Functional ATP receptors in rat anterior pituitary cells

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Villalobos, Carlos, Sara R. Alonso-Torre, Lucía Núñez, and Javier García-Sancho. Functional ATP receptors in rat anterior pituitary cells. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1963–C1971, 1997.—The effects of ATP and other nucleotides on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of single immunocytochemically typed anterior pituitary (AP) cells have been studied. ATP increased $[Ca^{2+}]_i$ in a large percentage (60–88%) of all five AP cell types: lactotropes, somatotropes, corticotropes, gonadotropes, and thyrotropes. Additivity experiments suggest the presence of at least two different receptors, one accepting both ATP and UTP (U receptor), producing Ca^{2+} release from the intracellular stores, and the other preferring ATP (A receptor), producing Ca^{2+} (and Mn^{2+}) entry. The characteristics of the U and A receptors were consistent with those of $P2Y_2$ and $P2X_2$, respectively, and their distribution in the different AP cell types was not homogeneous. The presence of other ATP receptors such $P2Y_1$ or $P2X_2/P2X_3$ heteropolymers in a small fraction of the cells cannot be excluded. Thus functional ionophoric P2X receptors, which are typical of neural tissue, are also present in the pituitary gland and could contribute to regulation of the gland's function.

P2X receptor; P2Y receptor; UTP; Ca^{2+} channels

ATP PLAYS AN IMPORTANT ROLE as an extracellular messenger for excitable cells (29). Nerve and muscle cells possess ionotropic ATP receptors (P2X) whose activation results in Ca^{2+} and Na^+ entry and depolarization of the plasma membrane. Metabotropic ATP receptors (P2Y) are widespread in different tissues and act through G protein-mediated activation of phospholipase C, inositol trisphosphate production, and Ca^{2+} release from the intracellular Ca^{2+} stores. At least seven members of each family, P2X and P2Y, have been cloned (13). Regarding agonist preferences, the P2X receptors and the $P2Y_1$ subtype prefer ATP over UTP, whereas both nucleotides are similarly able to activate the other P2Y receptors. 2-Methylthio-ATP (MeSATP) is a good substrate for the P2X and $P2Y_1$ receptors but a poor substrate for the other P2Y receptors (13).

Although P2X receptors are generally regarded as typical of excitable tissues, a strikingly high $P2X_2$ expression was found in the anterior pituitary (AP), and the functional role of the receptors at this site is unknown (3, 18). ATP has been shown to increase luteinizing hormone (LH) secretion by pituitary cells in primary culture (8). In addition, ATP is coreleased with pituitary hormones, leaving room for paracrine effects in neighboring cells (7). ATP is able to induce an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat lactotropes (5) and gonadotropes (8, 11). In both cases, the effects were attributed to Ca^{2+} release from the intracellular Ca^{2+} stores by stimulation of $P2Y_2$ receptors. A G protein-coupled $P2Y_2$ receptor that may be responsible for this effect has been cloned recently from a rat pituitary cDNA library (9). More recently, ATP has been reported to also induce Ca^{2+} entry in gonadotropes, probably through a P2X receptor (24). On the basis of the above observations, it has been suggested that P2 receptors may play a role in neuroendocrine regulation (10).

Here we combined Ca^{2+} imaging and immunocytochemical identification to study the effects of ATP on the five different AP cell types: lactotropes, somatotropes, corticotropes, gonadotropes, and thyrotropes. We find that, in addition to lactotropes and gonadotropes, the other kinds of secretory cells are also sensitive to ATP. Furthermore, we present evidence for involvement of both metabotropic and ionotropic receptors in the response to ATP.

MATERIALS AND METHODS

AP cells were obtained from 8- to 10-wk-old male Wistar rats, allowed to attach to 11-mm diameter polylysine-coated glass coverslips, and cultured for 2–3 days as described previously (28). Measurements of $[Ca^{2+}]_i$ were performed in cells loaded with fura 2 (15) by incubation for 60–90 min at room temperature with 5 μM fura 2-acetoxymethyl ester (AM) in standard medium composed of (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid-sodium, at pH 7.4. The coverslips were then mounted under the microscope (Diaphot; Nikon, Tokyo, Japan) in a chamber thermostated at 36°C and epi-illuminated alternately at 340 and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Robertbridge, East Sussex, UK) and analyzed using an Applied Imaging magical image processor (Newcastle, UK) with 32-megabyte video random-access memory. Eight video frames of each wavelength were averaged by hardware that had an overall time resolution of ~5 s for each pair of images at alternate wavelengths. A pixel-by-pixel ratio of consecutive frames obtained at 340- and 380-nm excitation was obtained, and $[Ca^{2+}]_i$ was estimated from this ratio by comparison with fura 2 standards. Test solutions were applied by continuous perfusion at 2–3 ml/min. This allowed >95% exchange of the medium bathing the cells within 5–10 s. Further details of these procedures have been provided previously (25, 28).

For identification of single cells according to the hormone they store, the coverslips were fixed with 4% paraformaldehyde at the end of the $[Ca^{2+}]_i$ measurements. Indirect immunofluorescence measurements using antibodies raised against one of the pituitary hormones were then performed. The field of interest was located by positioning a cross engraved in the

coverslip, as in the $[Ca^{2+}]_i$ experiment, and the fluorescence image was captured with the image processor. The image was digitalized, stored, and later moved and rotated in the computer as required to match exactly the images obtained in the $[Ca^{2+}]_i$ experiment. This procedure has been described in detail elsewhere (20, 28).

In some experiments, multiple sequential primary immunocytochemistry was performed using the abbreviated protocol that follows. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.3% Triton X-100 in the aforementioned solution for 3 min, and washed with PBS for 5 min. Ten percent goat serum in PBS was then added. After 5 min, the antibody against one of the AP hormones labeled with Oregon green 488 was added, and the incubation was continued for 15–30 min. After a washing, a fluorescence image was captured (excitation, 490 nm; emission, >510 nm) with the image processor. This process was repeated for a second (and even a third) fluorescent antibody against another AP hormone, and the resulting image was captured at the same camera gain. Finally, nuclei were stained with Hoechst 33258 (0.5 μg/ml, for 10 min), and another fluorescence image was acquired (excitation, 340 nm; emission, >420 nm). The first image was used as it was obtained for identification of the cells stained with the first antibody. The image corresponding to the cells stained with the second antibody was obtained by subtracting the first image from the second image. The image corresponding to the cells stained with the third antibody was obtained by subtracting the second image from the third image. We prepared fluorescent antibodies from antisera provided by the National Institute of Diabetes and Digestive and Kidney Diseases using Oregon green 488-isothiocyanate and following the labeling directions provided by the manufacturer. The antibody was then purified on a protein A Sepharose column (17) and used as described above (final dilution, 1:20 to 1:50, determined empirically for every antibody). The image from the fluorescence-stained nuclei facilitated definition of cellular boundaries in cells that were physically close.

