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# Adenosine 5'-Triphosphate (ATP) Receptors Induce Intracellular Calcium Changes in Mouse Leydig Cells

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**Cytoplasmic calcium ($[Ca^{2+}]_i$) changes evoked by adenosine 5'-triphosphate (ATP) were recorded in cultured individual Leydig cells within 10–18 h after cell dispersion. $[Ca^{2+}]_i$ was monitored using Fura-2AM loaded cells with a digital ratio imaging system. Five micromolars ATP induced biphasic $[Ca^{2+}]_i$ responses in most cells (94%, $n = 100$), characterized by a fast increase from a basal level (126 ± 5 n*M* SE, $n = 60$ cells) to a peak (5–7 times above basal levels) within seconds, followed by a slow decrease toward a plateau level (2–3 times above basal) within 5 min. The peak phase of the $[Ca^{2+}]_i$ response increased with ATP concentrations (1–100 μ*M* ATP) in a dose-dependent manner with an $IC_{50}$ of 5.9 ± 1.2 μ*M*, and it desensitized in a reversible manner with repeated application of 5 μ*M* ATP at < 5-min intervals. The $[Ca^{2+}]_i$ peak response was dependent on $Ca^{2+}$ release from an intracellular pool, whereas the plateau phase was dependent on extracellular $[Ca^{2+}]$. ATP did not appear to induce formation of nonspecific membrane pores, since stimulation for 10 min with ATP (10–100 μ*M*) in the presence of extracellular Lucifer yellow (LY) (5 mg/mL) did not result in dye loading of the cells. $[Ca^{2+}]_i$ transients were elicited by other adenosine nucleotides with an order of potencies (ATP > Adenosine diphosphate [ADP] > Adenosine > Adenosine monophosphate [AMP]) that was compatible with the expression of $P_2$ receptors. $[Ca^{2+}]_i$ responses were suppressed by the purinergic $P_2$ receptor antagonist, suramin. These results provide functional evidence for the expression of purinergic $P_2$ receptors in Leydig cells.**

**Key Words:** Testis; purinergic receptors; secretion; gap junctions.

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## Introduction

Extracellular adenosine 5'-triphosphate (ATP) is believed to regulate physiological processes, including cell contraction or relaxation, aggregation, cell growth, and secretion in numerous different cell systems, including neurons, smooth muscle (arterial and visceral), cardiac myocytes, endothelial, hematopoietic, and secretory cells. In these systems, ATP produces various effects, such as activation of ionic channels or induction of membrane pores, and/or activation of G-proteins, stimulation of phospholipid metabolism, and mobilization of intracellular $Ca^{2+}$ (reviewed in: Gordon, 1986; Burnstock 1990; Colman, 1990; El-Moatassim et al., 1992). These changes appear to be mediated by activation of a diversity of purinergic receptor types. Purinergic receptors were originally classified as types $P_1$ and $P_2$, where $P_1$ receptors were mainly sensitive to adenosine and $P_2$ receptors were most sensitive to ATP (Burnstock, 1978, 1990). More recently, $P_2$-purinoreceptors have been subdivided into at least four subtypes, $P_{2x}$, $P_{2y}$, $P_{2z}$, $P_{2t}$, according to actions underlying cell responses and the rank order of potency or new synthetic agonists (Gordon 1986; Cusak and Hournai, 1990).

The expression of the $P_{2z}$, receptor subtype has recently been suggested to be associated with the expression of the gap junction protein connexin43 (Cx43), possibly through an ATP-induced activation of conducting Cx43 hemichannels (Beyer and Steinberg, 1991). In the testis, the interstitial or Leydig cells that surround the spermatic cords directly communicate through large gap junctions, which express abundant Cx43 *in situ* and in vitro (Risley et al., 1992; Pérez-Armendariz et al., 1994, 1995, 1996; Varanda and Campos de Carvahlo 1994). For this reason, we have explored the possibility that Leydig cells might express purinergic $P_{2z}$-type receptor activity.

Moreover, ATP released from purinergic nerves or coreleased with other neurotransmitters at both sympathetic adrenergic and cholinergic synapses (Burnstock, 1972, 1978) has been proposed as the primary source of ATP for different cell types which express $P_2$ receptor activity. In

the testis, there is no consensus about autonomic innervation of Leydig cells (Hodson, 1970). Nevertheless, ultrastructural evidence of both direct and indirect autonomic innervation of these cells has now been provided in several species, including bird (Baumgarten and Holstein, 1968), reptile (Unsicker, 1973), telost (Gresik, 1973), and most recently mammals (Prince, 1992). Thus, anatomical studies on testis raise the possibility that ATP might be released or coreleased at sites of innervation, and has also led us to examine whether $P_2$ purinergic receptors are expressed in Leydig cells.

In this article, we provide functional evidence for the expression of $P_2$ receptors in Leydig cells. Our demonstration that $[Ca^{2+}]_i$ is quite sensitive to ATP and its metabolites suggests that in Leydig cells ATP may be physiologically important in regulating $Ca^{2+}$ homeostasis. The possible relevance of $P_2$ receptors for Leydig cell function is discussed. Part of these data have been presented in a preliminary form (Nadal et al., 1993).

