J. Pharm. Pharmacol. 1979, 31: 593–597 Received October 16, 1978

Non-competitive-non-equilibrium α-adrenoceptor blocking properties of *N*-benzyl iodoacetamide, betsamide*

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N-Benzyl iodoacetamide, betsamide, at 10 mg kg^{-1} i.v. blocked the hypertensive and contractile responses of the nictitating membrane of the cat to adrenaline. The blockade had a lag period before full development. Pretreatment of cats with betsamide for 7 or 18 h showed a non-equilibrium type of α-adrenoceptor blockade. The responses of the nictitating membrane to adrenaline were markedly depressed and did not recover after high doses of adrenaline. In the same cats, adrenaline caused a profound hypotension. The effect of betsamide lasted for at least 72 h. In the rat isolated vas deferens, 3×10^{-5} M betsamide non-competitively blocked the contractile responses to noradrenaline; the adrenoceptor blockade was less effective when betsamide was applied with noradrenaline. The blockade lasted for more than 24 h, and was not reversible after extensive washing. Betsamide antagonized the contractile effects of carbachol and 5-hydroxytryptamine on the rat vas deferens, but not the β-responses of the guinea-pig trachea to adrenaline and isoproenaline. Results are discussed in relation to a probable mechanism of action.

According to Baker (1967), a selective antagonist can be designed by linking an alkylating moiety to a carrier with selective affinity for specific receptor sites. It occurred to us to simplify the skeleton of dibenamine in an attempt to find α-blocking agents producing a long-lasting antagonism. For the chemically active moiety, we chose an iodoacetamide. This molecule has alkylating properties that might contribute to a further understanding of the long lasting blockade of α-adrenoceptors. The *N*-benzyl derivative of iodoacetamide, referred herein as betsamide, was the best representative of the derivatives synthesized in this project.

We now report on a pharmacological study aimed primarily at determining whether betsamide produced a long-lasting α-blockade as predicted by Baker's principle, and to contrast its mechanism of action with that postulated for the β-haloalkylamines. Results demonstrate that betsamide blocks the α-responses of catecholamines in a non-competitive, non-equilibrium manner. The long-lasting duration of action is of considerable pharmacological interest in the understanding of the kinetics of alkylation, and its site of interaction on the adrenoceptor.

* Partially supported by a grant from "Fundación Gildemeister," Santiago, Chile.

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METHODS

In vivo experiments. Eight adult cats (2·0–4·3 kg) were anaesthetized with 35 mg kg^{-1} sodium pentobarbitone i.p., tracheotomized and both vagi sectioned. Arterial blood pressure was monitored from the carotid artery using rigid polyethylene tubing connected to a strain gauge transducer attached to a Grass polygraph. A femoral vein cannula was used for bolus i.v. injections of drugs. Cats were heparinized once with 2500 IU i.v. shortly before drugs were given. Under these conditions, blood pressure was maintained at a constant level during 4–6 h. Simultaneously, in the same cats, the free borders of the enucleated nictitating membranes were connected by surgical suture to a force displacement transducer, Grass FT. 03, as described by Contreras & Huidobro (1970). At the beginning of each experiment, the nictitating membrane was given a basal resting tension of 0·8 g which was maintained without variation for at least 30 min before drug injection.

Two kinds of experiments were performed. In acute experiments betsamide was injected i.v. as a bolus dose and the effects of adrenaline on the blood pressure and nictitating membrane were compared before and after the injection. To study long-lasting effects of the drug, cats were pretreated with 10 mg kg^{-1} betsamide i.p. for 7 to 96 h before injection of adrenaline. The basic criteria for establishing α-adrenoceptor blockade in the cardiovascular system

in vivo was based on the reversal of the hypertension produced by injections of adrenaline (Ad). We defined the blockade of Ad responses when the hypertension produced by Ad 5 to 30 μg i.v. per cat, produced only hypotension or a brief hypertension of less than 20 mm Hg, followed by a marked hypotension. With cats pretreated with betsamide, the reversal of the Ad hypertension was the main indication of adrenoceptor blockade. The tension produced by the nictitating membrane on the application of Ad was compared with that obtained in control cats before the i.v. administration of betsamide.