In several experiments, Mn^{2+} was used as a Ca^{2+} surrogate for Ca^{2+} channels. This allowed direct study of plasma membrane channels without the interference of Ca^{2+} released from the intracellular Ca^{2+} stores (1, 14). Mn^{2+} entry assays were performed either in the whole population of cells or at the single cell level. For measurements in the whole cell population, the coverslips were introduced at a fixed angle (45°) into a quartz cuvette placed into the sample compartment of a fluorescence spectrophotometer that allowed rapid (30–300 Hz) alternation of up to six different excitation wavelengths (Cairn Research, Newnham, Sittingbourne, Kent, UK). Temperature was 30°C. Solutions were changed by simultaneous perfusion into the bottom and aspiration at the top of the quartz cuvette through tubing fitted outside of the optical pathway. Fluorescence emitted above 510 nm was measured and integrated at 1-s periods. Mn^{2+} entry was estimated from the quenching of the fura 2 fluorescence excited at 360 nm, a wavelength that is not sensitive to changes in Ca^{2+} concentration (16). Changes in $[Ca^{2+}]_i$ can be monitored simultaneously from the ratio of the fluorescences excited at 340 and 380 nm. This procedure has been described in detail elsewhere (26).

For Mn^{2+} entry measurements at the single cell level, the experiments were performed in the imaging system as described above for the $[Ca^{2+}]_i$ measurements. Because finding an isosbestic wavelength for Ca^{2+} is difficult in this case, quenching of fura 2 fluorescence by Mn^{2+} was estimated by the combined decrease of fluorescence at the two main excitation wavelengths, 340 and 380 nm. For these purposes, a total fluorescence (F_{total}) value was computed by adding the fluorescence at 340 nm (F_{340}), multiplied by a constant factor K, and the fluorescence at 380 nm (F_{380}). Because F_{340} increases with $[Ca^{2+}]$ and F_{380} decreases with $[Ca^{2+}]$, F_{total} becomes insensitive to $[Ca^{2+}]$ when a proper value is chosen for K (see Ref. 14 for detailed discussion). F_{total} images were obtained by adding, pixel by pixel, the consecutive frames obtained at 340 nm (multiplied by K) and at 380 nm (2).

Oregon green 488-isothiocyanate and fura 2-AM were obtained from Molecular Probes (Eugene, OR). Antibodies against rat prolactin (PRL) (rabbit, AFP425–10–91), β-thyroid-stimulating hormone (rabbit, AFP1274789), growth hormone (GH) (monkey, AFP4115), β-follicle-stimulating hormone (guinea pig, AFP85GP9691BFSHB), and anti-human adrenocorticotropic hormone (ACTH) (rabbit, AFP39013082) were generous gifts from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Child Health and Human Development, and the US Department of Agriculture (Rockville, MD). Fluorescein-labeled anti-rabbit, anti-guinea pig, or anti-monkey immunoglobulins were obtained from Sigma (London, UK). Nucleotides and their derivatives were from RBI Biochemicals (Natick, MA). Thapsigargin was from Alomon (Jerusalem, Israel). Other chemicals were either from Sigma or from Merck (Darmstandt, Germany).

RESULTS

ATP increases $[Ca^{2+}]_i$ in all five AP cell types. Perfusion with 10–100 μM concentrations of ATP elicited an increase in $[Ca^{2+}]_i$ in most of the AP cells present in the microscope field (62 ± 4%; mean ± SE of 39 experiments, n = 3,469 cells). Figure 1*A* illustrates the increase in $[Ca^{2+}]_i$ elicited by 1 μM ATP in a representative single cell. On removal of the nucleotide, $[Ca^{2+}]_i$ returned to the resting level. A second stimulation performed after a 5-min washing period produced a similar $[Ca^{2+}]_i$ increase. Figure 1*B* compares the average responses to different concentrations of ATP. The size of the $[Ca^{2+}]_i$ peak increased with ATP concentration, the half-maximal effect being reached at ~1 μM ATP.

The AP contains five main cell types, each one secreting a different pituitary hormone. To investigate the presence of ATP-sensitive cells within each cell type, immunocytochemical identification of the individual cell type was performed after stimulation with 50 μM ATP (in the same microscope field used for the $[Ca^{2+}]_i$ measurements; see MATERIALS AND METHODS for details). We have studied 1,845 AP cells in 22 independent experiments in which either lactotropes, somatotropes, corticotropes, gonadotropes, or thyrotropes were identified with the use of antibodies raised against their respective AP hormones. Results are summarized in Table 1. The ATP-sensitive cells were not restricted to a given cell type but distributed rather homogeneously within all five cell types, the percentage of sensitive cells ranging between 60 and 88%.

Effects of nucleotides other than ATP. Other nucleotides and nucleotide derivatives were also able to produce a $[Ca^{2+}]_i$ increase in AP cells. Figure 1*C* compares the effects of ATP and UTP averaged from the traces of 47 cells, and Fig. 1*D* compares the effects of ATP, α,β-methylene-ATP (α,β-MeATP), MeSATP, and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), averaged

