Effects of the Pesticide Amitraz and Its Metabolite BTS 27271 on Insulin and Glucagon Secretion From the Perfused Rat Pancreas: Involvement of α_{2D}-Adrenergic Receptors

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The study purpose was to investigate the direct effect of amitraz, a formamidine insecticide/acaricide, and its active metabolite BTS 27271 on insulin and glucagon secretion from the perfused rat pancreas. Amitraz and BTS 27271 (0.01, 0.1, 1, and 10 μmol/L) inhibited insulin secretion in a concentration-dependent manner. Amitraz increased glucagon secretion at 10 μmol/L, whereas BTS 27271 increased glucagon secretion at 1 and 10 μmol/L. Amitraz- and BTS 27271-induced decreases in insulin secretion and increases in glucagon secretion were not abolished during the 10-minute washout period. During the arginine treatment, both amitraz and BTS 27271 groups (0.1, 1, and 10 μmol/L) had lower insulin secretion and higher glucagon secretion than the control group. Idazoxan, an $\alpha_{2A/2D}$-adrenergic receptor (AR) antagonist, prevented the inhibitory effect of amitraz on insulin secretion in a concentration-dependent manner, but prazosin, an α_1- and $\alpha_{2B/2C}$-AR antagonist, failed to antagonize the effect of amitraz. These results demonstrate that (1) amitraz and BTS 27271 inhibit insulin and stimulate glucagon secretion from the perfused rat pancreas, (2) amitraz inhibits insulin secretion by activation of α_{2D}-ARs, since rats have α_{2D}- but not α_{2A}-ARs, and (3) amitraz and BTS 27271 may have a high binding affinity to the α_{2D}-ARs of pancreatic islets.

AMITRAZ (*N'*-[2,4-dimethylphenyl]-*N*-[(2,4-dimethylphenyl)imino]-*N* -methylmethanimidamide) is a formamidine insecticide/acaricide used to treat mange in animals[1] and to eradicate arthropods in agronomy and horticulture.[2] The insecticide/acaricide actions of amitraz may be mediated by octopamine receptors.[3-6]

Amitraz is rapidly absorbed, distributed, metabolized, and eliminated primarily via urine when administered orally to mammals. The degradation products present in the urine include *N'*-[2,4-dimethylphenyl]-*N*-methylformamidine (BTS 27271), 2,4-dimethylformanilide, 2,4-dimethylformanilide, 2,4-dimethylaniline, 4-formamido-3-methylbenzoic acid, 4-amino-3-methylbenzoic acid, and several unknown metabolites.[7] BTS 27271, one of the metabolites of amitraz, has been found to be more potent than amitraz with regard to its miticidal activity[8] and mammalian toxicity.[9-10]

Over 16 cases of amitraz toxicity in humans have been reported.[11-15] Symptoms include hyperglycemia, bradycardia, hypotension, miosis, headache, nausea, drowsiness, vomiting, polyuria, rash, stiffness, swelling, and coma. The toxic effects of amitraz in animals include hyperglycemia, central nervous system (CNS) depression, bradycardia, hypertension, polyuria, mydriasis, vomiting, delayed gastrointestinal transit, bloat, and colic.[16] The toxic effects of amitraz resemble those induced by the α_2-adrenergic receptor (AR) agonists xylazine and clonidine.[17] In addition, α_2-AR antagonists such as yohimbine, but not the α_1-AR antagonists, attenuate or block several of the effects of amitraz such as CNS depression,[18-19] bradycardia,[17,19-22] hypertension,[21,22] mydriasis,[17] delayed gastrointestinal transit,[20-21,23-24] and glucose intolerance.[25] Amitraz causes a dose-dependent inhibition of [^{3}H]clonidine and [^{3}H]yohimbine binding to α_2-ARs of mouse brain,[26] rat brain,[27] and dog platelets.[28] These findings suggest that the α_2-ARs mediate a number of the effects of amitraz and that the α_2-AR antagonists have the ability to block these effects.

Amitraz causes hyperglycemia and hypoinsulinemia when used topically,[29] orally,[30] and intravenously.[25] These effects of amitraz are similar to those induced by the α_2-AR agonists. In addition, yohimbine, an α_2-AR antagonist, antagonized the hypoinsulinemic effect of amitraz, whereas prazosin, an α_1-AR antagonist, did not.[25] In the β-cell line RINm5F, amitraz and its active metabolite BTS 27271 inhibit the intracellular cyclic adenosine monophosphate concentration and insulin secretion.[10] Pretreatment of RINm5F cells with pertussis toxin (PTX) antagonized the effect of amitraz and BTS 27271 on insulin secretion. These results suggest that amitraz and its active metabolite BTS 27271 induce hyperglycemia through inhibition of insulin secretion, which is mediated by α_2-ARs. One possible mechanism for this effect is through the inhibition of adenylate cyclase, an action mediated by PTX-sensitive G-proteins.[10]

The α_2-AR agonists such as oxymetazoline and clonidine stimulate glucagon secretion from α cells of the pancreatic islet in the rat. The α_2-AR antagonists, but not the α_1-AR antagonists, counteract the stimulatory effect on glucagon secretion.[31-34]

Since previous studies have not investigated the direct effect of amitraz and its active metabolite BTS 27271 on the secretion of insulin and glucagon from the pancreas, the present study was designed to address this question and to characterize the α-ARs that mediate the effect of amitraz on insulin secretion from the pancreas.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300 to 400 g) were used. The rats were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally), maintained at 37°C on a hot plate, and kept alive during the experiment. Pancreatic perfusion was performed as previously described.[35] Briefly,

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Submitted February 18, 1999; accepted May 25, 1999.

