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Research Paper

P2Y Receptor Mediated Modulation of Insulin Release by a Novel Generation of 2-Substituted-5′-*O*-(1-Boranotriphosphate)-Adenosine Analogues

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Purpose. A series of C2-substituted ATP analogues was previously shown to have potent insulin-secreting properties, yet with poor tissue-selectivity for the pancreatic β-cell. The present study was designed to evaluate the binding profile on β-cell membranes and the effects on insulin release and pancreatic vascular resistance of a second generation of $P2Y_1$ receptor agonists, based on C2-substitution of the adenosine 5′-*O*-(1-boranotriphosphate) scaffold.
Materials and Methods. Functional experiments were performed in the rat isolated pancreas model; binding studies with ATP-α-[^{35}S] were performed in membrane homogenates from the rat insulinoma INS-1 cell line. The diastereoisomers of the compounds are designated by A and B.
Results. Under 8.3 mmol l^{-1} glucose, 2-methylthio-ATP-α-B, A isomer, induced a biphasic and concentration dependent insulin response; its maximal efficacy reaches ninefold the baseline secretion and its EC_{50} is 28.1 nmol l^{-1}. No significant effect of this isomer was observed on vascular resistance, whereas the B isomer, which was a less potent insulin secretagogue, consistently induced a transient vasoconstriction. Interestingly, the insulin response induced by 2-methylthio-ATP-α-B, A isomer, was clearly glucose-dependent. This drug competes with ATP-α-[^{35}S] binding in a complex two sites interaction model, with a $K_{0.5}$ value of 17.7 nmol l^{-1}. 2-Chloro-ATP-α-B had a similar insulin-secreting profile as 2-methylthio-ATP-α-B, with a lower tissue-selectivity. The non-substituted ATP-α-B analog, A isomer, was less potent than the C2-substituted derivatives (A isomers) and had a vasorelaxant effect.
Conclusions. We conclude that 2-methylthio-ATP-α-B, A isomer, is a potent and tissue-selective P2Y receptor agonist with high efficacy. Its insulin-releasing action is glucose-dependent, which gives interest to this compound as a drug candidate for treating type 2 diabetes.

KEY WORDS: diabetes; insulin secretion; P2 receptor; P2Y receptor ligand.

INTRODUCTION

Structural analogues of ATP, with improved stability and selectivity, have been shown to increase insulin secretion through activation of P2Y receptors in isolated rat pancreas (1) and isolated human islets (2). The potential interest of these receptors as novel therapeutic targets for insulin-releasing antidiabetic drugs has been previously discussed (3); it is essentially accounted for by the K_{ATP} channel-independent coupling mechanism of the P2Y receptors (4), as opposed to the P2X receptors (5), and the implication of the cAMP-PKA-dependent pathway (6).

Various molecular subtypes of P2Y and P2X receptors are differentially expressed in the pancreas (7,8). A role for the $P2Y_1$ receptor subtype has been clearly evidenced in the maintenance of glucose homeostasis and insulin secretion in mice (9), even if the functional effects in that species are opposite (10,11) to those observed in rats, dogs and human tissues (3).

A substitution at the adenine C2 position and modifications of the polyphosphate chain of ATP have been reported to enhance insulin secreting activity (12). It has also been shown that a long C2 thioether substitution on ATP made the derivatives highly active, mainly at the P2Y receptor subtypes (13). A series of C2-substituted ATP analogues was previously developed in collaboration with the Department of Chemistry at Bar-Ilan University in Israel (14,15). These compounds were designed as enzymatically stable $P2Y_1$ specific ligands and shown to be potent and effective insulin secretagogues; however, they had poor tissue-selectivity for the pancreatic β-cell since they increased pancreatic vascular resistance. A novel series of nucleotides based on C2-substitution of the adenosine 5′-*O*-(1-boranotriphosphate) scaffold (ATP-α-B) was recently developed as stable $P2Y_1$ receptor agonists (16). The aim of the present study was to evaluate the binding profile on β-cell membranes and the functional effects on insulin release and pancreatic vascular resistance of this second generation of $P2Y_1$ receptor agonists.

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MATERIALS AND METHODS

Chemicals

All 2-substituted-5′-*O*-(1-boranotriphosphate)-adenosine derivatives were synthesized in the Department of Chemistry at Bar-Ilan University, Israel, using a method described by Nahum *et al.* (16), and were kindly supplied by Prof. B. Fischer. The two diastereoisomers were separated on high performance liquid chromatography. As discussed by these authors (16), their absolute configuration could not be established and, therefore, they are designated A and B. Each isomer of 2-methylthio-adenosine-5′-*O*-(1-boranotriphosphate) [2-methylthio-ATP-α-B] and of 2-chloro-adenosine-5′-*O*-(1-boranotriphosphate) [2-chloro-ATP-α-B] were tested; for adenosine-5′-*O*-(1-boranotriphosphate) [ATP-α-B], only the isomer A was tested.

