Effects of Field Stimulation on Cholinergic Fibers of the Pelvic Region in the Isolated Guinea Pig Ureter

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Abstract Electrical activities have been recorded in a preparation consisting of the pelvic region and the upper ureter of the guinea pig. Train field stimulation of the pelvic region evoked a train of nerve action potentials followed by a multiphasic smooth muscle action potential after a latency of about 2.5–8.0 sec. This smooth muscle response was abolished by tetrodotoxin and dibucaine, and also by cholinergic blocking agents. The response was, furthermore, inhibited by decreasing Ca^{2+} concentration and increasing Mg^{2+} concentration in Tyrode solution. This is therefore considered to be the response synaptically evoked by cholinergic fibers in the pelvic region.

Mechanical activities have been investigated on the same preparations by the Magnus method. Acetylcholine (10^{-7} – 10^{-4} g/ml) produced a group of twitch responses which were antagonized by cholinergic blocking agents. The responsiveness of the preparations to acetylcholine was markedly decreased by removal of the pelvic region.

Cholinesterase activities in the calyx-pelvis and upper ureter have been estimated by the biochemical method. A preparation consisting of calyx-pelvis exhibited twice the enzyme activity as compared with the upper ureter.

These results suggested that there is a cholinergic innervation in the pelvic region of the guinea pig ureter which also takes part in control of the ureteric activity.

Although many physiological and pharmacological studies on the ureteral function have been undertaken by various authors, the role of autonomic nervous system in the function of the ureter is not fully understood and controversial questions remain. Electron microscopic and histochemical studies showed the existence of cholinergic and adrenergic nerves along the entire length of the ureter (DUARTE-ESCALANTE *et al.*, 1969; ELBADAWI and SCHENK, 1969). On the other

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hand, the most prominent feature of the smooth muscle in the ureter was the abundance of close junctions between the muscle cells, and this was consistent with the findings of spike propagation demonstrated electrophysiologically and the absence of junction potentials (BOZLER, 1942b; BURNSTOCK and PROSSER, 1960; BENNETT *et al.*, 1962). Contraction waves of the ureter usually originate in the upper part of the urinary tract and are conducted towards vesicular end (BOZLER, 1942a; KOBAYASHI, 1965). Studies on the renal calyx and pelvis of a variety of species, including the guinea pig, have shown that the regions possess the structural characteristics which differ markedly from those observed in the ureter proper (GOSLING and DIXON, 1971; DIXON and GOSLING, 1971). These characteristics suggest the possibility that the calyx and pelvis may be specialized and possess additional or different functions from those of the ureter (TSUCHIDA *et al.*, 1977; MORITA *et al.*, 1978).

Recently, it has been demonstrated that the action potential originated from nervous elements was clearly recorded in the pelvic region and upper ureter of the guinea pig (YOSHIDA and KUGA, 1977). In the present study, using the pelvic region and upper ureter of the guinea pig, the relation between the neural action potential and the ureteric activity was investigated.

METHODS

Male guinea pigs weighing about 400–600 g were used for these experiments. After the animals were stunned and bled, the ureter was isolated together with the kidney. The renal parenchyma was removed under the dissection microscope, exposing the renal pelvis and calyx. The fat and connective tissues surrounding the ureter were removed. To exclude the spontaneous activity of the ureter, the calycinal region was removed. Accordingly, the preparation consisted of the renal pelvic region and upper ureter.

The experiments were carried out in an Acrylite chamber as shown in Fig. 1. Two pairs of electrodes were used in the chamber for stimulating and recording, and they were separated by an electrode for earth. These electrodes were of platinum wire of 0.4 mm diameter and fitted to an Acrylite plate placed on the top of the chamber. One side of the preparation was tied with silk thread and fastened to the end of Acrylite plate, while the other was connected with silk thread through a pulley to the transducer (Force-displacement transducer SB-1T-H, Nihon Kohden). The pelvic region of the preparation was always placed on the stimulating electrodes and the opposite side on the recording electrodes. Tension of about 50 mg was applied to the preparation. In the chamber, liquid paraffin was superimposed on Tyrode solution. The preparation was placed in liquid paraffin only when the action potential was recorded. The nerve and smooth muscle action potentials were evoked by the same stimulating electrodes and recorded by the same recording electrodes. To keep Tyrode solution and liquid paraffin at 37°C, the whole chamber was placed in a water bath controlled at $40 \pm 1^\circ\text{C}$.

