Chapter 22

Biophysical Methods to Analyze Direct G-Protein Regulation of Neuronal Voltage-Gated Calcium Channels

Norbert Weiss and Michel De Waard

Abstract

Neuronal voltage-gated calcium channels play an essential role for calcium entry into presynaptic endings responsible for the release of neurotransmitters. In turn, and in order to fine tune synaptic activity, numerous neurotransmitters exert a potent negative feedback over the calcium signal provided by G-protein-coupled receptors that can be recognized by characteristic biophysical modifications of the calcium current. There are two main biophysical approaches to analyze direct G-protein regulation of voltage-gated calcium channels: the so-called double-pulse method, which is indirectly assessed by the gain of current produced by a depolarizing prepulse potential, and the "subtraction" method that allows the analysis of G-protein regulation from the ionic currents induced by regular depolarizing pulses. The later method separates the ionic currents due to nonregulated channels from the ion currents that result from a progressive departure of G-proteins from regulated channels, thereby providing valuable information on the OFF kinetics of G-protein regulation. In this chapter, we introduce these "double pulses" and "subtraction" procedures for use primarily with single cells and also discuss the limitations inherent to these two approaches.

Key words Calciumchannel, Ca_v2 channel, G-protein-coupled receptor, G-proteins, Gβγ-dimer, Prepulse facilitation, Biophysical method

1 Introduction

Presynaptic voltage-gated calcium channels (VGCCs), primarily $Ca_v2.1$ and $Ca_v2.2$ channels, represent two of the most important players in the initiation of the Ca^{2+} signal by converting electrical impulses into intracellular Ca^{2+} elevations responsible for the release of neurotransmitters [6]. In turn, these channels are strongly regulated by a negative feedback mechanism provided by the activation of G-protein-coupled receptors (GPCRs) (for review, see [8, 36]). To date, up to 20 GPCRs have been described to modulate VGCCs (Table 1).

Direct inhibition of the Ca^{2+} channels occurs through the direct binding of G-protein βγ-dimer onto various structural molecular determinants of the Ca_v2-subunit [36]. At the whole

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Table 1
Neurotransmitter- and receptor-mediated G-protein modulation of Ca_v2 channels

Neurotransmitter	Receptor	Ca_v channel	Tissue/species	Reference
Ach	M4	$Ca_v2.2$	SCG/rat	Bernheim et al. [2]
	M2	$Ca_v2.1$ & $Ca_v2.2$	SCG/mouse	Shapiro et al. [39]
Adenosine	A1	$Ca_v2.2$	Ciliary ganglion/chicken	Yawo and Chuhma [50]
		$Ca_v2.2$	DRG/chicken	Kasai and Aosaki [29]
		$Ca_v2.1$ & $Ca_v2.2$	Cerebellum/rat	Dittman and Regehr [10]
		$Ca_v2.2$ & $Ca_v2.3$	Hippocampus (CA3 → CA1)/rat	Wu and Saggau [47]
ATP/ADP	P2Y	$Ca_v2.2$	SCG/rat	Brown et al. [5], Filippov et al. [16]
Dopamine	D2	HVA	DRG/chicken	Marchetti et al. [32]
Endocannabinoids	CB1	$Ca_v2.1$	SCG/rat	Garcia et al. [18]
		$Ca_v2.1$, $Ca_v2.2$ & $Ca_v2.3$	Cerebellum	Brown and Russell [4]
GABA	GABA B	$Ca_v2.1$ & $Ca_v2.2$	DRG/rat	Dolphin and Scott [12]
			DRG/chicken	Deisz and Lux [9], Grassi and Lux [22]
			Cerebellum/rat	Dittman and Regehr [10]
		$Ca_v2.2$ & $Ca_v2.3$	Hippocampus (CA3 → CA1)/guinea pig	Wu and Saggau [48]
		$Ca_v2.2$	SCG/rat	Filippov et al. [17]
Galanin	GalR1	$Ca_v2.2$	Hypothalamus/rat	Simen et al. [40]
Glutamate	mGluR1	$Ca_v2.2$	SCG/rat	Kammermeier and Ikeda [27]
LHRH	LHRH-R	$Ca_v2.2$	SCG/bullfrog	Elmslie et al. [15] Boland and Bean [3], Kuo and Bean [30]
Noradrenaline	α2-adrénergique	$Ca_v2.2$	SCG/bullfrog	Bean [1]
		$Ca_v2.2$	SCG/rat	Garcia et al. [19]
		Non-L	NG108-15	Docherty and McFadzean [11], McFadzean and Docherty [33]

