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PURINERGIC (P_2) RECEPTOR-OPERATED CALCIUM ENTRY INTO RAT THYROID CELLS+

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In the epithelial cell line FRT, derived from rat thyroid, extracellular ATP, at a concentration as low as $1x10^{-7}$ M, specifically increases cytosolic Ca^{++} two fold over the basal level of 255±45 nM. A maximum increase of 5 fold over basal is seen at $1x10^{-5}$ M ATP. The effect occurs in the absence of any measurable phosphatidyl inositol metabolism and requires the presence of extracellular Ca^{++}, but is independent of extracellular Na^{+}; it is duplicated by ATPγS but not by adenosine, AMP, ADP, AMP-PNP, AMP-CPP, or AMP-PCP. In the presence of the P_2-receptor antagonist suramin, the ATP induced Ca^{++} influx is completely inhibited, whereas Mg^{++}, La^{+++}, and verapamil are ineffective. It appears that the most likely (and unique) mechanism of ATP induced increase of cytosolic Ca^{++} in FRT cells is an increased influx through the activation of a P_2 receptor operated Ca^{++} channel.

ATP, at concentrations ranging from the micromolar to the millimolar range, is known to elicit a variety of cellular responses if Ca^{++} is present in the extracellular medium (1-3). Accordingly, evidence has been presented suggesting that extracellular ATP increases cytosolic Ca^{++} ($[Ca^{++}]i$) through mobilization and/or influx (4-7). The effects of ATP appear to be exerted through activation of P_2 purinergic receptors (2); however, a recent report has shown that multiple receptor-signal transduction systems are activated in FRTL-5 thyroid cells by extracellular ATP (8). The picture becomes more complicated since metabolites, such as adenosine and AMP may be involved through P_1 purinergic receptors and adenylyl cyclase modulation. Thus, it is difficult

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to define the role of $[Ca^{++}]i$ modulation as a causative element of extracellular ATP action or the consequence of the activation of multiple regulatory pathways. In the present study we show that in FRT thyroid cells (9), a cell line which retains the epithelial, polarized phenotype, but has lost sensitivity to TSH and adrenergic stimulation, extracellular ATP specifically induces Ca^{++} influx in the absence of any measurable activation of signal-transducing pathways and intracellular Ca^{++} mobilization. Similar effects of extracellular ATP have been described in very different cell systems such as smooth muscle cells (10), parotid acinar cells (11), thymocytes (12), and lacrimal acinar cells (13). We believe this is the first demonstration of receptor-operated Ca^{++} channels in cells of endocrine origin.

Materials and Methods

Materials. Indo-1 AM was purchased from Molecular Probes, Inc. (Eugene, OR); arachidonic acid [^{3}H], cat.#NET-298Z amd myo-inositol [^{3}H] cat.#NET-114A from Dupont-NEN; ATPγS from Boehringer Mannheim (Indianapolis, IN); U73122 was a gift from Dr. J. Bleasdale, Upjohn (Kalamazoo, MI). All other chemicals were from Sigma (St. Louis, MO).

Cells. FRT is an epithelial cell line derived from Fisher Rat Thyroids (9). Cells were cultured in Coons modified Ham's F12 medium supplemented with 5% calf serum. FRTL-5 cells (ATCC #CRL-8305) are also derived from Fisher Rat Thyroids but, at variance with the FRT, maintain thyroid differentiated functions and TSH sensitivity. The properties and culture conditions of FRTL-5 have been extensively described (14-15).

Measurements of Cytosolic Free Ca^{++}. Cells were loaded with Indo-1 acetoxymethylester (5 μM) for 30 min. at room temperature, on a rotating platform. The loaded cells were washed twice in HEPES buffered Hank's balanced salt solution (with or without 1.3 mM Ca^{++}), containing 0.2% bovine serum albumin (BSA) and 6 mM glucose. The final suspension was adjusted at $2x10^6$ cells/ml. To minimize interference of leaking dye with fluorescence measurements, 1 ml of cell suspension was centrifuged and the resulting pellet dispersed in 2 ml BSA-free buffer in an acryl cuvette which was placed in a thermostatted cell holder, equipped with a magnetic stirrer, of a PTI Deltascan Spectrofluorometer (Photon Technology International, Inc., South Brunswick NJ). Excitation was set at 350 nm, and emission was monitored as the ratio of intensities at 395 and 480 nm. Background fluorescence was determned on cells not loaded with the dye and automatically subtracted; $[Ca^{++}]i$ was calculated by the method of Grynkiewicz et al. (16).