In vitro experiments

Vas deferens. Rat vasa deferentia (Sprague-Dawley rats, 200–250 g) were dissected and placed in a 30 ml organ bath maintained at 37 °C with Tyrode solution and gassed with a mixture of 5% CO_2 in oxygen. Isometric contractions were registered as described by Miranda (1976). Agonists were applied for 30 s at 7 min intervals. Contractile responses were plotted as % of the maximal agonist response vs log agonist concentration. Agonist dose effect curves were obtained before and after the application of 1 or $3{\cdot}3 \times 10^{-5}$ M betsamide for 1, 2 or 15 min. To study the long lasting blockade, dose effect curves were repeated 2 or 24 h after exposure to betsamide, the tissue being rinsed 4–5 times every 15 min. As a control for this particular experiment, vasa deferentia treated with betsamide or with vehicle were stored overnight at 4 °C, and tested with noradrenaline (NA) 24 h after drug treatment. To investigate whether the blockade was related to the active site of the α-adrenoceptor, $9{\cdot}7 \times 10^{-5}$ M NA was added to the bath 1 min before addition of $3{\cdot}3 \times 10^{-5}$ M betsamide. After a 15 min exposure to betsamide plus NA, the tissues were rinsed and 2 h later dose-response curves to NA were repeated.

As a further demonstration of the non-equilibrium, irreversible nature of the blockade, three sets of vasa deferentia were exposed to $3{\cdot}3 \times 10^{-5}$ M betsamide for 15 min, then rinsed and implanted aseptically into the peritoneal cavity of a female rat. Paired control vasa were treated identically, except that betsamide was replaced by the vehicle. A week after the implant, vasa were removed from the peritoneum and mounted on an organ bath to generate NA dose-effect curves in paired sets of vasa deferentia.

Guinea-pig trachea. Tracheal strips were maintained in Krebs solution at 37 °C, and gassed with a mixture of 5% CO_2 in oxygen. (The composition of the Krebs solution in g litre^{-1} was NaCl 6·9; $NaHCO_3$, 2·1; glucose 1·8; KCl 0·36; $MgSO_4$ 0·29; KH_2PO_4 0·16; and $CaCl_2$ 0·37.) Isometric contractions or relaxations were recorded by means of a force displacement transducer (FT 0·03 C) connected to a Grass polygraph. To study catecholamine-induced relaxations, contractions were induced in the preparations by carbachol 100 ng ml^{-1}; once a plateau contraction was reached, varying concentrations of the catecholamines were applied to obtain dose-response curves. Carbachol was added at 20 min intervals, and the catecholamines 10 min after the addition of carbachol.

Drugs and chemicals. Acetylcholine chloride, (−)-noradrenaline hydrochloride, (−)-adrenaline bitartrate, 5-hydroxytryptamine creatine sulphate, carbachol chloride and isoprenaline hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The salts employed in the Tyrode solution were analytical grade purchased from E. Merck (Darmstadt, Germany). Betsamide was synthesized in two steps: first, the chloro derivative was obtained which, on transhalogenation, gave the *N*-benzyl iodoacetamide. Chemical purity was certified by thin layer chromatography on silica plates, and structure was confirmed by nuclear magnetic resonance analysis. Betsamide was dissolved in a 2:1 mixture of propylene glycol–water. This solvent was innocuous in the in vitro preparations, and caused a short-lived hypotensive response in the cat blood pressure.

Statistical analysis. Statistical differences were ascertained using the two-tailed Student's *t*-test. The degree of significance was set at P less than 0·05. In some experiments, the agonist ED50 and its 95% confidence limits, and potency ratio, were calculated according to Litchfield & Wilcoxon (1949).

RESULTS

Antagonism of adrenaline (Ad) on the cardiovascular system and nictitating membrane of the cat. Betsamide antagonized the effects of Ad on the cardiovascular system and the nictitating membrane of the cat. The adrenoceptor blockade had a slow onset; 60–90 min after an i.v. dose of 5 or 10 mg kg^{-1} betsamide, only a slight reduction in the effects of Ad on the nictitating membrane or blood pressure were found. The blockade was clearly demonstrable after pretreatment with betsamide for 7 h, as evidence by a marked reduction of the contractile effects of Ad on the nictitating membrane (Fig. 1). Likewise, 3 out of 3 cats pretreated with 10 mg kg^{-1} betsamide for 18 h reacted to i.v. injections of

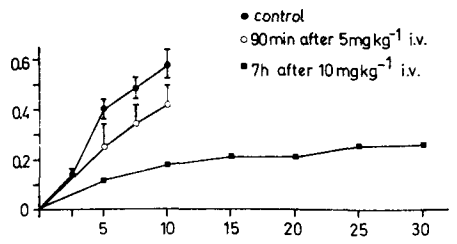

FIG. 1. Acute i.v. effect of betsamide on the adrenaline (Ad) responses of the nictitating membrane of the cat. Three cats were injected i.v. with 5 mg kg^{-1} betsamide. The responses of the nictitating membrane to the application of Ad were recorded before and after drug treatment. Open circles and squares represent mean tension developed by the membrane to the application of Ad; bars denote standard error of the mean. The curve with solid circles, denoted as control, was obtained from 3 cats before injection of betsamide. Solid squares represent responses from one cat pretreated with 10 mg kg^{-1} betsamide i.p. 7 h before the experiment. Ordinate: nictitating membrane tension (g). Abscissa: adrenaline (μg).

