dSLIP1, a Novel Protein which Interacts with Large-conductance Calcium-activated Potassium Channels

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A DISSERTATION

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

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Abstract

Large-conductance calcium-activated potassium channels (BK channels) are activated by depolarized membrane potential and elevated levels of intracellular calcium. BK channel activity underlies important physiological functions, such as the fast afterhyperpolarization (fAHP) in central neurons, transmitter and hormone release of neuron and gland cells, regulation of smooth muscle contraction, and regulation of secretion in exocrine cells. Electrophysiological studies have shown that native BK channels are regulated by a wide range of second messengers including several protein kinases and protein phosphatases (Chung et al., 1991; Ewald et al., 1985; Reinhart et al., 1991) and G-proteins (Cole and Sanders, 1989; Scornik et al., 1993; Toro et al., 1990). In addition, mammalian BK channels have a closely associated b subunit which modifies the calcium sensitivity of the channel, and may itself be the target for regulatory second messengers (Dworetzky et al., 1996; Hanner et al., 1997; McManus et al., 1995). Regulation of BK channel activity exerts a powerful modulation on neuronal excitability. Several mechanisms may influence the fAHP through indirect effects on BK channels. There is evidence that at least in some neuronal cell types, BK and voltage-dependent calcium channels (VDCCs) are closely associated, and may be physically coupled (Gola and Crest, 1993; Issa and Hudspeth, 1994; Robitaille et al., 1993). Other posttranslational modulatory effects on BK channels have been described, but the underlying molecular mechanisms have not yet been established (Subramony and Dryer, 1997; Subramony et al., 1996). For other voltage-gated potassium channels, a distinct beta subunit, KvBeta2, associates with the alpha subunit early in channel biosynthesis and exerts dramatic, chaperone-like effects on the alpha subunit, including stabilization and increased cell surface expression (Rettig et al., 1994; Shi et al., 1996).

To identify other proteins, such as Beta subunits, that interact with BK channels and influence BK expression, the C-terminal domain of a cloned BK channel (*dSlo*) was employed in screening a *Drosophilla* embryo cDNA library via a modified yeast two-hybrid approach, the interaction trap.

One of the novel clones identified, dSLIP1, specifically interacts with *Drosophila* and human BK channels and has partial homology to the PDZ domain of alpha1 syntrophin. The dSLIP1 and *dSlo* mRNAs are coincidentally expressed throughout the *Drosophila* nervous system, the two proteins interact in vitro, and they may be coimmunoprecipitated from transfected COS7 cells. Coexpression of dSLIP1 with *dSlo* or *hSlo* BK channels in *Xenopus* oocytes results in reduced macroscopic currents compared to expression of BK channels alone; current amplitudes may be rescued by coexpression with the C-terminal domain of *dSlo* channel which interacts with dSLIP1. Single channel recordings and immunostaining of transfected tissue culture cells suggest that dSLIP1 selectively reduces *Slo* BK currents by reducing the number of BK channels in the plasma membrane.

Chapter I: Introduction

Potassium channels, an overview

Ion channels are membrane proteins which form aqueous pores through the lipid bilayer. When open, ion channels coordinate the passage of a wide range of substrates, from ions to small proteins, through the electrically insulating membrane, and in this way produce and transduce electrical signals (Hille, 1992). However, ion channels are not always open. They are dynamic molecules which rapidly change between ion conducting (open) and non-conducting (closed) states, the process, called gating. In addition to the all-or-none gating mechanism, the frequency of gating may be modulated by other factors such as second messengers. Channels are ion-selective, an intrinsic property of the pore. When open, channel selectively allows particular classes of ions to flow through the pore, down the electrochemical gradient, while others are excluded. Gating and ion selectivity of different ion channel classes give rise to an integrated symphony of fluxes which regulates the electrical properties of excitable cells (Hille, 1992; Jan and Jan, 1989; Kandel et al., 1991).

Potassium channels are the most diverse class of ion channel and they participate in a wide range of biological functions such as the regulation of excitability, release of peptides or hormones from secretory cells, and the control of plant stomatal guard cell movement (Hille, 1992; Kandel et al., 1991). During the past decade, the combination of molecular biology and electrophysiology has greatly enhanced our understanding of the structure and function of all classes of ion channels. Indeed, the molecular diversity that has been revealed thus far surpasses the extent of variability anticipated by the previous decade of electrophysiological recordings of native channels. In some cases, heterologous expression of one polypeptide reconstitutes most properties of the native channel, while in other cases coexpression of several polypeptides is required (Sather et al., 1994). By comparing amino acid sequences and heterologous expression characteristics, cloned channel proteins may be divided into

different families. For example, the voltage-gated Na^+ and Ca^{2+} channels belong to a distinct family and are formed from one alpha subunit which folds into a fourfold pseudo-symmetrical array. Voltage-gated K^+ channels, on the other hand, are formed by the association of four subunits which may be the same or different, each subunit being similar to one of the internal repeats found in Na^+ or Ca^{2+} channels (Figure 1.1).

