Electrical effects of okadaic acid extracted from black sponge on rabbit sinus node

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- 1 Effects of okadaic acid on electrical responses and spontaneous activity in the dominant pacemaker cells of rabbit sinus node were investigated by use of microelectrode techniques.
- 2 Okadaic acid (10^{-5} M to 4×10^{-5} M) caused a shortening of cycle length of spontaneous firing (SPCL) accompanied by increases in both maximum upstroke velocity at phase 0 ($\dot{V}_{\max}$) and amplitude of action potential.
- 3 All of the effects of okadaic acid were relatively well preserved in a low- Ca^{2+} medium (0.12 mM). Okadaic acid restored the spontaneous activity of sinus node pacemaker cells even in a Ca^{2+} -deficient medium.
- 4 The effects of okadaic acid were markedly inhibited or abolished in a low Na^+ medium (24 mM or 70 mM) and in the presence of a slow channel blocking agent, verapamil (10^{-6} M).
- 5 In voltage-clamp experiments using a two-microelectrode technique, okadaic acid (10^{-5} M) caused an increase in the slow inward current without affecting the outward current. At a higher concentration (4×10^{-5} M), the drug increased the outward current.
- 6 These results indicate that okadaic acid causes an increase in spontaneous activity of sinus node pacemaker cells mediated by an enhancement of slow inward current (I_{si}) through verapamil-sensitive Ca^{2+} channels.

Introduction

Okadaic acid is a monocarboxylic acid ($\text{C}_{44}\text{H}_{66}\text{O}_{13}$) isolated from a common black sponge, *Halichondria* (Tachibana *et al.*, 1981). The molecular structure of this substance is reminiscent of the Ca^{2+} ionophores A-23187 and X-537A but its pharmacological properties are markedly different (Shibata *et al.*, 1982). Okadaic acid causes tonic and long-lasting contraction of smooth muscle even in the absence of Ca^{2+} (Shibata *et al.*, 1982; Shibata, 1987). In saponin-treated 'skinned' smooth muscle in the absence of Ca^{2+} (Ozaki *et al.*, 1987b), this agent causes a contraction. Recently, such a contraction has been attributed to myosin light-chain phosphorylation (Ozaki *et al.*, 1987; Bialojan *et al.*, 1988; Erdodi *et al.*, 1988) mediated through inhibition of myosin phosphatase activity by okadaic acid (Bialojan *et al.*, 1988; Erdodi *et al.*, 1988). In cardiac muscle, okadaic acid also causes an increase in the contractile force induced by electrical stimuli (Kodama *et al.*, 1986). This positive inotropic effect, unlike the case of vascular smooth muscle, was blocked by Ca^{2+} channel blockers and reducing $[\text{Ca}]_0$. Our previous study on guinea-pig isolated ventricular muscle (Kodama *et al.*, 1986) further suggested that this effect may be mediated by an enhancement of slow Ca^{2+} inward current (I_{si}) through the cell membrane. A similar effect of I_{si} was reported in isolated myocytes from guinea-pig cardiac ventricle (Hescheler *et al.*, 1988). This study suggested that the effect of okadaic acid on I_{si} is related to suppressed dephosphorylation through inhibition of protein phosphatase activity by this agent. This may result in facilitation of Ca^{2+} channel activation through an increase in phosphorylated Ca^{2+} channels. Thus, okadaic acid is a unique substance that exerts a cardiotonic effect probably through the inhibition of the protein phosphatases. In the present study, in order to evaluate the effectiveness of okadaic acid on spontaneous activity of the sinus node cell, we examined its action on electrical

responses of sinus node pacemaker cells where I_{si} plays an important role in the genesis of spontaneous activity and the action potential.

