The Journal of Experimental Biology 204, 3019–3026 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3395

Glucagon-like peptide isolated from the eel intestine: effects on atrial beating

Toshihiro Uesaka[1,3], Keiichi Yano[2], Seiji Sugimoto[2] and Masaaki Ando[1,*]

[1]*Laboratory of Integrative Physiology, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan,* [2]*Tokyo Research Laboratories, Kyowa Hakko Kogyo Co. Ltd, 3-6-6 Asahimachi, Machidashi, Tokyo 194-0023, Japan* and [3]*Department of Environment and Mutation, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan*

*Author for correspondence (e-mail: mando@hiroshima-u.ac.jp)

Accepted 13 June 2001

Summary

A new glucagon-like peptide was isolated from the intestine of the eel *Anguilla japonica*. The primary structure was determined by sequence analysis after cleavage with lysyl endopeptidase, quantitative amino acid analysis and fast atom bombardment mass spectrometry as HSQGTFTNDY^{10}SKYLETRRAQ^{20}DFVQWLMNSK^{30}-RSGGPT. Since its structure is similar to that of oxyntomodulins (OXMs) reported in various vertebrates, we named this peptide eel oxyntomodulin (eOXM). We found that eOXM enhanced the contractile force and the beating rate of the eel atrium in a dose-dependent manner. These effects of eOXM were not inhibited by betaxolol, a β_1-adrenoceptor antagonist, indicating that the actions of eOXM were independent of those of adrenaline. eOXM enhanced the intracellular Ca^{2+} concentration of the myocardium. The contractility of the eel atrium was greatly reduced after omitting Ca^{2+} from the bathing medium or after treatment with verapamil, a Ca^{2+} channel blocker. After inhibiting Ca^{2+} entry under these conditions, the inotropic effect of eOXM was markedly reduced, but the chronotropic effect was not altered significantly. These results indicate that the inotropic effect of eOXM is *via* a stimulation of Ca^{2+} influx but that the chronotropic effect may be independent of extracellular Ca^{2+}.

Key words: eel, oxyntomodulin, intestine, atrial beating, intracellular Ca^{2+}, extracellular Ca^{2+}, *Anguilla japonica.*

Introduction

Many bioactive peptides have been found in mammalian gut, and termed brain-gut peptides. Relatively few, however, have been isolated from teleost gut (Uesaka et al., 1994a; Uesaka et al., 1994b; Uesaka et al., 1995; Uesaka et al., 1996), although the existence of various peptides has been demonstrated immunohistochemically using antisera raised against mammalian peptides (Elbal and Agulleiro, 1986; Rombout et al., 1986; Abad et al., 1987). To clarify the role of gut peptides in teleosts, it is necessary to isolate the gut peptides and to examine their effects on fish organs.

We have determined that the eel atrium provides a suitable bioassay system, because it is both easy to prepare and is sensitive to peptides. The eel atrium can be easily isolated from the heart and beats spontaneously for more than 10 h in artificial saline. In addition, it has been demonstrated that the isolated eel atrium is sensitive not only to adrenaline or acetylcholine (ACh) (Yasuda et al., 1996), but also to eel neuropeptide Y (eNPY) (Uesaka, 1996). As a candidate intestinal hormone capable of enhancing atrial beating, we have isolated a glucagon-like peptide (HSQGTFTNDY^{10}SKYLETRRAQ^{20}DFVQWLMNSK^{30}RSGGPT) from the eel intestine. This peptide is shown to exhibit positive inotropic and chronotropic actions in the atrium. The inotropism is due to an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$).

Materials and methods

Purification and structural analysis

Isolation of a gut peptide

Japanese eels (*Anguilla japonica* Temminck & Schlegel) taken from a commercial source were decapitated and the gut excised immediately and stored at –40 °C. A boiled-water extract of eel gut (361 g wet mass) was prepared following a method previously described (Uesaka et al., 1994a; Uesaka et al., 1994b). The extract was applied to Sep-Pac C18 cartridges (Millipore, Milford, MA, USA). The retained material was eluted with 50 % (v/v) acetonitrile containing 10 % (v/v) 2-propanol and 0.1 % (v/v) trifluoroacetic acid (TFA), and the eluate was evaporated. The concentrated material was applied to a column of Toyopearl HW-40F (2.6 cm×100 cm; Tosoh, Tokyo, Japan) and eluted with 1 mol l^{-1} acetic acid and 10 % (v/v) 2-propanol at rate of 1.5 ml min^{-1}. Each fraction was assayed for its ability to enhance atrial contraction and beating rate.

Bioactive fractions were pooled and subjected to high-performance liquid chromatography (HPLC) separation (LC-6AD, Shimadzu, Kyoto, Japan). Retained material was eluted with a 50 min linear gradient of 0 %–90 % acetonitrile containing 10 % 2-propanol and 0.1 % TFA, and each fraction was bioassayed. The active fractions were applied to a C18 reverse-phase column (TSK ODS-80T_M, Tosoh) and eluted with a 100 min linear gradient of 15 %–35 % acetonitrile containing 5 % 2-propanol and 0.1 % TFA. The active fractions were further applied to a cation-exchange column (TSK CM-5PW, Tosoh) and eluted with a 35 min linear gradient of 0–0.35 mol l^{-1} NaCl in 10 % 2-propanol and 20 mmol l^{-1} phosphate buffer (pH 6.8). The bioactive peak was rechromatographed on the C18 reverse-phase column (TSK ODS-80T_M) with a 50 min linear gradient of 24 %–34 % acetonitrile containing 5 % 2-propanol and 0.1 % TFA. Final purification was performed using the same column under isocratic conditions, as shown in Fig. 1A, to give a single peak, EI-14.