Bovine serum albumin (Fraction V) was obtained from the Sigma Chemical Co. (St. Louis, MO, USA). ATP-α-S and ATP-α-[^{35}S] (specific radioactivity 1,250 Ci mmol l^{-1} at the reference date) were obtained from Perkin Elmer France. ADP-β-S and usual chemicals were obtained from Sigma-Aldrich France. Glass fiber filters (Whatman GF/B) were obtained from VWR France. The μBCA protein assay (Pierce) was obtained from Interchim France.

Functional Experiments in Isolated Perfused Pancreas

Experiments were performed in vitro in the isolated perfused pancreas from male Wistar albino rats (Charles River, France) fed ad libitum. Rats, weighing 340–360 g, were housed in a light-controlled room and given free access to food and water, in accordance with the national guidelines for the use and care of laboratory animals. After sodium pentobarbital anesthesia (60 mg kg^{-1} intraperitoneally), the pancreas was completely isolated from all the neighboring tissues, according to a technique previously described (17) and perfused through its own arterial system with a Krebs–Ringer bicarbonate buffer containing 2 g l^{-1} bovine serum albumin (BSA) and various concentrations of glucose: 2.8, 4.2, 5.0, 6.6 and 8.3 mmol l^{-1}. A mixture of O_2 (95%) and CO_2 (5%) was bubbled through this medium at atmospheric pressure. The pH of the solution was 7.35. The preparation was maintained at 37.5°C. Each organ was perfused at a constant pressure (40–50 cm water), selected so as to produce a flow rate of 2.5 ml min^{-1} at the start of the experiment; in these conditions, any change in the flow rate reflects a change in vascular resistance (18).

A 30 min adaptation period was allowed before the first sample was taken for insulin assay. A sample was taken 15 min later, at time 45 min. Then, an infusion of the ATP analogues was performed during 30 min. Pancreatic effluent fractions were collected during 1 min and measured in graduated test tubes, allowing the recording of pancreatic flow rate; an aliquot is then immediately frozen until insulin assay.

Assays

Insulin concentrations were determined by a radioimmunological method, using purified rat insulin as standard (Linco Research, St. Charles, MO, USA) and guinea pig antiporcine-insulin antiserum (ICN Biochemicals, Puteaux, France). The sensitivity of our assay was 0.06 ng ml^{-1}.

Data Expression and Statistical Analysis

Insulin output from perfused pancreas was determined by multiplying the hormone concentration in the effluent fraction by the flow rate and is expressed as nanograms per minute. Results are expressed as means ± standard error of the mean (SEM). For the kinetics of insulin secretion and vascular flow rate, the results are expressed as changes in relation to the value at time 45 min taken as 100%. For the determination of the concentration–response curves, the mean insulin output rate was calculated by dividing the area under the curve (AUC) for the drug infusion period by the number of minutes. The concentration–response curve for the insulin secretion induced by 2-methylthio-ATP-α-B (isomer A) and the effective concentration producing half maximal response (EC_{50}) were calculated with the Sigma Plot Software.

Binding Experiments on β-Cell Membrane Homogenates

Tissues and Homogenate Preparation

The rat insulinoma INS-1 cell line (19) was kindly provided by Prof. C. B. Wollheim (Geneva, Switzerland). The cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mmol l^{-1}L-glutamine, 10 mmol l^{-1} HEPES, 1 mmol l^{-1} sodium pyruvate and 50 μmol l^{-1} 2-mercaptoethanol (19). For binding experiments, INS-1 cells were plated in T150 culture flasks (Falcon) and grown until confluence 1 week later yielding about 10^8 cells per flask. Cells were scrapped and then homogenized for 20 s in a 50 mmol l^{-1} Tris/Hepes, pH 7.4 buffer, centrifuged for 20 min at 50,000 × *g*; the homogenization centrifugation steps were repeated a second time.

Binding Experiments

The homogenate (20 μg protein per assay) was incubated in the presence of 1 nmol l^{-1} ATP-α-[^{35}S] with or without 2-methylthio-ATP-α-B (isomer A). The non-specific binding was determined in the presence of 100 μmol l^{-1} ADP-β-S. The protein concentration of the homogenates was determined by the μBCA assay with bovine serum albumin (BSA) as standard. BSA standards were prepared in the same buffer as the samples to correct for possible buffer interference.

