British Journal of Pharmacology (1996) 118, 2220–2227

Thromboxane A_2 agonist modulation of excitatory synaptic transmission in the rat hippocampal slice

[1]Kuei-Sen Hsu & Wai-Ming Kan

Department of Pharmacology, College of Medicine, National Cheng-Kung University, Tainan 701, Taiwan

1 The effects of the selective thromboxane A_2 (TXA_2) receptor agonist I-BOP on neuronal excitability and synaptic transmission were studied in the CA1 neurones of rat hippocampal slices by an intracellular recording technique.

2 Superfusion of I-BOP (0.5 μM) resulted in a biphasic change of the excitatory postsynaptic potential (e.p.s.p.), which was blocked by pretreatment with SQ 29548, a specific antagonist of TXA_2 receptors. The inhibitory phase of I-BOP on the e.p.s.p. was accompanied by a decrease in neuronal membrane input resistance.

3 The sensitivity of postsynaptic neurones to glutamate receptor agonists, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or N-methyl-D-aspartate (NMDA), was unchanged by I-BOP (0.5 μM) pretreatment.

4 Bath application of Ba^{2+} (0.5 mM) prevented both the I-BOP-induced reduction of the neuronal membrane input resistance and the blockade of e.p.s.p. induced by I-BOP.

5 Intracellular dialysis of the hippocampal CA1 neurones with GDP (10 mM) significantly attenuated the I-BOP inhibition of e.p.s.p. and membrane input resistance. Incubation of the slices with either pertussis toxin (PTX, 5 $\mu g\ ml^{-1}$ for 12 h) or cholera toxin (CTX, 5 $\mu g\ ml^{-1}$ for 12 h) did not affect the biphasic action of I-BOP on the e.p.s.p. or the reduction of membrane input resistance induced by I-BOP.

6 Pretreatment of the slices with the protein kinase C (PKC) inhibitor, NPC-15437 (20 μM), abolished the biphasic modulation by I-BOP (0.5 μM) of the e.p.s.p. Intracellular application of a specific PKC inhibitor, PKCI 19–36 (20 μM), completely inhibited the I-BOP reduction of e.p.s.p. The specific cyclic AMP-dependent protein kinase (PKA) inhibitor, Rp-cyclic adenosine 3′,5′-monophosphate (Rp-cyclic AMPS, 25 μM), had no effect on the I-BOP action.

7 In this study we have demonstrated, for the first time, the existence of functional TXA_2 receptors in the hippocampus which mediate the effects of a TXA_2 agonist on neuronal excitability and synaptic transmission. Activation of the presynaptic TAX_2 receptors may stimulate the release of glutamate. Conversely, activation of postsynaptic TXA_2 receptors leads to inhibition of synaptic transmission resulting from a decrease in the membrane input resistance of the neurones. The pre- and postsynaptic actions of the TXA_2 agonist are both mediated by PTX- and CTX-insensitive G-protein-coupled activation of PKC pathways.

Keywords: Thromboxane A_2 receptor; excitatory synaptic transmission; G-protein; protein kinase C; hippocampus

Introduction

Thromboxane A_2 (TXA_2) is the major cyclo-oxygenase metabolite of arachidonic acid (AA) which has been shown to activate platelets and to be a potent vasoconstrictor (Reilly & FitzGerald, 1993). It is synthesized by platelets in response to specific stimuli by a variety of platelet agonists (Hamberg *et al.*, 1975; FitzGerald, 1991) and acts as an amplifying signal for other platelet activators such as thrombin. Recently, it has been found that under physiological conditions, roughly 80% of the urinary metabolites of TXA_2 derive from platelets and that these metabolites are greatly increased, coincident with ischaemia, in cases of coronary instability, reflecting platelet activation (Fitzgerald *et al.*, 1986; Reilly & FitzGerald, 1993). Hence, by regulating the activity of platelets and blood vessels, TXA_2 is thought to play a pathophysiological role in cerebral ischaemia, cardiovascular disease and shock (Pickard, 1981; Reilly & FitzGerald, 1993).

The role of TXA_2 in the cerebral ischaemic event has been of interest for a long time. Previous studies have shown that the level of brain TXB_2 (non-enzymatic degradation metabolite of TXA_2) increases 55 fold in cerebral ischaemia, and it has been postulated that this contributes to postischaemic hypoperfusion and the development of cerebral injury after ischaemia. Thus, it has been proposed that the elevation of TXA_2 levels in the brain is involved in the cerebral ischaemia-induced neuronal damage associated with cell death and subsequent functional deficiency (Shohami *et al.*, 1987; Tegtmeier *et al.*, 1990; Matsuo *et al.*, 1993). Many studies have investigated the effect of cerebral ischaemia on the production of TXA_2 in the brain; however, the effects of TXA_2 on electrophysiological properties of neuronal cells have not been clarified.

Recently much evidence has been shown that neuronal death triggered by ischaemia is a result of excessive facilitation of glutamatergic synaptic transmission in the brain (Rothman & Olney, 1986; Szatkowski & Attwell, 1994). During ischaemia, the extracellular concentration of glutamate has been found to increase from the normal, low micromolar value, to several hundred micromolar (Szatkowski & Attwell, 1994). Excessive activation of glutamate receptors on the neurones may promote a flow of events eventually leading to cell depolarization, massive Ca^{2+} influx, and neuronal death. Neurones in the CA1 region of the rat hippocampus have been shown to be particularly vulnerable to ischaemia (Pulsinelli *et al.*, 1982; Smith *et al.*, 1984) and the concentration of eicosa-

[1] Author for correspondence.

noids for the duration of ischaemia was correlated with the extent of neuronal damage following the insult (Westerberg *et al.*, 1987). Hence, the purpose of the present study was to determine whether the TXA_2-induced neuronal damage during cerebral ischaemia is due to the excessive enhancement of excitatory synaptic transmission. We investigated the effect of a TXA_2 receptor agoinst on neuronal excitability and excitatory synaptic transmission in the rat hippocampus by use of an intracellular recording technique in order to elucidate its mechanism of action. We found that the biphasic action of the TXA_2 agoinst used synaptic transmission, that could be attributed to the enhancement of glutamate release at the presynaptic site, and the reduction of membrane input resistance of the postsynaptic pyramidal cells were both via PTX- and CTX-insensitive G-proteins-coupled activation of PKC pathways. Hence, we provide electrophysiological evidence that functional pre- and postsynaptic TXA_2 receptors exist in the CA1 region of rat hippocampal slices.

Methods

Slice preparation and intracellular recordings

Rat hippocampal slices (500 μm thick) were isolated from male Sprague-Dawley rats (120–150 g) for intracellular recording by the procedure described previously (Hsu *et al.*, 1995; Hsu, 1996). A submerged recording chamber was used and the superfusing artificial cerebral spinal fluid (ACSF) solution was maintained at 32 ± 1°C. The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11 (pH 7.4); oxygenated with 95% O_2-5% CO_2. The chamber consisted of a circular well of a low volume (1–2 ml) and was constantly perfused at a rate of 2–3 ml min^{-1}. In experiments involving pertussis toxin (PTX) or cholera toxin (CTX) treatment, slices were incubated in ACSF solution containing PTX (5 μg ml^{-1}) or CTX (5 μg ml^{-1}) for at least 12 h before the recordings. Control preparations were treated with the same protocol in a PTX- or CTX-free ACSF solution. This experimental protocol was modified slightly from that described by Thomson & Gahiler (1992) and Huang & Gean (1994).