Supported in part by the Hashemite Kingdom of Jordan and Jordan University of Science and Technology.

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0026-0495/99/4811-0022$10.00/0

after cannulation of the celiac artery, the rat pancreas was immediately perfused with Krebs-Ringer bicarbonate (KRB) solution supplemented with 20 mmol/L HEPES, 5.5 mmol/L glucose, and 0.2% bovine serum albumin as a basal medium. The KRB was maintained at pH 7.4 and continuously aerated with 95% O_2:5% CO_2.

Experimental Design

The first 20 minutes of perfusion was considered an equilibration period. Subsequently, the effluent fluid was collected every minute from the cannula in the portal vein. The flow rate was set at 1 mL/min. In experiment 1, after a baseline period of 12 minutes, the medium containing amitraz (0.01, 0.1, 1, or 10 μmol/L) was administered for 10 minutes, followed by a washout period during which the basal medium was administered for 10 minutes. In experiment 2, after a baseline period of 12 minutes, the medium containing BTS 27271 (0.001, 0.01, 0.1, 1, or 10 μmol/L), the active metabolite of amitraz, was administered for 10 minutes, followed by a washout period during which the basal medium was administered for 10 minutes. In experiment 3, after a baseline period of 8 minutes, the pancreas was pretreated for 10 minutes with a medium containing the α-AR antagonist Idazoxan (0.1, 1, or 10 μmol/L) or prazosin (1 μmol/L). This was followed by the medium containing amitraz (1 μmol/L) and Idazoxan or prazosin for 10 minutes, and the basal medium for another 10 minutes for the washout period. In all experiments, 1 mmol/L arginine was administered at the end of the experiment for 8 minutes to stimulate insulin and glucagon secretion in order to confirm that the tissue retained the normal secretory capacity under the experimental conditions. The effluent fluids were kept at 4°C and subsequently assayed within 6 hours for insulin and glucagon using a radioimmunoassay as previously described.[36,37]

Test Agents

The following agents were used: amitraz (Agr Evo, Pikeville, NC), BTS 27271 HCl (Nor-Am Chemical, Wilmington, DE), idazoxan HCl (Rickitt & Colman Pharmaceutical, Kingston-Upon-Hill, England), and prazosin HCl (Pfizer, Groton, CT). All agents were dissolved in distilled H_2O except for Amitraz, which was dissolved in dimethyl sulfoxide to make 5-mmol/L stock solutions. These solutions were further diluted with KRB (basal medium) to attain the appropriate concentrations. Control experiments were performed following the same protocol except that KRB was used in place of the agents. Glucagon and rat insulin standards were donated by Eli Lilly Laboratories (Indianapolis, IN). Insulin antibody was donated by Dr V. Leclercq-Meyer of the Free University of Brussels, Belgium. Glucagon antibody was donated by Dr Joseph Dunbar of Wayne State University (Detroit, MI). ^{125}I-glucagon was purchased from Linco Research (St. Charles, MO).

Data Expression and Statistical Analysis

The effluent concentrations of insulin and glucagon were expressed as a percentage of the baseline level (mean ± SE of last five baseline values). The area under the curve (treatment period area, washout period area, and arginine period area) was calculated using a scanning program (Sigma Scan; Jandel, Corte Madera, CA). Data were analyzed using the SAS (SAS Institute, Cary, NC) Proc General Linear Means procedure. The factors were the agent (two levels), concentration (five levels), and area (three levels). The general design is a split-plot factorial design with repeated measures. Individual mean comparisons were calculated using the F test. The significance level was set at P less than .05.

RESULTS

Effects of Amitraz on Insulin Secretion

Insulin secretion remained constant during the first 32 minutes in the control group receiving 5.5 mmol/L glucose only. Administration of 1 mmol/L arginine in the control group increased insulin secretion by about 200% of the baseline level at the end of the experiment (Fig 1A to D). By calculating the areas under the curve for the amitraz treatments and comparing them with the corresponding area of the control group, amitraz (0.01, 0.1, 1, or 10 μmol/L) significantly inhibited insulin secretion in a concentration-dependent manner and reached a maximal inhibition of 33%, 47%, 60%, and 77% of the baseline level, respectively (Fig 1A to D). The effluent concentrations of insulin remained low during the 10-minute washout period in all groups, although it was expected that the effluent concentrations of insulin would return to the baseline level. Also by calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control group, the amitraz groups (0.1, 1, and 10 μmol/L) had significantly lower insulin secretion than the control group (Fig 1B to D).

Effects of Amitraz on Glucagon Secretion

The effluent concentrations of glucagon remained constant in the control group receiving 5.5 mmol/L glucose only. Administration of 1 mmol/L arginine to the control group increased glucagon secretion, with a peak of about 470% of the baseline level (Fig 2A to 2D). By calculating the areas under the curve for the amitraz treatments and comparing them with the corresponding area of the control group, amitraz (0.01, 0.1, or 1 μmol/L) did not cause any significant increases in glucagon secretion during the treatment and washout periods (Fig 2A to C). However, amitraz (10 μmol/L) significantly increased glucagon secretion, with a peak of 265% of the baseline level. The effluent concentrations of glucagon remained high during the 10-minute washout period. By calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control group, the Amitraz groups (0.1, 1, and 10 μmol/L) had significantly higher glucagon secretion than the control group (Fig 2B to D).