The binding experiments were performed by the rapid filtration method on a MR-24 Brandel cell harvester using GF/B filters. The radioactivity retained on filters was measured by a liquid scintillation method on a Rack Beta 1214 (LKB) counter without correction for counting efficiency. These two apparatus were kindly lent by Dr A. Privat (INSERM U336, Montpellier). Compounds were dissolved in the binding incubation buffer at a 10^{-2} mol l^{-1} concentration and then submitted to sequential dilutions in order to achieve concentrations in the incubations ranging from 10^{-9} to 10^{-5} mol l^{-1}.

Data and Statistical Analysis

In all experiments, each value is the mean of triplicates. Each experiment was replicated three to five times. Experimental data were analyzed with the Sigma Plot software according to a single or two sites interaction model. The probability of each model was determined by ANOVA and their comparison was performed by a bilateral Fisher's test that indicated which model was the more statistically probable.

RESULTS

Effects of 2-Methylthio-ATP-α-B on Insulin Secretion and Pancreatic Vascular Resistance

The administration of 2-methylthio-ATP-α-B (A isomer) in the presence of a slightly stimulating glucose concentration (8.3 mmol l^{-1}) resulted in an immediate and concentration dependent response in the concentration range of 0.0015–5 μmol l^{-1} (Fig. 1a, upper panel). The increase in glucose-induced insulin release was biphasic with a first 5-min peak followed by a second phase of sustained secretion. The results with isomer B (Fig. 1b, upper panel) are qualitatively similar to those registered with isomer A, but the compound is less potent.

According to its concentration–insulin response curve, the EC_{50} value of 2-methylthio-ATP-α-B (isomer A) is 28.1 nmol l^{-1}, and its maximal efficacy reaches ninefold the baseline secretion (Fig. 2); isomer B is clearly less potent.

On the other hand, there was no significant effect of 2-methylthio-ATP-α-B, isomer A on pancreatic vascular resistance (Fig. 1a, lower panel), whereas isomer B of the same compound consistently induced a transient vasoconstriction, at concentrations similar to those amplifying insulin secretion (Fig. 1b, lower panel).

As shown in Fig. 3 (upper panel), 2-methylthio-ATP-α-B, isomer A, does not increase insulin secretion from the isolated pancreas, when the glucose concentration in the medium is low (2.8 and 4.2 mmol l^{-1}). It induces only a small and