Intracellular recordings were made from CA1 pyramidal neurones by use of a glass microelectrode filled with 3 M potassium acetate (80–100 MΩ). Microelectrodes were pulled from microfibre 1.0 mm capillary tubing on a Brown-Flaming electrode puller (Sutter Instrument, San Rafael, CA, U.S.A.). A bipolar stimulating electrode was positioned in the stratum radiatum. Orthodromic stimuli were delivered with monophasic constant voltage pulse at 0.033 Hz from a Grass stimulator with an isolation unit. The stimulus intensity was adjusted to subthreshold for action potential initiation. Electrical signals were amplified by an Axoclamp-2A amplifier (Axon Co.) connected to a Data-6100 digital oscilloscope (Data Precision Co.) for data analysis and subsequent plotting of fast transient potential.

Statistical analysis

Throughout, results are expressed as mean ± s.e.mean. The significance of difference was evaluated by Student's two-tailed paired *t* test. Numbers of experiments are indicated by *n*. Probability values (*P*) of less than 0.05 were considered to be significant.

Drugs

[1S-(1α, 2β(5Z), 3α(1E, 3S*)]-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxabicyclo [2, 2, 1] heptan-2-yl]-5-heptenoic acid (I-BOP) and [1S-(1α, 2β(5Z), 3β, 4α)]-7-[3-(3-([2-(phenylamino) carbonyl] hydrazino)methyl)]-7-oxabicyclo [2, 2, 1] heptan-2-yl]-5-heptenoic acid (SQ 29548) were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). R-2, 6-diamino-N-[[1-(1-oxotridecy)-2-[piperidinyl]-methyl]-hexanamide dihydrochloride (NPC-15437), H-Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn-OH (PKCI 19-36) and guanosine 5'-diphosphate (GDP) were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). (±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), N-methyl-D-aspartic acid (NMDA), Rp-cyclic adenosine 3',5'-monophosphate (Rp-cyclic AMPS), baclofen, DL-amino-5-phosphonovaleric acid (DL-APV), tetrodotoxin and $BaCl_2$ were obtained from Sigma (St. Louis, MO, U.S.A.). All drugs were freshly prepared from stock solutions, dissolved in ACSF of known concentration. I-BOP was dissolved in ethanol as stock solution and kept frozen at −20°C until the day of the experiment. The concentration of ethanol in the perfusion medium ranged from 0.02 ~ 0.12%. In four control experiments, ethanol of these concentrations did not affect either the active or the passive properties of the recording neurones. PKCI 19-36 and GDP dissolved in the 3 M potassium acetate solution were applied intracellularly by allowing them to leak from the recording microelectrode for at least 40 min, according to the method described by Cerne *et al.* (1992).

Results

Effects of TXA_2 agonist on the excitatory postsynaptic potential, (e.p.s.p.)

The effects of the TXA_2 agonist on neuronal excitability and exitatory synaptic transmission were studied in the CA1 region of rat hippocampal slices by use of an intracellular recording technique. The data presented here were obtained from 168 stable intracellular recordings from rat hippocampal neurones with resting membrane potential more negative than −55 mV and action potential amplitude greater than 70 mV. The resting membrane potential and neuronal input resistance were -64.8 ± 0.6 mV ($n=42$) and 42.7 ± 1.2 MΩ ($n=42$), respectively.

Figure 1a shows an example of an intracellular recording of excitatory postsynaptic potential (e.p.s.p.) evoked by orthodromic stimulation of the Schaffer collateral-commissural pathway. In our previous study, we identified that this depolarizing synaptic response is mediated by both NMDA (~10%) and non-NMDA (~90%) glutamate receptors on the hippocampal CA1 neurones (Hsu *et al.*, 1995; Hsu, 1996). Superfusion of a specific TXA_2 agonst, I-BOP (0.5 μM) (Morinelli *et al.*, 1989), resulted in a biphasic change of the e.p.s.p. The e.p.s.p. significantly increased (168.0 ± 5.3%, $P<0.001$) during the initial 2–10 min and then decreased to 29.0 ± 5.7% ($P<0.001$) of the control ($n=12$). The initial increase of the e.p.s.p. occurred without any change in neuronal membrane potential or membrane input resistance. In contrast to the enhancement phase, the inhibition by I-BOP of the e.p.s.p. was accompanied by alternation of the passive cellular properties, i.e. a membrane hyperpolarization from -65.6 ± 0.5 mV to -75.8 ± 1.1 mV ($n=12$, data not shown) and a decrease in neuronal membrane input resistance from 41.9 ± 0.8 MΩ to 21.4 ± 1.2 MΩ ($n=12$) (Figure 1). The e.p.s.p. recovered within minutes after withdrawal of I-BOP, suggesting the reversibility of the effect of I-BOP. The biphasic action of I-BOP (0.1–3 μM) on the e.p.s.p. was dose-dependent (Figure 2a). The mean I-BOP modulation of the e.p.s.p. at steady state was (% of control ± s.e.mean): 0.1 μM, enhancement phase 132.1 ± 4.1% and inhibitory phase 110.1 ± 3.2% ($n=6$); 0.5 μM, enhancement phase 168.0 ± 5.3% and inhibitory phase 29.0 ± 5.7% ($n=12$); 3 μM, enhancement phase 121.3 ± 2.3% and inhibitory phase 11.3 ± 2.3% ($n=8$). Application of I-BOP at a low concentration (0.1 μM) produced only an increase of the e.p.s.p. amplitude. However, higher concentrations of I-BOP (0.5 and 3 μM) produced more pronounced depression of the e.p.s.p. Superfusion of SQ 29548 (5 μM), a recently developed high affinity antagonist of TXA_2

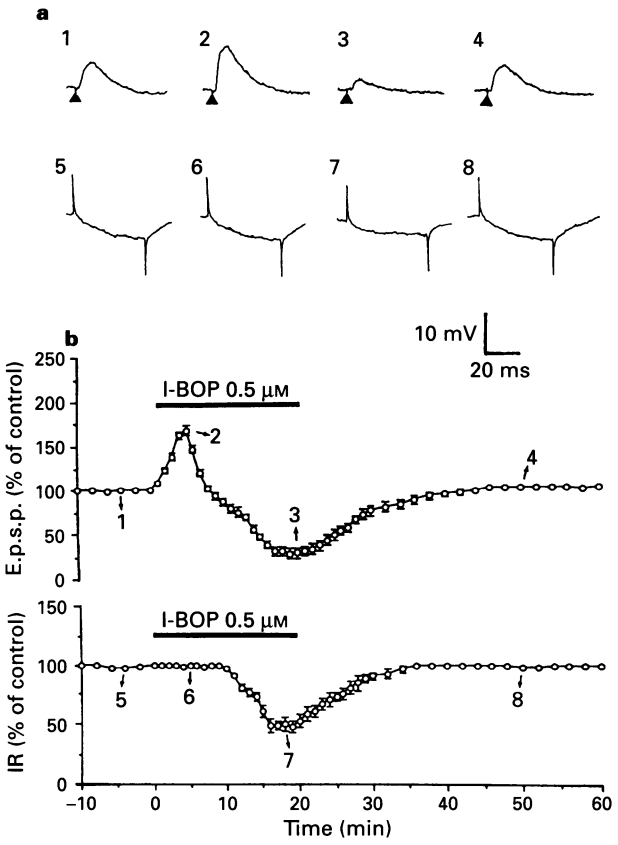

Figure 1 Effect of I-BOP on synaptic transmission. (a) A depolarizing excitatory postsynaptic potential (e.p.s.p.) could be evoked by the electrical stimulation of Schaffer collateral-commissural pathway. A hyperpolarizing current pulse was passed through the recording electrode (−0.2 nA, 50 ms) to monitor the membrane input resistance. The effect of I-BOP (0.5 μM) on the e.p.s.p. was biphasic and reversible. (b) Time course of the effect of 0.5 μM I-BOP on the e.p.s.p. Note that the biphasic change of the response is primary facilitation followed by depression of the e.p.s.p. The inhibition by I-BOP of the e.p.s.p. was accompanied by a substantial decrease in the membrane input resistance (IR). Filled triangles represent the point of synaptic stimulation. Bars denote the period of I-BOP application. Data are presented as mean ± s.e.mean ($n = 12$).