## Results

### *ATP induces $[Ca^{2+}]_i$ Transients in Leydig Cells*

In most Leydig cells maintained under resting conditions, basal $[Ca^{2+}]_i$ values were stably recorded for at least 30 min. Application of low extracellular ATP concentrations (1–100 $\mu M$) in the presence of 1 m$M$ $MgCl_2$ in the external media induced transient increases in $[Ca^{2+}]_i$ in most of the cells (94 of 100). Figure 1 shows traces representative of those recorded in most of the cells in response to 5 $\mu M$ ATP, where four phases are resolved. In the first phase, $[Ca^{2+}]_i$ increased from a basal level (126 ± 5 n$M$, SE, $n$ = 60 cells) to a peak (610 ± 98 n$M$, SE, $n$ = 10 cells) within 2–5 s. In the second phase, $Ca^{2+}$ levels decreased rapidly also within seconds. In the third phase, $Ca^{2+}$ levels decreased more slowly, tending to reach a plateau level (418 ± 39 n$M$, $n$ = 10 cells) within 5 min. The fourth phase was triggered by ATP removal, where $Ca^{2+}$ levels dropped and reached their resting values within 2–3 min.

### *Evidence that $[Ca^{2+}]_i$ Transients Induced by ATP in Leydig Cells are Receptor-Mediated*

Receptor-mediated responses are known to desensitize in response to repetitive applications at short intervals (Lefkowitz and Caron, 1988). In Leydig cells, the $Ca^{2+}$-ATP transients exhibit some slight heterogeneity in their threshold ATP concentration and shape among different cells from the same culture. However, when three repetitive applications of ATP were applied to the same cell separated by 5-min rinse periods, peak $[Ca^{2+}]_i$ responses recorded were of similar amplitude (Fig. 2A), in contrast to the plateau phases, which varied. When shorter rinse periods were allowed for recovery (2 min), a reversible decrease in the peak was induced (Fig. 2B), whereas the plateau phase was unaffected. Figure 2C shows the normalized mean percent change and SE in the amplitude of the peak (shaded bars) and plateau (open bars) $[Ca^{2+}]_i$-ATP

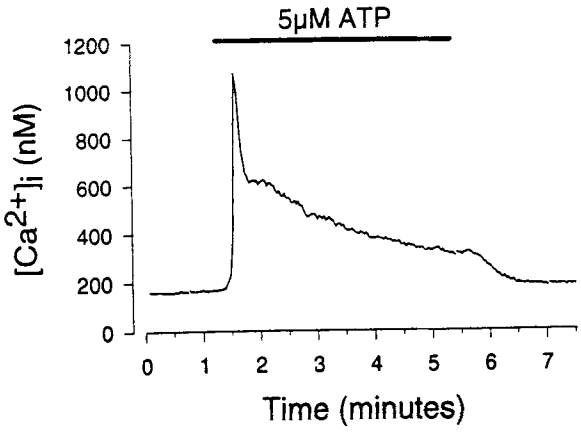


**Fig. 1.** ATP induces an increase in $[Ca^{2+}]_i$ in mouse Leydig cells. Ratiometric recording of $[Ca^{2+}]_i$ obtained from a Leydig cell loaded with Fura-2AM; $[Ca^{2+}]_i$ was calibrated by comparison of ratio values to calibration solutions (*see* Methods). Application of 5 $\mu M$ ATP in the external bath solution, during the time indicated by the line above recordings, induced a transient increase in $[Ca^{2+}]_i$ characterized by the following phases: At first $[Ca^{2+}]_i$ rapidly increased from a basal level to a peak (about seven times larger than basal levels) within 2–5 s, followed by a second phase where a partial rapid decrease in $Ca^{2+}$ levels occurred within seconds. Thereafter, phase three was identified by a change in the rate of decrease in $Ca^{2+}$ levels, where $Ca^{2+}$ decreased more slowly and tended to reach a plateau level within 5 min. Phase four was initiated after ATP removal, where $Ca^{2+}$ levels dropped to their resting levels within 1–2 min.

response recorded from a group of eight single cells. The peak amplitude decreased by 46 ± 10% (SE) when the interval between ATP applications was 2 min, whereas it was unaffected, compared to control responses, when the interval between ATP applications was 5 min. The plateau-phase amplitude was not significantly modified at both intervals. These results suggest that different mechanisms are determining the first and third phases of the response, as well as indicate that peak $[Ca^{2+}]_i$-ATP-induced response in Leydig cells desensitize. Thus, intervals >5 min were used to study other properties of the ATP-evoked $[Ca^{2+}]_i$ response.

Receptor-activated responses showed agonist dose dependence. Figure 3A shows the effect of different doses of ATP on the $[Ca^{2+}]_i$ recordings from a Leydig cell. ATP was added to the bath solution at the times indicated with the bars, at the doses written above the recordings. The peak $[Ca^{2+}]_i$-ATP response increased with higher concentrations of ATP. Figure 3B shows the concentration–response curve for the mean normalized peak (circles) and plateau (squares) $[Ca^{2+}]_i$-ATP response recorded from a group of isolated Leydig cells. Measurements were only included from cells where at least three concentrations of ATP were tested and the mean value of responses was calculated for at least five cells at each ATP concentration. The graph shows that the peak $[Ca^{2+}]_i$ exhibits dose dependence with a tendency to saturation above 100 $\mu M$