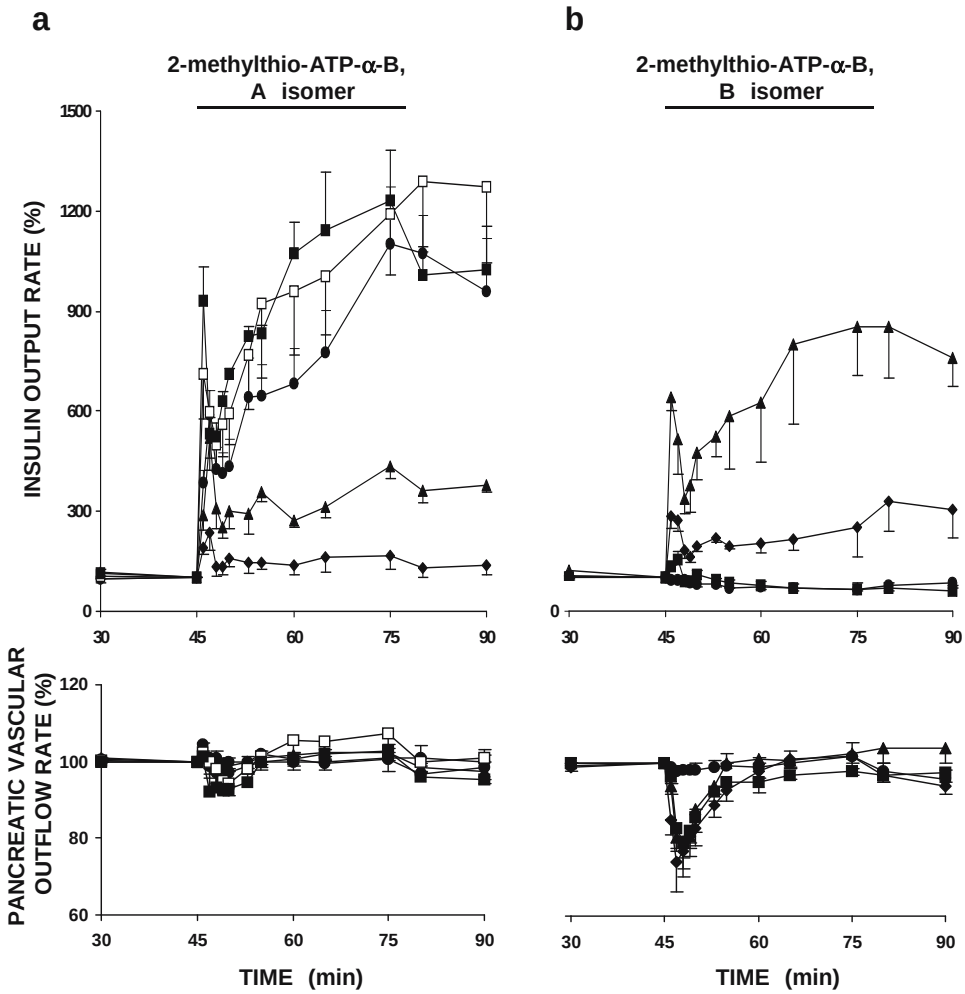

Fig. 1. (a) Insulin output (*upper panel*) and pancreatic vascular outflow (*lower panel*) induced by 2-methylthio-ATP-α-B, A isomer, at different concentrations: 0.0015 μmol l^{-1} (*filled diamond*), 0.005 μmol l^{-1} (*filled triangle*), 0.05 μmol l^{-1} (*filled circle*), 0.5 μmol l^{-1} (*open square*) and 5 μmol l^{-1} (*filled square*), in the presence of 8.3 mmol l^{-1} glucose. The results are the means ± SEM of three to five experiments. (b) Insulin output (*upper panel*) and pancreatic vascular outflow (*lower panel*) induced by 2-methylthio-ATP-α-B, B isomer, at different concentrations: 0.0015 μmol l^{-1} (*filled circle*), 0.015 μmol l^{-1} (*filled square*), 0.15 μmol l^{-1} (*filled diamond*), 1.5 μmol l^{-1} (*filled triangle*), in the presence of 8.3 mmol l^{-1} glucose. The results are the means ± SEM of three to four experiments.

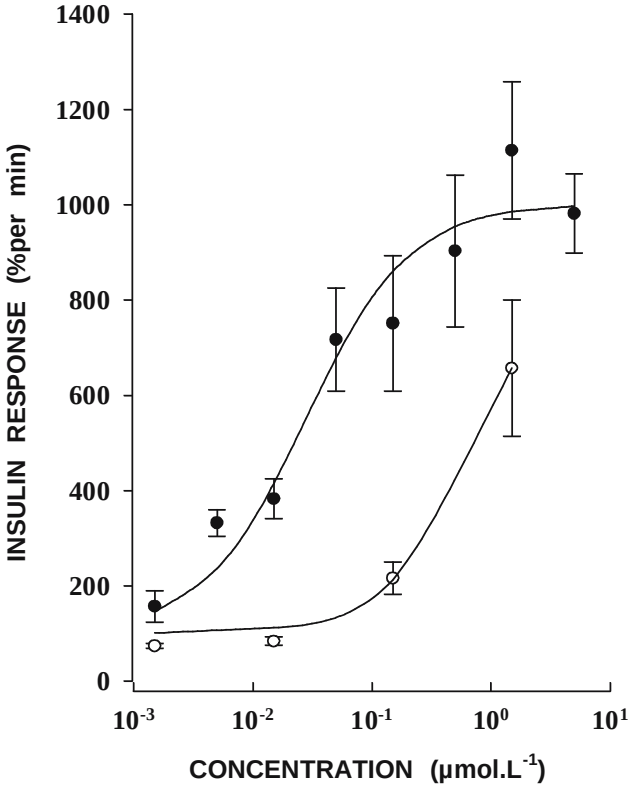

Fig. 2. Concentration–response curve for insulin secretion induced by the two isomers of 2-methylthio-ATP-α-B: isomer A (*filled circle*) and isomer B (*open circle*).

transient increase, in the presence of 5.0 mmol l^{-1} glucose. In contrast, there is a strong amplification of insulin release when the pancreas is stimulated by 6.6 or 8.3 mmol l^{-1} glucose. The glucose-dependence of the effect of 2-methylthio-ATP-α-B, isomer A, is shown in Fig. 3 (lower panel), for the first and the second phase of insulin secretion.

Effects of ATP-α-B (Isomer A) and 2-Chloro-ATP-α-B (Isomers A and B) on Insulin Secretion and Pancreatic Vascular Resistance

In the presence of 8.3 mmol l^{-1} glucose, all of the three compounds induced a biphasic and concentration dependent increase in insulin release, with a comparable pattern as that of 2-methylthio-ATP-α-B, isomer A (not shown). As shown in Fig. 4, isomers B of 2-methylthio-ATP-α-B and of 2-chloro-ATP-α-B are less potent than isomers A, and both display approximately the same potency as ATP-α-B, isomer A.

