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Effects of Trifluoperazine on the Activation of Cilia by 5-Hydroxytryptamine in *Mytilus edulis*

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ABSTRACT

1. The effects of trifluoperazine (TFP), a phenothiazine inhibitor of calmodulin, were studies on the activation of ciliary beat by 5-hydroxytryptamine (5-HT) in the lateral cells of the gill in *Mytilus edulis*. 2. The ciliary activation was totally blocked by 20 μm TFP. The inhibition was Ca-dependent. 3. At a concentration of 5-10 μm, TFP induced repetitive ciliary arrest responses of the lateral cells. 4. Deciliation was observed in the medium containing TFP at a concentration of 20 μm and above. 5. These results suggest that TFP increased the cytoplasmic Ca^{2+} of the ciliated cells and induced the ciliary arrest. Other possible mechanisms of the inhibition are discussed.

INTRODUCTION

Calmodulin (CaM) or calmodulin-like proteins mediate various kinds of regulatory activities in a wide range of cell types (Means and Dedman, 1980; Cheung, 1980; Means, Tash and Chafouleas, 1982). Within a single cell, CaM may control more than one type of response. In a protozoan cell such as *Paramecium* or *Tetrahymena*, for example, CaM appears to regulate such activities as the activation of the Ca^{2+} pump in the membrane, the ciliary reversal and the trichocyst discharge etc. (Satir, Garofalo, Gilligan and Maihle, 1980; Garofalo, Gilligan and Satir, 1983; Suzuki, Ohnishi, Hirabayashi and Watanabe, 1982).

In the gill of the mussel, the branchial nerve seems to control at least three different responses of the same cilia. Thus, repetitive stimulation given to the nerve at 2-5 pulses$\cdot s^{-1}$ increases the beat frequency of the lateral cilia. For this effect, 5-hydroxytryptamine (5-HT) is a putative transmitter substance released from the nerve terminals (Aiello, 1974). It has been reported that stimulation of the same nerve at 25-50 pulses$\cdot$s^{-1} caused a gradual decrease of the ciliary beat frequency lasting for 10 min or more (Paparo and Aiello, 1970). On the other hand, a single electric pulse given to the branchial nerve brings about an abrupt stoppage of the same cilia (Takahashi and Murakami, 1968). The ciliary arrest of the same type is induced by various stimuli that depolarize the lateral cell (Takahashi and Tsuchiya, 1971; Murakami and Takahashi, 1975a; Saimi, Murakami and Takahashi, 1983a; 1983b). The arrest response is Ca-dependent and most likely regulated by the level of cytoplasmic Ca^{2+} (Tsuchiya and Takahashi, 1972; Tsuchiya, 1977; Walter and Satir, 1978).

In detergent-treated models of the lateral cells the ciliary arrest is induced by an addition of Ca^{2+} to the medium (Tsuchiya, 1977; Walter and Satir, 1978) and the arrest is inhibited by a phenothiazine, trifluoperazine (TFP), which antagonizes the action of CaM (Levin and Weiss, 1976). It has been suggested that CaM may be involved in the regulatory mechanism underlying the arrest of the lateral cilia (Reed, Lebduska and Satir, 1982). In the gill of *Aequipecten irradians*, however, it has been reported that most of the CaM in cilia is localized not in the ciliary axonemes but in the detergent-solubilized fraction of the membrane-matrix and also that the effect of TFP on the ciliary arrest is non specific (Stommel, Stephens, Masure and Head, 1982). It seems likely that the principal function of CaM in some ciliated cells may be related to the activities of the membrane such as the Ca^{2+} transport by the Ca^{2+} pump (Vincenzi and Hinds, 1980; Stommel *et al*, 1982) and the formation of adenosine 3′, 5′-cyclic monophosphate (cAMP) by adenylate cyclase (Bradham and Cheung, 1980; Means *et al*, 1982). It is interesting in this connection that cAMP has been suggested to be involved in the activation of some cilia and flagella including the lateral cilia of *Mytilus* (Malanga and Poll, 1979; Means *et al*, 1982; Morisawa and Okuno, 1982; Murakami, 1983).