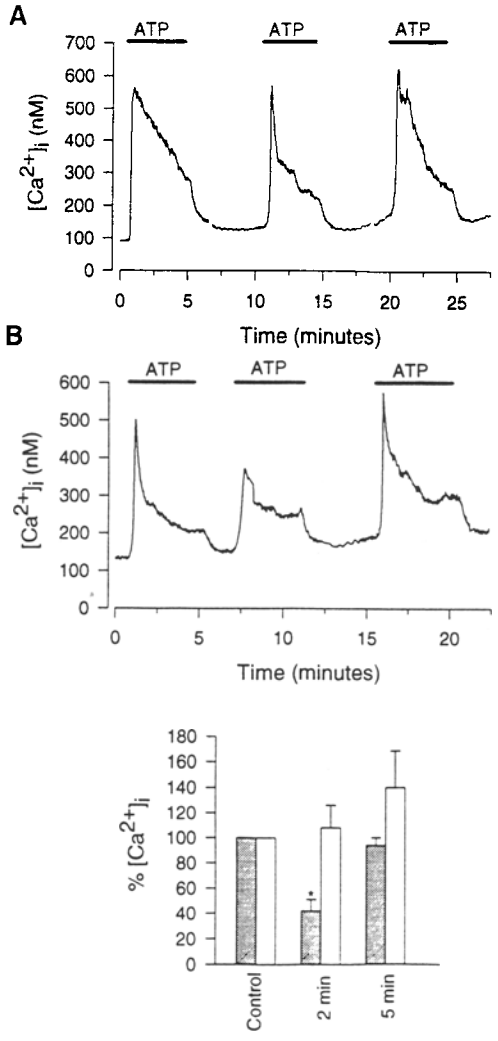


**Fig. 2.** ATP induces $[Ca^{2+}]_i$ transients that desensitize with repetitive applications. Ratiometric $[Ca^{2+}]_i$ recordings from a Leydig cell, calibrated as in Fig. 1. **(A)** When repetitive stimulations with ATP were applied to the cell and the interval between ATP stimulations was >5 min, the amplitude of the peak $[Ca^{2+}]_i$ transient was similar, although the amplitude of the plateau phase varied. **(B)** When the interval between ATP stimulations was 2 min the peak $[Ca^{2+}]_i$ induced by the subsequent application of ATP was decreased (43%), but after a 5-min rinse period the amplitude of the transient response was recovered (96%). **(C)** Histogram of the normalized peak (shaded bars) and plateau-phase (open bars) amplitudes of the $[Ca^{2+}]_i$ responses from a group of Leydig cells, recorded after repetitive stimulation with ATP at 2- and 5-min intervals. Plateau-phase amplitude was measured at 4 min of stimulation; measurements from eight cells of three different cell cultures. Note that a significant ($p < 0.005$, $t$-test) reduction in the amplitude of the peak $[Ca^{2+}]_i$ response (46 ± 10%) was found when the interval was short, and that the transient was restored to full amplitude when the interval was 5 min. In contrast, the amplitude of plateau phase was not consistently affected by these intervals of stimulation with ATP. Lines atop bars represent SE ($n = 8$).

ATP. A calculated $EC_{50}$ of ≈5.9 ± 1.2 μ*M* was obtained from the best fit of the data with the Michaelis equation. A change in the amplitude of the plateau phase was also apparently detected, but at a range of ATP concentrations two orders of magnitude lower.

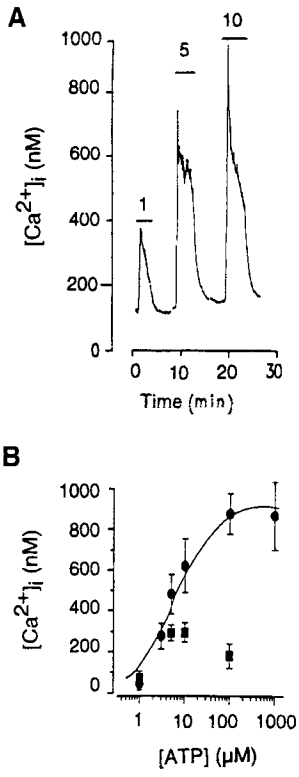


**Fig. 3.** Amplitude of peak $[Ca^{2+}]_i$ transient induced by ATP is dose-dependent. **(A)** $[Ca^{2+}]_i$ recordings from a single Leydig cell stimulated by different concentrations of ATP (indicated above the ATP-evoked $[Ca^{2+}]_i$ responses). Note that amplitudes of peak $[Ca^{2+}]_i$ responses increased with higher ATP concentrations. **(B)** Normalized concentration–response curve for peak (circles) and plateau (squares) $[Ca^{2+}]_i$-ATP responses. Data were obtained from cells where at least three different concentrations of ATP were tested ($n = 11$; 3 cultures), and mean value of peak and plateau responses for each concentration were calculated from at least five cells. Test solutions were applied at an interval rinsing period of 5 min. The mean amplitude of peak phase of the $[Ca^{2+}]_i$ response (circles) increased with ATP concentrations (1 to 100 μ*M* ATP) in a dose-dependent manner with an $IC_{50}$ of 5.9 ± 1.2 μ*M* calculated from the best fit with the Michaelis equation (solid line). In contrast, the plateau phase (squares) showed a different ATP dose–response relationship that did not increase at concentrations above 5 μ*M*. Bars are SEM.

Another characteristic of receptor-mediated responses is that they display agonist selectivity to molecules with similar structures. In order to determine whether the responses induced by ATP in Leydig cells were mediated by activation of $P_1$ or $P_2$ purinergic receptors, the effects of adenosine, adenosine monophosphate (AMP) and adenosine diphosphate (ADP) on $[Ca^{2+}]_i$ recordings were compared to those of ATP (Fig. 4). AMP had no effect even at concentrations as high as 1 m*M* (Fig. 4A). Adenosine (1 m*M*) induced only slight increases in $[Ca^{2+}]_i$, the amplitudes of which were 9.4% of the responses induced by 5 μ*M* ATP (Fig. 4B). ADP (10 μ*M*) also induced small $[Ca^{2+}]_i$ transients with amplitudes that were 44% of those induced by 10 μ*M* ATP (Fig. 4C). Thus, the pattern of agonist selectiv-

