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# External ATP mimics carbachol in initiating calcium mobilization from pancreatic β-cells conditioned by previous exposure to glucose

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**1** Exposure to ATP (2–200 μM) resulted in a prominent peak of $^{45}Ca$ efflux, when β-cell-rich pancreatic islets from *ob/ob*-mice were perifused with a $Ca^{2+}$-deficient medium. ADP and the stable α/β-methylene analogues of ATP and ADP also had stimulatory effects.

**2** The nucleotide initiation of $^{45}Ca$ efflux mimicked that obtained with carbachol both in requiring previous exposure to glucose and in being more pronounced after replacing extracellular $Na^+$ by $K^+$.

**3** It was possible to induce repeated peaks of stimulated $^{45}Ca$ efflux, when the exposure to ATP was interrupted with intervals of perifusion with glucose-containing media.

**4** The observations are consistent with the existence of $P_2$-purinoceptors in islets, suggesting that these receptors mediate a similar mobilization of calcium as noted when activating polyphosphoinositide breakdown with carbachol. In view of the high contents of ATP and ADP in the β-cell secretory granules, activation of $P_2$-purinoceptors should be considered as a possible mechanism for amplification of the initial insulin secretory response.

## Introduction

Measurements of the unidirectional efflux of $^{45}Ca$ from isolated pancreatic islets during perifusion with $Ca^{2+}$-deficient medium provide a sensitive means for studying mobilization of intracellular calcium. Using this technique it was found that carbachol activation of muscarinic receptors results in a marked initial mobilization of calcium from islets previously exposed to glucose (Hellman & Gylfe, 1986). The glucose dependence of the carbachol action also became evident when measuring the cytoplasmic $Ca^{2+}$ activity in the insulin-releasing RINm5F cells (Hellman *et al.*, 1986) or when using such cells for studying net fluxes of $Ca^{2+}$ with dual wavelength spectrophotometry (Gylfe & Hellman, 1986). With the latter approach it was actually possible to demonstrate that glucose induced the sequestration of $Ca^{2+}$ in the pool mobilized by carbachol.

External ATP has been found to stimulate insulin release in the presence of glucose in experiments with pieces of rabbit pancreas (Candela *et al.*, 1963) or the perfused rat pancreas (Sussman *et al.*, 1969; Loubatières *et al.*, 1972). After analysing the nucleotide specificity of this effect, it was proposed that the pancreatic β-cells are equipped with $P_2$-purinoceptors (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981). The present studies provide further support for this view, indicating that external ATP induces glucose-dependent mobilization of intracellular calcium similar to that obtained with muscarinic receptor activation.

## Methods

Adult *ob/ob*-mice were taken from a non-inbred colony (Hellman, 1965) and deprived of food overnight. The animals were killed by decapitation and pancreatic islets isolated by a collagenase technique. Previous studies have indicated that these islets contain more than 90% β-cells, which respond normally to glucose and other stimulators of insulin release (Hellman, 1970; Hahn *et al.*, 1974). The basal medium used for the experiments was a HEPES buffer with $Cl^-$ as the sole anion (Hellman, 1975). When analysing the effects of omitting $Na^+$, osmotic compensation was achieved by replacing NaCl with KCl and adjusting the pH with KOH instead of NaOH.

The dynamics of $^{45}Ca$ efflux and insulin release were studied by a previously described procedure (Gylfe & Hellman, 1978). The islets were loaded for 90 min at 37°C with 1.28 mM $^{45}Ca$ (391 Ci $mol^{-1}$) in the presence of 20 mM glucose. After two 3-min washes, batches of 8–10 islets were transferred to a 10 μl chamber and perifused at a constant rate of about 40 μl $min^{-1}$ with a

non-radioactive medium supplemented with albumin 1 mg ml$^{-1}$. When not otherwise stated, the islets were perifused at 37°C with a medium deprived of $Ca^{2+}$ and supplemented with 0.5 mM EGTA (final $Ca^{2+}$ concentration $<0.01$ μM). Details about the presence of glucose and other additives are given in the legends to the figures. In order to facilitate comparison, two or three chambers were perifused in parallel with different media using islets from the same animal. After perifusion the islets were removed from the chambers, freeze-dried overnight and weighed on a quartz fibre balance. $^{45}Ca$ was measured by liquid scintillation counting, and insulin assayed radioimmunologically with crystalline mouse insulin as a reference. The results are presented as percentages of the average $^{45}Ca$ efflux and insulin release recorded in the individual experiment between 50 and 60 min of perifusion. When analysing the interactions between ATP and carbachol, measurements of the radioactivity in the islets made it possible to calculate the fractional outflow rate, defined as the percentage release per min of the instantaneous islet content of $^{45}Ca$. The statistical significance of effects was assessed from paired *t* tests of experimental and control data obtained during parallel perifusions of islets from the same animal. The number of animals analysed is given in the legends to the figures.

