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Effects of proctolin on autonomic physiological functions in insects

KAREL SLÁMA[1], DANUTA KONOPIŃSKA[2] and WIESLAW SOBÓTKA[3]

[1]Laboratory of Ecological Pharmacology, INTERECO, Kladenská 11, 160 00 Praha 6, Czech Republic

[2]Institute of Chemistry, University of Wroclaw, ul. Joliot-Curie 14, 50383 Wroclaw, Poland

[3]Polish Academy of Sciences, Institute of Organic Chemistry, ul. Kasprzaka 44, 01224 Warszawa, Poland

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Abstract. The pharmacological effects of proctolin, [L-Dopa^2]-proctolin, [Phe(OMe)^2]-proctolin and [Cha(4-OMe)^2]-proctolin were studied on pupae of the mealworm, *Tenebrio molitor*. The results were obtained by prolonged monitoring of extracardiac pulsations in haemocoelic pressure. These pulsations revealed the functions of the autonomic nervous system (coelopulse). The administration of proctolin in 100 nM to 5 μM concentrations increased the amplitude of haemocoelic pressure pulsations up to six-fold, within a few minutes. With dosages corresponding to 1 μM concentrations, the effects of proctolin culminated between the 2nd and 3rd hour post-treatment, with a successive decline to the original level within the next 15–20 hrs. The frequency of pulsations and duration of resting periods were unaffected. The [L-Dopa^2]-proctolin analogue was slightly more effective than proctolin itself, although its action was retarded for 20–30 min. The remaining two proctolin derivatives were completely inactive.

Large doses of proctolin or [L-Dopa^2]-proctolin (more than 5 μM concentrations in the body) produced pathophysiological symptoms of "hyperproctolinism", manifested by paralysis of all haemocoelic pulsations for several hours. The findings of considerable in vivo persistence of proctolin are in conflict with some general views upon the rapid inactivation of proctolin by enzymes present in the haemolymph or tissues. It is possible that proctolin may be a valid neurohormone, involved in the regulation of homeostatic physiological functions, and this is discussed.

INTRODUCTION

Proctolin is certainly the best known and most investigated insect neuropeptide with pronounced myotropic functions (reviews by Starratt & Steele, 1980; O'Shea, 1986). In explanted neuromuscular preparations of various visceral organs, proctolin increases the amplitude of muscular contractions (Brown, 1977). Thus, for example, proctolin affects the heartbeat in isolated heart preparations (Miller, 1979), it acts on the peristaltic movements of the oviducts (Cook & Meola, 1978; Lange & Orchard, 1986) and, more relevant for this study, it increases the amplitude of contractions in some somatic skeletal muscles (Piek & Mantel, 1977; O'Shea, 1986). Once it was assumed (cf. O'Shea, 1982, 1986) that proctolin could act at a distance from its points of release, but in contrast to this assumption, the in vitro metabolic studies revealed extremely short biological half-lives of proctolin (see papers by Quistad et al., 1984; Starratt & Steele, 1984; Steele & Starratt, 1985; Schooley et al., 1987). It was concluded that proctolin should be rapidly hydrolysed by the aminopeptidase enzymes present in the haemolymph or tissues. The final conclusions were that proctolin was unlikely to be transported via haemolymph to any more distant targets, unless it had been somehow protected from enzymatic inactivation.

In order to obtain more information on the pharmacokinetic action of proctolin, we investigated its action on the autonomic nervous system (coelopulse, see Sláma, 1988). The coelopulse system regulates various homeostatic functions by means of rhythmically repeated pulsations in haemocoelic pressure. These pulsations are produced by contractions of the intersegmental somatic abdominal muscles. The system regulates circulation of haemolymph through the appendages, it controls inspiration or expiration of gases through the determined spiracles and it also controls osmolarity and water balance. In this work we have used previously described electronic methods for monitoring responses of the coelopulse system to proctolin. These techniques have been used for the determination of pharmacokinetic effects of various other chemicals (see Sláma, 1984, 1986, 1987, 1988; Sláma & Miller, 1988). In this study we report on the pharmacokinetic effects of some proctolin derivatives that have been previously used for structure-activity studies by Konopińska et al. (1986a,b, 1988a,b, 1988c).

MATERIALS AND METHODS

The effects of proctolin on haemocoelic pressure were recorded in 4-day-old pupae of *Tenebrio molitor* L., at room temperature (22–25°C). The stock material of larvae and pupae was maintained and staged at 27°C. The effects on extracardiac pulsations in haemocoelic pressure were measured on a multichannel electronic tensiometric unit (M-1000, Mikrotechna Co., Prague), using procedures described by Sláma (1984, 1988).

