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Short Communication

Analysis of the Molecular Evolution of *Listeria monocytogenes* Isolated from Japanese Meats and Environment

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SUMMARY: Food contaminated by *Listeria monocytogenes* is a problem on a worldwide level because it is a serious food-borne pathogen. Although 3 evolutionary divisions have been reported for *L. monocytogenes*, the evolution of Japanese isolates has not yet been clarified. Thus, in order to determine the lineage of these Japanese isolates, we classified and conducted phylogenetic analysis of 407 bp (position 1116-1522) of the *iap* gene derived from 88 isolates from Japanese listeriosis patients, foods and environment. The isolates were classified into 18 types commonly accompanied by serotypes, and the types were divided into 3 lineages. Our results suggest that these Japanese isolates belong to the 3 lineages of *L. monocytogenes* isolated in other countries.

Listeria monocytogenes causes a serious food-borne illness and there have been many reports of such outbreaks in the United States and Europe (1-3). In Japan, sporadic cases have occasionally been reported but no large outbreak has yet occurred. Contaminated food is believed to be the primary source of human exposure to *L. monocytogenes* and has been repeatedly linked to both sporadic cases and large outbreaks of listeriosis (4). Some Japanese food such as meat, milk and cheese, has also been contaminated by *L. monocytogenes* (5). We have previously reported the genetic diversity of the *iap* gene encoding an extracellular protein p60 among the isolates from various foods and listeriosis patients (6-8). In the present study, the *iap* gene cluster sequence from 88 isolates derived from meat, milk and the environment was used to establish a phylogeny for *L. monocytogenes* isolated in Japan.

The *L. monocytogenes* strains isolated from food, the environment and listeriosis patients during 1988-2003 are listed in Table 1. The isolates included 81 and 7 strains from food/environment and patients, respectively. The method of isolation has been described in previous reports (6,7). Serovars were determined using the Sahumy method developed in our laboratory (9), which gave the following numbers of isolates: serovar 1/2a, 32 strains; 1/2b, 26 strains; 1/2c, 4 strains; and 4b, 26 strains. The methods of determining the DNA sequence in the *iap* region (407 bp; position 1116-1522) from each isolate have been described in previous reports (6,7). The sequences were edited and aligned before comparison with the *L. monocytogenes* strain EGD-e (GenBank accession no. AL591824) using the DNAsis pro computer software (Ver.2.00.000.002; Hitachi Software Japan, Tokyo, Japan). Phylogenetic analysis and genetic distance estimation were also performed using DNAsis pro.

Eighty-nine isolates were classified into 19 types (Fig. 1) that differed in their EGD-e strain (0 type). Types 1 and 5-10 consisted of 15 isolates of serovar 1/2a only, type 3 included one isolate of serovar 1/2c, and then types 11 and 15-18 included 8 isolates of serovar 4b only. The remaining 5 types consisted of multiple serovars: 2 serovars each in type 2 (1/2a, 1/2c), type 4 (1/2a, 4b) and type 14 (1/2a, 1/2b), and then 3 serovars of 1/2a, 1/2b and 4b in types 12 and 13. The human isolates were classified into types 1, 5, 10 and 15-16 corresponding to the serovar. Thirteen of 18 types contained a sole serovar suggesting that the isolates from the Japanese foods and environment are commonly associated with the lineage, as suggested by a previous report (4).

Although three evolutionary divisions have been reported for *L. monocytogenes* (4,10), the details of this division have not yet been clarified for Japanese isolates. Nineteen types containing the EGD-e strain (type 0) were resolved into 3 large *L. monocytogenes* lineages in the present study: the A group (types 0-11), the B group (types 12-16) at a distance of 0.038, and the C group (types 17 and 18), containing serovar 4b only at a distance of 0.115. Groups A, B and C seem to correspond to Groups 2, 1 and 3, respectively, in the results of Ward et al. (4) and Rasmussen et al. (10), though different regions were analyzed in these two studies. These results suggest that the Japanese isolates also have 3 evolutionary streams.

The present results demonstrate that *L. monocytogenes* isolates from Japanese foods and the environment can be classified into 18 types commonly associated with serotypes, and that the identified types may be divided into 3 lineages similar to those of *L. monocytogenes* isolated in other countries.

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Table 1. Sources and serovars of the *L. monocytogenes* isolates

origin	serovar	No.	year	p[2]	strain
human[1]	1/2a	1	1989	S1	H1
		2	1988	S2	H3
		3	1996	T1	HM1
		4	1996	T1	HM2
		5	2002	F1	12H
	4b	6	1989	S3	H2
		7	2000	F2	11H
beef	1/2a	8	2000	T2	312B1
		9	1999	C1	173B3
	4b	10	1998	T3	82B1
		11	1998	S4	133B1
		12	2001	T4	392B1
		13	2003	T4	468B1
chicken	1/2a	14	1998	T4	80C1
		15	1989	S5	SC23
		16	1998	T4	89C5
		17	2000	T5	265C1
		18	2000	T6	268C1
		19	1999	S4	213C1
		20	1999	C1	221C1
		21	2000	T7	307C1
		22	1999	T5	186C1
		23	1999	T8	188C3
		24	1999	T6	223C3
	1/2b	25	1998	T4	72C1
		26	1996	T4	9C1
		27	1998	T4	79C1
		28	1996	S6	6C1
		29	1996	G	17C1
		30	1997	T4	66C3

origin	serovar	No.	year	p[2]	strain
chicken	1/2b	31	1997	T4	69C3
		32	1998	T4	74C1
		33	1998	T4	77C1
		34	1998	T4	87C1
		35	1998	T4	89C4
		36	1998	T4	91C3
		37	1998	T4	93C1
		38	1998	T4	95C1
		39	1998	T4	97C1
		40	1998	T4	99C4
		41	1998	T4	103C1
		42	1998	T4	105C2
		43	1998	T4	106C3
		44	1998	T4	109C1
		45	1998	T4	116C1
	1/2c	46	1996	C2	23C1
	4b	47	1997	S4	28C1
		48	1989	S5	SC32
		49	1997	T9	42C1
		50	1998	T9	125C1
		51	1997	T4	229C1
		52	2000	T3	295C1
		53	2001	T4	355C4
		54	2003	T4	490C1
pork	1/2a	55	1996	T4	11P1
		56	1998	T4	76P2
		57	1998	T4	78P5
		58	1999	S4	183P1
		59	1997	T3	63P1
		60	1998	T4	81P1

origin	serovar	No.	year	p[2]	strain
pork	1/2b	61	1998	T4	100P3
		62	1998	T4	102P1
		63	1998	T4	104P6
		64	1998	T4	112P4
		65	1998	T4	114P3
	1/2c	66	1998	T4	75P1
		67	1998	T4	76P1
		68	1998	T4	78P1
	4b	69	1998	T4	112P3
		70	2003	T4	489P1
sheep	1/2a	71		T[3]	IID566
	4b	72		T[3]	IID571
processed meat	1/2a	73	1989	S7	Hu53
	4b	74	1989	S7	Hu120
		75	1989	S7	Hu80
milk	1/2a	76	1989	S8	LM51
		77	1989	S8	LM41
		78	1989	S8	LM61
		79	1989	S8	LM71
		80	1989	S8	LM87
		81	1989	S8	LM103
cheese	4b	82	1989	S9	CH191
		83	1989	S9	CH115
		84	1989	S9	CH188
		85	1989	S9	CH190
environment	4b	86	1989	S9	CHF201
		87	1989	S9	CHF208
		88	1989	S9	CHF231

[1]: Isolates from listeriosis patients who caused meningitis and/or septicemia.
[2]: Different number shows the different place or shop. p, place; S, Saitama; T, Tokyo; F, Fukuoka; C, Chiba; G, Gunma.
[3]: The isolates were supplied from the University of Tokyo.

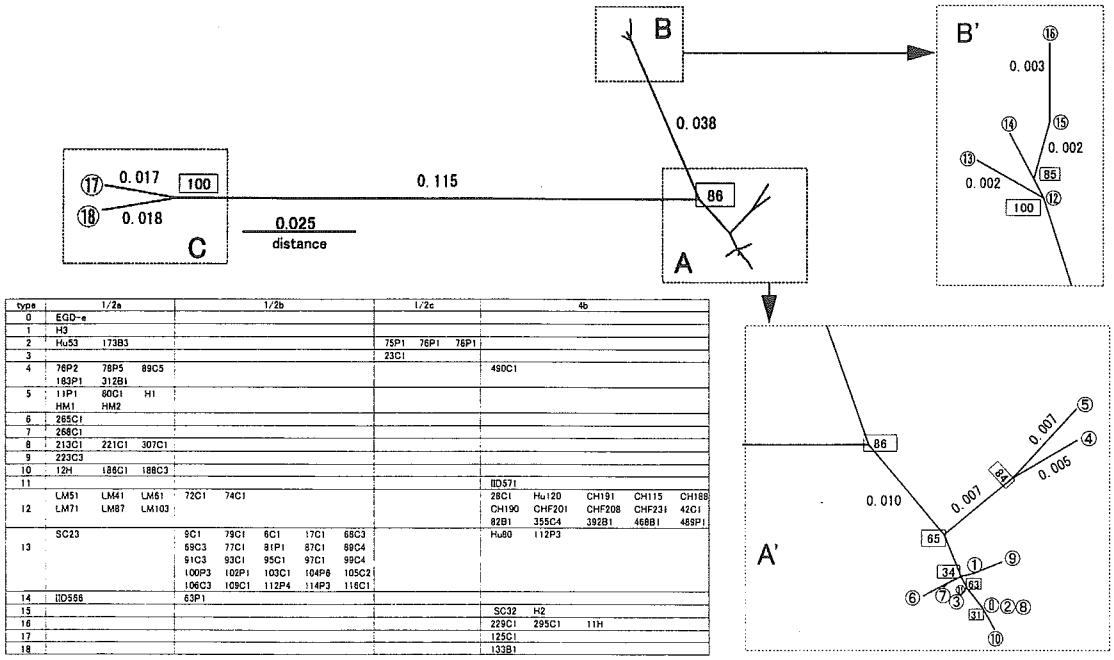

Fig. 1. Unrooted tree for *Listeria monocytogenes* isolated from Japanese foods and the environment. A' and B' show the magnified branches of A and B, respectively. Number, distance. Number in circle, type. Number in square, bootstrap rate (%). The percent was obtained by 1,000 neighbor-joining bootstrap replications.