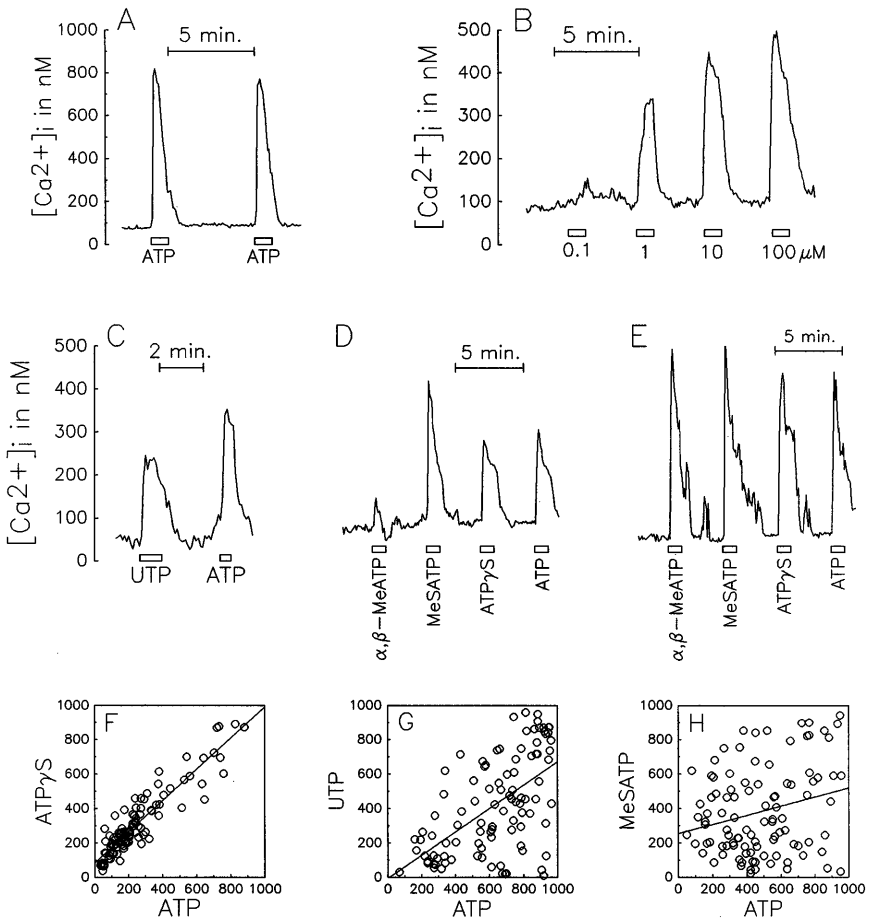

Fig. 1. Effects of ATP and other nucleotides on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of anterior pituitary (AP) cells. *A*: single cell stimulated twice with 1 μM ATP. *B*: average of traces of 120 single cells stimulated with different concentrations (0.1–100 μM) of ATP. *C*: comparison of effects of ATP and UTP, both at 50 μM; average of traces of 47 single cells. *D*: comparison of effects of α,β-methylene ATP (α,β-MeATP), 2-methylthio-ATP (MeSATP), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), and ATP, all at 50 μM; average of traces of 148 single cells. *E*: single cell from same experiment. *F–H*: correlation between effects of ATP and ATPγS (*F*), UTP (*G*), or MeSATP (*H*), all tested at 50 μM. Effects were quantified as increase of $[Ca^{2+}]_i$ (in nM) at peak after application of nucleotide. Lines fitted by least squares procedure. Values for correlation coefficient (r) were 0.92 (F, n = 102 cells), 0.58 (G, n = 100 cells), and 0.39 (H, n = 102 cells). Each experiment is representative of 2–5 similar ones.

from the traces of 148 cells. Other nucleotides tested were ADP and 2-chloro-ATP (ClATP) (results not shown). All the nucleotides and derivatives produced similar effects except α,β-MeATP, which was scarcely active. The 50% effective concentration values determined in experiments similar to those of Fig. 1*B* were 2 μM for UTP and 2.6 μM for MeSATP.

The poor response to α,β-MeATP in the average traces was because of the fact that only a few cells (4–10% in different experiments) responded to this nucleotide, but large responses were found in these sensitive cells (Fig. 1*E*). Further complexities in the action of other nucleotides were also disclosed by the single cell analysis. The correlation between the effects of ATP and ATPγS in each single cell was always very good (Fig. 1*F*), suggesting that both nucleotides are able to activate the same receptors. However, the correlation between the effects of ATP and either UTP (Fig. 1*G*) or MeSATP (Fig. 1*H*) was much worse. A possible explanation for this outcome would be the presence of more than one P2 receptor with different affinities for the different nucleotides and derivatives tested.

In the case of the ATP-UTP correlation (Fig. 1*G*), two dominant subpopulations may be defined: *1*) cells that respond similarly to both ATP and UTP, which are positioned near the diagonal of the plot, and *2*) cells that respond preferentially to ATP, which are positioned closer to the abscissa. This behavior could be rationalized in terms of one receptor (U receptor) that is equally well activated by both UTP and ATP and another receptor (A receptor) that is activated only by ATP. Cells in which U receptors dominate would correspond to the *subpopulation 1* described above, whereas cells possessing preferentially A receptors would make up *subpopulation 2*.

To test this working hypothesis, the additivity between the effects of UTP and ATP was studied in experiments in which the cells were stimulated first with UTP for 2 min and then with UTP + ATP for an

Table 1. *Response to ATP in the five AP cell types defined by immunocytochemical identification of the hormone they store*

Cell Type (Stored Hormone)	Percentage of All Cells	Cells Responding to ATP, %
Lactotrope (PRL)	58 ± 4	74 ± 3
Somatotrope (GH)	40 ± 7	74 ± 13
Corticotrope (ACTH)	18 ± 2	81 ± 5
Gonadotrope (FSH)	8 ± 1	88 ± 13
Thyrotrope (TSH)	2 ± 1	60 ± 25

Values are means ± SE of 3–6 independent experiments (n = 264–492 cells) for each antibody. In each experiment, cells were stained after cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements with 1 of the antibodies against 1 of the anterior pituitary (AP) hormones. PRL, prolactin; GH, growth hormone; FSH, follicle-stimulating hormone; TSH, thyroid-stimulating hormone.

additional 1 min. After a 5-min washing with control solution, the nucleotides were applied again in the reverse order. Results of a representative experiment are shown in Fig. 2. Figure 2*A* shows the average behavior of all the cells present in the microscope field. It is clear that ATP added on top of UTP was still able to produce an increase in $[Ca^{2+}]$, whereas the reverse did not apply. When analyzed at the single cell level, three main subpopulations could be identified: *1*) cells that responded only to ATP (Fig. 2*B*), *2*) cells that responded to both ATP and UTP as the first stimulus but to none of the nucleotides as the second stimulus [i.e., no response to the nucleotide that is added on top of the first nucleotide (Fig. 2*C*)], and *3*) cells that responded to both ATP and UTP as the first stimulus but only to ATP as the second stimulus (Fig. 2*D*). Figure 2*E* shows the relative sizes of each of these subpopulations, averaged in four similar experiments. The *subpopulation 3* (A + U) was always dominant. A subpopulation of cells responding only to UTP could not be identified. This outcome is consistent with the working hypothesis that postulates the existence of the two types of receptors (U and A) given that *subpopulation 1* had only A receptors, *subpopulation 2* had only U receptors, and *subpopulation 3* had both kinds.