Methods

Thirty five rabbits of either sex, weighing 1.5 to 2.0 kg, were anaesthetized by sodium pentobarbitone (30 mg kg^{-1} , i.v.). The hearts were excised rapidly and placed in oxygenated Krebs-Ringer solution to obtain small specimens of sinus node pacemaker tissue. Since the procedure has been given in detail in a previous paper (Kodama & Boyett, 1985), it is described here only briefly. The right atrium was separated from the remaining part of the heart and was opened by a longitudinal incision in the free wall to expose the endocardial surface. After the sinus node had been identified in the centre of the intercaval region, several strands of tissue (0.2–0.4 mm in width, 2 mm in length) were cut from the sinus node in a direction perpendicular to the crista-terminalis. They were trimmed with a razor blade leaving the endocardial surface intact, and tied by a fine silk thread to obtain small ball-like specimens composed of dominant pacemaker cells. The final size of these specimens was about 0.3 mm in diameter. The distance from the right branch of the sinoatrial ring bundle to the centre of the specimen was 1.0 to 1.5 mm. These specimens usually resumed their spontaneous activity 10–30 min after the ligatures were tied. Once the dissection procedure was complete, the specimens were transferred to another tissue bath, which was also superfused with Krebs-Ringer solution gassed with a mixture of 95% O_2 and 5% CO_2 at 30°C . The rate of solution flow was 5 ml min^{-1} . The composition of the solution was as follows (mM): NaCl 120.3, KCl 4.8, CaCl_2 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.3, KH_2PO_4 1.2, NaHCO_3 24.2 and glucose 5.5 (pH 7.4). In the low- Ca^{2+} solution, the CaCl_2 concentration was reduced to 0.12 mM, and in the Ca^{2+} -deficient solution the CaCl_2 was omitted. In the low- Na^+ solution, the Na^+ concentration was reduced to 70 mM or 24 mM by replacing NaCl with choline chloride or with lithium chloride, respectively.

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When choline chloride was used as a substitute, atropine sulphate (10^{-6} M) was added to the solution to eliminate cholinergic side-effects.

Transmembrane potentials were recorded with two glass microelectrodes filled with 3 M KCl, one intracellularly and the other extracellularly placed closely. Each electrode was connected by an Ag-AgCl wire to a high-input impedance buffer amplifier connected to a differential amplifier. The action potential was differentiated electronically in order to measure the maximum upstroke velocity ($\dot{V}_{max}$). Cycle length of spontaneous firing (SPCL) was determined by measuring the interval between two successive spikes of the first derivative of action potentials.

The preparations were allowed to equilibrate in the tissue bath for about 1 h before any tests were performed. Sixteen out of 43 ball-like specimens (37%) showed unstable or no spontaneous activity during this equilibration period and they were omitted. The remaining 27 specimens with regular and stable spontaneous activity were used for the present experiments.

In the voltage clamp experiments, a two-microelectrode voltage clamp technique was employed. Two conventional glass microelectrodes filled with 3 M KCl ($10\text{--}30\text{ M}\Omega$) were used, one to record the membrane potential and the other to apply the current intracellularly. The membrane potential and current were displayed on an oscilloscope (Kikusui Denshi, 5516 ST), and recorded on cassette tape recorder (TEAC, MR-10) and with a penrecorder (Nihon Kohden, RJG-4100). The principle of the feedback circuit was the same as that reported by Noma *et al.* (1980) and a virtual ground amplifier, essentially the same as that described by New & Trautwein (1972), was used. When both microelectrodes recorded identical action potentials, the preparation was clamped to -40 mV (holding potential) where the holding current was nearly zero (Noma *et al.*, 1979).

The following drugs were used: okadaic acid (Fujisawa, Tokyo, Japan); verapamil (Knoll, Whippany, West Germany); tetrodotoxin (TTX, Sankyo Tokyo, Japan). The preparations were superfused for 20–40 min with Krebs-Ringer solution containing these drugs. The okadaic acid was dissolved in absolute ethanol, and then diluted with deionized water. The

maximum concentration of ethanol in the superfusate was 0.4%.

Values were expressed as the mean $\pm$ s.e. unless otherwise stated. Statistical significance was evaluated by a paired *t* test, and *P* values of less than 0.05 were considered to indicate a significant difference. More details of each procedure are given in the Results.