Fig. 1. Diagram of the apparatus used for measurements of the smooth muscle and nerve action potential of the guinea pig ureter. Each electrode was separated by a distance of 2.3 mm. The earth was placed in the center of two pairs of electrodes. The chamber was placed in a $40 \pm 1^\circ\text{C}$ bath. For further explanation see text.

The normal Tyrode solution used in all experiments contained (mM): NaCl, 137.0; KCl, 2.7; CaCl_2 , 1.4; MgCl_2 , 1.1; NaH_2PO_4 , 0.4; NaHCO_3 , 12.0; glucose, 5.6. The pH of the solution was 7.5. The solution was gassed with pure oxygen. To study the effect of Ca^{2+} and Mg^{2+} concentrations on the initiation of the smooth muscle action potentials, the Ca^{2+} concentration was altered in a range between 0.17 and 1.36 mM, and the Mg concentration between 1.08 and 4.32 mM.

Stimulation was carried out with rectangular pulses from an electronic stimulator SEN-1101 with an isolator unit SS-101J (Nihon Kohden). Trains of 30–90 pulses were applied every 1 min, and the pulse strength was 1.5–5.0 V. The pulse frequency and width were kept constant throughout each experiment at 50 Hz and 0.5 msec, respectively. To get the response by direct stimulation of smooth muscle cells in the pelvic region, as an exception, stimulation consisting of 25.0 V or more and 10 pulses was also used. The nerve action potentials were displayed on an oscilloscope (VC-7 or VC-8, Nihon Kohden) through an AC-preamplifier and photographed by a trace-recording camera (UP-12, Iwatsu). Smooth muscle action potentials were recorded by a pen recorder.

The effect of acetylcholine on the pelvic region and upper ureter was examined using the Magnus method. The preparation isolated by the method described above was suspended in an organ bath (15 ml capacity) and attached to an isometric strain gauge (Force-displacement transducer SB-1T-H, Nihon Kohden) so as to record contractions of the pelvic region and upper ureter. Contractions were recorded on a pen recorder. The preparation was placed in Tyrode solution well gassed with pure oxygen, at 37°C , and given a resting tension of 50 mg. Following the equilibration for 60 min, drugs were added to the bath at the interval of 10 min.

The comparison of the cholinesterase activity in the calyx-pelvis and the upper ureter was performed by the following method; the preparations were divided into the calyx-pelvis and the upper ureter. Each preparation was rinsed in Tyrode solution, blotted and weighed. The tissues were homogenized with a glass homogenizer in 49-volume of cold Tyrode solution and homogenate was used as an enzyme source.

Assay of cholinesterase was as follows: cholinesterase activity was determined by the method of VOSS and SACHSSE (1970) with minor modification. The assay mixture contained 100 μ M of potassium phosphate buffer, pH 7.4, 1 μ M of acetylthiocholine or butyrylthiocholine and 0.1 ml of the enzyme on a final volume of 1.0 ml.; it was incubated at 37°C for 30 min. The enzyme reaction was stopped by the addition of 0.3 ml of 10% trichloroacetic acid solution and the mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was pipetted out and added to a mixture of 2 ml of DTNB solution* and 1 ml of 0.2 M potassium phosphate buffer, pH 8.0. The concentration of the DTNB-thiocholine complex was measured at 410 nm in a Hitachi 139 spectrophotometer.

Protein was estimated according to the method of LOWRY *et al.* (1951) using crystalline bovine serum albumin as standard.

RESULTS

The smooth muscle action potential with a prolonged latency

When a train of pulses was applied to the pelvic region, a multiphasic smooth muscle action potential recorded at a distance of 7 mm appeared with a prolonged latency (2.5–8.0), and a twitch tension was recorded simultaneously (Fig. 2). Because the response was recorded with external electrodes, it is difficult to define its shape, but the response had similar properties to those record in the smooth muscle of the guinea pig ureter by KURIYAMA *et al.*, (1967). It consists of the plateau potential and repetitive spikes superimposed on the plateau. As shown in Figs. 4 and 5, the repetitive spikes were also observed by the recording method used in this study, but they decreased in both number and amplitude with time. When number of stimulating pulses were varied between 30 and 90, the latencies

Fig. 2. The twitch tension (upper trace) and the smooth muscle action potential (lower trace) evoked by field stimulation consisting of 2.5 V, 50 Hz, 0.5 msec and 50 pulses.

* Fifty mg of 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in the mixture of equal volume of 0.2 M potassium phosphate buffer, pH 8.0 and 0.9% NaCl solution.

