KChIP1 and frequenin modify shal-evoked potassium currents in pyloric neurons in the lobster stomatogastric ganglion

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The transient potassium current (I\textsubscript{A}) plays an important role in shaping the firing properties of pyloric neurons in the stomatogastric ganglion (STG) of the spiny lobster, *Panulirus interruptus*. The *shal* gene encodes I\textsubscript{A} in pyloric neurons. However, when we over-expressed the lobster Shal protein by *shal* RNA injection into the Pyloric Dilator (PD) neuron, the increased I\textsubscript{A} had somewhat different properties from the endogenous I\textsubscript{A}. The recently cloned KChIPs (K-channel interacting proteins) can modify vertebrate Kv4 channels in cloned cell lines. When we co-expressed *hKChIP1* with lobster *shal* in *Xenopus* oocytes or lobster PD neurons, they produced A-currents resembling the endogenous I\textsubscript{A} in PD neurons: compared to currents evoked by *shal* alone, their voltage for half inactivation was depolarized, their kinetics of inactivation were slowed, and their recovery from inactivation was accelerated. We also co-expressed *shal* in PD neurons with lobster *frequenin*, which encodes a protein belonging to the same EF-hand family of Ca\textsuperscript{2+} sensing proteins as *hKChIP*. Frequenin also restored most of properties of the *shal*-evoked currents to those of the endogenous A-currents, but the time course of recovery from inactivation was not corrected. These results suggest that lobster shal proteins normally interact with proteins in the KChIP/frequenin family to produce the transient potassium current in pyloric neurons.
Introduction

Transient potassium currents, also known as A-currents ($I_A$), are active at subthreshold membrane potentials and help to control the excitability and firing properties of neurons and cardiac myocytes in vertebrate and invertebrate systems (Tsien and Carpenter 1978, Storm 1990, Tierney and Harris-Warrick 1992, Greenstein et al. 2000). $I_A$ plays important roles in shaping spike frequency by regulating the interspike interval, in setting the bursting rate in oscillatory neurons, and in modulating post-inhibitory rebound (Connor and Stevens 1971, Tierney and Harris-Warrick 1992, Kloppenburg et al. 1999, Greenstein et al. 2000). The pyloric network in the stomatogastric ganglion (STG) of the spiny lobster, *Panulirus interruptus*, contains 14 neurons that organize a rhythmic pumping and filtering movement of the foregut. All the neurons are conditional oscillators. It has been suggested that $I_A$ plays important roles in shaping the different firing patterns of the pyloric neurons (Hartline 1979, Tierney and Harris-Warrick 1992, Golowasch et al 1992, Guckenheimer et al 1993 Harris-Warrick et al. 1998). Even subtle changes in the conductance and kinetics of $I_A$ by application of low concentrations of the antagonist 4-aminopyridine (4-AP) or neuromodulators such as dopamine dramatically alter the firing properties of neurons in the network (Tierney and Harris-Warrick 1992, Harris-Warrick et al. 1995, Kloppenburg et al. 1999, Peck et al. 2001).

In *Drosophila*, two different genes in the *Shaker* family of voltage dependent potassium channel genes, *shal* and *shaker*, have been shown to encode A-type currents (Jan and Jan 1992, Pongs 1992, Jan and Jan 1997). These are equivalent to the vertebrate
Kv4 and Kv1 families of genes (Wei et al. 1990, Salkoff et al. 1992, Jan and Jan 1997). We have cloned these genes from Panulirus interruptus and showed that both encode an A-type current in Xenopus oocytes (Baro et al. 1996a, Baro et al. 1996b, Baro and Harris-Warrick 1998). However, several results argue that shal alone encodes I_A in the soma and neurites of pyloric neurons within the STG. First, only shal immunoreactivity was found in the soma and neuropil of pyloric neurons, while shaker immunoreactivity was selectively targeted to the axons of the neurons after they leave the ganglion (Baro et al. 2000). Second, there is a linear relationship between the number of shal transcripts within a neuron and the maximal conductance of its I_A measured from the soma (Baro et al. 1997), and there is a similar linear relationship between shal immunolabeling of the soma and maximal I_A conductance (Baro et al. 2000). Third, shal encodes currents in Xenopus oocytes that more closely resemble the endogenous I_A in pyloric neurons than shaker (Kim et al., 1997, 1998, Baro et al. 2001.).

