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Protein kinase C regulates ionic conductance in hippocampal pyramidal neurons: Electrophysiological effects of phorbol esters

(calcium-dependent potassium conductance/hippocampal slice/accommodation)

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ABSTRACT **The vertebrate central nervous system contains very high concentrations of protein kinase C, a calcium- and phospholipid-stimulated phosphorylating enzyme. Phorbol esters, compounds with inflammatory and tumor-promoting properties, bind to and activate this enzyme. To clarify the role of protein kinase C in neuronal function, we have localized phorbol ester receptors in the rat hippocampus by autoradiography and examined the electrophysiological effects of phorbol esters on hippocampal pyramidal neurons *in vitro*. Phorbol esters blocked a calcium-dependent potassium conductance. In addition, phorbol esters blocked the late hyperpolarization elicited by synaptic stimulation even though other synaptic potentials were not affected. The potencies of several phorbol esters in exerting these actions paralleled their affinities for protein kinase C, suggesting that protein kinase C regulates membrane ionic conductance.**

Protein kinase C, a phosphorylating enzyme stimulated by calcium and phospholipid, occurs ubiquitously in the body but is most concentrated in the brain (1–4). In peripheral tissues, protein kinase C may mediate cellular responses to membrane receptor stimulation; however, its role in the central nervous system is largely unknown (5–10). Phorbol esters, lipophilic compounds with inflammatory and tumor-promoting properties, may facilitate studies of protein kinase C, since they directly stimulate this enzyme (4, 11–13). Phorbol esters have been used as probes to demonstrate a role of protein kinase C in regulating effects of neurotransmitters on smooth muscle (14). To clarify the role of protein kinase C in neuronal function, we have now localized phorbol ester receptors in the rat hippocampus by autoradiography and studied the effects of phorbol esters on hippocampal pyramidal neurons by using intracellular recording techniques in the slice preparation *in vitro*. Phorbol esters potently affect neuronal electrophysiological properties, suggesting that protein kinase C regulates membrane ionic conductance.

MATERIALS AND METHODS

Autoradiography. Autoradiography was performed according to the methods of Palacios *et al.* (15) and of Nagle and Blumberg (16). Rats were perfused via the left ventricle with phosphate-buffered saline followed by 0.32 M sucrose. Eight-micrometer-thick brain sections were cut on a cryostat and thaw-mounted onto microscope slides coated with chrome alum/gelatin. Slide-mounted tissue sections were incubated at room temperature with 2–3 nM ^{3}H-labeled phorbol 12,13-dibutyrate (PBt_2) (New England Nuclear) for 45 min in buffer containing 50 mM Tris·HCl at pH 7.7 and 100 mM NaCl. To determine nonspecific binding, adjacent sections were incubated in the same medium with the addition of 1 μM unlabeled PBt_2. After incubation, tissue sections were washed for 4 min at 4°C, dipped in deionized water, and dried rapidly under a stream of cold dry air. After this labeling procedure, the slide-mounted tissue sections were juxtaposed to a tritium-sensitive film (Ultrafilm, LKB, Bromma, Sweden) in a standard x-ray film cassette and stored at 4°C. Autoradiograms were exposed for 7–14 days before development.

Receptor Binding Assay. Adult male Sprague–Dawley rats (250 g) were sacrificed by decapitation. The hippocampi were removed and homogenized with a Brinkmann Polytron in 100 vol of ice-cold buffer containing 50 mM Tris·HCl at pH 7.7 and 1 mM $CaCl_2$. The homogenates were centrifuged at 50,000 × *g* for 15 min and the pellets were washed by homogenization and repeated centrifugation. The final pellets were suspended in buffer at 600 μg of tissue per ml.

Binding assays were performed according to Driedger and Blumberg (17) with modifications. One milliliter of membrane suspension was incubated with 50 μl of [^{3}H]PBt_2 and 50 μl of drug or buffer. Tubes were incubated for 20 min at room temperature. Assays were terminated by suction filtration through Schleicher & Schuell no. 32 filters pretreated with 0.5% polyethyleneimine, followed by three washes with 3 ml of ice-cold 50 mM NaCl. Radioactivity retained by the filters was measured by liquid scintillation spectroscopy. Nonspecific binding was defined as binding of [^{3}H]PBt_2 in the presence of 5 μM unlabeled PBt_2.