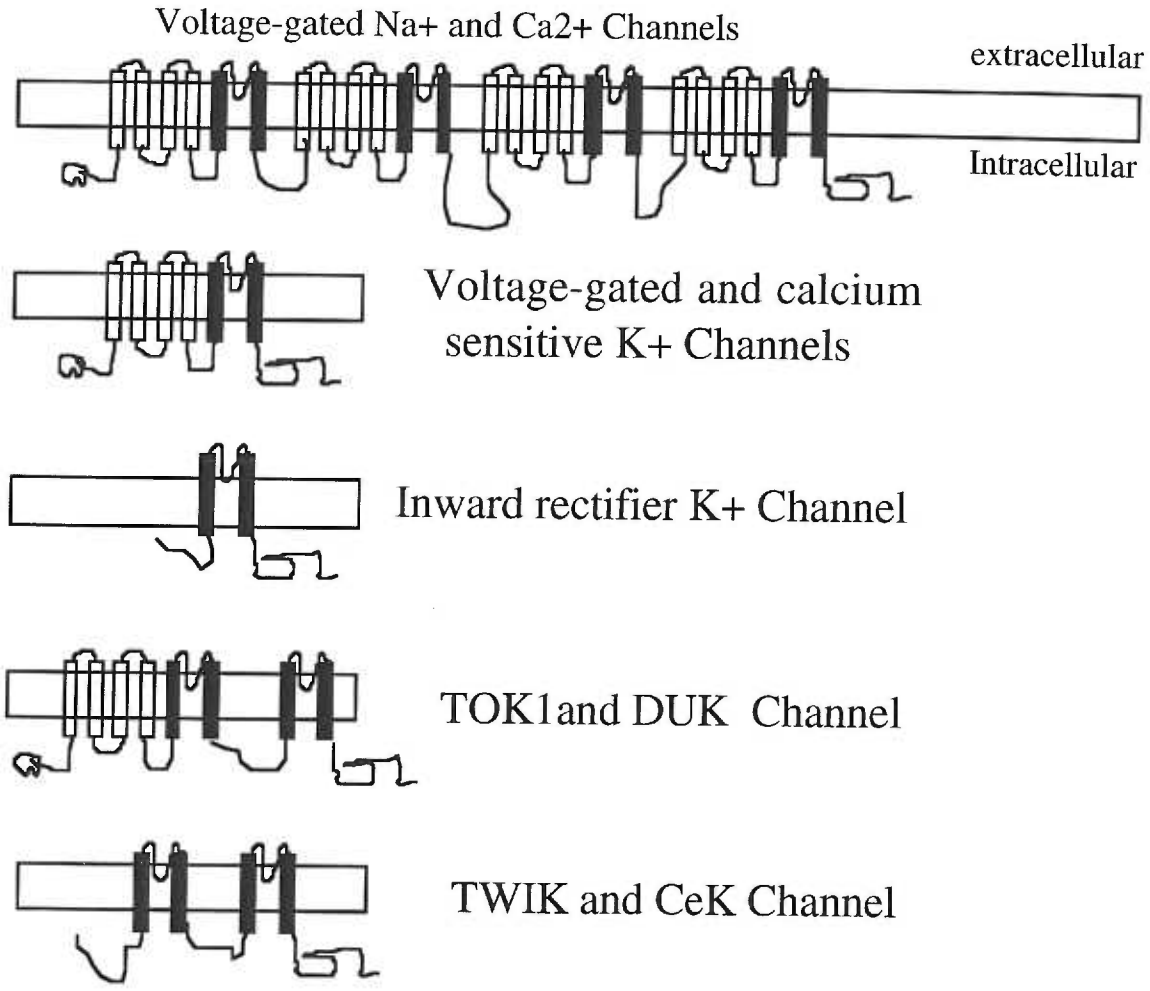

Figure 1.1 Representation of alpha subunits from four classes of potassium channels and their topological relationship to the alpha subunits of sodium and calcium channels. Voltage-gated sodium and calcium channel subunits contain four tandem structural units, each of which contains 6 transmembrane domains. Pore (P) domains are shown as membrane-embedded loops with flanking transmembrane domains (shown in black). All four potassium channels have a highly conserved P domain that is their unifying structural feature. The presumed P domains in sodium and calcium channels differ substantially among the four tandem structural units, and thus form a nonsymmetrical pore. The N- and C-terminal of all channels represented are presumed to be on the cytoplasmic side of the plasma membrane.

The first potassium channel cloned was the voltage-sensitive K^+ channel from *Drosophila*, the product of the *Shaker* gene (Papazian et al., 1987). This landmark study integrated molecular biology, genetics, and electrophysiology. Using a mutant fly, the *Shaker* fly, which lacks a voltage-gated potassium current (I_A) in flight muscle (Salkoff, 1983; Salkoff and Wyman, 1981; Solc et al., 1987), the cDNA encoding a functional *Shaker* channel was isolated and characterized (Papazian et al., 1987). Additionally, *Shaker* mRNA was synthesized in vitro, injected into *Xenopus* oocytes, and the resulting currents studied using the two-electrode voltage clamp configuration (Tempel et al., 1988). This series of manipulations has become a paradigm for the study of cloned ion channels. Using the cloned *Shaker* cDNA as a probe, many other voltage-dependent potassium channels have been isolated and characterized from *Drosophila* as well as other organisms including mouse, rat, and human. Following the molecular cloning of *Shaker*-like potassium channels, several other types of potassium channels, such as inward rectifiers, and calcium-sensitive potassium channels, were cloned (for review see, Jan and Jan, 1997). The functional diversity of potassium channels has been revealed by the molecular identification of several topologically distinct families, each with many members. Extensive studies on voltage-gated *Shaker* channel generate wealth of knowledge which provides a prototype for understanding the structure and function of potassium channels. In brief, the *Shaker* channel has six transmembrane domains (S1-S6) and a P-region between S5 and S6 with both N- and C-terminus residing intracellularly. It activates upon membrane depolarization through an intrinsic gating mechanism which involves the movement of charged residues primarily in S4 and S2, and inactivates after channel opening by two separate, yet interacting mechanisms, N- and C-type inactivation. Potassium channels are generally tetrameric. The assembly of the channel, either homo- or heteromeric, is determined by amino acids within the N-terminal domain of *Shaker*-like channels or the transmembrane domain region of the inward rectifiers. The GYG sequence within the P-region endows channel with exquisite selectivity for

potassium ions, and the permeation pathway is formed by the P-regions, the intracellular loop between S4 and S5, as well as the N-terminal domain of S6.