We have also expressed shal RNA by injection into pyloric neurons, and obtained an increased current that resembles the endogenous I_A (MacLean et al 1999, 2002). However, we show here that in a quantitative analysis, the increased I_A is not identical in its detailed biophysical properties to the endogenous I_A. In Pyloric Dilator (PD) neurons, the shal-expressed A-currents have a faster rate of inactivation, a slower time course of recovery from inactivation, and slightly hyperpolarized voltage dependence of inactivation.

These results raise the question of why the shal-encoded currents in pyloric neurons are different than the endogenous A-currents. In vertebrate neurons and cardiac myocytes, a similar discrepancy has been at least partly resolved by the finding of Kv
channel interacting proteins (KChIPs) (An et al. 2000). The KChIPs are a group of EF-hand calcium binding proteins, belonging to the neuronal calcium sensor-recoverin family. Co-expression of KChIP with Kv4 proteins in *Xenopus* oocytes, or the CHO and HEK 293 cloned cell lines, increased the channel density, slowed the inactivation kinetics and accelerated the rate of recovery from inactivation of Kv4 channels (An et al. 2000, Decher et al. 2001, Takimoto K et al. 2002). Following these studies, Nakamura et al. (2001) showed that frequenin (also called neuronal calcium sensor 1; NCS-1), a distant-related protein in the same family, can also regulate Kv4 currents. Similar to KChIP, frequenin can specifically bind to Kv4 channel proteins to increase the surface expression, accelerate the recovery from inactivation and slow the inactivation kinetics due to the increase in a slowly inactivating component of the current. However, the effect of frequenin on Kv4.2 is Ca\(^{2+}\) -dependent.

We hypothesized that the difference between endogenous I\(_A\) and *shal*-evoked currents in PD neurons was due to the lack of sufficient regulatory proteins in vivo. To test this, we have co-expressed lobster *shal* with the human gene, *hKChIP1*, as well as the lobster *frequenin* gene, in *Xenopus* oocytes and in PD neurons in the lobster STG. Co-expression of *hKChIP1* with *shal* altered the voltage inactivation properties as well as the kinetics of inactivation and rate of recovery from inactivation, to make the *shal* current identical to the endogenous I\(_A\). Co-expression of *shal* with *frequenin* produced many of the same changes in *shal* currents in PD neurons, but did not modify the rate of recovery from inactivation.
Materials and Methods

STG Dissection and PD cell identification

Pacific spiny lobsters (*Panulirus interruptus*) were purchased from Don Tomlinson Commercial Fishing (San Diego, CA) and maintained in artificial seawater at 16°C until use. Animals were anesthetized by cooling on ice for 30 minutes before dissection. The stomatogastric ganglion (STG) was dissected along with its motor nerves and associated commissural and esophageal ganglia, (Mulloney and Selverston, 1974) and pinned in a dish. The preparation was superfused continuously (3ml/min) with saline (16°C) containing (in mM): 479 NaCl, 12.8 KCl, 13.7 CaCl₂, 3.9 Na₂SO₄, 10.0 MgSO₄, 2glucose, and 11.1 Tris base, pH 7.35 (Mulloney and Selverston 1974). Extracellular recordings were made from identified motor nerves using glass suction electrodes. Individual somata were impaled with glass microelectrodes (10-25 MΩ, 3M KCl). The PD neurons were identified by the 1:1 correspondence of their intracellular action potentials with those recorded extracellularly on the PD motor nerve, and by their typical shape of membrane potential oscillations and synaptic inputs. (Kloppenburg *et al.*, 1999)

Microinjection of Neurons

*Shal* (L49135, Genbank) *Frequenin* (AF260780, Genbank) and *hKChIP1* (NM_014592, Genbank) RNAs were transcribed in *vitro* and capped using a T3 message mMachine kit (Ambion, Austin, TX, USA). The transcripts were cleaned using the RNeasy Mini kit (Qiagen, Valencia, CA). Following neuronal identification,
PD neurons were injected with an RNA solution containing 0.04% Fast Green using pressure pulses (40 psi, 0.2 Hz) driven by a home-made pressure injector and a pulse generator (Master-8, Jerusalem, Israel). The RNA solution contained 0.4 µg/µl Shal with or without 0.04µg/µl hKChIP1 or 0.01µg/µl frequenin. Various ratios of shal:hKChIP1 were tested, from 10:1 to 200:1 by weight, and all produced similar results. PD cells were injected with roughly equivalent amounts of RNA based on the color of the co-injected Fast Green. Fast Green alone was injected into control neurons, which were otherwise treated identically to the RNA-injected neurons. After injection, the ganglion, with attached nerves and the commissural and esophageal ganglia, was incubated in filter sterilized recording saline without Tris-base but with 5 mM HEPES 2g/l glucose, 50,000 unit/l penicillin and 50mg/l streptomycin at 16°C for 48-72 hrs to allow the expression of the proteins.

