oocytes. ATP evokes inward currents with fast rise time and moderate desensitization (Fig. 3A). ATP analogs and ADP also evoke inward currents with various potencies (Fig. 3B, Fig. 4). The order of agonist potency was ATP ADP 2-MeSATP $\gg \alpha\beta$-meATP (Fig. 4). The half-maximal effective concentration (EC_{50}) for ATP is 63 μM. UTP and 2-methylthio-ADP were ineffective (data not shown). The currents evoked by ATP (100 μM) were only partially inhibited by suramin at 500 μM (70% inhibition of maximum response) (Fig. 3C, Fig. 5A), the concentration required to completely block ATP-evoked currents in P_{2X-1}, P_{2X-2}, and P_{2X-3}. Both RB-2 (200 μM) and H_2DIDS (500 μM) also have a partial inhibitory effect on ATP-evoked currents in P_{2X-4} (Fig. 3D, E, Fig. 5B, C).

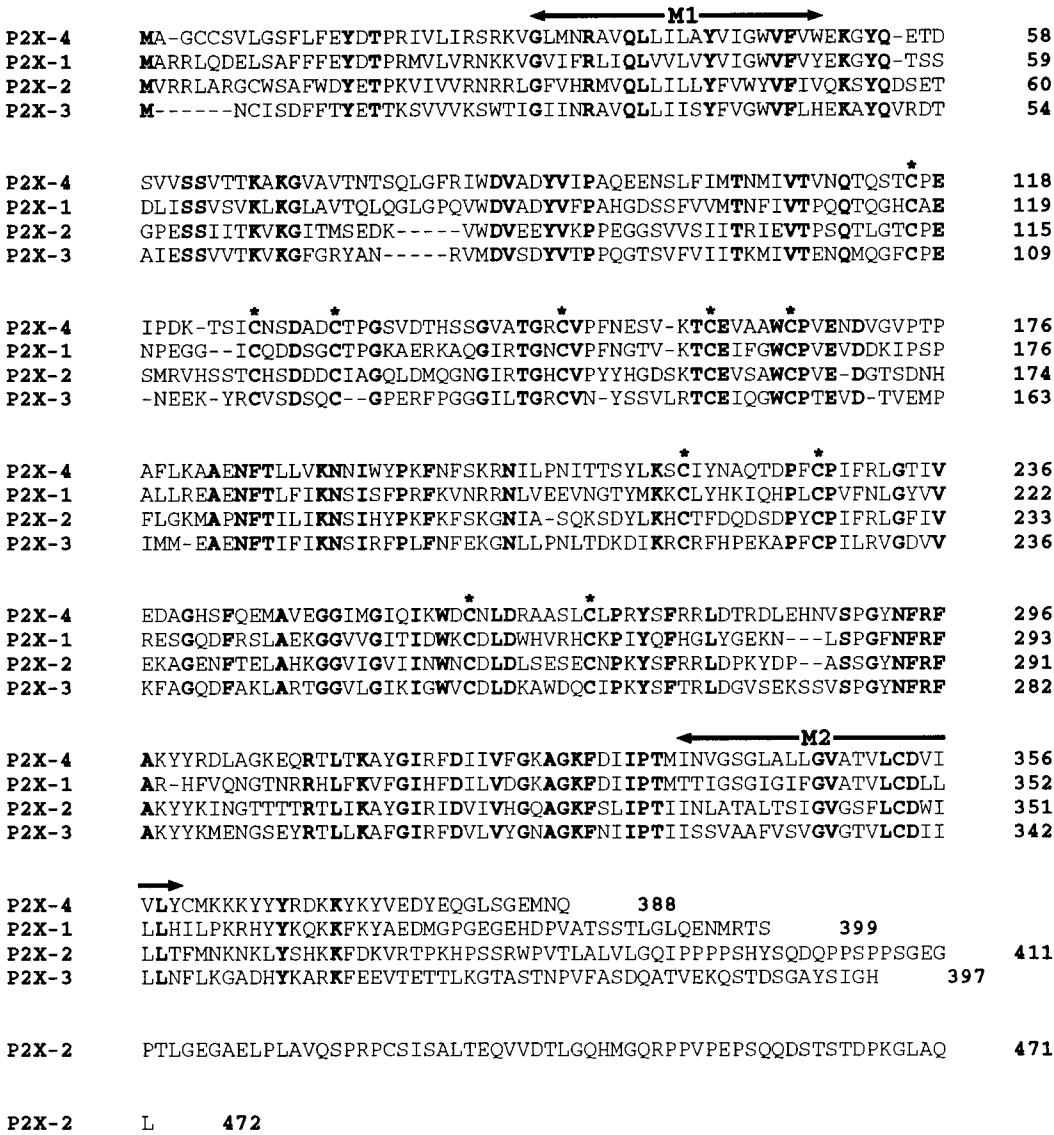

FIG. 1. Comparison of the amino acid sequences of the four members of the P_{2X} family. Amino acids are indicated in single-letter code. The identical amino acid residues among these proteins are shown in boldface type. Gaps introduced to generate this alignment are represented by dashes. The putative transmembrane (M1 and M2) regions are indicated. Ten conserved cysteine residues in the extracellular region are indicated by asterisks.