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Short Communication

Characteristics of *Listeria monocytogenes* Isolated from Imported Meat in Japan

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SUMMARY: The genomic structure of the *iap* region in *Listeria monocytogenes* (serovar 4b), isolated from chicken imported into Japan, was compared with those from Japanese strains. The isolate was similar to the Japanese strains in a comparatively new, rare group. Such strains might be imported from foreign countries.

Listeriosis, caused by *Listeria monocytogenes*, is known to be transferred via contaminated foods. The authors have been investigating isolates from the Japanese environment and raw non-processed foods since 1988, and have classified the isolates using the *iap* region, as reported by Köhler et al. (1) and Rasmussen et al. (2). Japanese *L. monocytogenes* strains have three evolutionary streams of A, B and C groups (3), similar to those in other countries (2,4). However, the numbers and rates for each lineage are not equal among the groups: A (32%; 28 isolates), B (66%; 58 isolates) and C (2%; only 2 isolates). The figures suggest that the first two lineages, the A and B groups, are dominant in Japan. We recently found one isolate that was very similar to the rare isolates belonging to the C group. Thus, in the present study, molecular biological analysis of this isolate was performed and its detailed genomic character was compared with those of Japanese isolates.

We attempted to isolate *L. monocytogenes* from a randomly selected total of 51 samples of raw and non-processed pork, beef and chicken, imported from 11 countries (USA, Canada, Denmark, Australia, Mexico, France, Thailand, Ireland, China, New Zealand and Brazil) from 2001 - 2003. While in bond, an appropriate volume of meat was removed under the routine quarantine condition of asepsis, put into sterilized plastic bags, and transferred to the laboratory through the Yokohama Quarantine Station, Yokohama, Japan. Details for the isolation methods have been described previously (5). One strain, serovar 4b, was obtained from chicken imported on 1 March 2001 from Correa (Rio Grande do Sul, Brazil). Chromosomal DNA was extracted from the strain and the target 687 bp (position 836 - 1522), within the *iap* gene of *L. monocytogenes*, was amplified by polymerase chain reaction (PCR) using two primers, SI3A and SI3B (5). Determination of the DNA sequence was performed for the 407 bp (position 1116-1522) in the amplified product. The sequence was edited and aligned, then compared with *L. monocytogenes* EGD-e (GenBank accession no. AL591824), and compared with 88 Japanese strains (3) using the computer software DNAsis pro (Ver. 2.00.000.002; Hitachi Software Japan, Tokyo, Japan). Phylogenetic analysis and the genetic distance estimation were also performed using DNAsis pro.

Figure 1 shows the typical cleavage pattern for the PCR amplified *iap* region 678 bp of serovar 4b isolates after digestion with *Fnu*4HI and *Alw*NI. In the figure, all strains, except No. 10, are Japanese strains of meat or human origin. Three similarly sized fragments were obtained from seven Japanese stains, except for strain Nos. 5, 8 and 10. Nos. 5 and 8 were isolated in 1998 from Japanese chicken and beef, respectively, while No. 10 (YC16C10) was an isolate from foreign chicken. After *Fnu*4HI digestion, three different isolates were cleaved into five smaller fragments, designated a through e. Although the size was different in each isolate, the size of No. 10 seemed to be similar to that of No. 8. After *Alw*NI digestion, only two fragments were obtained from Nos. 5 and 10, while in No. 8, three fragments of a size similar to α and γ, but smaller than β, were obtained. These results suggested that the rare isolates, Nos. 5 and 8, are closely related to the No. 10 isolate from imported chicken, though they were not the same type.

The nucleotide sequences of the three isolates were also

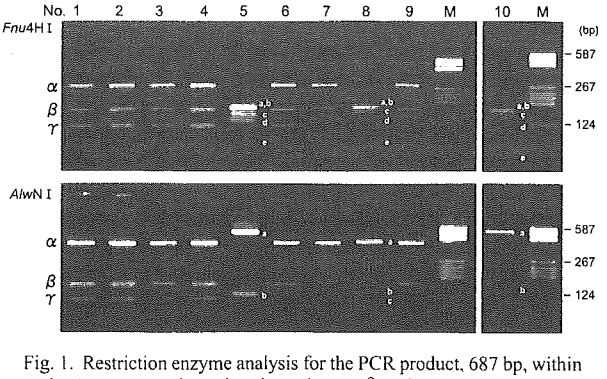

Fig. 1. Restriction enzyme analysis for the PCR product, 687 bp, within the *iap* gene region of each strain. α, β and γ correspond to the same fragments in the EGD-e strains with sizes of 288-, 175- and 2 mol bands for the 102- and 98-bp fragments by *Fnu*4HI, and 397, 161, 114 bp by *Alw*NI, respectively. The small letters a - e denote different bands in the Nos. 5, 8 and 10 isolates. Lane number (strain name, source, year), No. 1 (L97-28C1, chicken, 1997); No. 2 (L98-82B1, beef, 1998); No. 3 (L2K-295C1, chicken, 2000); No. 4 (L97-42C1, chicken, 1997); No. 5 (L98-125C1, chicken, 1998); No. 6 (L99-229C1, chicken, 1999); No. 7 (L98-112P3, pork, 1998); No. 8 (L98-133B1, beef, 1998); No. 9 (L2K-11H, human, 2000); No. 10 (YC16C10, chicken, 2001); M (size marker).

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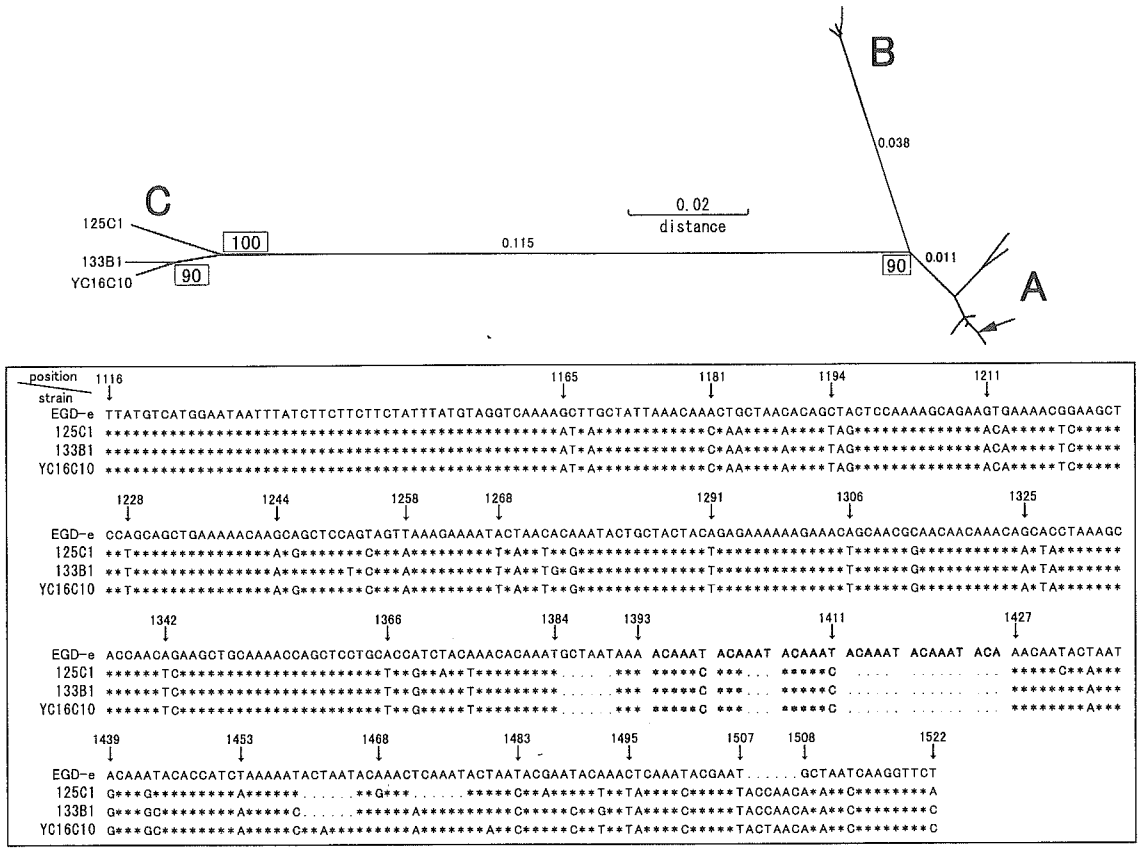

Fig. 2. Unrooted tree of *Listeria monocytogenes* isolated from meat and the nucleotide sequence (407 bp) within the *iap* region. The tree was established using 90 strains. Arrow in the solid circle: position of the EGD-e strain. Solid circles of A, B and C contain 28, 58 and 3 isolates, respectively. 125C1 and 133B1 strains isolated from raw and non-processed chicken and beef purchased from a shop in Japan. YC16C10 strain isolated from raw and non-processed chicken while in bond after importation from Brazil. Numbers in the tree correspond to distance. Numbers in squares represent the bootstrap rate (%). The frequency was recovered in 1,000 neighbor-joining bootstrap replications. The nucleotide sequence in the square: the small solid square shows a repeating structure. Asterisks and dots show the same nucleotide and nucleotide deletion, respectively.

characteristic, having more than 50 point-mutations and characteristic insertions and deletions in the *iap* region (Fig. 2), though Japanese isolates usually have mutations of less than 30 in this region (5). An unrooted tree for the diversity within the 407 bp *iap* region was originally established using the 88 Japanese and EGD-e strains (3); isolate No. 10 was inserted into this tree. Isolate No. 10 (YC16C10), together with Nos. 5 (125C1) and 8 (133B1), belongs to group C (Fig. 2, solid circle). In Figure 2, the solid circles A and B consist of 28 and 58 isolates containing the EGD-e, respectively. Lineage C was very far from lineages A and B, at a distance of 0.115, and No. 10 was closer to No. 8 than No. 5. Many foods have been imported into Japan from foreign countries. In the present study, we found a rare isolate from imported meat, though Japan has had no major outbreak of listeriosis, and we could find no references stating that such an isolate is common in other countries, including Brazil. The present results suggest that new types of *L. monocytogenes*, such as Nos. 5 and 8, might have been recently imported from other countries. The isolates having serovar 4b generally have a high virulence to humans, and have caused food poisoning in foreign counties. Thus, for the sake of food hygiene, the continuous inspection of Japanese commercial and imported meats is necessary.

