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Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes

J. Hescheler[1], G. Mieskes[2], J. C. Rüegg[3], A. Takai[3], and W. Trautwein[1]

[1] II. Physiologisches Institut der Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany
[2] Abteilung Klinische Biochemie, Zentrum für Innere Medizin, Universität Göttingen, D-3400 Göttingen, Federal Republic of Germany
[3] II. Physiologisches Institut der Universität Heidelberg, Im Neuenheimer Feld 326, D-6900 Heidelberg 1, Federal Republic of Germany

Abstract. The effects of a protein phosphatase inhibitor, okadaic acid (OA), were studied on membrane currents of isolated myocytes from guinea-pig cardiac ventricle. The whole-cell Ca^{2+} current (I_{Ca}) was recorded as peak inward current in response to test pulse to 0 mV. Extracellular application of OA (5–100 μM) produced an increase of I_{Ca}. The effect was markedly enhanced when the myocyte was pretreated with threshold concentrations of isoprenaline. I_{Ca} was increased from 11.3 ± 0.8 μA cm^{-2} to 19.0 ± 1.1 μA cm^{-2} ($n = 4$) by 5 μM-OA in the presence of 1 nM-isoprenaline. The delayed rectifier current was also slightly increased. Furthermore, the wash-out time of the β-adrenergic increase of I_{Ca} was markedly prolonged by OA. The β-adrenergic stimulation of cardiac Ca^{2+} current is thought to be mediated by cAMP-dependent phosphorylation. The present results strongly suggest that the effect of OA on I_{Ca} is related to inhibition of endogenous protein phosphatase activity which is responsible for the dephosphorylation process. By the isotope method, the inhibitory effect of OA on different types of phosphatase was compared. OA had a relatively high specificity to type 1-, and type 2A-phosphatases.

Key words: Cardiac muscle cell – Whole-cell clamp recording – Calcium current – Protein phosphatase inhibitor

Introduction

Okadaic acid (OA; $C_{44}H_{66}O_{13}$) is a toxin isolated from common marine sponges of the genus *Halichondria*. The chemical structure has been determined by Tachibana et al. (1981). Recently, it was reported that OA has a potent inhibitory effect on the protein phosphatase activity of smooth muscle extract and of a purified enzyme (Takai et al. 1987; Bialojan et al. 1988). In the heart muscle, OA increases the duration of action potential and enhances the contraction in cardiac papillary muscle, possibly due to the increase of calcium current (I_{Ca}; Kodama et al. 1986). Since cardiac calcium channels are thought to be regulated by cAMP-dependent phosphorylation, it is plausible that the effect of OA on cardiac muscle may be related to inhibition of protein phosphatase activity. Indeed, it has been shown that the β-adrenergic stimulation of I_{Ca} is antagonized by intracellular application of various types of protein phosphatase (Kameyama et al. 1986; Hescheler et al. 1987). Furthermore, the wash-out time of β-adrenergic stimulation is prolonged by intracellular perfusion with inhibitor 2 which selectively inhibits type 1-phosphatase (Hescheler et al. 1987; for classification of protein phosphatases, see Ingebritsen and Cohen 1983).

In the present experiments, therefore, we studied the effect of OA on I_{Ca} in myocytes from guinea-pig heart, and examined the specificity of the inhibitory effect of OA on protein phosphatases. The results show that the calcium current is markedly increased by OA especially in the presence of threshold concentrations of isoprenaline, and that OA strongly inhibits type 2-phosphatases as well as type 1-phosphatase.

Materials and methods

Single ventricular myocytes were isolated from hearts of adult guinea-pigs of either sex weighing 300–500 g according to the procedure described before (Taniguchi et al. 1981; Kameyama et al. 1985).

Tyrode's solution was of the following composition (in mM): NaCl, 112; $NaHCO_3$, 24; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.0; D-glucose, 10 and HEPES, 5. The solution was equilibrated wth 95% O_2 and 5% CO_2, and pH was 7.4. The pipette solution and the reaction mixture for enzyme assay contained (in mM): K-aspartate, 80; KCl, 50; KH_2PO_4, 10; $MgSO_4$, 1; HEPES, 5 and EGTA, 0.02 (pH was adjusted to 7.3–7.4 with KOH). For the pipette solution, 3 mM-Na_2ATP was supplemented. L(−)-Isoprenaline (Sigma, St. Louis, MO, USA) was dissolved in distilled water containing 1 mM-ascorbic acid. OA was dissolved in dimethylsulfoxide (DMSO) and added to solutions. The same amount [0.5% (v/v)] of DMSO was also added to control solutions. All experiments were made at 35 ± 1°C.