*Xenopus* oocyte expression

*Xenopus* oocytes were harvested and maintained as previously described (Baro et al. 1996b). Shal cRNA was diluted to a final concentration of 0.2 µg/µl and hKChIP1 to 0.02 µg/µl or frequenin at 0.005 µg/µl. Shal cRNA with or without hKChIP1 or frequenin was injected in 100 nl with a sterile glass microelectrode using a microinjector (NA-1, Sutter Instruments, San Rafael, CA, USA). The oocytes were cultured in sterilized ND96 solution (96 mM NaCl, 2mM KCl, 1.8 mM CaCl₂, 5mM HEPES and 1mM MgCl₂) with 1mM NaPyruvate and 0.1mg/ml Gentamycin at 16 °C. Recordings were typically made 24 hrs later.
Electrophysiology

**PD neurons:** After 2-3 days in organ culture, PD neurons were voltage-clamped using an Axoclamp 2B amplifier driven by pClamp8 software (Axon Instruments, Foster City, CA, USA). Microelectrodes were filled with 3 M KCl and had a tip resistance $\leq 8 \text{ M}\Omega$. To isolate PD neurons from most synaptic input and to isolate $I_A$ from most other currents, we superfused the ganglion with saline containing $10^{-7}$ M tetrodotoxin (TTX), $5 \times 10^{-6}$ M picrotoxin (PTX), $2 \times 10^{-4}$ M CdCl$_2$, $5 \times 10^{-3}$ M CsCl, and $2 \times 10^{-2}$ M tetraethylammonium chloride (TEA). The cells were held at -50 mV. The voltage dependence of activation was measured following a deinactivating prepulse to -120 mV for 400 ms with a series of 400 ms voltage steps from -60 mV to +30 mV in 10-mV increments. The data were leak subtracted using a P/6 protocol with steps opposite to the sign of activation. A control protocol for activation of non-$I_A$ currents was the same as the activation, but without the deinactivating step to -120 mV. The control protocol currents were digitally subtracted from the activation protocol currents to produce the isolated $I_A$. To measure the voltage dependence of inactivation, the cell was held at -50 mV where most of the $I_A$ was inactivated; a series of 1-s prepulses from -130 to -30 mV in 10 mV increments was given to remove the inactivation, followed by a 400 ms test pulse to 20 mV to determine the peak $I_A$ after each prepulse. The data were leak subtracted as described above. To measure the time course of recovery from inactivation, the cell was held at -50 mV and hyperpolarized to -120 mV for increasing step lengths before a test step to +20 mV to measure recovery of the current amplitude.

**Xenopus oocytes:** The oocytes were voltage clamped using a Geneclamp amplifier driven by Clampex 8.0 software (Axon Instruments, Foster City, CA, USA).
All recordings were made in standard ND96 solutions (Baro et al. 1996b). The A-current was recorded using similar protocols as in PD neurons, except that when recording the voltage-dependence of activation and inactivation, the membrane potential was held at -70 mV, and the prepulses lasted 1s to -90 mV for activation and 2s for inactivation.

**Current analysis:** The voltage dependence of activation of $I_A$ was determined by converting the peak current to a peak conductance, $g$, assuming $E_K = -94$ mV for oocytes and $E_K = -86$ mV for PD neurons (Hartline and Graubard 1992). The resulting $g/V$ curve was fitted to a third order ($n = 3$ for activation) and first-order ($n = 1$ for inactivation) Boltzmann equation of the form

$$\frac{g}{g_{\text{max}}} = \frac{1}{1 + e^{(V - V_{1/2})/s^n}},$$

where $g_{\text{max}}$ is the maximal conductance and $s$ is a slope factor. For the activation curve, $V_{1/2}$ is the voltage at which half-maximal activation of the individual gating steps occurs, assuming a third-order activation relation (Hodgkin and Huxley 1952). For the inactivation curve, $V_{1/2}$ is the voltage at which half the channels are inactivated.

The kinetics of inactivation of the current were analyzed by fitting the inactivating phase of the currents at +20 mV with the following equation:

$$I = I_o + I_f e^{-t/\tau_f} + I_s e^{-t/\tau_s},$$

where $\tau_f$ and $\tau_s$ are the time constants of fast and slow components of inactivation, and $I_f$ and $I_s$ are their amplitudes. $I_o$ is the non-inactivating component.