Structural analysis

The amino acid composition of the isolated peptide was determined by precolumn derivation with phenylisothiocyanate using a PICO-TAG amino acid analysis system (Millipore). The sequence of the N-terminal 32 residues was determined directly by automated Edman degradation using a gas-phase sequencer (PPSQ-10, Shimadzu). The sequence of residues 32–36 was determined after cleavage with lysyl endopeptidase; a small sample (approximately 30 pmol) was treated with 0.1 mol l^{-1} Tris-HCl (pH 9) and 2 mol l^{-1} urea for 6 h at 37 °C. The molecular mass of the entire molecule was obtained by fast atom bombardment mass spectrometry (JMX-HX110A, Jeol, Tokyo, Japan). A search for homologous sequences was performed with the Protein Information Resource database (National Biomedical Research Foundation, Washington, DC, USA).

Measurement of biological activity in the eel atrium

Japanese eels, weighing approximately 220 g, were kept in sea water (20 °C) for more than 1 week. After decapitation, the heart was rapidly excised and the atrium was isolated on ice. It was then tied with two cotton threads and connected to a force transducer (type 451996, Sanei, Tokyo, Japan). The details of this procedure have been described previously (Uesaka, 1996; Yasuda et al., 1996). The isolated eel atrium was bathed in artificial saline consisting of (in mmol l^{-1}): 118.5 NaCl, 4.7 KCl, 3.0 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$ and 10 sodium lactate, bubbled with a 95 % O_2/5 % CO_2 gas mixture (pH 7.4) at room temperature (24–28 °C). Although the pH of fish blood is approximately pH 7.9, equilibrated with 0.5 % CO_2 (Farrell, 1984), experiments in the present study were usually performed in the presence of 5 % CO_2 for convenience, since we have established that the effects of adrenaline, ACh, eNPY and eel oxyntomodulin (eOXM) were similar in the presence of 5 % CO_2 (pH 7.4) and 0.5 % CO_2 (pH 7.9) (T. Uesaka and M. Ando, unpublished observation). In Ca^{2+}-free Ringer's solution, 3.0 mmol l^{-1} $CaCl_2$ was replaced with 4.5 mmol l^{-1} NaCl. After preloading by 5.9 mN, spontaneous isometric contractions were converted into electrical signals by a transducer connected to a strain amplifier (6M82, Sanei) and these were recorded using an electric polyrecorder (EPR-10B, Toa, Tokyo, Japan). The rate of contraction was measured simultaneously using a tachometer (type 1321, Sanei). When examining atrial contractions in the absence of Ca^{2+}, the preparation was flushed twice with a Ca^{2+}-free Ringer's solution.

The effects of adrenaline (Sigma Chemical, St Louis, MO, USA), betaxolol (Mitsubishi Kasei, Tokyo, Japan) and verapamil HCl (Wako Pure Chemical, Osaka, Japan) were also examined.

Measurement of intracellular free Ca^{2+} concentration

$[Ca^{2+}]_i$ in the myocardium was measured as described previously (Uesaka, 1996). Briefly, the isolated atrium was cut into pieces in Ringer's solution and treated with collagenase (1 mg ml^{-1}; Wako Pure Chemical) in the same solution for 1 h at room temperature. After rinsing with Ringer's solution, the partially dispersed tissue was incubated with Calcium Green-1 acetoxymethyl ester (5 μmol l^{-1}; Molecular Probes, Eugene, OR, USA) and with Pluronic F-127 (0.05 %, Molecular Probes) as a surfactant for 40 min. The atrial cells were stuck onto a glass slide using coverslips, and mounted in a small chamber on the stage of an inverted microscope (Axiovert 135 MTV, Zeiss, Tokyo, Japan) and superfused with normal Ringer's solution. The fluorescent signal from Calcium Green-1 was recorded with a laser scanning confocal imaging system (MRC-600, Bio-Rad, Tokyo, Japan; dichroic reflecter 510LP; emission filter 515LP) equipped with a Kr–Ar laser (5470K, Ion Laser Technology, Salt Lake City, UT, USA; 488 nm for excitation). The intensity of the fluorescence was expressed in arbitrary units ranging from 0 to 255.

Statistical analyses

Data are reported as means ± S.E.M. *N* represents the number of preparations. The statistical significance of difference between means was examined using a Mann–Whitney *U*-test. The null hypothesis was rejected for $P<0.05$.

Results

Characterization of the purified peptide

Fig. 1A shows the final purification profile of the peptide, temporarily given the name EI-14, and Fig. 1B shows the biological activity of this peptide. It is clear that EI-14 gave a single peak (Fig. 1A), indicating the homogeneity of the peptide. When EI-14 was applied to eel atrium, both the contractile force and the beating rate increased gradually after a latent period (Fig. 1B). The sequence of EI-14 was analysed twice. The first trial gave the N-terminal 32 residues, although the detection of Arg^{18} and Trp^{25} was scanty (Table 1). The second trial confirmed the existence of Arg^{18} and Trp^{25}, while Arg^{31} and Ser^{32} were obscure. Combining these results, the