Adenosine-5'-triphosphate (ATP) was used as a sodium salt supplied either by Boehringer Mannheim GmbH, FRG, or Sigma Chemical Co., St Louis, MO, U.S.A. Other products from Boehringer were the sodium salts of adenosine-5'-monophosphate (AMP), adenosine-5-diphosphate (ADP) and guanosine-5'-triphosphate (GTP). Sigma also supplied adenosine, α/β-methylene analogues of ATP and ADP as well as the β/γ-methylene analogue of ATP.

## Results

Both ATP and ADP induced a prominent initial peak of $^{45}Ca$ efflux from islets perifused with medium deficient in $Ca^{2+}$ (Figure 1). This effect was critically dependent on the presence of glucose, being virtually absent in a medium devoid of the sugar. As shown from the inset in Figure 1a, the ATP-induced mobilization of $Ca^{2+}$ was not sufficient to cause a concomitant increase of insulin release. ATP and ADP

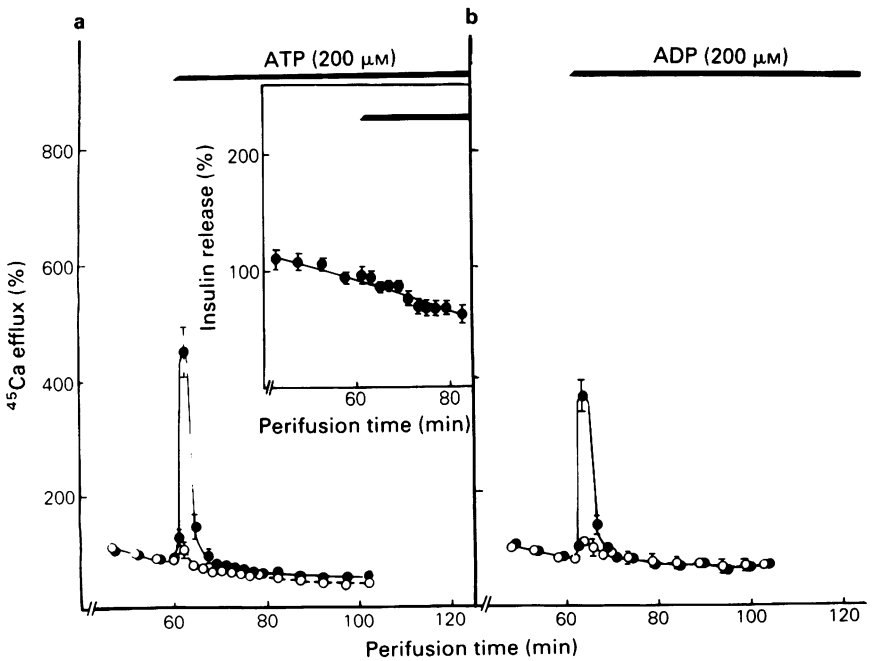


**Figure 1** Effects of ATP and ADP on $^{45}Ca$ efflux in the presence and absence of glucose. After loading with $^{45}Ca$, the islets were perifused with (●) or without (○) 20 mM glucose in a $Ca^{2+}$-deficient medium containing 0.5 mM EGTA. ATP (a) or ADP (b) was added at a concentration of 200 μM during the periods indicated by the horizontal bars. The sloping ends of these bars indicate the non-instantaneous change of concentration due to mixing. The inset in (a) shows insulin release during the initial peak of ATP-induced $^{45}Ca$ efflux obtained in the presence of glucose. Mean values for 4 experiments; s.e.mean shown by vertical lines.

were the most effective among tested nucleotides in promoting the efflux of $^{45}Ca$. In addition, 200 μM of the more stable α/β-methylene analogues provoked clear mobilization of calcium, but equimolar amounts of AMP, adenosine, GTP and β/γ-methylene ATP lacked distinct effects (Figure 2). Inclusion of $Ca^{2+}$ in the perifusion medium resulted in suppression of the nucleotide stimulation of the $^{45}Ca$ efflux. At physiological concentrations of $Ca^{2+}$, the effect of ATP was small, irrespective of the presence of glucose or whether the temperature was lowered to 20°C (Figure 3).