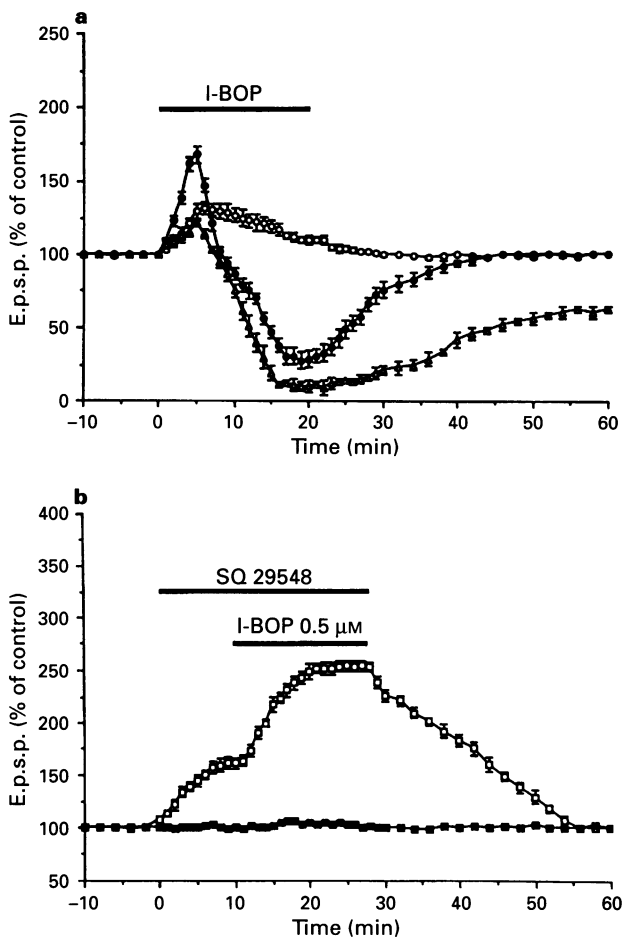

Figure 2 I-BOP modulates the e.p.s.p. in a dose-dependent manner which is blocked by SQ 29548. (a) The percentage changes of the e.p.s.p. are plotted as a function of time. The slices were superfused with I-BOP 0.1 μM (○), 0.5 μM (●) and 3 μM (△) for 20 min. Note that the higher concentration of I-BOP produced more pronounced depression of the e.p.s.p. (b) The modulation of the e.p.s.p. induced by I-BOP was blocked by SQ 29548 5 μM (□) and 20 μM (■), a selective TXA_2 receptor antagonist. Bars denote the period of application of the drugs. Data are presented as mean ± s.e.mean.

receptors (Morinelli *et al.*, 1989), produced a significant increase in the e.p.s.p. (153.2 ± 2.7% of control, $P < 0.001$) and completely blocked the reduction by I-BOP (0.5 μM) of the e.p.s.p. ($n = 6$) (Figure 2b). Application of a low concentration of SQ 29548 (5 μM) had little effect on the enhancement phase of the e.p.s.p. amplitude induced by I-BOP. However, at a high concentration (20 μM), SQ 29548 abolished both the enhancement and the depression of the e.p.s.p. induced by I-BOP (0.5 μM, $n = 7$) (Figure 2b), suggesting that SQ 29548 was not equally effective as an antagonist of the pre- and postsynaptic action of I-BOP. In addition, the I-BOP (0.5 μM)-induced reduction of the membrane input resistance was also abolished by SQ 29548 (5 μM) ($n = 8$, data not shown). These data demonstrate that the modulation of e.p.s.p. and membrane input resistance induced by I-BOP on hippocampal neurones is mediated by the activation of TXA_2 receptors.

To determine whether the biphasic action on e.p.s.p. induced by I-BOP is mediated by a pre- or postsynaptic mechanism, we examined the effects of I-BOP on the postsynaptic responses to exogenously applied AMPA and NMDA. The experiments were performed in the presence of tetrodotoxin (0.5 μM). As illustrated in Figure 3, application of AMPA (10 μM) or NMDA (10 μM) produced a membrane depolarization, 22.6 ± 3.6 mV ($n = 6$) and 20.5 ± 4.5 mV ($n = 6$), respectively. I-BOP (0.5 μM) pretreatment did not affect the AMPA-induced (21.5 ± 3.2 mV, $n = 6$) or NMDA-induced membrane depolarization (21.3 ± 3.1 mV, $n = 6$) (Figure 3) These results in the slice experiments were also confirmed in dissociated neurones; I-BOP (0.5 μM) had no significant effect on the membrane inward currents induced by pressure application of AMPA (100 μM, 98.7 ± 1.1%, $n = 8$) or NMDA (100 μM, 96.7 ± 2.6%, $n = 6$) on the acutely dissociated hippocampal CA1 neurones measured by use of whole-cell voltage-clamp recording at a holding potential of −60 mV (data not shown). These data suggest that the biphasic action of I-BOP on the e.p.s.p. in hippocampal CA1 neurones is not mediated by a postsynaptic action on either AMPA or NMDA receptors.

To determine whether the activation of postsynaptic potassium conductance is involved in the reduction of membrane input resistance induced by I-BOP, a specific inhibitor of inward rectifier K^+ channels, Ba^{2+} (Uchimura & North, 1990), was used. As depicted in Figure 4a, the effectiveness of Ba^{2+} treatment was confirmed by the finding that baclofen (10 μM), a specific γ-aminobutyric acid $(GABA)_B$ receptor agonist, failed to produce neuronal membrane hyperpolarization or decrease the membrane input resistance. In 4 untreated neurones, superfusion with 10 μM baclofen caused a −8.9 ± 1.2 mV membrane hyperpolarization. In the presence of Ba^{2+} (0.5 mM), baclofen produced little hyperpolarization (−0.3 ± 0.1 mV, $n = 6$). The effect of Ba^{2+} on the I-BOP action is summarized in Figure 4a. Bath application of Ba^{2+} (0.5 mM)

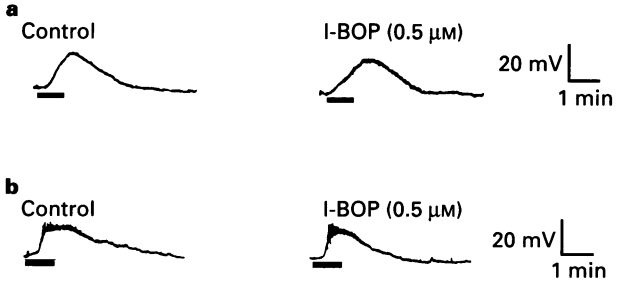

Figure 3 Effect of I-BOP on (a) AMPA- and (b) NMDA-induced membrane responses. Superfusion of either (a) AMPA (10 μM, 1 min) or (b) NMDA (10 μM, 1 min) evoked a membrane depolarization. Pretreatment of the slices with I-BOP (0.5 μM) for 20 min did not significantly affect either AMPA- or NMDA-induced depolarization. The experiment was performed in the presence of tetrodotoxin (0.5 μM). Bars denote the period of drug application.

prevented the reduction by I-BOP (0.5 μM) of the e.p.s.p. and the membrane input resistance (from 49.0±2.8% to 2.3±0.7%, $n=6$); however Ba^{2+} had no effect on the enhancement phase of the e.p.s.p. induced by I-BOP ($n=6$) (Figure 4a). These data suggest that the mechanism underlying the inhibitory effects of I-BOP on the e.p.s.p. and the membrane input resistance may be due to an increase in the postsynaptic potassium conductance and hypolarization of postsynaptic neurones.