Effects of BTS 27271 on Insulin Secretion

In a pattern similar to amitraz, BTS 27271, the active metabolite of amitraz, caused a concentration-dependent inhibition of insulin secretion. By calculating the areas under the curve for the BTS 27271 treatments and comparing them with the corresponding area of the control group, BTS 27271 (0.001 μmol/L) did not cause any significant inhibition of insulin secretion (data not shown). However, BTS 27271 administered at 0.01, 0.1, 1, or 10 μmol/L significantly inhibited insulin secretion, with a maximal inhibition of 34%, 52%, 72%, and 85% of the baseline level, respectively (Fig 3A to D). The effluent concentrations of insulin remained low in all groups during the 10-minute washout period. Also by calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control group, the BTS 27271 groups (0.1, 1, and 10 μmol/L) had significantly lower insulin secretion than the control group (Fig 3B to D).

Effects of BTS 27271 on Glucagon Secretion

By calculating the areas under the curve for the BTS 27271 treatments and comparing them with the corresponding area of the control group, BTS 27271 (0.01 and 0.1 μmol/L) did not

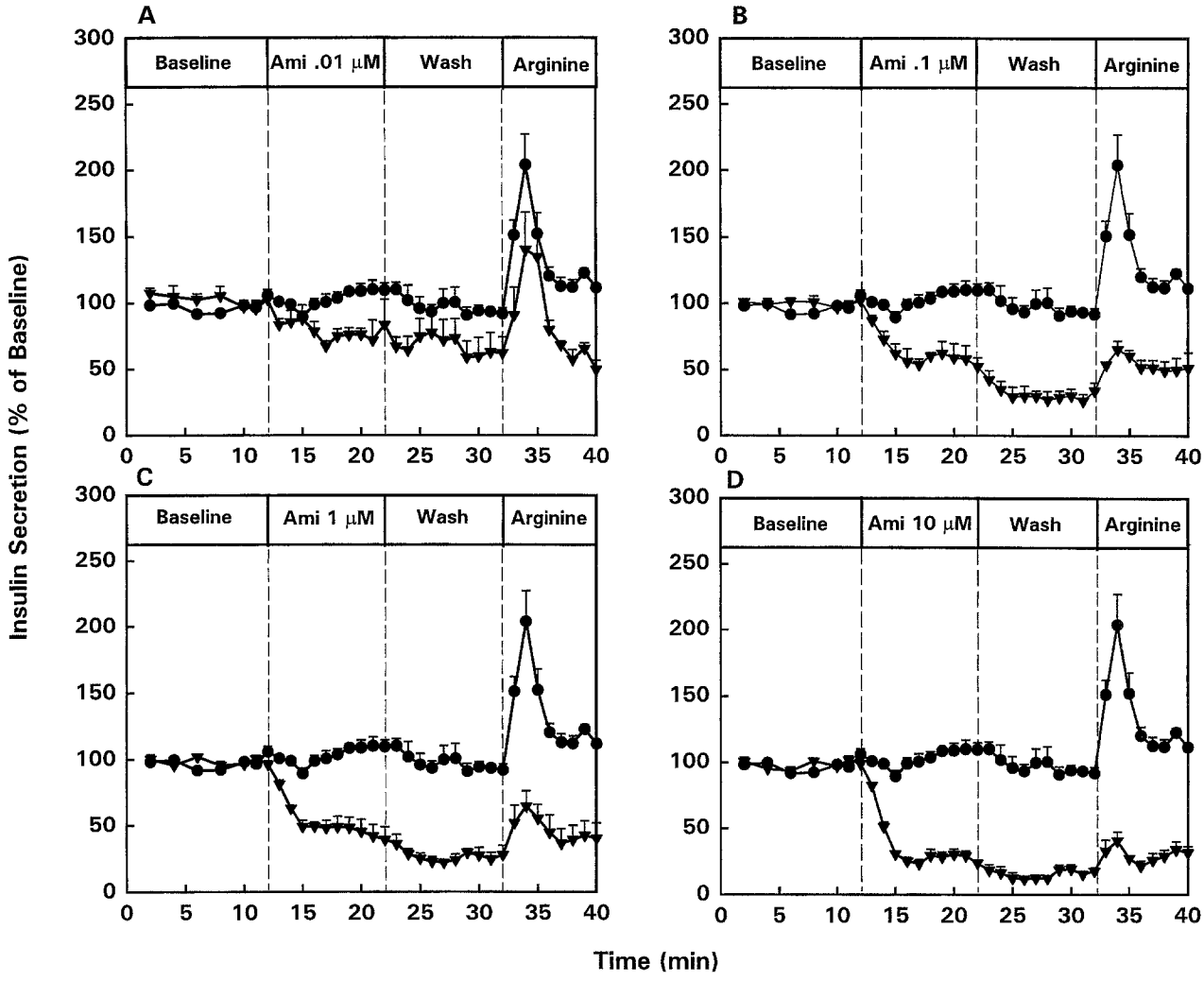

Fig 1. Effects of amitraz (Ami) on insulin secretion from the perfused rat pancreas. In these experiments, a 20-minute equilibration period preceded time 0. After a baseline period of 12 minutes, Ami was given for 10 minutes, followed by 10 minutes of the washout period (wash). At the end of the experiment, 1 mmol/L arginine was given for 8 minutes. Ami concentrations were 0.01 (A), 0.1 (B), 1 (C), and 10 μmol/L (D), respectively. By calculating the areas under the curve for Ami treatment and comparing them with the corresponding area of the control group, Ami (0.01, 0.1, 1, or 10 μmol/L) significantly ($P < .05$) inhibited insulin secretion. By calculating the areas under the curve for arginine treatment and comparing them with the corresponding area of the control group, the Ami groups (0.1, 1, and 10 μmol/L) had significantly ($P < .05$) lower insulin secretion than the control group. Values are the mean ± SE (n = 4-5). ●, Basal control; ▼, Ami.

significantly increase glucagon secretion during the treatment and washout periods (Fig 4A and B). However, BTS 27271 (1 and 10 μmol/L) significantly increased glucagon secretion, with a peak of 228% and 275% of the baseline level, respectively. The effluent concentrations of glucagon remained high during the 10-minute washout period in both groups (Fig 4C and D). By calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control group, the BTS 27271 groups (0.1, 1, and 10 μmol/L) had significantly higher glucagon secretion than the control group (Fig 4B to D).