Compounds to be tested were dissolved in insect Ringer solution and the amounts to be injected were adjusted to 2 μl volumes per pupa. Final concentrations of the hormones in the body were calculated on basis of 70% average water content from pupal weight. The test solutions were sucked inside the measuring needle of the hydraulic pressure transducer before initiation of the measurements (see Sláma 1976). The solution was separated from the haemolymph and hydraulic fluid by 1 μl of an inert mineral oil. Then, after several minutes or hours of control recording, the sample solution was injected into the body by means of the built-in Hamilton miniature valve connected to 701 microlitre syringe. The recordings continued usually for 5 to 24 hrs after the injection. Each concentration was tested on at least 5 pupae. The rate of the hormonal activity was assessed on the basis of changes in the frequency or amplitude of extracardiac pulsations.The methods for preparation of proctolin and its derivatives as well as the data on purity of the compounds used in this study have been described by Konopińska et al. (1986a, 1986b).

RESULTS

EXTRACARDIAC HAEMOCOELIC PULSATIONS IN NORMAL, 4-DAY-OLD PUPAE

The normal pattern of extracardiac pulsations in the pupae of *Tenebrio* undergoes chronological changes related to pupal-adult development. In the middle of the pupal instar, e.g., 3 to 5 days after pupal ecdysis (at 27°C), there occurs a rather constant pattern with very small individual variations, illustrated in Fig. 1. A more or less regular series of usually 6 to 10 pulsations are periodically repeated after 20–40 min periods of rest. The values of the baseline haemocoelic pressure are always subatmospheric in these pupae (0.2 to 0.5 kPa below local atmospheric pressure).

The haemocoel cavity of insect body represents a closed system with a more or less elastic integumental cover. Due to this, injection of any liquid material into the body would automatically increase the haemocoelic volume and, consequently, it would

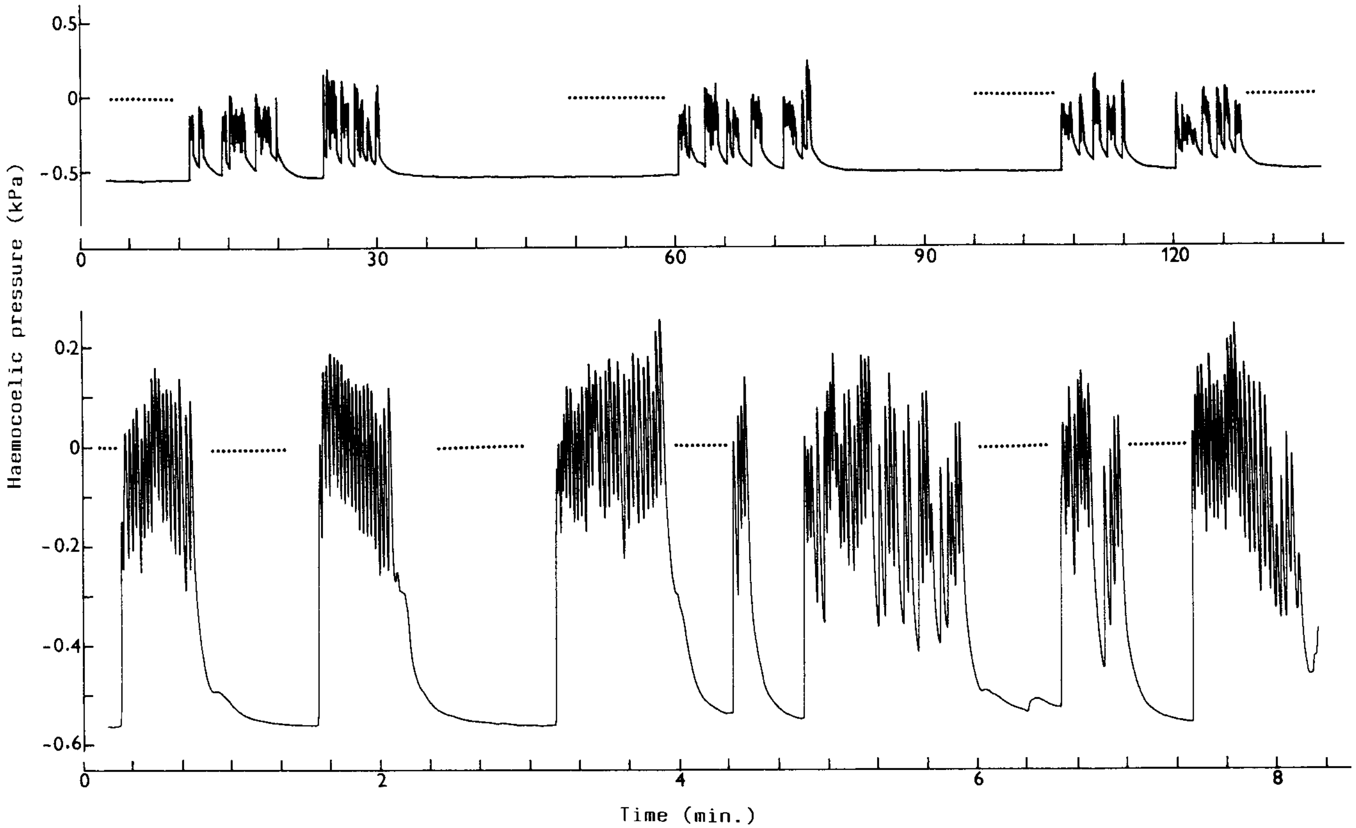

Fig. 1. Extracardiac haemocoelic pulsations in a normal, 4-day-old pupa of *Tenebrio molitor* at 25°C. Upper record shows regularly repeated series of th pulsations with subatmospheric baseline haemocoelic pressure during the periods of rest. Lower record shows one series of pulsations with expanded scales