On the other hand, ATP-α-B (isomer A) and 2-chloro-ATP-α-B (isomer A), at concentrations inducing a similar insulin response as 2-methylthio-ATP-α-B (isomer A), induced a sustained vasodilatation, contrasting with the transient vasoconstriction provoked by isomer B of 2-methylthio-ATP-α-B (Fig. 5).

Inhibition of ATP-α-[^{35}S] Binding to INS-1 Cells by Unlabeled ATP-α-S and 2-Methylthio-ATP-α-B (Isomer A)

ATP-α-[^{35}S] binding to INS-1 cell homogenate is accounted for by a high specific binding that is linear up to 1 mg protein ml^{-1} (non specific binding determined in the presence of 100 µmol l^{-1} unlabeled ATP-α-S is mainly due to a non-displaceable retention of the labeled drug to GF/B filters). The equilibrium is reached within 60–70 min at 25°C. At a 1 nmol l^{-1} concentration, ATP-α-[^{35}S] specific binding represents 93.41 ± 3.93 fmol/mg protein. Drug effects on ATP-α-[^{35}S] binding were investigated over a large concentration range by competition.

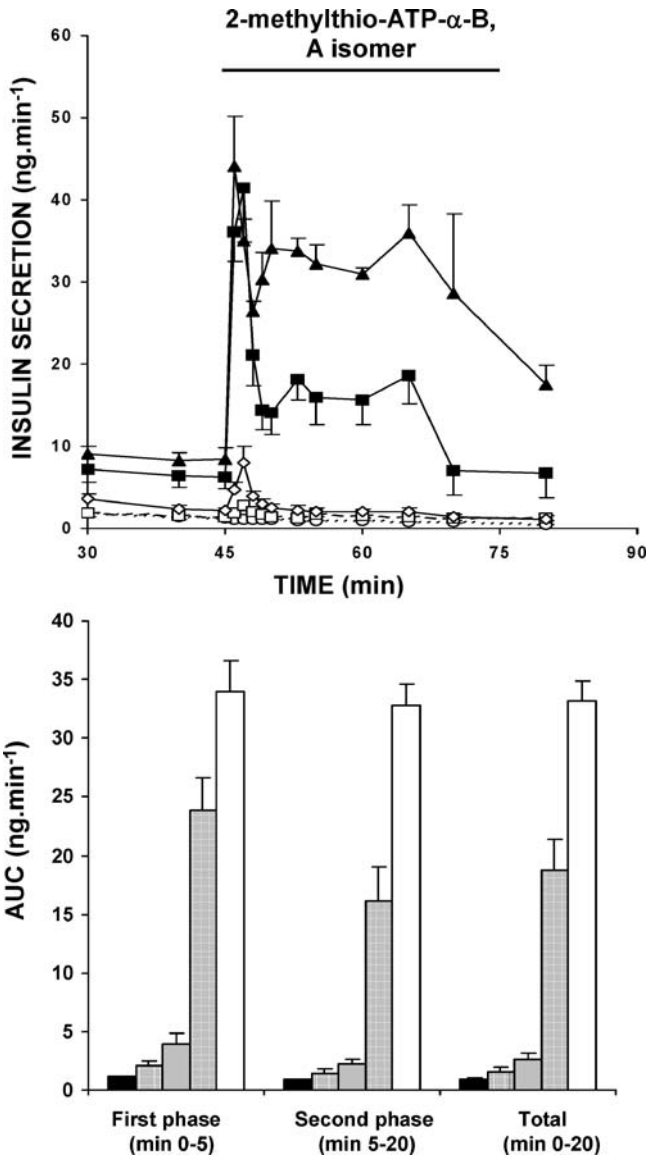

Fig. 3. *Upper panel*: insulin release in the presence of 2-methylthio-ATP-α-B (20 nmol l^{-1}), A isomer, at different glucose concentrations: 2.8 mmol l^{-1} (*open circle*), 4.2 mmol l^{-1} (*open square*), 5.0 mmol l^{-1} (*open diamond*), 6.6 mmol l^{-1} (*filled square*), 8.3 mmol l^{-1} (*filled triangle*). The results are the means ± SEM of five to six experiments. *Lower panel*: areas under the curve for the first phase (min 0–5), the second phase (min 5–20) and the total (min 0–20) insulin secretion induced by 2-methylthio-ATP-α-B (20 nmol l^{-1}), A isomer, in the presence of 2.8 mmol l^{-1} (*black column*), 4.2 mmol l^{-1} (column with *vertical lines*), 5.0 mmol l^{-1} (*grey column*), 6.6 mmol l^{-1} (column with *oblique lines*) and 8.3 mmol l^{-1} glucose (*white column*). The results are the means ± SEM of five to six experiments.