The concentration-dependence for the ATP-induced mobilization of $Ca^{2+}$ during perifusion with a $Ca^{2+}$-deficient medium containing glucose (20 mM) is shown in Figure 4. As little as 0.2 μM ATP was sufficient to induce a statistically significant mobilization of $Ca^{2+}$ ($P < 0.05$). The ATP stimulation of $^{45}Ca$ efflux was dependent on previous exposure to glucose rather than on the simultaneous presence of the sugar. Consequently, ATP was also able to mobilize calcium when glucose was removed 6 min before the introduction of the nucleotide (Figure 5a). It was possible to induce repeated peaks of $^{45}Ca$ efflux, when the exposure to ATP was interrupted with intervals of perifusion with a glucose-containing medium. The second peak was lower than the original one, even when considering the decline of the response obtained with the prolongation of perifusion (Figure 5b). Whilst the ATP stimulation became more pronounced when $Na^+$ was replaced with $K^+$ in the perifusion medium, it was somewhat reduced after lowering the temperature to 20°C (Figure 6). Other experiments indicated a nearly complete disappearance of the ATP stimulation after removing $Mg^{2+}$ from the perifusion medium or inhibiting phosphoinositide hydrolysis with 10 mM neomycin (Figure 7).

Figure 8 presents the results obtained when exposing the islets to ATP before and after carbachol. Previous exposure to ATP resulted in marked reduction of the amounts of $^{45}Ca$ mobilized by ATP. The response to ATP was suppressed compared with non-carbachol exposed islets also when the efflux rate was related to the islet content of $^{45}Ca$. After exposure to carbachol the increase of the fractional outflow rate obtained with ATP was $0.64 \pm 0.11\%$, $min^{-1}$ (mean value ± s.e.mean), corresponding to a reduction by $1.33 \pm 0.50\%$, $min^{-1}$ ($P < 0.05$). When introducing the compounds in the opposite order, ATP depressed the stimulation obtained with carbachol by

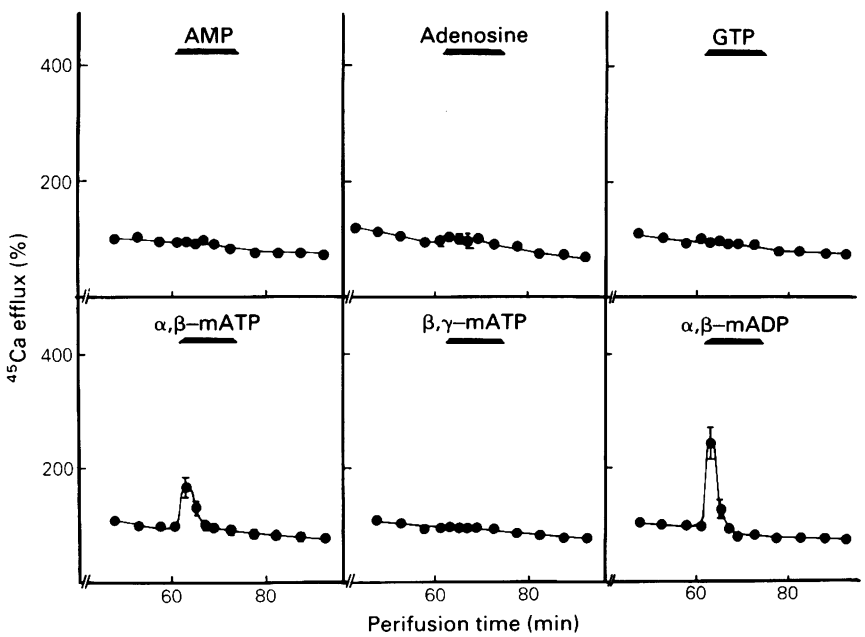


**Figure 2** Effects of different nucleotides on $^{45}Ca$ efflux in the presence of glucose. After loading with $^{45}Ca$ the islets were perifused with a $Ca^{2+}$-deficient medium containing 0.5 mM EGTA and 20 mM glucose. During the periods indicated by the horizontal black bars the islets were exposed to 200 μM of either AMP, adenosine, GTP, α/β-methylene ATP (α,β-mATP), β/γ-methylene ATP (β,γ-mATP) or α/β-methylene ADP (α,β-mADP). Mean values for 4–5 experiments; s.e.mean shown by vertical lines.