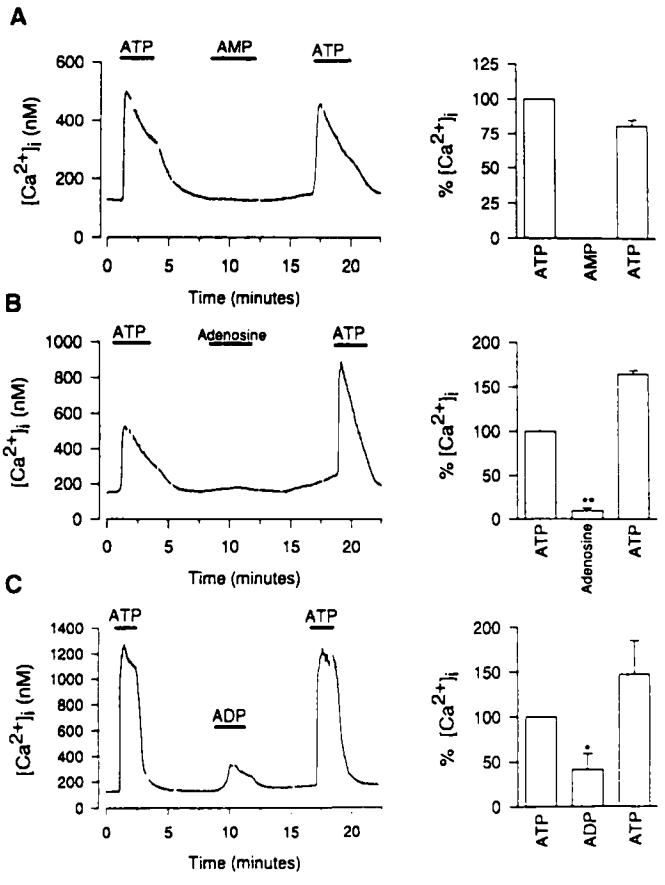


**Fig. 4.** Agonist specificity of ATP-activated $[Ca^{2+}]_i$ responses. (Left) $[Ca^{2+}]_i$ recordings from different individual cells (**A, B,** and **C**), and (right) normalized amplitude of peak $[Ca^{2+}]_i$ transients from groups of Leydig cells, obtained during stimulation with ATP and other adenine nucleotides. **(A)** AMP was inactive at concentrations of 1 m*M*, whereas characteristic responses were induced by 5 μ*M* ATP. Similar responses were recorded from other six cells. **(B)** Adenosine (1 m*M*) induced only slight responses (left), that were 9.4 ± 3.1% (SE $n = 3$) of responses to 5 μ*M* ATP. **(C)** ADP at 10 μ*M* induced responses that were 44 ± 19% (SE) of those induced by the same concentration of ATP ($n = 5$ cells). By applying the Student's *t*-test to the data, the responses induced by ADP and adenosine were significantly reduced compared with responses induced by ATP ($p < 0.05$ = * and $p < 0.005$ = **, respectively). This pattern of agonist selectivity (ATP > ADP > adenosine > AMP) is compatible with that of a $P_2$ receptor.

ity for $[Ca^{2+}]_i$ transients induced by adenosine nucleotides in Leydig cells (ATP > ADP > Adenosine > AMP) is compatible with that of the $P_2$ receptor.

Suramin has been shown to be a competitive antagonist of $P_2$ receptors (Evans et al., 1992; Wiley et al., 1993; Hoiting et al., 1990). The effect of this molecule was evaluated on the $[Ca^{2+}]_i$-ATP response in Leydig cells. A small traction of cells (<5%), such as the one shown in Fig. 5, exhibited a slowly developing $[Ca^{2+}]_i$ response. Cells were repetitively stimulated with 5 μ*M* ATP at 5-min intervals. Three minutes before the second application of ATP, 300 μ*M* suramin was added to the Ringer solution, and its

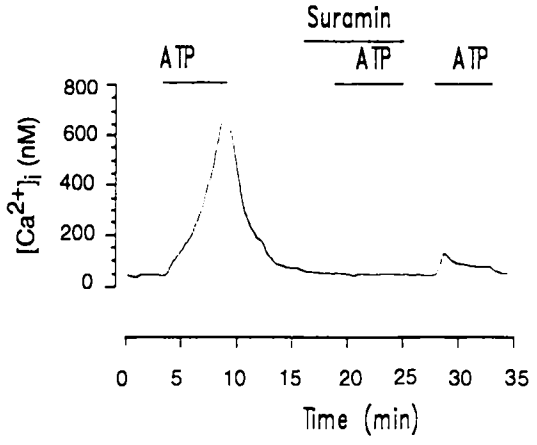


**Fig. 5.** ATP-evoked $[Ca^{2+}]_i$ responses are reversibly blocked by suramin. Three pulses of ATP (5 μ*M*) were applied to a Leydig cell at the times indicated by the lines atop $[Ca^{2+}]_i$ recordings. Two minutes before the second stimulation with ATP, 300 μ*M* suramin was applied to the perfusion bath and maintained during the application of ATP. In the presence of suramin, ATP did not evoke a $[Ca^{2+}]_i$ response. This effect was slowly reversible after washout of the drug; partial recovery is illustrated at the end of this recording.

presence in the bath was maintained during the application of this nucleotide. Under this condition, 5 μ*M* ATP did not elicit a $[Ca^{2+}]_i$-ATP response. Four minutes after the washout of the drug, the $[Ca^{2+}]_i$-ATP response was partially restored. Similar abolition of the ATP response was found in other Leydig cells after exposure to this drug ($n =$ 15, 3 cell cultures).