We report here that TFP strongly inhibits the ciliary activation of the lateral cilia of *Mytilus* by 5-HT. The inhibition is Ca-dependent. The results suggest that CaM may play multiple roles in the lateral cells. The possible role of CaM in the regulatory systems of the ciliary motility is discussed in relation to the effect of TFP on the ciliated cells.

MATERIALS AND METHODS

Most of the experiments were carried out on the lateral cilia on the gill filaments of *Mytilus edulis*. A few observations were made on the latero-frontal cilia and the cilia in the ciliary junctions between the gill-filaments (ciliary pads). The specimens were collected near the Misaki Marine Biological Station, brought to the laboratory in Tokyo and kept in an aquarium of circulating natural sea water (water temperature, 19°C).

A gill-filament was isolated from the descending lamella of a demibranch. The isolated filament was split into two lateral halves along its entire length to expose the basal side of the ciliated epithelium. One of the half gill-filament preparations was then placed straight in a perfusion vessel with its lateral side facing downward. The vessel had a capacity of about 0.4 ml. It was continuously perfused with artificial sea water (ASW, see below) at a rate of 1.6 ml$\cdot$min^{-1}.

In order to monitor the frequency of the ciliary beat photoelectrically (Takahashi and Tsuchiya, 1971), the microscopic image of the cilia was projected onto a photomultiplier tube (Toshiba, MS-9S) through a pinhole in a screen placed in front of the tube. The photoelectric current was amplified and displayed on a cathode ray oscilloscope. The frequency of its fluctuations caused by the ciliary beat was counted directly on the screen of the oscilloscope or by means of an electronic counter (Takahashi and Tsuchiya, 1971).

The ASW used had the following composition (in mM): MaC1, 434; KC1, 10; $CaC1_2$, 10; $MgC1_2$, 53; N-(2-hydroxyethyl)-piperazine-N′-2-ethane sulfonic acid (HEPES), 10 (pH 7.5). To obtain the Ca-free ASW, the $CaC1_2$ in the ASW was replaced with an osmotically equivalent amount of NaC1. The Ca-free ASW containing 1 mM ethyleneglycol-bis(β-aminoethyl ether)N, N′-tetraacetic acid (EGTA) (EGTA-ASW) was usually applied after washing the preparation with Ca-free ASW. ASW containing 10^{-7} M 5-HT was used to stimulate the cilia to beat.

All the experiments were carried out at room temperature (22°-24°C).

RESULTS

Unless stimulated with 5-HT, the lateral cilia on the half gill-filament preparation were usually quiescent. An addition of 5-HT to the medium stimulated the cilia to beat (Aiello, 1974). The threshold concentration of 5-HT was between 10^{-10} and 10^{-9} M. The cilia started beating within 20 s after the onset of perfusion with 10^{-7} M 5-HT. The beat frequency of the activated cilia reached a plateau after a min or so. The maximal frequency was about 25 s^{-1} (range, 21.4-32.5 s^{-1}).

The activation of ciliary beat by 5-HT was inhibited by TFP. The preparation was immersed in the medium containing TFP at various concentrations (5, 10, 15, 20, 25 μm) prior to the application of 5-HT. The test solution for the ciliary activation contained the same concentration of TFP in addition to 10^{-7} M 5-HT. The number of cells activated by 5-HT decreased as the concentration of TFP was increased (Fig. 1). The maximal beat frequencies of the cilia activated by 5-HT are plotted against the concentration of TFP in Fig. 2. The ciliary beat was irregular in the medium containing 5 or 10 μm TFP; transient and local arrests of the cilia were often observed. In 15 μm TFP, the frequency of the activated beat was reduced to about a half. No ciliary beat was induced by 5-HT in the presence of 20 μm TFP.

Observations were made on the changes of ciliary activity following an addition of 20 μm TFP to the medium containing 10^{-7} M 5-HT in which the lateral cilia had been activated. Local and transient arrest responses and a synchronous, rather than metachronous, beating along the length of the gill filament started about 1.5 min after the addition of TFP. The beat frequency decreased and the cilia finally stopped within 3 min. A further exposure to 20 μm TFP induced a detachment of up to 30 percent of the lateral cilia.