Assay for Inositol Phosphates. Cells grown in 6-well plates were incubated with [^{3}H]myoinositol (2 μCi/ml) for 24 hours, washed twice with Hank's solution, treated with LiCl (10mM) for 10 min. at 37°, and rewashed. Test agents were then added for the times indicated, and the reaction terminated by the addition of 3 ml ice cold 4.5% $HClO_4$-Hank's solution (2:1 vol/vol). Each plate was chilled for 30 min. and scraped. Cells were centrifuged, the supernatant's pH adjusted to 8.0 with KOH and stored at -70° C. [^{3}H]Inositol metabolites were fractionated by sequential elution from a Dowex AG 1-x8 column and the radioactivity in each fraction measured (17). The amount of each inositol phosphate is presented as total counts/minute in each well.

Results and Discussion

The resting $[Ca^{++}]i$ in FRT cells is 255 ± 45 nM ($n = 5$). Extracellular ATP rapidly increases $[Ca^{++}]i$ as shown by the sharp increase of Indo-1 fluorescence (Fig. 1A). The effect is dose dependent, thus ATP causes a doubling of free $[Ca^{++}]i$ at 0.1 μM, and up to a 5 fold increase at 10 μM (Fig. 1B). We have not attempted to measure the effect of higher concentrations of ATP since complex membrane

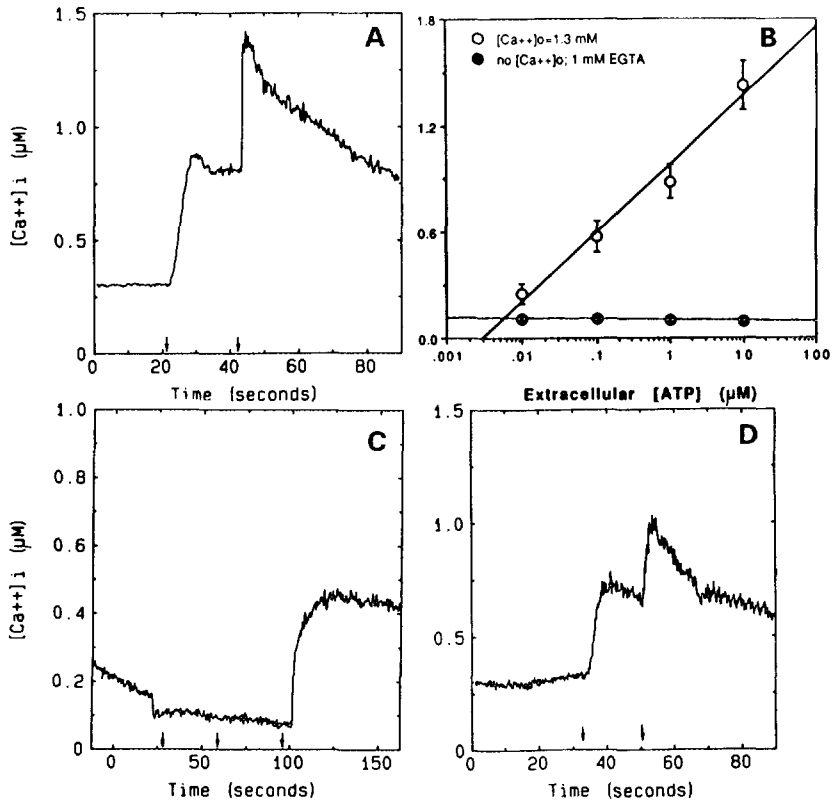

Figure 1. Changes of [Ca^{++}]i in Indo-1 AM loaded FRT cells after addition of ATP, ionomycin, or ATPγS. Panel A: cells are in Hank's containing 1.3 mM Ca^{++}; the left and right arrows indicate addition of ATP at 1 and 10 μM, respectively. Panel B: concentration dependent effect of ATP in the presence of 1.3 mM Ca^{++} (○), or in the absence of Ca^{++} and the presence of 1 mM EGTA (●). Panel C: cells are in Hank's without Ca^{++} and 1 mM EGTA; the arrows from left to right indicate addition of ATP at 1 and 10 μM; and ionomycin 1 μM,respectively. Panel D: cells are in Hank's containing 1.3 mM Ca^{++}; the left and right arrows indicate the addition of ATPγS to 1 and 10 μM,respectively. The data shown are representative of at least 3 experiments yielding essentially identical results.