increase mechanical pressure inside the body. Such a common, nonspecific effect of Ringer injection (2 μl of Ringer plus 1 μl of the outer oil septum) is shown in Fig. 2. It is important to stress that in all these experiments, which have been classified as ineffective, the amplitude of the haemocoelic pulsations never surpassed the pre-injection values more than twice. In addition, the nonspecific effects were always most pronounced closely after the injection, disappearing within 2 or 3 hours.

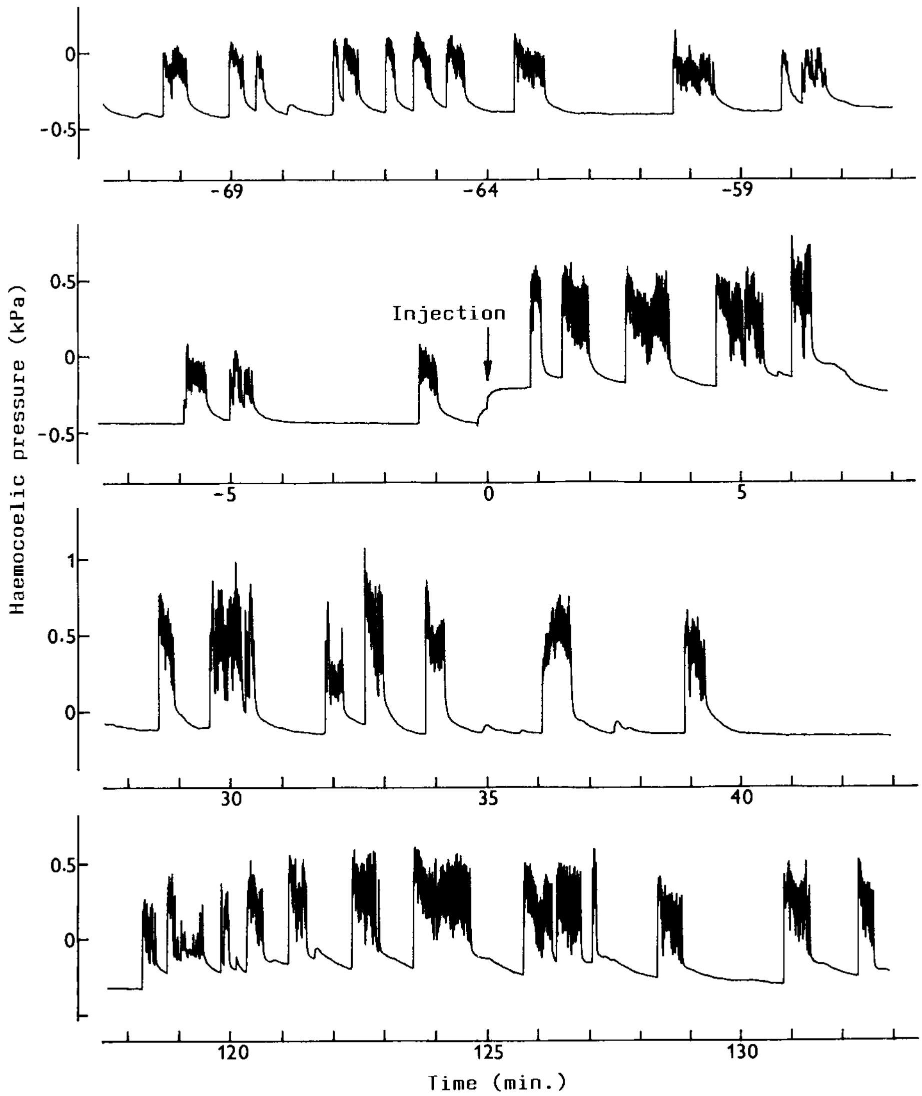

Fig. 2. Nonspecific elevation of haemocoelic pressure produced by injection of Ringer solution (2 μl of Ringer plus 1μl of inert oil from tip of the measuring needle) in a 4-day-old pupa of *Tenebrio* at 25°C.