Student’s $t$-tests were used to assess statistical significance. Throughout this paper, all calculated values are reported as means $\pm$ SE.
Results

Injection of Shal RNA enhances I_A

In each STG, there are two PDs with almost identical electrophysiological properties. Thus, one PD neuron, injected only with Fast Green, can serve as an internal control for the other, RNA-injected PD neuron. After injection, the ganglion, with the appropriate motor nerves and associated commissural and esophageal ganglia attached, was cultured in sterilized HEPES- buffered saline at 16°C for 48-72 hrs to allow expression of the proteins.

The I_A in control PD neurons after 48-72 hrs culture had almost identical voltage and kinetic properties as the currents recorded in PD neurons from acutely dissected STG preparations (Baro et al., 1996 and 1997, Table 1, Fig. 1). The amplitude of endogenous I_A was around 300 nA at +20 mV (Fig 1A(i)). Activation of I_A was voltage-dependent. The normalized peak conductance/voltage relation was fitted with a third-order Boltzmann relation (Equation 1). The voltage of half maximal activation of the individual gating particles was around -41 mV (n=10; Table 1), corresponding to a half-maximal activation of the current itself at -13.6 mV; the slope factor was -20 mV (Fig 1B(i)). The current inactivated with time in a voltage-dependent manner. To determine the voltage dependence of inactivation, pre-pulses from -130 to -30 mV were given, followed by a step to +20 mV; the peak currents were plotted against the pre-pulse voltages and fit by a first order Boltzmann equation (Fig. 1B(ii)). The V_{1/2} for inactivation was -63 mV with a slope factor of 5.1 mV (n=10, Fig. 1B(ii)). The kinetics
of inactivation for this current was rapid and biphasic, and was fit by equation 2. At +20 mV, approximately 52% of the peak current inactivated with a slow time constant of 81 ms, while the remaining current inactivated with fast time constant of 20 ms (Table 1, Fig. 1A(ii)). The A-currents were mostly inactivated when the membrane potential was held at -50 mV; when the PD neurons were stepped to -120 mV, inactivation was removed with a single exponential time course; the time constant was about 18 msec (Fig. 2).

In the PDs that were injected with shal RNA, the amplitude of I_A more than doubled 48-72 hrs following injection (7.2 µS in the shal-injected PDs vs. 3.4 µS in control PDs; p<0.01, Fig 1A(i)). However, the newly expressed currents had somewhat different biophysical parameters from the endogenous I_A. The voltage dependence of activation of the exogenous I_A was similar to the endogenous current (Fig. 1B(i)), but the voltage of half-inactivation showed a trend to hyperpolarize by 5 mV and its slope factor was increased by 1.7 mV (p<0.05) (Fig 1B(ii)). Although the fast and slow time constants of inactivation did not change, the percentage of current inactivating with τ_slow decreased from 52% to 36% (p<0.01, Table 1). As a consequence, the I_A in shal-injected PD neurons inactivated more quickly than control neurons, which is clearly seen when the amplitude-normalized currents are superimposed (Fig 1A(ii)). Finally, the rate of recovery from inactivation during a prepulse to -120 mV was significantly slowed by 56% (p<0.05; Table 1, Fig 2)

**hKChIP1 alters the shal currents expressed in Xenopus oocytes**
As described previously by Baro et al (1996), 24 hrs after injection into Xenopus oocytes, shal RNA produced a transient A-type current resembling the I_A in pyloric neurons (Fig 3A; n= 7). The voltage of half maximal activation of the individual gating particles was around -41 mV (n=5; Table 1), corresponding to a half-maximal activation of the current itself at −13.6 mV (n=5); the slope factor was −18 mV. The V_{1/2} for inactivation was −73 mV with a slope factor of 7 mV (n=7; Fig 3B). The kinetics of inactivation for this current was rapid and biphasic. At +20 mV, approximately two thirds of the peak current inactivated with a fast time constant of 35 msec, while the remaining current inactivated with a slow time constant of 139 ms (Table 1, Fig. 1A3A). The shal currents were mostly inactivated when the membrane potential was held at -50 mV; when the oocytes were stepped to −90 mV, removal of inactivation occurred with a time constant of about 470 ms (Fig 3C).