Fig. 3. Relationship between number of pulses and latencies in the smooth muscle response. The preparation was stimulated by train of 30–90 pulses. The other stimulus parameters were kept constant at 2.5 V, 50 Hz, and 0.5 msec. Abscissa, number of pulses; ordinate, latencies (sec).

between the beginning of stimulation and the smooth muscle response decreased with increasing the number of pulses (Fig. 3).

Relation between the nerve and smooth muscle action potential

PROSSER *et al.* (1955) recorded neural action potential in the ureter of the rat and termed it “prespike,” but they did not make its function clear. In the previous report we described experiments where, in the pelvic region and upper ureter of the guinea pig, action potentials originated from nerve elements could be recorded and the physiological properties of the nerve were investigated (YOSHIDA and KUGA, 1977). In the present experiments the relation between the nerve and smooth muscle action potential was investigated. When a train of stimuli consisting of 50 pulses of 1.5–5.0 V was applied to a preparation every 1 min, a train of nerve action potentials were observed and the smooth muscle action potential followed after the prolonged latency (Fig. 4A). MnCl_2 (2×10^{-3} g/ml) abolished the smooth muscle action potential while leaving the nerve action potentials unaffected (Fig. 4B). When tetrodotoxin (5×10^{-7} g/ml) was added to Tyrode solution after washing out MnCl_2 , both the nerve and smooth muscle action potentials were abolished (Fig. 4D). These results indicate that the mechanism of the action of the two agents are quite different; since Mn^{2+} is believed to suppress the generation of Ca-dependent action potentials, the inhibition by MnCl_2 is probably a direct action on the smooth muscle cells; since tetrodotoxin generally blocks the nerve excitation but not the smooth muscle excitation, the inhibition by tetrodotoxin is likely to be an indirect action on the smooth muscle.

Fig. 4. Effects of Mn^{2+} and tetrodotoxin on the evoked nerve and smooth muscle action potentials. Left hand traces show nerve action potentials on an oscilloscope and right hand traces show smooth muscle action potentials by a pen recorder. The nerve action potentials show the initial five responses out of a train of fifty responses. A, control; B, in the presence of $MnCl_2$ (2×10^{-3} g/ml); C, after washing out $MnCl_2$; D, in the presence of tetrodotoxin (5×10^{-7} g/ml); E, after washing out tetrodotoxin. Trains of pulses were applied at the bars under the pen recordings.

Effects of various blocking agents

The smooth muscle action potential was abolished by cholinergic blocking agents, atropine and hyoscine (5×10^{-7} g/ml, Fig. 5B), but not affected by either α - or β -blocking agents, such as phentolamine, phenoxybenzamine, dichloroisoproterenol and propranolol, or ganglion blocking agents, such as tetraethylammonium and hexamethonium.

Effects of Ca^{2+} and Mg^{2+} concentration on the initiation of the smooth muscle action potential

It is well known that Ca^{2+} and Mg^{2+} seriously affect transmitter release at the neuromuscular junction. Decreasing the Ca^{2+} concentration and increasing the Mg^{2+} concentration have been found to decrease greatly the amount of transmitter released by stimulation not only in skeletal muscle (DEL CASTILLO and ENGBAER, 1954) but also in smooth muscle (KURIYAMA, 1964). As described previously, many pulses were required to excite smooth muscle cells of the pelvic region by a train stimulation. The number of pulses necessary to produce the smooth muscle action potential varied from preparation to preparation. The initiation of the smooth muscle action potential was tested in each concentration

Fig. 5. Effect of hyoscine on the evoked nerve and smooth muscle action potentials. The traces are the same as those in Fig. 4. A, control; B, in the presence of hyoscine (5×10^{-7} g/ml); C, after washing out.

of Ca^{2+} and Mg^{2+} by varying the number of pulses at a constant voltage.

Either decreasing the Ca^{2+} concentration or increasing the Mg^{2+} concentration in the medium increased number of pulses were required to evoke the action potential in the smooth muscle, indicating that the generation of action potentials was suppressed. At the concentration of 0.17 mM CaCl_2 or 4.32 mM MgCl_2 the action potentials were abolished leaving only the responses by direct stimulation of the smooth muscle (Table 1). When Ca^{2+} and Mg^{2+} concentration of Tyrode solution were altered simultaneously to half the normal $[\text{Ca}^{2+}]_o$ * and to twice the normal $[\text{Mg}^{2+}]_o$,* the response was completely abolished. By placing the preparation to normal solution the action potential recovered immediately.