The characteristics of *L. monocytogenes* isolates from imported chicken were investigated, and the strain was found to belong to a minor lineage in Japan.

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Original Article

Comparison of Genomic Structures in the Serovar 1/2a *Listeria monocytogenes* Isolated from Meats and Listeriosis Patients in Japan

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SUMMARY: Foodborne disease by *Listeria monocytogenes*, serovar 1/2a has recently been reported in many countries. Although contamination by this bacteria is also known to be gradually spreading among the marketed foods of Japan, there is little information on relation between listeriosis and food contamination. In the present study, the characteristics of the genomic structures of serovar 1/2a were compared among the isolates from marketed meats and listeriosis patients. Several isolates from meats purchased at the same shop on different days had the same genomic structure, and prolonged contamination was suggested by the conditions in the shop. Genomic structures of one strain isolated from meat were identical to those of two isolates from a patient. Another isolate was obtained from meats purchased at two different shops, and this isolate was also identical to that of the isolates from another patient. These findings suggest that the isolates from meat may have caused the listeriosis in the patients, and that the strains may have somehow traveled between the shops.

INTRODUCTION

Contamination by *Listeria monocytogenes* has been reported to induce a foodborne disease, listeriosis, in Europe and the U.S. since the 1980s (1-3). The incidence rate is different among districts (4,5), and the rates per million have been estimated to be 2 to 8 in the U.S. and Europe, 10.9 in Spain (6) and 1.6 to 14.7 in France (7), though the occurrence is usually lower than those of salmonellosis and vibriosis. In Japan, wide contamination by *L. monocytogenes* has also been found in marketed meats, fish and processed foods, and the incidence rate of listeriosis in Japanese has been estimated as 0.65 per million per year (8). In spite of these low incidence rates, listeriosis has a high mortality of more than 20% (9) due to the existence of high-risk groups (10) such as pregnant women, neonates and diabetics.

Thirteen serovar-types have been identified using the combination of somatic- and flagella-antigens, and the strain of serovar 1/2c is dominant (approximately 60%) in the environment (11). However, the isolation rates of serovars 1/2a, 1/2b and 1/2c in food are almost equal (approximately 20-30%). Further, the restricted serovars of 1/2a, 1/2b and 4b have frequently been isolated from patients (7,12-14). In human listeriosis, the isolation rates of serovars 4b and 1b (present serovar: 1/2b) in Japan were 60.6 and 31.0%, respectively, over the 26-year period of 1958-1984, but other serovars were rare during this period (15). These rates seem to have changed in recent years; the rates of serovars 4b, 1/2a and 1/2b in Japan were reported as 56, 26.5 and 18%, respectively (16). Similar phenomena have been observed in other countries. For example, the rates of serovars 4b, 1/2a and 1/2b were 29-89.7%, 3.4-41.9% and 3.5-42.3%, respectively, in Italy (17), and similar increased were observed in a study on 11 countries including Finland in the 1990s (18). The rates of serovar 4b were the highest in 11 of 12 countries and those of serovar 1/2a were the second in 10 countries. In Finland the rates of serovar 1/2a and 4b were 42.3 and 57.7% in 1989 (18). However, in another report (19), the number of human listeriosis cases caused by serovar 1/2a increased from 22% in 1990 to 67% in 2001, and those caused by serovar 4b decreased from 61 to 27%, respectively. Thus, elucidation of the relation between the serovar 1/2a strains from the field and those isolated from patients is needed to clarify the epidemiology of *L. monocytogenes* before the further spread of serovar 1/2a-induced listeriosis in Japan.

The *iap* gene encoding an extracellular protein p60 has genetic diversity in the EGD-*L. monocytogenes* strain (20,21). The authors have reported that the combination of restriction fragment length polymorphism (RFLP) analysis of the chromosomal DNA extracted from *L. monocytogenes* and analysis of the diversity in a part of the *iap* gene is useful for discrimination among the *L. monocytogenes* isolates from foods and humans (22-24). In the present study, the characteristics of the genomic structures of serovar 1/2a were compared among the isolates from marketed meats and listeriosis patients using the combination of RFLP analysis of the extracted chromosomal DNA and the multiple sequence alignments for the polymerase chain reaction (PCR) products of

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the *iap* gene.

MATERIALS AND METHODS

Isolation, identification and serotyping of *L. monocytogenes*: Meat-origin isolates: A total of 20 *L. monocytogenes* isolates, all of serovar 1/2a, were used in the present study. Seventeen isolates were from unprocessed pork, chicken and beef purchased between June 1996 and June 2000 at 8 different shops in the Tokyo metropolitan area, Chiba and Saitama Prefectures. UVM broth (Becton, Dickinson and Company, Sparks, Md., USA) and Palcam (Merck, Darmstadt, Germany) agar were used for the pre-culture broth and the selection of *Listeria* spp., respectively. Identification was performed by the usual method described in the previous reports (22-24), and the serovar of the isolates was then determined by the Sahumy method (25).

Human-origin isolates: Two strains of serovar 1/2a were isolated in 1996 from the blood and feces of one listeriosis patient with septicemia using a method similar to that used here for meat. One isolate from the spinal fluid of a listeriosis patient with meningitis was provided in 2000 by the First Department (Neurology) of Internal Medicine, School of Medicine, Kurume University.

RFLP analysis of chromosomal DNA extracted from *L. monocytogenes* isolates: The chromosomal DNA was extracted and purified from the isolated strains as previously described (22-24). The DNA was digested with the restriction enzymes *Ban*III, *Pst*I, *Xba*I, *Sal*I, *BamH*I and *Bgl*II according to the manufacturer's instructions (Toyobo, Tokyo, Japan). The reactants were separated on 0.8% agarose gel followed by staining with ethidium bromide (EtBr). DNA fragments on the gel were visualized using an UV transilluminator, and a photograph was taken of each gel.

Determination of the nucleotide sequence: A part of the *iap* gene (810 bp) which contains the repeated variable nucleotide sequences was amplified by PCR using a pair of primers, SI3A (5′-ACTGGTTTCGTTAACGGTAAA-3′) and SI4B (5′-TTTAGTGTAACCAGAGCAATC-3′). The amplified products were then cycle-sequenced (554 bp) using a pair of dye* primers, SI4AD (5′-AATACGGTGTTTCTGT TCAAG*-3′) and SI4BD (5′-TTTAGTGTAACCAGAGCA ATC*-3′), with a DNA sequencer (Hitachi SQ-5500; Hitachi, Tokyo, Japan) as previously described (22-24). A part of the determined nucleotide sequences, 407 bp between positions 1116 and 1522, was used for a comparative analysis against the SV1/2a EGD (EGD) strain reported by Kohler et al. (20) (GenBank accession number X52268).

RESULTS

RFLP analysis of chromosomal DNA extracted from *L. monocytogenes* isolates: The chromosomal DNAs extracted from the 20 isolates were digested with the 6 restriction enzymes *Pst*I, *Xba*I, *Ban*III, *Sal*I, *BamHI* and *Bgl*II, and the RFLP analysis was then attempted by agarose gel electrophoresis. Figures 1a and 1b show the typical digested patterns by *Pst*I and by 3 restriction enzymes, respectively. The patterns from 20 isolates were classified into 8 different types, a to h, and the same classification was obtained after the digestion by all 6 restriction enzymes (data not shown). In the following description the numbers correspond to the strain numbers shown in the Figures.

The digested patterns in the 2 isolates (Nos. 1 and 2) from one patient were identical (type a), but a different pattern (type b) were seen in isolate No. 3 from the other patient. The patterns of the former 2 human isolates were indentical to those of Nos. 4 and 8 from pork and chicken, respectively, purchased in the same store but in a different year. The latter pattern from No. 3 (the other human isolate) was indentical to those of 2 isolates, Nos. 14 and 20 from chicken purchased in the same year but from different stores. Although other patterns from the meat isolates were not matched with those of human isolates, a pattern (type c) was identified in 5 isolates, Nos. 5, 6 and 9 from one store and Nos. 10 and 19 from two other stores and a pattern (type d) was identified in 4 isolates, Nos. 7, 11, 13 and 17 from different stores. However, the RFLP patterns from the other 4 isolates, Nos. 12, 15, 16 and 18, were not matched with those of any other isolates, and thus were classified into types e to h, respectively.

