Biochem. J. (1990) 265, 203–211 (Printed in Great Britain)

Extracellular ATP increases cytoplasmic free Ca^{2+} concentration in clonal insulin-producing RINm5F cells

A mechanism involving direct interaction with both release and refilling of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool

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Effects of extracellularly applied ATP (added as disodium salt) on stimulus–secretion coupling were investigated in clonal insulin-producing RINm5F cells. Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$), electrical activity, membrane potential, formation of $InsP_3$ and insulin release were measured. Addition of ATP in a Ca^{2+}-containing medium promoted a rapid rise in $[Ca^{2+}]_i$, which was followed by a slow decline towards the basal level. In a Ca^{2+}-free medium, the ATP-induced increase in $[Ca^{2+}]_i$ was smaller, but still enough to elicit insulin secretion. Upon normalization of the extracellular Ca^{2+} concentration, the response to ATP recovered instantaneously. The presence of glucose in the incubation medium was a prerequisite to obtain a pronounced effect of ATP in the absence of extracellular Ca^{2+}. However, glucose did not enhance the response to ATP in a Ca^{2+}-containing medium. The effect of ATP was dose-dependent, with a clearly detectable increase in $[Ca^{2+}]_i$ at 1 μM and a maximal response being obtained at 200 μM-ATP. The response to ATP was unaffected by activating adenylate cyclase by forskolin, but was abolished by 10 nM of the phorbol ester phorbol 12-myristate 13-acetate. The effects of ATP on $[Ca^{2+}]_i$ could not be accounted for by a generalized increase in plasma-membrane permeability, as evident from the failure of the nucleotide to increase the fluorescence of the nuclear stain ethidium bromide. After stimulation with ATP there was an increase in membrane potential, in both the absence and the presence of extracellular Ca^{2+}. Blockage of the voltage-activated Ca^{2+} channals with D-600, in a Ca^{2+}-containing medium, decreased the effect of ATP on $[Ca^{2+}]_i$ slightly. Patch-clamp measurements using the cell-attached patch configuration revealed that the RINm5F cells produce spontaneous action potentials, the frequency of which increased markedly on addition of ATP. Whole-cell recordings demonstrated that the increase in spike frequency was not associated with the development of an inward current, but was rather accountable for by a decrease in the activity of the ATP-regulated K^+ channels. Addition of 200 μM-ATP stimulated phospholipase C activity, as evident from the formation of $InsP_3$, both in the absence and in the presence of extracellular Ca^{2+}. Thus in the absence of extracellular Ca^{2+} the stimulatory effect of ATP on insulin release can be explained by $InsP_3$-induced mobilization of intracellularly bound Ca^{2+}. Hence, in the RINm5F cells extracellular ATP acts in a manner similar to other Ca^{2+}-mobilizing agents. In addition, it is suggested that ATP stimulation activates a mechanism leading to direct refilling of the $InsP_3$-sensitive Ca^{2+} pool by extracellular Ca^{2+}.

INTRODUCTION

Extracellularly applied ATP affects a variety of biological processes such as secretion, regulation of membrane potential, ionic permeabilities and cell proliferation ([1–11]; for a review see [12]). These effects are in many cases specific for ATP and not mimicked by other adenosine compounds or purine nucleotides. To account for the various actions of the nucleotide and put them into a physiological context, the existence of purinergic receptors has been proposed [13,14]. However, the effects of ATP might also be secondary to a fairly generalized increase in membrane permeability to inorganic ions and small organic molecules [15,16]. Indeed, ATP has been utilized to permeabilize mast cells reversibly, a procedure demanding the absence of extracellular bivalent cations [17,18]. In 1985 Dubyak and de Young performed an extensive study in Ehrlich ascites-tumour cells, demonstrating that micromolar concentrations of extracellular ATP induced a rapid and large increase in $[Ca^{2+}]_i$; the increase was due to both intracellular mobilization and influx over the plasma membrane of Ca^{2+} [7, 19]. Although a more general permeability increase induced by the nucleotide was excluded, the mechanism

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; PLC, phospholipase C; BSA, bovine serum albumin; PKC, protein kinase C.

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underlying the influx of Ca^{2+} was not clarified. In this context it is noteworthy that micromolar concentrations of ATP have been reported to activate a receptor-operated Ca^{2+} channel in arterial smooth-muscle cells [20]. Mobilization of intracellular Ca^{2+} in the Ehrlich ascites cells was found to result from PLC activation and thereby generation of InsP_3 [19]. The extent to which this activation of PLC was due to the nucleotide interacting with a specific purinergic receptor was not clarified.

In the present study we were interested in clarifying whether extracellularly applied ATP affected the regulation of $[Ca^{2+}]_i$ in clonal insulin-producing RINm5F cells and, if that was the case, the possible underlying mechanisms.

MATERIALS AND METHODS

Cell culture

Clonal insulin-producing RINm5F cells were grown in 50 ml Nunclon flasks containing 10 ml of RPMI 1640 medium supplemented with 10% (v/v) NU-Serum or 10% (v/v) fetal-calf serum, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. At confluency, which was obtained ater 4–5 days of culture, cell suspensions were prepared by trypsin treatment as previously described [21]. The cell suspensions were incubated for at least 1 h before any of the experimental procedures. During the incubation time, the culture flasks were shaken gently to keep the cells in suspension. For the patch-clamp experiments, cells were plated on to coverslips and kept in tissue culture for about 2 h before the measurements.