DISCUSSION

In this paper, we describe the cloning and pharmacological characterization of a fourth member of the P_{2X} family, P_{2X-4}. The four P_{2X} receptor subtypes so far identified vary in size from 388 amino acids (P_{2X-4}) to 472 amino acids (P_{2X-2}); P_{2X-1} and P_{2X-3} are 399 and 397 residues, respectively. While the two putative transmembrane regions are well conserved among the four P_{2X} receptor subtypes, the intracellular amino- and carboxyl-terminal regions and an extracellular region are divergent among the four subtypes, suggesting that these regions are involved in the determination of the pharmacological properties of each subtype.

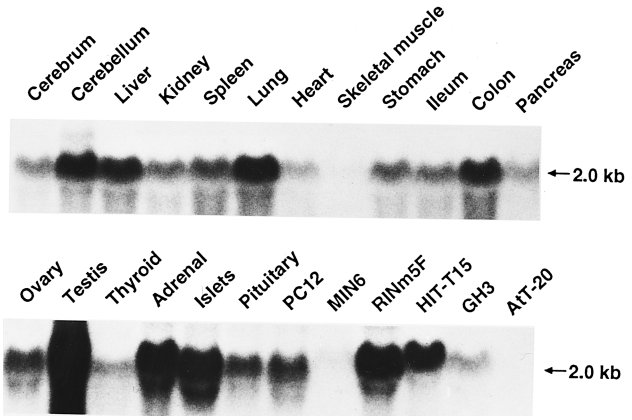

FIG. 2. RNA blot analysis of P_{2X-4} mRNA in various rat tissues and hormone-secreting cell lines. For autoradiography the nylon membrane was exposed to X-ray film with an intensifying screen at −80°C for 3 days. The size of the transcript is indicated.

