Synergistic Effects of Cyclic AMP-Related Vasodilators and the Phosphatase Inhibitor Okadaic Acid

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ABSTRACT—The phosphatase inhibitor okadaic acid at 100 nM slowly but completely inhibited high K⁺-induced contraction in the rat aorta ($t_{1/2}$ = 118.9 min). High K⁺-induced contraction was partially inhibited (to 37–65%) by 1 μ M forskolin, 100 μ M dibutyryl cyclic AMP, 100 nM atrial natriuretic peptide, 1 μ M nitroglycerin, 10 nM sodium nitroprusside, 300 pM nicardipine or 100 nM verapamil. The rate of relaxation due to okadaic acid became faster when the contraction was partially inhibited by these compounds. Augmentation of the relaxation was greater with forskolin and dibutyryl cyclic AMP than with the other inhibitors. These results support the suggestion that okadaic acid inhibits phosphatase to augment the phosphorylation due to cyclic AMP-dependent kinase, resulting in smooth muscle relaxation.

Keywords: Okadaic acid, Cyclic AMP, Smooth muscle (vascular)

Okadaic acid is a potent inhibitor of protein phosphatases, inhibiting type 2A phosphatase at lower concentrations and type 1 phosphatase at higher concentrations (1, 2). In smooth muscle, okadaic acid has concentration-dependent dual effects. At concentrations higher than 1 μ M, okadaic acid induces contraction (3, 4) by phosphorylating the myosin light chain (5). At lower concentrations, in contrast, okadaic acid inhibits contractions induced by high K⁺-depolarization and receptor agonists (6–8). Since the characteristics of the inhibitory effect of okadaic acid are similar to those of cyclic AMP-related relaxants such as forskolin and dibutyryl cyclic AMP (db-cAMP), it has been suggested that okadaic acid may inhibit phosphatases and thus increase the cyclic AMP-dependent protein phosphorylation (6). If this is the case, the effect of okadaic acid should be augmented when the cyclic AMP level is increased, and testing this hypothesis was the purpose of the present experiments.

Male Wistar rats (200–250 g) were killed by a sharp blow on the neck and exsanguination. The thoracic aorta was isolated and cut into transverse strips (2-mm-wide and 5-mm-long). Endothelium was removed by gently rubbing the intimal surface with a finger moistened with physiological salt solution which contained 136.9 mM NaCl,

5.4 mM KCl, 1.5 mM CaCl₂, 1.0 mM MgCl₂, 23.8 mM NaHCO₃, 0.01 mM ethylenediaminetetraacetic acid and 5.5 mM glucose. High K⁺ solution was made by substituting 60 mM NaCl with equimolar KCl. These solutions were saturated with a 95% O₂ and 5% CO₂ mixture at 37°C to maintain the pH at 7.4.

Okadaic acid was donated by Dr. D. Uemura, Shizuoka University and nitroglycerin, by Nippon Kayaku Co., Tokyo. Other chemicals used were forskolin, nicardipine, verapamil (Sigma Chemical Co., St. Louis, MO, USA); db-cAMP (Yamasa Shouyu, Tokyo); α -human atrial natriuretic peptide (Peptide Institute, Osaka); and sodium nitroprusside (Wako Pure Chemicals, Osaka). Results of the experiments are shown by the mean $\pm$ S.E.M. of the data.

Contractile force was measured with a force-displacement transducer (Nihon Kohden, Tokyo). The muscle preparation was equilibrated for 30 min in a 10-ml bath. High K⁺ was applied three times, 10 min each, at a 15-min interval, before starting the experimental procedures. When the contractile force due to the fourth application of high K⁺ reached a steady level, okadaic acid was added. As shown in Fig. 1A, 100 nM okadaic acid slowly and completely relaxed the high K⁺-induced contraction. The time required to relax the contraction by 50% (half-time or $t_{1/2}$) was 118.9 ± 5.5 min ($n=12$). Lower concentrations of okadaic acid inhibited the contraction more slowly ($t_{1/2} > 120$ min), whereas a higher concentration

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Fig. 1. Effect of 100 nM okadaic acid (OA) on 65.4 mM K^+ -induced contraction. (A) Okadaic acid was added when the contraction induced by high K^+ reached a steady level. (B) and (C) During the high K^+ -induced contraction, 100 nM verapamil or 1 μ M forskolin was added, and then okadaic acid was added. Muscle contraction before the addition of okadaic acid (T) and time needed to induce a 50% relaxation ($t_{1/2}$) are shown by arrows.

(300 nM) more rapidly inhibited the contraction with a shorter $t_{1/2}$ (58.9 ± 1.9 min, $n=4$). From these results, we decided to use $t_{1/2}$ rather than contractile force as an indicator of the inhibitory effect of okadaic acid.