Effects of acetylcholine to the pelvic region and upper ureter

The smooth muscle action potential with the prolonged latency is evoked only by repetitive stimulation of the pelvic region at a relatively low voltage. Since the action potential of smooth muscle is always associated with a twitch tension (KIL and KJEKSHUS, 1966; Fig. 2), the effect of acetylcholine on the twitch tension of the pelvic region and upper ureter was examined by the Magnus method.

At first, the responsiveness to acetylcholine was compared with and without the pelvic region. In the preparation with the pelvic region 10^{-7} g/ml acetylcholine evoked twitch responses and as the acetylcholine concentrations increased from 10^{-7} to 10^{-4} g/ml, the number of twitch responses increased gradually

* $[\text{Ca}^{2+}]_o$ and $[\text{Mg}^{2+}]_o$ exhibit the Ca^{2+} and Mg^{2+} concentration in Tyrode solution, respectively.

Table 1. Effects of Ca^{2+} and Mg^{2+} concentration on the initiation of the smooth muscle action potentials. Each effect of them was tested by separate experiments.

Stimulation		Ca concentration (mm)				Mg concentration (mm)			
V	No. of pulses	1.36	0.98	0.34	0.17	1.08	2.16	3.24	4.32
2.5	30	+	—	—	—	+	—	—	—
"	40	+	+	—	—	+	+	—	—
"	50	+	+	—	—	+	+	—	—
"	60	+	+	+	—	+	+	—	—
"	70		+	+	—		+	—	—
"	80			+	—			+	—
"	90			+	—			+	—
25.0	10	+	+	+	+	+	+	+	+

The lowest line shows the direct stimulation of the smooth muscle as a control.

The appearance and the absence of response by stimulation consisting of 2.5 V, 30–90 pulses, and 25.0 V, 10 pulses (the lowest line) are expressed as +, and —, respectively.

1.36 mm of Ca^{2+} concentration and 1.08 mm of Mg^{2+} concentration are normal in Tyrode solution.

(Fig. 6A–D). By removal of the pelvic region, the twitch responses to the concentration up to 10^{-5} g/ml acetylcholine disappeared, and only 10^{-4} g/ml acetylcholine evoked twitch responses (Fig. 6D). Thus the sensitivity of the pelvic region to acetylcholine is much higher as compared with the upper ureter. The

Fig. 6. Effects of acetylcholine on the pelvic region and upper ureter. Upper and lower traces exhibit the responses in the preparations with pelvic region and without pelvic region, respectively. Applied acetylcholine concentrations in the bath are expressed as g/ml.

twitch responses to acetylcholine in both preparations were antagonized by cholinergic blocking agents (not shown).

Comparison of the cholinesterase activity of the calyx-pelvis and upper ureter

Because the sensitivity of the pelvic region to acetylcholine is much higher as compared with the upper ureter, it is possible that the cholinesterase activity in the pelvic region is higher than in the upper ureter. Therefore, the cholinesterase activity in the calyx-pelvis and the upper ureter was measured by the biochemical method.

As shown in Table 2, although the whole tissue of calyx-pelvis and upper ureter were used for measuring the enzyme activity, the preparation containing the calyx-pelvis exhibited two times enzyme activity as compared with the upper ureter. This supports that the sensitivity to acetylcholine in the pelvic region is much higher than in the upper ureter.

DISCUSSION

When extracellular field stimulation was applied to the pelvic region, smooth muscle action potentials with prolonged latencies could be recorded. Since this response disappeared by suppressing the nerve action potentials, it is considered that there may be some synaptic correlation between the nervous elements and the smooth muscle in the pelvic region. It is worth noting that, as compared with the other part of the ureter, in the pelvic region the smooth muscle action potential can be evoked by the stimulation of much lower voltage, which accorded with rheobase (about 1.5 V) obtained in the strength-duration relationship for evoking the action potential in the nerve elements (YOSHIDA and KUGA, 1977). In addition, substances such as tetrodotoxin (Fig. 4) and dibucaine which block nerve excitation, as well as cholinergic blocking agents, for example, hyoscine (Fig. 5) and atropine, abolished the smooth muscle action potential. These facts suggest that the response may be evoked by nerve action potentials which are due to excitation of cholinergic nerve fibers in the pelvic region.

As shown in Fig. 3 the latencies of the responses shortened when the number of pulses per train was increased. In this case, the conduction velocity of excitation of the smooth muscle in the ureteral part was not altered at all (not shown). Therefore, this may indicate that the pelvic region is responsible for the extremely

Table 2. Comparison of the cholinesterase activity in the calyx-pelvis and upper ureter.