TXA_2 agonist modulates synaptic transmission via a G-protein coupled mechanism

It has been claimed that the TXA_2 receptor is linked via a pertussis toxin (PTX)- and cholera toxin (CTX)-insensitive G-protein (Reilly & FitzGerald, 1993). Thus, G-proteins could be involved in I-BOP action on neuronal excitability and synaptic transmission in the hippocampus. This hypothesis was tested by studying the internal dialysis of hippocampal neurones with GDP, an inhibitor of G-proteins (Kucera & Rittenhouse, 1988;

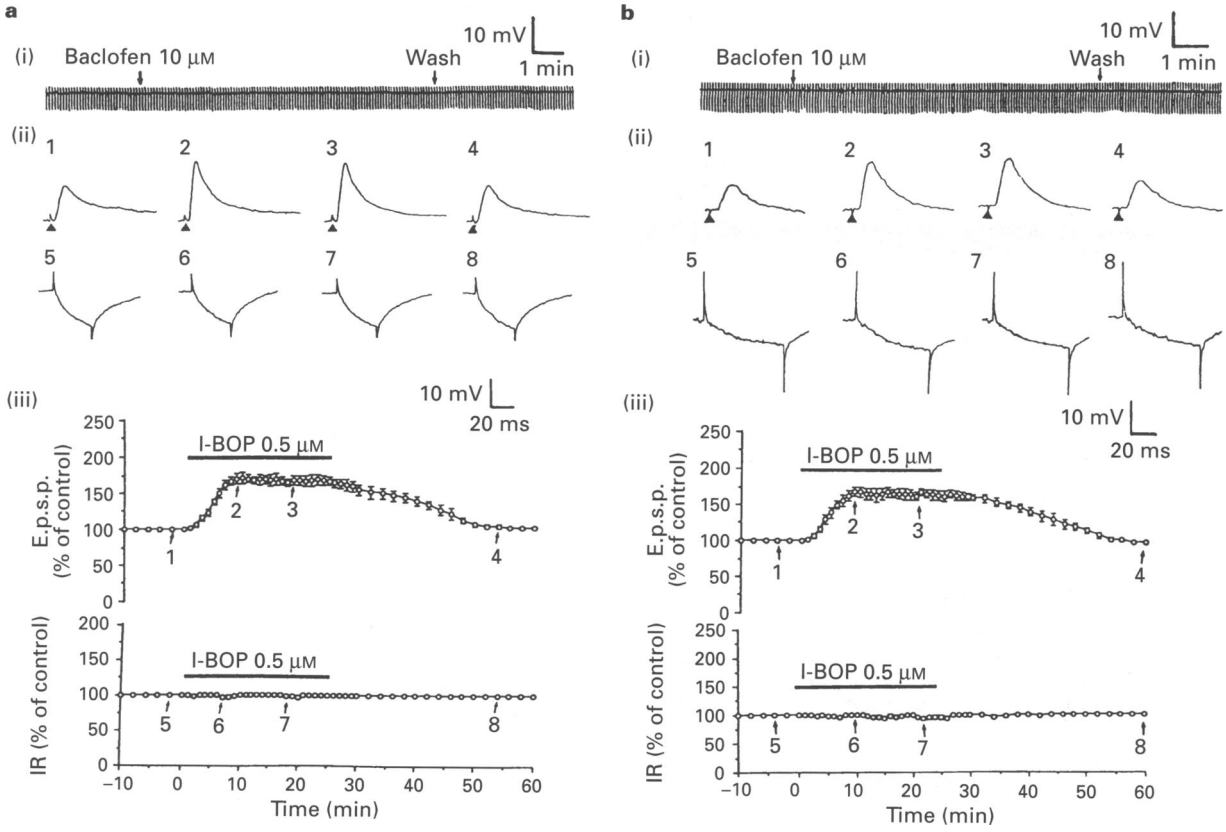

Figure 4 Effects of barium (Ba^{2+}) and GDP on the biphasic action of I-BOP on the synaptic transmission. (a(i)) Chart record illustrating the effect of baclofen (10 μM) on a hippocampal neurone in the presence of Ba^{2+} (0.5 mM). During the experiment, −0.2 nA hyperpolarizing current pulses were injected to monitor the membrane input resistance. The results indicate that baclofen failed to affect the membrane potential and the input resistance on the hippocampal neurone in the presence of Ba^{2+}. During the recording, 10 μM DL-APV was applied in the perfusing ACSF solution to reduce the Ba^{2+}-induced increase in background spontaneous synaptic activity. (a(ii) and a(iii)) Sixty minutes after washout of baclofen with Ba^{2+} (0.5 mM)- and DL-APV (10 μM)-containing ACSF solution, application of I-BOP (0.5 μM) to the same cell resulted in an increase in the e.p.s.p. amplitude but failed to change the neuronal membrane input resistance (IR). This indicates that Ba^{2+} significantly attenuated the I-BOP-induced depression of the e.p.s.p. and the membrane input resistance. (b(i)) Chart record illustrating the effect of baclofen (10 μM) on the hippocampal neurone after intracellular application of GDP (10 mM) for 40 min. During application of baclofen, −0.2 nA hyperpolarizing current pulses were injected to monitor the membrane input resistance. Baclofen failed to affect the membrane potential and the input resistance on the hippocampal neurone intracellularly dialyzed with GDP (10 mM). (b(ii)) After application of I-BOP (0.5 μM) to the same cell as shown in the b(i), there was an increase in the e.p.s.p. but no change in the neuronal membrane input resistance. (b(iii)) Intracellular application of GDP (10 mM, $n=8$) significantly abolished the inhibition of the e.p.s.p. and the input resistance induced by I-BOP (0.5 μM). In contrast, the enhancement effect of I-BOP on the e.p.s.p. remained unaffected. Solid triangles represent the point of stimulation. Bars denote the period of I-BOP application. Data are presented as mean±s.e.mean. The changes of the e.p.s.p. and the membrane input resistance (IR) induced by I-BOP are expressed as a percentage of control.

Orellana *et al.*, 1989; Doroshenko, 1991). During dialysis of the hippocampal neurones with 10 mM GDP which prevents the activation of G-proteins, the depression of the e.p.s.p. and membrane input resistance induced by I-BOP (0.5 μM) were abolished in all eight neurones tested (Figure 4b). This demonstrates that the inhibition by I-BOP of the e.p.s.p. and membrane input resistance occurs through the activation of GTP-binding proteins. The effectiveness of GDP treatment was confirmed by the finding that baclofen, a specific $GABA_B$ receptor agonist (10 μM), failed to produce neuronal membrane hyperpolarization or decrease the membrane input resistance (Huang & Gean, 1994). To determine the identity of the G-proteins that mediate the I-BOP action on synaptic transmission, hippocampal slices were pretreated with either PTX (5 $\mu g\,ml^{-1}$) or CTX (5 $\mu g\,ml^{-1}$) for 12 h (Huang & Gean, 1994; Thomson & Gahiler, 1992). The effects of I-BOP on e.p.s.p. and the input resistance in PTX- and CTX-pretreatment hippocampal slices are summarized in Figure 5a and b. As shown in Figure 5a(i), both the membrane hyperpolarizing and the membrane input resistance decreasing effects normally produced by baclofen (10 μM) were blocked in hippocampal CA1 neurones of PTX-pretreated slices. This indicates that the activity of G-proteins expressed in neurones, which are sensitive to PTX (such as G_i or G_0), was blocked by our pretreatment protocol. During superfusion of 0.5 μM I-BOP, the e.p.s.p. amplitude significantly increased (158.7±3.7%) during the initial 2–10 min and then decreased to 36.4±3.9% in PTX-pretreated neurones ($n=10$). In addition, the membrane input resistance was reduced from 42.7±1.2 MΩ to 22.3±0.9 MΩ in the presence of 0.5 μM I-BOP ($n=10$). Therefore, PTX treatment did not affect the action of I-BOP. Incubation of the slices with CTX (5 $\mu g\,ml^{-1}$) also did not change the biphasic modulation of the e.p.s.p. induced by I-BOP. In the CTX-pretreated slices, the initial increase of the e.p.s.p. induced by I-BOP (0.5 μM) was 161.3±4.7% and the inhibitory phase was 38.2±3.2% of the control ($n=10$). Moreover, the membrane input resistance was reduced from 43.2±1.1 MΩ to 21.6±0.7 MΩ in the presence of I-BOP (0.5 μM, $n=10$) in the hippocampal neurones of CTX-pretreated slices (Figure 5b). Hence as neither PTX ($n=10$) nor CTX ($n=10$) appeared to affect the I-BOP (0.5 μM)-induced modulation of synaptic transmission in the hippocampus, this effect of I-BOP is mediated by PTX- and CTX-insensitive G-proteins.