Expression of $P_{2z}$ receptor subtype has been found in a restricted number of cells. $P_{2z}$ receptor activation by ATP[4] has been found to induce membrane pores that allow the passage of molecules with an $M_r$ as high as 900 Dalton, including Lucifer yellow (LY) (457 Dalton) and Fura-2 (636 Dalton) within minutes (Cockcroft and Gomperts, 1980; Steinberg et al., 1987; Tatham and Lindau, 1990; Wiley et al., 1993). To test whether ATP induced membrane pores in Leydig cells at the doses used in our experiments, control and ATP (10–100 μ*M*)-stimulated cells were incubated in Ringer media in the presence of 10 m*M* LY, a concentration previously used to show penetration of this dye into other cell types through ATP-induced membrane pores (Steinberg et al., 1987). After 15–30 min of incubation with 10–100 μ*M* ATP, some sparsely distributed cells showed LY uptake, but most Leydig cells were not dye-loaded (Fig. 6). Thus, $P_{2z}$ receptors do not appear to participate in the responses of Leydig cells to ATP at the concentrations and under the conditions used in our experiments.

### *$[Ca^{2+}]_i$ ATP Responses in Leydig Cells are Partially Dependent on External $Ca^{2+}$*

As shown above, the peak and plateau phase amplitude showed different ATP sensitivities, and were unequally affected by repetitive stimulation at short intervals, sug-

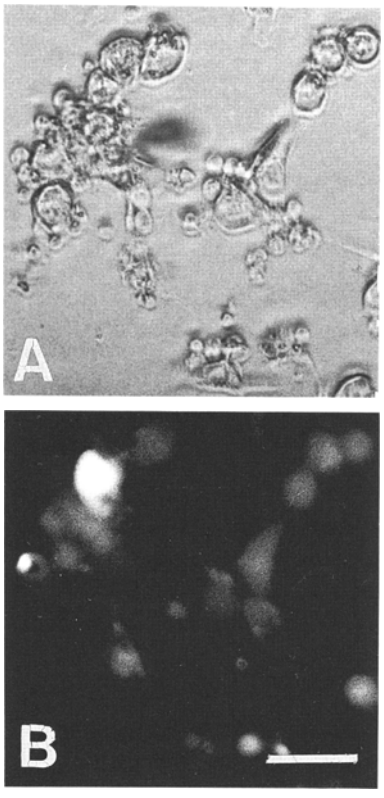


**Fig. 6.** ATP does not induce Lucifer yellow uptake in Leydig cells. **(A)** Phase-contrast and **(B)** fluorescent micrographs of cultured Leydig cells. Cells were incubated in normal Ringer media in the presence of 10 m*M* LY, a concentration previously used to show penetration of this dye into other cell types through ATP-induced membrane pores (Steinberg et al., 1987). Then, 100 μ*M* ATP was added to the incubation medium and cells were observed under fluorescent microscopy. After 15 min of incubation with ATP, some sparsely distributed cells, possibly with a certain degree of cell membrane damage, showed LY uptake (arrows), but most Leydig cells were not dye-loaded. In the large cell clump, two cells at most were dye-loaded without further cell-to-cell dye transfer to their neighbors, possibly as a consequence of previous cell uncoupling. Calibration bar 40 μ*M*.

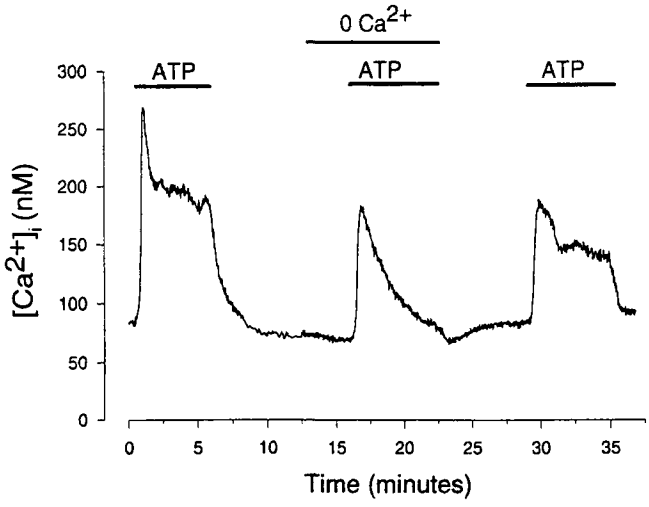


**Fig. 7.** The plateau phase of the ATP-evoked intracellular $Ca^{2+}$ response in Leydig cells is partially dependent on the presence of external $Ca^{2+}$. $[Ca^{2+}]_i$-ATP recordings from a Leydig cell initially bathed in external solution containing 1 m*M* $CaCl_2$. During the time indicated by the bar atop the recording, the perfusion solution was changed to one without $Ca^{2+}$ and with 1 m*M* EGTA. Under this condition, the basal $[Ca^{2+}]_i$ level was slightly decreased (most evident on rinsing with $Ca^{2+}$-containing solution at about 3 min), and the amplitude of the plateau responses was markedly attenuated. The response partially recovered on return to $Ca^{2+}$-containing solution. Similar results were found in 15 different cells.

gesting that both components of the response were determined by different cell mechanisms. As an initial approach to identify them, $[Ca^{2+}]_i$ responses were compared when performed in the presence or total absence of extracellular $Ca^{2+}$. Figure 7 shows $[Ca^{2+}]_i$ recordings obtained from a Leydig cell in the presence of 1 m*M* $CaCl_2$ in the extracellular solution. Under this condition, 5 μ*M* ATP induced the characteristic peak and plateau-phase $[Ca^{2+}]_i$ responses. After 5 min of rinsing with normal bathing solution, the solution was changed, at the time indicated with the bar, to one containing 1 m*M* EGTA and no added $Ca^{2+}$. Under these conditions the basal $[Ca^{2+}]_i$ level was slightly decreased, and subsequent application of 5 μ*M* ATP induced a $[Ca^{2+}]_i$ response whose plateau phase was abolished. Nevertheless, the size of the peak response was almost unchanged when compared with the peak response recorded after returning to a $Ca^{2+}$-containing solution. The plateau-phase response was partially restored on return to $Ca^{2+}$-containing solution. These results indicate that ATP evokes $Ca^{2+}$ release from intracellular stores (peak phase) as well as $Ca^{2+}$ influx from extracellular medium (the plateau phase ).