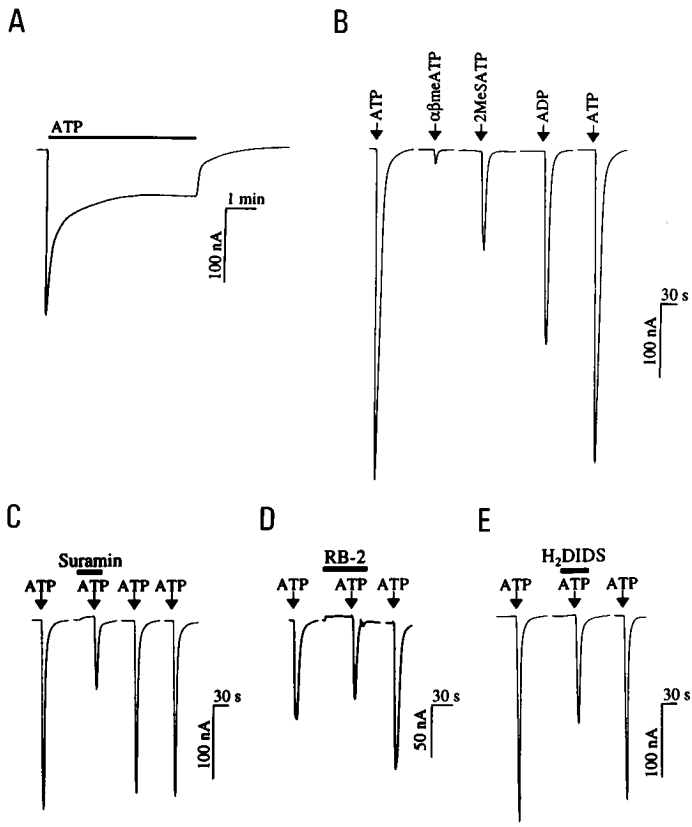

FIG. 3. Pharmacological and electrophysiological properties of the P_{2X-4} expressed in *Xenopus oocytes.* A, A representative trace of currents evoked by ATP (100 μM). B, A representative trace of currents evoked by ATP (100 μM), $\alpha\beta$meATP (100 μM), 2MeSATP (100 μM), ADP(100 μM), and ATP (100 μM). C–E, Effects of various ATP receptor antagonists on ATP-evoked currents. Concentrations of suramin, RB-2, and H_2DIDS used are 500 μM, 200 μM, and 500 μM, respectively. Agonists and antagonists were applied for the period indicated by arrows (3 sec pulse) or horizontal bars.

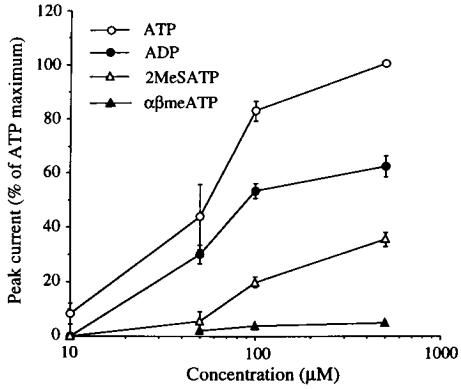

FIG. 4. Dose–response curves for ATP, ADP, 2MeSATP, and $\alpha\beta$meATP. EC_{50} for ATP is 63 μM. Current responses to the agonists are expressed as % of peak currents induced for ATP by 500 μM (ATP maximum). Values (mean ± SEM) were obtained from measurements of 4–6 oocytes (2–5 separate experiments) for each concentration.

RNA blot study has shown that the tissue expression pattern of P_{2X-4} is distinct from those of P_{2X-1}, P_{2X-2}, and P_{2X-3}. P_{2X-4} is widely expressed in various peripheral tissues as well as in brain. In contrast, the sites of P_{2X-1}, P_{2X-2}, and P_{2X-3} expression are restricted to certain tissues: the major sites of expression of P_{2X-1}, P_{2X-2}, and P_{2X-3} are vas deferens and urinary bladder; pituitary, vas deferens, and spinal cord; and dorsal-root ganglion, respectively. Interestingly, P_{2X-4} mRNA is expressed in all of the endocrine tissues examined, as well as in the various hormone-secreting cell-lines, suggesting that P_{2X-4} mediates ATP-induced biological responses in endocrine cells such as hormone release. We have suggested recently that ATP co-released with insulin from pancreatic β-cells stimulates insulin secretion from pancreatic β-cells (4). Li et al. have shown that extracellular ATP stimulates insulin secretion (18). It is possible, therefore, that the P_{2X-4} in pancreatic β-cells participates in stimulus-secretion coupling in insulin release.

Pharmacological characterization of the cloned P_{2X-4} expressed in *Xenopus* laevis oocytes shows that, although ATP-evokes inward currents in a dose-dependent manner, the pharmacological properties of P_{2X-4} are different from those of P_{2X-1}, P_{2X-2}, and P_{2X-3}, in several respects. First, the