Exposure to 10^{-7} M 5-HT in the presence of 25 μm TFP failed to activate the lateral cilia. After about 5 min, 40 percent of the lateral cilia and more than 80 percent of the latero-frontal cilia were detached. The basal part of the latero-frontal cilia remained in the preparations after the deciliation. The detachment site of the lateral cilia could not be seen. The lateral cilia that were left attached after the treatment with 20 μm TFP for 10 min scarcely recovered the beat when washed with normal ASW and then stimulated with 10^{-7} M 5-HT. On the other hand, more than 90 percent of the lateral cilia recovered after a 10 min exposure to 15 μm TFP. No deciliation was observed in 15 μm TFP.

There were obvious differences in the response to TFP among the different kinds of cilia on the same preparation. The latero-frontals seemed less sensitive to

TFP than the laterals, so far as the motility was concerned. The reverse was true for the deciliation. The cilia of the ciliary pads (interfilamentar junctions) kept beating apparently normally in 20 μm TFP. The medium containing 100 μm TFP killed all the cells in the preparations within a min.

Chlorpromazine (CPZ) was less effective than TFP. An addition of 50 μm CPZ to the medium containing 10^{-7} M 5-HT induced the lateral cilia to beat irregularly; transient arrest response and synchronized beating appeared repeatedly. The maximal beat frequency of the cilia was about 60 percent of the control without CPZ.

The inhibitory effect of TFP on the ciliary activation by 5-HT was Ca-dependent. In Fig. 3 ciliary beat frequencies are plotted against the time after the addition of 10^{-7} M 5-HT. It has been known that the lateral cilia can be activated by 5-HT without extracellular Ca^{2+}, or in EGTA-ASW (Murakami and Takahashi, 1975b). In EGTA-ASW containing 20 μm TFP more than 80 percent of the lateral cells were activated by 5-HT. The maximal frequency of the activated cilia was 60-75 percent of the control without TFP (Fig. 3). No deciliation was observed in EGTA-ASW containing 20 μm TFP.

DISCUSSION

In the present study we found that TFP strongly inhibits the activation of the lateral cilia by 5-HT (Figs. 1 and 2). The inhibitory action of TFP in Ca-dependent (Fig. 3). TFP is phenothiazine known to inhibit the action of CaM (Levin and Weiss, 1976; Satir *et al*, 1980). Multifunctional actions of CaM on protozoan cells have been reported several times (Satir *et al*, 1980; Suzuki, Ohnishi, Hirabayashi and Watanabe, 1982). On the other hand, the regulation of the ciliary arrest response is the only function of CaM so far investigated in the ciliated cells in the gill of the bivalves (Reed, Lubduska and Satir, 1982; Stommel *et al*, 1982). If TFP inhibits the ciliary arrest, the ciliary beat would not be interrupted by the ciliary arrest in the presence of TFP. The present results were rather reverse in appearance: the ciliary beat was arrested in the medium containing 5-HT and TFP.

We may interpret the results in three ways: First, TFP may directly inhibit the mechanism by which 5-HT stimulates the ciliary beat. CaM has been known as a regulator of adenylate cyclase (Bradham and Cheung, 1980). It has been suggested that cAMP is involved in the regulatory mechanism of flagellar and ciliary motility (Malanga and Poll, 1979; Means *et al*, 1982; Morisawa and Okuno, 1982; Murakami, 1983). Second, TFP may inhibit the Ca^{2+} pump in the membrane and thus increases the level of the intracellular Ca^{2+}. It has been reported that an increase in the concentration of Ca^{2+} over 10^{-6} M inhibits the activity of adenylate cyclase irrespective of the presence or absence of TFP (Potter, Piascik, Wisler, Robertson and Johnson, 1980). The 5-HT-induced activation of cilia is possibly inhibited by an increase in Ca^{2+} through a block of adenylate cyclase. Third, if TFP induces an increase in Ca^{2+} and if TFP does not inhibit the mechanism of ciliary arrest under the conditions of the present experiment, the ciliary beat would be stopped by the Ca-dependent arrest response, even though the mechanism of ciliary activation by 5-HT is intact. It has been repeatedly demonstrated that the ciliary arrest response of the lateral cilia can be induced in the presence of 5-HT (Takahashi and Murakami, 1968; Saimi *et al*, 1983a; 1983b).