The involvement of protein kinase C involves in the agonist action of TXA_2

It has been well documented that TXA_2 receptor stimulation in platelets is linked through a G-protein to phospholipase C (PLC) activation which hydrolyzes membrane phosphoinositides, resulting in the release of two intracellular messengers,

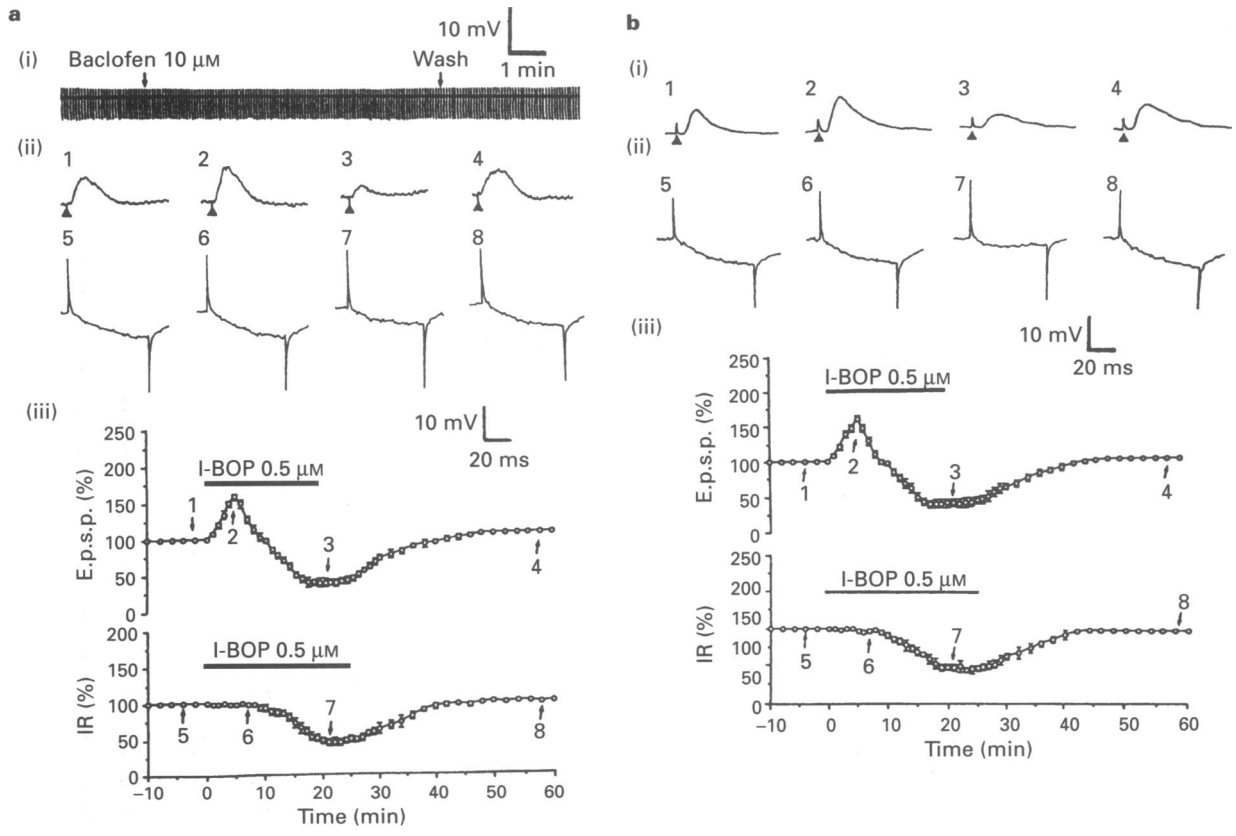

Figure 5 Effects of pertussis toxin (PTX) and cholera toxin (CTX) on the biphasic action of I-BOP on synaptic transmission. (a(i)) Chart record illustrating the effect of baclofen (10 μM) on a hippocampal neurone which was exposed to PTX (5 $\mu g\,ml^{-1}$) for 12 h. During the recording, −0.2 nA hyperpolarizing current pulses were injected to monitor the membrane input resistance. Baclofen did not affect the membrane potential and the input resistance on neurones pretreated with PTX. (a(ii)) Application of I-BOP (0.5 μM) to the same cell as shown in a(i) resulted in a biphasic modulation of the e.p.s.p. amplitude and a reduced membrane input resistance. (a(iii)) PTX-pretreatment (5 $\mu g\,ml^{-1}$, $n=10$) did not affect the modulation of the e.p.s.p. and the depression of neuronal membrane input resistance induced by I-BOP (0.5 μM). (b(i) and b(ii)) Neither the biphasic action on the e.p.s.p. nor the depression of membrane input resistance induced by I-BOP was affected by the CTX-pretreatment (5 $\mu g\,ml^{-1}$, $n=10$). The hippocampal slices were pretreated with PTX (5 $\mu g\,ml^{-1}$) and CTX (5 $\mu g\,ml^{-1}$) for 12 h before the recordings. Solid triangles represent the point of stimulation. Bars denote the period of I-BOP application. Data are presented as mean±s.e.mean. The changes of the e.p.s.p. and the membrane input resistance (IR) reduced by I-BOP are expressed as the percentage of control.

inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Reilly & FitzGerald, 1993). These, in turn, release intracellular Ca^{2+} and stimulate protein kinase C (PKC), respectively. Thus, PKC activation could be involved in the I-BOP modulation of the e.p.s.p. and the membrane input resistance on the hippocampal neurones. This hypothesis was tested by pretreatment of the hippocampal slices with the PKC inhibitor, NPC-15437 (Sullivan *et al.*, 1992). As shown in Figure 6a, superfusion of NPC-15437 (20 μM) resulted in a transient reduction of the e.p.s.p. and subsequent application of 0.5 μM I-BOP failed to affect the e.p.s.p. in all 6 slices tested ($105.0 \pm 2.3\%$, $n=6$, $P<0.001$). In addition, the reduction of neuronal membrane input resistance induced by I-BOP (0.5 μM) was also abolished by the pretreatment with NPC-15437 (20 μM, $n=6$) (data not shown). Further evidence supporting the hypothesis that PKC activation is involved in the depression by I-BOP of the e.p.s.p. and the membrane input resistance was obtained by internal application of the specific PKC inhibitor, PKCI 19–36 (20 μM) to the hippocampal CA1 neurones; this abolished the I-BOP induced inhibition of the e.p.s.p. ($n=8$) (Figure 6b). In contrast, the enhancement effect of I-BOP on the e.p.s.p. was significantly potentiated by internal dialysis of PKCI 19–36 (20 μM) (from $168.0 \pm 5.3\%$ to $212.3 \pm 3.9\%$, $n=6$, $P<0.001$) (Figure 6b). These data indicate that both the presynaptic enhancement and the postsynaptic inhibition of excitatory synaptic tranmission induced by I-BOP are mediated by the activation of PKC pathways. To test whether the activation of protein kinase A (PKA) is involved in the effect of I-BOP; Rp-cyclic AMPS (a potent inhibitor of PKA) was used (Rothermel *et al.*, 1984). As shown in Figure 6c, application of Rp-cyclic AMPS (25 μM) alone decreased the e.p.s.p. below baseline values ($89.7 \pm 2.1\%$ of the control, $n=6$) but failed to affect the biphasic action of I-BOP (0.5 μM) on e.p.s.p., suggesting that PKA activation is not involved in the effects of I-BOP on hippocampal CA1 neurones.