Media

In the patch-clamp experiments, the extracellular solution was composed of (mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$ and 5 Hepes/NaOH (pH 7.4). For investigation of inward Ca^{2+} currents, the pipette was filled with an intracellular solution composed of (mM): 150 *N*-methyl-D-glucamine, 110 HCl, 10 EGTA, 5 Hepes, 1 $MgCl_2$ and 3 Mg–ATP (pH 7.15). This solution replaces the cytoplasm in the whole-cell recordings. In some experiments 0.5 mM-GTP was included in the pipette solution. In the whole-cell experiments, investigating the effect of extracellular ATP on the K^+ conductance, the pipette contained (mM): 125 KCl, 30 KOH, 10 EGTA, 5 Hepes, 2 $CaCl_2$, 1 $MgCl_2$ and 0.3 Mg–ATP or 0.2 Mg–ATP + 0.1 GTP (pH 7.15). The basal medium used in all other experiments was composed of (mM): 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$ or no added Ca^{2+}, 1.2 $MgCl_2$ and 25 Hepes/NaOH (pH 7.4) [22]. The medium was supplemented with 1 mg of bovine serum albumin/ml, except in the membrane-potential measurements with bisoxonol. This is because bisoxonol binds to the albumin and thereby increases basal fluorescence, which decreases the sensitivity of the method.

Recordings of electrical activity and measurements of $[Ca^{2+}]_i$

The procedures for the patch-clamp experiments, using the cell-attached patch and whole-cell configurations, were as described previously [23]. Qualitative changes in membrane potential were assessed by using the fluorescent dye bisoxonol [24]. The dye was used at a concentration of 150 nM, and test substances were added when a stable fluorescence signal had been obtained. The excitation and emission wavelengths were 540 and 580 nm respectively. For the measurements of $[Ca^{2+}]_i$, suspensions of RINm5F cells were incubated for 30 min with 2.5–5 μM-quin-2 acetoxymethyl ester. This resulted in loadings of 1–4 nmol of quin-2/10^6 cells, assuming that 1 mg dry wt. corresponds to 4×10^6 cells [25]. The excitation and emission wavelengths were 340 and 490 nm respectively. All fluorescence recordings were performed in an Aminco–Bowman spectrofluorometer, slightly modified to allow constant stirring of the cell suspensions, at 37 °C, as described previously [23]. The traces shown are representative for experiments performed with at least three different cell preparations.

Production of inositol phosphates

Cell suspensions were labelled with [2-^{3}H]inositol during a 24 h incubation period and then washed twice in the incubation buffer, which contained 11 mM-glucose, 1 mM-inositol and either 1.28 mM or no added Ca^{2+}. The cell suspensions were then transferred to conical glass tubes (about 1×10^6 cells/100 μl of the respective buffer) and incubated for 1 min at 37 °C in the absence or presence of 200 μM-ATP. Incubations were terminated by addition of 20 ml trichloroacetic acid (60%, v/v), and to separate water-soluble inositol phosphates from inositol containing phospholipids, 2 ml of chloroform/methanol/HCl (200:100:1, by vol.) was added to each cell suspension. After addition of 0.5 ml of redistilled deionized water and subsequent mixing, the tubes were centrifuged at 670 *g* for 1 min, to allow the separation of the aqueous phase (1 ml) containing the inositol phosphates, which was then transferred to new glass tubes and neutralized with 1 M-NaOH. Ins*P*, InsP_2 and InsP_3 were separated by ion-exchange chromatography (1 ml column of Dowex 1×8; 200–400 mesh, formate form) as described in ref. [26]. The radioactivities of the fractions containing the different inositol phosphates were then determined by liquid-scintillation counting.

Measurements of insulin release

The dynamics of insulin release were studied by perifusing about 5×10^6 cells mixed with Bio-Gel P-4 polyacrylamide beads in a 0.5 ml column at 37 °C [27]. The flow rate was 0.2 ml/min, and 1 min fractions were collected and assayed for insulin radioimmunologically, with crystalline rat insulin as the standard.

Measurements of cell permeability

These were performed with ethidium bromide, a compound that fluoresces when in contact with nucleic acids [18]. The concentration of dye was 25 μM, and about 2×10^6 cells were used in each experiment. Excitation and emission wavelengths were 365 and 590 nm respectively. The fluorescence recordings were made in an Aminco–Bowman spectrofluorometer as described above. The trace shown is typical for experiments performed with three different cell preparations.

Materials

ATP (disodium salt), quin-2 acetoxymethyl ester, PMA, EGTA and ethidium bromide were from Sigma, St. Louis, MO, U.S.A. [2-^{3}H]Inositol was bought from Amersham, and D-600 was a gift from Knoll A.G., Ludwigshafen am Rhein, Germany. Crystalline rat insulin was a gift from Novo Industry, Copenhagen, Denmark, whereas ^{125}I-labelled insulin and forskolin

were generously supplied by Swedish Hoechst, Stockholm. NU-Serum was from Collaborative Research, Bedford, MA, U.S.A., and culture flasks were purchased from Nunc, Roskilde, Denmark. Bio-Gel P-4 was from Bio-Rad, Richmond, CA, U.S.A.