Effects of α-AR Antagonists on Amitraz-Induced Inhibition of Insulin Secretion

Amitraz (1 μmol/L) alone inhibited insulin secretion by 45% ± 7% of the baseline level (Fig 1C). By calculating the areas under the curve for the amitraz-antagonist treatments and comparing them with the corresponding area of the amitraz-alone group, pretreatment of the pancreas with the $\alpha_{2A/2D}$-AR antagonist idazoxan (0.01 μmol/L) for 10 minutes did not significantly change the amitraz-induced inhibition of insulin secretion (Fig 5A), whereas idazoxan (0.1 and 1 μmol/L) antagonized the effect of amitraz on insulin secretion in a concentration-dependent manner (Fig 5B and C). Idazoxan (0.1 μmol/L) abolished and attenuated this effect during the treatment and washout periods, respectively. Idazoxan (1 μmol/L) abolished the effect of amitraz during both the treatment and the washout periods. Prazosin (1 μmol/L), and α_1- and $\alpha_{2B/2C}$-AR antagonist, failed to antagonize the effect of amitraz. By calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control groups, idazoxan (0.1 and 1 μmol/L) abolished the inhibitory

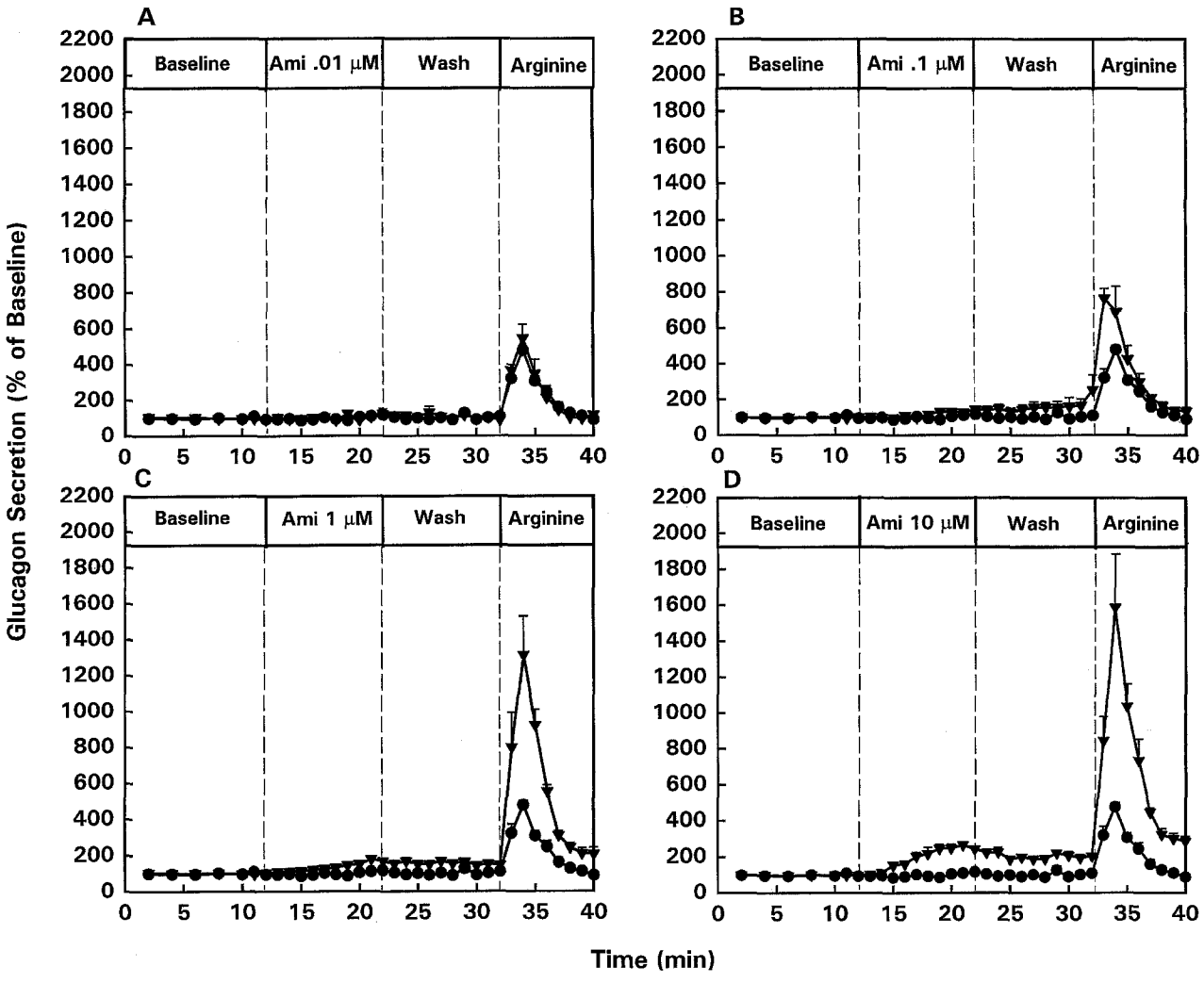

Fig 2. Effects of amitraz (Ami) on glucagon secretion from the perfused rat pancreas. The treatment protocol was the same as in Fig 1. Ami concentrations were 0.01 (A), 0.1 (B), 1 (C), and 10 μmol/L (D). By calculating the areas under the curve for Ami treatment and comparing them with the corresponding area of the control group, only Ami 10 μmol/L significantly ($P < .05$) increased glucagon secretion. By calculating the areas under the curve for arginine treatment and comparing them with the corresponding area of the control group, the Ami groups (0.1, 1, and 10 μmol/L) had significantly ($P < .05$) higher glucagon secretion than the control group. Values are the mean ± SE (n = 4-5). ●, Basal control; ▼, Ami.