Discussion

In this study, we have provided the first electrophysiological evidence that functional pre- and postsynaptic TXA_2 receptors exist in the CA1 region of rat hippocampal slices. The TXA_2 agonist at a low concentration (0.1 μM) produced enhancement of the e.p.s.p.; however, at higher concentrations (0.5 and 3 μM), it could produce a biphasic change of the e.p.s.p. The biphasic effect of the TXA_2 agonist on the excitatory synaptic transmission has been attributed to the enhancement of glutamate release at the presynaptic site and the reduction of membrane input resistance of the postsynaptic pyramidal cell via PTX- and CTX-insensitive G-proteins-coupled to activation of the PKC pathway.

Mechanism of action of pre- and postsynaptic TXA_2 receptors

The results of the present study provide evidence that the initial increase of the e.p.s.p. amplitude induced by the TXA_2 agonist (I-BOP, 0.5 μM) was due to an increase of glutamate release from presynaptic terminals. Two sets of data support this conclusion. Firstly, the initial increase of the e.p.s.p. induced by I-BOP (0.5 μM) occurred with negligible changes in resting membrane potential (RMP) or input resistance of the postsynaptic neurones. Secondly, postsynaptic responses induced by AMPA and NMDA in the slice preparation and in acutely dissociated hippocampal CA1 neurones were unchanged by I-BOP (0.5 μM) pretreatment. The mechanism underlying the increase in the evoked release of glutamate through the activation of presynaptic TXA_2 receptors remains to be established. The most likely possibility is that the activation of PKC by I-BOP (see below) may directly phosphorylate voltage-dependent Ca^{2+} channels and increase the activity of Ca^{2+} channels, resulting in an increased interterminal Ca^{2+} level and the release of glutamate. We cannot

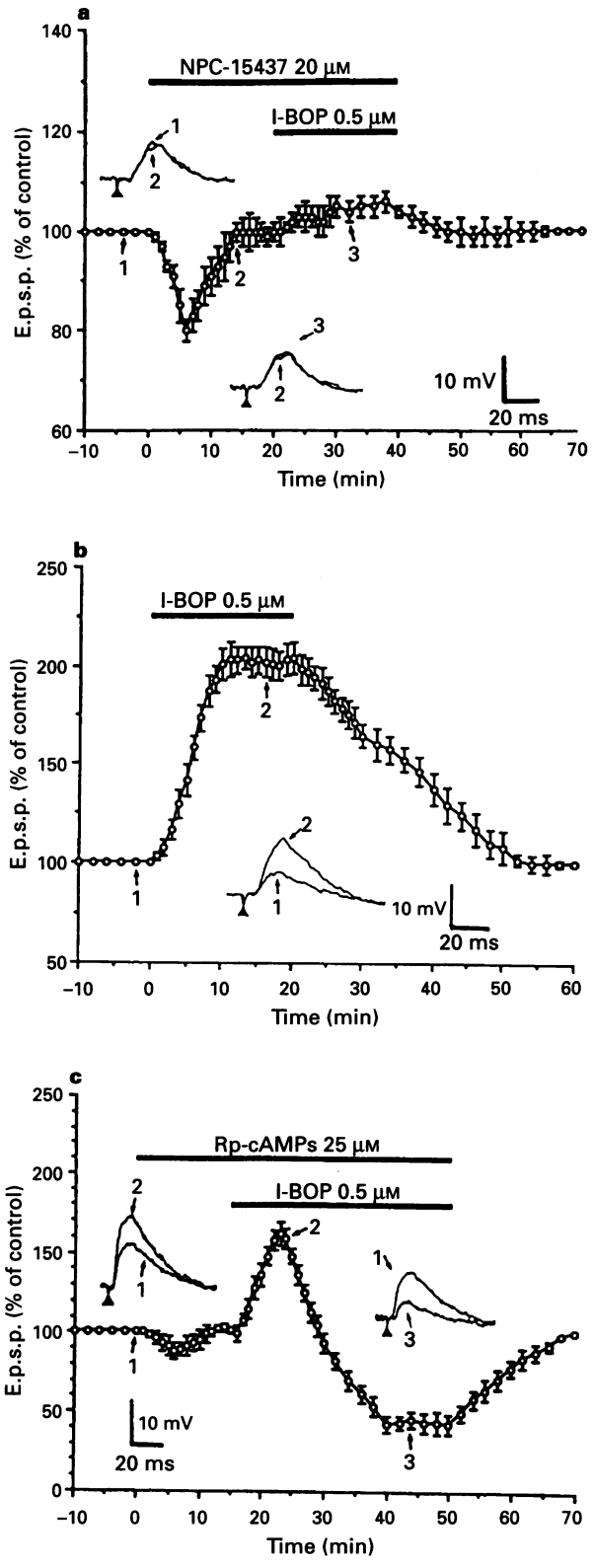

Figure 6 Protein kinase C (PKC) inhibitors block the modulation of I-BOP on the synaptic transmission. (a) In the presence of a specific PKC inhibitor, NPC-15437 (20 μM, $n=6$), the I-BOP modulation of the e.p.s.p. was significantly attenuated. (b) Intracellular application of PKCI 19–36 (20 μM, $n=6$) abolished the inhibition by I-BOP of the e.p.s.p. but potentiated the enhancement effect of I-BOP. (c) Pretreatment with the protein kinase A (PKA) inhibitor, Rp-cyclic AMPS (25 μM, $n=6$), did not significantly affect the biphasic action of I-BOP (0.5 μM) on the e.p.s.p. Solid triangles represent the point of synaptic stimluation. Bars denote the period of delivery of different treatment as indicated. Data are presented as mean ± s.e.mean.

rule out the possibility that PKC activation may alter the release process by changing the properties of synaptic vesicle proteins, or by altering some other aspect of the vesicular fusion mechanism described by Parfitt & Madison (1993) in rat hippocampus. Our results are in agreement with the earlier suggestion that PKC activator, phorbol esters, enhance the synaptic transmission in hippocampal slices via a presynaptic mechanism. It has shown that phorbol esters enhance synaptic transmission without changing the sensitivity of postsynaptic pyramidal cells to iontophoretically applied glutamate (Malenka *et al.*, 1986). On the other hand, the decrease of the membrane input resistance caused by the activation of the postsynaptic TXA_2 receptors certainly contributes to depression of the e.p.s.p. induced by I-BOP. This action alone could account for the depression of the excitatory synaptic transmission that was produced by I-BOP. Firstly, in virtually every neurone tested, depression of the e.p.s.p. by I-BOP persisted at time when I-BOP-induced decreases in the membrane input resistance were detectable. Secondly, Ba^{2+} (0.5 mM), a specific inhibitor of inward rectifier K^+ channels (Uchimura & North, 1990), prevented both the I-BOP-induced reduction of the membrane input resistance of hippocampal CA1 neurones and the depression of the e.p.s.p. induced by I-BOP. Thirdly, dialysis of the hippocampal neurones with GDP (10 mM) or PKCI 19–36 (20 μM) which prevented the I-BOP-induced depression of the membrane input resistance, also abolished the depression of the e.p.s.p. induced by I-BOP in all neurones tested. All of these results suggest that I-BOP reduces excitatory synaptic transmission of rat hippocampal CA1 neurones via its depression of the membrane input resistance.