THE EFFECTS OF PROCTOLIN

Administration of proctolin in doses corresponding to 0.1–5.0 μM concentrations in the body caused enormous elevation of the amplitude of extracardiac pulsations. For comparison, the usual measures of the pulsations in a normal, untreated, 4-day-old pupa of *Tenebrio* were: amplitude 0.3 to 0.5 kPa with the frequency between 0.5 and 0.6 Hz (at 25°C). After injections of proctolin, however, the pulsation amplitude rose to 2.5 kPa or even more, e.g., a 5- to 6-fold increase. By contrast, the frequency of individual contractions of each pulse remained more or less unchanged, suggesting absence of the chronotropic effects. An excellent example of these pharmacokinetic actions of proctolin can be found in Fig. 3. In contrast to the literature data suggesting rapid in vivo proctolin inactivation, the pharmacological symptoms of proctolin, as shown in Fig. 3, surprisingly persisted for several hours.

In most of our measurements we used dosages corresponding to 1μM concentrations of proctolin in the body (uninterrupted 48 hr recordings with 16 individual pupae). In this case the largest amplitudes of the pulsations occurred between 2–3 hours after the injections. Later, the enormously increased pulsation amplitudes successively diminished, reaching the original pre-treatment values usually after 10 to 15 hrs. All pupae injected with the dosages corresponding to 1μM concentrations of proctolin survived the treatment without remarkable developmental disturbances and emerged later as normal adults.

THE EFFECT OF LARGE AND SMALL DOSES OF PROCTOLIN

Large doses of proctolin (5–10 μM final concentrations in the body) caused almost immediate and severe paralysis of all muscles functioning in the coelopulse system. The paralysis was characterized by complete absence of all extracardiac pulsations for several hours. Curiously enough, there was no remarkable elevation of the baseline haemocoelic pressure during the period of the paralysis. This indicates that the overdosage of proctolin did not produce any tetanic-like or tonic contractions of the intersegmental abdominal muscular sheath.

Recordings performed during the above described period of paralysis revealed the presence of special, so far unseen, oscillations in haemocoelic pressure. These oscillations had very low frequency (3–5 per min) and rather small amplitude (below 50 Pa). They were most probably caused by unnatural overstimulation of the peristaltic movements of some visceral organs. In most cases the paralysis was reversible. In general, it may be regarded as a physiological syndrome of "hyperproctolinism". The termination of paralysis after several hours of inactivity occurred instantly with a series of extracardiac pulsations of great amplitude. The post-paralysis pulsations of large amplitude again continued for 10 or 15 hrs, which also confirms previous statements about long persistence of the proctolin effects.

Smaller doses of proctolin, from 100 to 10 nM concentrations in the body, also induced considerable elevation of pulsation amplitude, as shown in Fig. 4. Even in this case of low dosages, the maximum physiologial effectiveness occurred between the 2nd and the 3rd hr after the injection, although the effects usually disappeared much sooner, within 10 hrs after the injection.

The electronic method used in this study enabled prolonged monitoring of the effects of proctolin in the range of rather small, 1 to 100 nM concentrations in a number of