RESULTS

Fig. 1(*a*) shows that addition of 200 μM-ATP to suspensions of quin-2-loaded RINm5F cells, incubated in the presence of 1.3 mM-Ca^{2+} and 20 mM-glucose, caused a rapid increase in $[Ca^{2+}]_i$, which slowly returned to near basal levels. When the cells were depolarized with 25 mM-K^+, there was again an increase in $[Ca^{2+}]_i$, which could be reversed by blocking the voltage-activated Ca^{2+} channels with D-600. In the continuous presence of D-600 (Fig. 1*b*), the increase in $[Ca^{2+}]_i$ in response to ATP was less pronounced, whereas the effect of K^+ was absent. When cells were stimulated in the absence of extracellular Ca^{2+} (Fig. 1*c*), the effect of ATP was smaller, but still clearly detectable. Despite the restricted release of Ca^{2+}, insulin release was elicited under the latter conditions, as shown in Fig. 1(*d*). These experiments demonstrate that part of the increase in $[Ca^{2+}]_i$, in response to ATP, is due to release of Ca^{2+} from internal stores. It is known that the presence of glucose prevents the loss of Ca^{2+} from intracellular stores, probably by maintaining the intracellular ATP levels, when extracellular Ca^{2+} is low [28]. This is compatible with the fact that the effect of ATP in a Ca^{2+}-free medium was hardly detectable in the absence of glucose, whereas there was a clear increase in $[Ca^{2+}]_i$ in the presence of the sugar (see Table 1 and Fig. 1*c*). Interestingly, the presence of glucose had a tendency to dampen the increase in $[Ca^{2+}]_i$ in response to ATP at 1.3 mM extracellular Ca^{2+}. This might be explained by an increased Ca^{2+} buffering by the cells in the presence of the sugar.

To determine the sensitivity of the cells to ATP, a dose–response curve was established (see Fig. 2). An effect of $[Ca^{2+}]_i$ was detected between 0.2 and 1 μM,

Table 1. Effect of ATP on $[Ca^{2+}]_i$, as measured with quin-2, under a variety of experimental conditions

Values are given as maximal net increases in $[Ca^{2+}]_i$ from the resting level immediately before addition of ATP (200 μM). Values are means ± S.E.M. for four experiments. Statistical significances were calculated by Student's *t* test for paired (comparison within the groups) or unpaired (comparison between the groups) data: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns (not significant, $P \geqslant 0.05$).

Condition	Increase in $[Ca^{2+}]_i$ (nM)	
1.28 mM-Ca^{2+}	344 ± 99*	ns
1.28 mM-Ca^{2+}, 20 mM-glucose	139 ± 28*	
No Ca^{2+}, 0.5 mM-EGTA	6 ± 2	***
No Ca^{2+}, 0.5 mM-EGTA, 20 mM-glucose	54 ± 6**	

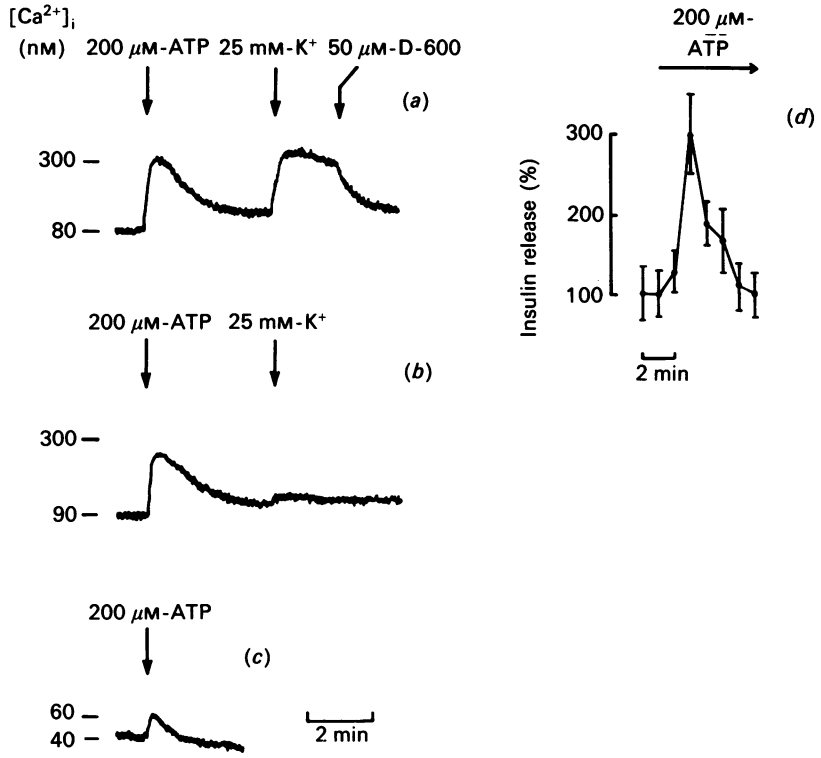

Fig. 1. Effect of 200 μM-ATP on $[Ca^{2+}]_i$ in the presence of 20 mM-glucose at 1.28 mM-Ca^{2+} (*a* and *b*) or no added Ca^{2+} and 0.5 mM-EGTA (*c*)

In (*b*), 50 μM-D-600 was present from the beginning of the experiment. (*d*) Insulin release from perifused RINm5F cells under the same conditions as in (*c*). The mean value of the insulin release during the first 5 min was set to 100%, and all values are given relative to this value. The arrow indicates the introduction of ATP. Values are means ± S.E.M. for three experiments.

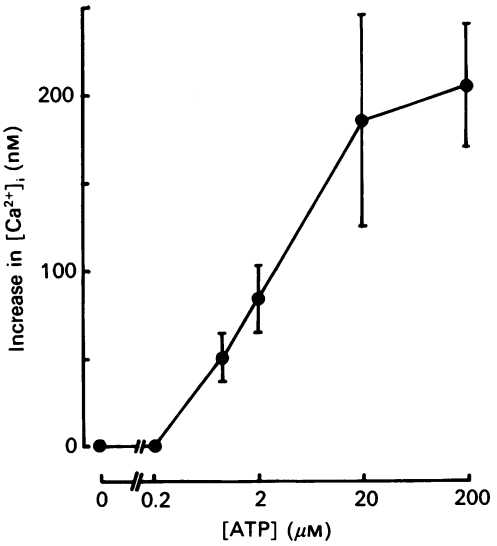

Fig. 2. Dose-response of the ATP-induced increase in $[Ca^{2+}]_i$

The experiments were performed at 1.28 mM-Ca^{2+} in the absence of glucose. Values are given as net increases in $[Ca^{2+}]_i$ above the resting level immediately before the addition of ATP. Values are means ± S.E.M. for five experiments.