The interactions of UTP and MeSATP were also studied in experiments of the same design as those of Fig. 2, and the results are illustrated in Fig. 3. In the average trace (Fig. 3*A*), both MeSATP and UTP were able to increase $[Ca^{2+}]_i$ after stimulation with the other nucleotide. Cells responding either to both nucleotides (Fig. 3*B*, 63% of the responding cells) or selectively to one of them (Fig. 3, *C* and *D*; 9 and 22% of the responding cells, respectively) were identified. In all the cases, the responding cells were also sensitive to ATP. The results could be rationalized within our working hypothesis if MeSATP were able to activate A receptors but not U receptors. The interactions of MeSATP and ATP (Fig. 4) were also consistent with this interpretation. ATP was able to increase $[Ca^{2+}]_i$ after treatment with MeSATP in some cells (Fig. 4, *B* and *C*; 21 and 63% of the responding cells, respectively), and all the cells that responded to ATP after stimulation with MeSATP were also sensitive to UTP. This suggests that the additional response to ATP results from the presence of U receptors. MeSATP had little effect when applied on top of ATP (Fig. 4*A*). Only a few cells responded to MeSATP added on top of ATP (not shown). We refer to the possible significance of this minor subpopulation in the DISCUSSION.

Calcium entry and calcium release from the intracellular stores. To investigate the relative contributions of Ca^{2+} entry and Ca^{2+} release from the intracellular stores and the $[Ca^{2+}]_i$ increase induced by ATP and other nucleotides, we compared the effects obtained in Ca^{2+}-free and Ca^{2+}-containing media. Figure 5*A* shows that ATP and UTP elicited a similar $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium (60 ± 6 and 73 ± 6 nM, respectively; mean ± SE of 10 independent experiments), suggesting that both nucleotides are able to release Ca^{2+} from the intracellular stores. However, the effect in Ca^{2+}-

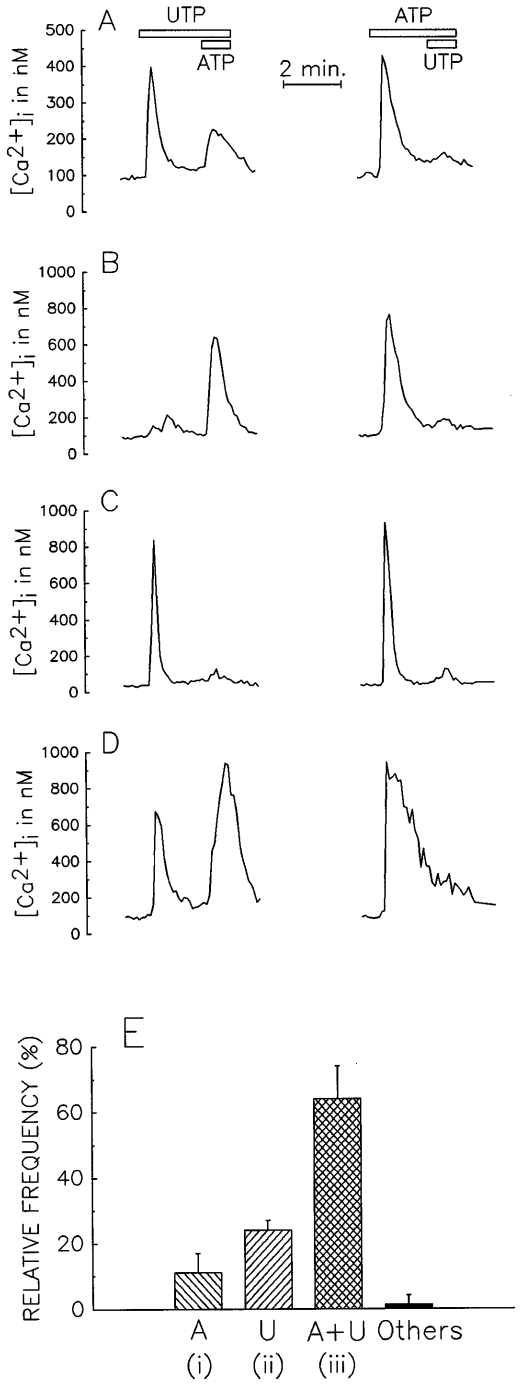

Fig. 2. Additivity between effects of UTP and ATP. Concentrations were 50 μM for both nucleotides. Cells were first stimulated with UTP for 2 min, and then perfusion was switched to a solution containing both UTP and ATP for an additional 1 min; after a 5-min washing with control solution, cells were first stimulated with ATP and then with solution containing both ATP and UTP. *A*: average of traces of 47 single cells. *B–D*: traces corresponding to 3 different single cells. *E*: relative sizes, expressed as a percentage of all cells responding to nucleotides, of the following subpopulations: A, responding only to ATP; U, responding only to nucleotide added first (either ATP or UTP); A + U, responding to UTP and also to ATP added after UTP; others, cells that could not be included within above categories. Results from 4 independent experiments (123 single cells responding to nucleotides). See text for interpretation of results in terms of different types (A or U) of ATP receptors.

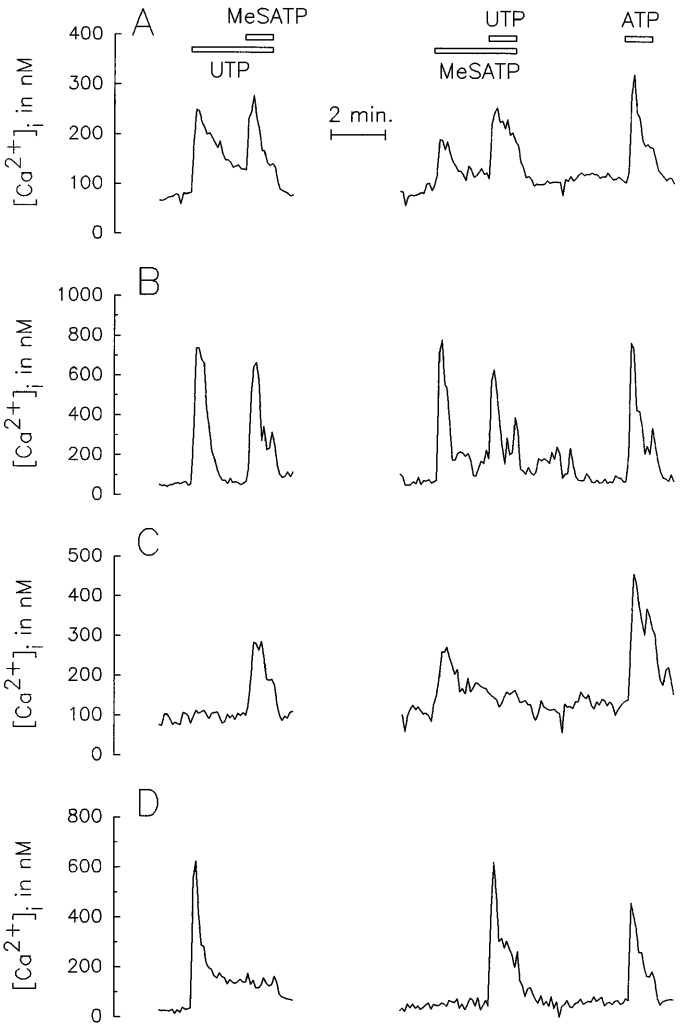

Fig. 3. Additivity between effects of UTP and MeSATP. Concentrations were 50 μM for all nucleotides. Cells were first stimulated with UTP for 2 min, and then perfusion was switched to a solution containing both UTP and MeSATP for an additional 1 min; after a 5-min washing with control solution, cells were first stimulated with MeSATP and then with solution containing both MeSATP and UTP. At end of experiment, cells were stimulated with ATP. *A*: average of traces of 24 single cells. *B–D*: traces corresponding to 3 different single cells.