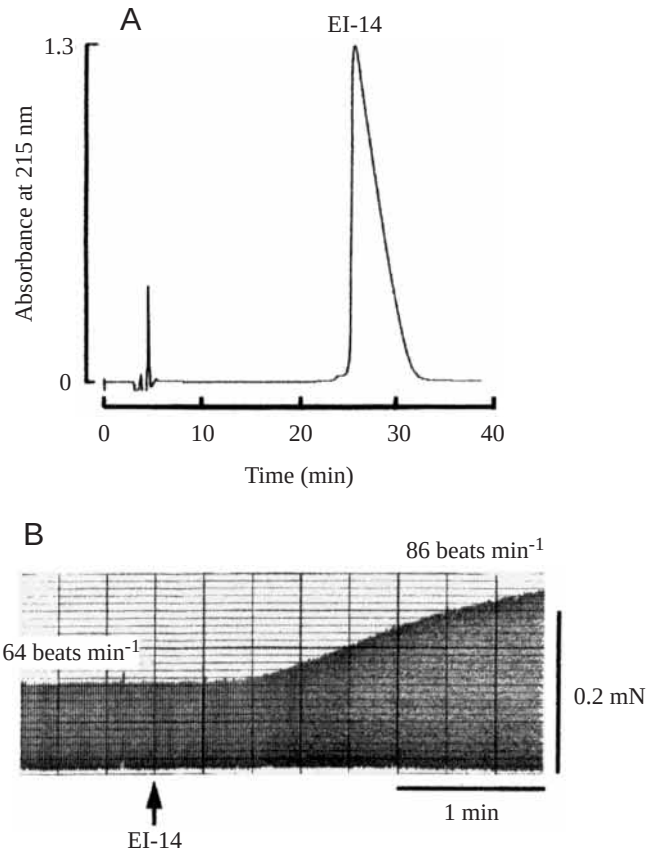

Fig. 1. Final HPLC purification of EI-14 obtained from eel gut. (A) The active fraction obtained by chromatographic steps was subjected to reverse-phase HPLC (TSK ODS-80T$_M$ column) and eluted isocratically with 26.5 % acetonitrile in 5 % 2-propanol and 0.1 % trifluoroacetic acid (pH 2.2). The flow rate was 0.5 ml min^{-1}. (B) Effects of purified EI-14 on atrial beating and contractile force.

N-terminal 32 residues of EI-14 was determined as HSQGTFTNDY^{10}SKYLETRRAQ^{20}DFVQWLMNSK^{30}RS (Table 1).

After digestion of EI-14 with lysyl endopeptidase, three peptides were obtained. Of these three peptides, two contained fragments of the above 32 residues; HSQGTFTNDYSKYLET and AQDFVQWLMNS. However, another peptide had the sequence (R)(S)GGPT (Table 1). Comparing this sequence with the result of the second trial, this peptide was considered to be the C-terminal fragment of EI-14. We concluded, therefore, that the entire sequence of EI-14 is HSQGTFTNDY^{10}SKYLETRRAQ^{20}DFVQWLMNSK^{30}RSGGPT. This conclusion was supported by quantitative amino acid analysis and molecular mass spectrometry.

Table 2 shows the amino acid composition of EI-14, which was almost identical to that expected from the sequence analysis described above. The relative molecular mass (M_r) of EI-14 (4207±1) was almost identical to that predicted from the sequence (M_r=4207). To identify the structure, a peptide consisting of the 36 amino acid residues was synthesized using the sequence result. Fig. 2 compares the synthesized peptide (S) with native EI-14 (N) on the same HPLC. The retention time of the synthesized peptide was identical to that of the native EI-14 in both reverse-phase and cation-exchange HPLC. When a mixture of native and synthesized peptide was applied, only a single peak was observed with both types of HPLC.

Effects of EI-14 on atrial beating

The isolated eel atrium continues to beat spontaneously at a constant rate for more than 10 h in artificial saline. The basal atrial contractile force was 1.9±0.2 mN and the beating rate was 60.5±1.9 beats min^{-1} (N=36). When synthesized EI-14 (10^{-7} mol l^{-1}) was added to the bathing fluid, both the contractile force and the beating rate increased gradually as in the case of the native EI-14 (Fig. 1B). This increase in both the force and the contraction rate were concentration-dependent, with a threshold of 10^{-9} mol l^{-1} and a maximal effect at 3×10^{-7} mol l^{-1} (Fig. 3). Similar enhancements in both the force and the rate were observed after blocking β_1-adrenoceptors with betaxolol. In the presence of betaxolol (10^{-5} mol l^{-1}), the effects of adrenaline were completely abolished, confirming previous results (Uesaka, 1996). Under the same conditions, however, the synthetic EI-14 (10^{-7} mol l^{-1}) increased the contractile force by 2.9±0.4 mN and the beating rate by 24.0±3.3 beats min^{-1} (N=6, P<0.01).

Effects of EI-14 on the intracellular free Ca^{2+} concentration

$[Ca^{2+}]_i$ showed spontaneous oscillations in the eel atrium (Fig. 4), with an oscillatory frequency of 20–30 cycles min^{-1}. After application of synthetic EI-14, the peak level of $[Ca^{2+}]_i$ doubled, but the frequency tended to decrease (to 18–25 cycles min^{-1}).

Table 1. *Amino acid sequence of EI-14*

	Amino acid residue						
	5	10	15	20	25	30	35
N-term-1	H S Q G T F T N D Y S K Y L E T R (R) A Q D F V Q (W) L M N S K R S						
N-term-2	H S Q G T F T N D Y S K Y L E T R R A Q D F V Q W L M N S K (R) (S) G G P						
Lys-endo	(R) (S) G G P T						

Residues are represented by the single-letter code.
Residues in parentheses denote insufficient levels for detection.
N-term-1 and -2, N-terminal analysis trials 1 and 2, respectively.
Lys-endo shows the sequence of one peptide generated after lysyl endopeptidase digestion.

Table 2. *Amino acid composition of EI-14*

	Asx	Glx	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Ile	Leu	Phe	Trp	Lys	Cys
Observed	3.0	3.4	3.9	3.3	0.9	3.4	3.9	1.4	1.2	1.9	1.5	0.8	–	2.1	1.9	ND	2.0	ND
Expected	4	4	4	3	1	3	4	1	1	2	1	1	0	2	2	1	2	0

Observed values are normalized to Lys=2.0.
Expected values are obtained from the sequence in Table 1.
A dash denotes not detected; ND, not determined.