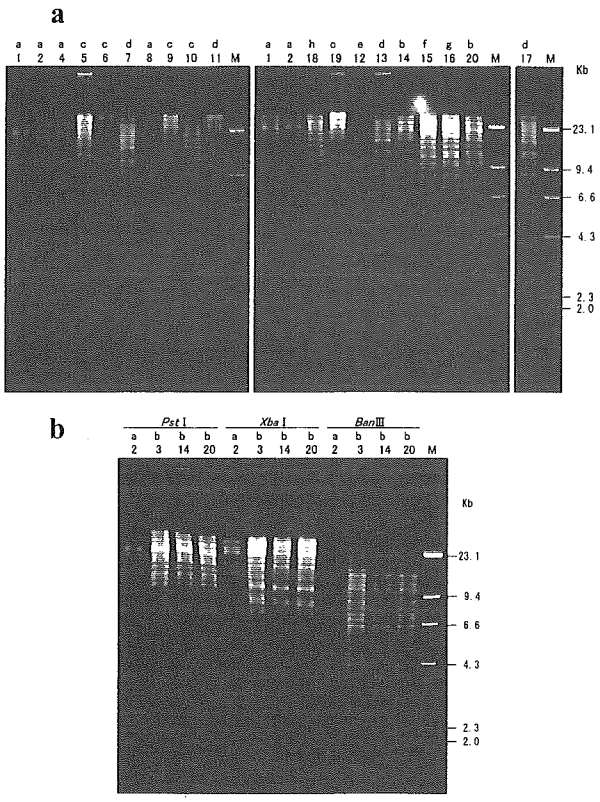

Fig. 1. RFLP analysis of the *Listeria monocytogenes* isolated from meats and patients. Typical digested patterns by *Pst*I (a) and *Pst*I *Xba*I and *Ban*III (b). Small letters a to d on the strain numbers show the same cleavage pattern. Number (in parentheses) and strain name: (1) L96-HM1, (2) L96-HM2, (3) L2K1-12H, (4) L96-11P1, (5) L98-76P2, (6) L98-78P5, (7) L98-104P5, (8) L98-80C1, (9) L98-89C5, (10) L99-183P1, (11) L99-213C1, (12) L99-173B3, (13) L99-221C1, (14) L99-186C1, (15) L2K-265C1, (16) L99-223C3, (17) L2K-268C1, (18) L2K-307C1, (19) L2K-312B1, (20) L99-188C3.

Comparison of the nucleotide sequences: The sequences determined for all the strains are shown in Fig. 2 in comparison with that of the reference SV1/2a EGD strain. Although no nucleotide deletion was observed in the region of these isolates, the isolates showed variations in nucleotide substitutions and insertions, and in the number of repeated sequences.

A total of 12 nucleotide substitutions were observed in 15 of the isolates investigated compared with that of the EGD

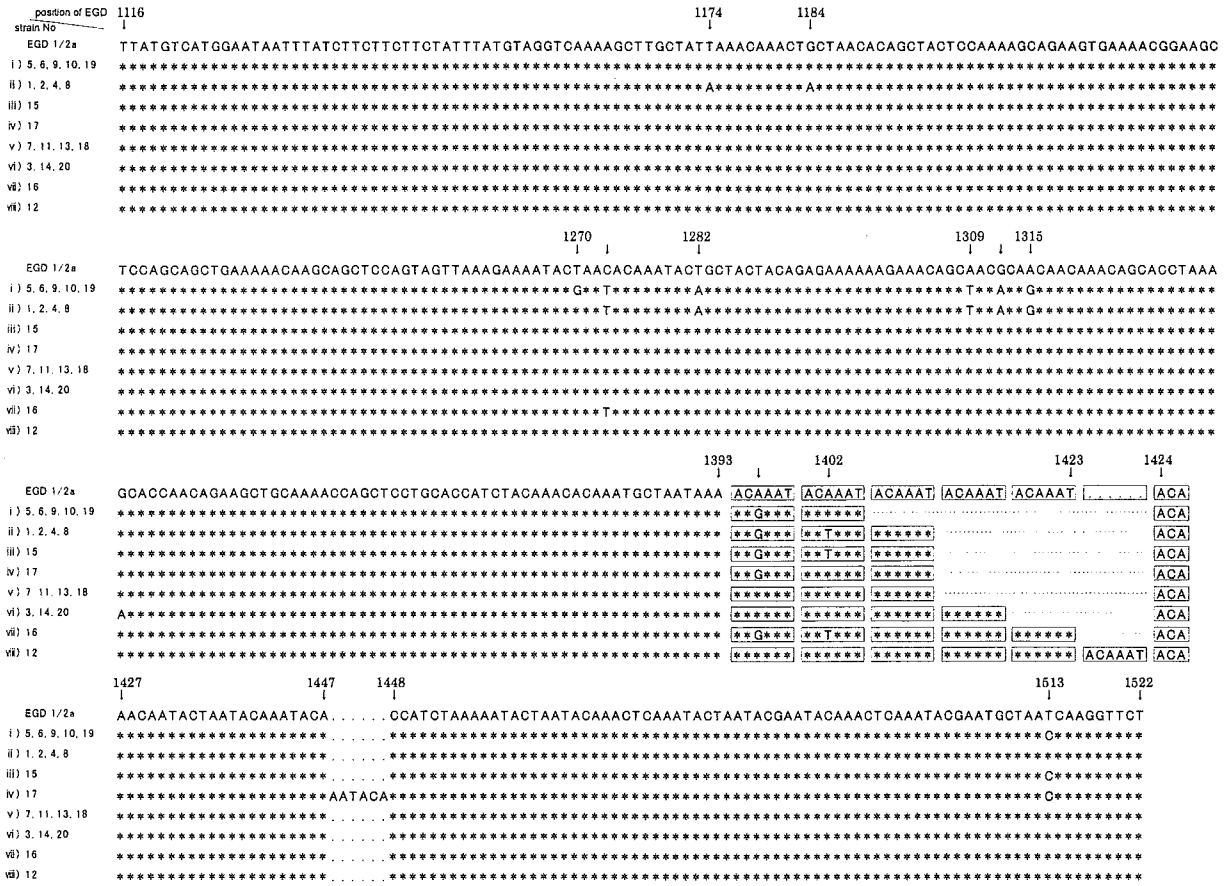

Fig. 2. Nucleotide sequences of *iap* region (407bp) in serovar 1/2a *Listeria monocytogenes* isolates. Number and strain name are in Fig. 1.

strain, while 5 isolates, Nos. 7, 11, 13, 18 (type v) and 12 (type viii) had no nucleotide substitution. The number of nucleotide substitutions in each isolate ranged from 1 to 9. The 20 isolates were consequently classified into 7 types from the nucleotide substitutions. Only one isolate, No. 17, had a 6-nucleotide insertion of AATACA between positions 1447 and 1448 downstream of the repeated sequence.

The repeated sequence consisted of 6 nucleotides (ACAAAT) for one unit and ended with ACA located at positions 1394 to 1426 in the EGD strain, and the EGD strain had 4 cycles. Nucleotide substitutions within the structure consisted of an A to G substitution at position 1396 in 12 isolates, and an A to T substitution at position 1402 in 6 of these 12 isolates. The number of repeated sequences ranged from 1 to 5, and the 20 isolates were classified into 5 types based on these repetitions.

Consequently, the 20 isolates were classified into 8 types based on the nucleotide substitutions, a single insertion and the repeated structures.

DISCUSSION

Table 1 shows the summary of the details for the isolates and the results. These isolates were classified into 9 types, I to IX, though 8 types were obtained from the RFLP analysis of chromosomal DNA. This is due to disagreements in the types of IV, V and VI. The cleavage pattern type d was divided into 2 types of IV containing 3 isolates and V of No. 17. This phenomenon is not strange because RFLP analysis cannot detect a 6-nucleotide insertion, and also perhaps 2-nucleotide substitution in No. 17 if the nucleotide does not relate with the cutting site. Then, although types IV and VI had the same nucleotide sequence, the type VI of No. 18 had a different cleavage pattern of h because the analysis of the nucleotide sequence cannot detect a difference in other regions due to the analysis of short PCR product. Although discriminations of bacteria using the RFLP and the PFGE analysis of a PCR product and/or a chromosomal DNA have frequently been performed in epidemiology after a foodborne disease such as salmonellosis (26) and listeriosis (27,28), the present results suggest that the analysis by RFLP only, and perhaps also that by PFGE only, are not sufficinet for an accurate discrimination among the strains. Thus, more detailed gene analysis for the discrimination of *L. monocytogenes* strains would be made possible by the combination of RFLP analysis of the chromosomal DNA and determination of the sequence for the PCR product.

In case A, 2 isolates, Nos. 4 and 8, and 3 isolates, Nos. 5, 6 and 9, were identified as the same type, types I and III, respectively, suggesting that contamination by these 2 strains was prolonged in this shop because these meats were purchased on different days. It is particularly important that the former strains of type I were identified as the same type with the isolates from a patient. A similar identification was also obtained among an isolate from the other patient, No. 14 and No. 20 from chicken (type II). The virulence of

Table 1. Genomic characteristics and details of *Listeria monocytogenes* used in the present study

Case	No.	Sample	Date	Strain	Cleavage pattern	Nucleotide substitution	Number of repeat structure	Insertion	Delation	Type
human	1	Blood	960318	L96-HM1	a	9	2	0	0	I
	2	Feces	960408	L96-HM2	a	9	2	0	0	I
	3	Spinal fluid	020904	L2K1-12H	b	1	3	0	0	II
A	4	Pork	960624	L96-11P1	a	9	2	0	0	I
	5	Pork	980210	L98-76P2	c	8	1	0	0	III
	6	Pork	980211	L98-78P5	c	8	1	0	0	III
	7	Pork	980417	L98-104P5	d	0	2	0	0	IV
	8	Chicken	980213	L98-80C1	a	9	2	0	0	I
	9	Chicken	980311	L98-89C5	c	8	1	0	0	III
B	10	Pork	990612.	L99-183P1	c	8	1	0	0	III
	11	Chicken	990620	L99-213C1	d	0	2	0	0	IV
C	12	Beef	990613	L99-173B3	e	0	5	0	0	VII
	13	Chicken	990620	L99-221C1	d	0	2	0	0	IV
D	14	Chicken	990613	L99-186C1	b	1	3	0	0	II
	15	Chicken	000611	L2K-265C1	f	3	2	0	0	VIII
E	16	Chicken	990620	L99-223C3	g	3	4	0	0	IX
	17	Chicken	000611	L2K-268C1	d	2	2	1	0	V
F	18	Chicken	000619	L2K-307C1	h	0	2	0	0	VI
G	19	Beef	000611	L2K-312B1	c	8	1	0	0	III
H	20	Chicken	990614	L99-188C3	b	1	3	0	0	II

the isolates from meats was not examined in the present study. Although many Japanese do not eat raw meat except fish, our results show the risk of foodborne disease from meat. The chicken isolates may be related to sporadic listeriosis in Japanese because 3 of 4 isolates (Nos. 8, 14 and 20 in types I and II) were from chicken.