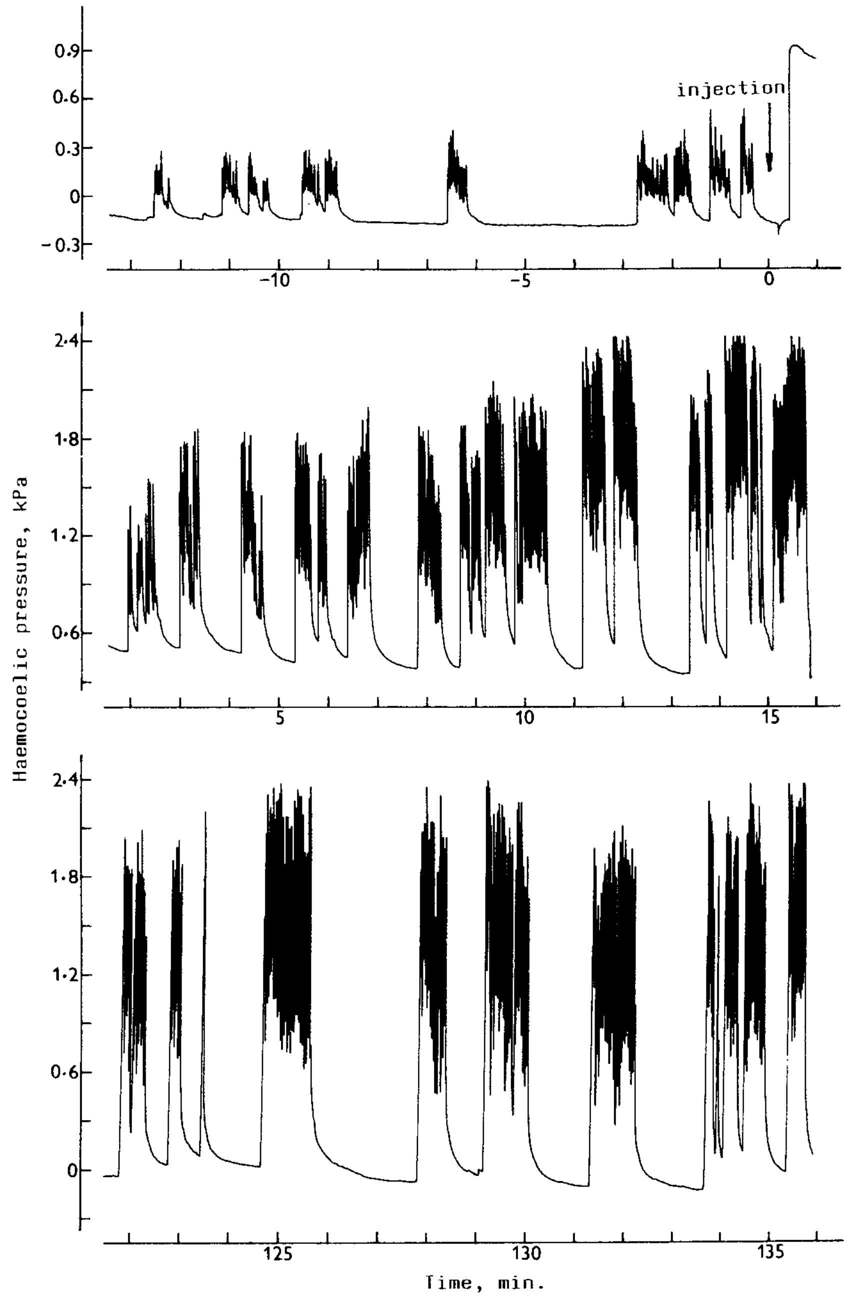

Fig. 3. The effect of proctolin (1 μM final concentration in the body) on extracardiac haemocoelic pulsations in 4-day-old pupa of *Tenebrio* at 24°C.

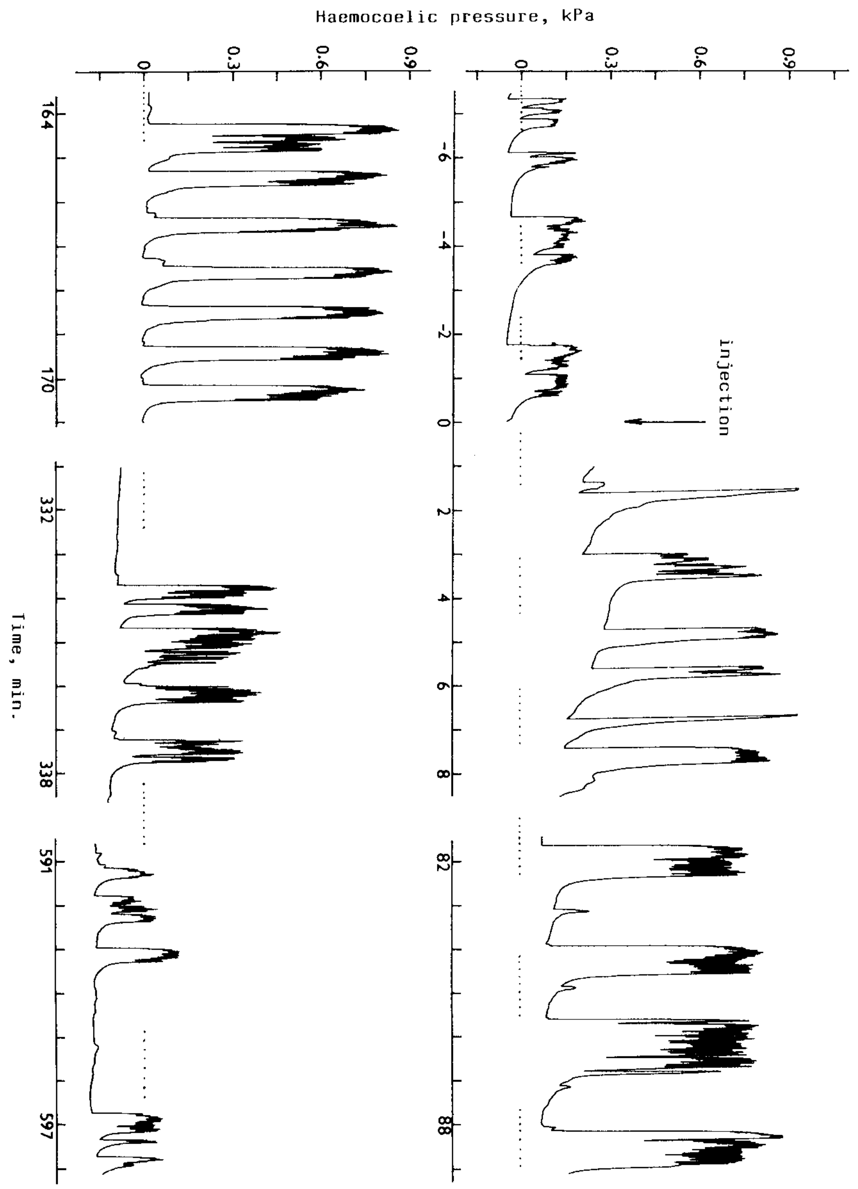

Fig. 4. Reversible effects of small concentrations of proctolin (100 μM final concentration in the body) on haemocoelic pulsations in 4-day-old pupa of *Tenebrio* at 24°C.