The deciliation induced by 20 or 25 μm TFP may indicate an increase in the intracellular Ca^{2+} (Satir *et al*, 1980). Spontaneous repetitions of the ciliary arrests were observed in the solution containing 5 or 10 μm TFP, or 50 μm CPZ which is another phenothiazine inhibitor of CaM (Levin and Weiss, 1976). Ionophores such as X537A and A23187 induce similar repetitions of the ciliary arrest (Murakami and Takahashi, 1975b; Satir, 1975; Walter and Satir, 1978). Deciliation and repetitive arrests have also been induced in the gill cilia of *Aequipecten irradians* by TFP though at a higher concentration (more than 200 μm, Stommel *et al*, 1982).

The present results seem to suggest the third possibility mentioned above: that is, a transient increase in the Ca^{2+} level induces the ciliary arrest, at least in the medium containing less than 15 μm TFP. It remains to be determined, however, whether the level of cAMP is affected under the present experimental conditions; the triggering of the ciliary arrest may occur in parallel with the inhibition of the 5-HT-activation of cilia. It would be an interesting hypothesis that both CaM and cAMP are involved in the regulation of the activity of the lateral cilia. Mobilization of intracellular Ca^{2+} (Paparo and Murphy, 1975) and an increase in the level of cAMP (Malanga and Poll, 1979) have been reported to occur when the gill is stimulated by 5-HT.

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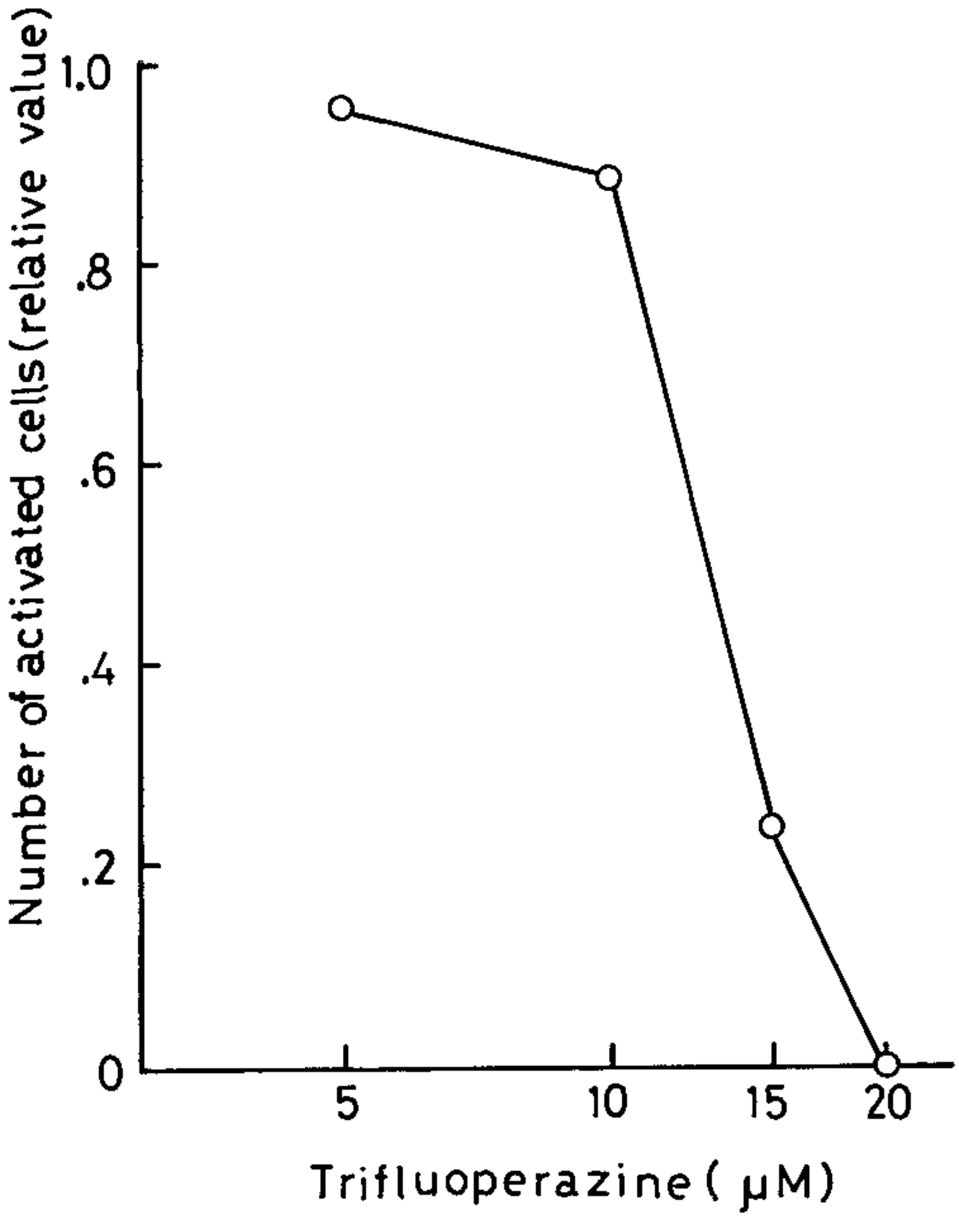