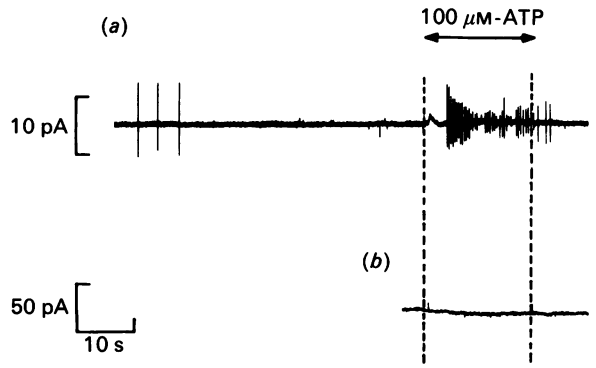

Fig. 3. Effect of ATP on the electrical activity in RINm5F cells

(*a*) Cell-attached patch recording at the cell's membrane potential. ATP was added to the bath during the interval indicated by the arrow. (*b*) Recording after establishing the whole-cell configuration in the same cell. The cell membrane potential was held at −70 mV.

whereas half-maximal stimulation was observed at about 2 μM-ATP. Addition of 200 μM-ATP elicited a maximal response. The concentration of ATP that produces a detectable effect might be even lower than observed here, since it is likely that intracellular quin-2 decreases the effects on $[Ca^{2+}]_i$ because of its Ca^{2+}-buffering capacity. Extracellularly applied ATP also activated PLC in the RINm5F cells. As shown in Table 2, there was a significant increase in the production of InsP_3 in both the presence and the absence of extracellular Ca^{2+}. Addition of ATP also enhanced the production of InsP_2 in a Ca^{2+}-containing medium.

Fig. 3(*a*) shows a patch-clamp recording performed in the cell-attached configuration. Many cells produced spontaneous action potentials, seen as biphasic current deflections, as previously observed also by other investigators [29]. The action potentials in the RINm5F cells are known to involve inward Na^+ and Ca^{2+} currents and a delayed outward K^+ current [30]. In this cell, addition of 100 μM-ATP markedly increased the spike frequency (Fig. 3*a*). The effect was completely reversible upon removal of the nucleotide, and re-addition of ATP again induced an increase in spike frequency several times (results not shown). Fig. 3(*b*) shows a whole-cell recording from the same cell. Whether in the presence or absence of GTP, application of ATP failed to elicit any inward currents (cf. [20]), which could have accounted for the increase in spike frequency. Moreover, addition of ATP did not directly affect the Ca^{2+} inflow through the voltage-activated Ca^{2+} channels (result not shown). The same negative results were obtained in several cells from different preparations. Nevertheless, in view of the rapid onset and reversal of the effects produced by ATP, it appears likely that the nucleotide affects spike frequency in the RINm5F cells by interaction with an ion channel. An increased spike frequency could also result from the inhibition of an outward current. Fig. 4 shows a recording in which small hyper- and de-polarizing pulses from the holding potential of −70 mV were used to monitor changes in the activity of K^+ channels, which constitute the RINm5F-cell resting conductance. Addition of 100 μM extracellular ATP decreased the K^+ conductance when applied shortly after wash-out of ATP, seen as an increase in K^+ conductance. This decreasing effect of ATP could not be repeated by a second pulse of the nucleotide, which may be due to the wash-out of intracellular coupling factors. Fig. 5 shows that 200 μM-ATP caused a depolarization in the RINm5F cells, in both the presence and the absence of extracellular Ca^{2+}. This effect, however, was not as pronounced as that induced by 25 mM-K^+. Nevertheless, in view of the small effect of D-600, the ATP-induced depolarization and

Table 2. Production of inositol phosphates after 1 min stimulation with ATP in the presence of 11 mM-glucose

Values (d.p.m./10^6 cells) are means ± S.E.M. for *n* experiments. Statistical significances were calculated by Student's *t* test for paired data: $*P < 0.05$, $**P < 0.01$ versus control.

Condition	Addition	Ins*P*	InsP_2	InsP_3	*n*
1.28 mM-Ca^{2+}	Control	688 ± 199	301 ± 99	724 ± 184	5
1.28 mM-Ca^{2+}	200 μM-ATP	654 ± 204	370 ± 104*	1002 ± 180**	5
No Ca^{2+}, 0.5 mM-EGTA	Control	1075 ± 304	182 ± 20	429 ± 63	7
No Ca^{2+}, 0.5 mM-EGTA	200 μM-ATP	1068 ± 312	245 ± 43	533 ± 84*	7

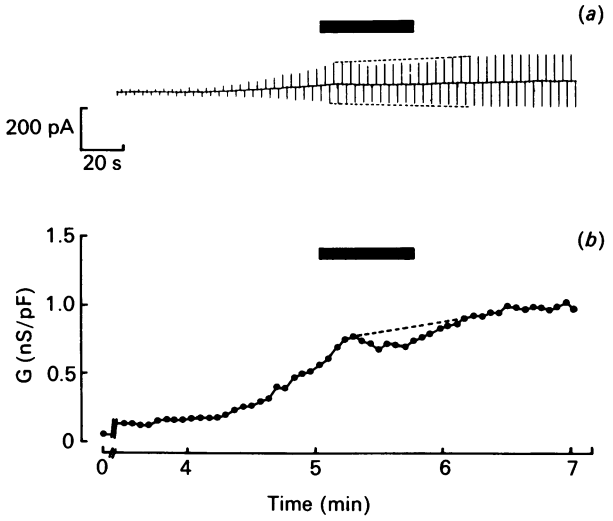

Fig. 4. Effect of ATP on the increase in K^+ conductance caused by wash-out of intracellular ATP in the whole-cell configuration