Intracellular Recording from Hippocampal Slices. Slices were obtained from male Sprague–Dawley rats by using techniques that have been described in detail elsewhere (18, 19). One 400-μm-thick slice was held submerged in the recording chamber at 30°C. Temperature was regulated by a heating–cooling module (Cambion, Cambridge, MA) and was monitored within 1 mm of a slice by a hypodermic thermistor probe. Other slices were maintained in an incubation chamber at room temperature (21–22°C). The standard physiological saline was saturated with 95% O_2/5% CO_2 and consisted of (in mM): NaCl, 122.6; KCl, 5.4; $CaCl_2$, 2.5; $MgSO_4$, 2.0; NaH_2PO_4, 1.2; $NaHCO_3$, 26.2; and glucose, 10. The recording chamber provides constant perfusion and allows switching between salines via a stopcock. Individual cells were studied in control saline, after switching to an experimental saline, and after returning to control saline.

Intracellular recordings were made with fiber-filled glass microelectrodes that contained 2 M potassium methylsulfate and had impedances of 40–60 MΩ, measured at 135 Hz. Conventional intracellular recording techniques were used. A balanced-bridge circuit was used for altering the membrane potential by passing current through the recording electrode.

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Abbreviations: AHP, afterhyperpolarization; dPiBt, 12-deoxyphorbol 13-isobutyrate; EPSP, excitatory postsynaptic potential; IPSP, inhibitory postsynaptic potential; LHP, late hyperpolarizing potential; PAc_2, phorbol 12,13-diacetate; PBt_2, phorbol 12,13-dibutyrate.

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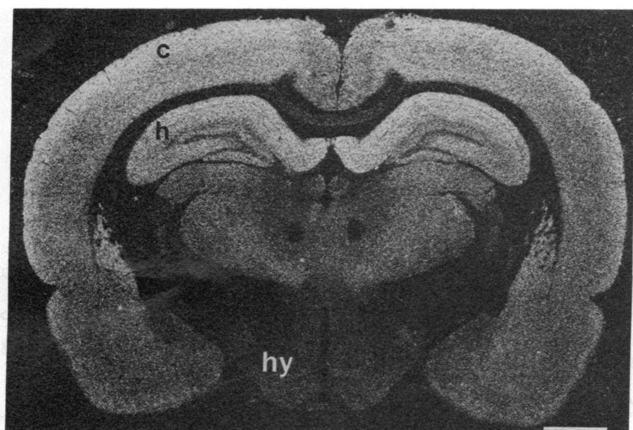

FIG. 1. $[^3H]PBt_2$ autoradiography of the rat hippocampus. This coronal section through the rat hippocampus was processed for autoradiography with $[^3H]PBt_2$. The hippocampus (h) and cerebral cortex (c) were densely labeled, but portions of the hypothalamus (hy) contained low levels of labeling. Incubation of adjacent sections with 1 μM PBt_2 completely eliminated the labeling. (Scale bar = 1.5 mm.)

Experiments were done on more than 30 CA1 neurons from 25 rats. Cells were stable from 1 to 5 hr, with resting membrane potentials of −55 to −70 mV and input resistances of 30–70 MΩ. Cells were activated either directly, with current injection via the bridge circuit, or synaptically, by electrical stimulation in the stratum radiatum ("orthodromic"). One-hundred millisecond depolarizing current pulses were used to elicit four or five action potentials and a long-lasting afterhyperpolarization (AHP). Cell input resistance during the slow phase of the AHP was determined by measuring the voltage deflection produced by a 100-msec 0.25-nA hyperpolarizing current pulse starting 300 msec after the end of the depolarizing pulse. Data were collected on an FM tape recorder and a chart recorder. Fast potentials were recorded by photographing the face of an oscilloscope. All potential measurements were made by hand.

Atropine sulfate (Sigma), cimetidine (Smith Kline & French), propranolol (Sigma), tetraethylammonium chloride (Eastman Kodak), and tetrodotoxin (Sigma) were used as indicated. To avoid introducing solvent effects, only the water-soluble phorbol compounds phorbol 12,13-diacetate (PAc_2), 12-deoxyphorbol 13-isobutyrate (dPiBt), and phorbol (LC Services, Woburn, MA) were used.