adrenaline with a profound hypotension. Fig. 2 shows the results of one of these experiments. Adrenaline produced only hypotension in these cats; in addition, the contractile effects of Ad on the nictitating membrane were significantly reduced by more than 50% compared with the effects of Ad on control cats not treated with betsamide. The adrenoceptor blockade produced by a dose of 10 mg kg^{-1} betsamide i.p. lasted for at least 3 days. In two cats pretreated with betsamide for 72 h, the i.v. administration of increasing doses of Ad caused marked hypotension similar to the results illustrated in Fig. 2. In one cat, 96 h after pretreatment, Ad

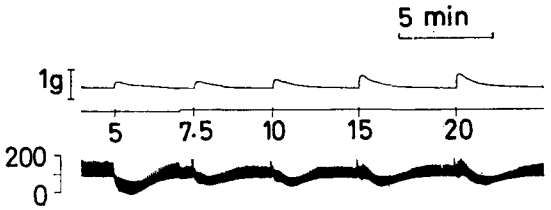

FIG. 2. Effect of adrenaline (Ad) on the nictitating membrane and blood pressure of a cat pretreated with 10 mg kg^{-1} betsamide i.p. 18 h before the experiment. Male cat, 4300 g. Upper record: nictitating membrane contractions. Lower panel: carotid blood pressure. Calibrations on left: blood pressure in mm Hg, tension in g. At the signals, doses of A (μg/cat) injected i.v. Note that although in this case there is no control for the effect of Ad on the blood pressure before drug treatment, all the doses produced profound hypotension as opposed to the well-known dose-related hypertensive effects of Ad in non-treated cats. The nictitating membrane contractions were reduced by about 50% when compared with the control group shown in Fig. 1.

caused graded hypertension, an indication that the blockade was possibly being overcome. A dose of 10 mg kg^{-1} betsamide itself did not cause significant changes in the cat blood pressure nor in the tension of the nictitating membrane within 1 h of injection.

Antagonism of the noradrenaline contractile responses in the rat vas deferens by betsamide. Betsamide antagonized the contractile responses of NA or Ad in the rat vas deferens, provided a lag period was allowed for the blockade to develop. In general, the blockade was not only dose-dependent, but it varied with the time the tissue was left in contact with betsamide. Two hours after a 15 min incubation with $3{\cdot}3 \times 10^{-5}$ M betsamide, the dose-response curve of NA was shifted to the right and was flat (Fig. 3). Proportionally less adrenoceptor blockade was observed when the tissues were incubated with

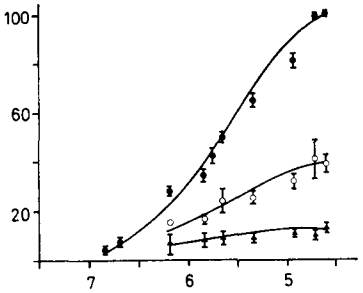

FIG. 3. Effect of $3{\cdot}3 \times 10^{-5}$ M betsamide on the rat vas deferens responses to noradrenaline. Dose response curves were obtained before (solid circles), 120 min after a 15 min incubation with betsamide (open circles), and 24 h later (solid, black triangles). The maximal effect of NA obtained for each preparation on day zero served as the 100% response for all dose effect curves. Each point represents the mean ± s.e.m. of 8 different experiments. Control, vehicle treated preparations, maintained in Tyrode solution for 24 h at 4 °C, exhibited a noradrenaline dose-effect curve superimposable on that represented by the open circles, and was omitted for practical purposes. Ordinate: % response. Abscissa: −log [NA].

betsamide for 1 or 2 min. The blockade was long-lasting, persisting for at least 24 h indicating that it is not easily reversible (Fig. 3). Vehicle-treated tissues stored at 4°C for 24 h exhibited a reduced response to NA compared with zero time, showing some degree of tissue deterioration. Nevertheless, the blocking effect of betsamide was evident since the NA dose response curve was flat (Fig. 3).