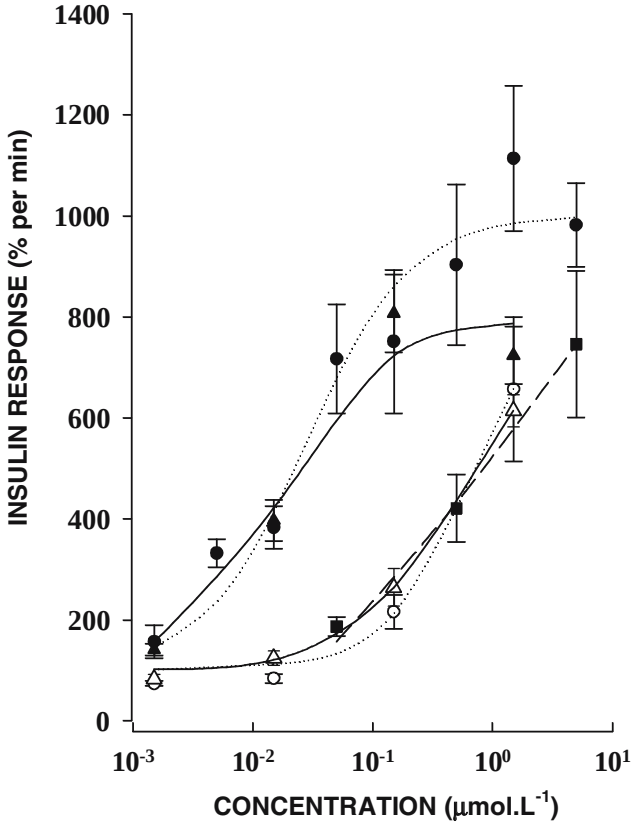

Fig. 4. Concentration-response curves of insulin secretion induced by 2-methylthio-ATP-α-B, isomers A (*filled circle*, *dotted line*) and B (*open circle*, *dotted line*); ATP-α-B, isomer A (*filled square*, *broken line*); and 2-chloro-ATP-α-B, isomers A (*filled triangle*, *full line*) and B (*open triangle*, *full line*).

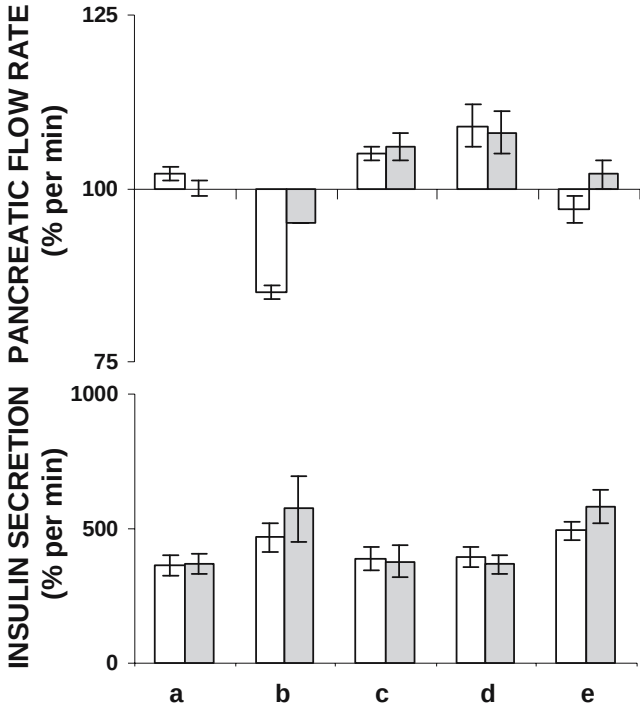

Fig. 5. Areas under the curve (percent of baseline) for the first 5 min (*open square*) and the total 20 min (*filled square*), calculated for the changes in pancreatic flow rate (*upper panel*) and insulin secretion (*lower panel*), for the different compounds: (a) 2-methylthio-ATP-α-B, isomer A (0.015 µmol l^{-1}); (b) 2-methylthio-ATP-α-B, isomer B (1.5 µmol l^{-1}); (c) ATP-α-B, isomer A (0.5 µmol l^{-1}); (d) 2-chloro-ATP-α-B, isomer A (0.015 µmol l^{-1}) and (e) 2-chloro-ATP-α-B, isomer B (1.5 µmol l^{-1}).

