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Evaluation of cryogenine on rat paw thermal oedema and rat isolated uterus

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Summary

1. Significant inhibition of oedema formation caused by thermal injury was observed for calcium carbaspirin, phenylbutazone, hydrocortisone, cryogenine and indomethacin when given daily beginning 2 days prior to thermal exposure and afterwards. Several anti-inflammatory drugs, including cryogenine, failed to reduce thermal oedema significantly when given as single doses 1 h prior to the thermal injury.

2. Kinetic experiments on the rat isolated uterus demonstrated that cryogenine, chlorpromazine, and flufenamic acid were, in part, competitive inhibitors of synthetic bradykinin, while indomethacin and tetrabenazine showed only non-competitive antagonism.

Introduction

The anti-inflammatory activity of cryogenine (an alkaloid obtained from the Mexican plant *Heimia salicifolia* Link and Otto)† was documented initially by Kaplan, Wolke & Malone (1967). Trottier & Malone (1969) have investigated the antihistamine, antiacetylcholine and anti-5-hydroxytryptamine activities of cryogenine on smooth muscle preparations. The present study was undertaken to determine the antibradykinin activities of cryogenine *in vivo* and *in vitro*. The activity of cryogenine was compared with that of several clinically used anti-inflammatory compounds and of certain other agents known to possess anti-inflammatory activity in most experimentally-induced and pathologically-generated inflammations (Collier, Holgate, Schachter & Shorley, 1959; Collier & Shorley, 1963; Laurence & Bacharach, 1964; Bonta & De Vos, 1967; Collier, James & Piper, 1968; Greenbaum, Carrara & Freer, 1968; Kellermeyer & Graham, 1968; Nies & Melmon, 1968; Schachter, 1969). We have reinvestigated thermal oedema (Rocha e Silva & Antonio, 1960) as a model for experimental inflammation and have observed the time-course of thermal trauma on rats' hind paws. Possible drug-receptor specificities of the noted antibradykinin activity were studied on the isolated rat uterus.

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† The cryogenine (mol. wt.=435·53) used in this study was isolated from plant material by Dr. John K. Brown, Department of Pharmacognosy, University of the Pacific, Stockton, California, and should not be confused with the trade name product Cryogénine (phenylsemicarbazide, mol. wt.=151·2), a specialty of Lumière of Lyons, France, and distributed by Laboratoires Sarbach of Châtillon, France. For the structure of alkaloidal cryogenine, see Trottier & Malone (1969).

Methods

Thermal oedema

Adult male Sprague-Dawley rats (140–200 g, Simonsen-A) were deprived of food 1–2 h prior to oral administration of agents suspended or dissolved in 0·25% agar and given at a constant volume of 10 ml/kg. Controls received only the agar vehicle. One hour after dosing, the rats were anaesthetized with sodium pentobarbitone (45 mg/kg, i.p.). Both control and treated animals (8/group) were placed around the perimeter of a water bath (46·5° C) so that the left foot extended into the bath to a level marked just above the ankle. The bodies of the animals rested on a fibre-board surface extended away from the bath so as to avoid heating the entire animal. The rats were removed from the apparatus after 30 min and rectal temperatures recorded 5–10 min later. One hour after removal from the bath, the animals were decapitated and the hind feet amputated just above the ankle joint. Oedema was assessed by calculating the percentage difference between the weights of the swollen foot and the non-heated foot of the same animal (the feet were weighed to the nearest mg).

In multiple-dose studies, rats were dosed orally once a day for 2 days prior to heating the paw, a third dose was given 1 h before foot immersion, and one daily dose thereafter for 3 days. Foot volumes were recorded by a plethysmograph daily starting 1 h after removal from the bath. The rats' paws were immersed in a mercury well up to a reference mark inked on the ankle. The displacement of mercury was transmitted through a transducer (Statham P23BB) and recorded using an electric-stylus recorder (Beckman type RS Dynograph).