Results

Transmembrane action potential under control conditions

The observed transmembrane potentials always showed a smooth transition from phase 4 to phase 0 (Figure 1). Furthermore, there were no changes in the shape of the action potential that were suggestive of a shift of a pacemaker site within the small specimens. These observations suggest that there was a nearly uniform distribution of transmembrane potential within the specimens. This is to be expected because the diameter (about 0.3 mm) of the small specimens was less than the space constant (0.5–0.8 mm) of this tissue (Bonke, 1973; Seyama, 1976). The amplitude of the action potential (APA), maximum diastolic potential (MDP), $\dot{V}_{max}$ and SPCL under control conditions are presented in Tables 1 to 3. These values are similar to those recorded from the leading pacemaker site of the intact rabbit sinus node (Bleeker *et al.*, 1980; Kodama & Boyett, 1985).

Treatment of the preparations with TTX (10^{-6} M) for 30 min did not affect the shape of action potential (not shown in Figures or Tables). This may mean that the fast sodium inward current (I_{Na}) does not contribute to the action potentials of these preparations.

Effects of okadaic acid

Effects of okadaic acid on action potentials were examined in five preparations bathed in the normal medium. Okadaic acid at 10^{-5} M caused a shortening of SPCL (increase in firing rate) and an increase in $\dot{V}_{max}$ (Figure 1, Table 1), but had no effect on either the amplitude of the action potential (APA) or the maximum diastolic potential (MDP). Okadaic acid at a higher

Figure 1 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells. (a) Control; (b) 10^{-5} M okadaic acid; (c) 4×10^{-5} M okadaic acid. Panels (b) and (c) were recorded 20 min after application of okadaic acid. The upper trace represents the action potential and the lower trace shows the first derivative of the action potential.

Table 1 Effects of okadaic acid on the membrane action potentials of sinus node cells

	APA (mV)	MDP (mV)	$\dot{V}_{max}$ (Vs ⁻¹)	SPCL (ms)
Control	83.6 $\pm$ 1.8	69.1 $\pm$ 1.0	8.1 $\pm$ 0.6	462 $\pm$ 18
Okadaic acid (10^{-5} M)	87.1 $\pm$ 2.7	69.3 $\pm$ 0.8	11.2 $\pm$ 1.0*	378 $\pm$ 19*
Okadaic acid (4×10^{-5} M)	93.5 $\pm$ 3.0*	70.6 $\pm$ 0.6	13.9 $\pm$ 1.9*	324 $\pm$ 5*

Values are means $\pm$ s.e. (*n* = 5) before and 20 min after application of okadaic acid; APA: action potential amplitude; MDP: maximum diastolic potential; $\dot{V}_{max}$: maximum upstroke velocity at phase 0; SPCL: spontaneous firing cycle length. *Significantly different from the values of control at *P* < 0.05.

concentration (4×10^{-5} M) caused a significant increase in APA, but had no effect on MDP.

The onset of action of okadaic acid was approximately 6 min after application of the substance and the maximum effect was observed with 20 min. After washing out the drug, all the parameters of the action potential gradually returned to the control levels within 30 min.

Effects of the vehicle (ethanol) were examined in three preparations. The highest concentration of ethanol used in the present study (0.4%) had no effect on either the action potential or spontaneous activity.

Low-calcium medium

In eight preparations, the effects of okadaic acid were examined in either a low Ca^{2+} or a Ca^{2+} -deficient medium. When the preparations were superfused with Krebs-Ringer solution containing 0.12 mM Ca^{2+} for 20 min, APA and $\dot{V}_{\max}$ were progressively decreased, resulting in the complete cessation of spontaneous activity at the resting membrane potential (-44.4 ± 4.0 mV, $n = 4$). In such preparations, the treatment with okadaic acid (4×10^{-5} M) restored regular spontaneous action potentials. APA, MDP and $\dot{V}_{\max}$ of these action potentials were all considerably less than those observed under the control conditions, but SPCL was comparable to the control value (Figure 2, Table 2). Similar restoration of spontaneous activity by okadaic acid, but with much smaller APA and

$\dot{V}_{\max}$, was observed in the Ca^{2+} -deficient medium (Figure 2, Table 2).