For electrophysiological measurements, the isolated cells were transferred into a small chamber (0.3 ml in volume) which was perfused with Tyrode's solution at a rate of 5 ml min^{-1}. Membrane currents were recorded under voltage clamp condition as whole-cell currents (Hamill et al. 1981). The pipette had a resistance of 1–2 MΩ when filled with the pipette solution. The resting membrane potential of the cells was -81 ± 2 mV ($n = 60$). The Na^+ current was inacti-

Offprint requests to: A. Takai

vated by applying a prepulse of 100 ms duration to − 40 mV which was followed by a test pulse of 300 ms duration. The stimulation rate was 0.2 Hz. The Ca^{2+} current (I_{Ca}) and the delayed rectifier current were determined as peak inward current and current 300 ms after onset of the test pulse, respectively. For calculation of the current density, the membrane area was estimated from the membrane capacity assuming a specific capacity of 1 μF cm^{-2}.

The catalytic subunits of phosphatase-1 (type 1) and phosphatase-2A (type 2A) were prepared from rabbit skeletal muscle as described by Tung et al. (1984). The aortic protein phosphatase which has a subunit structure comprising three polypeptides was isolated from bovine aorta as described before (DiSalvo et al. 1985). According to Cohen's classification (Ingebritsen and Cohen 1983), this enzyme is a type 2A-phosphatase since it is not affected by either inhibitor 1 or inhibitor 2 (DiSalvo et al. 1983) and has a 5 – 10 times higher myosin light chain phosphatase activity than phosphorylase a phosphatase activity (Bialojan et al. 1985). Protein phosphatase-2C (type 2C) was purified from rabbit liver according to McGowan and Cohen (1987). Protein phosphatase-2B (= calcineurin; type 2B) from bovine brain was kindly provided from Prof. C. Klee (NIH, Bethesda, MD, USA).

The phosphatase activities were determined by measuring the rate of phosphate liberation from bovine heart myosin light chains (Blumenthal et al. 1978) which had been ^{32}P-phosphorylated (DiSalvo et al. 1983) by chicken gizzard myosin light chain kinase (Walsh et al. 1983). For the assay of phosphatase-1 and phosphatase-2C, the reaction mixture was supplemented with 2 mM-$MnCl_2$ and 11 mM-magnesium acetate, respectively. The activity of phosphatase-2B was measured in the presence of 0.1 mM-$CaCl_2$ and 0.2 μM-calmodulin (from porcine brain; Boehringer, Mannheim, FRG). The reaction was started by adding the substrate (^{32}P-labelled myosin light chains) to the reaction mixture containing phosphatase and OA, and stopped by addition of ice-cold 10%-trichloroacetic acid. In all cases, the concentration of phosphatase was such that less than 5% of the substrate (4 μM) was dephosphorylated during the reaction time of 10 min.

Numerical data were presented as mean ± SEM, and difference was evaluated by the Student's *t*-test. Dose-inhibition relations were fitted to the Hill function by the least-squares method, and compared by a method of covariance analysis (Snedecor and Cochran 1980).

OA, isolated from the black sponge *Halichondria okadai*, was kindly provided by Dr. Y. Tsukitani (Fujisawa Pharmaceutical Co., Ltd., Tokyo, Japan).

Results

Figure 1 shows the typical effect of OA on membrane currents of the isolated cardiac myocyte. Under control conditions, the Ca^{2+} current (I_{Ca}) measured as peak inward current (Fig. 1A) had a V-shaped current-voltage relation (Fig. 1B; ●). The maximum of the curve occurred at the test pulse to 0 mV (Fig. 1 B). The threshold potential and apparent reversal potential were about − 40 mV and 60 mV, respectively. Before application of OA, the myocyte was superfused with a threshold concentration (1 nM) of isoprenaline. By this pre-treatment, I_{Ca} was increased about 10% (Fig. 1B; ■). When 5 μM-OA was extracellularly applied in addition, the I_{Ca} increased to about twice the control amplitude (19 μA cm^{-2}) within 10 min (Fig. 1B; ▲; see also Table 1). The maximal I_{Ca} was recorded with the same test pulse potential (0 mV) as in the control. The threshold potential and reversal potential were not changed by OA (Fig. 1B). The time course of inactivation of I_{Ca} was also unaffected (Fig. 1A). On the other hand, the delayed rectifier current was slightly increased by OA (Fig. 1A, B). These effects of OA on the ionic currents were completely reversed within 15 min when OA was removed. Thus, OA had very similar effects to those of high concentrations (> 50 nM) of isoprenaline (cf. Kameyama et al. 1985).