perturbations, which include permeabilization to ions, are likely to be triggered (18). The ATP dependent rise in free [Ca^{++}]i is the result of increased influx; this is unequivocally demonstrated by data presented in Fig. 1B which show that no variation of Indo-1 fluorescence could be measured in response to ATP challenge, when no extacellular Ca^{++} was added and traces of the metal were chelated by 1 mM EGTA. However, these latter conditions, did not deplete intracellular Ca^{++} stores since ionomycin (1 μM), added to the cells after ATP, still induced a significant, albeit modest, rise of [Ca^{++}]i (Fig. 1C).

The effect of ATP could be duplicated by the hydrolysis resistant analog ATPγS with nearly equal potency (Fig. 1D); this suggests that induction of Ca^{++} influx does not involve ATPase activity. Several other purine nucleosides and nucleotides were tested; these included: 5'-adenylyl-imidodiphosphate (AMP-PNP), two isomers of

Table 1: Effect of Suramin and Ca^{++} channel blockers on FRT cells free cytosolic Ca^{++} in the absence or in the presence of 10 μM ATP

		$[Ca^{++}]i$ (nM)	
Compound	Concentration (μM)	Basal	+ATP
None	--	255±45	1427±152
Suramin	300	250±32	217±19
$LaCl_3$	10	251±37	1389±181
$LaCl_3$	100	248±22	1358±106
$CdCl_2$	500	239±58	1402±143
$MgCl_2$	10,000	247±34	1409±161
Nifedipine	10	253±43	1399±137
Verapamil	10	232±33	1418±132

methylene-adenosine triphosphate (AMP-CPP and AMP-PCP), benzoyl-benzoyl adenosine-5′ triphosphate, adenosine, AMP and ADP, which were all ineffective at concentrations up to 100 μM. Thus, a true potency order, to characterize pharmacologically the receptor involved, could not be defined. However, the lack of effect of adenosine, AMP and ADP would be consistent with a P_2 purinergic receptor. We have tested this directly studying the effect of suramin on ATP induced Ca^{++} influx. Suramin is an anti-trypanosomiasis agent which competitively inhibits P_2-agonist induced responses (19, 20). When FRT cells were previously exposed to 300 μM suramin for 10 minutes, ATP and/or ATPγS challenge completely failed to induce Ca^{++} influx (Table 1).

It has been reported that ATP stimulates different receptor coupled signaling pathways in FRTL-5 thyroid cells; these include activation of phospholipase C and inhibition and activation of adenylyl cyclase thus interfering with TSH actions (10). At variance with the FRTL-5 cells, which maintain thyroid differentiated functions and sensitivity to TSH, FRT cells do not express TSH receptor and display altered signaling pathways (21). Thus, it was of interest to investigate whether increased phospholipase C activity was involved with the mechanism whereby ATP induces Ca^{++} influx. We have approached this by using the compound U-73122. This aminosteroid has been shown to be an effective inhibitor for phospholipase C dependent reactions in several cell types (22). Under conditions identical to those depicted in Fig. 1A, pre-incubation with 1 μM U-73122 appears to have no appreciable effect on Ca^{++} influx (Fig. 2A); a modest decrease of the magnitude of