To determine whether the increase in $[Ca^{2+}]_i$ is due to an influx of Ca^{2+} from the extracellular medium or to a release of Ca^{2+} from intracellular stores, Ca^{2+} in the bathing medium was omitted. In the absence of external Ca^{2+}, both the contractile force and the beating rate gradually decreased and the inotropic effect of EI-14 was markedly reduced (Fig. 5B). A similar reduction in the inotropic effect of EI-14 was also observed in the presence of verapamil (10^{-5} mol l^{-1}), a Ca^{2+} channel blocker (Fig. 5C). However, the positive chronotropic effect of EI-14 was retained after the inhibition of Ca^{2+} entry (Fig. 5B,C).

Discussion

The present study determined the primary structure of a new glucagon-like peptide (HSQGTFTNDY^{10}SKYLETRRAQ^{20}-DFVQWLMNSK^{30}RSGGPT) isolated from the Japanese eel intestine. The N-terminal 29 residues were identical to the eel glucagon isolated from the European eel pancreas (Conlon et al., 1988). Since the structure of the new peptide is similar to oxyntomodulins (OXMs) isolated from a variety of vertebrates (Table 3) and since glucagon is processed from the OXM in the mammalian intestine (Plisetskaya and Mommsen, 1996), it is plausible to designate this peptide (EI-14) eel OXM (eOXM). Although Conlon et al. (1988) isolated another OXM-like peptide from European eel pancreas (Conlon et al., 1988), its sequence is slightly different from that reported in our study (see Table 3). It is probable that processing of the preproglucagon protein differs between the intestine and the pancreas, as described by Plisetskaya and Mommsen (Plisetskaya and Mommsen, 1996). Eel oxyntomodulin is similar to that of alligator gar (*Lepisosteus spatula*) (Pollock et al., 1988b) or bullfrog (*Rana catesbeiana*) (Pollock et al., 1988a), but differs from that of elasmobranchs, with 73 % identity with dogfish (*Scyliorhinus canicula*) (Conlon et al., 1994) and only 58 % identity with ratfish (*Hydrolagus colliei*) (Conlon et al., 1987). Although the structures are not presented in Table 3, Raufman et al. (Raufman et al., 1992) also isolated two truncated glucagon-like peptides from paddlefish (*Polyodon spathula*) consisting of 31 amino acid residues, one

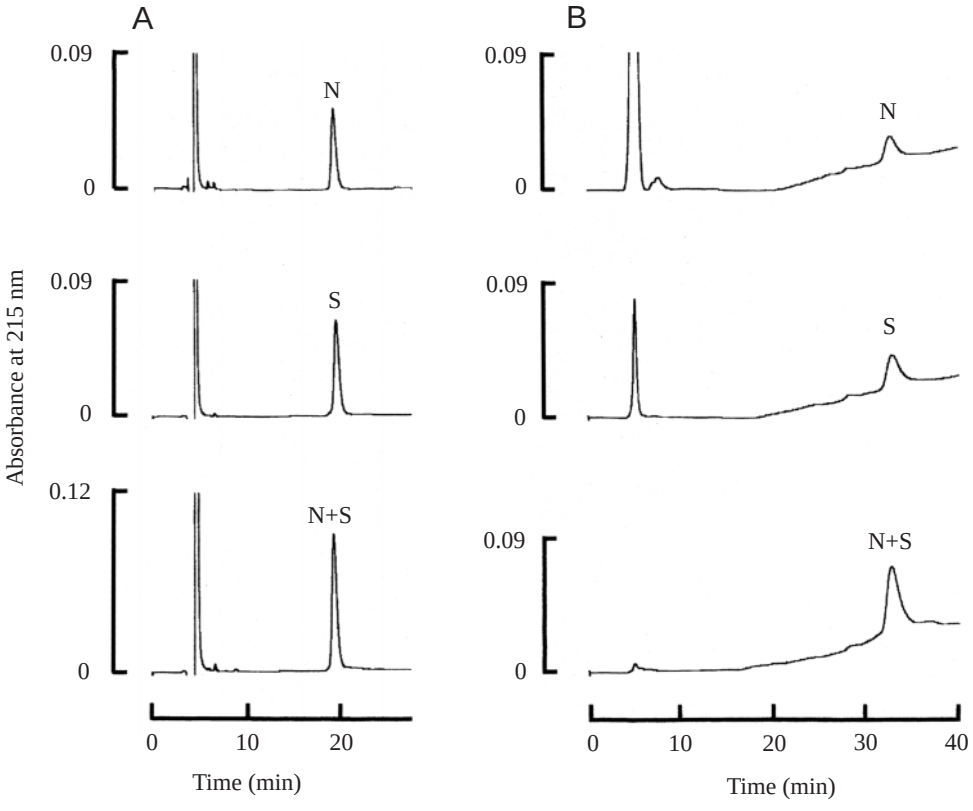

Fig. 2. A comparison of the chemical characteristics of native (N) and synthetic (S) EI-14. (A) Reverse-phase chromatograms of N, S and N+S using the TSK ODS-120T column. N, S and N+S were eluted with a 50 min linear gradient of 20 %–30 % acetonitrile in 10 % 2-propanol and 0.1 % trifluoroacetic acid. The flow rate was 0.5 ml min^{-1}. (B) Cation-exchange chromatograms. Each peptide was eluted with a 25 min linear gradient of 0-0.5 mol l^{-1} NaCl in 10 % ethanol and 20 mmol l^{-1} phosphate buffer (pH 6.7). The flow rate was 0.5 ml min^{-1}.