On the other hand, the same strains were isolated from meats purchased at different shops, suggesting that there were some routes for moving the strain between shops of D and H (type II), among shops A, B and G (type III), and among shops A, B and C (type IV). However, no information was obtained for these shops. Accumulation of information on the possible relation might be useful for surveillance and management of the risk, though the virulence is, of course, unknown for any of these isolates except type II. Further investigation will be needed for the management and/or HACCP of the meat safety in Japan.

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Full Paper

Inhibitory Mechanism of Monensin on High K^+-Induced Contraction in Guniea-Pig Urinary Bladder

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Abstract. In this study, we examined the inhibitory mechanism of monensin on high K^+-induced contraction in guinea-pig urinary bladder. The relaxant effect of monensin (0.001 – 10 μM) was more potent than those of NaCN (100 μM – 1 mM) and forskolin (3 – 10 μM). Monensin (0.1 μM), NaCN (300 μM), or forskolin (10 μM) inhibited high K^+-induced contraction without decreasing $[Ca^{2+}]_i$ level. Monensin and NaCN remarkably decreased creatine phosphate and ATP contents. Monensin and NaCN inhibited high K^+-induced increases in flavoprotein fluorescence, which is involved in mitochondrial respiration. Forskolin increased cAMP content but monensin did not. Monensin increased Na^+ content at 10 μM but not at 0.1 μM that induced maximum relaxation. In the α-toxin-permeabilized muscle, forskolin significantly inhibited the Ca^{2+}-induced contraction, but monensin did not affect it. These results suggest that the relaxation mechanism of monensin in smooth muscle of urinary bladder may be an inhibition of oxidative metabolism.

Keywords: monensin, urinary bladder, phosphocreatine, Na^+ content

Introduction

Monensin is a polyether, monocarboxylic molecule used as a Na^+ ionophore in cellular physiology and pharmacology. Some studies have shown that monensin caused cardiotonic effects (1 – 4). Pressman and Painter (5) considered that the cardiotonic effects of monensin are caused by an increase in intracellular sodium concentration and the resultant stimulation of sodium-calcium exchange. On the other hand, it has been reported that monensin inhibited the high K^+- or receptor agonist-induced contraction in some smooth muscles (6 – 9).

Smooth muscles have been classified into phasic and tonic muscles on the bases of their electrophysiological and mechanical behaviors (10 – 12). Phasic smooth muscles, including ileum, urinary bladder, uterus, vas deferens, etc., produce contraction often associated with electrical spikes. Depolarization with high K^+ elicits an initial phasic contraction followed by a sustained contraction (13). In contrast, tonic smooth muscles such as aorta and trachea generally produce contraction without electrical spikes. A high K^+-induced depolarization elicits a sustained contraction with the slow onset of contraction. The contractile diversity among different smooth muscles appears to be attributed to variations in the dependence on oxidative metabolism (14, 15), as well as cellular protein expression (16 – 21) and electrophysiological properties (22, 23).

In guinea-pig taenia coli, which is classified as a phasic muscle, monensin inhibited the high K^+-induced contraction by inhibiting oxidative adenosine triphosphate (ATP) production (6). It was suggested that monensin inhibited the 5-hydroxytryptamine-induced contraction due to a change in intracellular sodium concentration in canine coronary artery, which is classified as a tonic muscle (7). These data imply that mechanism of monensin-induced relaxation in phasic muscle is inhibiting oxidative ATP production and that in tonic muscle is related to Na^+ movement.

In the present study, we attempt to characterize the

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inhibitory mechanism of monensin on high K^+-induced contraction in guinea-pig urinary bladder, a phasic muscle. The investigation was performed by measuring muscle tension, intracellular Ca^{2+} ($[Ca^{2+}]_i$) level, phosphocreatine (PCr) and ATP contents, oxidized flavoprotein as an indicator of mitochondrial respiration, pyridine nucleotides as indicator of glycolytic activity, cAMP content, Na^+ content, and tension development of α-toxin permeabilized muscle in the urinary bladder.

Materials and Methods

Muscle preparations and tension measurement

Male guinea pigs (Hartley strain, 300 – 400 g; Funabashi Farm, Funabashi) were bled after stunning, and then the urinary bladder was quickly removed. Trigumun vesicae, superficial tissue, fat, and mucous layer were removed. Longitudinal strips of urinary bladder were about 10 – 15 mm in length and 3 – 5 mm in width. The muscle strips were incubated with physiological salt solution (PSS) containing 136.8 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 5.6 mM glucose. PSS was aerated with 95% O_2 and 5% CO_2 to adjust pH to 7.2 at 37°C. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals of Nippon Veterinary and Animal Science University.

Muscle tension was recorded isometrically. One end of each strip was bound to a glass holder and the other end was connected with a silk thread to a strain-gauge transducer (TB-611T; Nihon Kohden, Tokyo) in an organ bath containing PSS with resting tension of 3 g. The muscle strips were equilibrated for 30 min to obtain a stable contractility induced by hyperosmotically added 65 mM KCl (H-65 K^+). The Na^+-deficient, high K^+ solution (I-154 K^+) was made by substituting an equimolar amount of K^+ for Na^+ in the PSS. As it has been reported that Na^+-free PSS decreases utilization of extracellular glucose in guinea-pig urinary bladder (24), 5.5 mM pyruvate was added in I-154 K^+ for an energy supply. The developed tension was expressed as a percentage, by assigning the values at rest in normal PSS to be 0% and those at a steady level of contraction in H-65 K^+ or I-154 K^+ to be 100%.

Simultaneous measurement of a muscle contraction and $[Ca^{2+}]_i$ level

$[Ca^{2+}]_i$ level was measured simultaneously with a muscle contraction as reported previously (25). Muscle strips were incubated with PSS containing 5 μM fura2/AM for overnight at 4°C. Cremophor EL (at 0.02%), a noncytotoxic detergent, was also added to increase the solubility of fura2/AM. One end of the muscle was pinned to the bottom of the organ bath, which was filled with 8 ml of PSS, and the other end was attached to the transducer with silk thread. The muscle strip was kept horizontally in the organ bath. The muscle strip was alternately excited with light at 340 and 380 nm through a rotating filter wheel, and the 500-nm emission was measured through a band-pass filter with a fluorometer (CAF-100; Japan Spectroscopic Co., Ltd., Tokyo), and their ratio (F340/F380) was recorded as an indicator of $[Ca^{2+}]_i$. The fluorescence ratio was expressed as a percentage, by assigning the values at rest in normal PSS to be 0% and those at a steady level of contraction in isosmotically added 65 mM KCl (I-65 K^+) just before an application of each relaxant to be 100%. We determined muscle tension and $[Ca^{2+}]_i$ level 40 min after the addition of monensin and 20 min after addition of NaCN or forskolin.

Assay of creatine phosphate and adenosine triphosphate

PCr and ATP contents in the muscle strips were measured by high-performance liquid chromatography (HPLC) as reported previously (26). The muscles were incubated with H-65 K^+ for 15 min and then with monensin for 50 min, NaCN for 20 min, or forskolin for 20 min. These treatment times were chosen since the relaxation responses to these agents reached a plateau. After the incubation, the muscles were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 6% perchloric acid (0.9 ml). The homogenate was centrifuged at 15,000 × g for 5 min and the supernatant neutralized with 0.2 ml of 2 M $KHCO_3$. The neutralized extracts were centrifuged once more and 20 μl supernatant was applied to HPLC.

The HPLC system (Shimadzu Corp., Kyoto) consisted of a pump (LC-10AT), a system controller (SCL-10A), an auto injector (SIL-10AF), a column oven (CTO-10A), and wave length-selectable detector (SPD-10Ai) set at 216 nm. Chromatography was performed by a μRPC C2/C18 ST (4.6-mm internal diameter and 100 mm length; Amersham Pharmacia Biotech, Little Chalfont, UK) column using mobile phases of 50 mM KH_2PO_4 and 5 mM terabutylammonium hydrogen sulphate (TBAHS) (pH 6.0, buffer A), and 50 mM KH_2PO_4, 5 mM TBAHS and 40% methanol (pH 6.0, buffer B). Flow rate was 1.0 ml/min and the elution started with 65% buffer A. Then, for 14 min, buffer B was increased at a rate of 2.5%/min. This was followed by elution with 70% buffer B for 20 min and then with 100% buffer A for 10 min. These procedures were programmed with the system controller. The sensitivity of the detecter was usually set at 1.0 a.u.f.s. and the oven temperature at 40°C. PCr and ATP contents are

expressed as μmol/g wet weight.