Previously, we have shown that the relaxant effects of forskolin and db-cAMP are inversely proportional to the magnitude of contractile tension; that is, the relaxant effect is greater when the contraction before the addition of the relaxant was smaller (9). To examine if the inhibitory effect of okadaic acid is also modified by the magnitude of contraction, we partially inhibited the high K^+ -induced contraction by adding Ca^{2+} channel blockers. As shown in Fig. 1B, 100 nM verapamil was added during the high K^+ -induced contraction which inhibited the contraction to a new steady level of $36.5 \pm 2.7\%$ ($n=4$). Then 100 nM okadaic acid was sequentially added. In the presence of verapamil, the rate of okadaic acid-induced relaxation became slightly faster. $T_{1/2}$ was decreased by 19.2 min or to 85.0% of the $t_{1/2}$ in the absence of verapamil. Nicardipine (100 pM) showed a similar effect as verapamil, inhibiting the high K^+ -induced contraction to 52.7% and decreasing $t_{1/2}$ to $82.2 \pm 6.1\%$ ($n=4$). The

Fig. 2. Effects of various inhibitors on high K^+ -induced contraction and the relaxant effect of okadaic acid (OA). The experiment was done as shown in Fig. 1. The inhibitor was added during the high K^+ -induced contraction, and the new steady level (T in Fig. 1) is plotted on the abscissa, taking the contraction before the addition of inhibitor as 100%. Okadaic acid was then added, and the rate of relaxation ($t_{1/2}$ in Fig. 1) is plotted on the ordinate, taking the $t_{1/2}$ in the absence of inhibitor as 100%. Each point represents mean of 4–5 experiments, and S.E.M. is shown by vertical and horizontal bars. The correlation coefficient for the regression line is 1.000 for cyclic AMP-related relaxants (1 μ M forskolin and 100 μ M db-cAMP: filled circles), 0.990 for cyclic GMP-related relaxants (100 μ M atrial natriuretic peptide [ANP], 10 nM sodium nitroprusside [SNP] and 1 μ M nitroglycerin: open circles), and 0.922 for Ca^{2+} channel blockers (100 nM verapamil and 300 pM nicardipine: filled squares).

relationship between the magnitude of contraction (T in Fig. 1) and potency of okadaic acid ($t_{1/2}$ in Fig. 1) in Fig. 2 shows that the relaxant effect of okadaic acid is greater when the magnitude of contraction before the addition of okadaic acid was smaller. The similarity between the effects of okadaic acid and cyclic AMP-related vasodilators supports the suggestion that okadaic acid inhibits phosphatase to augment the phosphorylation due to cyclic AMP-dependent kinase.

In the next experiments, we determined if the increase in cyclic AMP level further augments the effect of okadaic acid. To increase the cyclic AMP level, we added forskolin or db-cAMP during the high K^+ -induced contraction. As shown in Fig. 1C, addition of 1 μ M forskolin inhibited the high K^+ -induced contraction to $49.2 \pm 6.1\%$ ($n=4$). In the presence of forskolin, the rate of okadaic acid-induced relaxation became faster and shortened $t_{1/2}$ to $37.5 \pm 1.4\%$ ($n=4$). The effect of db-cAMP was similar to that of forskolin. The relationship between the magnitude of contraction (T in Fig. 1) and potency of okadaic acid ($t_{1/2}$ in Fig. 1) is shown in Fig. 2. The slope of the correlation was steeper in the presence of cyclic AMP-related

relaxants than in the presence of Ca^{2+} channel blockers, suggesting that the inhibitory potency of okadaic acid is greatly augmented when the cyclic AMP level is increased.

To determine if the inhibitory effect of okadaic acid is selectively augmented by cyclic AMP, we examined the effects of cyclic GMP. To increase the cyclic GMP level, we used 10 nM sodium nitroprusside, 100 nM atrial natriuretic peptide and 1 μM nitroglycerin. As shown in Fig. 2, these compounds inhibited the high K^{+} -induced contraction to 45.4–64.7% and shortened $t_{1/2}$ to 65.1–82.0% of the respective control level. The slope of the correlation for cyclic GMP-related relaxants is only slightly steeper than that for Ca^{2+} channel blockers, suggesting that cyclic GMP has only a small, if any, potentiating effect on okadaic acid-induced relaxation. It is generally believed that cyclic GMP activates protein kinase to phosphorylate functional proteins and relaxes smooth muscle. However, the site of action of cyclic GMP seems to be different from that of cyclic AMP because cyclic GMP relaxes smooth muscle with little effect on myosin phosphorylation (11), and cyclic AMP but not cyclic GMP augmented the effect of okadaic acid (present result). It is possible that okadaic acid inhibits the phosphatase antagonizing the cyclic AMP-dependent kinase with little effect on the phosphatase antagonizing the cyclic GMP-dependent kinase.

In the rat aorta, the major mechanism of the effect of cyclic AMP on high K^{+} -induced contraction is to decrease the Ca^{2+} sensitivity of contractile elements without changing the cytosolic Ca^{2+} level (9). Cyclic AMP has been shown to inhibit myosin phosphorylation to relax smooth muscle (10). This effect may be antagonized by the increase in cytosolic Ca^{2+} (9), and this may be the reason why the relaxant effect of cyclic AMP is stronger on a smaller contraction (or in the presence of lower cytosolic Ca^{2+} level). In the present experiments, we found that the inhibitory effect of okadaic acid is also stronger when the contraction was partially inhibited by Ca^{2+} channel blockers or nitro-vasodilators. Furthermore, the inhibitory effect of okadaic acid was more strongly augmented when the contraction was partially inhibited by cyclic AMP-related relaxants. These results support the suggestion that okadaic acid inhibits phosphatases and thus augments cyclic AMP-dependent phosphorylation to induce vasodilation. This effect may be antagonized by the increase in cytosolic Ca^{2+} level and augmented by the increase in cyclic AMP level.

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