The blockade was not selective for α-adrenoceptor agonists on the rat vas deferens. Two h after a 15 min incubation with $3{\cdot}3 \times 10^{-5}$ M betsamide, the dose-response curves for carbachol and 5-HT were displaced to the right about 10-fold and the maximal

response reached up to 50% of the initial response. However, in spite of the non-specificity of blockade in the rat vas deferens, noradrenaline protected the α-adrenoceptor from betsamide's antagonism. The application of $9{\cdot}7 \times 10^{-5}$ M NA with $3{\cdot}3 \times 10^{-5}$ M betsamide significantly reduced by about 20% the α-adrenoceptor blockade produced by betsamide, in agreement with Furchgott (1954) and Graham et al (1971).

As a further indication that the adrenoceptor blockade produced by betsamide was long-lasting, and non-equilibrium in nature, experiments were conducted in tissues treated with betsamide and maintained for 7 days in the peritoneal cavity of a rat. Results indicate that after this prolonged storage, the vehicle-treated tissues were still capable of producing dose-dependent contractile responses, while the betsamide-treated vasa were almost unresponsive to NA. Fig. 4 illustrates paired control and betsamide treated vasa. The contractile responses observed in the vehicle-treated vasa were much less vigorous than those obtained in the same preparations on day zero.

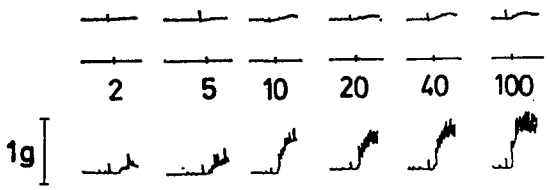

FIG. 4. Noradrenaline responses on vas deferens maintained for a week on the peritoneum of a recipient female rat after a 15 min exposure to $3{\cdot}3 \times 10^{-5}$ M betsamide. Upper panel: betsamide-treated vas deferens, lower panel: paired vehicle-treated vas deferens. At the signal, applications of noradrenaline in μg/bath. Calibration on left, valid for both panels. (On day zero, the vehicle-treated vas deferens developed a slightly higher muscular tension in response to noradrenaline than the contralateral, betsamide treated, vas deferens).

Effect of betsamide on the guinea-pig trachea. Responses to catecholamines in the trachea were not modified by $3{\cdot}3 \times 10^{-5}$ M betsamide. From Table 1, the ED50's of isoprenaline or Ad were not significantly altered in the presence of betsamide. Maximal agonists responses were attained, in contrast to the non-competitive kinetics seen in the rat vas deferens to the application of NA or other agonists.

Table 1. Effect of $3{\cdot}3 \times 10^{-5}$ M betsamide on the β-responses of catecholamines on the guinea-pig trachea.

	ED50 × 10^{-6} M (95% confidence limits) Before	After	Ratio*
Isoprenaline (6)[a]	0·175 (0·08–0·35)	0·50 (0·13–1·84)	2·85
Adrenaline (4)	2·5 (0·85–7·37)	6·25 (1·60–24·35)	2·50

*ED50 ratio after/before, in both cases $P > 0{\cdot}05$, not statistically significant.
[a] Number in parentheses refers to the number of experiments.

DISCUSSION

Both the in vivo and in vitro results indicate that betsamide produces a long-lasting, non-competitive α-adrenoceptor blockade. The reversal of Ad hypertension in cats pretreated with dibenamine for several days has long been considered as classical evidence for in vivo non-equilibrium α-adrenoceptor blockade (Nickerson & Goodman 1947). Pretreatment with betsamide for 18 h up to 3 days clearly demonstrates that the antagonism of adrenaline's effect on the blood pressure is long lasting. The time and dose-dependent kinetics of the noradrenaline antagonism in the rat vas deferens also support the hypothesis. The prolonged lag period required to demonstrate the antagonism in vivo and in vitro is probably related to the mechanism of the blockade; that most likely involves the generation of an active species. The active metabolite could be generated spontaneously or through enzyme catalysis. We favour the formation of a carbacation generated by the liberation of iodine in situ. The cation would attack nucleophylic groups in the close vicinity of the α-adrenoceptor causing the blockade. However, because of the high chemical reactivity, the cation will attack other sites apart from the α-adrenoceptor. In fact, the blockade is not selective for the α-adrenoceptor, although apparently it seems not to alter β-adrenergic responses in the guinea-pig trachea.