Mediation of the TXA_2 agonist action by G-proteins

The possible involvement of G-proteins in the inhibition by I-BOP of the neuronal excitability and the synaptic transmission was tested by intracellular dialysis of the postsynaptic pyramidal cells with GDP. Internal dialysis of 10 mM GDP, which inhibits the activation of G-proteins (Orellana *et al.*, 1987), resulted in the loss of the reduction of e.p.s.p amplitude and membrane input resistance induced by I-BOP (0.5 μM) (Figure 4a). This demonstrates the involvement of a G-protein-coupled mechanism in the reduction of the synaptic transmission and the neuronal excitability induced by postsynaptic TXA_2 receptor activation. Pertussis toxin (PTX) and cholera toxin (CTX) are popularly used for implicating G-proteins in specific functions (Birnbaumer, 1992). In this study, to determine the identity of the G-proteins that mediate the I-BOP-induced modulation of neuronal excitability and synaptic transmission, the hippocampal slices were pretreated with either PTX (5 $\mu g\ ml^{-1}$) or CTX (5 $\mu g\ ml^{-1}$) for 12 h (Thomson & Gahiler, 1992; Huang & Gean, 1994). We found that neither PTX- nor CTX-pretreatment affected the actions of I-BOP on the e.p.s.p. and neuronal membrane input resistance. This suggests that the TXA_2 agonist modulates the neuronal excitability and the synaptic transmission in the CA1 region of the rat hippocampal slices via a PTX- and CTX-insensitive G-protein. Hence, the mechanism of action of the TXA_2 agonist in the hippocampus is similar to that previously found by Carlson *et al.* (1989) and Takahara *et al.* (1990) in human platelets; they showed that TXA_2-induced platelet shape change, secretion and aggregation occurred via a PTX- and CTX-insensitive G-protein. This G-protein has not been fully characterized, but is thought to belong to the G_z or the G_q class of G-proteins.

The involvement of PKC in the pre- and postsynaptic activation of TXA_2 receptors

To determine whether TXA_2 agonist activation of a G-protein(s) and coupling, which result in the modulation of neuronal excitability and the synaptic transmission, involves intracellular mediators such as protein kinase C (PKC) and a cyclic AMP-dependent protein kinase (PKA), we examined the effects of two specific inhibitors of PKC and one specific inhibitor of PKA on the action of I-BOP. We found that NPC-15437, a specific membrane permeable inhibitor of PKC, significantly abolished the effect of I-BOP on the e.p.s.p. (Sullivan *et al.*, 1991; 1992). Further evidence supporting the role of PKC activation in the effect of I-BOP on the e.p.s.p. was obtained by intracellular application of PKCI 19–36, a synthetic pseudosubstrate inhibitor of PKC (Bell & Burns, 1991). The mechanism of action by which the enhancement effect of I-BOP on the e.p.s.p amplitude was potentiated by internal dialysis of the pyramidal cells with PCKI 19–36 is not yet resolved and remains to be elucidated. Recently, it has been shown that PKA activation may also be indirectly ivolved in the TXA_2 receptor activation (Heemskert *et al.*, 1994). To test this possibility we examined the effect of a specific inhibitor of PKA on the action of I-BOP. The specific membrane-permeable and competitive inhibitor of PKA (Dostmann *et al.*, 1990; Frey *et al.*, 1993), Rp-cyclic AMPS which competes with cyclic AMP for the binding site on the regulatory subunit of PKA, was used. Pretreatment of the hippocampal slices with Rp-cyclic AMPS had no effects on the biphasic modulation by I-BOP of the e.p.s.p. Taken together, these data suggest that the biphasic modulation by I-BOP of the e.p.s.p. and the reduction of membrane input resistance by I-BOP on the hippocampal CA1 neurones are mediated by activation of PKC but not of PKA. It is clear that the pre- and postsynaptic TXA_2 receptors share a pharmacologically indistinguishable intracellular mechanism (PKC activation) to exert their actions in the hippocampus.

In the present study, we demonstrated that TXA_2 receptors exist on the hippocampal neurones; but the kind of cells supplying TXA_2 to the neurones remain to be elucidated. Pearce *et al.* (1989) showed that ATP released TXA_2 from rat astrocytes via activation of purinoceptors and similar results were also confirmed by Nakahata *et al.* (1992) with rabbit astrocytes. Therefore, the cell supplying TXA_2 to neurones might be astrocytes. Moreover, the brain vascular bed might be another source of TXA_2 for neurones, since Shirahata *et al.* (1988) indicated that endothelium-derived contracting substance in brain vascular bed might be a TXA_2-like substance.

In conclusion, our findings suggest that the TXA_2 agonist, I-BOP regulates the excitatory synaptic transmission in the hippocampus by a complex neuromodulatory mechanism. I-BOP, at a low concentration enhances glutaminergic synaptic transmission in the hippocampal CA1 neurones primarily by increasing neurotransmitter release from presynaptic terminals. This effect probably represents the significant mechanisms of TXA_2-induced neuronal death in cerebral ischaemia, since the excessive facilitation of glutaminergic synaptic transmission in the brain may promote a flow of events eventually leading to cell depolarization, massive Ca^{2+} influx, and neuronal death (Rothman & Olney, 1986; Szatkowski & Attwell, 1994). Conversely, at higher concentrations, I-BOP reduced the membrane input resistance of the hippocampal CA1 neurones and produce a depression of the excitatory synaptic transmission. These effects may result in the reduction of neuronal excitability which then affect higher brain functions including learning and memory. It has been found that when the membrane input resistance is reduced by drugs (e.g. $GABA_B$ receptor against baclofen), a decrease in inhibitory transmission as well as excitatory transmission is observed (Thompson & Gahwiler, 1992). In the present study, we did not test the effect of I-BOP on the inhibitory synaptic transmission in the hippocampal CA1 neurones. Whether the TXA_2 agonist could also modulate the inhibitory synaptic transmission remains unknown. Additional studies are required to test this possibility.

We are grateful to Dr C.C. Huang for critical comments on the manuscript. This work was supported by research grants from National Science Council, Taiwan, R.O.C. (NSC 84-2331-B006-106 and NSC 85-2331-B006-095).

References

BELL, R. & BURNS, D. (1991). Lipid activation of protein kinase C. *J. Biochem.*, **266,** 4661–4664.

BIRNBAUMER, L. (1992). Receptor-to effector signalling through G protein: roles for $\beta\gamma$ dimers as well as α subunits. *Cell*, **71,** 1069–1072.

CARLSON, K.E., BRASS, L.F. & MANNING, D.R. (1989). Thrombin and phorbol esters cause the selective phosphorylation of a G protein other than G_i in human platelet. *J. Biol. Chem.*, **264,** 13298–13305.

CERNE, R., JIANG, M. & RANDIC, M. (1992). Cyclic adenosine-3',5'-monophosphate potentiates excitatory amino acid and synaptic responses of rat dorsal horn neurons. *Brain Res.*, **596,** 111–123.

CHEN, S.T., HSU, E.L., HOGAN, E.L., HALUSHKA, P.V., LINET, O.I. & YATSU, F.M. (1986). Thromboxane, prostacyclin, and leukotrienes in cerebral ischemia. *Neurology*, **36,** 446–470.

DOROSHENKO, P. (1991). Second messengers mediating activation of chloride current by intracellular GTP-γ-S in bovine chromaffin cells. *J. Physiol.*, **436,** 725–738.

DOSTMANN, W.R.G., TAYLOR, S.S., GENIESER, H.H., JASTEROFF, B., DOSKELOND, S.O. & OGRIED, D. (1990). Probing the nucleotide binding sites of cAMP-dependent protein kinases I and II with analogs of adenosine 3',5'-cyclic phosphothioates. *J. Biol. Chem.*, **265,** 10484–10491.