Calcium activated potassium channels

In 1958, Gardos demonstrated that elevated internal calcium induced potassium flux from red blood cell ghosts (Gardos, 1958). Subsequently, Meech showed that microinjection of calcium induced hyperpolarization of an *Aplysia* neuron and this was due to the increase of a potassium conductance (Meech, 1972). Voltage-clamp, patch-clamp and lipid-bilayer studies indicated that there are several different subtypes of calcium-activated potassium channels prevalent in neurons, muscle and other type of cells (Latorre et al., 1989). Calcium-activated potassium channels may be divided into three classes based on single-channel conductance, voltage-dependence of gating, and sensitivity to blockers: BK, large-conductance, calcium and voltage activating channels; SK, small-conductance, and IK, intermediate-conductance channels, activated only by calcium. BK channels were the first which were functionally examined in great detail, as they are easily recognized and may be efficiently incorporated into planar lipid bilayers (Adams et al., 1982; Marty, 1981; Pallotta et al., 1981). BK channels have a single channel conductance between 100-300 pS and typically need micromolar concentrations of calcium to open. Moreover, they also require depolarization, calcium and voltage work together to gate BK channels. Thus at more depolarized potentials, less calcium is needed while at more hyperpolarized potentials, more calcium is required (Figure 1.2; (Barrett et al., 1982). More recently, it has been shown that BK channels may open without calcium but under dramatic voltage conditions, exceeding 200 mV (Cui et al., 1997), indicating that BK channels are actually voltage-gated and calcium modulated. BK channels are blocked by millimolar concentrations of external TEA (tetrathylammonium) and some of them are also sensitive to nanomolar concentrations of external CTX (charybdotoxin) or IBX (iberiotoxin).

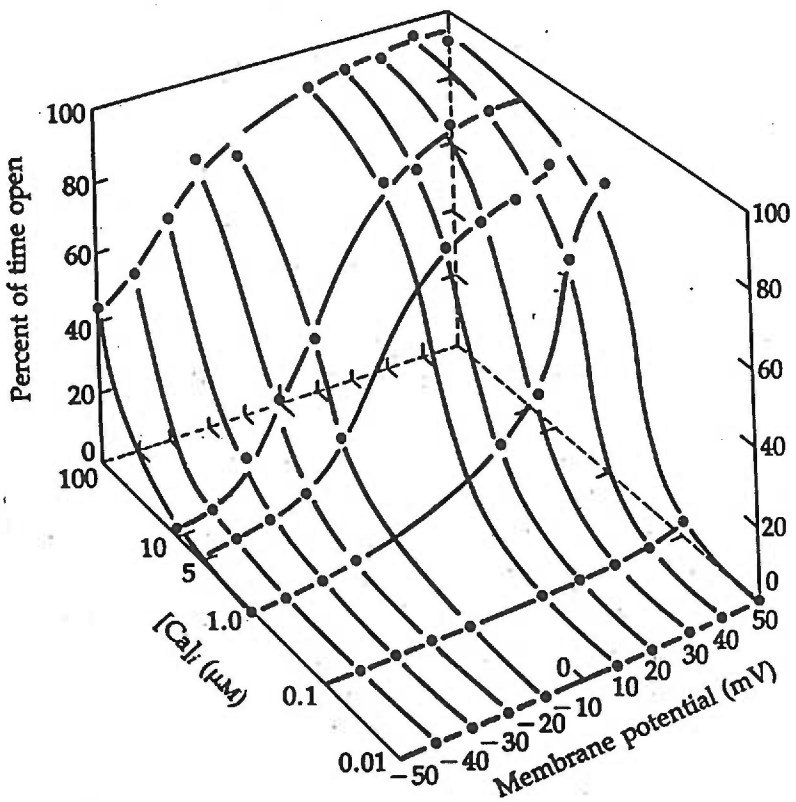

Figure 1.2. Voltage and calcium-dependence of the BK Channel.

The plot shows the percent of time open versus membrane potential and free $[Ca^{2+}]_i$ for single BK channels. The data were calculated from long records of inside-out patches from rat myotubes, so that known $[Ca^{2+}]$ could be readily applied to the intracellular face. T = 21°C (Barrett et al., 1982)

Small-conductance calcium-activated potassium channels have been less well studied, but possess single-channel conductance of 5-20 pS and their open probability depends solely on internal calcium and not transmembrane potential. SK channels require significantly less internal calcium for gating than BK channels, requiring submicromolar concentrations. SK channels are not sensitive to TEA, CTX, or IBX, but some are blocked by picomolar concentrations of apamin. Single SK channels were first recorded from cultured neonatal rat skeletal muscle (Blatz and Magleby, 1986) and subsequently have been shown in a variety of excitable and non-excitable cells (Sah, 1996); this family has recently been cloned (Kohler et al., 1996). In neurons, SK channels are thought to underlie the slow after-hyperpolarization (sAHP) and spike frequency adaptation (Hille, 1992). SK channels are modulated by second messengers which result from activation of many neurotransmitter or hormone receptors (Sah, 1996). Finally, intermediate-conductance calcium-activated potassium channels (IK channels) are the least well characterized, and have unit conductance between 20-80 pS. They are gated only by internal calcium and are not voltage-dependent, and their calcium sensitivity is similar to SK channels. IK channels are not sensitive to TEA, IBX, or apamin, but are blocked by CTX and the antifungal agent, clotrimazole. The Gardos channel is an IK channel, and has recently been cloned (Ishii et al., 1997).

BK channels

The distinctive hallmarks of BK channels are: 1) under physiological conditions, they require both calcium and voltage for activity and, 2) they possess large single channel conductances with rates of ion flux exceeding the rates of free diffusion. Though BK channels have very large conductances, they are still highly selective to potassium ions with a permeability ratio for potassium to sodium of more than 100. This indicates that BK channels have multi-ion pores with a fixed negative charge at the channel entrances (see review of (Latorre et al., 1989). Potassium ion repulsion in the multiply-occupied pore, together with the high local

potassium ion concentrations created by negative charges at channel vestibules and depolarization greatly increase the rate of flux through the pore.