containing medium was larger for ATP (see Fig. 5*A* for a typical experiment; 306 ± 26 nM compared with 125 ± 13 for UTP, mean ± SE of 10 experiments), indicating that the relative contribution of Ca^{2+} entry is larger for ATP. The $[Ca^{2+}]_i$ increase induced by MeSATP was also very much reduced by removal of external Ca^{2+} (by 75 ± 2%, mean ± SE of 4 experiments, results not shown).

The relative contribution of the intracellular Ca^{2+} stores was also assessed in experiments in which the stores were emptied of Ca^{2+} by treatment with the endomembrane adenosinetriphosphatase inhibitor thapsigargin (23). It has been shown previously that a 5-min treatment with this drug is enough to achieve full emptying of the intracellular Ca^{2+} stores (26). Fig. 5*B* illustrates representative results. Thapsigargin treatment strongly inhibited the action of UTP (by 62%), had a smaller effect on the action of ATP (32% inhibition), and had little effect on the actions of ClATP (24% inhibition) and MeSATP (3% inhibition).

The effects of ATP and other nucleotides on Ca^{2+} entry were assessed directly by using Mn^{2+} as a surrogate of Ca^{2+} for Ca^{2+} channels. It has been shown previously that Mn^{2+} is able to permeate several types of Ca^{2+} channels, for example, L-type voltage-gated channels (26, 27) and *N*-methyl-D-aspartate receptor-operated channels (21, 28). Figure 6, *A–C*, illustrates the effects of UTP and ATP on Mn^{2+} entry in a representative experiment performed with the imaging system. The ratio of the fluorescences excited at 340 and 380 nm (an index of $[Ca^{2+}]_i$; see MATERIALS AND METHODS) was measured simultaneously and is shown as dotted lines. The average trace for all the cells (Fig. 6*A*) shows that both nucleotides were able to produce an acceleration of Mn^{2+} entry, although this effect was much more marked for ATP. The dominant cell subpopulation was the one illustrated in Fig. 6*B*, in which both UTP and ATP produced an increase in $[Ca^{2+}]_i$ but only ATP produced an acceleration of Mn^{2+} entry. A smaller fraction of cells responded only to ATP (Fig. 6*C*), and another fraction, still smaller, responded to UTP with an acceleration of Mn^{2+} entry (not shown). The categories represented in Fig. 6, *B* and *C*, made up 78% of the cell population.

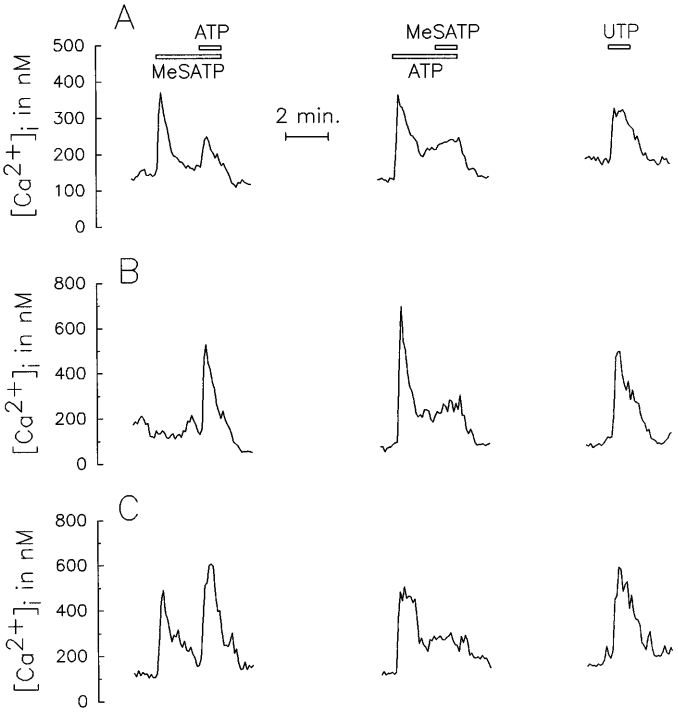

Fig. 4. Additivity between effects of ATP and MeSATP. Concentrations were 50 μM for all nucleotides. Cells were first stimulated with MeSATP for 2 min, and then perfusion was switched to solution containing both MeSATP and ATP for an additional 1 min; after a 5-min washing with control solution, cells were first stimulated with ATP and then with solution containing both ATP and MeSATP. At end of experiment, cells were stimulated with UTP. *A*: average of traces of 55 single cells. *B* and *C*: traces corresponding to 2 different single cells.

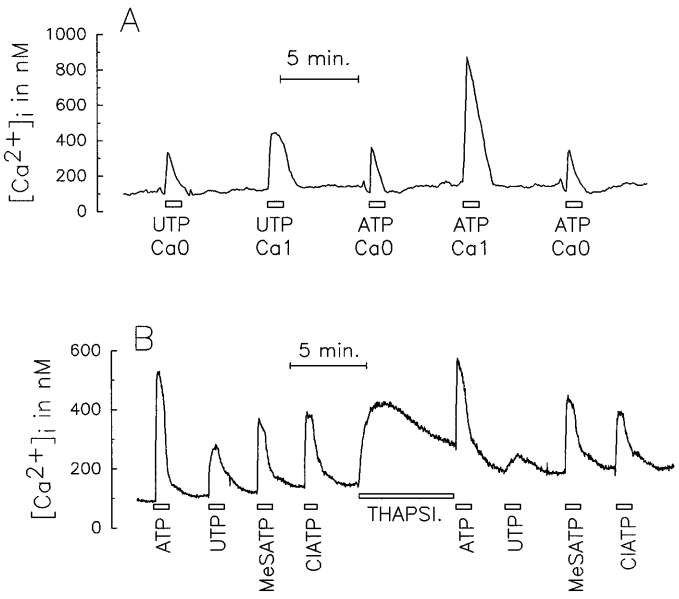

Fig. 5. Effects of Ca^{2+} removal (*A*) and pretreatment with thapsigargin (*B*) on $[Ca^{2+}]_i$ increases induced by ATP and other nucleotides. Concentrations were 50 μM for all nucleotides. *A*: effects of UTP and ATP in Ca^{2+}-free medium (Ca0, containing 500 μM EGTA added 30 s before the nucleotide) and in regular, Ca^{2+}-containing medium (Ca1) are compared. Trace shown is average of 49 single cells. Experiment is representative of 4 similar ones. Six additional experiments were performed in the Cairn spectrophotometer. *B*: cells were first stimulated with several nucleotides, as shown, then treated with 500 nM thapsigargin for 5 min and stimulated again. Experiment performed in the Cairn spectrophotometer, representative of 2 similar ones. ClATP, 2-chloro-ATP.