(*a*) The cell was held at −70 mV and current responses to alternating 20 mV hyper- and de-polarizing pulses were used to monitor changes in the activity of the ATP-regulated K^+ channels. The black bar indicates the introduction of 100 μM-ATP in the bath when applied shortly after wash-out of ATP, as evidenced by increased K^+ conductance. The delay is due to the dead-space of the perifusion system. (*b*) Effect of ATP on the K^+ conductance resulting from wash-out of intracellular ATP (note the slight decrease). Similar effects were observed in three other cells. The black bar indicates the introduction of ATP. The broken lines in both (*a*) and (*b*) represent extrapolated values expected in the absence of additions. Times are given in minutes after formation of the whole-cell configuration. The experiments were performed in the presence of 0.3 mM-ATP or 0.2 mM-ATP + 0.1 mM-GTP in order to facilitate detection of possible changes in activity of ATP-regulated K^+ channels. A similar change in channel activity in the intact cell (0 min) should increase spike frequency.

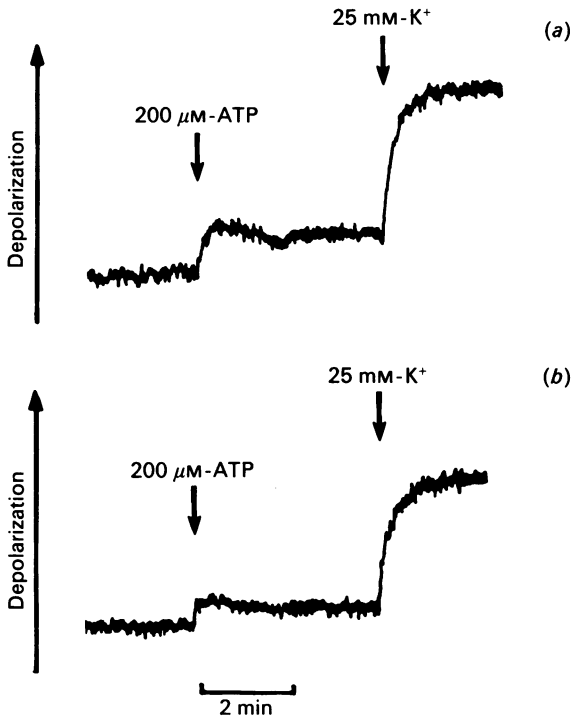

Fig. 5. Effects of ATP and K^+ on the membrane potential of RINm5F cells in the presence of 20 mM-glucose

Qualitative changes in membrane potential were monitored with the fluorescent dye bisoxonol. An increase in fluorescence denotes depolarization of the cells. (*a*) 1.28 mM-Ca^{2+}; (*b*) no Ca^{2+} and 0.5 mM-EGTA.

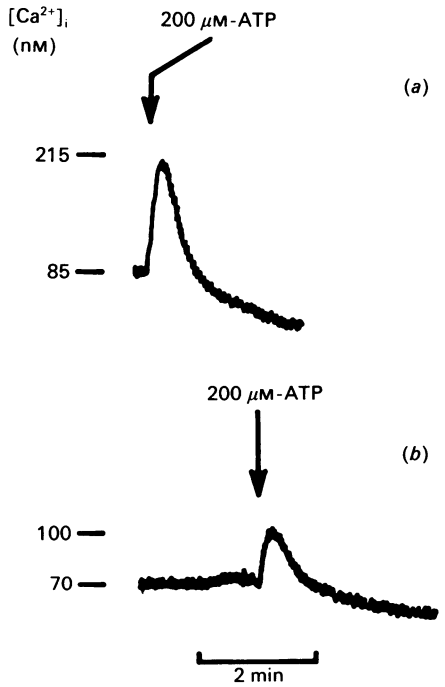

Fig. 6. Effect of ATP on $[Ca^{2+}]_i$, as measured with quin-2 in suspensions of RINm5F cells, after addition of 2 mM-EGTA to a medium containing 1.28 mM-Ca^{2+} and 20 mM-glucose

ATP (200 μM) was added 12 s (*a*) or 2 min (*b*) after the addition of EGTA.

thereby Ca^{2+} influx through voltage-activated Ca^{2+} channels can only account for a minor part of the nucleotide-induced increase in $[Ca^{2+}]_i$ (cf. Fig. 1*b*).

To determine the extent to which release of intracellularly bound Ca^{2+} contributed to the observed effects of ATP on $[Ca^{2+}]_i$, different experimental protocols were used. In the initial experiments, a low concentration of $LaCl_3$ was added to the Ca^{2+}-containing medium (buffered with Tris base instead of Hepes). Theoretically, this approach should make it possible to study intracellular Ca^{2+} release in a Ca^{2+}-containing medium under conditions where Ca^{2+} influx is efficiently blocked and intracellular Ca^{2+} stores remain filled [7,31]. However, it seemed that ATP formed a complex with $LaCl_3$ and such an interaction made the results difficult to interpret (not shown). To avoid this type of problem we used another approach. The cells were incubated in Ca^{2+}-containing medium supplemented with 20 mM-glucose, and to lower the external Ca^{2+} concentration promptly to the μM range, 2 mM-EGTA was added. The cells were subsequently stimulated with 200 μM-ATP (Fig. 6). When added 12 s after removal of extracellular Ca^{2+}, the