effect of Amitraz (1 μmol/L) on 1-mmol/L arginine-induced insulin secretion (Fig 5B to D).

DISCUSSION

This is the first study to demonstrate the direct effect of amitraz and BTS 27271, the active metabolite of amitraz, on insulin and glucagon secretion from the pancreas. These results are consistent with the findings of previous in vivo[17,19,21,38] and clonal β-cell line[10] studies with respect to the effects of amitraz and BTS 27271 on insulin secretion. However, none of the previous studies demonstrated the effects of amitraz and BTS 27271 on glucagon secretion.

Amitraz and BTS 27271 (0.01 to 10 μmol/L) caused a concentration-dependent inhibition of insulin secretion, and the effect of BTS 27271 was slightly more potent than that of amitraz. These results are consistent with those of another study that demonstrated the inhibitory effect of amitraz and BTS 27271 on insulin secretion from the rat β-cell line RINm5F.[10] The other metabolites of amitraz were not examined in the present study, as it is known that BTS 27271 is the only metabolite of amitraz that inhibits insulin secretion from RINm5F cells.[10]

Amitraz and BTS 27271 increased glucagon secretion. These results were not unexpected, since other studies showed that α_2-AR agonists such as oxymetazoline and clonidine stimulate glucagon secretion.[31-34] However, the groups that were exposed to amitraz and BTS 27271 showed enhanced arginine-induced glucagon secretion despite the washout (10 minutes) of the pesticide. In vivo administration of amitraz caused binding to α_2-ARs in the rat brain that persisted at least 48 hours, although this binding was eliminated by six in vitro washings.[27] The enhancement of arginine-induced glucagon secretion could be