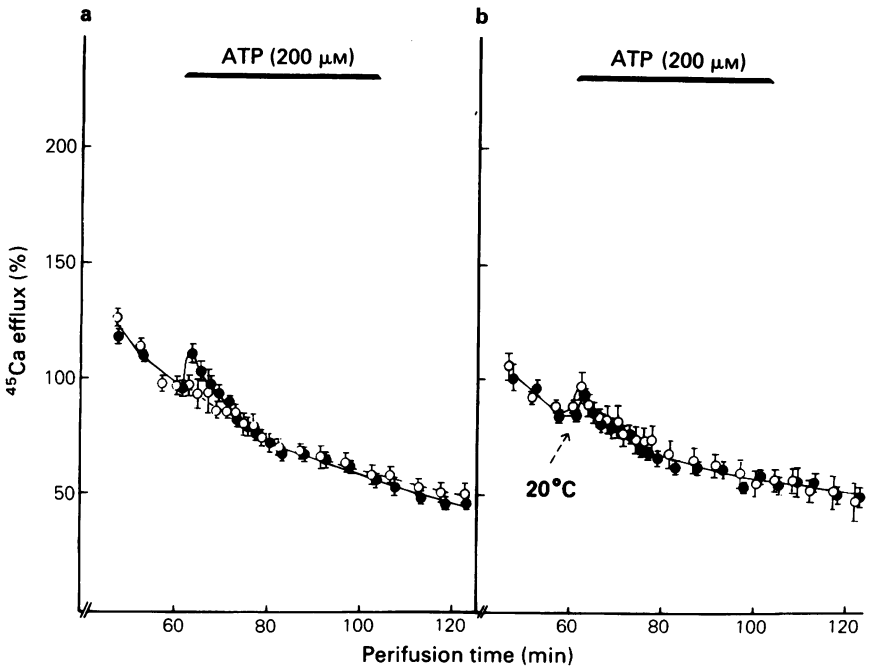


**Figure 3** Effect of ATP on $^{45}Ca$ efflux in the presence of physiological concentrations of $Ca^{2+}$. After loading with $^{45}Ca$, the islets were perifused in the presence of 5 (a) or 20 mM (b) glucose with a medium containing 1.28 mM $Ca^{2+}$. In (a) the islets were exposed (●) or not (○) to 200 μM ATP. In (b) the islets were exposed to 200 μM ATP either at the normal temperature of 37°C (○) or after reducing the temperature to 20°C (●). Mean values for 5 experiments; s.e.mean shown by vertical lines.

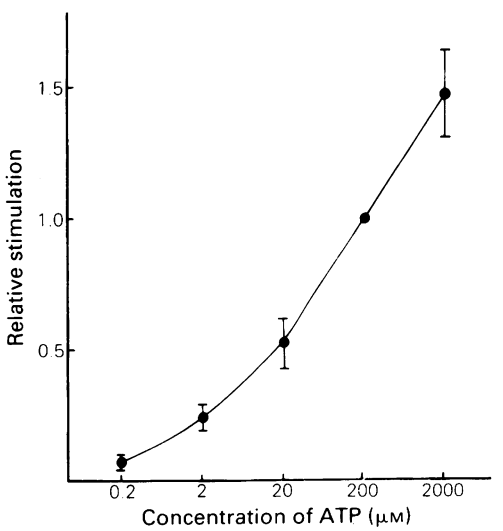


**Figure 4** Relative stimulation of $^{45}Ca$ efflux obtained with different concentrations of ATP. In experiments designed as in Figure 1 the islets were exposed to ATP after 60 min of perifusion with a $Ca^{2+}$-deficient medium containing 0.5 mM EGTA and 20 mM glucose. The symbols denote maximal increments obtained with ATP relative to the effect of 200 μM of this nucleotide observed in parallel experiments with islets from the same animal. Mean values for 7–8 experiments; s.e.mean shown by vertical lines.