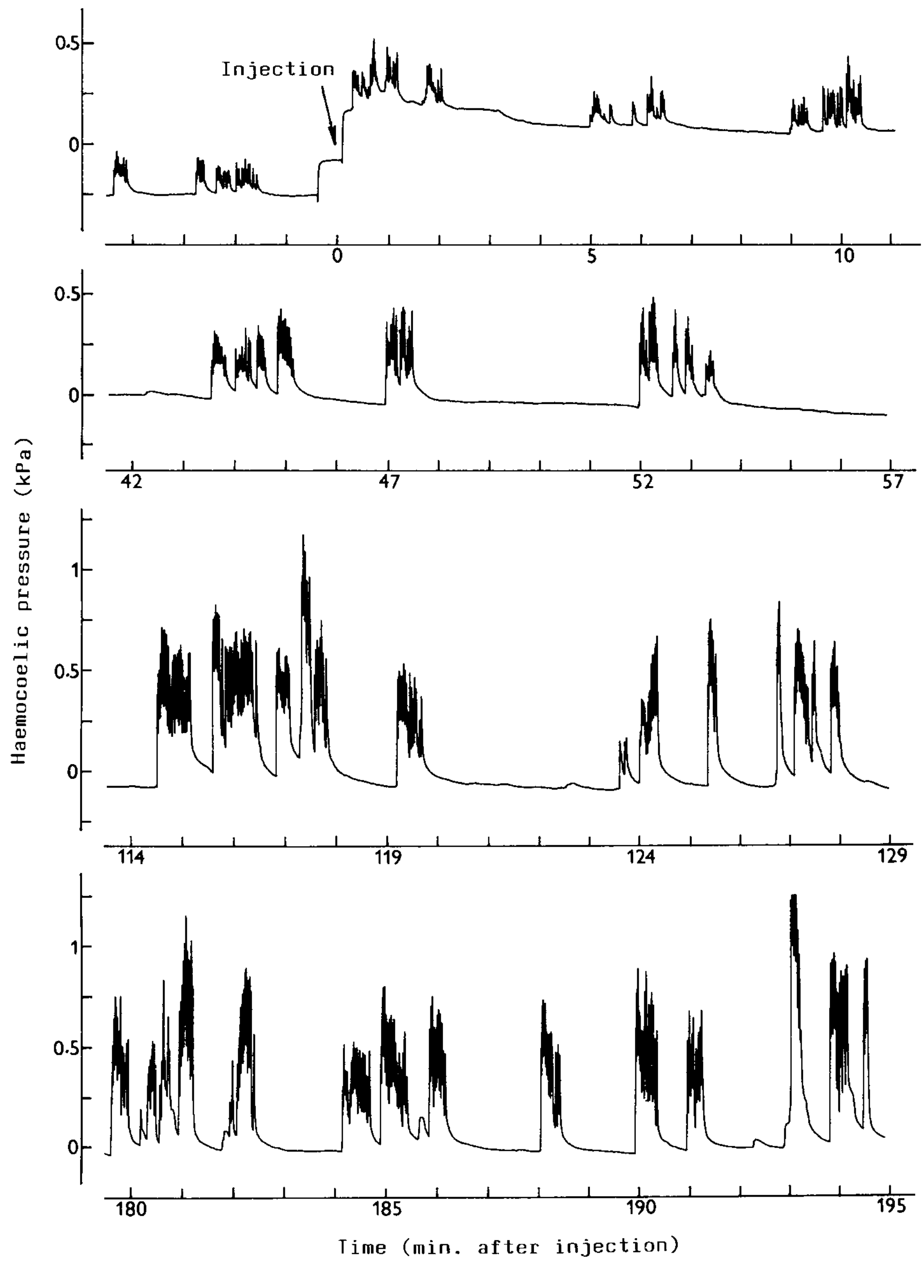

Fig. 5. Delayed effects of [L-Dopa[2]]-proctolin on extracardiac haemocoelic pulsations in 4-day-old pupa of *Tenebrio* at 25°C (1μM final concentration in the body).

individual pupae (n = 36 specimens). The selected record in Fig. 4 shows typical pharmacodynamic responses obtained with 10–100 nM concentrations of proctolin. The responses obtained with 1 nM concentrations were not uniform. In 3 out of 9 realized recordings with this concentration we found some indications of positive effects, while the 6 remaining records did not permit distinction between the real positive effects from the nonspecific effects caused by Ringer injections alone. We conclude, therefore, that 1 nM concentrations of proctolin may be just on the margin of pharmacological effectiveness of proctolin in *Tenebrio*.