TABLE 1. *Effects of orally administered compounds given 1 h prior to the development of thermal oedema (30 min exposure) in the rat*

Test agent (salt)	Dosage (mg/kg)	% thermal oedema Control	% thermal oedema Treated	% reduction oedema	*P*	°C change rectal temperature	*P*
Hydrocortisone (acetate)	100	82·8± 7·8*	80·4± 5·3	−2·8	>0·50	−0·11	>0·50
Carbaspirin (calcium)	400	90·8± 8·9	100·0± 5·3	+10·1	>0·25	+0·57	>0·10
Phenylbutazone	200	98·8± 7·5	91·9± 6·7	−7·0	>0·50	+0·04	>0·50
Indomethacin	50	97·6± 5·5	81·2± 7·0	−16·8	>0·50	+0·08	>0·50
Flufenamic acid	200	99·1± 9·8	83·1±11·6	−16·2	>0·25	+0·08	>0·50
Chlorpromazine (HCl)	50	93·9± 7·8	55·4± 8·3	−41·0	<0·01	−1·01	<0·05
Tetrabenazine	150	93·3±11·8	47·7± 8·3	−48·9	<0·001	−1·45	<0·05
Benzocaine	500	100·0± 9·9	71·8± 8·2	−28·2	<0·05	−1·33	<0·005
Morphine (sulphate)	500	93·5± 7·4	67·2± 8·0	−28·1	<0·05	+0·92	<0·001
Reserpine	150†	92·2± 5·7	91·2± 7·9	−1·0	>0·50	−0·93	<0·025
Cryogenine	250	90·6± 7·3	76·7± 8·3	−15·3	>0·10	−0·88	<0·05

* Figures represent the mean of 7–8 determinations ±1 S.E. Each determination was made 1 h after termination of thermal exposure. † Not given as a single dose, but as 50 mg/kg daily for three days prior to thermal challenge.

TABLE 2. *Effects of multiple-dose premedication on thermally-induced pedal oedema (weight) in the rat*

Test agent	Dosage (mg/kg)	% thermal oedema	% reduction oedema	*P*
Agar vehicle	—	87·1± 5·3*	—	—
Cryogenine	100	65·8± 6·4	−25·3	<0·025
Indomethacin	25	56·0±11·0	−36·7	<0·025
Flufenamic acid	50	95·1± 9·2	+9·2	>0·25

Doses listed were administered orally daily for 2 days and 1 h before exposure to heat. Oedema was determined 1 h after removal from the bath. * Figures are the mean ±1 S.E.

In another type of experiment, animals were dosed orally 2 days and 1 h before thermal exposure. One hour after heating, the rats were killed and the feet amputated and weighed as previously described. In all multiple-dose studies, the animals were allowed free access to food and water.

Isolated rat uterus

Uterine horns obtained from virgin rats primed with 1 mg/kg diethylstilboestrol injected s.c. 24 h previously, were suspended in 49 ml de Jalon's solution at 31° C and aerated with 95% O_2 and 5% CO_2. Methods followed the cumulative techniques described by van Rossum (1963). When antagonists could be dissolved only with the aid of acid or base, the appropriate control responses were obtained with synthetic bradykinin which had been incubated for 10 min with the same concentration of acid or base. Whenever possible, the antagonists were diluted in de Jalon's solution in order to minimize pH and ionic changes when introduced into the perfusion bath.

Agonist affinity (pD_2) for bradykinin was calculated from the pooled concentration-response curves using the methods of van Rossum (1963) and Ariëns (1964). The affinity constant for each competitive antagonist (pA_2) was calculated from the