nucleotide still elicited a marked peak in $[Ca^{2+}]_i$ (Fig. 6*a*), although more transient than that observed in the presence of extracellular Ca^{2+} (cf. Fig. 1*b*). Moreover, this increase in $[Ca^{2+}]_i$ was drastically decreased when the cells were incubated in the Ca^{2+}-deprived medium for 2 min before ATP addition (Fig. 6*b*). In Fig. 7 we investigated the dependence of the InsP_3-sensitive Ca^{2+} pool on extracellular Ca^{2+}, under conditions where the Ca^{2+} channels were blocked by D-600. Raising the external Ca^{2+} concentration from about 10 μM to 1.3 mM induced an increase in $[Ca^{2+}]_i$, which settled at around 130 nM (Fig. 7*a*). Subsequent addition of 200 μM-ATP produced a response similar to that previously demonstrated (cf. Fig. 1*b*). When the nucleotide was added 1 min or more before Ca^{2+} (Fig. 7*b*), the Ca^{2+}-induced rise in $[Ca^{2+}]_i$ was similar to that observed in the absence of ATP. However, simultaneous addition of Ca^{2+} and ATP produced a larger increase than Ca^{2+} alone, corresponding to a transient peak maximal at about 1 min after the addition, before returning to the resting level normally observed in the presence of extracellular Ca^{2+} (Fig. 7*c*). Hence the ATP response was immediately restored in the latter situation (cf. Fig. 1*b*). The data suggest that release and refilling of the InsP_3-sensitive Ca^{2+} pool occur almost simultaneously (Fig. 7*c*). From these experiments, it might be suggested either that ATP very transiently increases the permeability to Ca^{2+} or, more likely in view of the patch-clamp data, that most of the response observed also in a Ca^{2+}-containing medium is due to release of intracellularly bound Ca^{2+}. If the latter is true, the filling of the InsP_3-sensitive Ca^{2+} pool is accounted for by a direct Ca^{2+} influx from the extracellular space.

By performing experiments such as those described in Table 3, an attempt was made to obtain more detailed information about the mechanisms whereby extracellularly applied ATP influences Ca^{2+} metabolism in RINm5F cells. When the intracellular level of cyclic AMP was increased, by stimulating the adenylate cyclase

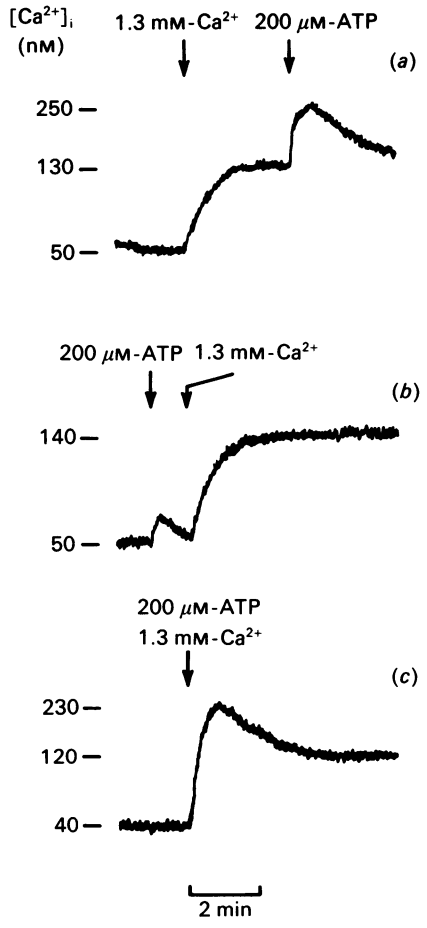

Fig. 7. Effect of ATP and 1.3 mM-Ca^{2+} on $[Ca^{2+}]_i$ in suspensions of RINm5F cells, as measured with quin-2

The voltage-activated Ca^{2+}-channel blocker D-600 was present at 50 μM throughout all the experiments.

Table 3. Effect of ATP on $[Ca^{2+}]_i$ as measured with quin-2 at 1.28 mM-Ca^{2+} in the absence of glucose

Suspensions were preincubated with various agents for the lengths of time given in the Table. Values are means ±S.E.M. for *n* experiments. Statistical significances were calculated by Student's *t* test: $^{**}P < 0.01$ versus control.

Conditions	Increase in $[Ca^{2+}]_i$ (nM)	*n*
Control	214±35	9
5 μM-forskolin, 3 min	276±94	5
10 nM-TPA, 3 min	4±4**	4

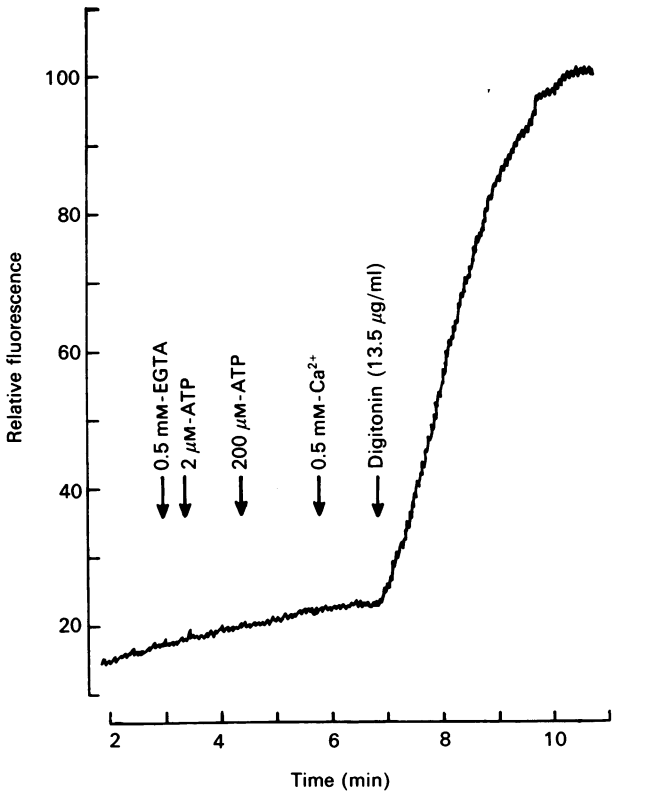

Fig. 8. Effect of EGTA, ATP and digitonin on membrane permeability of RINm5F cells incubated in a Ca^{2+}-deficient medium