As illustrated in Fig. 6, ATP-α-[^{35}S] binding inhibition by unlabeled ATP-α-S is accounted for by a complex interaction to INS-1 cell homogenate. The modeling of this inhibition according to a single site interaction is statistically less probable than a more complex two sites interaction ($P < 0.001$ in favor of the two sites model). This two sites interaction model allows determining that the high affinity component represents roughly 40% of the specific binding and the low affinity component the 60% remaining binding. The inhibitory constants $K_{0.5}$ (apparent dissociation constants) for high and low affinity sites are 8 nmol l^{-1} and 1.4 µmol l^{-1}, respectively.

As observed with ATP-α-S, inhibition of ATP-α-[^{35}S] binding by 2-methylthio-ATP-α-B (isomer A) is better accounted for by a two sites interaction model ($P < 0.001$) with the same proportions of high and low affinity sites. $K_{0.5}$ values were 17.7 nmol l^{-1} and 2.5 µmol l^{-1} for high and low affinity sites respectively.

DISCUSSION

The present study reports the functional effects of a novel generation of 2-substituted-5′-*O*-(1-boranotriphosphate)-adenosine analogues on insulin secretion and pancreatic vascular resistance. The results clearly show that among these derivatives, 2-methylthio-ATP-α-B (A isomer) is a very potent and effective insulin secretagogue with a much better tissue-selectivity than its structural analogues (present study) and previously tested 2-thioether 5′-*O*-(1-thiotriphosphate) adenosine derivatives (14,15).

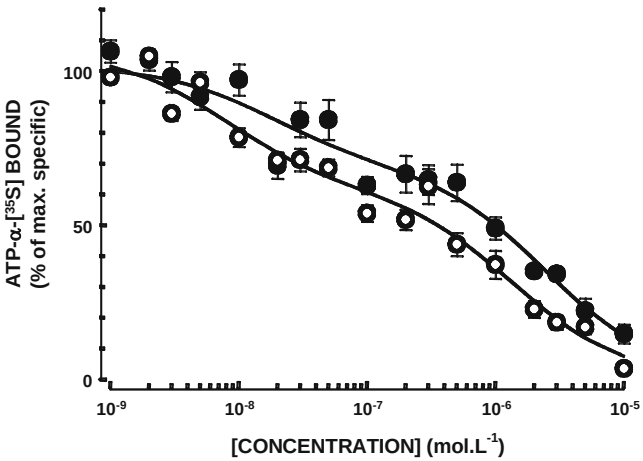

Fig. 6. Inhibition of ATP-α-[^{35}S] binding to INS-1 cell membrane homogenates by unlabeled ATP-α-S (*open circle*) or 2-methylthio-ATP-α-B, isomer A (*filled circle*). Data are the mean of three to five independent experiments, each performed in triplicates. For each P2Y receptor agonist, a two sites interaction is statistically the most relevant ($P < 0.001$, bilateral Fischer's test).

The chemical synthesis of these new entities, based on C2-substitution of the adenosine 5′-*O*-(1-boranotriphosphate) scaffold, was previously reported by Nahum *et al.* (16). These compounds were shown to be chemically highly stable and resistant to hydrolysis by ecto-nucleoside triphosphate diphosphohydrolase (NTPDase), which is a prerequisite for an ATP analogue with potential pharmacological interest.

In our conditions in the isolated rat pancreas, both 2-methylthio- and 2-chloro-ATP-α-B derivates (isomers A) were very potent insulin secretagogues, with an EC_{50} of 28.1 nmol l^{-1} for the former. This potency appears to be approximately fivefold higher than that of ADP-β-S (20), which has been shown to be 100 times more potent than ATP. This relative potency versus ATP mimics that of the first generation of 2-substituted ATP derivatives, namely 2-hexylthio-ATP-α-S (14) and 2-benzylthio-ATP-α-S (15). These data on the secretory potency of these new analogues are in agreement with the results concerning their potency as $P2Y_1$ receptor agonists evaluated by induction of stored Ca^{2+} release by HEK 293 cells stably transfected with rat-brain $P2Y_1$ receptors (16). Furthermore, diastereoisomers A of both 2-methylthio- and 2-chloro-ATP-α-B were more potent insulin secretagogues than their corresponding B counterparts, which is again in accordance with the relative potency in the $P2Y_1$-induced Ca^{2+} releasing action (16). It remains to be determined whether the 2-methylthio-ATP-α-B secretory effect is coupled to Ca^{++} release and/or to cAMP increase, as previously demonstrated for ATP-α-S (6). The new C2-substituted ATP-α-B derivatives also display a very high efficacy, with a maximal response for 2-methylthio-ATP-α-B (isomer A) reaching ninefold the baseline insulin secretion, which is clearly higher than that of ADP-β-S in similar conditions (20). In any case, whatever the mechanism of action, it should be tightly coupled to glucose metabolism and glucose-dependent amplification pathways, as evidenced by the glucose dependence of the insulin-releasing effect.