THE EFFECTS OF [L-DOPA^2]-PROCTOLIN

Pharmacological action of this proctolin derivative was almost identical with the effects of proctolin, including maximum effectiveness between 2–3 hours after the injection as well as persistence for several hrs. At low or medium concentrations (10–1000 nM, n = 18 measurements; see also Fig. 6), the [L-Dopa^2]-proctolin was slightly more effective than proctolin. In most cases we observed some differences in the early stages of pharmacokinetic action between the two hormones. The effects of [L-Dopa^2]-proctolin became visible

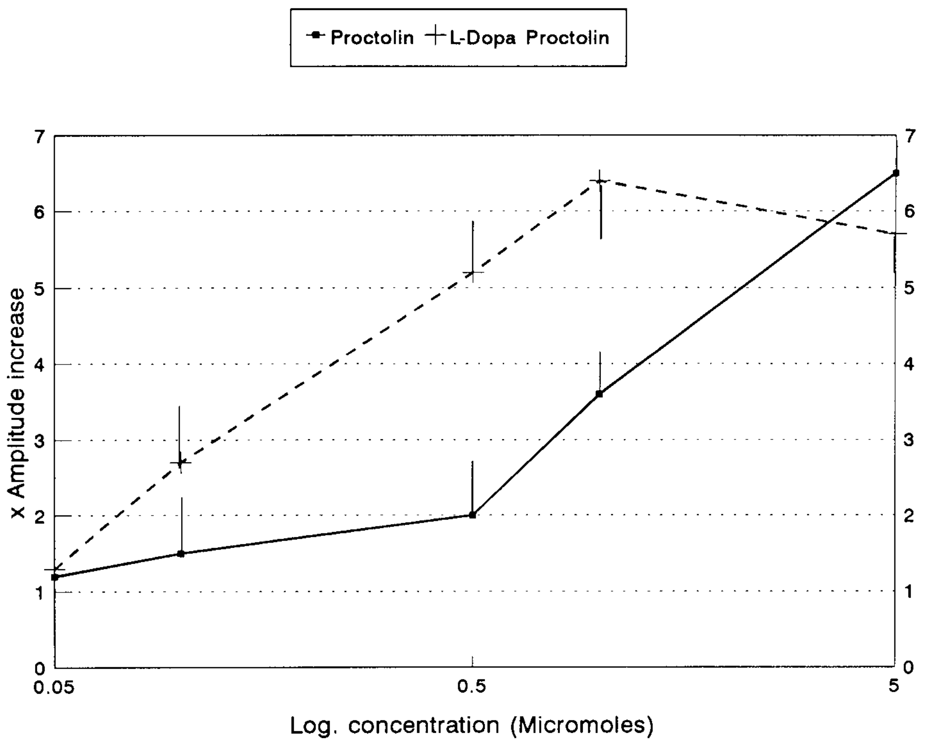

Fig. 6. Dose-response relationships in the effects of proctolin and [L-Dopa^2]-proctolin on extracardiac haemocoelic pulsations. The values indicate multiples of the increased amplitude of the haemocoelic pulsations in the interval between 2nd and 3rd hr after the injection. Vertical bars indicate S.E.M., n = 5–8 specimens in each group.

on the tensiometric records after some 20–30 minutes delay. This retarded pharmacological action of [L-Dopa^2]-proctolin can be observed in Fig. 5, and may indicate, among other things, a possibility of metabolic conversion of a hormonal analogue into the real hormone.

SOME STRUCTURE-ACTIVITY RELATIONSHIPS

The two remaining derivatives of proctolin, i.e. [Phe(p-OMe)^2]- and [Cha(4-OMe)^2]-proctolin, were virtually inactive within the range of 100 nM to 10 μM concentrations. In order to evaluate the effectiveness of the two active compounds, we have calculated the dose-response curves shown in Fig. 6. The values indicate multiples of the increased amplitude of the extracardiac pulsations between hours 2 and 3 after the injection. These results show that within the most effective range of 0.1, 0.5 and 1.0 μM concentrations the [L-Dopa^2]-proctolin has been more effective. The values for 5 μM concentrations are not quite correct since they have been affected by the described paralysis associated with pathophysiological symptoms of hyperproctolinism.

DISCUSSION

The results presented in this study reveal several pharmacologically important facts: 1. Proctolin injections increase the amplitude of extracardiac haemocoelic pulsations (e.g., increase physiological contractions of the somatic intersegmental abdominal muscles) in exactly similar manner as in the in vitro neuromuscular preparations (see Starratt and Steele, 1980 for review); 2. The effects produced by physiological amounts of proctolin persist in the body of *Tenebrio* pupae for many hrs with maximum responses between the 2nd and 3rd hr after the injection; 3. Large doses of proctolin cause pathophysiological syndromes of hyperproctolinism, which are manifested by prolonged paralysis of all functions of the autonomic nervous system. These facts provide clear experimental evidence that proctolin is a real neurohormone with potential ability to regulate the homeostatic physiological functions in distant target tissues.