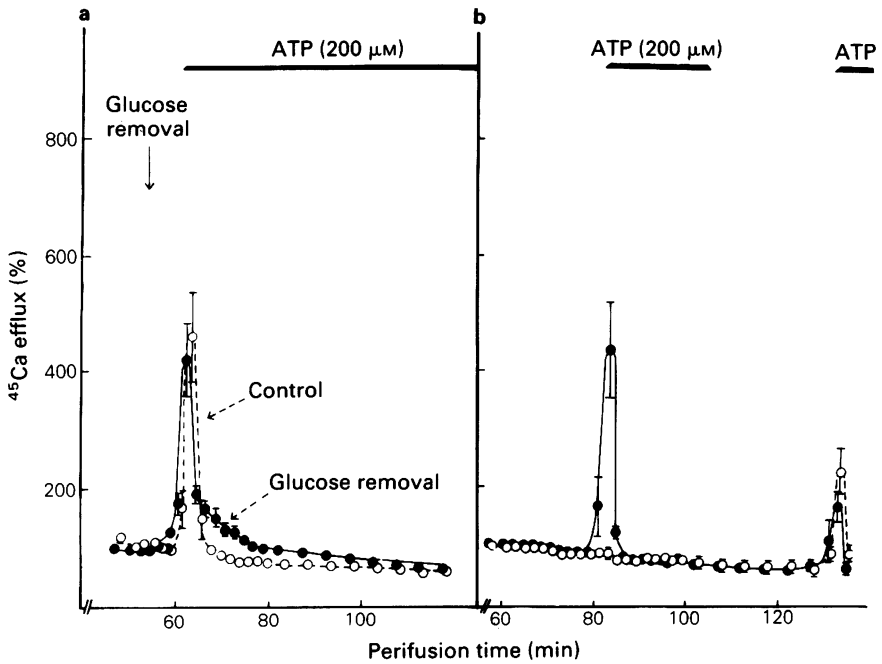


**Figure 5** Effects of ATP on $^{45}Ca$ efflux after removal of glucose (a) or repeated exposure to the nucleotide (b). After loading with $^{45}Ca$, the islets were perifused with a $Ca^{2+}$-deficient medium containing 0.5 mM EGTA. In (a) the effect of 200 μM ATP 6 min after removal of 20 mM glucose (●) is compared with that obtained on islets continuously exposed to the same concentration of glucose (○). Panel (b) indicates the effects obtained with 200 μM ATP in the presence of 20 mM glucose, when the islets were exposed once (○) or twice (●) to the same concentration of the nucleotide. Mean values for 5 experiments; s.e.mean shown by vertical lines.

$1.12 \pm 0.44\%$, $min^{-1}$ ($P < 0.05$). These significances were even higher ($P < 0.01$) with Wilcoxon's non-parametric matched-pairs test, since suppression was found in all of the 8 experiments in both series.

## Discussion

In the present study ATP stimulated the radioactive efflux from pancreatic islets preloaded with $^{45}Ca$ to apparent isotopic equilibrium and perifused with a medium essentially lacking $Ca^{2+}$. It is evident from previous experiments where $La^{3+}$ was used for displacing $^{45}Ca$ bound to the exterior of the cells, that only minimal amounts of such calcium remain after 40 min of perifusion (Hellman, 1978; Flatt *et al.*, 1980). Since the pool of superficial $^{45}Ca$ can be expected to be particularly small in the presence of EGTA, there is no doubt that the calcium mobilized by external ATP originates from intracellular stores. External ATP may mobilize intracellular calcium by different mechanisms. The nucleotide has been found to increase the passive permeability non-specifically both of transformed cells and normal secretory cells like mast cells (Heppel *et al.*, 1985). Since this increase of the cell permeability seems to be critically dependent on divalent cations (Bennett *et al.*, 1981; Gomperts, 1983), it was pertinent to note that absence of $Mg^{2+}$ did not enhance but actually suppressed the ATP effect. Another reason for rejecting the view that the ATP action reflects a fairly generalized permeability increase is, that ADP was equally effective in promoting $^{45}Ca$ efflux from isolated islets.