Since different P2Y receptors are expressed in various tissues and organs (21), and P2Y agonists are known to affect vascular tone, the tissue-selectivity of the new compounds was considered. The model used in the present study offers the opportunity to monitor within the same experiment the effects of agonists over time on both insulin secretion and pancreatic vascular tone (18). Most of the structural analogues which have been evaluated so far in that model show no or poor tissue selectivity in that they both induce insulin release and alter vascular resistance in the same concentration range, either relaxing (20) or increasing vascular tone (14,15). The most selective derivative as yet was 2-methylthio-ATP (1) but this compound is readily metabolized. In the present study, 2-methylthio-ATP-α-B (A isomer) proved to be highly selective since no significant vascular effect was registered at concentrations strongly effective at enhancing insulin secretion. In contrast, B isomer induced a transient but consistent vasoconstriction whereas the 2-chloro analogue induced a sustained vasodilatation.

Despite the fact that the P2Y receptors present on pancreatic β-cells display some characteristics of the $P2Y_1$ subtype and were proposed as $P2Y_1$-like by Ralevic and Burnstock (22), these receptors also exhibit some differences (15) and the precise molecular subtype(s) still remain(s) to be elucidated. The high potency of the 2-substituted-ATP-α-B derivatives (A isomers) enhancing insulin release, and the relatively lower potency of the B isomers, also recorded for Ca^{2+} release in $P2Y_1$ receptor transfected cells (16), is a strong additional argument to suggest the presence of a $P2Y_1$ receptor subtype on the pancreatic β-cell. Furthermore, these new compounds have been recently reported to be highly specific agonists for $P2Y_1$ as compared with $P2Y_2$ receptor subtypes (23). In addition, recent results in $P2Y_1$ receptor knock-out mice suggest that this receptor subtype plays a physiological role in the maintenance of glucose homeostasis, at least in part through regulation of insulin secretion (9), even if in that species it induces an inhibitory control of insulin secretion (11). We could recently identify by RNA amplification and protein determination the presence of $P2Y_1-$, $P2Y_2-$, $P2Y_4-$ and $P2Y_6-$ receptor subtypes in the INS-1 β-cell line (24).

The complexity of that issue is further illustrated by the results we have obtained in our binding experiments. The analysis of the competition between ATP-α-[^{35}S] and unlabelled ATP-α-S shows that ATP-α-S binds to two distinct sites with a high affinity component representing approximately 40% of the specific binding. Similar results were obtained by Schäfer and Reiser (25) on synaptosomal membranes from rat brain cortex. However, this result contrasts with data previously reported by Laubinger and Reiser (26), assuming the presence of a single binding site for ATP-α-S in rat lung although different P2 receptor subtypes are expressed in that tissue. 2-methylthio-ATP-α-B (A isomer) also interacts with ATP-α-[^{35}S] binding sites according to a two-site model. Its affinity value for the high affinity site is in the same range as ATP-α-S. Taking into account the effectiveness at low concentrations of both compounds, it can be postulated that the high affinity ATP-α-[^{35}S] binding site is involved in glucose-induced insulin secretion. However, relative potencies of ATP-α-S and 2-methylthio-ATP-α-B (A isomer) for insulin secretion are inverted compared to their affinities in the binding assay. Such a discrepancy suggests that the different sites detected in binding experiments have different functional relevance. The relative importance of each of the receptor subtypes ($P2Y_1-$, $P2Y_2-$, $P2Y_4-$ and $P2Y_6-$) in the binding pattern and in the functional response to P2Y receptor agonists remains to be clearly established. The two binding components detected with ATP-α-[^{35}S] might represent mean interactions with different receptor subtypes, thus explaining this lack of correlation between drug affinities and drug actions.

CONCLUSION

In conclusion, the $P2Y_1$ receptor agonist 2-methylthio-ATP-α-B, A isomer, amplifies insulin release from the rat pancreas in a glucose-dependent manner; such an effect results from a complex interaction of the agonist with P2Y receptor(s) involving both high and low affinity binding sites. 2-Methylthio-ATP-α-B displays a very high potency and efficacy, as well as a very good β-cell selectivity as compared to pancreatic vascular tissue. Therefore, this compound may be considered as a potentially attractive drug candidate for the treatment of type 2 diabetes.

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