Coexpression of shal with hKChIP1 significantly altered the properties of the resulting A-currents in Xenopus oocytes. Compared with the current evoked by shal alone, the maximum conductance of the shal + hKChIP1 current more than doubled (106.9 µS in shal +hKChIP injected cells vs 50.8 µS in shal alone; p<0.01, Fig 3A). When co-injected with hKChIP1, the voltage of half maximal activation of shal currents was significantly (p<0.01) shifted by -7mV in the hyperpolarizing direction, while the voltage of half -inactivation was significantly (p<0.01) shifted by 6 mV in the depolarizing direction and its slope factor became slightly steeper (Fig 3B, Table 1). With regard to the kinetics of inactivation, the slow and fast time constants of inactivation for shal were not statistically different from the shal+hKChIP1 current (p> 0.1). However, the relative contribution of the slowly inactivating component was dramatically increased
from 30% to 94% (p<0.01, Table 1). As a consequence, the overall time course of inactivation slowed down, as can be seen when the currents are normalized to the same amplitude and superimposed (Fig 3A(ii)). In addition, co-injection with hKChIP1 almost doubled the rate of recovery from inactivation of the shal current at –90 mV (Fig 3C, Table 1). All these biophysical changes generated by co-injection of hKChIP1 with shal would increase the effective I_A in the subthreshold voltage range (below –45 mV), which in turn would hyperpolarize the membrane potential and reduce the excitability of the cell. The increased peak conductance, slowed inactivation kinetics, faster recovery from inactivation and more negative activation voltage induced by co-expression of hKChIP1 with shal are similar to those observed when KChIPs were co-expressed with mammalian Kv4 channels in CHO cells and Xenopus oocytes (An et al. 2000).

Co-expression of hKChIP1 with shal in PD neurons produces an A-current that closely resembles the endogenous A-current.

We then co-injected shal RNA with hKChIP1 into lobster PD neurons under the same culture conditions as described above. After 48-72 hrs, co-injection produced a large I_A when compared to the endogenous A-current (Fig 4A(i). The maximum conductance of the I_A produced by shal +hKChIP injection was significantly different from control values (7.6±2.0 μS vs. 3.4± 0.6 μS; p<0.01), but was not significantly different from shal alone injection (7.6 μS vs 7.2 μS, respectively). However, all of the biophysical parameters of the current were closer to those of the endogenous I_A in PD neurons, compared to shal injection only (Fig. 4; Table 1). Co-injection of hKChIP1 with shal did not alter the V_Acl significantly (Fig. 4B(i)), but it shifted the voltage for half-
inactivation back to a more depolarized value with a steeper slope, which was almost identical to the native current values (p>0.4; Fig. 4B(ii), Table 1). Once again the inactivation rate constants did not change, but the percentage of current inactivating with \( \tau _{s} \) increased from 34% to 45%, not significantly different (p>0.15) from the native current value of 52%. This caused the enhanced \( I_A \) to inactivate at a rate very similar to \( I_A \) in control PD neurons when amplitude-normalized and superimposed (Fig. 4A(ii)). Finally, compared to shal over-expressing neurons, the co-injection significantly reduced the time constant for recovery from inactivation to a value not significantly different from that seen in native currents (Fig 5). As seen in Table 1, none of the voltage and kinetic parameters of \( I_A \) in \( shal + hKChIP1 \)-injected neurons are significantly different from \( I_A \) parameters in the paired control PD neurons. These data show that co-injection of \( Shal + hKChIP1 \) RNA can reproduce the endogenous \( A \)-currents in PD neurons. Injection of \( hKChIP1 \) RNA alone into PD neurons did not significantly alter the amplitude or biophysical parameters of the endogenous \( I_A \) (n=4; data not shown).