Permeability changes were monitored by using the fluorescent dye ethidium bromide, which increases its fluorescence upon binding to nucleic acid.

with 5 μM-forskolin, the effect of ATP on $[Ca^{2+}]_i$ was unaffected. However, if instead PKC was activated with 10 nM-PMA, the increase in $[Ca^{2+}]_i$ normally evoked by the nucleotide was almost undetectable. The latter finding was not unexpected, since it has been reported that activation of PKC with PMA interferes with the formation of InsP_3 in RINm5F cells as well as in other cell types [32–34].

To exclude the possibility that extracellular ATP caused unspecific permeabilization of the plasma membrane of the RINm5F cells, permeability changes were monitored with ethidium bromide. Concentrations of ATP up to 200 μM, added to cells incubated in the absence of extracellular Ca^{2+}, had no detectable effect on their permeability, as shown in Fig. 8. The continuous slow increase in ethidium bromide fluorescence observed was probably due to cells being damaged by the continuous stirring of the suspension.

DISCUSSION

It is well established that glucose has to be metabolized in order to evoke insulin release in normal pancreatic β-cells [35,36]. It is currently believed that metabolism leads to an increase in the cytoplasmic ATP concentration, which then brings about a closure of ATP-regulated K^+ channels [23,37,38]. This results in depolarization, opening of voltage-activated Ca^{2+} channels, an increase in $[Ca^{2+}]_i$ and eventually insulin release [23]. The RINm5F cells are also equipped with ATP-regulated K^+ channels [39]. However, in these cells glucose fails to promote insulin release [40], whereas glyceraldehyde has a pronounced stimulatory effect [41], suggesting a disturbance in their glucose metabolism. Since glyceraldehyde stimulation has been shown to increase the cellular content of 1,2-diacylglycerol [42], and direct activation of PKC by PMA or 1,2-didecanoylglycerol was found to close the ATP-regulated K^+ channels, it was suggested that these channels are operated by PKC in the RINm5F cells [42]. Nevertheless, when discussing effects of glyceraldehyde in these cells, it should be remembered that this compound lowers intracellular pH, which might in itself decrease the activity of these channels [43,44]. In accordance with the effect of PKC activation on the ATP-regulated K^+ channels, we have previously found that PMA induces a small depolarization in the RINm5F cells [21]. Since extracellularly applied ATP was found to activate PLC and thereby generate not only InsP_3 but also 1,2-diacylglycerol, the modest depolarization observed here might be explained by an activation of PKC. Our results favour the notion that stimulation with extracellular ATP leads to closure of the ATP-regulated K^+ channels (cf. [42]). Considering that ATP did not evoke an inward current, it seems reasonable to speculate that the nucleotide induced activation of PKC and thereby a closure of the ATP-regulated K^+ channels. This would result in a small depolarization, which may well be sufficient to explain the observed increase in spike frequency (cf. [29] and [30]). Indeed, PKC activation with PMA has been reported to increase the electrical activity of glucose-stimulated β-cells [45]. Recently, it was demonstrated that glucose and tolbutamide, probably through their action on the ATP-regulated K^+ channels, can modulate spike frequency in persistently depolarized normal β-cells [46,47].

The dose-dependence and also the effects of extracellular ATP on $[Ca^{2+}]_i$ in RINm5F cells, constituting both an extra- and an intra-cellular Ca^{2+} component, were similar to those previously demonstrated in other cell types [1,7,48–50]. In accordance with the effects in rat aortic myocytes, pancreatic islets and Ehrlich ascites cells [19,48,51], the intracellular component of the RINm5F cells was mediated through the formation of InsP_3 and Ca^{2+} release from the endoplasmic reticulum or a specific InsP_3-sensitive Ca^{2+} pool such as the calciosome [52]. This finding is in contrast with a previous study, where an effect of ATP on the formation of Ins$P$, but not InsP_2 and InsP_3, could be observed [51]. This might reflect that RINm5F cells in various laboratories are different. The ATP effect in normal β-cells is probably mediated through purinergic (P_2) receptors [1,50,51].