## Discussion

### *ATP-Evoked $[Ca^{2+}]_i$ Responses Result from Activation of $P_{2x}$ or $P_{2y}$ Receptors*

Here we have demonstrated that extracellular ATP application induces prominent biphasic $[Ca^{2+}]_i$ transients in Leydig cells. Our findings differ from a previous report where ATP was not found to induce any changes in $[Ca^{2+}]_i$ levels in suspensions of Leydig cells loaded with Quin-2 (Sullivan and Cooke, 1986). Differences with this last study may have arisen from a different degree of expression or state of activation of $P_2$ receptors in Leydig cells, which may vary depending on the methods used to obtain the cells or have resulted from differences in recording conditions.

Our results indicate that $[Ca^{2+}]_i$-ATP transients in Leydig cells are not attributable to the formation of nonspecific membrane pores, characteristic of $P_{2z}$ receptor subtype, since we did not detect LY uptake from the external medium during stimulation with ATP for up to 30 min. In addition, the $[Ca^{2+}]_i$ transients were elicited with relatively low concentrations of ATP in the presence of 1 m*M* $MgCl_2$ in the external medium, whereas activation of $P_{2z}$ recep-

tors, under these conditions, has been found to require high concentrations of ATP ( 100 μ*M*–5 m*M*) (Steinberg et al., 1987; Tatham and Lindau, 1990; Wiley et al., 1993). Moreover, $Ca^{2+}$ transients in Leydig, as shown here, have several characteristics similar to those found in other cell types that express purinergic $P_{2x}$ and/or $P_{2y}$ receptor subtypes: first, the amplitude and temporal course of the biphasic $Ca^{2+}$ pattern (Sasakawa et al., 1989; Dorn, et al., 1989; Soltoff et al., 1990; Li et al., 1991; Niitsu, 1992), second, a low ATP $EC_{50}$ (5.9 μ*M*) (Sasakawa et al., 1989; Honore et al., 1991), third, its agonist selectivity (Cusack and Hourani, 1990; Burnstock 1990), fourth, its suppression by the $P_2$ antagonist suramin (Hoiting et al., 1990; Evans, 1992; Wiley et al, 1993), and fifth, a response refractoriness to repetitive stimulation at short intervals (2 min) (Charest et al., 1985; Dorn et al., 1989). Thus, results presented here strongly suggest that Leydig cells express purinergic $P_{2x}$ and/or $P_{2y}$ receptor subtypes.

### *Possible Mechanisms Involved in the Response*

Results presented here demonstrate that the first phase of the peak $[Ca^{2+}]_i$-ATP transient mainly results from $Ca^{2+}$ mobilization from an intracellular pool. $P_2$ receptors in different systems are known to be coupled to guanine nucleotide regulatory protein (G-protein) and to phospholipases (PL) C, A, and D (Okajima et al, 1989; Bocckino et al., 1989; Exton, 1990). Activation of PLC by ATP has been found to induce phosphoinositide turnover, $Ca^{2+}$ mobilization from endoplasmic reticulum, and stimulation of protein kinase C (Martin and Harden, 1989; Sasakawa et al., 1989; Van der Merwe et al., 1989; Tenneti and Talamo, 1993). Thus, it is likely that in Leydig cells, the peak $[Ca^{2+}]_i$-ATP response mainly results from the activation of mechanisms secondary to phosphoinositide breakdown.

Peak $[Ca^{2+}]_i$-ATP response in Leydig cells exhibited refractoriness to repetitive stimulation at short intervals (2 min) that fully recovered after 5 min. This recovery time is also within the range found for ATP effects in other cell types (Charest et al., 1985; Dorn et al., 1989). For other type of receptors, refractoriness in the response has been found to reflect the minimum time required for replenishment of intracellular $Ca^{2+}$ reservoirs through membrane calcium influx (Mc Donough et al., 1988), or from other forms of desensitization of agonist-induced response, such as receptor uncoupling to G-protein secondary to its phosphorylation (Harden, 1983; Lefkowitz and Caron, 1988) or its sequestration (Toews et al., 1984). For the $P_2$ receptors, little is known at the present time, although evidence exists suggesting that the response may desensitize as a result of receptor alteration or of its uncoupling to G-protein (Martin and Harden, 1989). Thus, in Leydig cells, it is possible that the second phase or initial fast rate of decay of $Ca^{2+}$ levels, results from a certain degree of response desensitization besides activation of $Ca^{2+}$ extrusion mechanisms, such as the $Na^+$-$Ca^{2+}$ exchange and $Ca^{2+}$ pump (Carafoli, 1991).

The third phase, where the rate of decrease in $Ca^{2+}$ levels decelerates and tends to plateau, as demonstrated here, is mainly determined by $Ca^{2+}$ influx through the membrane from the external media. $Ca^{2+}$ influx through activation of a variety of membrane channels is the other most common effect known to be induced by $P_2$ receptors in different cell types, including secretory cells (Burnstock 1990; Colman, 1990; El-Moatassim et al., 1992). Hence, it is possible that in Leydig cells, ATP also activates membrane channels. In support of this hypothesis in preliminary recordings, we have found that ATP activates within seconds biphasic inward currents that also desensitize with repetitive stimulations. Thus, third phase may result from the equilibrium between the activation of ionic membrane channels and $Ca^{2+}$ extrusion mechanisms mentioned above. After ATP removal, membrane channels may rapidly be turned off, as indicated by the rapid drop in $Ca^{2+}$ levels toward its resting values during the fourth phase. This last phase is likely to be mainly determined by the activity of the $Na^+$-$Ca^{2+}$ transporter and the $Ca^{2+}$ pump.