RESULTS

Phorbol ester receptors in the rat forebrain were visualized by autoradiography (Fig. 1). The density of phorbol ester receptors varied widely among grey matter areas, as reported by Nagle and Blumberg for the mouse brain (16). The hippocampus, cerebral cortex, and striatum were densely labeled, while large areas of the hypothalamus had low levels of activity. White matter tracts also displayed very low levels of labeling. Since the hippocampus is enriched in phorbol ester receptors, the hippocampal slice preparation is particularly well suited for examining the electrophysiological effects of these compounds.

Intracellular recording from hippocampal CA1 pyramidal neurons, during bath application of 250 nM PAc_2 for 15–30 min, revealed a small membrane depolarization (2.8 ± 2.7 mV; mean and SD) accompanied by a slight increase in input resistance (14.5 ± 4.1%; n = 6). In half of these cells, slow (<2-Hz) spontaneous action potential firing was also elicited.

The AHP following current-induced activation of a pyramidal neuron includes a fast and a slow phase. These components can be further distinguished by their differential sensitivity to experimental manipulations, demonstrating that the slow AHP is a calcium-dependent potassium potential (20–24). Application of 250 nM PAc_2 dramatically reduced the amplitude of the slow AHP (Fig. 2). dPiBt, a phorbol ester with higher affinity for receptors, was more potent than PAc_2 in reducing slow AHP amplitude, while phorbol, the parent alcohol, was inactive in both physiological and receptor-binding assays (Table 1). Since dPiBt and PAc_2 gave qualitatively identical results, we focused on one, PAc_2, for the remainder of the study.

At the peak of the slow AHP, input resistance decreased by 36 ± 8% (n = 6) as previously reported (25). However, when the slow AHP was almost completely blocked by PAc_2, peak input resistance fell by only 6 ± 2%, suggesting that PAc_2 acts by blocking the conductance changes underlying the slow AHP rather than by shifting its reversal potential. Phorbol esters were relatively selective in blocking the slow rather than the fast AHP. A similar pattern of AHP inhibition in CA1 pyramidal neurons is elicited by acetylcholine acting on muscarinic receptors (26), norepinephrine on β-receptors (21) and histamine on H-2 receptors (27). Conceivably, the inhibitory effect of phorbol esters on the slow AHP could have been mediated by release of these transmitters from terminals present in the CA1 region. However, the muscarinic antagonist atropine (1 μM; n = 3), the β-adrenergic blocker propranolol (20 μM; n = 3), and the histamine H-

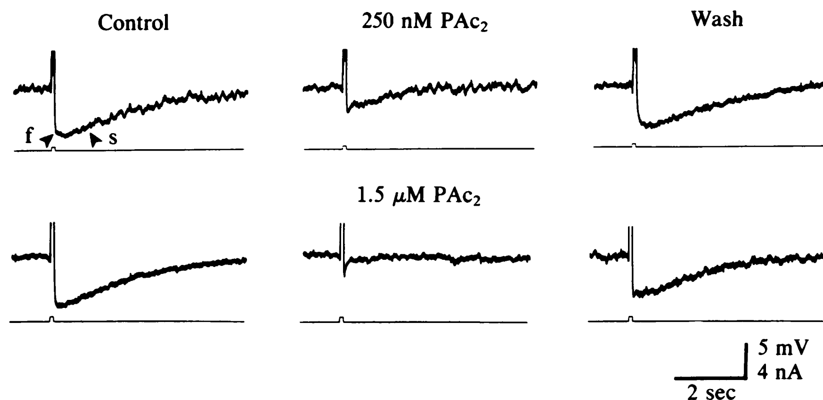

FIG. 2. Blockade of the slow AHP by PAc_2. After a short train of spikes elicited by a 100-msec depolarizing current pulse, pyramidal neurons undergo a biphasic AHP, consisting of fast and slow phases, designated f and s, respectively, in the upper left tracing. Both 250 nM and 1.5 μM PAc_2 produced a selective reversible blockade of the slow AHP. The effects of the lower concentration (upper row) were recorded after 35 min; those of the higher concentration (lower row), after 15 min; recovery was observed after 60 and 75 min. The resting membrane potentials were −54 and −60 mV, respectively. Action potentials are truncated in all figures. Membrane potential tracings are shown directly above current tracings in each pair.