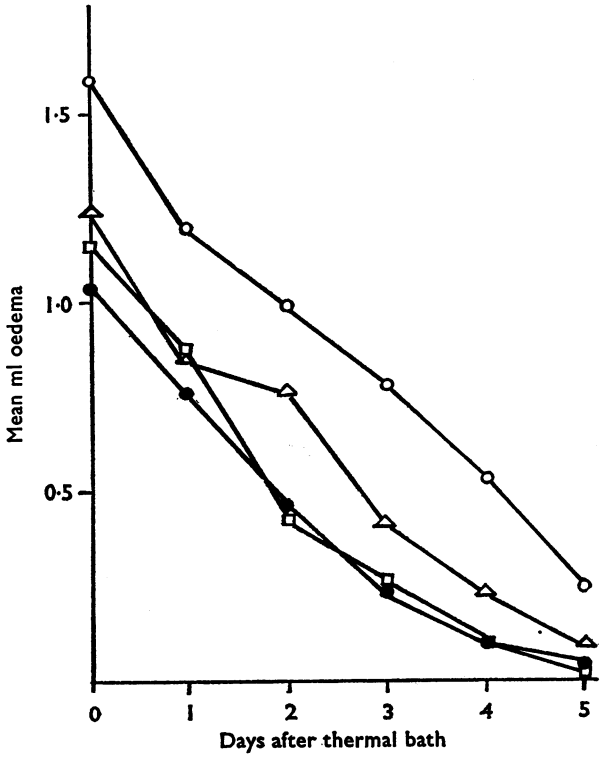

FIG. 1. Time-course of thermal oedema (volume); 1 h after removal from the bath designated here as 0 time. Drug dosing was initiated 2 days prior to heat exposure and terminated 3 days later. Agar vehicle control, 10 (ml/kg)/day (○——○); calcium carbaspirin, 200 (mg/kg)/day (△——△); phenylbutazone, 100 (mg/kg)/day (□——□); hydrocortisone, 50 (mg/kg)/day (●——●). Percentage oedema 1 h after removal from bath ±1 S.E. (observed *P*): agar vehicle=116·0±6·6; calcium carbaspirin=90·7±6·8 (<0·025); phenylbutazone=81·7±6·7 (<0·005); hydrocortisone=77·4±7·4 (<0·005).

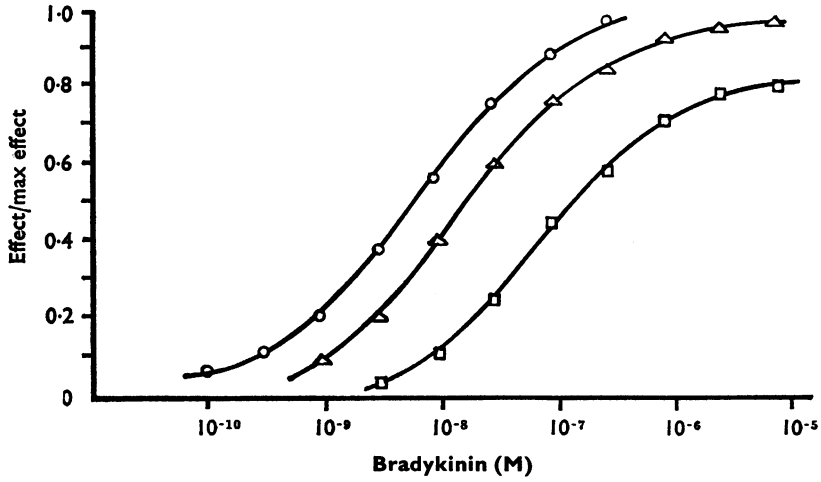

FIG. 2. Log concentration-response curves on isolated rat uterus. Bradykinin alone (○——○); bradykinin after 10 min incubation with 10 μM (△——△) or 30 μM flufenamic acid (□——□). Other incubation concentrations are not shown for reasons of clarity.

horizontal displacements of the bradykinin concentration-response curve after 10 min incubations with various concentrations of the antagonist. The affinity constant for each non-competitive antagonist (pD'_2) was calculated from the depressions of the maximum of the bradykinin concentration-response curve after 10 min incubations with various concentrations of the antagonist.