	Acetylcholinesterase	Butyrylcholinesterase
Calyx-pelvis	2.04 ± 0.11 (5)	1.04 ± 0.18 (5)
Upper ureter	1.12 ± 0.10 (5)	0.67 ± 0.08 (5)

Enzyme activities were expressed as μM thiocholine/mg of protein/30 min. The numbers in parentheses denote the number of ureters examined.

prolonged latency. KOSTERLITZ and LYDON (1971) reported in the studies on the impulse transmission in the myenteric plexus-longitudinal muscle preparation of the guinea pig ileum that the response of the smooth muscle increased in amplitude and decreased in latency when, instead of a single pulse, train of pulses were used as stimulus. As a possible explanation they suggested that the amount of acetylcholine released from the myenteric plexus was increased by a train stimulus. The increased amount of acetylcholine will excite more muscle cells and increase the diameter of functional bundle (BURNSTOCK and PROSSER, 1960). If the same relationship holds for the experiments described in this paper, the increased amount of acetylcholine released by many pulses will shorten the latency. The response with the prolonged latency has a different nature from that to direct stimulation, because the latency of response to direct stimulation remained the same while changing the number of pulses for stimulation. However, the mechanism for the prolonged latency remains unknown (YOSHIDA and KUGA, 1977).

The effects of the Ca^{2+} and Mg^{2+} concentration of the initiation of the smooth muscle action potential was likely to be similar to those reported for the neuromuscular transmission of the skeletal muscle and other smooth muscles. Although it is known in the taenia coli and the vas deferens that Ca^{2+} deficiency depolarizes the membrane and excess Mg^{2+} hyperpolarizes it (BÜLBRING and KURIYAMA, 1963; KURIYAMA, 1964), this may not be the cause for inhibiting the smooth muscle action potential in the present study, because the change of the Ca^{2+} and Mg^{2+} concentration used are totally minor. This is obvious from the fact that the response to direct stimulation appeared under these condition. Therefore, the results shown in Table 1 suggest the possibility that the variation in the Ca^{2+} and Mg^{2+} concentration interferes with the release of the transmitter from nerve endings.

The effect of acetylcholine on the pelvic region and upper ureter provided evidence that the density of acetylcholine receptors in the pelvic region is higher than in the upper ureter. FINBERG and PEART (1970), in the experiments recording the contraction of circular muscle in the isolated pelvis, found that acetylcholine was without effect, suggesting that the acetylcholine receptor does not exist in the circular muscle of the pelvis. The fact indicates that acetylcholine receptors may exist in the longitudinal muscles, because the preparations consisting of longitudinal and circular muscles used in the present experiments respond to acetylcholine evidently. A marked decline of the sensitivity to acetylcholine by removal of the pelvic region is compatible with the fact that there is relatively rich innervation on a specific region of smooth muscles in the pelvic region (DIXON and GOSLING, 1971). ELBADAWI and SCHENK (1969) and DUARTE-ESCALANTE *et al.* (1969) showed the existence of cholinergic and adrenergic nerves along the entire length of the ureter. The latter group also pointed out that a type of concentrated neuron-like or ganglion cell has been found at the lower part of the renal pelvis and that the cholinergic innervation is better developed than the

adrenergic one at the ureteropelvic junction. These facts consist with the present results that the pelvic region is different from the other part of the ureter in sensitivity to acetylcholine and that, in spite of the existence of cholinergic nerves in the upper ureter, receptors reacting to acetylcholine are extremely few as compared to the pelvic region. Accordingly, the observations in the Magnus method are consistent with findings in the intact dog by KIL and KJEKSHUS (1966) and finding on the existence of cholinergic receptors and of cholinergic fibers in the pelvic region, as well as the higher cholinesterase activity in the calyx-pelvis.

There are several reports indicating that ureteric innervation is sympathetic (BOYARSKY *et al.*, 1967; KAPLAN *et al.*, 1968). However, many histochemical and electron microscopic studies showed the existence of cholinergic nerve fibers as well as adrenergic ones (DUARTE-ESCALANTE *et al.*, 1969; ELBADAWI and SCHENK, 1969; DIXON and GOSLING, 1971; GOSLING and DIXON, 1971; SCHULMAN, 1976). The present results suggest the possibility that the cholinergic system also takes part in the pelvic innervation, and support the observations of histochemical and electron microscopic studies. It is considered that the ureteric activity is controlled by the specific region consisting of the calyx and pelvis, the activity of which may be regulated by the cholinergic nervous system.

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