Low-sodium medium

In the other eight preparations, the effects of okadaic acid were tested in the presence of low concentrations of Na^+ . When the preparations were superfused with Krebs-Ringer solution containing 70 mM Na^+ for 20 min, APA, MDP and $\dot{V}_{\max}$ were decreased and SPCL was prolonged significantly. In such preparations okadaic acid (4×10^{-5} M) caused a shortening of SPCL, but the other parameters of the action potential (MDP, APA, and $\dot{V}_{\max}$) were not significantly affected (Figure 3, Table 3).

When the preparations were exposed to Krebs-Ringer solution containing 24 mM Na^+ , APA and $\dot{V}_{\max}$ were progressively decreased, resulting in complete cessation of spontaneous activity at the resting potential (-35.6 ± 3.1 mV, $n = 4$). In such preparations, okadaic acid (4×10^{-5} M) failed to restore the spontaneous activity (Figure 3, Table 3). The resting potential was also unaffected by this substance.

Influence of verapamil

Effects of okadaic acid were examined in four preparations pretreated with a cardiac slow-channel blocker, verapamil.

Figure 2 Effects of okadaic acid (OA) on the membrane action potential of sinus node pacemaker cells in low- Ca^{2+} and Ca^{2+} -free medium. Upper and lower panels show experiments in a low- Ca^{2+} (0.12 mM) and Ca^{2+} -free medium respectively. (a) Control; (b) 20 min after exposure to the low- Ca^{2+} or the Ca^{2+} -free medium; (c) 20 min after application of okadaic acid (4×10^{-5} M) in either low- Ca^{2+} or Ca^{2+} -free medium.

Table 2 Effects of okadaic acid (4×10^{-5} M) on the sinus node cell in low calcium and calcium-free medium

	APA (mV)	$\dot{V}_{\max}$ (Vs^{-1})	SPCL (ms)
Control	85.5 ± 6.0	10.5 ± 1.9	384 ± 31
Low Ca^{2+} medium (0.12 mM)	0	0	—
Low Ca^{2+} medium + okadaic acid	$60.8 \pm 4.3^*$	$2.2 \pm 0.1^*$	$433 \pm 51^*$
Control	84.0 ± 5.6	10.3 ± 1.5	376 ± 18
Ca^{2+} -free medium	0	0	—
Ca^{2+} -free medium + okadaic acid	$11.1 \pm 2.2^*$	$0.3 \pm 0.1^*$	$411 \pm 33^*$

Values are means $\pm$ s.e. ($n = 4$). The preparations were exposed to low calcium and calcium-free medium for 20 min and okadaic acid (4×10^{-5} M) was further applied for 20 min.

* Significantly different from the values before application of okadaic acid at $P < 0.05$.

Abbreviations are the same as in Table 1.

Figure 3 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells in a low Na^+ medium (70 and 24 mM). Upper and lower panels show experiments in medium containing 70 and 24 mM Na^+ respectively. (a) Control; (b) 20 min after exposure to a low Na^+ medium; (c) 20 min after application of okadaic acid (4×10^{-5} M) in a low Na^+ medium.

Representative experiments are shown in Figure 4. The 30 min pretreatment with verapamil (10^{-6} M) resulted in a cessation of spontaneous activity at the resting potential (-37.8 ± 1.1 mV, $n = 4$). In these preparations, okadaic acid (4×10^{-5} M) failed to restore spontaneous activity.

Influence of propranolol and atropine

Effects of okadaic acid were also examined in preparations pretreated with propranolol (10^{-6} M), a β -adrenoceptor

antagonist, and atropine (10^{-6} M), a acetylcholine receptor antagonist. Neither atropine nor propranolol had any effect on electrical responses to okadaic acid (data not shown).