**Coexpression of shal with lobster frequenin partially restores the properties of A-currents in PD neurons**

We used several sets of degenerate primers, based on the sequences of the vertebrate \( KChIP \) genes, in RT-PCRs with lobster RNA to try to clone the lobster \( KChIP \) homolog, but without success. However, one primer set did yield a fragment of the lobster \( frequenin \) gene. This is another protein in the same family of EF-hand calcium-binding proteins as \( KChIP \), and the \( Panulirus interruptus \) homolog had already been previously cloned by Jeromin et al. (1999). We co-injected \( shal \) and \( frequenin \) RNAs into
Xenopus oocytes, but *frequenin* significantly suppressed the expression of shal A-current in these cells (data not shown). However, when we co-expressed *shal* and *frequenin* in lobster PD neurons, the resulting increase in $I_A$ was only slightly (and not significantly) suppressed (5.5 µS in *Frequenin + shal* vs 6 µS in *shal* alone injected cells). *Frequenin* significantly modified the *shal*-evoked currents to more closely resemble the endogenous $I_A$ (Fig 6A, Table 1). Compared to the currents evoked by *shal* alone, the *shal+frequenin*–evoked current had similar voltage dependence of activation (with a slightly steeper slope for activation), but a more depolarized voltage for half-inactivation (-62.1 mV in *Frequenin + shal* vs -68 mV *shal* alone injected cells, $p<0.05$, Table 1, Fig 6B(i)). In addition, the currents produced by co-expression of *shal* and *frequenin* had slower inactivation kinetics compared to the *shal*-evoked currents, (Fig.6A(i)), due to the larger percentage of slowly inactivating current.(56% vs 36%, Table 1). All these parameters were significantly different from those obtained with *shal* injection alone ($p<0.05$), and not significantly different from the parameters of the endogenous $I_A$ (Fig. 6A(ii), B(ii); Table 1). However, unlike *hKChIP1*, *frequenin* co-injection failed to accelerate the time constant for release from inactivation of the *shal*-evoked currents (Fig.6C). The value of this time constant was around 24 ms, which was somewhat faster but statistically similar to *shal*-evoked currents (28 ms, Fig. 6C(i), Table 1) but significantly slower than endogenous currents (18 ms, $p<0.05$; Fig. 6C(ii), Table 1).

**Discussion**

Electrophysiological, pharmacological and modeling studies all show that $I_A$ plays an important role in setting the activity states and firing pattern of the 6 cell types in
the pyloric network (Tierney 1992, Harris-Warrick et al 1995; Golowasch 1999, Kloppenburg et al. 1999, Goldman 2001.). Our earlier studies suggested that lobster shal encodes I_A in pyloric neurons (Baro et al. 1997, Baro and Harris-Warrick 1998, MacLean et al., 1999, Baro et al. 2000.). However, when injected by itself into PD neurons, shal RNA produces an A-type current which, although similar to the endogenous I_A, differs in several parameters. The currents generated by shal in PD neurons inactivate significantly more rapidly, recover from inactivation significantly more slowly and have a slightly hyperpolarized V_1/2 for inactivation than the native A-currents.

One possible explanation for the failure of injected shal RNA to reproduce the endogenous I_A in PD neurons is that there are a rate-limiting number of some auxiliary proteins that normally interact with shal proteins in the neurons. A number of auxiliary proteins have been reported that interact with different Shaker family potassium channels and modify their gating, kinetics and localization in vivo and in vitro (Heinemann et al. 1994; Rettig et al. 1994, Pongs et al. 1999). The recently cloned KChIPs are a group of calcium binding proteins that can interact with vertebrate shal-related Kv4 channels and modify their properties to become more similar to native currents in different cell types (An et al. 2000. Decher et al. 2001, Rosati et al. 2001). For example, hKv4.3 is known to generate the I_to1 current in human ventricular cells, but hKv4.3 alone failed to reproduce this current in heterologous cell lines; in particular, the artificial current had a much slower rate of recovery from inactivation. Co-expression of hKChIP2 with hKv4.3 in Xenopus oocytes produced A-currents much more similar to the ventricular I_to1 (Decher et
al. 2001). Thus, we hypothesized that co-injection of *shal* with a KChIP protein into PD neurons would restore normal properties to the *shal*-evoked $I_A$.

Our results show that lobster *shal* can indeed interact with KChIP proteins to restore normal properties of the resulting A-current in PD neurons. In fact, all the significant differences between the endogenous $I_A$ and the additional current generated by injection of *shal* alone are corrected by co-injection of hKChIP1 with *shal* RNA. Most significantly, the voltage for half inactivation is shifted in the depolarized direction, the ratio of slowly to rapidly inactivating current is increased, and the rate constant for recovery from inactivation is accelerated compared to the *shal*-induced current. As a consequence, none of the biophysical parameters of $I_A$ in the *shal*+ hKChIP1-injected neurons are significantly different from the endogenous $I_A$ in the control PD neurons. This demonstrates that the shal current can be modified by auxiliary proteins.

However, despite repeated efforts, we have not yet successfully cloned the lobster *KChIP* homolog. Using *KChIP*- specific degenerate primers, we did clone a fragment of the lobster *frequenin* gene, which encodes another small calcium sensor protein in the same superfamily as KChIPs (Jeromin et al., 1999). Compared to the KChIP proteins, frequenin is involved in a number of different biological processes. It can interact with many proteins, from phosphatidylinositol 4-kinase to $Ca^{2+}$ channels, resulting in modification of exocytosis and Golgi transport in different systems (Pongs *et al.*, 1994; Hendricks *et al.*, 1999; Walch-Solimena and Novick, 1999; Wang *et al.*, 2001; Koizumi *et al.*, 2002). The mechanisms underlying many of its functions are still unclear.
Recent research showed that frequenin could also modify the properties of the vertebrate Kv4 channel in a similar way as KChIPs (Nakamura et al. 2001).