FITZGERALD, D.J., CATELLA, F. & FITZGERALD, G.A. (1986). Platelet activation in unstable coronary disease. *N. Engl. J. Med.*, **315,** 983–989.

FITZGERALD, G.A. (1991). Mechanisms of platelet activation; thromboxone A_2 as an amplifying signal for other agonists. *Am. J. Cardiol.*, **68,** 11B–15B.

FREY, U., HUANG, Y.Y. & KANDEL, E.E. (1993). Effects of cAMP stimluate a late stage of LTP in hippocampal neurons. *Science*, **260,** 1661–1664.

HAMBERG, M., SVENSSON, J. & SAMUELSSON, B. (1975). Thromboxanes- a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.*, **75,** 2294–2298.

HEEMSKERT, J.W., FEIJGE, M.A., SAGE, S.O. & WALTER, U. (1994). Indirect regulation of Ca^{2+} entry by cAMP-dependent and cGMP-dependent protein kinases and phospholipase C in rat platelets. *Eur. J. Biochem.*, **223,** 543–551.

HSU, K.S. (1996). Characterization of dopamine receptors mediating inhibition of excitatory synaptic transmission in the rat hippocampal slice. *J. Neurophysiol.*, (In press).

HSU, K.S., HUANG, C.C. & GEAN, P.W. (1995). Voltage- and use-dependent block by 1-methyl-4-phenylpyridinium ion (MPP^+) of N-methyl-D-aspartate-activated currents in rat hippocampal neurons. *Neurosci. Lett.*, **189,** 17–20.

HUANG, C.C. & GEAN, P.W. (1994). Paired-pulse depression of the N-methyl-D-aspartate receptor-mediated synaptic potentials in the amygdala. *Br. J. Pharmacol.*, **113,** 1029–1035.

KUCERA, G.L. & RITTENHOUSE, S.E. (1988). Inhibition of GDP-β-S of agonist-activated phospholipase C in human platelets requires cell permeabilization. *Biochem. Biophys. Res. Commun.*, **153,** 417–421.

MALENKA, R., MADISON, D.V. & NICOLL, R.A. (1986). Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature*, **321,** 175–177.

MATSUO, Y., IZUMIYAMA, M., ONODERA, H., KUROSAWA, A. & KOGURE, K. (1993). Effect of a novel thromboxane A_2 receptor antagonist, S-1452, on postischemic brain injury in rats. *Stroke*, **24,** 2059–2065.

MORINELLI, T.A., OATIS, J.E., OKWU, A.K., MAIS, D.E., MAYEUX, P.R., MASUDA, A., KNAPP, D.R. & HALUSHKA, P.V. (1989). Characterization of an ^{125}I-labeled thromboxane A_2/prostaglandin H_2 receptor agonist. *J. Pharmacol. Exp. Ther.*, **251,** 557–562.

NAKAHATA, N., ISHIMOTO, H., KURITA, M., OHMORI, K., TAKAHASHI, A. & NAKANISHI, H. (1992). The presence of thromboxane A_2 receptors in cultured astrocytes from rabbit brain. *Brain Res.*, **583,** 100–104.

ORELLANA, S., SOLSKI, P.A. & BROWN, J.H. (1987). Guanosine 5'-O-(thiotriphosphate)-dependent inositol trisphosphate formation in membrane is initiated by phorbol ester and protein kinase C. *J. Biol. Chem.*, **262,** 1638–1643.

PARFITT, K.D. & MADISON, D.V. (1993). Phorbol esters enhance synaptic transmission by a presynaptic, calcium-dependent mechanism in rat hippocampus. *J. Physiol.*, **471,** 245–268.

PEARCE, B., MURPHY, S., JEREMY, J., MORROW, C. & DONDANA, P. (1989). ATP-evoked Ca^{2+} mobilisation and prostanoid release from astrocytes: P-purinergic receptors linked to phosphoinositide hydrolysis. *J. Neurochem.*, **52,** 971–977.

PICKARD, J.D. (1981). Role of prostaglandins and arachidonic acid derivatives in the coupling of cerebral blood flow to cerebral metabolism. *J. Cereb. Blood Flow Metab.*, **1,** 361–384.

PULSINELLI, W.A., BRIERLY, J.B. & PLUM, F. (1982). Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann. Neurol.*, **11,** 491–498.

REILLY, M. & FITZGERALD, G.A. (1993). Cellular activation by thromboxane A_2 and other eicosanoids. *Eur. Heart J.*, **14,** 88–93.

ROTHERMEL, J.D., JASTORFF, B. & PARKER-BOTELHO, L.H. (1984). Inhibition of glucagon-induced glycogenolysis in isolated rat hepatocytes by the Rp disastereomer of adenosine cyclic 3',5'-phosphorothioate. *J. Biol. Chem.*, **259,** 8151–8155.

ROTHMAN, S.M. & OLNEY, J.W. (1986). Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann. Neurol.*, **19,** 105–111.

SHIRAHATA, H., USUI, H., KURAHASHI, K., FUJIWARA, M. & FUKUI, K. (1988). Endothelium-dependent contraction induced by nicotine in isolated canine basilar artery-possible involvement of a thromboxane A_2 (TXA_2) like substance. *Life Sci.*, **42,** 437–445.

SHOHAMI, E., JACOBS, T.P., HALLENBECK, J.M. & FEUERSTEIN, G. (1987). Increased thromboxane A_2 and 5-HETE production following spinal chord ischemia in the rabbit. *Prostaglandins Leukot. Med.*, **28,** 169–181.

SMITH, M.L., AUER, R.N. & SIESJO, B.K. (1984). The density and distribution of ischemic brain injury in the rat following 2–10 min of forebrain ischemia. *Acta Neuropathol.*, **64,** 319–332.

SULLIVAN, J.P., CONNOR, J.R., TIFFANY, C., SHEARER, B.G. & BURCH, R.M. (1991). NPC-15437 interacts with the C1 domain of protein kinase C. An analysis using mutant PKC constructs. *FEBS Lett.*, **285,** 120–123.

SULLIVAN, J.P., CONNOR, J.R., SHEARER, B.G. & BURCH, R.M. (1992). "2,6-Diamino-N-([1-(1-oxotridecyl)-2-piperidinyl]-methyl)hexanamide (NPC-15437): A novel inhibitor of protein kinase C interacting at the regulatory domain. *Mol. Pharmacol.*, **41,** 38–44.

SZATKOWSKI, M. & ATTWELL, D. (1994). Triggering and execution of neuronal death in brain ischemia: two phases of glutamate release by different mechanisms. *Trends Neurosci.*, **17,** 359–365.

TAKAHARA, K., MURRAY, R., FITZGERALD, G.A. & FITZGERALD, D.J. (1990). The response to thromboxane A_2 analogues in human platelets. *J. Biol. Chem.*, **265,** 6836–6844.

TEGTMEIER, F., HEISTER, W.U., SCHELLER, H.D., NIKOLOV, R. & HOLLER, M. (1990). Eicosanoids in rat brain during ischemia and reperfusion-correlation to DC polarization. *J. Cereb. Blood Flow Metab.*, **10,** 358–364.

THOMSON, S.M. & GAHILER, B.H. (1992). Comparison of the actions of baclofen at pre- and postsynaptic receptors in the rat hippocampus in vitro. *J. Physiol.*, **451,** 329–345.

UCHIMURA, N. & NORTH, R.A. (1990). Muscarine reduces inward rectifying potassium conductance in rat nucleus accumbens neurons. *J. Physiol.*, **422,** 369–380.

WESTERBERG, E., DESHPANDE, J.K. & WEILOCH, T. (1987). Regional difference in arachidonic acid release in rat hippocampal CA1 and CA3 regions during cerebral ischemia. *J. Cereb. Blood Flow Metab.*, **7,** 189–192.

(Received March 11, 1996
Revised April 17, 1996
Accepted April 30, 1996)