In many cell types, emptying of the intracellular Ca^{2+} stores activates a plasma membrane pathway for Ca^{2+} entry (capacitative Ca^{2+} entry; see Ref. 22). This mechanism has been shown to operate in GH_3 pituitary cells (26) and in rat lactotropes (4). It could be argued that the acceleration of Mn^{2+} entry induced by ATP may be secondary to the emptying of the intracellular Ca^{2+} stores and the activation of capacitative Ca^{2+} entry (see Ref. 26 for a similar mechanism in GH_3 cells) rather than to activation of receptor-operated channels. To test this point, we studied the effect of ATP in cells whose intracellular Ca^{2+} stores were first emptied of Ca^{2+} by treatment with thapsigargin (Fig. 6*D*). ATP also produced an acceleration of Mn^{2+} entry under these conditions. Figure 6, *E* and *F*, illustrates the effects of other nucleotides also in thapsigargin-treated cells. Both MeSATP and ClATP increased Mn^{2+} entry, whereas UTP had little effect under these conditions. These results suggest that the increase in Ca^{2+}-Mn^{2+} entry observed in some cells after treatment with UTP (Fig. 6*A*) may be a result of the activation of the capacitative pathway on depletion of the Ca^{2+} stores. However, the entry of Ca^{2+}-Mn^{2+} induced by ATP, MeSATP, or ClATP cannot be fully explained by this mechanism.

Distribution of responses to UTP and MeSATP in different AP cell types and in freshly prepared cells. To estimate the relative distribution of U and A receptors among the different cell types, we compared the $[Ca^{2+}]_i$ responses to sequential stimulation with 20 μM UTP and 10 μM MeSATP. Corticotropes, somatotropes, and

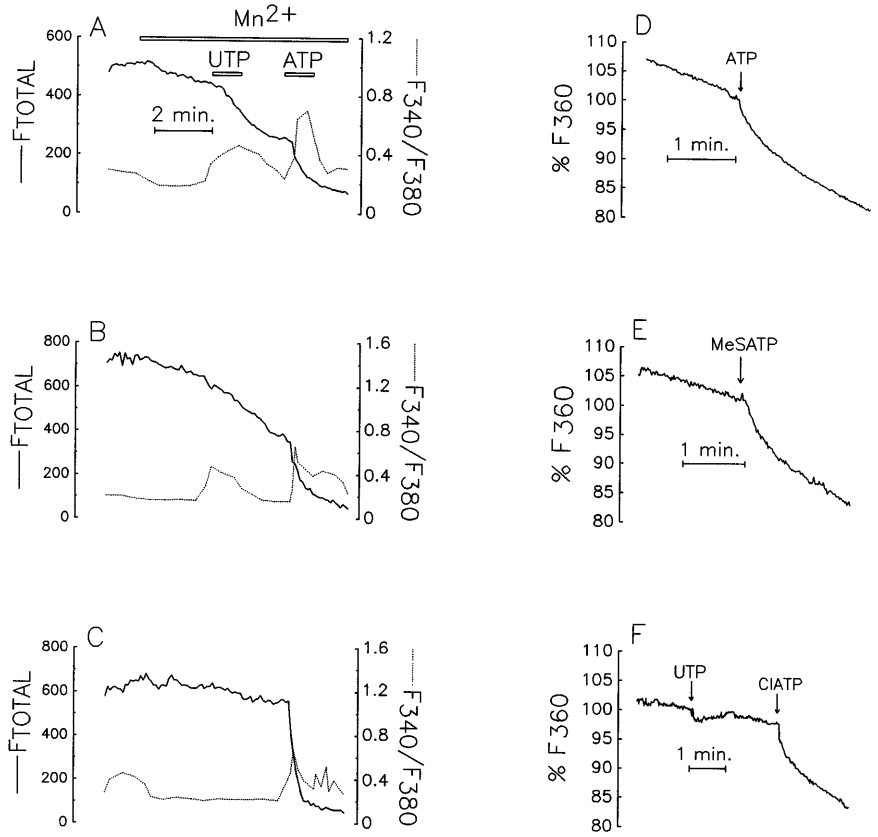

Fig. 6. Effects of ATP and other nucleotides on entry of Mn^{2+}. Concentrations were 50 μM for all nucleotides. *A–C*: experiment performed in imaging system. Ratio of fluorescence at 340 nm to fluorescence at 380 nm (F_{340}/F_{380}, dotted lines) is index of changes in $[Ca^{2+}]_i$, whereas decrease of total fluorescence (F_{total}, solid lines) reflects quenching of fura 2 fluorescence by entering Mn^{2+} (see MATERIALS AND METHODS). Concentration of Mn^{2+} (in Ca^{2+}-free medium) was 0.2 mM. *A*: average of traces from 27 single cells. *B* and *C*: traces from 2 single cells. This experiment is representative of 4 similar ones. Note that ratio values may become inaccurate when F_{total} goes to very low values, as at the end of traces; experiments performed in the Cairn spectrophotometer. Readings of fluorescences excited at 340, 360, and 380 nm were taken simultaneously. Decrease of F_{360} (insensitive to Ca^{2+}) reflects quenching of fura 2 fluorescence by entering Mn^{2+} (see MATERIALS AND METHODS). Cells had been first treated with thapsigargin (500 nM) for 6 min (not shown). Concentration of Mn^{2+} (in Ca^{2+}-free medium) was 0.2 mM. Effects of ATP (*D*), MeSATP (*E*), and UTP and ClATP (*F*) are shown. Experiments are representative of 2–4 similar ones.

lactotropes present in the same microscope field were identified by multiple sequential immunocytochemistry with the use of the fluorescent primary antibodies and the abbreviated protocol described in MATERIALS AND METHODS. Figure 7*A* illustrates this approach. In the left column, the images corresponding to ACTH-, GH-, and PRL-containing cells are shown. The bottom panel shows the nuclei staining in the same field. The right column shows $[Ca^{2+}]_i$ images from the same experiment taken at rest or during stimulation with either UTP or MeSATP and the contours of the four cells present in the field. Combining all this information, we can study the responses to each nucleotide separately in each cell type. Thus, in Fig. 7*A*, *cell 1* was a corticotrope that showed no response to UTP and a very weak response to MeSATP. *Cell 2* was a somatotrope and responded only to MeSATP. *Cell 3* was a lactotrope and responded only to UTP. Finally, *cell 4* did not react with any of the antibodies and responded to both UTP and MeSATP.