Simultaneous measurement of muscle contraction and oxidized flavoprotein (FPox) fluorescence or reduced pyridine nucleotide (PNred) fluorescence

FPox or PNred fluorescence was measured simultaneously with a muscle contraction as reported previously (27). The muscle strip was also kept horizontally in the organ bath, as described for the measurement of $[Ca^{2-}]_i$ level. The muscle strip was excited with light at 450 nm and the 530 nm emission was measured with a fluorometer (CAF-100) to detect FPox fluorescence. It was excited with light at 340 nm and the 470 nm emission was measured with a fluorometer to detect PNred fluorescence. FPox or PNred fluorescence was expressed as a percentage, by assigning the values at rest in normal PSS to be 0% and those at a steady level of contraction in H-65 K^+ just before an application of monensin or NaCN to be 100%. Effects of monensin and NaCN on muscle tension and FPox or PNred fluorescence were determined 40 min and 20 min after addition of agents, respectively.

Assay of cAMP content

cAMP content in the muscle strips was measured by enzyme immunoassay as reported previously (28). After incubation of the muscles with monensin for 50 min, NaCN for 20 min, and forskolin for 20 min in the presence of H-65 K^+, the muscles were rapidly frozen in liquid nitrogen and stored at −80°C until homogenized in 6% trichloroacetic acid (0.4 ml). The homogenate was centrifuged at 3,000 × *g* for 15 min and the supernatant was washed with 1.5 ml of water-saturated diethylether four times; the cAMP content was assayed by using an enzyme immunoassay kit (Amersham Pharmacia Biotech). cAMP contents are expressed as pmol/g wet weight.

Measurement of Na^- content

Na^- content in muscle strips was measured by the 'Li-method' as reported previously (6). After incubation with test solution (normal PSS plus 60 mM KCl and 0.1 or 10 μM monensin, at 37°C), the muscles were exposed to Li solution (169.5 mM LiCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES, and 5.6 mM glucose) at 0.5°C and pH 7.2 for 30 min in order to remove extracellular Na^-. The tissues were blotted between filter papers, weighed, and ashed overnight at 180°C in a quartz tube with 0.5 ml of a mixture of equal amounts of HNO_3 (61%) and $HClO_4$ (60%). Na^- content in the sample was measured by flame photometer (atomic absorption photometer, Hitachi-Z8200: Hitachi, Tokyo). Na^- content is expressed as mmol/kg wet weight.

Measurement of muscle tension in α-toxin permeabilized smooth muscle

A thin longitudinal muscle strip, 5.0 mm in length and 0.2 mm in width, was prepared from the isolated urinary bladder. The muscle strip was held horizontally in an 1-ml organ bath. One end of each strip was fixed and the other end was connected to a strain-gauge transducer. The muscle tension was isometrically recorded. Permeabilized muscle was prepared by treating the urinary bladder strips with *Staphylococcus aureus* α-toxin (80 μg/ml, 30 min), as described previously (29). The relaxing solution contained 130 mM potassium propionate, 4 mM $MgCl_2$, 4 mM ATP, 20 mM tris-maleate, 2 mM creatine phosphate, 10 U/ml creatine phosphokinase, and 2 mM ethyleneglycol bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA). A 3-μM Ca^{2-} (pCa 5.5) solution was prepared using Ca^{2+}-EGTA buffer composed of 2 mM EGTA and 1.51 mM $CaCl_2$. The required Ca^{2+}-concentrations were calculated assuming the apparent binding constant of Ca^{2+}-EGTA complex to be 10^6 M^{-1} at pH 6.8 (30). The solutions were adjusted to pH 6.8 at 24°C with KOH (0.1 N).

Chemicals

Chemicals used were monensin and forskolin (Sigma-Aldrich, St. Louis, MO, USA); sodium cyanide (NaCN) (Wako Pure Chemical, Osaka); fura2/AM (Dojindo Laboratories, Kumamoto); and cremophor EL (Nacalai Tesque, Kyoto).

Statistics

Values are expressed as the mean ± S.E.M. and statistical analyses were performed by Student's *t*-test.

Results

Effects of monensin, NaCN, and forskolin on a high K^+-induced contraction

H-65 K^+ showed a phasic contraction, following a sustained one. When the H-65 K^--induced contraction reached a steady level, monensin (0.001 – 10 μM) was added. Although 0.001 μM monensin had little effect, the higher concentrations inhibited the H-65 K^+-induced contraction (Fig. 1A). The results indicate that the rate of relaxation was dependent on the concentration of monensin. NaCN (100 – 1000 μM) or forskolin (3 – 10 μM) also inhibited the H-65 K^--induced contraction in a concentration dependent manner (Fig. 1: B and C). The relaxant effect of monensin was more potent than those of NaCN and forskolin.

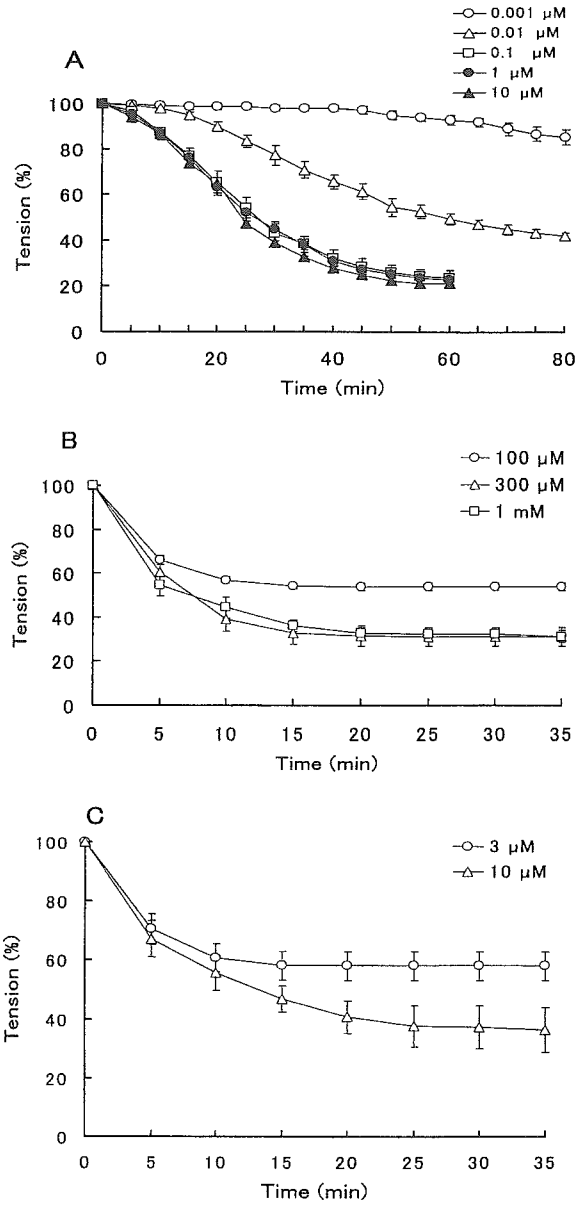

Fig. 1. Effects of monensin (A), NaCN (B), or forskolin (C) on the contraction induced by high K^+ in urinary bladder. Ordinate: relative tension. The contractions induced by high K^+ just before the application of each agent were taken as 100%. Abscissa: time (min) of exposure to each agent. Each point represents the mean of 4 – 8 preparations. Vertical bars indicate the S.E.M.

Effects of monensin, NaCN, or forskolin on an elevated $[Ca^{2+}]_i$ level by high K^+

The effects of monensin, NaCN, or forskolin on $[Ca^{2+}]_i$ level were measured simultaneously with muscle tension, using a fluorescent Ca^{2+} indicator, fura2. I-65 K^+ induced a muscle contraction and elevated $[Ca^{2+}]_i$ level indicated by F340/F380. Monensin (0.1 μM) showed a significant inhibition in the I-65 K^+-induced contraction without a decrease in $[Ca^{2+}]_i$ level (33.4 ± 4.3% for tension, $P<0.01$; 93.9 ± 3.2% for $[Ca^{2+}]_i$ level, no significance, 40 min after addition of monensin) (Fig. 2A).

NaCN (300 μM) decreased muscle contraction induced by I-65 K^+ significantly, but did not change $[Ca^{2+}]_i$ level (36.5 ± 1.1% for tension, $P<0.01$; 95.3 ± 1.5 % for $[Ca^{2+}]_i$ level, no significance, 20 min after addition of NaCN) (Fig. 2B). Forskolin (10 μM) also showed a significant inhibition in the I-65 K^+-induced contraction with an insignificant decrease in $[Ca^{2+}]_i$ level (37.9 ± 1.2% for tension, $P<0.01$; 93.1 ± 2.8% for $[Ca^{2+}]_i$ level, no significance, 20 min after addition of forskolin) (Fig. 2C).

Effects of monensin, NaCN, or forskolin on PCr and ATP contents

Effects of monensin (0.01, 0.1 μM), NaCN (300 μM), or forskolin (10 μM), on PCr and ATP contents are summarized in Fig. 3. Monensin and NaCN decreased PCr and ATP contents below the control in the presence of H-65 K^+, significantly. On the other hand, forskolin did not affect PCr and ATP levels significantly.

Effects of monensin or NaCN on change of FPox or PNred fluorescence

Effects of monensin or NaCN were investigated on FPox or PNred fluorescence measured simultaneously with muscle contraction. H-65 K^+ induced a muscle contraction and elevated FPox fluorescence (Fig. 4A). Monensin (0.1 μM) inhibited the H-65 K^+-induced muscle contraction and increase in FPox fluorescence significantly (42.1 ± 3.1% for tension, 25.3 ± 2.2% for FPox fluorescence, $P<0.01$, respectively) (Fig. 4A). NaCN (300 μM) also inhibited the H-65 K^+-induced muscle contraction and increase in FPox fluorescence significantly (43.8 ± 1.7% for tension, 30.8 ± 2.6% for FPox fluorescence, $P<0.01$, respectively) (Fig. 4B).