Membrane currents

Effects of okadaic acid on the slow inward current and on the outward currents were investigated in four preparations by a two-microelectrode voltage clamp technique. Representative experiments are shown in Figure 5. A depolarization step

Table 3 Effects of okadaic acid (4×10^{-5} M) on the sinus node cells in low sodium medium

	APA (mV)	$\dot{V}_{\max}$ (Vs^{-1})	SPCL (ms)
Control	81.6 ± 5.8	7.7 ± 2.2	411 ± 43
Low (70 mM) Na^+ medium	48.4 ± 6.4	1.2 ± 0.3	800 ± 69
Low (70 mM) Na^+ medium + okadaic acid	50.4 ± 7.6	1.4 ± 0.3	$504 \pm 27^*$
Control	83.3 ± 2.1	7.2 ± 0.6	489 ± 44
Low (24 mM) Na^+ medium	0	0	—
Low (24 mM) Na^+ medium + okadaic acid	0	0	—

Values are means $\pm$ s.e. ($n = 4$). The preparations were exposed to low sodium (70 and 24 mM) medium for 20 min and okadaic acid (4×10^{-5} M) was further applied for 20 min.

* Significantly different from the values before application of okadaic acid at $P < 0.05$.

Abbreviations are the same as in Table 1.

Figure 4 Effects of okadaic acid (OA) on the action potential of sinus node pacemaker cells pretreated with verapamil. (a) Control; (b) 30 min after application of verapamil (10^{-6} M); (c) 20 min after application of okadaic acid (4×10^{-5} M).

Figure 5 Effects of okadaic acid (OA) on membrane currents of sinus node pacemaker cells. Right panel: voltage clamp traces during depolarization (from -40 mV to -10 mV). Left panel: current-voltage relation for I_{si} (open symbols) and I_{out} at the end of depolarization step (closed symbols) in the absence (circles) and the presence of okadaic acid at 10^{-5} M (triangles) and 4×10^{-5} M (squares).

from the holding potential of -40 mV to -10 mV elicited a slow inward current (I_{si}) followed by a gradually increasing outward current (I_{out}). When the membrane potential was returned to -40 mV, an outward tail current (I_{tail}) was recorded. The amplitude of I_{si} was estimated by taking the difference between the inward peak of I_{si} and the current level at 200 ms after the onset of the depolarization step. The amplitude of I_{out} was estimated by the shift by the steady-state current level at the holding potential. After treatment with okadaic acid (10^{-5} M) the amplitude of I_{si} was significantly increased ($117 \pm 3.0\%$ of control, $n = 4$), while both I_{out} at the end of the depolarization step and I_{tail} were unaffected. At a higher concentration of the drug (4×10^{-5} M), I_{si} was further increased ($141 \pm 5.6\%$ of control, $n = 4$) and both I_{out} and I_{tail} were enhanced.

Discussion

In the present study small ball-like specimens of sinus node tissue were used to investigate the effects of okadaic acid. These preparations are considered to be composed of dominant pacemaker cells of the sinus node because the specimens were obtained from a restricted area of sinus node: the centre of the intercaval region, 1.0 to 1.5 mm away from the right branch of the sinoatrial ring bundle on the medial border of the crista terminalis. Previous reports have shown that the leading (or dominant) pacemaker sites of rabbit sinus node are located in this area (Bleeker *et al.*, 1980; Kodama & Boyett, 1985). It has also been previously found that most of the electrophysiological properties of the pacemaker cells of the intact sinus node are well preserved in such very small specimens (Kodama & Boyett, 1985). TTX (10^{-6} M) had no effect on either the transmembrane action potential or the spontaneous firing rate of the preparations, both of which indicate little or no contribution of the fast sodium inward current (I_{Na}) to the electrical activity of the preparations. TTX has been shown to have little effect on dominant pacemaker cells (Strauss *et al.*, 1977; Irisawa, 1978).

Okadaic acid at concentrations above 10^{-5} M caused a significant shortening of cycle length of spontaneous firing (SPCL) and an increase in both V_{max} and amplitude of action potentials (APA). It is well known that the upstroke phase of the action potentials of the dominant pacemaker cells of mammalian sinus nodes is generated by an activation of slow inward current (I_{si}). I_{si} has also been shown to play an impor-

tant role in the development of pacemaker depolarization (diastolic depolarization) especially in its later phase (Noma *et al.*, 1980; Yanagihara *et al.*, 1980; Noble, 1984). In the present experiments, the spontaneous activity of the preparations was completely suppressed by a slow-channel blocking agent, verapamil (10^{-6} M), and okadaic acid failed to overcome this inhibitory effect of verapamil. Voltage clamp data also revealed that okadaic acid markedly augmented I_{si} . These facts suggest that the effect of okadaic acid on sinus node pacemaker cells is mediated by the enhancement of I_{si} by the agent.