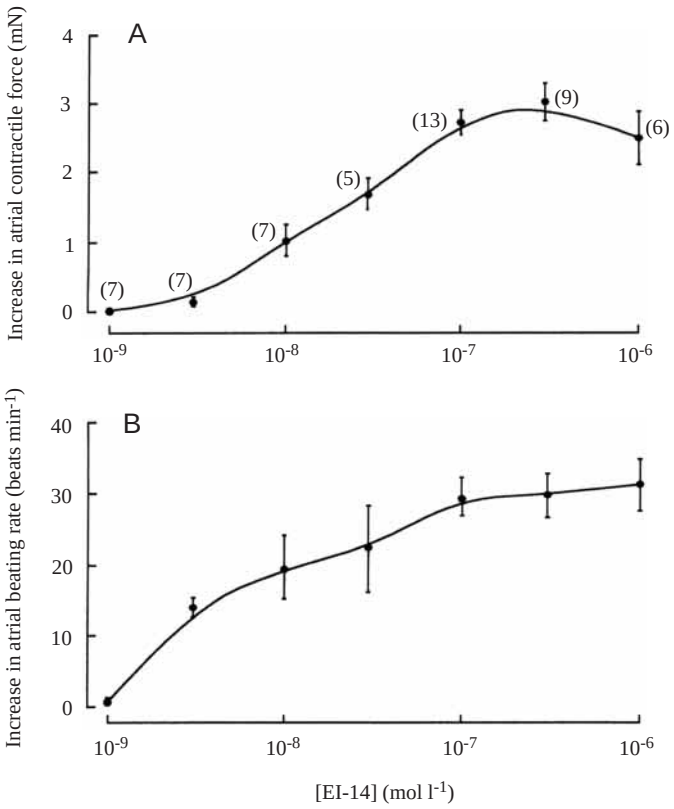

Fig. 3. Concentration–response curve for the effects of synthetic EI-14 on atrial beating. (A) The change in contractile force after addition of EI-14 plotted against its corresponding concentration (logarithmic scale). The control value before addition of EI-14 was 14.34±1.95 mN (*N*=13). Values are means ± S.E.M. The number of experiments at each concentration is indicated in parentheses. (B) The effect of EI-14 on atrial beating rate. The control value was 69.5±6.6 beats min^{-1} (*N*=13). The sample size is the same as in A.

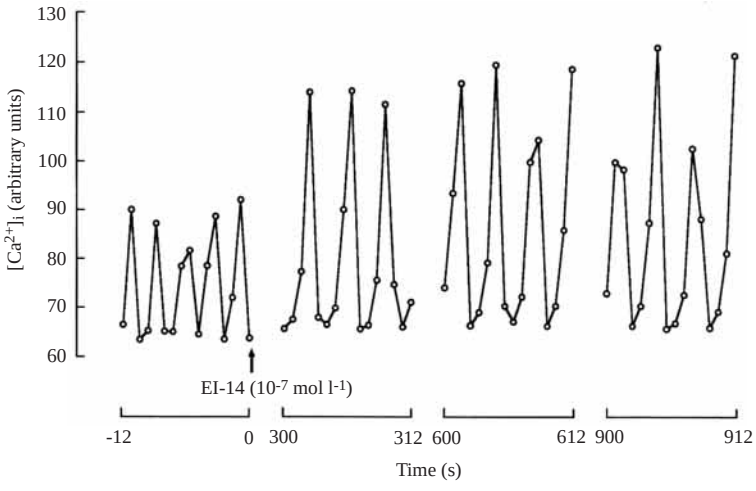

Fig. 4. Oscillation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a region of the atrial myocardium. The $[Ca^{2+}]_i$ was determined by measuring the brightness of all pixels (*N*=1200) in the region, and the mean brightness ± S.E.M. was obtained. Error bars were all smaller than the size of the symbol. EI-14 (10^{-7} mol l^{-1}) was applied at time zero.

with 71 % identity and the other 68 % identity to eOXM. With the exception of the elasmobranch data, the information given in Table 3 suggests that the N-terminal sequence is relatively conserved and the C-terminal sequence variable among vertebrates. The N-terminal sequence (glucagon sequence) may, therefore, be an essential structure for the action of OXMs in vertebrates.

There is general agreement that glucagon increases cardiac contractile force and heart rate. Farah and Tuttle (Farah and Tuttle, 1960) first established the positive inotropic effect of glucagon in a dog heart–lung preparation. Thereafter, the effect of glucagon on cardiac contractility was confirmed and reproduced in anaesthetised animals, isolated heart preparations and cultured heart cells (Parmley et al., 1968; Smitherman et al., 1978; Chernow et al., 1986; Iwanij and Hur, 1987; Mery et al., 1990). There have, however, been reports of species differences in the response of the heart to glucagon. Dogs, rats and cats are responsive to glucagon, whereas in guinea pig, rabbit and pig the effect of the peptide has been questioned (Prasad, 1975; MacLeod et al., 1981; Farah, 1983). The difference in response to glucagon is explained by the species difference in the endopeptidase that degrades glucagon into a miniglucagon [glucagon-(19-29), an active form of glucagon] (Pavoine et al., 1991). It has, however, been reported that the effects of glucagon on arterial blood pressure and heart rate in rats are smaller than those of glucagon-like peptide-1-(7-36) amide (Barragan et al., 1994). These results suggest that the C-terminal structure of glucagon-like peptides is also important for positive inotropic and chronotropic effects on the heart.

The eel glucagon-like peptide (eOXM) has positive inotropic and chronotropic effects on the eel atrium (Fig. 1B, Fig. 5A). Although these effects of eOXM are similar to those of adrenaline (Yasuda et al., 1996), eOXM appears to stimulate atrial beating independently of the β_1-adrenoceptors. In the presence of betaxolol, a β_1-adrenoceptor antagonist, the effects of eOXM remain (present study), while the effects of adrenaline are completely blocked, as previously described (Uesaka, 1996).