NPY	Y2	Non-L $\mathrm{Ca_v}2.2$	SCG/rat SCG/rat	Plummer et al. [35] Toth et al. [43]
Opioids – enkephalins	μ	$\mathrm{Ca_v}2.2$	NG108-15	Kasai [28]
Opioids – dynorphins	κ	Non-L	DRG/rat	Bean [1]
Serotonin	5HT-1A	Non-L	Spinal neuron/lamprey	Hill et al. [23]
Somatostatin	SS-R	$\mathrm{Ca_v}2.2$	DRG/rat	Ikeda and Schofield [26]
			SCG/rat	Ikeda and Schofield [25]
Substance P	NK1	$\mathrm{Ca_v}2.2$	SCG/rat	Shapiro and Hille [38]
VIP	VIP-R	$\mathrm{Ca_v}2.2$	SCG/rat	Zhu and Ikeda [52]

SCG superior cervical ganglion, *DRG* dorsal root ganglion cell level, this regulation is recognized by various phenotypical modifications of the Ca^{2+} current, including a decrease of the inward current amplitude [3, 49], and in some cases a depolarizing shift of the voltage-dependence curve of current activation [1], and a slowing of activation [32] and inactivation kinetics [51]. In addition, short highly depolarizing voltage step, usually applied around +100 mV before the current eliciting pulse ("double-pulse" protocol), is sufficient to reverse, at least partially, most of the landmarks of G-protein inhibition. This protocol produces a so-called prepulse facilitation [13, 24, 37]. While the inhibition of the Ca^{2+} current has been attributed to the direct binding of G-protein βγ-dimer to the Ca_v2-subunit (referred as "ON" landmark for the onset of the inhibition), all the other landmarks including the slowing of current kinetics and prepulse facilitation can be described as variable time-dependent dissociation of Gβγ-dimer from the channel (referred as "OFF" landmarks for the arrest of the inhibition) and consequent recovery from G-protein inhibition [14, 41, 44]. Hence, proper attribution and precise quantitative evaluation of "ON" and "OFF" landmark parameters are necessary to assess the sensitiveness of a given calcium channel/GPCR complex and most importantly provide essential insight into the dynamic regulation of presynaptic calcium channels by G-proteins and GPCRs.

In this chapter, we provide a step-by-step illustration of the two main analytical methods that can be used to extract and describe the main parameters of "ON" and "OFF" G-protein landmarks. It is assumed that the reader already masters specific cell culture preparations and basic single cell patch-clamp recordings.

2 Methods

2.1 Biophysical Analysis of G-Protein Regulation by the "Double-Pulse" Method

The electrophysiological protocol classically used in the "double-pulse" method is shown in Fig. 1a. Initially introduced by Scott and Dolphin [37] and then widely adopted [13, 24], the method consists of comparing the peak current amplitude elicited by a given test pulse before (P1) and after (P2) application of a depolarizing prepulse of variable voltages and durations, both in control and G-protein-activated conditions. An example of current recordings is shown in Fig. 1b for $Ca_v2.2/\beta_3$ channels expressed in *Xenopus oocytes* in response to a 500 ms long test pulse elicited at 10 mV and a prepulse at 70 mV of variable durations. Notably, in control condition, a significant extent of current inactivation is produced by application of depolarizing prepulses as evidenced by a net decline of the peak current amplitude. In contrast, under G-protein activation, prepulse applications induce a current facilitation as evidenced by net-increased peak current amplitudes that