1) BK channels are voltage-activated and calcium-modulated

At single channel level, the duration of the open and closed events reflect the time the channel spends in different conformational states. By fitting the distributions of either open or closed interval duration under steady-state conditions with a number of exponential components with maximum likelihood, the minimum number of open and closed kinetic states involved in gating may be estimated. Meanwhile, by fitting the channel open probability measured at different calcium concentrations with Hill equation, Hill coefficient (n) is obtained providing a minimum estimate of the number of calcium ions required to activate the channel. This approach was used to study the kinetics of BK channels (Methfessel and Boheim, 1982; Moczydlowski and Latorre, 1983). The kinetic studies suggest that BK channels undergo very complex conformation changes between multiple open and closed states, and channel opening can occur with 1, 2, 3, or more Ca^{++} ions bound to the channel; the apparent mean duration of the openings increases with the number of bound calcium ions (Blatz and Magleby, 1987; Latorre et al., 1989; McManus, 1991). Although most of the open/closed states involve Ca^{++} binding, there is at least one voltage-dependent, rate-limiting conformational change associated with channel gating that is separate from Ca2+ binding (Cui et al., 1997; Meera et al., 1996).

The kinetics of activation and deactivation of macroscopic currents evoked by voltage steps applied to inside-out patches excised from *Xenopus* oocytes expressing cloned *mSlo* BK channels and bathed with a fixed concentration of intracellular calcium are well described by a single exponential function. Indeed, this relationship is consistent over a wide range of voltages and internal Ca2+ concentrations ([Ca]i). Activation rates increase with voltage and with [Ca]i and, importantly, approached saturation at high [Ca]i. Deactivation rates generally

decrease with lower [Ca]i and hyperpolarized voltage, and also approached saturation at high [Ca]i. Plots of macroscopic conductance as a function of voltage under different calcium concentrations are well fit with a Boltzmann equation and reveal that the essential effect of altering calcium is to shift the midpoint of the relationship along the voltage axis, without altering the slope. Taken together with the saturating behavior at high calcium concentrations and the single exponential nature of the kinetics at a fixed calcium concentration, the data imply that BK channels are fundamentally gated by voltage, and that calcium ions modulate the voltage-dependence of the channels. This has been verified, as steps to very depolarized potentials in the absence of calcium (<5 nM), ~200 mV, do indeed, evoke BK currents (Cui et al., 1997). The voltage-dependent opening of the channels require an equivalent 1.1e to 1.8e gating charge movement calculated by fitting the macroscopic conductance-voltage relationships at different calcium concentrations with a Boltzmann equation (Cui et al., 1997; Stefani et al., 1997).

2) BK channel distribution and proposed physiological function

BK channels have been models for studies of permeation and gating due to their large unit conductance. BK channels have been recorded from variety of tissues and cell types such as neurons (Adams et al., 1982), striated and smooth muscle (Latorre et al., 1982; Pallotta et al., 1981), endocrine and exocrine glands (Marty, 1981; Petersen and Maruyama, 1984), kidney (Christensen and Zeuthen, 1987; Gitter et al., 1987), as well as hepatocytes (Jenkinson et al., 1983), epithelium (Rae et al., 1990), fibroblast (Stockbridge and French, 1989), and macrophage (Gallin, 1984) and inner ear hair cells (Fuchs, 1996; Jiang et al., 1997; Tucker and Fettiplace, 1995). They have not, however, been recorded from heart. Although, BK channels couple electrical signals (voltage dependence) with metabolic signals (internal calcium), in different cell types BK channels appear to exhibit different calcium sensitivities, ranging from 10 nM to 100 uM (Latorre et al., 1989; McManus, 1991). However, these distinctions must be viewed in the context of experimental protocol, as

voltage will influence the apparent calcium sensitivity and so far all cloned BK channels demonstrate mM calcium sensitivities when measured under similar conditions (see below). Insight into the functional roles of BK channels has been facilitated by application of BK channel blockers. Based on studies of native BK channels, they are believed important in several physiological functions, such as the fast afterhyperpolarization in central neurons, transmitter and hormone release, and regulation of smooth muscle contraction.

In neurons, action potential repolarization determines the rate of repetitive firing. The classical view of action potential repolarization in squid axon was initially ascribed to the activation of delayed rectifier potassium channels, subsequent to sodium channel inactivation (Hodgkin and Huxley, 1952). Since these initial studies, it has become clear that a wide array of potassium channel subtypes participate in shaping the repolarizing and hyperpolarizing phases of the action potential. In bull sympathetic neurons, two separate potassium currents contribute to spike repolarization and the main contributor is the rapidly activating, calcium-dependent, TEA-sensitive BK current. Treatments which block calcium entry decreased the rate of action potential repolarization (Adams et al., 1982; MacDermott and Weight, 1982). Using a more selective blocker of BK channels, CTX, it was shown in rat hippocampal CA1 pyramidal cells that each action potential is followed by an afterhyperpolarization with four distinct kinetic components. Block of the fast component using CTX, presumably BK channels, resulted in a significantly broadened action potential and reduction of the amplitude of the fast after hyperpolarization (fAHP; Storm, 1987). In studies of *Helix* ganglia U cells, repolarization following an action potential is predominantly due to the activation of BK channels. BK currents were induced by the surge of calcium current concomitant with spike upstroke, facilitated by the colocalization of BK and calcium channels within calcium domains. Although the submembrane calcium concentration could rise to 50-80 mM, calcium diffusion was quite limited and BK channels located relatively further from calcium channels remained quiescent (Gola and Crest, 1993). These distal BK channels were proposed to

constitute a safety mechanism, triggered only upon large, cytotoxic calcium influx. In response to a prolonged depolarization, U cells fire a limited number of spikes of decreasing amplitude, similar to phasic neurons. Therefore, in U cells BK channel participate in a fast process (spike repolarization) as well as long-lasting events (frequency regulation; (Crest and Gola, 1993).

Small arteries and arterioles remain partially contracted and may then further constrict or dilate. The contraction of smooth muscle is initiated by calcium influx through voltage-dependent calcium channels on the cell membrane; smooth muscle contractility is tightly regulated by membrane potential. Activation of potassium channels hyperpolarizes the cell and induces closure of voltage-dependent calcium channel, leading to vasolilation (Nelson and Quayle, 1995). Unlike cardiac and skeletal muscle, which use calcium released from intracellular stores to trigger contraction, smooth muscle uses calcium sparks originated from ryanodine-sensitive calcium channels in the sarcoplasmic reticular (SR) to facilitate vasodilation through activation of BK channels. The calcium release is brief and highly localized just under the cell membrane which has little direct effect on spatially averaged $[Ca^{2+}]i$ regulating contraction, and its primary function is to activate the BK channels which are appropriately positioned (Nelson et al., 1995). In these cells, BK channels function as endogenous calcium detectors to translate the calcium signal into membrane potentials, and this has attracted many pharmaceutical companies to develop therapeutical drugs for vascular diseases.