Table 1. Relative potencies of phorbol esters in rat hippocampus

Drug	Affinity for phorbol ester receptors K_i, nM	Inhibition of slow AHP		
		Dose, nM	n	% inhibition
dPiBt	75 ± 7	100–150	3	60 ± 13
PAc_2	460 ± 90	250	6	51 ± 10
		500–1000	4	85 ± 18
		1500	3	98 ± 4
Phorbol	>100,000	50,000–100,000	3	−2 ± 3

The affinity (K_i) of these drugs for specific [^{3}H]PBt_2 binding sites was determined in homogenates of rat hippocampus. K_i values are reported as the mean ± SEM (n = 3). The percent inhibition of the slow AHP (mean ± SD) produced after 30 min of drug application was calculated from measurements of the slow AHP at 400 msec after the end of a 100-msec depolarizing current pulse.

2 antagonist cimetidine (10 μM; n = 2) all failed to reverse PAc_2's blockade of the slow AHP, suggesting that these transmitters are not involved.

In response to a prolonged depolarizing pulse, hippocampal pyramidal cells do not fire action potentials at a constant rate. Rather, following the initial cluster of spikes, there is a distinct pause, which is accompanied by a "sag" in membrane potential and followed by a markedly decreased rate of firing ("accommodation"). The inhibitory influence exerted by the slow AHP is thought to contribute to accommodation. During application of 250 nM PAc_2, accommodation was greatly reduced (Fig. 3*B*). At a time when the slow AHP was blocked, the number of spikes produced by a 2-sec depolarizing current pulse increased from 6.5 ± 1.8 to 26.6 ± 10 (n = 6). Effects of 250 nM PAc_2 were completely reversible with washing.

The effects of higher concentrations of PAc_2 were quantitatively and qualitatively different from the effects of 250 nM PAc_2 described above. Perfusion with 1–1.65 μM PAc_2 for 20–30 min resulted in a peak membrane depolarization of 6.6 ± 3.2 mV and an increase in resting input resistance of

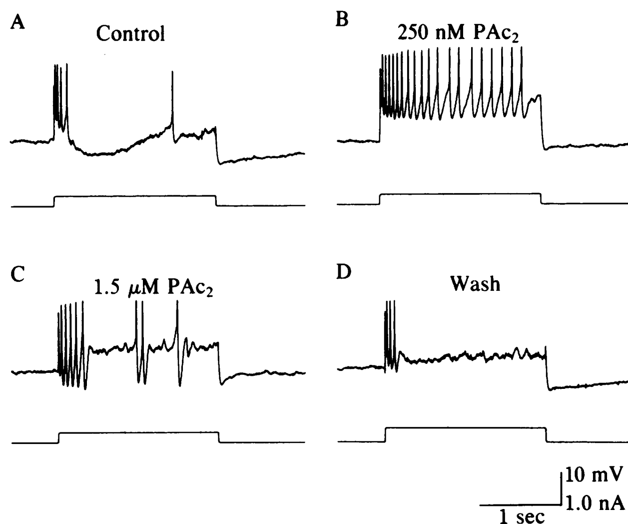

FIG. 3. Effects of PAc_2 on accommodation. As shown in *A*, a 2-sec depolarizing current pulse produces an initial cluster of spikes followed by a sag in membrane potential and a resumption of action potential firing at a decreased rate. In *B*, administration of 250 nM PAc_2 for 30 min produced a dramatic increase in the number of spikes produced by the same current pulse as in *A*. Administration of 1.5 μM PAc_2 for 30 min to the same cell decreased the number of action potentials and induced an increase in the undershoots following individual action potentials (*C*). Washing with control saline for 60 min decreased the size of the undershoots toward original levels (*D*). Resting membrane potential in *A* and *B* was −60 mV, in *C* and *D* it was −54 mV.