### *Heterogeneity in the $[Ca^{2+}]_i$-ATP Response*

$Ca^{2+}$ responses shown here exhibit some heterogeneity in their threshold ATP concentration and shape. Regarding with its shape, peak width variation was most readily observed. Width of the peak response is determined by the amount of expression, kinetics of activation and deactivation, as well as the different regulatory properties of the mechanisms involved in the first three phases of the response (Fig. 1). Width variation among $[Ca^{2+}]_i$-ATP response was detected between different Leydig cells, but also within the same cell. As indicated in Fig. 2A, for three repetitive pulses of ATP applied to the same cell, first-phase responses were of similar amplitude. In contrast amplitude of the third phase of the response was variable. Thus, it is possible that when the amplitude of the third phase was largest, it temporally overlapped and occluded the fast component of decay in $Ca^{2+}$ levels or second phase, increasing the apparent width of the response. The mechanisms underlying variation of the amplitude of the third phase are presently unknown, but may result from the different regulatory properties of the various components involved in this phase of the response. In addition, heterogeneity in $[Ca^{2+}]_i$-ATP response among different Leydig cells may also result from the existence of subpopulations of cells that exhibit a variable degree of expression of the different cell mechanisms involved in the response, as has been found in other gland cells (Horta et al., 1991; Hiriart and Ramirez Mendelez, 1991). Finally, differences may also result from different metabolic state of the cells resulting from their recent dispersion as well as from variations in the general state of animals used to prepare each cell culture. Identification and characterization of all mechanisms participating in $[Ca^{2+}]_i$-ATP-induced response is required to clarify this aspect.

### *Possible Sources of ATP Release in the Interstitial Space of the Testis*

Results presented here suggest that ATP may function as either neurotransmitter or neuromodulator at nerve terminals found in the interstitial space of the testis. ATP released from purinergic and sympathetic terminals has been proposed as the primary source of ATP for the different cell types that express $P_2$ receptor activity (Burnstock, 1972, 1978). In the testis, evidence for both indirect and direct innervation of Leydig cells has been found in various species, including cells from mammalian testis (*see* Introduction). In human testis, ultrastructural studies suggest that in addition to the cholinergic (type I) and adrenergic (type II) terminals, there is a third group of nonadrenergic, noncholinergic nerve endings (Prince, 1992). However, morphological studies have not yet addressed whether ATP is present at some of these types of terminals. Interestingly, the nonadrenergic and noncholinergic type of nerve terminals have also been found in other parts of the male reproductive system, such as vas deferens and epididymis (Gu et al., 1983), where there is also evidence that ATP activates $P_2$ receptors (Friel, 1988).

Other possible sources of ATP in the testicular interstitial space may include ATP released from secretory granules of mast cells (Uvnas, 1974), which represent a second cell type coexisting with Leydig cells in this space (Gaytan et al., 1992). Alternatively or in addition, ATP may be locally released from a population of cells that are periodically renewed, as is suggested by the presence of macrophages, which also normally coexist with Leydig cells in this space (Raburn et al., 1993).

### *Possible Biological Consequences of External Stimulation with ATP in Leydig Cells*

$P_2$ receptors are ubiquitously expressed in a great variety of cells as indicated in the Introduction. However, only recently in a few of them, their functional consequences have been identified. In secretory cells, $P_2$ receptor activation has been found to induce $[Ca^{2+}]_i$ transients and ionic membrane fluxes in different primary exocrine (Soltoff et al., 1990; Sasaki and Gallacher, 1992; Vincent, 1992) and endocrine (Sugiyama, 1971; Loubatieres-Mariani et al., 1979; Sasakawa et al., 1989; Geschwind et al., 1989) peptidergic cells, as well as in derived cell lines (Geschwind et al., 1989; Okajima et al., 1989; Li et al., 1991; Sela et al., 1991). In some of them, where they have been studied, these changes were found associated with an increase in fluid and/or peptide secretion. Moreover, in adrenocortical cells, ATP has also been found to enhance steroidogenesis (Niitsu, 1992). Here it is demonstrated that $[Ca^{2+}]_i$ in Leydig cells is finely regulated by ATP, and that large transients could be elicited by low ATP concentrations. Since steroidogenesis is a $Ca^{2+}$-dependent process (Hall, 1988), it is possible that activation of $P_2$ receptors stimulates steroidogenesis and testosterone release. Although such a function of ATP was not detected in a previous report (Sullivan and Cooke, 1986), in that work $[Ca^{2+}]_i$-ATP changes were also not detected, and consequently, the negative evidence does not yet rule out such a possibility. Independently of the possible stimulatory role of ATP on steroidogenesis, expression of $P_2$ receptors in Leydig cells may also be relevant for other cell functions (*see* Introduction), such as for pH regulation (Puceat et al., 1993) and control of cell growth (Fang and Wu, 1993). Future experiments will address these possible roles of ATP in testicular function.