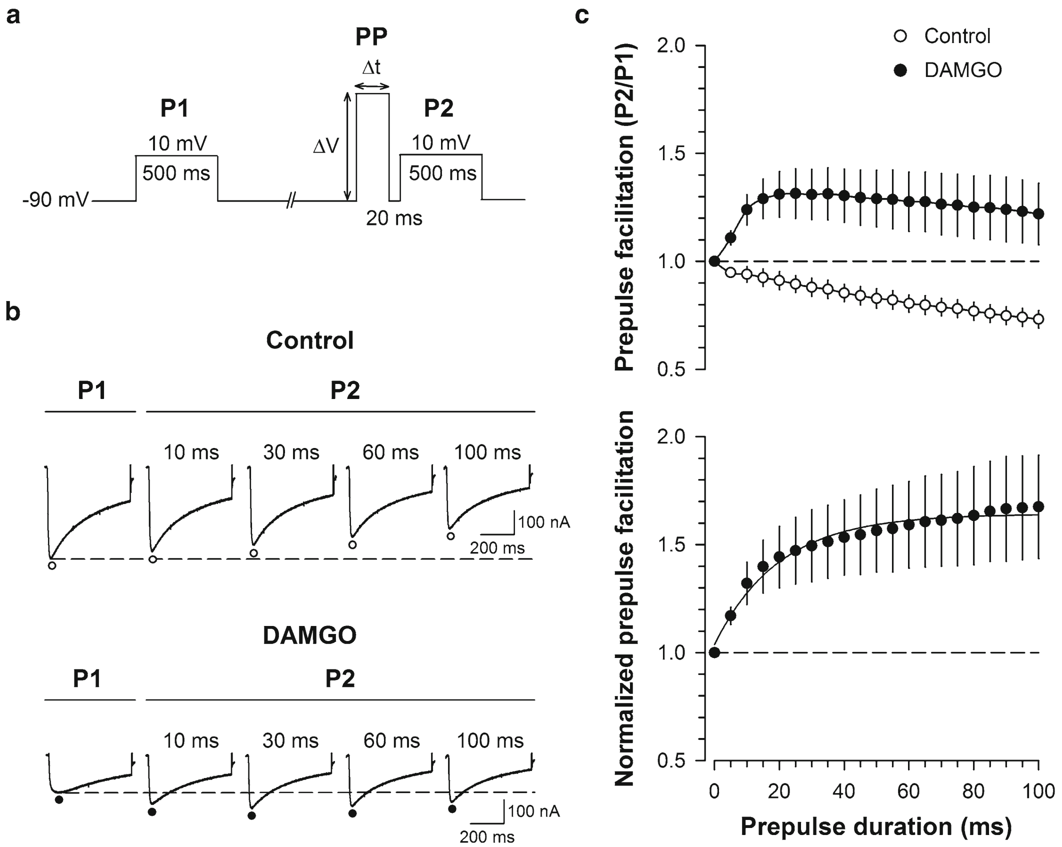

Fig. 1 Analysis of G-protein regulation by the "double-pulse" method. (**a**) Experimental protocol to measure prepulse-induced facilitation using the "double-pulse" method. P1 and P2, eliciting current pulses; *PP* prepulse. (**b**) Representative current traces recorded before and after G-protein activation by DAMGO, elicited by 500 ms P1 at 10 mV and 500 ms P2 at 10 mV following PP at 70 mV of variable durations. (**c**) Peak current amplitude ratio (P2/P1) for control (*open circles*) and DAMGO (*filled circles*) conditions (*top panel*). Normalized prepulse facilitation $(P2/P1_{DAMGO})/(P2/P1_{Control})$ to eliminate the prepulse-induced inactivation component resulting to the net prepulse facilitation kinetic. Fitting the result by a single exponential provides the time constant τ and maximal extent of prepulse facilitation (current recovery) (*bottom panel*) (Reproduced from Weiss and De Waard [45])

usually progressively decline with longer depolarizing prepulses. Under those conditions, elicited P2 currents are affected by a gain of current resulting from the dissociation of G-proteins from the channel (recovery from inhibition) and a loss of current due to channel inactivation induced by depolarizing prepulses. For short duration prepulses, the gain of current is predominant, whereas the tendency is inverted by increasing prepulse duration, at time points where G-protein dissociation saturates but channel inactivation increases. The control condition contains only the prepulse-induced inactivation component, whereas both the facilitation component and the inactivation component are present under

G-protein activation. Figure 1c illustrates the average behavior of normalized peak currents (P2/P1) plotted as a function of prepulse duration for both control and G-protein-activated conditions for a prepulse potential of 70 mV. In order to eliminate the inactivation component and isolate the net facilitation component under G-protein activation condition, the evolution of P2/P1 ratio observed under G-protein activation is normalized with regard to the evolution of P2/P1 ratio measured in control condition. The resulting result can then be best fitted by a single exponential function, providing the time constant of G-protein dissociation from the channel (t) and the maximal extent of current facilitation (current recovery). While τ provides important information about the kinetics of G-protein regulation, the extent of current facilitation assed by the "double-pulse" method indirectly gives access to the maximal amplitude of current inhibition that the activation of G-protein produced.