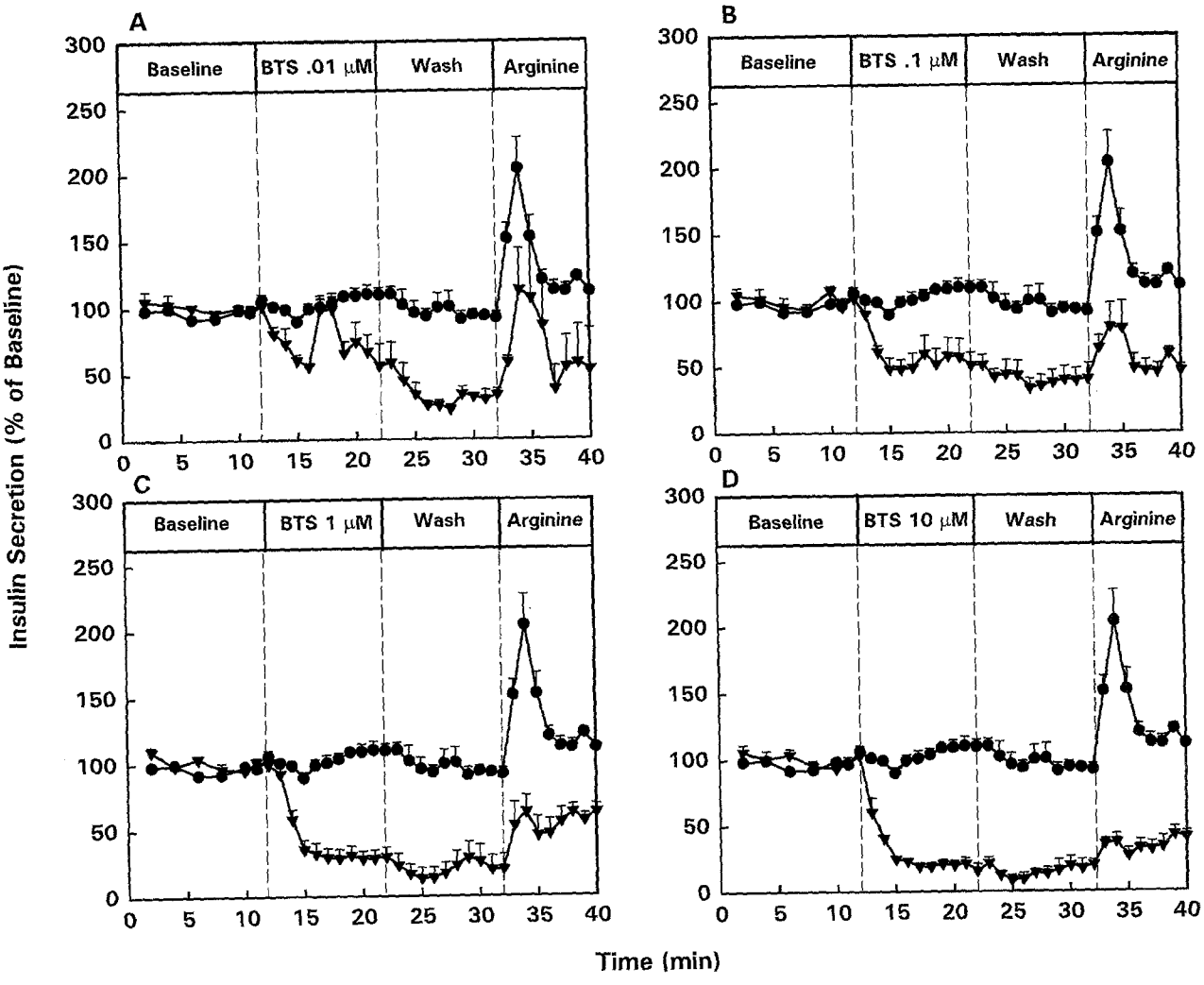

Fig 3. Effects of BTS 27271 (BTS) on insulin secretion from the perfused rat pancreas. The treatment protocol was the same as in Fig 1. BTS concentrations were 0.01 (A), 0.1 (B), 1 (C), and 10 μmol/L (D). By calculating the areas under the curve for BTS treatment and comparing them with the corresponding area of the control group, BTS (0.01, 0.1, 1, or 10 μmol/L) significantly ($P < .05$) inhibited insulin secretion. By calculating the areas under the curve for arginine treatment and comparing them with the corresponding area of the control group, the BTS groups (0.1, 1, and 10 μmol/L) had significantly ($P < .05$) lower insulin secretion than the control group. Values are the mean ± SE (n = 3-5). ●, Basal control; ▼, BTS.

due to a high binding affinity of amitraz and BTS 27271 to their receptors. Further studies in receptor affinity are needed to prove or disprove this speculation.

Amitraz is a highly lipid-soluble compound that is rapidly absorbed through cutaneous tissues,[15,16,29] and thus it is potentially hazardous to veterinarians, animal caretakers, and pet owners. Over 16 cases of amitraz toxicity in humans have been reported in which hyperglycemia was the most prominent sign.[11-15] Since hyperglycemia is found in most human cases of amitraz poisoning, exposure to this pesticide would be expected to pose a greater hazard to patients with type 2 diabetes, who already have impaired insulin secretion and/or utilization. α_2-AR agonists cause a greater suppression of insulin secretion from human patients with type 2 diabetes versus normal subjects.[39,40] Exposure of patients with type 2 diabetes to amitraz and its active metabolite may reduce their insulin secretion and further hamper glucose metabolism. The potential development of diabetes due to amitraz and BTS 27271 is not known and should be further investigated.

In both the amitraz and BTS 27271 treatment groups, the inhibition of insulin secretion persisted. The effluent insulin concentrations remained low during the 10-minute washout period, although they were expected to return to the baseline level after the washout of the pesticide. To answer the question of whether this effect is due to an insufficient washout time, we prolonged the washout period to 20 minutes; however, the effluent concentrations remained low and did not return to the baseline level (data not shown). Based on these results, we speculate that this phenomenon may be due to a high binding affinity of amitraz and BTS 27271 to their receptors. The effluent concentrations of glucagon also remained high during the 10-minute washout period for both the amitraz- and BTS