When analysing the nucleotide specificity of the $Ca^{2+}$ mobilization it became evident that it fulfils the criteria for an effect mediated by $P_2$-purinoceptors (Burnstock, 1978; Burnstock & Kennedy, 1985; Katsuragi & Furukawa, 1985). Consequently, not only ATP and ADP but also their more stable α/β-methylene analogues induced mobilization, whereas AMP, adenosine and β/γ-methylene ATP were without obvious effects. Measurements of the release of insulin and glucagon from the perfused rat pancreas have provided evidence for the existence of $P_1$-purinoceptors in the $\alpha_2$-cells and $P_2$-receptors in the β-cells (Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981; Chapal *et al.*, 1984). It should be emphasized that pancreatic islets from *ob/*

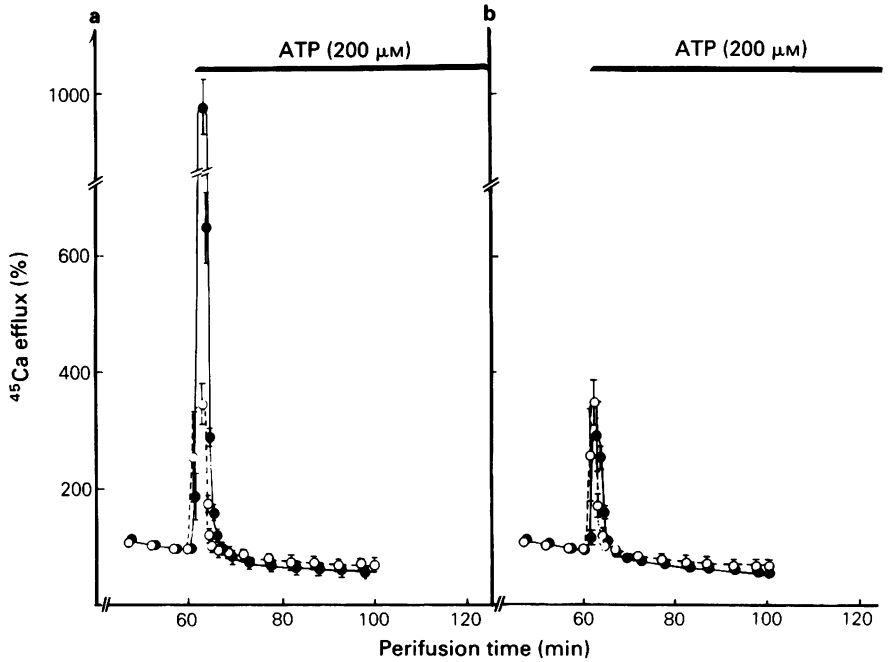


**Figure 6** Effects of ATP on $^{45}Ca$ efflux after removal of $Na^+$ (a) or lowering the temperature to 20°C (b). After loading with $^{45}Ca$ the islets were perifused with a $Ca^{2+}$-deficient medium containing 0.1 mM EGTA and 20 mM glucose. In (a) are shown the effects obtained with 200 μM ATP, when $Na^+$ was iso-osmotically replaced by $K^+$. In (b) the effect of 200 μM ATP was tested at a temperature of 20°C. Open symbols denote control experiments performed at 37°C with the ordinary concentration of $Na^+$. Mean values for 4 experiments; s.e. mean shown by vertical lines.

*ob*-mice are practically devoid of the glucagon-producing $\alpha_2$-cells (Hellman, 1965).

The exposure to ATP resulted in a pattern of initial calcium mobilization similar to that observed after muscarinic receptor activation (Hellman & Gylfe, 1986). Thus, like that of carbachol the effect was not only dependent on previous exposure to glucose, but also became more pronounced when replacing extracellular $Na^+$ with $K^+$. In support of the idea that $P_2$-purinoceptor agonists mobilize calcium by the same mechanism as muscarinic agents, it was possible to demonstrate mutual interactions between ATP and carbachol. An important effect of activating muscarinic receptors in the pancreatic islets is polyphosphoinositide breakdown (Best & Malaisse, 1984; Morgan *et al.*, 1985), an event which supposedly induces rapid mobilization of calcium from the endoplasmic reticulum mediated by inositol 1,4,5-trisphosphate (Prentki & Wollheim, 1984). Although there is so far no direct evidence that the $P_2$-purinoceptor is linked to the formation of inositol 1,4,5-trisphosphate in the pancreatic β-cells, extracellular ATP has been found to trigger rapid breakdown of phosphatidylinositol 4,5 bisphosphate in both hepatocytes (Creba *et al.*, 1983) and Ehrlich ascites tumour cells (Dubyak, 1986). In both types of cells the response to ATP was associated with rapid mobilization of calcium from intracellular stores, resulting in a rise of its cytoplasmic concentration (Dubyak & De Young, 1985; Charest *et al.*, 1985). In support for the idea that the ATP-induced mobilization of the islet calcium was mediated by polyphosphoinositide breakdown, the stimulated $^{45}Ca$ efflux was suppressed by neomycin. The latter compound has been found to block the polyphosphoinositide metabolism in other tissues by binding directly to the mono- and diphosphates of phoshatidylinositol (Orsulakova *et al.*, 1976; Carney *et al.*, 1985).