Table 1. Effect of OA on calcium current (I_{Ca})

Control	10.1 ± 0.6 ($n = 4$)
1 nM-ISP	11.3 ± 0.8 ($n = 4$)
1 nM-ISP + 5 μM-OA	19.0 ± 1.1 ($n = 4$)
Control	9.9 ± 0.7 ($n = 5$)
50 nM-ISP	14.5 ± 0.9 ($n = 5$)
50 nM-ISP + 5 μM-OA	24.0 ± 1.3 ($n = 5$)

In the same type of experiments as in Fig. 1, 5 μM-OA was applied in the presence of 1 nM- or 50 nM-isoprenaline (ISP). The values of I_{Ca} (μA cm^{-2}) are presented as mean ± SEM

Threshold concentrations of isoprenaline were necessary for lower concentrations (1 – 5 μM) of OA to increase the amplitude of I_{Ca}. The membrane currents were only slightly affected by OA (1 – 5 μM) in the absence of isoprenaline; the increase of I_{Ca} was within 10%. On the other hand, the effect on I_{Ca} was enhanced when OA (5 μM) was applied in the presence of 50 nM-isoprenaline (Table 1).

Higher concentrations (> 10 μM) of OA, however, did increase the I_{Ca} even in the absence of adrenergic stimulation. The effect was especially marked when OA was intracellularly applied. In the experiment shown in Fig. 2, the pipette was filled with the pipette solution containing 50 μM-OA. I_{Ca} measured as peak inward current in response to the test pulse potential of 0 mV (inset) was plotted against the time. The measurement was started 15 s after achievement of the whole-cell configuration (0 min). The I_{Ca} increased form 10.3 ± 0.7 μA cm^{-2} to a steady level of 23.7 ± 1.1 μA cm^{-2} ($n = 3$) within 2 – 3 min.

As shown in Fig. 3, the wash-out of the effect of isoprenaline was markedly slowed down in the presence of OA. Isoprenaline (50 nM) was applied for 2 min and washed out either in the absence (control) or in the presence of OA (5 μM; extracellular application). In the control, 50 nM-isoprenaline increased I_{Ca} from 10.7 ± 0.4 μA cm^{-2} to 22.7 ± 0.8 μA cm^{-2} ($n = 8$), and this was completely reversed when isoprenaline was removed. I_{Ca} was increased to 27.0 ± 1.1 μA cm^{-2} ($n = 4$) as isoprenaline was again applied together with 5 μM-OA. The time required to wash out 75% of the response to isoprenaline was 132 ± 11 s ($n = 8$) in the control, and this was prolonged to 394 ± 45 s ($n = 4$) by 5 μM-OA.

Figure 4 shows the inhibitory effect of OA on the myosin light chain phosphatase activity of various protein phosphatases. Among phosphatases examined, the catalytic subunit of skeletal type 2A-phosphatase ($2A_c$) was most potently inhibited; the concentration of OA required to obtain a 50% inhibition (ID_{50}) was 1 nM. OA also inhibited the aortic type 2A-phosphatase ($2A_1$; $ID_{50} = 100$ nM) and the catalytic subunit of type 1-phosphatase (1; $ID_{50} = 500$ nM).