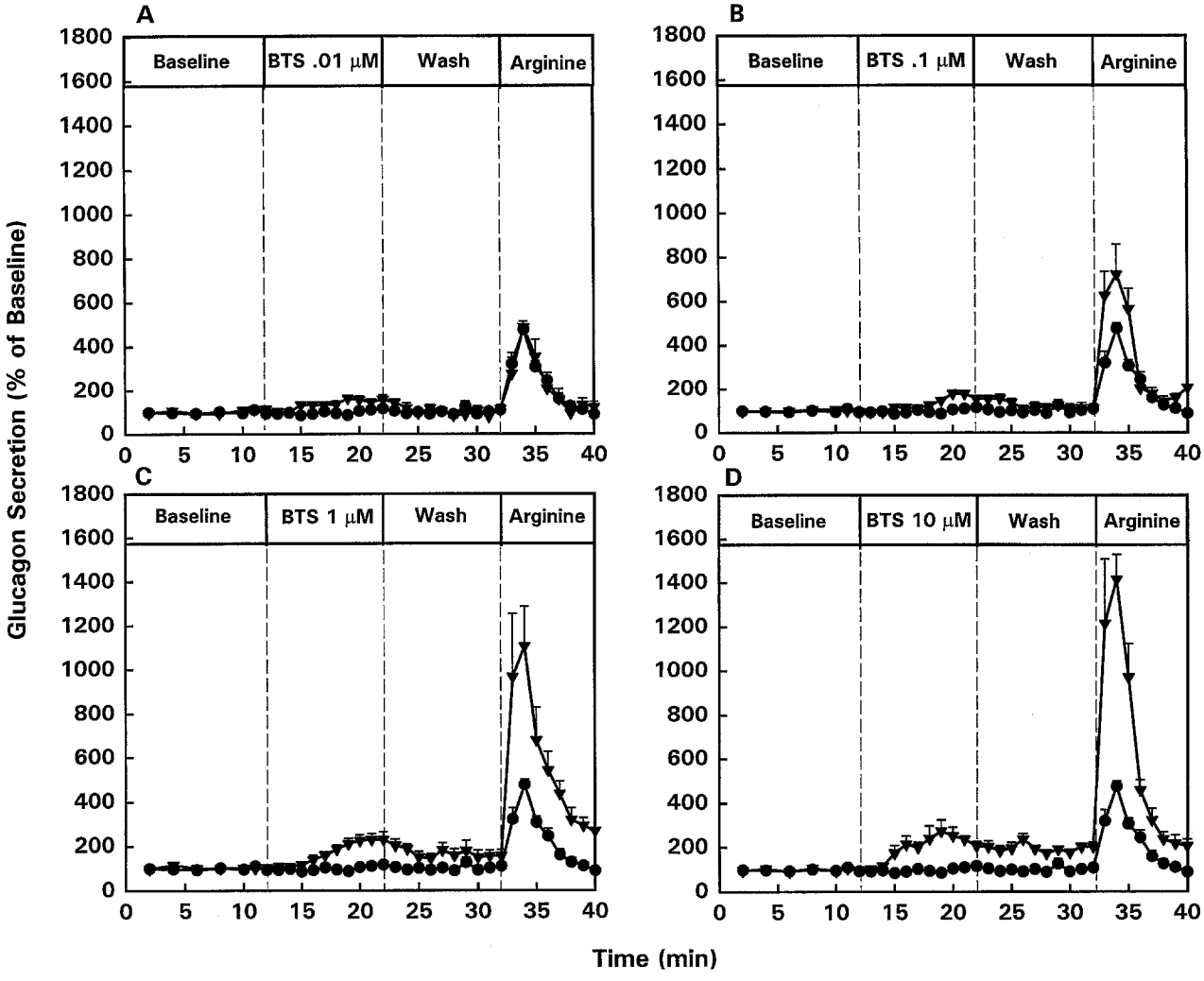

Fig 4. Effects of BTS 27271 (BTS) on glucagon secretion from the perfused rat pancreas. The treatment protocol was the same as in Fig 1. BTS concentrations were 0.01 (A), 0.1 (B), 1 (C), and 10 μmol/L (D). By calculating the areas under the curve for BTS treatment and comparing them with the corresponding area of the control group, only BTS 10 μmol/L significantly ($P < .05$) increased glucagon secretion. By calculating the areas under the curve for arginine treatment and comparing them with the corresponding area of the control group, the BTS groups (0.1, 1, and 10 μmol/L) had significantly ($P < .05$) higher glucagon secretion than the control group. Values are the mean ± SE (n = 3-5). ●, Basal control; ▼, BTS.

27271-treated groups. The persistent effect of amitraz and BTS 27271 on the secretion of insulin and glucagon should prompt careful handling of this pesticide to avoid the toxicity.

By calculating the areas under the curve for the arginine treatment and comparing them with the corresponding area of the control group, groups that were exposed to amitraz and BTS 27271 (0.1, 1, and 10 μmol/L) had significantly lower insulin secretion than the control group. However, when insulin secretion was expressed as the percentage increase above the pre-arginine value, groups that were previously exposed to amitraz and BTS 27271 were not significantly different from the control groups. All groups had approximately a 200% increase over the pre-arginine level in response to 1 mmol/L arginine. Thus, the effect of amitraz and BTS 27271 on arginine-induced insulin secretion depends on how the data are expressed.

Idazoxan, an $\alpha_{2A/2D}$-AR antagonist, blocked the inhibitory effect of amitraz on insulin secretion in a concentration-dependent manner, but prazosin, an α_1- and $\alpha_{2B/2C}$-AR antagonist, failed to antagonize the effect of amitraz. These results suggest that $\alpha_{2A/2D}$-ARs mediate the inhibitory effects of amitraz on insulin secretion. Our present findings are consistent with those of previous reports in which α_2-ARs mediated the actions of amitraz in animals[17,19,21,38] and a β-cell line.[10] The $\alpha_{2A/2D}$-ARs are orthologs, ie, a species may have either α_{2A} or α_{2D} but not both; rodents have α_{2D}-ARs but not α_{2A}-ARs.[41] Thus, α_{2D}-ARs should be the receptors that mediate the inhibition of insulin in rodents.

We did not study the effect of idazoxan or prasozin on the amitraz-induced increase in glucagon secretion, because we used amitraz at a concentration of 1 μmol/L, which decreased