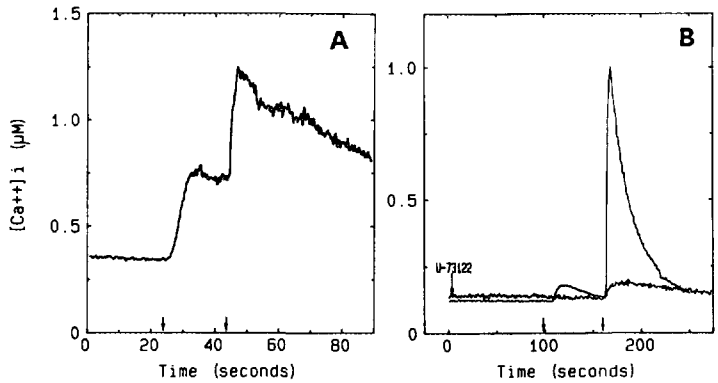

Figure 2. The effect of U-73122 on ATP induced $[Ca^{++}]i$ rise in FRT and in FRTL-5 cells in Hank's containing 1.3 mM Ca^{++}. Panel A: FRT cells exposed to 1 μM U-73122 for 3 minutes, prior to the addition of ATP to 1 and 10 μM (left & right arrows),respectively. Panel B: FRTL-5 cells exposed (as indicated) or not exposed to 1 μM U73122 prior to the addition of 10 μM ADP and ATP (left & right arrows),respectively.

the phenomenon could be measured at 5 μM U-73122 (data not shown). However, at 1 μM U-73122 inhibition of ATP induced $[Ca^{++}]i$ rise in FRTL-5 cells was nearly complete (Fig. 2B). It appears that whereas both cell strains display P_2 purinergic receptor activity the signal transduction pathways coupled to these receptor are different, at least insofar as phospholipase C activation is concerned. This was confirmed by direct measurements of inositol trsiphosphate (IP_3) in both cell strains after ATP challenge. As anticipated ATP (5 μM) induced a rapid, substantial increase in IP_3 production in FRTL-5 cells whereas no effect was shown in FRT cells (Fig. 3). These observations strongly suggest that ATP induced $[Ca^{++}]i$ rise in FRT cells does not occur through activation of phospholipase C and IP_3 signaling. The question then arises as to which is the mechanism of ATP action. This has to be confined to Ca^{++} influx since, as shown above, ATP stimulated $[Ca^{++}]i$ rise in FRT cells does not occur in the absence of extracellular Ca^{++}. Therefore, increased Ca^{++} entry must be the result of Ca^{++} channels opening. We used several approaches in order to identify the channel/s involved (Table 1). Magnesium, a potent blocker of N-methyl-D aspartate (NMDA) activated channels (23), did not block ATP induced Ca^{++} influx at concentration up to 10 mM. The organic Ca^{++} blockers verapamil (10 μM) and the dihydropyridine nifedipine (10 μM) did not affect significantly the resting $[Ca^{++}]i$ nor did they reduce the effect of ATP. The inorganic blockers La^{+++} and Cd^{++} (both at 0.1 mM) were likewise ineffective (Table 1). These findings would argue against the involvement of NMDA type or voltage-gated Ca^{++} channels (24, 25), although direct voltage measurements should be carried out for a definitive answer. The involvment of a Na^{+}-Ca^{++} exchanger seems highly unlikely since the effect of ATP on Ca^{++} influx

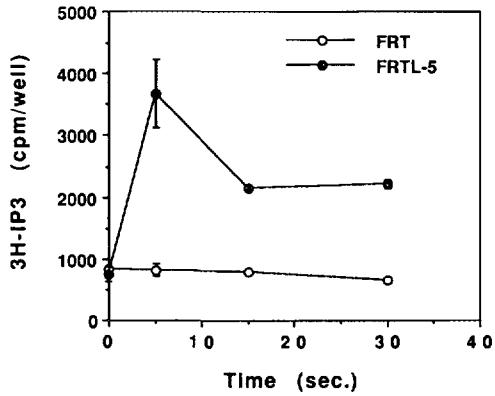

Figure 3. Time dependence of the effect of 5 μM ATP on ^{3}H-IP$_3$ production in FRT (○) and in FRTL-5 (●) cells. Data points represent mean ± SD of 3 separate assays. See methods for details.

was independent of Na^+ concentration between 0 and 140 mM (data not shown); also, the lack of inhibition by La^{+++}, a powerful inhibitor of Na^+-Ca^{++} exchange (26) would argue against this possibility. We believe that the data presented support the existence in thyroid cells of P_2 purinergic receptor-operated channels selective for Ca^{++} which become active upon ligand binding, under conditions of physiological ionic gradient. It is conceivable that similar channels are operative also in the functionally diversified FRTL-5 cells in which ATP induced Ca^{++} influx has been demonstrated (27). However,in these cells multiple signaling pathways are active, most notably phospholipase C activation and phosphoinositide cascade, such that resolving the contribution of strictly ligand-gated Ca^{++} influx is much more complicated.

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