It is well documented that halomethylketones are very reactive compounds, and have been used extensively to modify biological macromolecules (Schramm & Lawson 1963; Lawson & Schramm 1965; Webb 1966). However, in spite of the non-specific chemical reactivity of betsamide, which could affect cellular metabolic processes, betsamide blocked the α-receptor. Furthermore, the blockade was reduced in the presence of noradrenaline. The protection experiments could be interpreted as suggesting that betsamide probably interacts (apart from all the α-receptor unspecific sites) near the 'active site' of the α-adrenoceptor, competing with noradrenaline. The implication is that betsamide must interact initially with the α-receptor in a competitive way and later, as a second step of blockade, in a non-competitive, non-equilibrium fashion. The nature of the long-lasting non-competitive effect is

probably related to covalent (irreversible alkylation) binding to the α-receptor. This aspect of betsamide's blockade is analogous to that of the β-haloalkylamines (Nickerson & Gump 1949; Graham & Lewis 1954; Triggle 1965; Furchgott 1966). In fact, the kinetics and the selectivity of blockade of these two compounds are almost identical.

The mechanism of an irreversible or non-equilibrium blockade of the α-receptor by betsamide is apparently similar to that of dibenamine and related mustards. There are many features common to both drugs: the prolonged in vivo and in vitro effect, the lag period before completion of blockade, the limited selectivity of blockade, and the relatively low potency as α-adrenoceptor blocking agents (Nickerson 1957). Previous studies suggested the possible involvement of a sulphyhydryl group on the α-receptor which could be sensitive to irreversible, covalent attack by α-blocking agents (Harvey & Nickerson 1954; Lippert & Bellau 1973; Huidobro-Toro & Carpi 1976; Salman et al 1976). The interaction of betsamide with this sulphyhydryl group on the α-receptor is certainly compatible with present results, and the chemistry of an iodoacetamide derivative.

In conclusion, betsamide constitutes a further example demonstrating the validity of Baker's principle in drug design. Efforts are in progress to design compounds with a higher selectivity of drug action. Even though betsamide does not present advantages over dibenamine as a blocking agent, it is of interest that both share apparently a similar mechanism of action in spite of different chemical structures and reactivities.

Acknowledgements

We are indebted to Dr Manuel Ruiz for his help and encouragement in the chemical synthesis of betsamide and Dr F. Gatta (Istituto Superiore Di Sanita, Roma) for the quantitative analysis and n.m.r. spectra of betsamide.

REFERENCES

Baker, B. R. (1967) Design of active site-directed irreversible enzyme inhibitors. John Wiley and Sons New York

Contreras, E., Huidobro, F. (1970) Br. J. Pharmacol. 39: 789–797

Graham, J. D. P., Ivens, C., Lever, J. D., McQuiston, R., Spriggs, T. L. B. (1971) Br. J. Pharmacol. 41: 278–284

Graham, J. D. P., Lewis, G. P. (1954) Br. J. Pharmacol. 9: 68–75

Furchgott, R. T. (1954) J. Pharmacol. Exp. Ther. 111: 265–284

Furchgott, R. F. (1966) in: Harper, N. J., Simmonds, A. (eds): Advances in Drug Research Academic Press: New York, p 2

Harvey, S. C., Nickerson, M. (1954) J. Pharmacol. Exp. Ther. 112: 274–290

Huidobro-Toro, J. P., Carpi, A. (1976) Arch. Int. Pharmacodyn. Ther. 222: 180–192

Lawson, W. B., Schramm, H. J. (1965) Biochemistry 4: 377–386

Lippert, B., Bellau, B. (1973) in: Usdin, E., Snyder, S. H. (eds.) Frontiers of Catecholamine Research. Pergamon, Oxford, p. 369

Litchfield, J. T., Wilcoxon, F. (1949) J. Pharmacol. Exp. Ther. 96: 99

Miranda, H. (1976) Arch. Int. Pharmacodyn. Ther. 221: 223–234

Nickerson, M. (1957) Pharmacol. Rev. 9: 246

Nickerson, M., Goodman, L. S. (1947) J. Pharmacol. Exp. Ther. 89: 167–185

Nickerson, M., Gump, W. S. (1949) J. Pharmacol. Exp. Ther. 97: 25–47

Salman, A. N., Chai, H., Miller, D. D., Patil, P. N. (1976) Eur. J. Pharmacol. 36: 41–48

Schramm, H. M., Lawson, W. B. (1963) Z. Physiol. Chem. 332: 97–100

Triggle, D. J. (1965) in: Harper, N. J., Simmonds, A. (eds), Advances in Drug Research. Academic Press, New York. p. 173

Webb, J. L. (1966). Enzyme and Metabolic Inhibitors. Vol. II, p. 635 and Vol. III, p. 1. Academic Press New York