In endocrine cells which conduct electrical action potentials, such as gland cells or neurons, secretion is triggered by elevated intracellular free calcium levels. The source of calcium ions which gates BK channels is influx through voltage-gated calcium channels which open upon membrane depolarization, such as occurs during the rising phase of the action potential (Reuter, 1983). As the influx of calcium elevates intracellular levels and the membrane

remains depolarized during the plateau and falling phase of the action potential, BK channels are activated, extruding potassium ions and repolarizing the membrane. This, in turn, impedes further calcium influx and limits secretion (Petersen and Maruyama, 1984). By using physiological and morphological techniques, Robitaille, et al, provided convincing evidence of colocalization between voltage-gated calcium channels and BK channels which control transmitter release at the frog neuromuscular junction (Robitaille et al., 1993).

The mechanism of regulated secretion operative in exocrine cells, which do not fire action potentials, is different from the one described above. For cells such as pancreatic acini, salivary glands, sweat glands, lacrimal glands, gastric mucosa and tracheal epithelium, fluid secretion is in regulated by hormonal and/or neuronal influence and is associated with release of intracellular potassium, followed by potassium reuptake; blocking of any of these steps greatly reduces stimulant-evoked secretion (Petersen and Maruyama, 1984). The secretory process begins with the stimulation of secretagogues such as acetylcholine or noradrenaline, or indirectly from nerve terminals, which through signal transduction pathways, generate inositol trisphosphate (IP3) and cyclic ADP-ribose which mediate the release of calcium from intracellular stores (Gerasimenko et al., 1996; Kasai and Petersen, 1994; Thorn et al., 1993; Toro and Stefani, 1991). Rising calcium levels activate BK channels, evoking potassium extrusion and fluid secretion (Maruyama et al., 1983).

Elegant studies of vertebrate inner ear hair cells provided the most powerful integrated understanding of how different channels, including BK channels, collaborate to tune chemical and electrical signals (Fuchs, 1996). Mechanical input causes the opening of non-selective cation channels which causes depolarization. The depolarized potential opens voltage-gated calcium channels in the basolateral membrane, and the influx of calcium triggers the release of transmitters onto associated afferent dendrites. At the site of transmitter release (the active zone), synaptic vesicles are colocalized with clustered voltage-gated

calcium channels and BK channels (Issa and Hudspeth, 1994), and it is the systematic differences in the number and kinetics of BK channels which underlie the tonotopically organized electrical tuning of the hair cells in some vertebrates (Wu and Fettiplace, 1996).

Slowpoke

Electrophysiological studies have employed specific ion channel blockers to dissect and identify the different channel types underlying excitability (Hille, 1992). One prototypic preparation is the *Drosophila* dorsal longitudinal flight muscles (DLM) and larval body wall muscles. Five prominent extrajuctional ionic currents are observed in the mature larval muscle. The very first current develops in mid-embryonic stages, an inward voltage-dependent calcium current (I_{ca}). Rapidly thereafter, two voltage-activated outward potassium currents, a fast transient current (I_A, *Shaker*), and a slowly activating current (I_K) appear. Very late in embryogenesis, two more ionic currents develop, a fast transient Ca^{2+}-activated potassium current (I_{CF}, *Slowpoke*), and then a slow noninactivating current (I_{CS}). Both the earlier Ca^{2+}-independent and later Ca^{2+}-activated transient potassium currents underlie the fast component of spike repolarization. In the adult, the later current appears to largely supplant the earlier current in this role (Broadie and Bate, 1993; Salkoff, 1985).

1) The*Slo* mutation

Mutants with impaired motor functions were initially used to analyze the particular channels disrupted for these functions. One of the mutants, *Shaker,* which displays leg-shaking behavior under ether anesthesia, lacks I_A (Salkoff, 1983). Elkins et al. used EMS-mutagenized flies and found another mutant which is partially paralyzed at 38°C, with uncoordinated movement and inability to climb a cylinder. Even at 18°C, they display abnormal locomotor behavior, preferring to walk rather than to fly, or fly only in short hops (Elkins et al., 1986). This mutant, *Slowpoke* (*Slo*), is recessive and maps to the right arm of the third chromosome at position 86, clearly distinct from *Shaker*. Although the two mutants

have similar phenotypes under ether anesthesia, *Slo* flies show much more motion dysfunction than *Shaker* flies (Trout and Kaplan, 1973). Electrophysiological studies of *Slo* flies revealed that DLM spikes were abnormally lengthened compare to wild type flies (Elkins et al., 1986). To further analyze the basis of the *Slo* defect, two-electrode voltage-clamp experiments were carried out on DLMs. In wild type flies, the current elicited above -40 mV has several components: a) a fast inward calcium current (I_{ca}), followed by, b) a transient outward potassium current carried by two separated components, a voltage-gated current (I_A), and a calcium-sensitive current (I_{CF}; Salkoff, 1983), and c) a slowly-activating, second outward current, which may also have two components, I_K and I_{CS}, (Elkins and Ganetzky, 1988; Gho and Mallart, 1986; Singh and Wu, 1989). The I_A component may be selectively eliminated by 4-aminopyridine (4-AP) or by *Shaker* mutations. In contrast, *Slo* flies demonstrate a markedly reduced early outward potassium current, and this reduction is not due to *Shaker*. In *Slo* flies the early transient component can be blocked with 4-AP (blocks I_A), and in the *Slo-shaker* double mutant, all the early transient outward currents were eliminated. These results indicate that the missing component in *Slo* flies is I_{CF}. Furthermore, the activation and steady-state kinetic properties of I_A are similar in *Slo* and wild type flies when the recordings are performed in calcium-free solution, and the BK channel blocker charybdotoxin had no effect on the transient outward current in *Slo* flies, but inhibited all of the early outward currents in *Shaker* flies, further confirming that the *Slo* mutation eliminated I_{CF}.