Note 1 Extracting parameters of G-protein regulation using the "double-pulse" method implies that control and G-protein-inhibited channels inactivate at the same rate and with a same extent. It also implies that the voltage dependence of this inactivation is not altered by G-protein inhibition. If this condition is not fulfilled, then the normalization procedure is flawed by approximation. So far, little information is available about the inactivation properties of the inhibited channel, but evidence points to the fact that G-protein-inhibited channels inactivate slower than control channels [15].

2.2 Biophysical Analysis of G-Protein Regulation by the "Subtraction" Method

In contrast to the "double-pulse" method, the "subtraction" method avoids the use of depolarizing prepulses and is not affected by possible alteration in channel inactivation kinetics induced by G-protein binding. This method extracts parameters of G-protein regulation from ionic currents elicited by regular depolarizing pulses by separating the ionic currents due to nonregulated channels from the ionic currents that result from the progressive unbinding of G-proteins from the regulated channel (current recovery). A step-by-step illustration of this method is illustrated in Fig. 2 using a representative example of a $Ca_v2.2/\beta_3$ channel expressed in *Xenopus oocytes* inhibited by application of the μ-opioid receptor agonist DAMGO.

1. Control ($I_{Control}$) and DAMGO-inhibited (I_{DAMGO}) currents, recorded before and after μ-opioid receptor activation, respectively, were triggered by a test pulse at 10 mV (Fig. 2a).
2. Subtracting I_{DAMGO} from $I_{Control}$ provides $I_{Inhibited}$, the amount of inhibited current upon G-protein activation (Fig. 2b, blue trace). The time course of the inhibited current is affected by both the recovery from G-protein inhibition that occurs