Results

Thermal oedema

As reported by previous investigators (Rocha e Silva & Antonio, 1960; Starr & West, 1967), exposure of the rat paw to 46·5° C caused marked oedema and multiple petechiae. As shown in Table 1, several agents were tested for their anti-inflammatory activity when given orally 1 h prior to heat exposure; in experiments with chlorpromazine, tetrabenazine, morphine and reserpine, the dose of sodium pentobarbitone was reduced to 33 mg/kg to compensate for the central depressant activity of these neuroleptic agents. Reserpine and tetrabenazine produced palpebral ptosis; and the rats vocalized when handled indicating hyperaesthesia. After tetrabenazine, dorsal muscle tone was visibly increased. Morphine-treated animals were moderately cataleptic and benzocaine caused blanching of the extremities. Significant reduction of oedema formation was seen only with those agents affecting rectal temperature. However, while cryogenine and reserpine lowered rectal temperature, no reduction of oedema was observed.

In the multiple-dose study (Fig. 1), oedema formation was significantly inhibited by calcium carbaspirin, phenylbutazone, and hydrocortisone 1 h after removal from the bath. Paw swelling in these groups remained well below control levels during the entire observation period and the feet manifested less severe exfoliative lesions. Multiple dosing with cryogenine and indomethacin (Table 2) also produced an anti-inflammatory effect 1 h after heating.

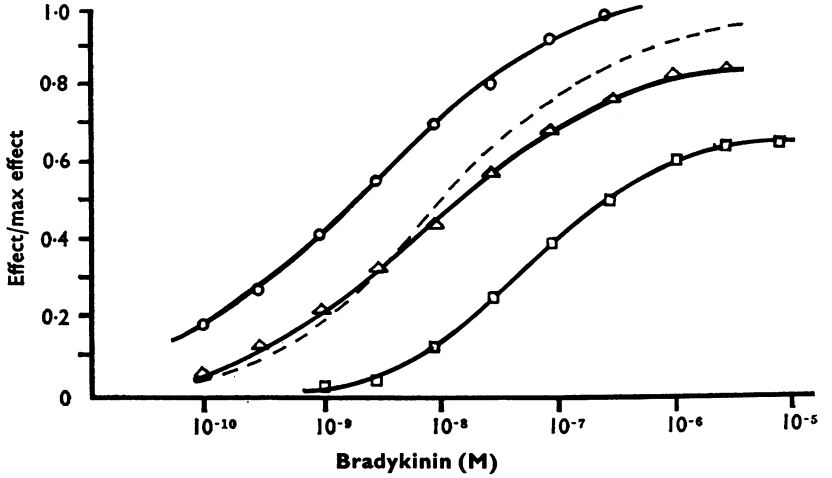

FIG. 3. Log concentration-response curves on isolated rat uterus. Bradykinin alone (○——○); bradykinin response after 10 min incubation with 30 μM (△——△) or 100 μM cryogenine (□——□). The broken line represents the bradykinin response after incubation with 1 ml of 0·2% acetic acid—the amount equivalent to that used to solubilize 100 μM cryogenine. Other incubation concentrations are not shown for reasons of clarity.