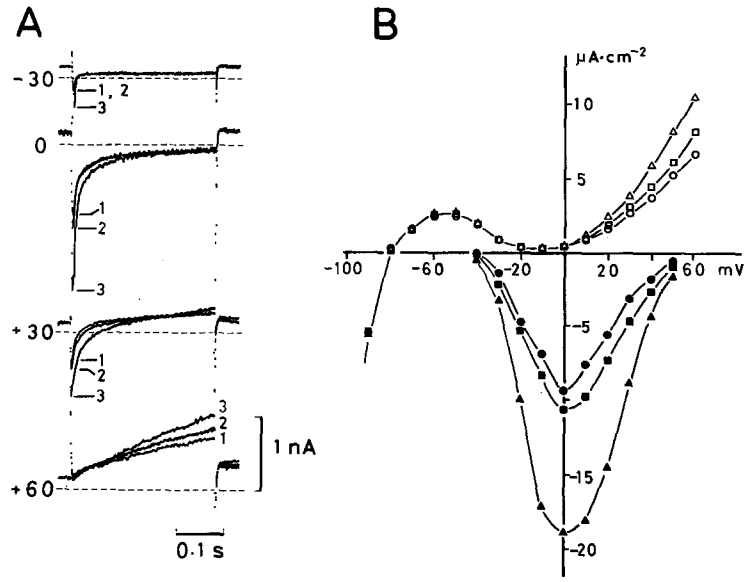

Fig. 1A, B
Effect of OA on membrane currents. **A** Traces of current response to 4 different test potentials (in mV; −30, 0, 30 and 60). Three traces are superimposed: 1, control; 2, 1 nM-ISP; 3, 1 nM-ISP + 5 μM-OA. Zero current level is indicated by *broken line*. **B** Current-voltage relations obtained in the same cell as in **A**. ● and ○, control; ■ and □, 1 nM-ISP; ▲ and △, 1 nM-ISP + 5 μM-OA. The peak inward current (*closed symbols*) and the current 300 ms after the onset of test pulse (*open symbols*) were plotted

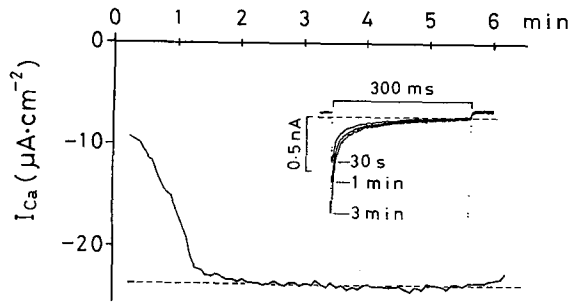

Fig. 2. Effect of intracellular application of OA on calcium current. The pipette was loaded with the pipette solution containing 50 μM-OA. A whole-cell clamp recording. Test pulses to 0 mV were repetitively applied at the rate of 0.2 Hz, and calcium current (I_{Ca}; peak inward current) was plotted against time. At 0 min, the membrane under the tip of pipette was raptured by suction. Note the quick increase of I_{Ca}. *Inset:* original current traces at 30 s, 1 min and 3 min are superimposed

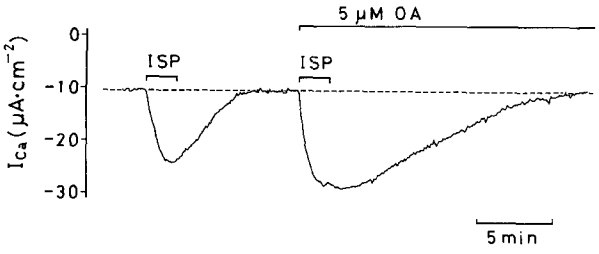

Fig. 3. Effect of OA on β-adrenergic response. In the same type of recording as in Fig. 2, isoprenaline (ISP; 50 nM) was superfused in the absence and presence of 5 μM-OA. Note the larger β-adrenergic response and the marked prolongation of the wash-out time in the presence of OA

Calcineurin (2B) was inhibited to a lesser extent (ID_{50} = 10 μM), whereas type 2C-phosphatase (2C) was not affected by up to 10 μM-OA. In these enzymatic experiments, the reaction was started by adding ^{32}P-labelled myosin light chains (4 μM) to OA-treated phosphatase (see Materials and methods). The control activities and the dose-inhibition relations were not significantly changed when the reaction

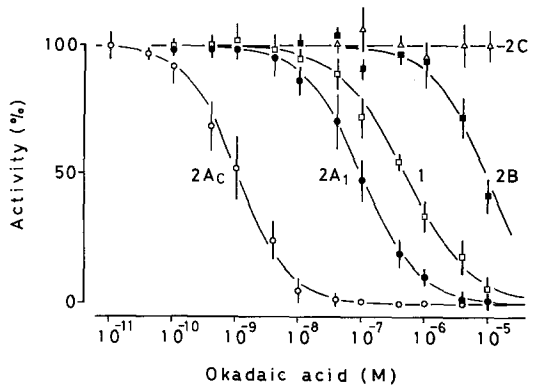

Fig. 4. Effect of OA on various types of protein phosphatase. Dose-inhibition relations. The names of the protein phosphatases are abbreviated as $2A_c$ (○, skeletal catalytic subunit); $2A_1$ (●, aortic polymolecular enzyme); 1 (□); 2B (■, calcineurin) and 2C (△). The myosin light chain phosphatase activities are presented as percentage of the control values (in nmol P_i min^{-1} mg^{-1} protein): phosphatase-1, 2.1 ± 0.2; $2A_c$, 104.3 ± 1.9; $2A_1$, 32.1 ± 3.5; 2B, 1.7 ± 0.2 and 2C, 92.2 ± 4.1 ($n = 6-8$). Each point is average of 3–4 data. *Vertical bars* indicate SEM

was initiated by addition of phosphatase to the reaction mixture containing the substrate and OA.