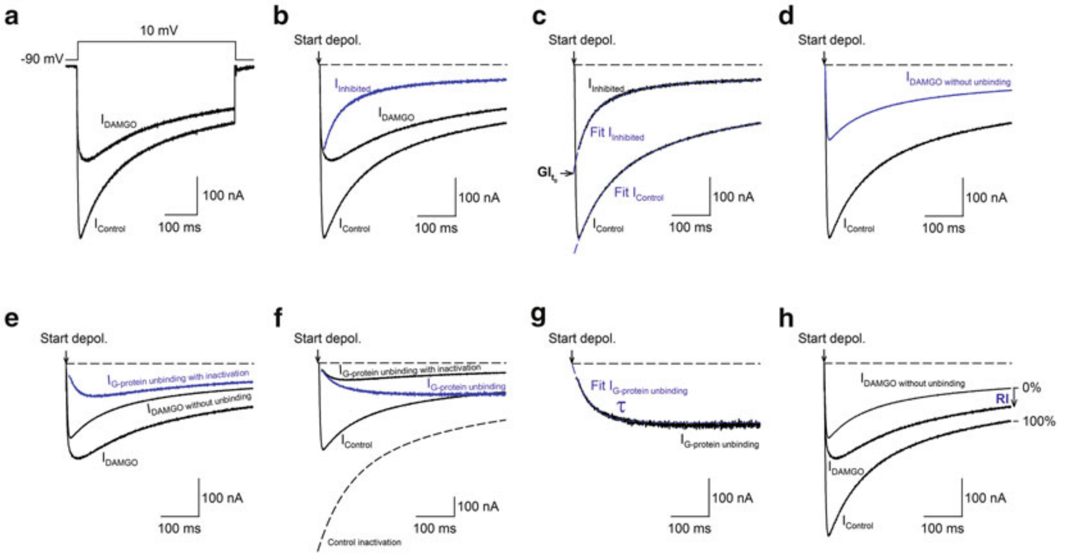

Fig. 2 Step-by-step illustration of the analysis of G-protein regulation by the "subtraction" method. (**a**) Representative current traces elicited at 10 mV for control (I_{Control}) and DAMGO (I_{DAMGO}) conditions. (**b**) Inhibited current ($I_{\text{Inhibited}}$, *blue trace*) under G-protein activation obtained by subtracting I_{DAMGO} from I_{Control}. The *dashed line* represents the zero current level and the *arrow* the start of the depolarization. (**c**) I_{Control} and $I_{\text{Inhibited}}$ were extrapolated to $t = 0$ ms (start of the depolarization) by fitting traces (*blue dashed lines*) with a single and double exponential, respectively, in order to determine the maximal extent of G-protein inhibition ($\text{GI}t_0$). (**d**) Estimate of the fraction of control currents that is present in I_{DAMGO} ($I_{\text{DAMGO without unbinding}}$, *blue trace*) and that is due to a population of control channels. It is obtained by the following equation: $I_{\text{Control}} \times (1 - \text{GI}t_0)$. (**e**) The fraction of I_{DAMGO} current that recovers from G-protein inhibition (G-protein-inhibited channel population) is shown in *blue* ($I_{\text{G-protein unbinding with inactivation}}$) obtained by subtracting $I_{\text{DAMGO without unbinding}}$ from I_{DAMGO}. (**f**) The kinetics of G-protein dissociation ($I_{\text{G-protein unbinding}}$, *blue trace*) from G-protein-inhibited channels is obtained by dividing $I_{\text{G-protein unbinding with inactivation}}$ by the normalized control inactivation component (*dashed line* obtain by fitting I_{Control} with a single exponential). (**g**) Fit of $I_{\text{G-protein unbinding}}$ (*dashed blue line*) by a single exponential decrease provides the time constant τ of G-protein dissociation from the channel. (**h**) Measure of the percentage of recovery from G-protein inhibition (RI) at the end of 500 ms pulse at 10 mV by $\text{RI} = (I_{\text{DAMGO}} - I_{\text{DAMGO without unbinding}})/(I_{\text{Control}} - I_{\text{DAMGO without unbinding}}) \times 100$ (Reproduced from Weiss and De Waard [45])

during the current eliciting pulse (conversion of G-protein-inhibited channels toward non-inhibited channels) and by the voltage-dependent inactivation of the channel that occurs during the eliciting pulse.

Note 2 One assumption is made that G-protein-bound channels do not undergo openings. It is worth to mention that ion-conducting openings of presumably G-protein-bound channels were initially proposed [7, 31], which could potentially affect the kinetics of $I_{\text{Inihibited}}$. However, openings of G-protein-bound channels remain difficult to assess directly and would require further investigation.

3. At the start of the eliciting pulse ($t = 0$ ms), there has been no recovery from G-protein inhibition, no opening from G-protein-bound channels, and inactivation has not taken place yet. Hence, in order to estimate the maximal extent of current inhibition produced by G-protein activation, I_{Control} and $I_{\text{Inhibited}}$ traces are extrapolated to $t = 0$ ms with a single and double exponential function, respectively (Fig. 2c, fits in blue). Fitting $I_{\text{Inhibited}}$ to $t = 0$ ms provides the first parameter of G-protein regulation termed GIt_0 for G-protein-induced current inhibition at the start of the depolarization and represents the maximal extent of current inhibition before any recovery process takes place ($\text{GI}t_0 = I_{\text{Inhibited}}\ t_0 / I_{\text{Control}}\ t_0 \times 100$ when expressed as percentage).
4. Applying this percentage of G-protein inhibition to I_{Control} results in $I_{\text{DAMGO without unbinding}}$, the theoretical current that would result from G-protein inhibition if the dissociation of G-proteins from the channel during the eliciting pulse did not occur at all (Fig. 2d, blue trace).
5. Subtracting $I_{\text{DAMGO without unbinding}}$ from I_{DAMGO} provides $I_{\text{G-protein unbinding with inactivation}}$ (Fig. 2e, blue trace). This current contains both the gain of current due to G-protein dissociation from the inhibited channels (recovery from inhibition) and inactivation of the gained current.