In the foregoing studies we have investigated the effects of various drugs, hormones, insecticides and other compounds on the autonomic physiological functions in *Tenebrio* (Sláma, 1986, 1987). Some neurotoxic materials, such as organophosphorus or carbamate insecticides, induce positive pressure peaks of large amplitude. In contrast to the present results obtained with the neurohormone proctolin, however, the peaks induced by insecticides were always accompanied by unnatural, tetanic convulsions and irritation-like spasms. In addition, there was a complete disappearance of the regular physiological pattern of the pulsations in haemocoelic pressure. So far, the only in vivo effects similar to proctolin (see Fig. 3) have been found by Provansal-Baudez & Sláma (1985). In this case an enormous elevation of the amplitude of extracardiac pulsations occurred in response to extracts from the perisympathetic neurohaemal organs of *Periplaneta*, which are now generally known to be rich in proctolin and other neuropeptides.

The prolonged persistence of the effects of proctolin in *Tenebrio* pupae has been quite unexpected, because most previous studies suggested that proctolin was rapidly broken down and inactivated by aminopeptidase enzymes present in the haemolymph (Quistad et al., 1984; Starratt & Steele, 1984; Steele & Starratt, 1985). According to Isaac (1986), the aminopeptidases involved in proctolin degradation in *Schistocerca* have the K_m constants around 1 μM concentrations, whereas physiological effects of proctolin appear already at the nanomolar levels. Insect endocrinologists actually observed similar disproportions between the in vitro calculated rapid enzymatic breakdown in contrast to prolonged in vivo half-life in the case of juvenile hormone (Sláma & Jarolím, 1981). Moreover, vertebrate endocrinology has many examples to show that peptidic hormones acting at nanomolar levels do not become inactivated by enzymatic degradation. They are inactivated mainly by physical factors like filtration through the membranes, spontaneous hydrolysis or excretion (Hanc, 1959). The pupa of *Tenebrio* represents special immobile metamorphic stage with relatively low total body metabolism, reduced circulation of haemolymph and reduced excretion. These factors may be partly responsible for the unusually prolonged persistence of the pharmacological effects of proctolin in the body.

In a previous study Konopińska et al. (1988c) described the in vitro cardioexcitatory activity of a series of proctolin analogues in which the Tyr moiety in position 2 of the peptide chain was replaced by Phe(p-NH_2), Phe(p-NMe_2), Phe(p-NO_2), Phe(p-OMe), L-Dopa or Cha(4-OMe). The effects were tested on the explanted heart preparations of *Periplaneta* and *Tenebrio*. The L-Dopa analogue was five times more active in *Periplaneta*, but it had only half of the activity of proctolin in *Tenebrio*. The Phe(p-OMe) analogue, which was completely inactive in our in vivo assays, was more active than proctolin in both the above species and, finally, the Cha(p-OMe) analogue was inactive in all cases. Thus, there is a similarity between the in vivo and in vitro effectiveness of proctolin and [L-$Dopa^2$]-proctolin, but apparently this does not apply to [Phe(p-OMe)]-proctolin analogue.

The pathophysiological symptoms of hyperproctolinism, caused by overdosage of proctolin, are not new in insect endocrinology. They closely resemble the pathophysiological symptoms of hyperecdysonism, first described by Ohtaki & Williams in 1970. Hyperproctolinism clearly indicates that proctolin, like ecdysone and ecdysteroids, represents a category of peripheral hormones, which are used for modulation of already running programmes, e.g. for the homeostatic control of growth, metabolism and development. In contrast, the centrally produced hormones are lacking these more or less quantitative properties, because they act according to the general biological "all or nothing" rule. In this case, as for example in the case of juvenile hormone, even a 500 million times overdosage may still be without actual pathophysiological symptoms (cf. Sláma et al., 1974).

The phenomenal effects of proctolin on the functioning of the autonomic coelopulse system clearly suggest that a real physiological role of this neurohormone in insect body may primarily depend on regulation of various homeostatic mechanisms, e.g., mechanisms preventing excess or deficiency (in the sense of physiological definition used by Wigglesworth, 1964). The intersegmental abdominal muscles of the pupae of *Tenebrio* constitute a major part of the abdominal somatic muscular sheath. They are responsible for all actively regulated changes in haemocoelic pressure. And the presence of proctolin in somatic muscles has been well documented (Anderson et al., 1988). In addition, there exists convincing experimental evidence from other systems that proctolin is not only present, but is really affecting functions of the somatic muscles (O'Gara, 1990).

Further physiological coincidences, suggesting involvement of proctolin in regulation of autonomic physiological functions, can be derived from considerable accumulation of proctolin in the thoracic ganglia of the ventral nerve cord (cf. Lange et al., 1988). The most essential regulatory centres of the autonomic nervous system are just located within the thoracic ganglia (Sláma et al., 1979). Thus, in addition to pharmacological evidence presented in this paper, there exist well based neurochemical and neuroanatomical arguments in favour of the association of proctolin with the functions of the coelopulse system.

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