Fig. 1. Effect of trifluoperazine (TFP) on the number of lateral cells activated by 10^{-7} M 5-HT. The numbers of active and inactive cells were either directly counted when they were limited, or estimated by the length of the active and inactive regions along the gill axis as fractions of the total (ca. 50 cells or ca. 850 µm) in the microscopic field. The preparations were immersed in the medium containing TFP for 3 to 4 min prior to the perfusion with the medium containing 5-HT as well as TFP at the same concentration. All the cells were activated to a plateau within a min in the control medium without TFP. The estimations were made 3 to 5 min after the onset of the perfusion. Each point is the mean value of measurements on 3 to 7 preparations from different mussels.

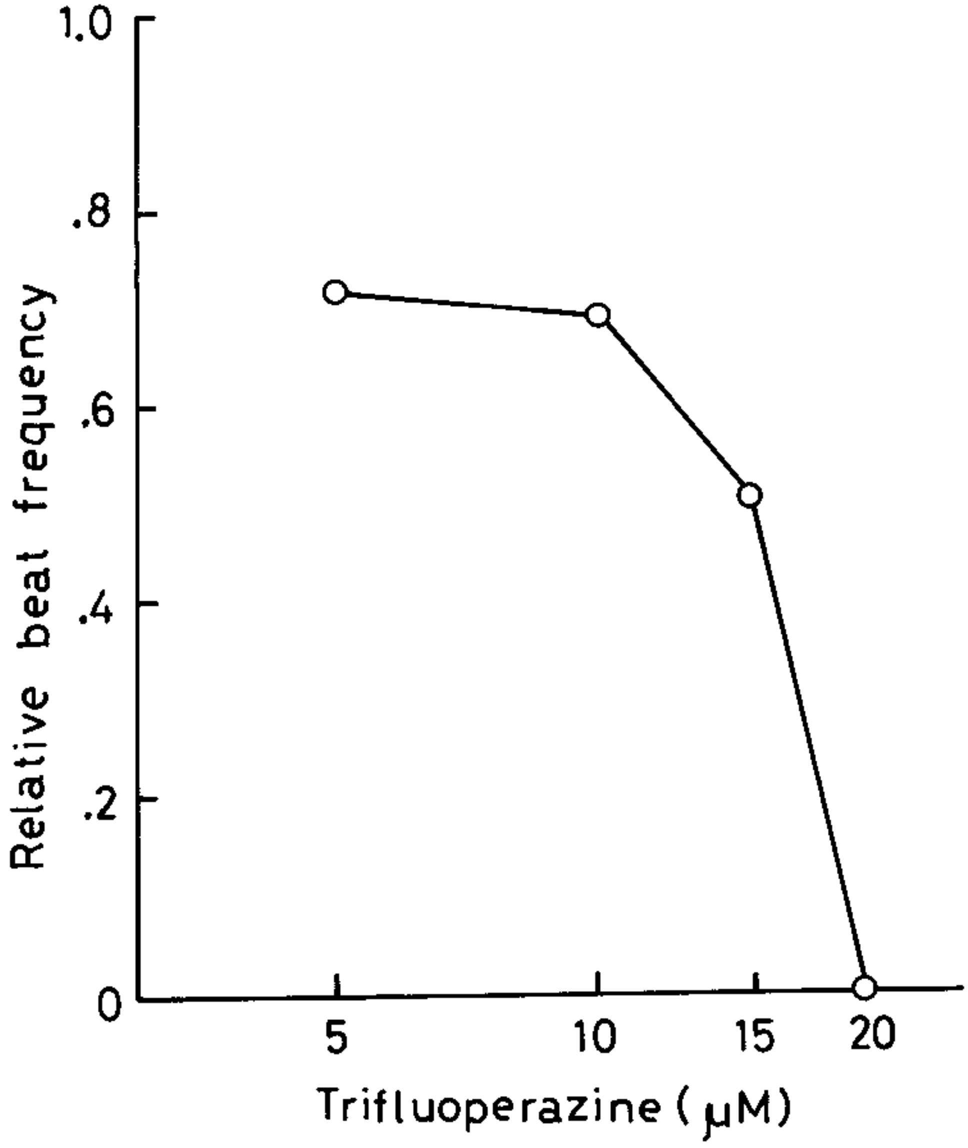

Fig. 2. Effect of trifluoperazine (TFP) on the maximal frequency of the lateral cilia activated by 10^{-7} M 5-HT. The beat frequency of the plateau observed in each solution is normalized with respect to that of the