Discussion

It has been reported that OA prolongs the duration of action potential and increases the contraction in cardiac papillary muscle (Kodama et al. 1986). The present whole-cell clamp experiments have shown that OA strongly and reversibly enhances the stimulating effect of isoprenaline on I_{Ca}, which is thought to be mediated by the cAMP-dependent phosphorylation system. The time required to wash out the effect of isoprenaline is markedly prolonged by micromolar concentrations of OA. Furthermore, OA has a potent inhibitory action on protein phosphatase activities. These observations strongly support the idea that OA produces its stimulating effect on cardiac muscle through suppression of dephosphorylation process resulting in enhanced phosphorylation

of channel related protein(s), which in turn increases the opening probability of calcium channels (see Reuter et al. 1983; Brum et al. 1984; Flockerzi et al. 1986).

Prolongation of wash-out time of the effect of isoprenaline is also produced by intracellular application of inhibitor 2 which specifically inhibits type 1-phosphatase (Huang and Glinsmann 1976), indicating that endogenous type 1-phosphatase is at least partly responsible for reversal of the β-adrenergic response of cardiac cells (Hescheler et al. 1987). However, even in the presence of inhibitor 2, the adrenergic effect is slowly reversed when isoprenaline is removed. Intracellular application of inhibitor 2 itself has only a slight effect on the membrane current while adenosine 5′-(3-thiotriphosphate) (ATPγS) which leads to a phosphatase-resistant thiophosphorylation produces a slow but large increase of I_{Ca} (Kameyama et al. 1986; Hescheler et al. 1987). These previous observations suggest the existence of other phosphatase(s) related to the dephosphorylation of Ca^{2+} channels. The present results with isolated phosphatases have shown that OA strongly inhibits type 2A-phosphatases and type 1-phosphatase. In the absence of β-stimulation, I_{Ca} is markedly increased by more than 10 μM-OA which nearly abolishes both type 1- and type 2A-phosphatase activities. These findings suggest that type 2A-phosphatase(s) as well as type 1-phosphatase may be involved in the downward regulation of the β-adrenergic response.

The increase of I_{Ca} by OA occurs much faster than that by ATPγS. This is not unexpected ATPγS is a relatively poor substrate for protein kinases compared with ATP, and therefore thiophosphorylation proceeds much slower than normal phosphorylation (see Cassidy et al. 1979). On the other hand, the interaction of OA with phosphatases seems to be a faster process. The rates of dephosphorylation are not changed when the enzyme reactions are started by adding phosphatase to the reaction mixture containing substrate and OA instead of addition of substrate to OA-treated phosphatase (see Results). The fast increase of I_{Ca} produced by OA suggests that the intracellular protein kinase activity is considerably high even in the unstimulated condition.

The affinity of OA is remarkably different for various types of protein phosphatase. The skeletal type 2A-phosphatase is about 5000 times more strongly inhibited by OA than type 1-phosphatase, although there is a striking sequence homology between these enzymes (see Berndt et al. 1987). The three-dimensional conformation of phosphatase proteins may be an important factor for binding of OA. The catalytic subunit of skeletal type 2A-phosphatase is about 100 times more susceptible to OA than the aortic polymolecular phosphatase. This may imply that the aortic phosphatase has a different catalytic subunit from that of the skeletal phosphatase. It is also possible that the non-catalytic subunits makes the catalytic subunit less accessible to OA. The molecular mechanism of phosphatase inhibition by OA remains to be further studied.

To our knowledge, OA is the first exogenous substance that potently and reversibly inhibits the protein phosphatases. The effect of OA on the membrane currents can be produced by extracellular application, indicating a relatively high membrane permeability. This is a unique characteristic of OA, compared with the endogenous protein phosphatase inhibitors and ATPγS which are membrane-impermeable.

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