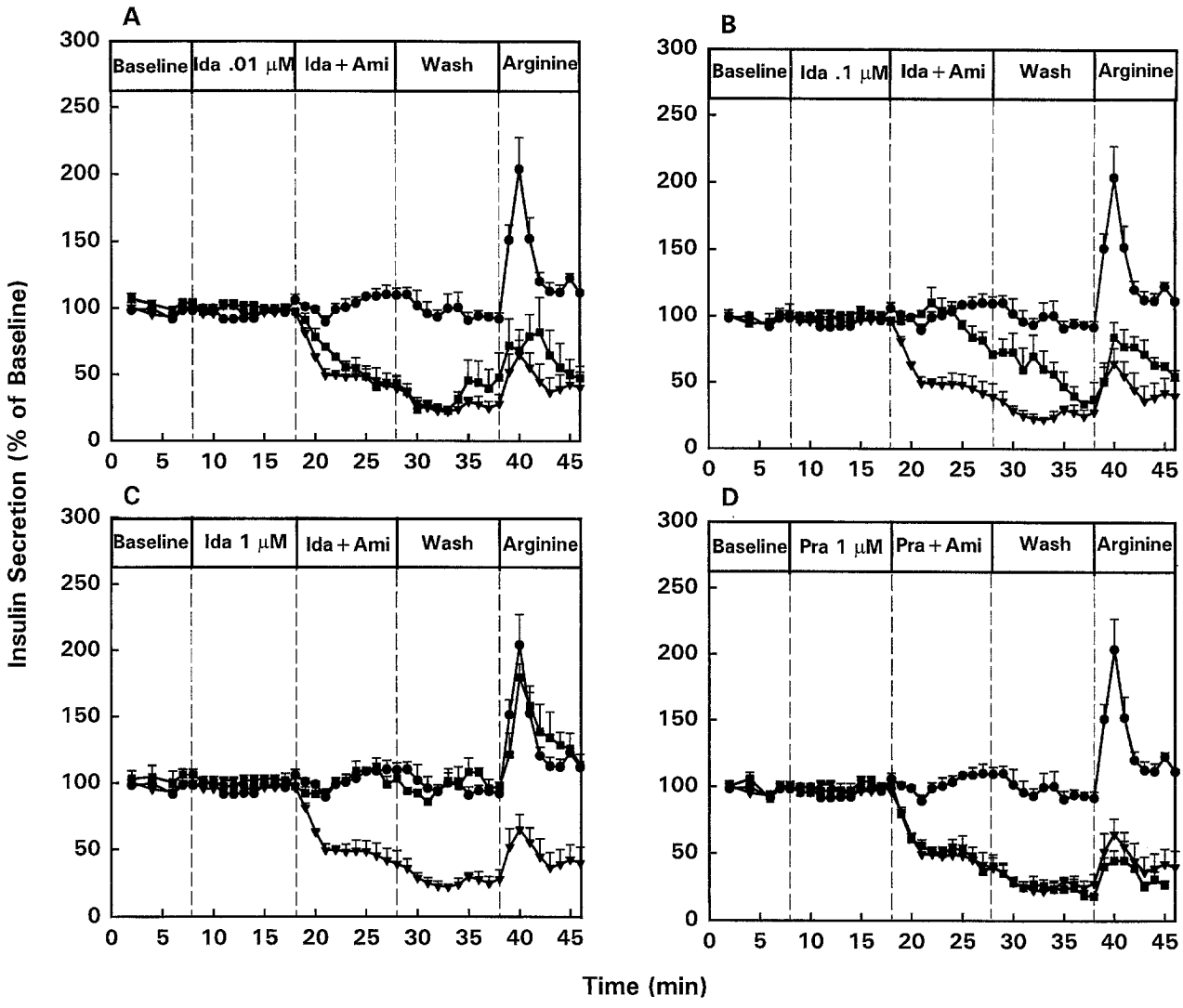

Fig 5. Effects of α-AR antagonists on amitraz (Ami)-induced inhibition of insulin secretion from the perfused rat pancreas. In these experiments, a 20-minute equilibration period preceded time 0. After a baseline period of 8 minutes, the $\alpha_{2A/2D}$-AR antagonist Idazoxan (Ida) or the α_1-AR antagonist, prazosin (Pra) were given for 10 minutes. This was followed by the medium containing Ami (1 μmol/L) and Ida or Pra for 10 minutes, and the basal medium for another 10 minutes for the washout period. At the end of the experiment, 1 mmol/L arginine was given for 8 minutes. By calculating the areas under the curve for the Ami-antagonist treatments and comparing them with the corresponding area of the Ami-alone group, Ida (0.01 μmol/L) did not significantly change the Ami-induced inhibition of insulin secretion (A), whereas Ida (0.1 and 1 μmol/L) antagonized the effect of Ami on insulin secretion in a concentration-dependent manner (B and C, respectively). Pra (1 μmol/L) failed to antagonize the effect of Ami (D). By calculating the areas under the curve for arginine treatment and comparing them with the corresponding area of the control groups, Ida (0.1 and 1 μmol/L) abolished the inhibitory effect of Ami (1 μmol/L) on 1-mmol/L arginine–induced insulin secretion (B and C). Values are the mean ± SE (n = 4). ●, Basal control; ■, α-antagonists (Ida or Pra); ▼, Ami.

insulin secretion by 45% ± 7% of the baseline level but did not cause significant increases in glucagon secretion. We intended to use amitraz (10 μmol/L) for the antagonism study, but idazoxan (1 μmol/L) failed to antagonize the effects of amitraz at this concentration (data not shown).

Imidazoline binding sites on pancreatic β cells have been reported.[42-44] A number of α_2-AR agonists (eg, oxymetazoline and clonidine), α_2-AR antagonists (eg, efaroxan, DG-5128, and idazoxan), and the nonselective α-AR antagonist phentolamine are imidazoline compounds. It is well established that imidazoline compounds such as phentolamine,[45] efaroxan,[46] and DG-5128[47] stimulate insulin secretion. In the present study, idazoxan (≤1 μmol/L) failed to stimulate insulin secretion. These results are consistent with those of another study demonstrating that idazoxan failed to alter insulin secretion or arginine-induced glucagon secretion.[48]

Our findings suggest that amitraz inhibits insulin secretion by activation of $\alpha_{2A/2D}$-ARs. Amitraz and BTS 27271 inhibit arginine-induced insulin secretion and enhance arginine-induced glucagon secretion. These effects may result from the high binding affinity of amitraz and BTS 27271 to the α_2-ARs of pancreatic islets. Since amitraz exerts α_2-AR agonistic

activities, specific α_2-AR antagonists may be useful in treating amitraz toxicity including hyperglycemia and hypoinsulinemia. The results of several human studies indicate a possible role of α_2-ARs in the impaired β-cell function in type 2 diabetic patients.[49-51] It is likely that these patients are more sensitive to amitraz than normal subjects. Further studies are needed to prove or disprove this hypothesis.

ACKNOWLEDGMENT

We wish to thank Laverne Escher for technical assistance.

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