The observation of a reduced nucleotide stimulation of $^{45}Ca$ efflux after raising extracellular $Ca^{2+}$ to 1.28 mM should not be taken to indicate that less calcium is mobilized. Several studies have demonstrated significant ATP stimulation of insulin release in the presence of physiological concentrations of $Ca^{2+}$ (Candela *et al.*, 1963; Sussman *et al.*, 1969; Loubatières *et al.*, 1972; Loubatières-Mariani *et al.*, 1979; Chapal & Loubatières-Mariani, 1981). It is therefore likely that the reduction of the $^{45}Ca$ efflux with increase of extracellular $Ca^{2+}$ reflects a depletion of radioactivity in intracellular stores mediated by $Ca^{2+}$-$Ca^{2+}$ exchange. A suppression of $^{45}Ca$ mobilization with inclusion of $Ca^{2+}$ in the perifusion medium is not unique for activation of $P_2$-purinoceptors but can

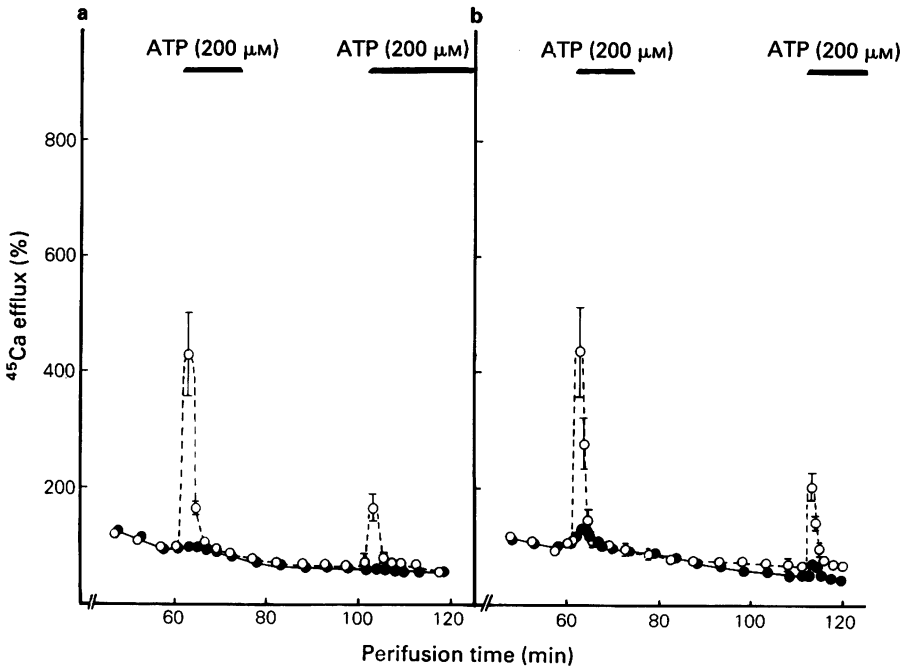


**Figure 7** Effects of ATP on $^{45}Ca$ efflux after removal of $Mg^{2+}$ (a) or addition of neomycin (b). After loading with $^{45}Ca$, the islets were perifused with a $Ca^{2+}$-deficient medium containing 20 mM glucose. The effects obtained with 200 μM ATP in the absence (●) or presence (○) of 2.2 mM $MgCl_2$ in medium supplemented with 1 mM EDTA are shown in (a). In (b) 10 mM neomycin was included (●) or not (○) in medium supplemented with 0.5 mM EGTA. Mean values for 4–6 experiments; vertical lines show s.e.mean.