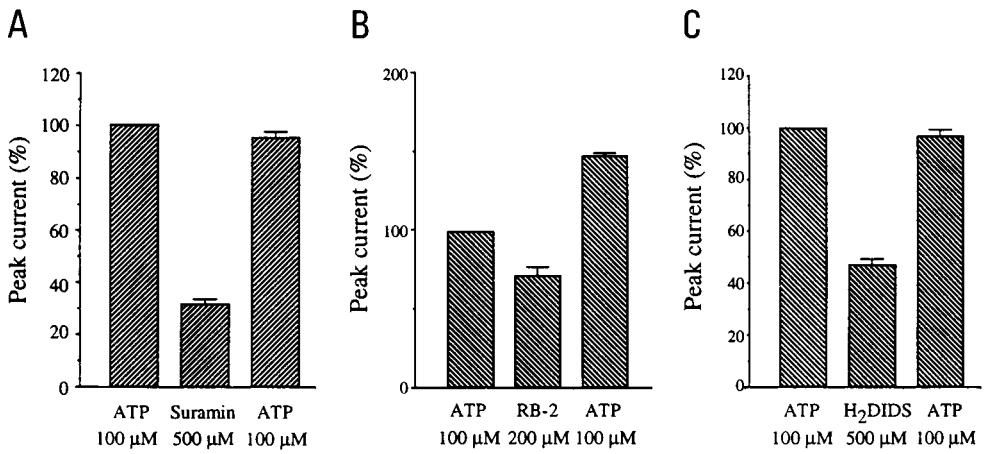

FIG. 5. Summary of the maximum inhibition of ATP-evoked currents by various ATP antagonists. A, suramin. B, RB-2. C, H_2DIDS Inhibition by each antagonist is expressed as % of control (currents evoked by ATP at 100 μM). Right column in each panel represents reversible ATP response after washout of each antagonist. Values (mean ± SEM) were obtained from measurements of 3–6 oocytes (2–4 separate experiments).

rank order of agonist potency for P_{2X-4} is ATP ADP 2MeSATP $\gg$ $\alpha\beta$meATP; for P_{2X-1}, 2MeSATP $\geq$ ATP $\alpha\beta$meATP ADP (9); for P_{2X-2}, ATP $\approx$ 2MeSATP ($\alpha\beta$meATP and ADP are ineffective for P_{2X-2}) (10); and for P_{2X-3}, 2MeSATP $\gg$ ATP $\geq$ $\alpha\beta$meATP $\gg$ ADP (11). While the half maximal response (EC_{50}) to ATP for P_{2X-1} and P_{2X-3} is 0.6 μM and 1.2 μM, respectively (5, 9, 11,), the EC_{50} for P_{2X-4} is 63 μM, similar to that for P_{2X-2} (60 μM) (5, 10). Second, ATP desensitizes P_{2X-4} moderately, but strongly desensitizes P_{2X-1} (9) and does not desensitize P_{2X-2} at all (10). Third, ATP-evoked currents in P_{2X-4} are only partially blocked by the ATP receptor antagonist suramin at 500 μM, the concentration required to completely block the effect of ATP in P_{2X-1}, P_{2X-2}, and P_{2X-3} (9–11). In addition, the other antagonists, RB-2 and H_2DIDS (19), also have a partial inhibitory effect on the ATP-evoked currents in P_{2X-4}. These results show clearly that the pharmacological properties of P_{2X-4} are different from those described of the previously cloned P_{2X} receptors (9–12). During the course of this study, Bo et al. have reported the cloning and pharmacological characterization of P_{2X-4}, but the detailed peripheral tissue distribution of P_{2X-4} has not yet been described (20). Although the amino acid sequence which they describe is identical to ours, the pharmacological characterizations of P_{2X-4} are different. They have shown the order of agonist potency to be ATP 2MeSATP ADP $\approx$ $\alpha\beta$meATP and suramin and RB-2, rather, potentiating ATP-evoked currents. We have found that the order of agonist potency is ATP ADP 2MeSATP $\gg$ $\alpha\beta$meATP and that ATP evoked-currents are potentiated by the second application of ATP after washout of RB-2 (Fig. 3D, Fig. 5B). The reason for the discrepancy between our results and theirs is unclear at present, but is due probably to the experimental conditions used. Since a recent study has suggested that the P_{2X} receptor may form a heterooligomer of different subunits (12), exogenously introduced P_{2X-4} into heterologous cells also may couple to other P_{2X} receptor subtypes or an unidentified subtype natively expressed in the cells. It is possible, therefore, that P_{2X-4} exhibits differing pharmacological properties, depending on the heterologous cells used. Coexpression studies should clarify this issue.

The cloning and pharmacological characterization of P_{2X-4} should provide a better understanding of the mechanisms of ATP-induced signal transduction in non-neuronal cells as well as in neuronal cells.

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