Electrophysiological studies of the wild type and mutant flies have dissected the electrical components and elucidated the physiological functions of individual current during transduction of electrical signals. In DLMs, current-clamp studies showed that the elimination of I_A by *Shaker* mutations had no effect on delayed excitation, repolarization, or interspike interval. In contrast, specific elimination of I_{CF} by *Slo* mutations caused complete loss of delayed excitation, prolonged repolarization, and increased interspike interval. The DLM

spikes evoked by endplate potentials in response to stimulation of the motor neuron at frequencies corresponding to that of normal flies were also as prolonged as those observed under current-clamp conditions. It appears that I_{CF} plays the major role both in action potential repolarization and delayed excitation in DLMs, and I_A which is inactivated after the first spike and cannot recover is not required during a train of action potentials. In the absence of I_{CF}, repolarization is accomplished, eventually, by I_K. When I_K and I_{CS} blocked by quinidine, spike duration lasts as long as several seconds. When all K^+ currents have been eliminated, Ca^{2+} current inactivation and ion transporter/pump systems may terminate the prolonged muscle spike (Elkins and Ganetzky, 1988; Singh and Wu, 1990). During development, I_{CF} is the last current to appear. At the time of eclosion there is a dramatic increase in I_{CF} and it replaces I_A as the major repolarization current. So, in newly eclosed flies, I_A plays an important role in regulating the duration, amplitude, and frequency of DLM spikes. Consequently, newly eclosed *Shaker* flies, lacking of I_A have the same DLM phenotype as *Slo* adults, lacking of I_{CF}. Analysis of larval presynaptic motor terminae suggest that the four distinct potassium currents are also involved in repolarizing presynaptic terminals. The significant differences between DLMs and presynaptic terminal are that in presynaptic terminae, the depolarization depends on a presynaptic calcium current, which displays only slight voltage-dependent inactivation, and that both I_A and I_{CF} are crucial repolarization currents, each of them capable of substituting for the other in *Slo* or *Shaker* flies, whereas in DLMs, I_{CF} is dominant current for repolarization (Gho and Ganetzky, 1992).

In summary, electrophysiology studies of the *Drosophila* dorsal longitudinal flight muscles (DLM) reveal that five current components are involved in action potential generation and repolarization, with each component playing a distinct role in the process. Two fly mutations, *Shaker* and *Slo*, selectively eliminate voltage-dependent I_A and calcium-dependent I_{CF} currents, respectively, causing impairment of motor function. Molecular cloning demonstrate

that the *Shaker* locus encodes a component of voltage-dependent potassium channel, and the *Slo* locus encodes a component of a calcium-activated potassium channel (see below).

2) Molecular cloning of the *Slo* gene

The detailed genetic map of *Drosophila*, coupled with the remarkable number of mutant fly strains permits genetic crosses to localize genes to relative small pieces of genomic DNA. Using fragments of these pieces of genomic DNA as probes on Northern blots from wild type and mutant flies may identify transcripts which are different between wild type and mutant, making them candidates as transcripts derived from the gene of interest. The suspect fragment of genomic DNA may then be used to probe a cDNA library, isolating the relevant cDNA clone. Nucleotide and amino acid sequence information, together with functional expression studies may permit identification of the desired cDNA clone. This strategy was successfully used in 1987 to clone the first potassium channel gene, the *Shaker* gene (Kamb et al., 1987; Papazian et al., 1987; Timpe et al., 1988). Subsequent homology screens attempting to identify an I_{CF} component, have identified a wide range of voltage-dependent potassium channel clones from many species, but none encoded a calcium-activated potassium channel, suggesting that the *Shaker* sequence and the *Slo* sequence are not highly homologous. However, using this same molecular genetic strategy with *Slo* flies, a fragment of the *Slo* gene and cDNA were isolated by Ganetsky and colleagues (Atkinson et al., 1991). The predicted amino acid sequence of the partial *Slo* cDNA clone predicted a likely potassium channel, based upon overall transmembrane topology and the presence of a conserved pore-loop sequence (Atkinson et al., 1991). However, the initial cDNA clone did not yield functional channels when expressed in *Xenopus* oocytes. An additional N-terminal coding sequence was subsequently isolated, including an initiator methionine codon, and this clone did yield functional calcium-activated potassium channels when expressed in *Xenopus* oocytes (Adelman et al., 1992). Moreover, it was demonstrated that multiple *Slo* cDNA splice variants are derived from the *Slo* gene. The overall transmembrane and splicing

topology of *dSlo* is presented in Figure 1.3. The *dSlo* polypeptides are approximately 1200 amino acids long, about twice as long as the *Shaker* coding sequence, with at least six putative transmembrane domains and a P segment; both the N- and large C-termini reside intracellularly. The conserved transmembrane core is followed by a C-terminal region of high alternative splicing variability, yielding a total of 144 variants if all possible exon combinations are permitted; no evidence for restricted combinations were found.