On the other hand, H-65 K^+-induced increase in PNred fluorescence was not affected by the presence of monensin or NaCN (data not shown). These results suggest that monensin inhibits mitochondrial respiration, but not glycolytic activity.

Effects of monensin or forskolin on cAMP content

Forskolin (10 μM) increased cAMP content above the control in the presence of H-65 K^+ as shown in Fig. 5 (control, 60.7 ± 5.4 pmol/g wet weight; forskolin, 118.3 ± 4.6 pmol/g wet weight). However, monensin (0.1 μM) did not change cAMP content (59.1 ± 4.0 pmol/g wet weight).

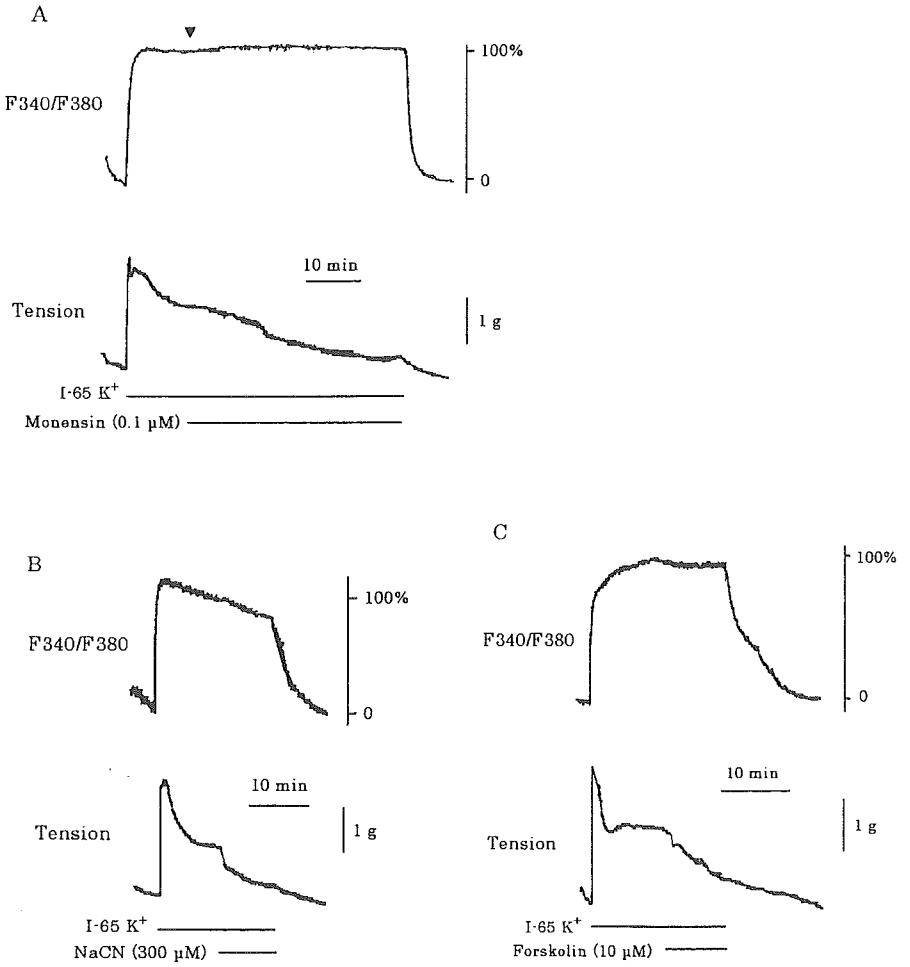

Fig. 2. Effects of monensin (A), NaCN (B), or forskolin (C) on the I-65 K^+-induced increases in $[Ca^{2+}]_i$ level (F340/F380, upper trace) and muscle tension (lower trace). The increases in $[Ca^{2+}]_i$ level induced by I-65 K^+ just before an application of each agent was taken as 100%. Each trace of typical results from 4 experiments.

Effects of monensin on a contraction in Na^+-deficient, high K^+ (I-154 K^+) solution

Exposure of the muscle to I-154 K^+ solution resulted in a rapid rise followed by sustained contraction reaching a steady level within 15 min. As we have reported that Na-free PSS decreases utilization of extracelluar glucose in guinea-pig urinary bladder (24), 5.5 mM pyruvate was added in I-154 K^+ for an energy supply. The sustained contraction induced by I-154 K^+ or H-65 K^+ was in the same magnitude (At 15 min, the I-154 K^+-induced contraction was 104.5 ± 6.5% of H-65 K^+-induced contraction, no significant difference). Such a sustained contraction in I-154 K^+ was inhibited by monensin (0.1 μM) at 25 – 60 min, as was the case with the H-65 K^+. However, monensin inhibited I-154 K^+-induced contraction more rapidly than H-65 K^+-induced contraction (Fig. 6).

Effect of monensin on Na^+ content

Effect of monensin was investigated on the cellular Na^+ content in H-65 K^+-depolarized muscle. As shown in Fig. 1, 0.1 and 10 μM monensin both inhibited H-65 K^+-induced contraction to the same degree. In the H-65 K^+ solution, the cellular Na^+ content did not change at 20, 40, and 60 min after application of 0.1 μM monensin. However, the cellular Na^+ content increased at 20, 40, and 60 min after application of 10 μM monensin (Fig. 7).

Effects of monensin or forskolin on Ca^{2+}-induced contractions in permeabilized muscle

As shown in Fig. 8, 3 μM Ca^{2+} induced a sustained contraction in α-toxin-permeabilized muscle. At 30 min, monensin (0.1 μM) inhibited the high K^+-induced contraction to 43.2 ± 5.1% in the intact muscle (Fig. 1A). However, monensin (0.1 μM) did not affect

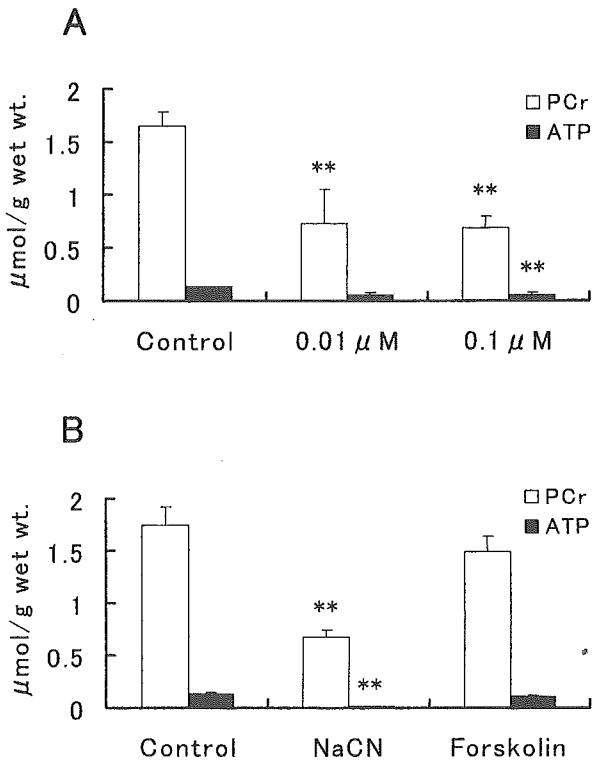

Fig. 3. Effects of monensin (0.01 and 0.1 μM) (A) and NaCN (300 μM) or forskolin (10 μM) (B) on PCr and ATP contents in the presence of high K^+. Control: 50 min (A) or 20 min (B) after the application of high K^+, respectively. Monensin was applied for 50 min. NaCN or forskolin was applied for 20 min. The data are expressed as the means ± S.E.M. Vertical bars indicate the S.E.M. **: Significant difference from each respective control with $P<0.01$.

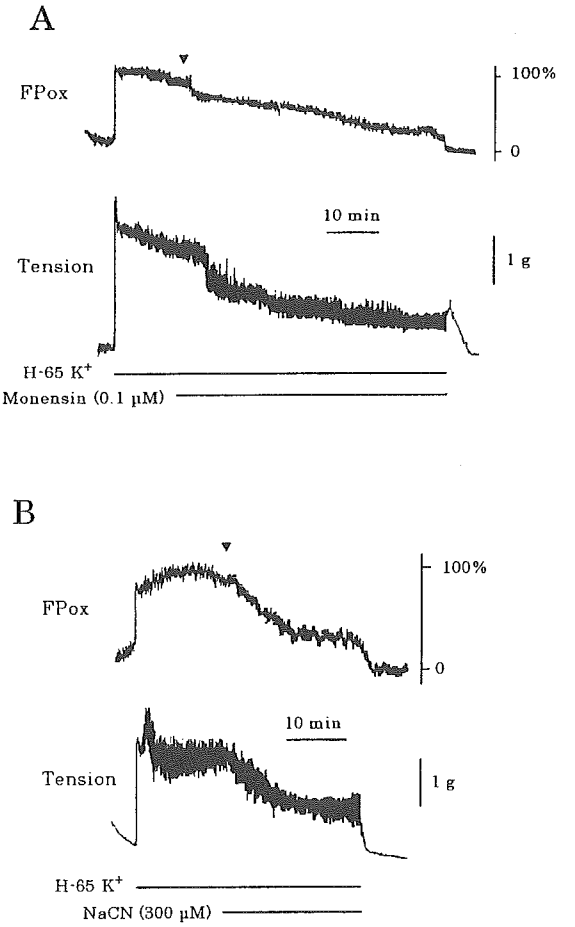

Fig. 4. Effects of monensin (A) or NaCN (B) on high K^+-induced muscle tension (lower trace) and the increases in oxidized flavoprotein (FPox) fluorescence (upper trace). The increase in FPox fluorescence induced by high K^+ just before an application of each agent was taken as 100%. Trace of typical results from 4 experiments.

the Ca^{2+}-induced contraction for 30 min in the permeabilized muscle (Fig. 8A). At 10 min, NaCN (300 μM) inhibited the high K^+-induced contraction to 39.0 ± 5.3% (Fig. 1B), but NaCN (300 μM) did not affect the Ca^{2+}-induced contraction for 10 min (Fig. 8B). However, forskolin (10 μM) significantly inhibited the Ca^{2+}-induced contraction to 70.8 ± 3.1% at 10 min (Fig. 8C).