We first looked at the possible interaction between *shal* and *frequenin* by co-injection into *Xenopus* oocytes. However, *frequenin* significantly depressed the current evoked by co-injection with *shal*. This failure might be due to species-specific differences in post-translational modification of the proteins. For example, Nakamura et al (2001) showed that the effect of frequenin on Kv4 channels was Ca^{2+} -dependent. Therefore it would not be surprising that some post-translational modification is involved in the interaction between lobster frequenin and shal, which is not efficiently mimicked in *Xenopus* oocytes.

To avoid inter-species incompatibilities, we decided to co-inject lobster *frequenin* with lobster *shal* in the lobster PD neurons. When this was done, the resulting $I_A$ was significantly different from that seen with *shal* alone, and most biophysical parameters were similar to the control $I_A$. Specifically, the voltage for half-inactivation and kinetics of inactivation were not significantly different from the control $I_A$ parameters. The only parameter that was not corrected by frequenin was the rate of recovery from inactivation, which remained slower than the endogenous current. We are not sure why this one parameter remained unchanged. One possibility is that multiple auxiliary proteins interact with lobster shal, including frequenin and additional unknown proteins. One of these might be the lobster homolog of *KChIP*. Nakamura et al (2001) also showed that when co-expressed with Kv4.2 in oocytes, frequenin had a greater effect on the kinetics of inactivation, whereas KChIP1 had a stronger effect to enhance surface expression and accelerate recovery from inactivation. Patel et al (2002) also suggested
that different protein motifs in KChIP2 affect inactivation and recovery from inactivation of Kv4.3 differently. Therefore these two functions may be separate. In PD neurons, frequenin may only be involved in the modification of inactivation kinetics and voltage dependence of the shal current, while other proteins may shape the recovery from inactivation.

Although we still do not completely understand all of the factors that shape the shal potassium currents in pyloric neurons, our results support our earlier hypothesis that the \textit{shal} gene encodes $I_A$ in the soma and neuropil of lobster pyloric neurons. We previously used molecular and immunocytochemical methods to show that \textit{shal} mRNA and protein levels are linearly correlated to the amplitude of $I_A$, and the shal protein is present in appropriate locations in the STG neurons and neuropil (Baro et al. 1996a; Baro et al. 1996b, Baro and Harris-Warrick 1998). Our ability to correctly mimic $I_A$ by co-injection of \textit{shal} with \textit{hKChIP1} and, to a significant extent, lobster \textit{frequenin} provides an important additional step in demonstrating that \textit{shal} encodes lobster $I_A$. Our results suggest that shal proteins may interact with frequenin and/or other KChIP-like proteins to produce the natural transient potassium current in pyloric neurons. Our results are also the first to show that frequenin/KChIP proteins can modify shal channels in functional neurons.
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Table 1 effects of KChIP1 on shal A-currents

<table>
<thead>
<tr>
<th></th>
<th>Act V$_{1/2}$ (mV)</th>
<th>s(Act) (mV)</th>
<th>Inact V$_{1/2}$ (mV)</th>
<th>s(Inact) (mV)</th>
<th>Inact $\tau_{fast}$ (ms)</th>
<th>Inact $\tau_{slow}$ (ms)</th>
<th>% $\tau_{slow}$</th>
<th>$\tau_{rec}$ (ms)</th>
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</thead>
<tbody>
<tr>
<td>PD neuron</td>
<td></td>
<td></td>
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<tr>
<td>Control n=10</td>
<td>-40.7±8.4</td>
<td>-20±2.7</td>
<td>-63.5±8.4</td>
<td>5.1±0.65</td>
<td>20±2.3</td>
<td>80±9.4</td>
<td>52.4±6.1</td>
<td>18.0±5.5</td>
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<tr>
<td>Shal n=9</td>
<td>-37.3±4.0</td>
<td>-17.1±1.1*</td>
<td>-67.9±2.8</td>
<td>6.9±0.97*</td>
<td>17.6±1.7</td>
<td>83.1±15.5</td>
<td>35.7±8.7**</td>
<td>28±9.2*</td>
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<tr>
<td>Shal+KChIP n=6</td>
<td>-36.5±1.4</td>
<td>-18.0±0.7*</td>
<td>-62.0±1.4*</td>
<td>5.5±0.36*</td>
<td>18.0±3.0</td>
<td>72.1±5.9</td>
<td>45.9±6.7*</td>
<td>16.0±4.5*</td>
</tr>
<tr>
<td>Shal +Freq n=7</td>
<td>-40.4±3.4</td>
<td>-19.8±1.4*</td>
<td>-62.1±2.5**</td>
<td>6.0±1.2</td>
<td>19.8±1.5</td>
<td>86.8±17.6</td>
<td>56.5±12.5</td>
<td>24.3±5.6*</td>
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<tr>
<td>Oocyte</td>
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<tr>
<td>Shal n=7</td>
<td>-40.7±2.6</td>
<td>-18.4±1.6</td>
<td>-73.1±2.4</td>
<td>6.6±0.62</td>
<td>34.7±10</td>
<td>138.6±25</td>
<td>30.5±11</td>
<td>465±143</td>
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<td>Shal+Kchip n=6</td>
<td>-47.5±2.2*</td>
<td>-17.3±1.0</td>
<td>-67.1±2.3*</td>
<td>4.5±0.55**</td>
<td>39.4±27</td>
<td>146.5±37</td>
<td>94.2±17**</td>
<td>243±120*</td>
</tr>
</tbody>
</table>