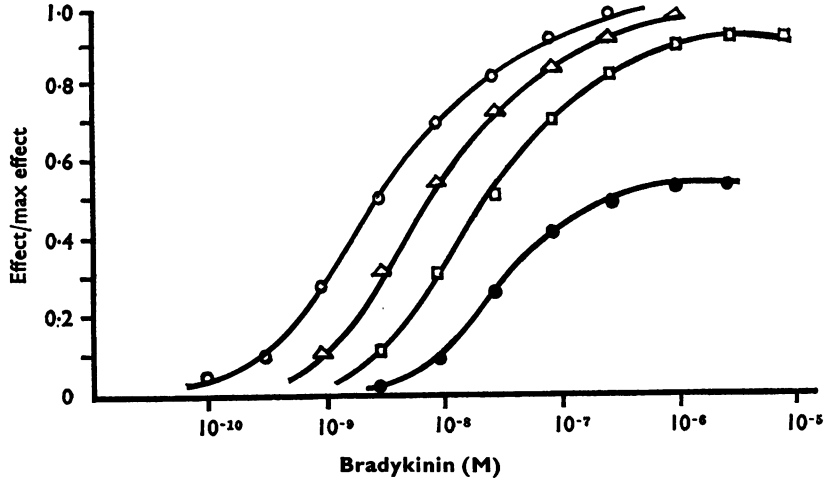

FIG. 4. Log concentration-response curves on isolated rat uterus. Bradykinin alone (○——○); bradykinin response after 10 min incubation with 1 μM (△——△), 3 μM (□——□), or 10 μM chlorpromazine HCl (●——●). Other incubation concentrations are not shown for reasons of clarity.

Isolated rat uterus

The results indicated that 1–100 μM incubation concentrations of calcium carbaspirin and phenylbutazone did not inhibit the bradykinin-induced contractions. Flufenamic acid (Fig. 2) produced both competitive and non-competitive activity. Cryogenine (Fig. 3) showed mixed inhibitory activity characterized by a reasonably parallel shift of the bradykinin concentration-response curve to the right and a consistent depression of maximum contractility of the tissue. Chlorpromazine pro-

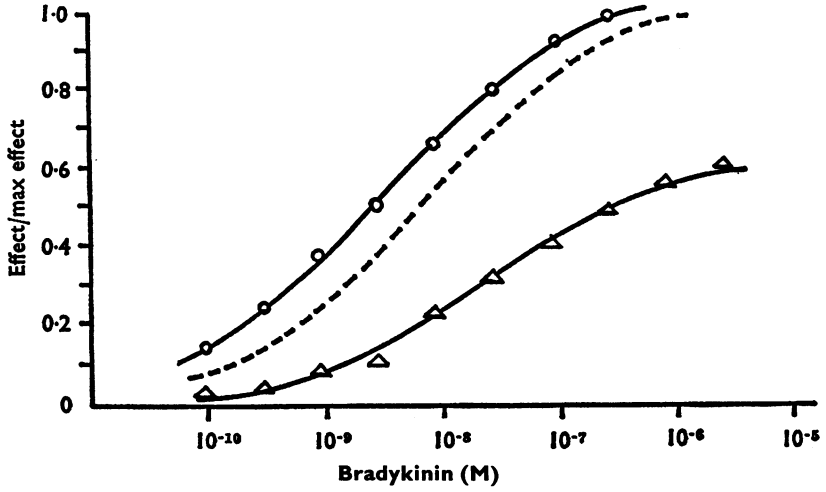

FIG. 5. Log concentration-response curves on isolated rat uterus. Bradykinin alone (○——○); bradykinin response after 10 min incubation with 100 μM indomethacin (△——△). The broken line represents the bradykinin response after incubation with 240 mcg NaOH—the amount equivalent to that used to dissolve the indomethacin. Lower incubation concentrations of indomethacin are not shown for reasons of clarity.