Eel oxyntomodulin increases $[Ca^{2+}]_i$ in the eel myocardium (Fig. 4). Similar increases in $[Ca^{2+}]_i$ are observed after treatment of the eel atrium with adrenaline or neuropeptide Y (Uesaka, 1996). It is thus plausible that eOXM enhances contractility *via* an increase in $[Ca^{2+}]_i$. In the eel atrium, contraction appears to be induced by entry of Ca^{2+} from the extracellular fluid, since atrial contractility is markedly reduced in Ca^{2+}-free Ringer's solution or after treatment with verapamil, a Ca^{2+} channel blocker (Fig. 5). In general, heart contractions in poikilotherms can be induced by an influx of Ca^{2+} from the extracellular fluid. Electron microscopy of a number of poikilotherm hearts demonstrates both a sparsity of sarcoplasmic reticulum and an absence of transverse tubules (Santer, 1985). Furthermore, contraction of the amphibian (Bers, 1985) and teleost

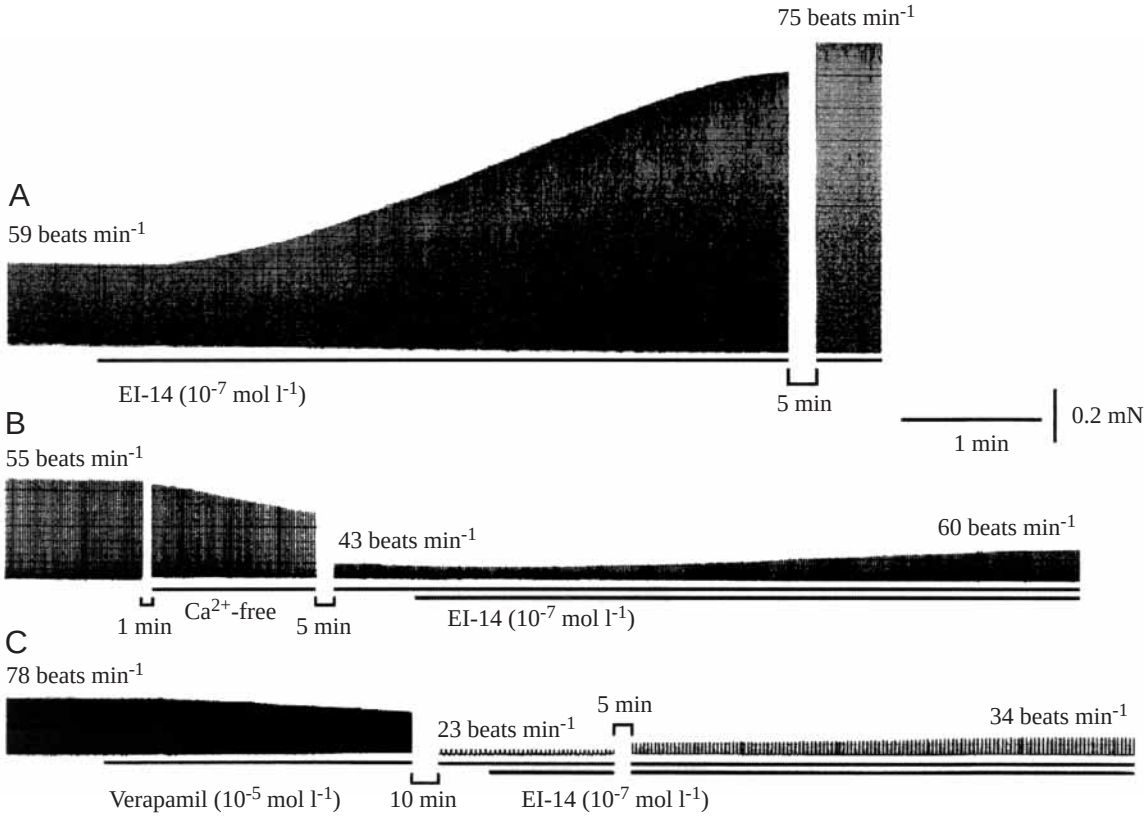

Fig. 5. Effects of extracellular Ca^{2+} on atrial beating. (A) Representative control response after treatment with synthetic EI-14 in normal Ringer's solution. EI-14 (10^{-7} mol l^{-1}) was applied during the period indicated by the horizontal bar. (B) Effects of EI-14 in the absence of Ca^{2+} in the bathing medium. Ca^{2+} was omitted during the period indicated by the horizontal bar (Ca^{2+}-free). (C) Effects of EI-14 after pretreatment with verapamil. Verapamil (10^{-5} mol l^{-1}) was applied during the period indicated by the horizontal bar.

Table 3. *Primary structure of oxyntomodulin-like peptides in vertebrates*

	Amino acid residue							Identity
	5	10	15	20	25	30	35	(%)
Teleosts								
Eel (intestine)[a]	H S Q G T F T N D Y S K Y L E T R R A Q D F V Q W L M N S K R S G G P T							
Eel (pancreas)[b]	- - - - - - - - - - - - - - Q - M K Q - - - L - - - - - - - - - - N - N S S							75
Alligator gar (pancreas)[c]	- - - - - - - - - - - - - - - D - - - - - - - - - - - - - - S T - - - - - I -							89
Elasmobranchs								
Dogfish (pancreas)[d]	- - E - - - - S - - - - - M D N - - - K - - - - - - - - S T - - N -							73
Ratfish (pancreas)[e]	- T D - I - S S - - - - - - - D N - - T K - - - - - - - L S T - - N - A N -							58
Amphibians								
Bullfrog (pancreas)[f]	- - - - - - - S - - - - - - - D S - I S							86
Mammals								
Pig (intestine)[g]	- - - - - - - S - - - - - - - D S - - - - - - - - - - - - - - - T - - N K N N I A							73
Human (intestine)[h]	- - - - - - - S - - - - - - - D S - - - - - - - - - - - - - - - T - - N R N N I A							73

The organs from which the peptides are derived are given in parentheses.
Residues are represented by the single-letter code.
Dashes indicate residues identical to eel oxyntomodulin.
Levels of sequence identity are indicated on the right.
[a]Present study; [b]Conlon et al., 1988; [c]Pollock et al., 1988b; [d]Conlon et al., 1994; [e]Conlon et al., 1987; [f]Pollock et al., 1988a; [g]Bataille et al., 1982; [h]Bataille et al., 1981.