* significantly different from shal expressed cells, p<0.05
+ significantly different from control p<0.05
** very significantly different from shal expressed cells, p<0.01
++ very significantly different from control p<0.01
Figure 1 Shal produces large A-currents in PD neurons.

A(i): $I_A$ measured at +20 mV after a prepulse to –120 mV from control and shal-injected PD neurons; in A(ii), the currents are scaled to show their difference in inactivation rate.

B: Plots of $g/g_{\text{max}}$ vs voltage for activation (B(i)) and inactivation (B(ii)) of A-currents in control (□) and Shal (△).
Figure 2 *Shal-evoked* currents in PD neurons have slower recovery from inactivation than endogenous currents.

A. Current traces from vehicle (A(i)) and shal (A(ii)) injected PD neurons. The voltage protocol is shown at the top. B. Plots of the time course of recovery from inactivation in control (□) and shal (△).
Figure 3  **hKChIP1 alters the Shal currents expressed in Xenopus oocytes.**

A(i): Oocytes were injected with *Shal* or *Shal+hKchip1* RNA. Currents were triggered by a 200 ms pulse to +20 mV after a prepulse to –90 mV. A(ii): The currents in A(i) are scaled to show the difference in inactivation rates. B: Plots of $g/g_{\text{max}}$ vs voltage for activation and inactivation of A-currents in *Shal* (○) or *Shal+hKChIP1* (●) expressing cells. C. Plots of the time course of recovery from inactivation at –90 mV from Shal (○) or Shal+hKChip1 (●) expressing cells.
Figure 4 Coexpression of shal with hKChIP1 produces an A-current similar to the endogenous A-current in PD neurons.

A(i): $I_A$ measured at +20 mV after a prepulse to −120 mV from control and shal+hKchip-injected PD neurons; in A(ii) the currents are scaled to compare their inactivation rate. B(i) and B(ii): Plots of $g/g_{\text{max}}$ vs voltage for activation (B(i)) and inactivation (B(ii)) of A-currents in control (□) and shal + hKChIP1 (○). The control and Shal+hKChIP1 curves are almost completely overlapping.
Figure 5 *hKChIP1* accelerates the recovery from inactivation of the *shal*-encoded A-currents in PD neurons.

A. Current traces from vehicle (A(i)) and *shal*+*hKChIP1* (A(ii)) injected PD neurons. The voltage protocol is the same as shown in Fig. 2. B. Plots of the time course of recovery from inactivation in control (□) and *shal* +*hKChIP1* (○). The control and *shal*+*hKChIP1* curves are almost completely overlapping.
Figure 6 Frequentin modifies the shal-evoked currents resemble endogenous A-currents in PD neurons

A: Scaled $I_A$ currents measured at $+20$ mV after a prepulse to $-120$ mV from frequentin +shal or shal (A(i)) and frequentin +shal or vehicle (A(ii)) injected PD neurons to compare their inactivation kinetics. B(i), B(ii): Plots of $g/g_{max}$ vs voltage for activation (B(i)) and inactivation (B(ii)) of A-currents in control (□), shal (△) and shal + frequentin (○). C: Plots of the time course of recovery from inactivation in shal (△) or vs. shal + frequentin (○) (C(i)) and shal + frequentin (○) or vs. vehicle (□) (C(ii)) injected cells.