Discussion

There are two relaxing mechanisms of smooth muscle by monensin: 1) a change in intracellular Na^+ content (7), 2) an inhibition of oxidative metabolism in guinea-pig taenia coli (6). The latter mechanism was based on the evidence that monensin inhibited high K^+-induced contraction with the decreases in oxygen consumption and ATP contents (6). It has been thought that the PCr/creatine kinase system plays a role in the transport of high energy phosphates from the mitochondrial compartment to the sites of energy utilization, correlating with oxidative metabolism in mammalian smooth muscle (15, 31, 32). In the present experiments, monensin and NaCN decreased PCr and ATP contents below the control in the presence of H-65 K^+, significantly. On the other hand, Ozaki et al. (27) reported that the high K^+-induced contraction was accompanied by an increase in FPox fluorescence or PNred fluorescence in guinea-pig taenia coli. They suggested that the changes in FPox and PNred fluorescence represent mitochondrial respiration activity and glycolysis activity, respectively. In the present study, NaCN, an inhibitor of mitochondrial respiration, and monensin both inhibited significantly H-65 K^+-induced contraction and increase in FPox fluorescence. These data suggest that the inhibitory mechanisms of monensin on high K^+-induced contraction in the guinea-pig urinary bladder

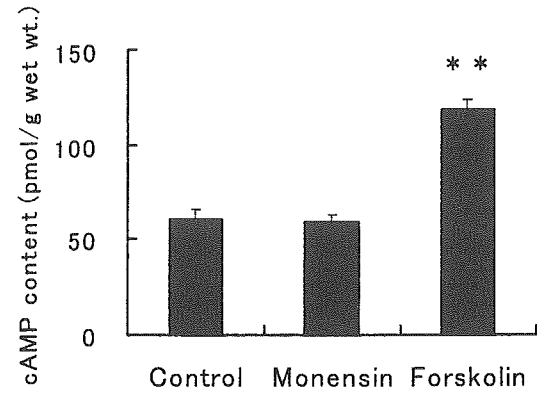

Fig. 5. Effects of monensin or forskolin on cAMP contents. Preparations were precontracted with H-65 K^- for 20 min and were treated with monensin or forskolin. Control was treated with H-65 K^- for 15 min. Monensin (0.1 μM) or forskolin (10 μM) was applied at 50 min or 20 min. Each point represents the mean of 4 experiments. Vertical bars indicate the S.E.M. **: Significant difference from each respective control with $P<0.01$.

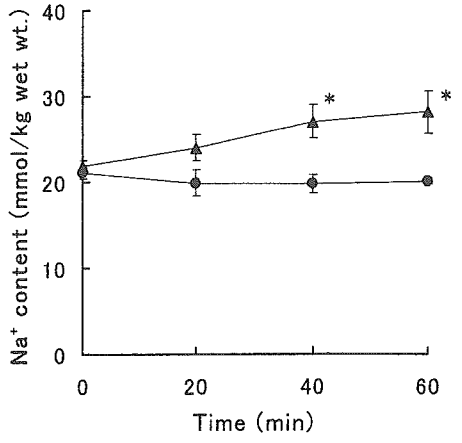

Fig. 7. Effects of monensin on Na^+ contents. Preparations were precontracted with H-65 K^+ for 15 min and were treated with 0.1 μM monensin (circle) or 10 μM (triangle). Each point represents the mean of 4 experiments. Vertical bars indicate the S.E.M. *: Significant difference from each time with $P<0.05$.

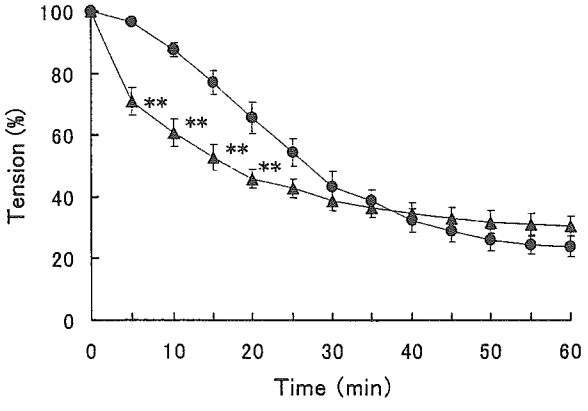

Fig. 6. Effects of monensin on the contraction induced by H-65 K^- or I-154 K^- in urinary bladder. Ordinate: relative tension. The contractions induced by H-65 K^- (circle) or I-154 K^- (triangle) just before the application of each agent were taken as 100%. Abscissa: time (min) of exposure to each agent. Each point represents the mean of 5 – 6 preparations. Vertical bars indicate the S.E.M. **: Significant difference from each time with $P<0.01$.

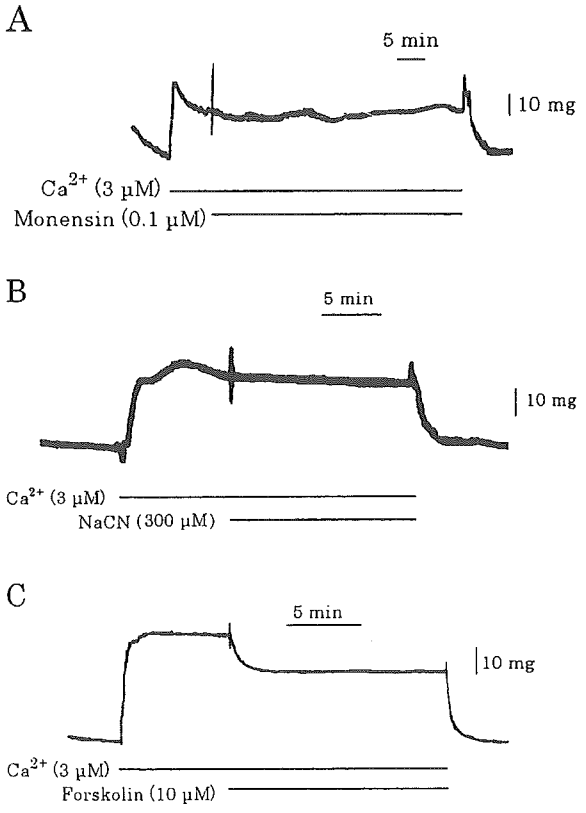

Fig. 8. Effects of monensin (A). NaCN (B), or forskolin (C) on the Ca^{2+}-induced contraction in α-toxin permeabilized muscle. Each trace of typical results from 3 – 4 experiments. The muscles were contracted by 3 μM Ca^{2+}. After the contraction reached plateau, monensin (A), NaCN (B), or forskolin (C) was added.

may be mainly by the inhibition of mitochondrial respiration. Recently, the urinary bladder isolated from the rat was reported to be fairly resistant to hypoxic conditions bubbled with nitrogen instead of oxygen (33). This result is different from our present data. There are some possible explanations for this difference. Presumably, the action of agents such as cyanide and monensin is somehow different from that of hypoxia, or the different species may bring about a different response. The energy metabolism related to the contractility of smooth muscle in urinary bladder remains to be clearly

elucidated.

In the present study, monensin, NaCN, and forskolin remarkably inhibited the high K^+-induced contraction without decreasing $[Ca^{2+}]_i$ level. There are two possible explanations in this phenomenon: 1) decreasing Ca^{2+} sensitivity of the contractile protein and 2) an inhibition of oxidative metabolism. It has been suggested that cAMP and cGMP-related agents inhibited high K^+-induced contraction by decreasing Ca^{2+} sensitivity of the contractile protein in some smooth muscles (3). In bovine trachea, it has been reported that forskolin increased cAMP content and inhibited high K^+-induced contraction by decreasing Ca^{2+} sensitivity of the contractile protein (34). In the present experiments, forskolin also increased cAMP content and inhibited Ca^{2+}-induced contraction in α-toxin permeabilized muscle of guinea-pig urinary bladder. However, monensin did not increase cAMP content (Fig. 5). Moreover, monensin and NaCN did not inhibit Ca^{2+}-induced contraction (Fig. 8). On the other hand, it has been shown that hypoxia and oxidative metabolic inhibition reduced high K^+-induced contraction without decreasing $[Ca^{2+}]_i$ level in some smooth muscles (35). It has been reported that cyanide inhibited high K^+-induced contraction without changing $[Ca^{2+}]_i$ level in guinea-pig ileum (36). These data indicate that monensin-induced relaxation is not due to decreasing Ca^{2+} sensitivity of the contractile protein but due to an inhibition of oxidative metabolism.

Kishimoto et al. (6) has shown that monensin relaxed remarkably the Na^+-deficient, high K^+-induced contraction and monensin (0.1 μM) induced a small increase in Na^+ content in normal solution in guinea-pig teania coli. They concluded that the action of monensin in the inhibition of high K^+-induced contraction of the guinea-pig taenia coli is not related to the change in intracellular Na^+ concentration. In this study, monensin (0.1 μM) was similarly potent to relax the H-65 K^+- and I-154 K^+-induced contraction in guinea-pig urinary bladder (Fig. 6). Furthermore, monensin increased Na^+ content at 10 μM but not at 0.1 μM, a concentration that induced maximum relaxation in guinea-pig urinary bladder (Fig. 7). These results suggest that the relaxation induced by a lower concentration of monensin in phasic muscles is not related to the change in intracellular Na^+ content.

In conclusion, it is suggested that monensin potently inhibited high K^+-induced contraction due to an inhibition of oxidative metabolism in the guinea-pig urinary bladder.

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