The effects of okadaic acid on the sinus node action potential were markedly attenuated or nearly abolished in a low- Na^+ medium, whereas they are relatively well preserved in a low- Ca^{2+} medium. Okadaic acid restored spontaneous activity of sinus node cells even in a Ca^{2+} -deficient medium. These results can be explained by the mechanism through Na^+ - Ca^{2+} exchange. In a low Na^+ medium, the intracellular Ca^{2+} concentration may have been increased to the inhibition of Na^+ - Ca^{2+} exchange mechanism (Mullins, 1981). Intracellular Ca^{2+} accumulation would lower the Ca^{2+} concentration gradient across the cell membrane, and secondarily induce various changes in membrane properties, such as an increase in K^+ conductance (Isenberg, 1977), or Ca^{2+} -induced inactivation of the slow channel (Tsien, 1983). These secondary changes may inhibit or counteract I_{si} carried by Ca^{2+} . Thus, lowering $[Na]_o$ may inhibit slow inward current across cell membrane. The involvement of the ion selectivity of the slow channel may also be considered. Noma & Irisawa (1976) and Noma *et al.* (1977) suggested that the slow inward current of the sinus node cell is carried by Na^+ as well as by Ca^{2+} . Okadaic acid may increase I_{si} carried not only by Ca^{2+} but also by Na^+ . In either the low- Ca^{2+} or the Ca^{2+} -deficient medium, therefore, okadaic acid can increase I_{si} carried by Na^+ , because the normal Na^+ concentration gradient across the cell membrane may have been maintained under these conditions. In a low Na^+ medium, the decreased gradient of Na^+ concentration may reduce the contribution of Na^+ to I_{si} . However, since a recent study in bull-frog atrial cells (Campbell *et al.*, 1988) has suggested that I_{Ca} is carried almost exclusively by Ca^{2+} ions, it seems more likely that the present effect of low $[Na]_o$ is mediated through a Na^+ - Ca^{2+} exchange mechanism. In our previous experiments with guinea-pig papillary muscles (Kodama *et al.*, 1986) the prolongation of action potential duration by okadaic acid was enhanced in a low- Ca^{2+} medium, while it was nearly abolished in a low- Na^+ medium. The present results seem analogous to those earlier findings.

Since a deactivating K^+ outward current is thought to be one component of the development of pacemaker depolarization, a decrease in this current may also cause a positive chronotropic effect on sinus node pacemaker cells. However, in the present voltage clamp experiments, K^+ outward current was increased. Therefore, K^+ outward current seems unlikely as a possible mechanism for the action of okadaic acid.

The possibility that okadaic acid causes release of neurotransmitters from nerve terminals in the sinus node which, then, modulate the spontaneous activity may be excluded, since neither a β -adrenoceptor nor a ACh receptor antagonist had any effect on the response of the tissue to okadaic acid.

In conclusion, the present study has revealed that okadaic acid has an intrinsic effect on sinus node dominant pacemaker cells, resulting in an increase in spontaneous activity. It was also suggested that this positive chronotropic effect is mediated by the enhancement of slow inward current through verapamil-sensitive Ca^{2+} channels. Recently, Hescheler *et al.* (1988) demonstrated that okadaic acid potently inhibited the protein phosphatases. They also observed an increasing effect of okadaic acid on cardiac I_{Ca} in the presence of isoprenaline which stimulates I_{Ca} through cyclic AMP-dependent phosphorylation of Ca channels (Reuter, 1983; Brum *et al.*, 1984). From these results, they have assumed that the effect of okadaic acid on I_{Ca} is related to suppression of phosphatase

activity resulting in inhibition of dephosphorylation of phosphorylated Ca channel proteins which enhances I_{Ca} . The present results would also be consistent with such an assumption. Okadaic acid is a useful tool for studying the involvement of protein phosphatase activity in the regulation of

cardiac function, such as spontaneous activity and contractility.

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