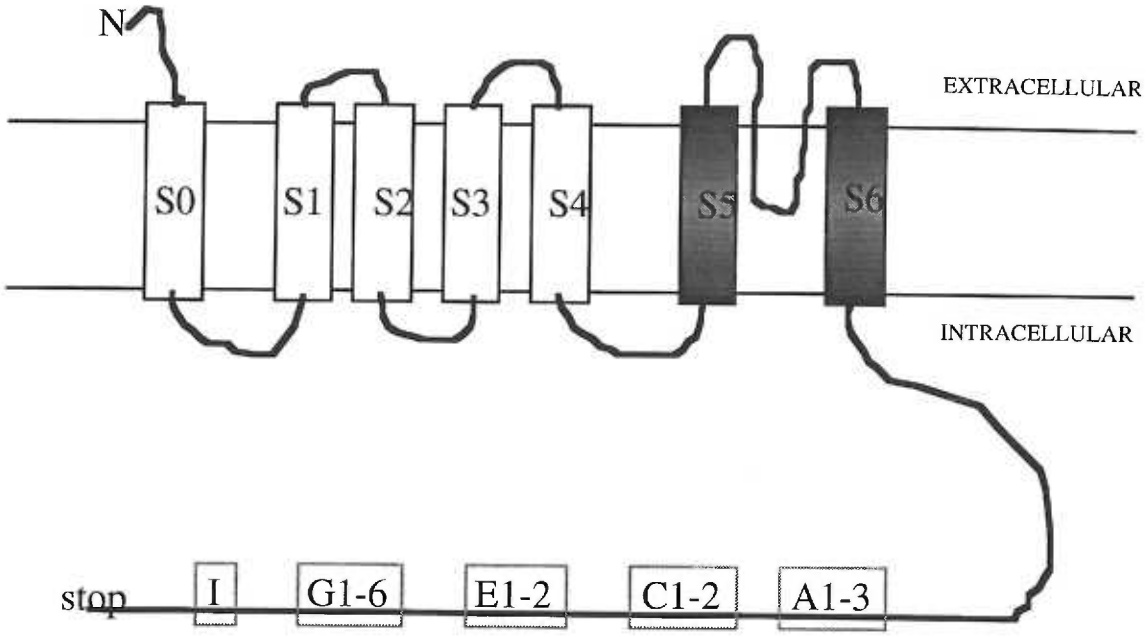

Figure 1.3. Proposed membrane topology of *dSlo*. *dSlo* contains a seventh transmembrane domain (S0) that leads to an exoplasmic N terminus compared with *Shaker*-like potassium channels which have six transmembrane domains (S1-6). The splicing cassettes (A, C, E, G, I) at the long C-terminal are also shown. Numbers refer to the number of splicing variants identified in *Drosophila*.

The functional significance of the alternatively splice cassettes was examined. Comparison of two splice variants (A1/C2/E2/G5/I0 and A1/C2/E1/G3/I0) demonstrated that in both cases the channels have a unit conductance of 126 pS, channel activation is rapid and the open probability increases steeply with both depolarization and elevated intracellular calcium concentrations. However, they have markedly different calcium sensitivities (Adelman et al., 1992). Analysis of several other splice variants indicated that the main functional difference between the A1 and A3 cassettes is in unit conductance, whereas the main difference in properties between the E1 and E2 cassettes is in calcium sensitivity. Activation kinetics were different between G3 and G5, but not consistently in different in A and E box backgrounds. These results suggest that the variable C-terminal region of the *Slowpoke* channel subunit comprises modular, yet interactive functional domains which influence the essential features of unit conductance, calcium sensitivity, and gating (Lagrutta et al., 1994).

The *Slo* channel is a tetramer, consisting of four subunits (Shen et al., 1994), and is very sensitive to external TEA. The mutation Y308V in the pore region dramatically reduced TEA sensitivity. Y308 resides at a position analogous to that in voltage-gated potassium channels where the presence of a Y endows high TEA sensitivity. This result suggests that the *Slo* channel has a similar external pore structure to voltage-gated potassium channels. The fact that *Slo* currents observed in *Xenopus* oocytes revealed most of the important properties of the native I_{CF} recorded in *Drosophila* DLMs and larval body wall muscles, demonstrated that *Slowpoke* is the major component of the native *Drosophila* BK channel. However, two differences should be noted: first, the calcium sensitivity of the *Slo* channel expressed in oocytes is less than that of BK channels in most other cells including DLMs, and second, during sustained depolarization, I_{CF} inactivates but the *Slo* current in oocytes shows little inactivation (Gho and Mallart, 1986; Singh and Wu, 1990). These differences might due to a) differences in recording conditions, b) modulation of native channels by metabolic signals

not present in oocytes or, 3) heterologous subunits intrinsic to the native BK channel (Adelman et al., 1992).

In summary, molecular cloning reveals that *dSlo* encodes the major, if not the only, component of I_{CF} which functions as a calcium and voltage-activated large conductance potassium channel when expressed in *Xenopus* oocytes. The predicted secondary structure of the *dSlo* channel is similar to that of the *Shaker* channel. Varieties of *dSlo* isoforms are generated by mRNA alternative splicing, generating a diversity of BK channels. Additional proteins may contribute to BK channel diversity.

3) Cloning of BK channels from other species

Using *dSlo* sequence as probe, the mouse BK channel (*mSlo*) cDNA was isolated from brain and skeletal muscle (Butler et al., 1993). Similar to *dSlo*, *mSlo* mRNA has multiple variants produced by alternative splicing of at least four exons. Electrophysiology studies of the mouse brain-derived clone mbr5 showed that the *mSlo* channel is potassium selective over sodium, its activation is dependent on both intracellular calcium concentration and membrane potential, and it has a single channel conductance of 272pS. Like the native BK channels, *mSlo* currents are blocked by charybdotoxin, iberiotoxin, and TEA. *mSlo* channels are more sensitive to calcium than *dSlo*; at 10 uM calcium, half-maximal activation(V_{half}) of *mSlo* was reached at +23 mV, whereas no activity of *dSlo* channels was recorded below +50 mV at the same calcium concentration (Butler et al., 1993).

Several isoforms of the BK channel have been cloned from human brain by homology screening (*hSlo*; (Tseng-Crank et al., 1994). The open reading frames encode proteins ranging from 1154 to 1195 amino acids, and all possess significant homology to the *Slowpoke* gene products in *Drosophila* and mouse. All isoforms are generated by alternative RNA splicing of a single gene at four sites located in the large carboxy-terminal portion of the

protein. The individual *hSlo* mRNA isoforms are expressed differentially throughout human peripheral tissues and within different regions of the central nervous system. Heterologous expression of seven different *hSlo* clones in *Xenopus* oocytes shows that each clone encodes voltage and calcium-activated K+ channels with single channel conductances of 200-220pS. Splice variants differ significantly in their calcium sensitivity; at 10uM calcium, the V_{half} is about -5mV for hbr3 and about -18mV for hbr5; both are more sensitive to calcium than *mSlo* (Tseng-Crank et al., 1994). A remarkable feature of *mSlo* and *hSlo* is that they contain a stretch of triplet repeat nucleotides coding for a polyserine near the 5' end; the repeats in *hSlo* are longer than in *mSlo*. These repeats are found in many higher eukaryotic genomes and the expansion of such repeats either at 5' or 3' ends of genes is believed to cause several pathological conditions (Horwitz, 1997; Koshy and Zoghbi, 1997; Lindblad and Schalling, 1996; Perutz, 1996).