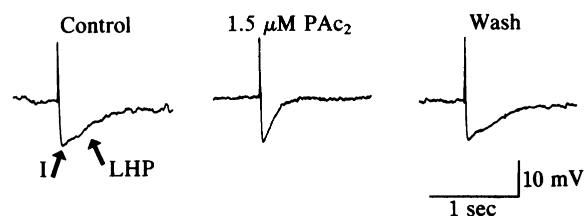

FIG. 4. Blockade of the LHP by PAc_2. Orthodromic stimulation of a CA1 pyramidal neuron elicits an EPSP and a spike followed by a fast IPSP designated I on the left and a slower LHP as indicated. Application of 1.5 μM PAc_2 for 25 min selectively blocked the LHP in a reversible manner. Recovery was observed 55 min later. Resting membrane potential was −55 mV in all traces.

22.1 ± 9.7% (n = 7). During longer application of these higher concentrations of PAc_2, the membrane potential and resistance decreased to near control levels. With high concentrations of PAc_2 or with dPiBt (100–400 nM; n = 4), the number of spikes elicited during a 2-sec pulse transiently increased and then, even though the slow AHP had not returned, the number of spikes decreased back toward control levels. This "paradoxical accommodation" was associated with a decreased frequency of spike firing in the initial cluster, as well as loss of the sag in membrane potential (Fig. 3*C*). Furthermore, although the observation was difficult to quantify, the undershoots following single action potentials appeared to be enhanced markedly in 1.5 μM PAc_2 (Fig. 3*C*), even when corrections were made for the change in resting membrane potential.

We investigated the effects of phorbol esters on synaptic transmission by examining the excitatory postsynaptic potential (EPSP) and the inhibitory postsynaptic potential (IPSP) as well as the late hyperpolarizing potential (LHP) evoked by electrical stimulation of afferent fibers (23, 28). These potentials were not affected by 250 nM PAc_2. At higher concentrations, 1–1.5 μM, the LHP, which is a synaptically activated potassium-dependent potential, was completely blocked (n = 7) before any effects on the EPSP or the IPSP were evident (Fig. 4). With prolonged high dose application, the EPSP and the IPSP did diminish somewhat; however, the reduction coincided with a decrease in input resistance. dPiBt at 100–150 nM (n = 3), but not phorbol at 50–100 μM (n = 3), blocked the LHP in a similar fashion.

Almost all of the effects of high dose PAc_2 were reversible, but there was a wide range in the rate with which the various effects reversed. The LHP recovered first, followed by the slow AHP, and finally by accommodation. Recovery of the latter was frequently seen only after 2–3 hr of washing. At this time, the action potential pattern of firing during a 2-sec pulse was essentially the same as in controls. However, even after 3 hr of washing, the membrane potential sag after the initial cluster of spikes did not fully recover.

Application of PAc_2 (1.5 μM) for up to 1 hr did not affect sodium-dependent action potentials. Action potential amplitude and duration, measured at the control resting potential by repolarizing the cell with current injection, were apparently unaltered. PAc_2 (250 nM) had two effects on presumed calcium-dependent action potentials studied in the presence of tetrodotoxin (0.5–1.0 μM) plus tetraethylammonium chloride (5–10 mM) (n = 4). PAc_2 actually enhanced individual calcium spike amplitudes and slightly shortened spike duration. As expected, since it is a calcium-dependent potassium potential (20, 29), the slow AHP following individual calcium spikes was abolished.

DISCUSSION

Phorbol esters affected several electrophysiological properties of CA1 hippocampal pyramidal neurons. The relative potencies of phorbol esters in producing these effects paral-

lel their affinities for phorbol ester receptors. In view of the strong evidence that phorbol esters bind to protein kinase C, these findings imply that protein kinase C activation by phorbol esters mediates these electrophysiological effects.

Inflammatory and tumor-promoting subtypes of phorbol ester receptors have been distinguished in homogenates of mouse skin with receptor-binding techniques (30). In the present study, dPiBt, a phorbol ester that is highly selective for the inflammatory subtype of receptor, was very potent in displacing PBt_2 binding and in producing electrophysiological effects. dPiBt has also been found to be active in several isolated smooth muscle preparations (14). Together, these results suggest that phorbol ester receptors in excitable tissues, both peripheral and central, belong to the inflammatory subtype.