Note 3 The kinetics of the $I_{\text{G-protein unbinding with inactivation}}$ current are apparent since the gain of current is affected by inactivation, whereas inactivation is itself altered by the gain of current. Since the gained current results from the conversion of G-protein-inhibited channels toward non-inhibited channels, the real inactivation kinetics should be similar to the one of the non-inhibited channels. The amplitude of $I_{\text{G-protein unbinding with inactivation}}$ current will also depend on what extent inactivation of the channel may undergo during the depolarization when still in the G-protein-inhibited state. However, this inactivation will be significantly less than with a high depolarizing prepulse as the one that is applied in the "double-pulse" method.

6. In order to extract the net G-protein dissociation component, $I_{\text{G-protein unbinding with inactivation}}$ is divided by a normalized curve that depicts the inactivation of non-inhibited channels obtained by fitting I_{Control} by a single exponential function (Fig. 2f, dashed line). The resulting current $I_{\text{G-protein unbinding}}$ (Fig. 2f, blue trace) reflects the net kinetics of G-protein dissociation from the channel and reaches a stable plateau where no G-protein dissociation occurs anymore.
7. The kinetic τ of G-protein dissociation from the channel is obtained by fitting $I_{\text{G-protein unbinding}}$ by a decreasing single exponential function (Fig. 2g, blue dashed line). This time constant

represents the second essential parameter of G-protein regulation of voltage-gated calcium channels.

8. Finally, in order to get an estimate of the maximal fraction of G-protein-inhibited channels that recover from inhibition during the eliciting pulse, the percentage of current that had recovered from inhibition (RI) is measured such that: $\mathrm{RI} = 100 \times (I_{\mathrm{DAMGO}} - I_{\mathrm{DAMGO\ without\ unbinding}})/(I_{\mathrm{Control}} - I_{\mathrm{DAMGO\ without\ unbinding}})$ at a time point where $I_{\mathrm{G\text{-}protein\ unbinding}}$ reaches a plateau. RI represents the third critical parameter that describes the calcium channel regulation by G-proteins.

3 Concluding Remarks

The biophysical analysis of direct G-protein regulation of voltage-gated calcium channels has been largely performed using the "double-pulse" method. This technique is easy to apply in both primary neurons in culture and heterologous expression systems including various mammalian cell lines and *Xenopus oocytes* and has been widely recognized and accepted. However, this approach makes the postulate that nonregulated and G-protein-inhibited channels inactivate with the same kinetics. Currently, because of technical difficulties to experimentally investigate this feature, there are no clear data in the literature supporting this assumption. In contrast, it is likely that G-protein bound channels inactivate at a slower rate than nonregulated channels, potentially introducing a significant bias to this procedure. This likelihood stems from the fact that Gβγ-dimers bind predominantly on one channel determinant that has been involved in the control of inactivation [42]. In contrast, the "subtraction" method does not require that G-protein-bound channels inactivate with the same kinetics than nonregulated channels. Moreover, this method does not require the application of a depolarizing prepulse that is usually applied along with an interpulse that provides an incentive for G-protein reassociation with the channel, therefore underestimating the real extent of G-protein dissociation. The "subtraction" analysis is exclusively based on current traces elicited at regular membrane voltages, before and after G-protein activation. Most importantly, this method allows the analysis of G-protein regulation at physiological membrane potential, providing a better understanding of the physiological dynamics of the regulation. It uncovers the importance of the offset of G-protein regulation in physiological processes rather than exclusively putting the emphasis on the onset of G-protein inhibition. This is a particularly important aspect of G-protein regulation knowing that neuronal networks undergo a significant extent of tonic G-protein activation. On the other hand, an inherent limitation of this approach is that it is limited to a range of membrane potentials where ionic currents can be effectively measured. Although this method has been developed and validated on heterologous expressed channels, it is likely that it can also be suitable for analyzing G-protein regulation of voltage-gated calcium channels in native neuronal environment.

In summary, both of the described methods are not model independent and are both affected by their intrinsic assumptions and/or limitations. However, they provide similar qualitative information about the kinetics of the G-protein regulation and are therefore extremely informative in terms of how G-protein-coupled receptors dynamically regulate voltage-gated calcium channel in health and diseased state. Indeed, mutations in the genes encoding VGCCs linked to neurological disorders including hemiplegic migraine type 1 have been shown to alter direct G-protein regulation of mutated channels [20, 21, 34, 46]. Hence, perfect analysis of G-protein regulation of mutated channels not only contributes to our understanding of the associated channelopathies but also represent important signaling information for potential new therapeutic strategies.

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