(Driedzic and Gesser, 1988) ventricle appears to be relatively insensitive to ryanodine, a blocker of the sarcoplasmic reticulum Ca^{2+} release. More directly, Mery et al. (Mery et al., 1990) demonstrated, using a patch-clamp technique, that the Ca^{2+} current is increased by glucagon in frog and rat ventricular myocytes. It is likely therefore, that eOXM enhances Ca^{2+} influx, enhancing $[Ca^{2+}]_i$, which enhances the contractile force of the eel atrium. However, the chronotropic effect of eOXM is still present after Ca^{2+} entry has been inhibited with Ca^{2+}-free solutions or verapamil. This indicates that the chronotropic effect of eOXM is independent of extracellular Ca^{2+}. Eel oxyntomodulin might stimulate beating rate by increasing levels of other intracellular mediators, such as cyclic AMP. The fact that the oscillations in $[Ca^{2+}]_i$ are much slower than the beating rate in our preparations may be because of damage to the pacemaker cells by collagenase and/or Pluronic F-127.

Eel oxyntomodulin may act as a hormone, being produced in the intestine and targeted to the heart. It is not known what triggers release of eOXM from the intestine, but a recent finding in the rat small intestine is of value for reference. When the lumen of the small intestine is perfused with $200\,mmol\,l^{-1}$ NaCl Ringer's solution (hyperosmotic), guanylin (an intestinal peptide composed of 15 amino acid residues) secretion into the lumen increased threefold, accompanied by a slight increase in uroguanylin secretion (Kita et al., 1999). Uroguanylin is another intestinal peptide of 15 amino acid residues and is considered to be a hormone targeting the kidney and pancreas (Nakazato et al., 1998), while guanylin is a luminocrine substance (Forte and Currie, 1995). If similar factors, such as concentrated NaCl, stimulate eOXM secretion from the intestine into the circulation, eOXM may enhance the contractile force and beating rate of the heart.

This research was supported in part by Grants-in-Aid for Scientific Research (C) no. 08640864 and JSPS fellowship no. 3524 from the Ministry of Education, Science, Sports and Culture, Japan, and also by the Fisheries Agency of Japan.

References

Abad, M. E., Peeze Binkhorst, F. M., Elbal, M. T. and Rombout, J. H. W. M. (1987). A comparative immunocytochemical study of the gastro-entero-pancreatic (GEP) endocrine system in a stomachless and a stomach-containing teleost. *Gen. Comp. Endocrinol*. **66**, 123–136.

Barragan, J. M., Rodriguez, R. E. and Blazquez, E. (1994). Changes in arterial blood pressure and heart rate induced by glucagon-like peptide-1-(7-36) amide in rats. *Am. J. Physiol*. **266**, E459–E466.

Bataille, D., Gespach, C., Tatemoto, K., Marie, J. C., Caudray, A. M., Rosselin, G. and Mutt, V. (1981). Bioactive enteroglucagon (oxyntomodulin): present knowledge on its chemical structure and its biological activities. *Peptides* **2**, 41–44.

Bataille, D., Tatemoto, K., Gespach, C., Jonvall, H., Rosselin, G. and Mutt, V. (1982). Isolation of glucagon-37 (bioactive enteroglucagon/oxyntomodulin) from porcine jejuno-ileum. Characterization of the peptide. *FEBS Lett.* **146**, 79–86.

Bers, D. M. (1985). Ca influx and sarcoplasmic reticulum Ca release in cardiac muscle activation during postrest recovery. *Am. J. Physiol.* **248**, H366–H381.

Chernow, B., Reed, L., Geelhoed, G. W., Anderson, M., Teich, S., Meyerhoff, J., Beardsley, D., Lake, C. R. and Holaday, J. W. (1986). Glucagon: endocrine effects and calcium involvement in cardiovascular actions in dogs. *Circulation Shock* **19**, 393–407.

Conlon, J. M., Dafgard, E., Falkmer, E. and Thim, L. (1987). A glucagon-like peptide, structurally related to mammalian oxyntomodulin, from the pancreas of a holocephalan fish, *Hydrolagus colliei*. *Biochem. J.* **245**, 851–855.

Conlon, J. M., Deacon, C. F., Hazon, N., Henderson, I. W. and Thim, L. (1988). Somatostatin-related and glucagon-related peptides with unusual structural features from the European eel (*Anguilla anguilla*). *Gen. Comp. Endocrinol.* **72**, 181–189.

Conlon, J. M., Hazon, N. and Thim, L. (1994). Primary structures of peptides derived from proglucagon isolated from the elasmobranch fish, *Scyliorhinus canicula*. *Peptides* **15**, 163–167.

Driedzic, W. R. and Gesser H. (1988). Differences in force–frequency relationships and calcium dependency between elasmobranch and teleost hearts. *J. Exp. Biol.* **140**, 227–241.

Elbal, M. T. and Agulleiro, B. (1986). An immunocytochemical and ultrastructural study of endocrine cells in the gut of a teleost fish, *Sparus auratus* L. *Gen. Comp. Endocrinol*. **64**, 339–354.

Farah, A. E. (1983). Glucagon and heart. In *Handbook of Experimental Pharmacology*, vol. II (ed. P. Lefevre), pp. 553–609. Berlin: Springer-Verlag.