Phorbol esters selectively blocked the slow calcium-dependent potassium component of the AHP. Since this action is shared by several transmitters and phorbol esters potentiate the release of transmitter substances (31, 32), the possibility arose that the slow AHP blockade is mediated indirectly by transmitter release. We were unable to address this question by decreasing extracellular calcium concentration and blocking synaptic transmission, because this manipulation directly inhibits the slow AHP. Instead, we have demonstrated that antagonists to several transmitters known to inhibit the slow AHP do not block the action of PAc_2. Conceivably, the inhibition of the slow AHP by phorbol esters involves release of other transmitters. However, PAc_2 (250 nM), which blocks the slow AHP, did not affect any of the three synaptic potentials that we examined. Thus, if PAc_2 blocks the slow AHP by releasing another transmitter, then it would have to produce a rather specific effect on the release of that agent.

Autoradiographic studies of phorbol ester receptors in the rat brain revealed a heterogeneous distribution among gray matter zones and low densities in white matter areas, consistent with a neuronal localization of these receptors. In other autoradiographic studies, lesions of intrinsic hippocampal neurons with quinolinic acid eliminated the major proportion of phorbol ester binding in the hippocampus (unpublished observations). Accordingly, the blocking effects of phorbol esters on the slow AHP may be exerted directly on pyramidal cells by reduction of calcium influx or inhibition of some subsequent step. In *Aplysia* bag cells, phorbol 12-myristate 13-acetate, a potent phorbol ester, increases the height of calcium-dependent action potentials (33). In our studies of pyramidal neurons, calcium spike amplitude was enhanced, but duration was shortened by PAc_2. Since the amount of calcium influx cannot be inferred from the size of the calcium spike, voltage-clamp techniques will be necessary to determine the net effect on calcium influx before this issue can be resolved. Since activation of cyclic AMP-dependent protein kinase also inhibits the slow AHP (21), it will be interesting to learn how the blocks caused by activation of protein kinase C and cyclic AMP-dependent protein kinase are related.

Accommodation of pyramidal cell firing during a prolonged depolarizing pulse has been attributed to two factors (34): (*i*) the calcium-dependent potassium conductance, which causes the slow AHP, and (*ii*) the M-current, a voltage-dependent potassium current sensitive to block by muscarine (35). The effect of PAc_2 on accommodation was biphasic. Low concentrations blocked accommodation, consistent with the observed inhibition of the slow AHP. However, at higher concentrations of PAc_2, the neurons showed a paradoxical accommodation to a prolonged depolarizing pulse, despite continued blockade of the slow AHP. We suggest that this paradoxical accommodation involves the activation or enhancement of a voltage-dependent conductance.

Phorbol esters block the LHP selectively and completely whereas we know of no other agents reported to do so. Whether the antagonism is pre- or postsynaptic remains unclear. If presynaptic, then the cells responsible for producing the LHP probably differ from those producing the other synaptic potentials studied, such as the GABAergic interneurons (GABA, γ-aminobutyric acid). If, on the other hand, the block of the LHP is postsynaptic, then phorbol esters should be useful tools for determining the LHP neurotransmitter and its mechanism of action.

At present a large body of evidence links protein kinase C to the polyphosphoinositide system. Stimulation of the polyphosphoinositide system by neurotransmitters triggers the formation of inositol trisphosphate and diacylglycerol, which directly activates protein kinase C (5, 36). Since stimulation of muscarinic cholinergic receptors in the hippocampus activates the polyphosphoinositide system (37), protein kinase C could mediate the effects of acetylcholine. Indeed, the effects produced by low concentrations of PAc_2 do resemble those caused by acetylcholine (26), since both substances depolarize hippocampal pyramidal neurons, increase membrane resistance, and block the slow AHP. However, muscarinic agonists elicit intense spike activity not observed with PAc_2 even at higher concentrations. Perhaps, in the absence of inositol trisphosphate, phorbol esters only partially reproduce the effects of muscarinic receptor stimulation. In various smooth muscle systems, phorbol esters potently influence acetylcholine-, histamine-, serotonin-, and bradykinin-elicited contractions, all of which act via phosphoinositides (14). These earlier findings, along with the present results, strongly support a role for protein kinase C in synaptic transmission and in mediating modulatory actions of neurotransmitters, for example, inhibition of calcium-dependent potassium conductance.

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