also be seen after exposing the islets from *ob/ob*-mice to the muscarinic receptor agonist carbachol (Hellman & Gylfe, 1986). The $^{45}Ca$ efflux occurring with external ATP corresponded to about 25% of that obtained when introducing equimolar carbachol in similarly designed experiments (Hellman & Gylfe, 1986). Considering the less pronounced effect of ATP, it is not surprising the nucleotide failed to stimulate insulin release during perifusion with a $Ca^{2+}$-deficient medium. In the absence of glucose, carbachol has been found to initiate a late phase of stimulated $Ca^{2+}$ efflux secondary to a rise of intracellular $Na^+$ (Hellman & Gylfe, 1986). There may well be an increase of intracellular $Na^+$ also when the β-cells are exposed to ATP, although not sufficient to produce a delayed stimulation of $^{45}Ca$ efflux. In Ehrlich ascites tumour cells, micromolar concentrations of ATP have effects similar to carbachol in raising intracellular $Na^+$ by activation of an amiloride-sensitive $Na^+$-$H^+$ exchange system (Wiener *et al.*, 1986).

The physiological role of the $P_2$-purinoceptor mediating mobilization of intracellular calcium in the pancreatic β-cells is still not clear. For long, an exclusively intracellular function was attributed to ATP, primarily because so little could be detected in extracellular fluids. However, it can be predicted that a local accumulation of nucleotides occurs in the vicinity of the β-cells. In association with the transmitter action of ATP, this nucleotide has been found to be released after stimulation of peripheral nerves (White, 1985). Another and perhaps more important source of ATP is the β-cells themselves. Glucose stimulation of insulin release has been found to enhance significantly the release of ATP from isolated rat islets (Leitner *et al.*, 1975). Indeed, analyses of insulin secretory granules from a transplantable rat insulinoma have indicated concentrations of ATP and ADP of no less than 3.5 and 5.1 mM respectively (Hutton *et al.*, 1983). The activation of the $P_2$-purinoceptor may therefore be important in amplifying the secretory response. Further studies will have to elucidate to what extent the initial phase of glucose-stimulated insulin release can be accounted for by the ability of the $P_2$-purinoceptor to mobilize calcium incorporated in response to glucose.

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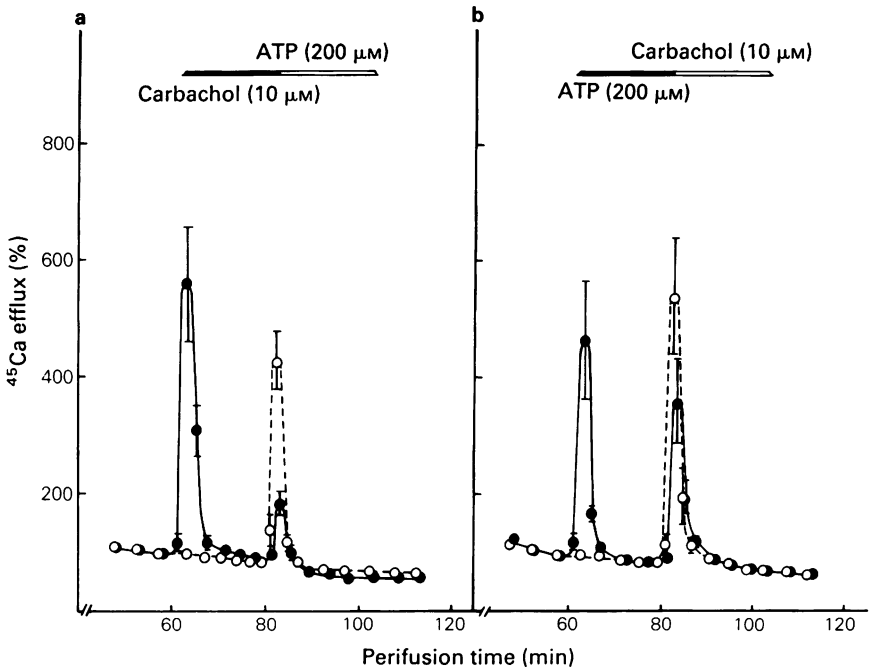


**Figure 8** Effects of sequential exposures to carbachol and ATP on $^{45}Ca$ efflux. After loading with $^{45}Ca$ the islets were perifused with a $Ca^{2+}$-deficient medium containing 0.5 mM EGTA and 20 mM glucose. In (a) exposure to 10 μM carbachol (open bar) at 60 min was followed by exposure to 200 μM ATP (filled bar) at 80 min. In (b) the substances were added in the reverse order. Broken lines refer to experiments with exposure to ATP or carbachol only at 80 min. Mean values for 8 experiments; vertical lines show s.e.mean.

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