Farah, A. E. and Tuttle, R. (1960). Studies of the pharmacology of glucagon. *J. Pharmacol. Exp. Thr*. **129**, 49–55.

Farrell, A. P. (1984). A review of cardiac performance in the teleost heart: intrinsic and humoral regulation. *Can. J. Zool*. **62**, 523–536.

Forte, L. R. and Currie, M. G. (1995). Glucagon: a peptide regulator of epithelial transport. *FASEB J.* **9**, 643–650.

Iwanij, V. and Hur, K. C. (1987). Development of physiological responsiveness to glucagon during embryogenesis of avian heart. *Dev. Biol.* **122**, 146–152.

Kita, T., Kitamura, K., Sakata, J. and Eto, T. (1999). Marked increase of guanylin secretion in response to salt loading in the rat small intestine. *Am. J. Physiol.* **277**, G960–G966.

Macleod, K. M., Rodgers, R. L. and McNeill, J. H. (1981). Characterization of glucagon-induced changes in rate, contractility and cyclic AMP levels in isolated cardiac preparations of the rat and guinea pig. *J. Pharmacol. Exp. Ther*. **217**, 798–804.

Mery, P. F., Brechler, V., Pavoine, C., Pecker, F. and Fischmeister, R. (1990). Glucagon stimulates the cardiac Ca^{2+} current by activation of adenylyl cyclase and inhibition of phosphodiesterase. *Nature* **345**, 158–161.

Nakazato, M. Yamguchi, H., Date, Y., Miyazato, M., Kangawa, K., Goy, M. F., Chino, N. and Matsukura, S. (1998). Tissue distribution, cellular source, and structural analysis of rat immunoreactive uroguanylin. *Endocrinol.* **139**, 5247–5254.

Parmley, W. W., Glick, G. and Sonnenblick, E. H. (1968). Cardiovascular effects of glucagon in man. *N. Engl. J. Med*. **279**, 12–17.

Pavoine, C., Brechler, V., Kervran, A., Blache, P., Le-Nguyen, D., Laurent, S., Bataille, D. and Pecker, F. (1991). Miniglucagon [glucagon-(19–29)] is a component of the positive inotropic effect of glucagon. *Am. J. Physiol*. **260**, C993–C999.

Plisetskaya, E. M. and Mommsen, T. P. (1996). Glucagon and glucagon-like peptides in fishes. *Int. Rev. Cytol.* **168**, 187–257.

Pollock, H. G., Hamilton, J. W., Rouse, J. B., Ebner, K. E. and Rawitch, A. B. (1988a). Isolation of peptide hormones from the pancreas of the bullfrog (*Rana catesbeiana*). *J. Biol. Chem.* **263**, 9746–9751.

Pollock, H. G., Kimmel, J. R., Ebner, K. E., Hamilton, J. W., Rouse, J. B., Lance, J. B. and Rawitch, A. B. (1988b). Isolation of alligator gar (*Lepisosteus spatula*) glucagon, oxyntomodulin, and glucagon-like peptide: amino acid sequences of oxyntomodulin and glucagon-like peptide. *Gen. Comp. Endocr*. **69**, 133–140.

Prasad, K. (1975). Glucagon-induced changes in the action potential, contraction, and Na^+-K^+-ATPase of cardiac muscle. *Cardiovasc. Res*. **9**, 355–365.

Raufman, J.-P., Singh, L., Singh, G. and Eng, J. (1992). Truncated glucagon-like peptide-1 interacts with exendin receptors on dispersed acini from guinea pig pancreas. *J. Biol. Chem.* **267**, 21432–21437.

Rombout, T. H. W. M., Van Der Grinten, C. P. M., Peeze Binkhorst, F. M., Taberne-Thiele, J. J. and Schooneveld, H. (1986). Immunocytochemical identification and localization of peptide hormones in the gastro-entero-pancreatic (GEP) endocrine system of the mouse and a stomachless fish, *Barbus conchonius*. *Histochem*. **84**, 471–483.

Santer, R. M. (1985). Morphology and innervation of the fish heart. *Adv. Anat. Embryol. Cell Biol.* **89**, 1–102.

Smitherman, T. C., Osborn, R. C. and Atkins, J. M. (1978). Cardiac dose-response relationship for intravenously infused glucagon in normal intact dogs and man. *Am. Heart J*. **96**, 363–371.

Uesaka, T. (1996). Synergistic action of neuropeptide Y and adrenaline in the eel atrium. *J. Exp. Biol.* **199**, 1873–1880.

Uesaka, T., Yano, K., Sugimoto, S. and Ando, M. (1996). Effects of eel neuropeptide Y on ion transport across the seawater eel intestine. *Zool. Sci*. **13**, 341–346.

Uesaka, T., Yano, K. Yamasaki, M. and Ando, M. (1994a). Glutamate substitution for glutamine at position 5 or 6 reduces somatostatin action in the eel intestine. *Zool. Sci*. **11**, 491–494.

Uesaka, T., Yano, K., Yamasaki, M., Nagashima, K. and Ando, M. (1994b). Somatostatin-related peptide isolated from the eel gut: effects on ion and water absorption across the intestine of the seawater eel. *J. Exp. Biol.* **188**, 205–216.

Uesaka, T., Yano, K., Yamasaki, M. and Ando, M. (1995). Somatostatin-, vasoactive intestinal peptide-, and granulin-like peptides isolated from intestinal extract of goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol*. **99**, 298–306.

Yasuda, M., Uesaka, T., Furukawa, Y. And Ando, M. (1996). Regulation of atrial contraction in the seawater-adapted eel, *Anguilla japonica*. *Comp. Biochem. Physiol.* **113A**, 165–172.