Figure 7*B* shows the average responses of all the cells of each kind present in the microscope field. The smallest average responses to both UTP and MeSAP were observed in corticotropes. The largest response to MeSATP was observed in lactotropes. Somatotropes showed $[Ca^{2+}]_i$ increases of intermediate size. All three cell types showed only modest responses to UTP. The group containing the remaining cells, negative to all three antibodies, showed the largest response to UTP, and the response to MeSATP was as large as in lactotropes (Fig. 7*B*). This group of cells must have contained gonadotropes and thyrotropes. A study of the traces of individual cells showed that, as found in nonidentified AP cells (Fig. 2), many of the cells were sensitive to both nucleotides, and a smaller fraction was sensitive to only one of them.

The cell-by-cell study allowed us to estimate the fraction of cells within each cell type that was sensitive to each nucleotide and the typical amplitude of the responses. Results from three independent experiments are summarized in Table 2. The fraction of cells responding to MeSATP was 51–53% in corticotropes and larger (84%) in lactotropes. The fraction of cells responding to UTP was uniformly small in all three cell types (24–29%). The nonidentified cell group was the one responding best to UTP (62%) and also gave a very good response to MeSATP (80%). The differences in the size of the responses ($\Delta[Ca^{2+}]_i$), expressed as the fraction of the average response of all the cells in Table 2, were even larger. This indicates that the typical size of the $[Ca^{2+}]_i$ peak observed in the responding corticotropes and somatropes was smaller than in the other responding cells.

To check that the responses to the nucleotides observed in AP cells were not an artifact induced by culture, we repeated some experiments in freshly isolated cells that were allowed to attach to the polylysine coverslips for 1 h. Stimulation of these cells with nucleotides gave results similar to those obtained with the primary cultures. Thus stimulation with 20 μM

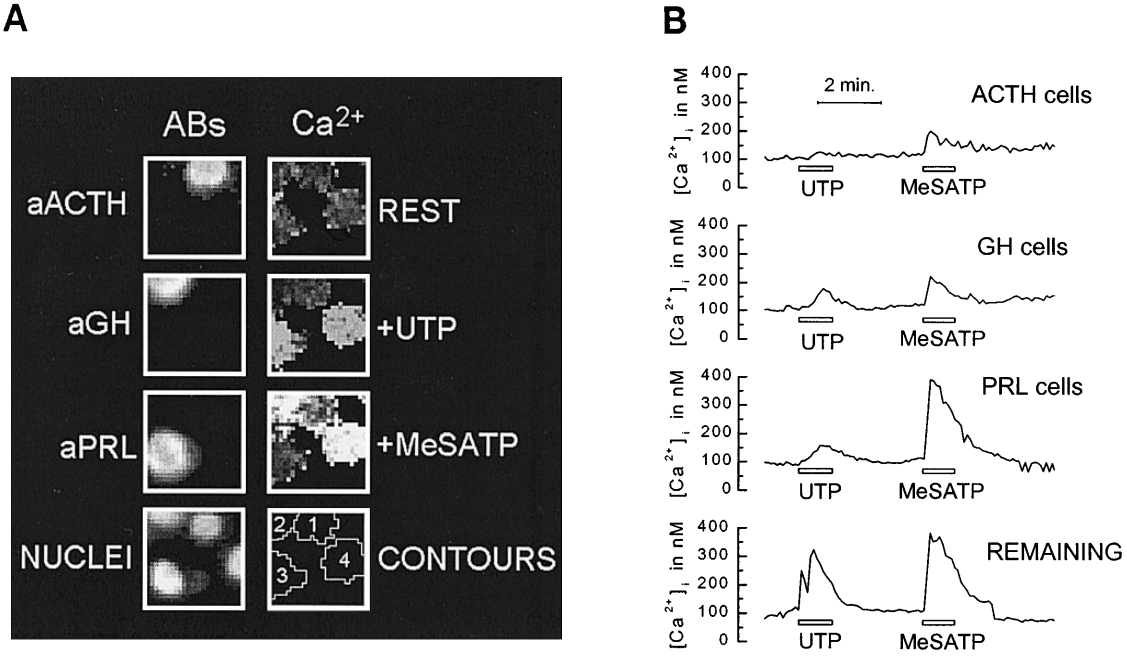

Fig. 7. Responses to UTP and MeSATP in individual corticotropes, somatotropes, and lactotropes. *A*, *left* (labeled ABs, for antibodies): immunocytochemical identification of 3 main cell types, with the use of fluorescent primary antibodies, is illustrated (see MATERIALS AND METHODS for details and text for further explanations). Same 30 × 30 μm section of microscope field is shown stained with different antibodies or with nuclear stain Hoechst 33258. *A*, *right*: 3 images of same field taken during $[Ca^{2+}]_i$ measurements before (Rest) or after stimulation with either 20 μM UTP or 10 μM MeSATP. Larger Ca^{2+} concentrations appear as brighter areas of gray. *B*: averaged $[Ca^{2+}]_i$ traces for 9 corticotropes (ACTH cells), 21 gonadotropes (GH cells), 34 lactotropes (PRL cells), and 12 cells that did not stain with any of the antibobies against the first 3 cell types (Remaining) from the same experiment as in *A*.