Despite elaborate experiments, we cannot at present give a full account of how extracellular Ca^{2+} contributes to the ATP-induced rise in $[Ca^{2+}]_i$. However, our data demonstrate that it cannot be explained by unspecific leakage of Ca^{2+} into the cell. The patch-clamp analyses did not favour either the existence of specific ATP-operated Ca^{2+} channels [20] or any major influx of Ca^{2+} through voltage-activated Ca^{2+} channels. The latter finding is compatible with both the membrane-potential recordings and the measurements of $[Ca^{2+}]_i$ in the presence of D-600. The existence of an extracellular component is suggested from experiments demonstrating a more pronounced ATP-induced increase in $[Ca^{2+}]_i$ in the presence than in the absence of extracellular Ca^{2+}. As is obvious from the present study, a decrease in the extracellular Ca^{2+} concentration results in a lowering of $[Ca^{2+}]_i$ and a restricted filling of the InsP_3-sensitive Ca^{2+} pool. A normalization of the extracellular Ca^{2+} concentration restores $[Ca^{2+}]_i$ within about 2 min, and, in connection with this, also the InsP_3-sensitive Ca^{2+} pool. When the extracellular Ca^{2+} concentration was raised simultaneously with ATP stimulation, the normal response to the nucleotide was observed. Hence it seems likely that the InsP_3-sensitive Ca^{2+} pool was replenished instantly, when the extracellular Ca^{2+} concentration was normalized. Since we do not favour the idea that ATP stimulation activates a major influx of Ca^{2+} into the cytoplasm, and thereby subsequent refilling of the InsP_3-sensitive Ca^{2+} pool, we suggest that this refilling is rather due to a mechanism related to that postulated by Putney [53], namely that the emptying of the InsP_3-sensitive Ca^{2+} pool activates Ca^{2+} influx across the plasma membrane, by a so far unknown mechanism, directly into this pool. The presence of extracellular Ca^{2+} should then consequently allow continuous refilling, resulting in a more pronounced InsP_3-induced Ca^{2+} release. Thus the ATP-induced increase in $[Ca^{2+}]_i$ in the RINm5F cells, also in the presence of extracellular Ca^{2+}, could be due almost entirely to release of intracellularly bound Ca^{2+}. The model postulated by Putney was originally designed to explain agonist-induced mobilization of Ca^{2+} in non-excitable cells, but might consequently apply also to excitable cells. Whereas the InsP_3-sensitive Ca^{2+} pool in resting non-excitable cells is relatively stable in the absence of extracellular Ca^{2+} [53,54], this does not apply to the RINm5F cells, where this pool was rapidly diminished in Ca^{2+}-deficient media. Hence there seem to exist differences in the regulation of the InsP_3-sensitive Ca^{2+} pool between various cell types.

In a number of studies dealing with the regulatory

mechanisms of phosphoinositide metabolism, it has been suggested that there exists a certain 'cross-talk' between the adenylate cyclase and PLC systems [55–57]. Irrespective of whether activation of PKC only inhibits $Ins P_3$ formation [32] or also interacts at other sites in the signal-transduction pathway, the ATP-induced increase in $[Ca^{2+}]_i$ in the RINm5F cells was drastically inhibited after stimulation with PMA. It was recently demonstrated that a cyclic-AMP-mediated phosphorylation of the $Ins P_3$ receptor restricted the ability of the trisphosphate to release Ca^{2+} from microsomes [57]. However, an increase in intracellular cyclic AMP levels simultaneously enhanced Ca^{2+} uptake into the $Ins P_3$-sensitive Ca^{2+} pool, and thus compensated for the diminished potency of $Ins P_3$ to release Ca^{2+} [57]. The latter effect is compatible with the $[Ca^{2+}]_i$ results obtained under the present experimental conditions, demonstrating no effect of a direct activation of the adenylate cyclase system by forskolin on the ATP response.

It is well known that insulin release in response to glucose stimulation is biphasic [36]. From time to time it has been suggested that the first phase of insulin release involves mobilization of intracellularly bound Ca^{2+} [36]. Although there is no conclusive evidence supporting such a mechanism in glucose-stimulated insulin release, it is noteworthy that we have previously demonstrated both a carbamoylcholine-induced increase in $[Ca^{2+}]_i$ and insulin release in normal pancreatic β-cells in the absence of extracellular Ca^{2+} [28]. Under similar experimental conditions, we can now demonstrate that the ATP-induced increase in $[Ca^{2+}]_i$ in the RINm5F cells is paralleled by an increase in insulin release. Both the effect of carbamoylcholine in normal β-cells and that of ATP in RINm5F cells are accounted for by the activation of PLC and thereby the formation of $Ins P_3$. In this context it should be noted that these cell types display somewhat different characteristics with regard to the $Ins P_3$-sensitive Ca^{2+} pool [28]. It is thus possible to elicit a sustained intracellular release of Ca^{2+} with $Ins P_3$ in permeabilized RINm5F cells [58], whereas in permeabilized normal β-cells this pool seems to be desensitized to $Ins P_3$, or its Ca^{2+} is redistributed to pools not sensitive to $Ins P_3$ [28]. Such a difference might also explain the more transient effects observed with carbamoylcholine in the normal β-cells [59] compared with the extended response to ATP in the RINm5F cells, under similar experimental conditions. Possibly this might reflect a more differentiated regulation of the intracellular Ca^{2+} movements in normal β-cells.

The present study demonstrates that the interaction of extracellular ATP with the stimulus–secretion coupling in RINm5F cells can be explained by the fact that this nucleotide acts in a manner similar to other Ca^{2+}-mobilizing agonists. Moreover, it is suggested that ATP stimulation, by an as yet unidentified mechanism, directly activates the refilling of the $Ins P_3$-sensitive Ca^{2+} pool with extracellular Ca^{2+}.

This work was supported by the Swedish Medical Research Council (19X-00034 and 12X-08647), the Swedish Diabetes Association, the Nordic Insulin Foundation, the Swedish Hoechst Diabetes Research Foundation, Åke Wibergs Stiftelse, Syskonen Svenssons Fond, Magn. Bergvalls Stiftelse, O.E. och Edla Johanssons Fond, Stiftelsen Lars Hiertas Minne, the Bank of Sweden Tercentenary Foundation, Tore Nilsons Foundation for Medical Research, Clas Groschinskys Minnesfond, Familjen Ernfors Stiftelse, Aage and Louis-Hansens Memorial Foundation, Novo Industry, Farmitalia Carlo Erba, Funds of the Karolinska Institute and the medical Faculty of Gothenburg University. P. R. holds a postdoctoral fellowship at the Swedish Medical Research Council.

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Received 20 June 1989/4 September 1989; accepted 7 September 1989