Local stimulation of Leydig cells is expected *in situ* if ATP is released from any of the possible sources discussed above. Nevertheless, important amplification of $Ca^{2+}$ signals is expected to occur by transferring the elevated $Ca^{2+}$ from one cell to its neighbors through their numerous gap junction channels formed with Cx43 (Risley et al., 1992; Varanda and Campos de Carvahlo, 1994; Pérez-Armendariz et al., 1994, 1995, 1996), which are known to be permeable to $Ca^{2+}$ (Christ et al., 1992). In preliminary experiments, local stimulation with ATP to one cell of a clump, through a high-resistance patch pipet, induced synchronous changes in $[Ca^{2+}]_i$ in the other cells, suggesting its transfer through gap junctions. If future experiments confirm these observations, an extensive modulatory role of ATP might be expected in the possible cell functions mentioned before.

## Methods

### *Experimental Preparation*

As an initial step to study ATP effects on Leydig cells, we used a rapid nonenzymatic technique for dissociation of interstitial cells slightly modified from the one described by Kawa in 1987, which we had previously characterized (Pérez-Armendariz et al., 1996). Owing to its briefness, it is likely that this method favors unaltered receptor expression in cell membranes. Briefly, testes were dissected from adult CD1 mice and the tunica albuginea was removed. Three testes were placed in 10 mL of Dulbeco's Modified Eagle's Medium (DMEM) in a 50-mL tube and were gently shaken for 4 min at room temperature until the seminiferous tubules became slightly loose, but not separated. The resulting exudate was enriched in Leydig cells. Cells were washed three times by centrifugation. The final pellet from a number of testis was suspended in a small volume of DMEM to adjust a density of $10^6$ cells/mL. Then, 1 mL of cell suspension was plated in glass coverslips and maintained in DMEM with 10% fetal bovine serum for 10–18 h in 95% $O_2$ and 5% $CO_2$ at 34°C before use.

### *Cell Identification and Functional State*

Using the dispersion technique mentioned before, we had previously found a significant enrichment of Leydig cells, as indicated by detection of 70–84% 3 β-hydroxy steroid dehydrogenase-positive cells, and a significant increase in testosterone production in cell cultures maintained for 8, 24, and 36 h, in the presence of lutenizing,

human chorionic gonadotropin hormones or dibutiryl ciclic AMP (Pérez-Armendariz et al., 1996). In the present article, ATP studies were done in cells cultured for 10–18 h, a period of time where we have confidence about their survival and functional integrity. For $[Ca^{2+}]_i$ recordings, Leydig cells were further selected by identifying their characteristic abundant cytoplasmic cholesterol droplets, visualized as birefringent bodies under phase-contrast optics (Christensen, 1975; Kawa, 1987). $[Ca^{2+}]_i$ recordings were obtained within 2 h after cells were exposed to a Kreb's Ringer medium. Within this period of time, cells had been found to maintain stable resting membrane potential and channel properties (Pérez-Armendariz et al., 1994). $[Ca^{2+}]_i$ recordings were obtained within 1 h after dye loading and 40 min after initiation of fluorescence excitation, conditions under which cells are able to keep stable $[Ca^{2+}]_i$ basal levels.

### *Cell Loading*

To load Leydig cells, the acetoxymethyl ester form of Fura-2 (Fura-2/AM; Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide (DMSO) to form a 1-m*M* stock solution. Leydig cells plated on coverslips were removed from culture media and incubated in a normal Ringer solution containing 5 μ*M* Fura-2/AM without $Ca^{2+}$ with a final DMSO concentration of 0.05% for 40 min at room temperature. Cells were washed by transferring the coverslips to a dish containing normal Ringer solution, where they were gently shaken 5–10 times. This step was repeated three times, to eliminate detritus and nonattached remaining cells. After the rinsing period, coverslips were transferred to a recording chamber mounted on an inverted microscope equipped with an intensified CCD Camera for $Ca^{2+}$ measurements.

### *$[Ca^{2+}]_i$ Measurements*

In each field, Leydig cells were epi-illuminated alternately at 340 and 380 nm using a computer-controlled filter wheel (Sutter Instruments, Novato, CA) containing appropriate excitation optical filters that was synchronized with the recording camera. The fluorescence emitted by single Leydig cells in each field was selected; images were acquired at emission wavelengths >500 nm using an intensified CCD camera (Quantex) and were stored for further analysis using the Image 1 AT/FL hardware/software package (Universal Imaging, Media, PA) on a Dell System 325 computer equipped with an optical disk drive for mass storage.

Fluorescence measurements were corrected for background fluorescence and dark camera current. Four video frames at each wavelength were averaged, obtaining an overall time resolution of 2 s for each pair of images at alternating wavelengths. The resulting images were ratioed (340/380) pixel by pixel to produce ratio images, and $[Ca^{2+}]_i$ was calculated by interpolation into a lookup table constructed by imaging Fura-2-free acid in the presence of known $Ca^{2+}$ concentrations using the same microscope objective.

### *Experimental $Ca^{2+}$ Recording Conditions*

The recording chamber (0.5-mL vol) was connected to a gravity perfusion system. Recordings under basal conditions were obtained when cells were bathed with a normal saline solution that was continuously perfusing the bath. When changes in solution were applied, the media in the recording chamber were exchanged completely within 10 seconds. Experiments were carried out at room temperature (22°C) in an external Ringer solution with the following composition: 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 2 m*M* $NaHCO_3$, 10 m*M* HEPES, pH 7.36, unless otherwise indicated.

### *Dye Experiments*

Leydig cells were incubated for 10 min in dye solution containing 5 mg of LY/mL of normal Ringer solution (≈10 m*M*, 0.5% w/v), in the presence or in the absence of 10 and 100 μ*M* ATP. After this time, cells were rinsed several times in normal external solution, dye uptake was visualized in an inverted microscope using conventional fluorescence microscopy with Xenon illumination and FITC filters, and the percentage of cells taking up dye was determined.

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