control in the normal artificial sea water without TFP (range 21.4-32.5 s^{-1}). The estimations were made 3 to 5 min after the onset of the perfusion. Each point is the mean of the values on 3 to 7 preparations from different mussels.

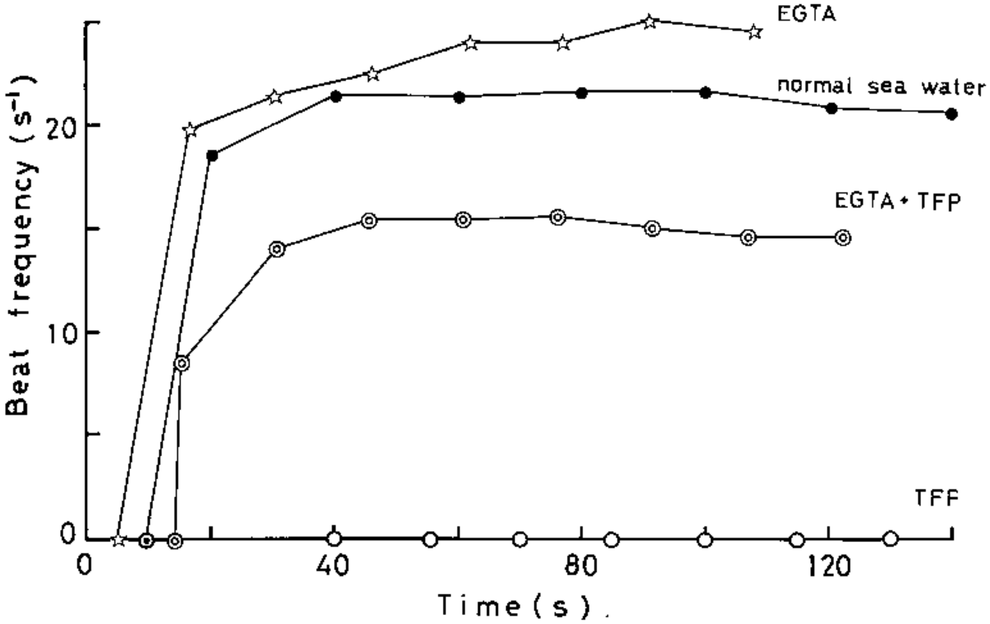

Fig. 3. Ca-dependence of the effect of trifluoperazine (TFP) on the ciliary activation by 5-HT. A typical example of a series of experiments; beat frequencies of the lateral cilia are plotted against the time in s after the onset of the perfusion with the 5-HT solutions under 4 different conditions. The cilia were activated by 5-HT (10^{-7} M) in the Ca-free artificial sea water containing 1 mM EGTA and 20μm TFP (EGTA + TFP; double circles), while 20μm TFP totally blocked the action of 5-HT when added to the normal (10 mM Ca) artificial sea water (TFP; open circles). The controls in the normal artificial sea water (normal sea water; solid circles) and in the Ca-free artificial sea water containing 1 mM EGTA (EGTA; stars) are also shown in the figure.