Table 2. *Comparison to responses to UTP and MeSATP in corticotropes, somatotropes, and lactotropes*

		Response to UTP		Response to MeSATP	
Cell Type	*n*	%	$\Delta[Ca^{2+}]_i$	%	$\Delta[Ca^{2+}]_i$
All cells	236	34	1.00 ± 1.94	69	1.00 ± 1.14
Corticotrope	17	24	0.39 ± 0.98	53	0.30 ± 0.36
Somatotrope	84	29	0.66 ± 1.54	51	0.38 ± 0.67
Lactotrope	90	27	0.80 ± 1.64	84	1.67 ± 1.22
Remaining	45	62	2.25 ± 2.83	80	1.10 ± 1.07

$\Delta[Ca^{2+}]_i$ values are means ± SD. AP cells were stimulated sequentially with 20 μM UTP and 10 μM 2-methylthio-ATP (MeSATP). After $[Ca^{2+}]_i$ measurements, cells were stained sequentially with fluorescent antibodies against ACTH, GH, and PRL and were analyzed individually. "Remaining" refers to cells that were negative to all 3 antibodies. Peak size ($\Delta[Ca^{2+}]_i$) was estimated as the difference of the average $[Ca^{2+}]_i$ values just before and after the stimulus (1-min integration periods), expressed as fraction (mean ± SD) of $\Delta[Ca^{2+}]_i$ value obtained in whole cell population. $\Delta[Ca^{2+}]_i$ values (in nM) for all cells were 59 ± 115 UTP and 162 ± 184 MeSATP. Percent of cells responding within each type was estimated by counting cells in which $\Delta[Ca^{2+}]_i$ was >20 nM.

UTP, 10 μM MeSATP, and 10 μM ATP produced a $[Ca^{2+}]_i$ response in 22, 51, and 53% of the cells, respectively (188 cells in 3 different experiments). The size of the responses was also similar to the size obtained with cultured cells. Therefore, the results obtained here with primary cultures are likely to represent the physiological behavior of AP cells.

DISCUSSION

We show here that functional ATP receptors are present in all five cell types of AP. The percentage of cells responding to ATP varied between 60 and 88% in the different cell types. Thus our results confirm previous reports on the presence of ATP receptors in lactotropes (5) and gonadotropes (8, 11, 24) and extend them to the other cell types. Because pituitary hormone release is a Ca^{2+}-triggered process, our results suggest that ATP may induce secretion of all five pituitary hormones. ATP has been shown previously to induce secretion of LH (7, 24). In addition, ATP is coreleased with pituitary hormones (7, 24). This leaves room for paracrine interactions among different pituitary cells. Taken together, these results suggest that ATP receptors may play a role in the control of pituitary function.

The results from the additivity experiments shown here (Figs. 2–4) suggest the presence of at least two different ATP receptors, one that is activated equally well by UTP and ATP (U receptor) and another that shows a strong preference for ATP over UTP (A receptor). ATPγS activates both receptors, whereas MeSATP and ClATP seem to prefer A receptors. Many of the cells possess both kinds of receptors, although either A or U receptors may predominate in a smaller subpopulation of cells (Fig. 2). Regarding the distribution of A and U responses among the different AP cell types, we find that the distribution of A receptors is larger in lactotropes and in another subpopulation that must include gonadotropes and thyrotropes (Table 2). Somatotropes and corticotropes presented smaller A-type responses both by the fraction of responding cells and by the typical amplitude of the responses. The U-type responses were limited to a smaller fraction of cells (<30%) in lactotropes, somatotropes, and corticotropes. The AP cell subpopulation not included in the above kinds of cells showed a much larger U-type response (Fig. 6 and Table 2). This suggests that either gonadotropes or thyrotropes must have a large density of U-type receptors. These differences in ATP receptor distribution among different AP cell types may be relevant for the control of hormone secretion.

The characteristics of the U receptor described here were consistent with those of a $P2Y_2$ receptor. The increase in $[Ca^{2+}]_i$ observed on stimulation of these receptors in Ca^{2+}-free medium (Fig. 5*A*) indicates that the nucleotide is able to induce release of Ca^{2+} from the intracellular stores. As a matter of fact, most previous studies attributed the effects of ATP in pituitary to metabotropic ($P2Y_2$) receptors inducing Ca^{2+} release from the intracellular Ca^{2+} stores (5, 7, 8, 11) mediated by inositol trisphosphate production (12). In the same line, a $P2Y_2$ receptor has been identified recently within a rat cDNA pituitary library (9). We find that UTP also induces some Ca^{2+} (Mn^{2+}) entry (Fig. 6), but a large part of it may be regarded as secondary to the activation of the capacitative pathway on emptying of the Ca^{2+} stores.

It is clear, however, that a large component of the $[Ca^{2+}]_i$ increase induced by ATP, MeSATP, and ClATP is a result of Ca^{2+} entry through a noncapacitative pathway because *1*) the $[Ca^{2+}]_i$ increase was largely decreased after removal of external Ca^{2+} (Fig. 5*A*), *2*) the increase was still observed after emptying of the intracellular Ca^{2+} stores by treatment with thapsigargin (Fig. 5*B*), and *3*) direct evidence of the opening of a plasma membrane pathway for entry was provided by the acceleration of Mn^{2+} entry (Fig. 6). This component of the ATP action was associated with the A receptors defined above, with low reactivity for UTP. The phenomenological properties of this component, including low desensitization (Fig. 1*A*), fit with the properties of the $P2X_2$ receptor whose mRNA has been detected recently in the AP (4, 18). ATP has also been reported to induce Ca^{2+} entry in gonadotropes, probably through a P2X receptor (24). Thus AP cells would be an exception to the rule that P2X receptors are restricted to "excitable" (nerve and muscle) cells. Alternatively, it might be reasonable to include AP cells (and perhaps other secretory cells) within the excitable category, because they possess voltage-dependent ion channels and are able to generate action potentials and their activity is modulated by neurotransmitters.

The A component of the response to ATP in pituitary cells could still contain minor contributions of other P2 receptors. Thus the presence of $P2Y_1$ receptors with high affinity for MeSATP (13) cannot be excluded. On the other hand, the presence of P2X receptors other than $P2X_2$ seems necessary to explain the response to α,β-MeATP found in a small fraction of cells (Fig. 1*E*). A likely candidate is the $P2X_3$ receptor, which is sensitive to this nucleotide (6, 19) and whose mRNA has been

detected in pituitary tissue, although at very low levels (6). The existence of a few cells responding to MeSATP added on top of ATP is also consistent with this idea, because $P2X_2$ and $P2X_3$ can form heteropolymers that are sensitive to α,β-MeATP, like $P2X_3$, but show little desensitization, like $P2X_2$ (19). Other P2X receptors, most of which are sensitive to MeSATP (13), may contribute to the A response. Such a polymorphism may be responsible for the rather bad correlation found at the single cell level for the actions of ATP and MeSATP (Fig. 1*H*).

In summary, we provide evidence for the presence of at least two types, ionotropic and metabotropic, of functional ATP receptors in all five types of AP cells. Experiments with freshly isolated cells suggest that the results obtained with primary cultures are not artifactual but are representative of the physiological behavior of AP cells. Because ATP is coreleased with several AP hormones, the presence of ATP receptors suggests the possibility of paracrine interactions among the different pituitary hormones secreted. Further studies are required, however, to assess the physiological relevance of ATP receptors for the regulation of pituitary function.

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