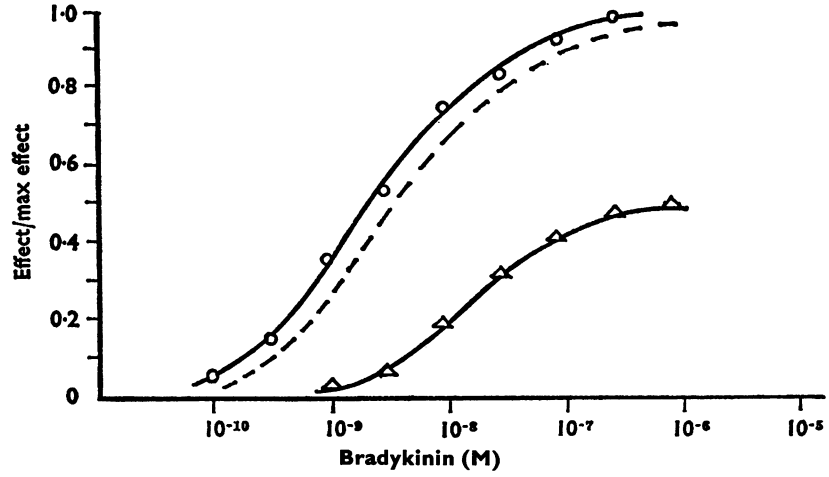

FIG. 6. Log concentration-response curves on isolated rat uterus. Bradykinin alone (○——○); bradykinin response after 10 min incubation with 100 μM tetrabenazine (△——△). The broken line represents the bradykinin response after incubation with 1 ml of 0·148% HCl—the amount equivalent to that used to dissolve the tetrabenazine. Lower incubation concentrations of tetrabenazine are not shown for reasons of clarity.

TABLE 3. *Affinity constants of antagonists* (10 *min incubation*) *against bradykinin* (pD_2 = 8·5, S.D. = 0·14) *on isolated rat uterus*

Antagonist (salt)	pA_2	pD'_2
Carbaspirin (calcium)	—	—
Phenylbutazone	—	—
Indomethacin	—	3·8
Flufenamic acid	5·6	3·8
Chlorpromazine (HCl)	5·6	4·9
Tetrabenazine	—	4·0
Cryogenine	5·7	3·7

duced definite competitive antagonism, and in the highest concentration some non-competitive activity (Fig. 4). Indomethacin and tetrabenazine showed only non-competitive antagonism (Figs. 5 and 6). Table 3 summarizes the quantitative evaluation of the types of antagonism seen.

Discussion

Thermal trauma is a model of inflammation which is particularly difficult to inhibit by orally administered drugs. Starr & West (1967) reported detectable anti-inflammatory activity against thermal oedema with intraperitoneal injections of clinically-established anti-inflammatory agents. We assumed that these agents would also be effective if administered orally. The oral route is preferable in this instance since the possibility of counterirritation from parenteral injections is eliminated. In the present study, the clinically useful drugs were ineffective orally unless the animals were primed by daily dosing beginning 2 days before thermal exposure. It appears that effective tissue levels must be attained well before and during the inflammatory challenge. We suspected that the centrally-active agents used by Rocha e Silva & Antonio (1960) were effective in thermal oedema because of lowered body temperature. However, the present investigation failed to reveal any correlation between a compound's ability to lower body temperature and its ability to inhibit the oedema following thermal injury. Certain centrally acting drugs may suppress or delay experimentally induced inflammations (Arrigoni-Martelli, Tóth, Segre & Corsico, 1967; Brown, Kissel & Lish, 1968; Kelsey & Frishmuth, 1968; Svanes, 1968) by lowering systemic blood pressure and thus reducing vascular leakage . If thermal injury is to be employed as a model for investigating true anti-inflammatory activity, the present authors suggest that multi-dose premedication be used.

The evidence that thermal-oedema is, in part, mediated by bradykinin (Rocha e Silva & Antonio, 1960) is supported by the finding that certain of the compounds effective *in vivo* also possessed anti-bradykinin activity *in vitro*. However, phenylbutazone and calcium carbaspirin, which were effective against thermal oedema, did not possess anti-bradykinin activity in the isolated uterus preparation. Simke, Graeme & Sigg (1967) have demonstrated that both of these agents are antagonists of bradykinin-induced bronchoconstriction in the anaesthetized guinea-pig. On the other hand, they are not effective bradykinin antagonists in the guinea-pig isolated lung preparation as described by Aarsen (1966).

In the isolated rat uterus, chlorpromazine, cryogenine, and flufenamic acid antagonized bradykinin competitively and within a very close range of molecular potency. These compounds cannot be considered specific bradykinin antagonists since they also antagonize other pharmacologically active substances. Indomethacin and tetrabenazine exhibited only non-competitive activity in the isolated uterus; therefore, the ability of a compound to reduce thermal oedema and its antibradykinin activity in rat uterus cannot be correlated.

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