Several additional BK channel clones have been isolated from a variety of tissues and species (Dworetzky et al., 1994; Jiang et al., 1997; McCobb et al., 1995; Pallanck and Ganetzky, 1994; Perez et al., 1993; Vogalis et al., 1996). When these clones were expressed either in oocytes or in cell lines, they generated potassium channels with similar single-channel conductance and pharmacology as native BK channels. However, one notable distinction between cloned and native BK channels is their calcium sensitivity, the cloned channels generally being less calcium sensitive.

4) Beta subunit for BK channels

Biochemical purification of the BK channel from bovine tracheal smooth muscle, employing ^{125}I-CTX as a marker and conventional chromatographic techniques, revealed that the purified channel preparations contained two components of 62 and 31 KD (Garcia-Calvo et al., 1994; Knaus et al., 1994). Amino acid sequence analysis showed that the 62 KD component was derived from the bovine *Slo* gene. The 31 KD subunit was a highly

glycosylated 191 amino acid protein encoded by a novel gene, and both components could bind to CTX (Knaus et al., 1995). The deduced amino acid sequence from the bovine cDNA clone indicated that the beta subunit has two putative transmembrane domains separated by an extracellular loop, and bears little sequence homology to subunits of known ion channels. Immunoprecipitation experiments confirmed that the beta subunit may be coprecipitated by BK-specific antibodies, and *visa versa* (Knaus et al., 1994). When the *mSlo* alpha and bovine beta subunits were coexpressed in oocytes, beta subunits conferred increased calcium sensitivity compared to channels composed of alpha subunits alone. While the slope of activation was not altered with different calcium concentrations, the voltage midpoint was shifted to more negative potentials by almost 90 mV at 10 uM calcium. The alpha+beta *Slo* channels reflect the range of calcium sensitivity reported for native BK channel from brain and smooth muscle (McManus, 1991). Both homomeric channels (alpha) and heteromeric channels (alpha+beta) exhibited similar sensitivities to block by TEA and CTX, however the alpha+beta subunits channel was also sensitive to dehydrosoyasaponin (DHS-I) a natural, potent agonist of BK channels, while the homomeric alpha channel was insensitive (McManus et al., 1995). The alpha and beta subunit interaction showed some specificity, since the *dSlo* was unaffected by the coexpression of this mammalian beta subunit. The region responsible for beta subunit regulation was mapped to the N-terminal 41 amino acids. This region includes a newly identified transmembrane domain S0 which transports the N-terminal of *Slo* channels outside the cell (Meera et al., 1997). The S0 transmembrane domain apparently functions as a separable domain, as coexpression of human S0 with truncated *dSlo* which lacks S0 was able to transfer the beta subunit regulation by the *hSlo* region to this *dSlo* (Wallner et al., 1996). In the other studies, coexpression of the human beta subunit (*hSlo* beta) with *hSlo* in oocytes or HEK293 cells showed that besides the shift of calcium sensitivity, the kinetics of the channels are also changed, such as a marked slowing of BK channel activation and relaxation, and significant reduction in slow inactivation. In addition, *hSlo* currents in oocytes were more sensitive to iberiotoxin than were *hSlo* + *hSlo* beta

currents, and the potency of blockade by the alkaloid BK blocker tetrandrine was much greater on *hSlo* + *hSlo* beta currents compared with *hSlo* currents alone. Furthermore, the modulation of BK channel activity by phosphorylation was affected by the presence of the *hSlo* beta subunit. Application of cAMP-dependent protein kinase (PKA) increased the P_o of *hSlo* channels but decreased the P_o of *hSlo* + *hSlo* beta channels (Dworetzky et al., 1996).

In adult rat *rSlo* mRNA is widely distributed (Chang et al., 1997). Immunocytochemistry studies show that *rSlo* channels are highly concentrated in terminal areas of prominent fiber tracts consistent with its targeting into a presynaptic compartment, which implies an important role in neural transmission (Knaus et al., 1996). In marked contrast to expression of BK *rSlo* mRNA, expression of BK beta mRNA is relatively low and predominantly peripheral. In rat brain, BK beta mRNA occurs only in a few discrete populations of neurons that also express BK alpha mRNA. These results indicate that the major type of BK channel in the brain, unlike the alpha-beta channel type in aortic and tracheal smooth muscle, is devoid of the beta subunit (Chang et al., 1997). It also raises the provocative issue of whether BK beta subunits serve other functions or other *Slo* subunits exist in CNS.

In rat, two BK channel variants with same single-channel conductance have been recorded from distinct populations of adrenal chromaffin cells. One BK current is rapidly inactivating (BKi) and is found in about 75-80% of rat chromaffin cells, while the remainder of cells express a mix of inactivating and non-inactivating current or mostly non-inactivating, BKs current (Lingle et al., 1996). Homology cloning of *mSlo* cDNAs from rat chromaffin cells revealed two *rSlo* variants, but neither exhibited inactivation when expressed in oocytes. One of the *rSlo* variants contains a cysteine-rich 59-amino acid insertion, reminiscent of zinc-finger domains, at C-terminal region equivalent to the G-box of *dSlo*. This *rSlo* channel is more sensitive to calcium with a V_{half} of -7.7mV than the one without the cysteine-rich region (V_{half} of 42.8mV, at 10uM calcium). The cloned *rSlo* had similar sensitivity to CTX

(Kd=2.2nM at +60mV), to BKs in native rat chromaffin cells implying that the cloned *rSlo* is probably BKs rather than BKi. In mammalian Kv channels which do not show N-type inactivation, coexpression of the beta1 subunit induces rapid inactivation (see above). However, the beta subunit of bovine BK channel had no effect on the inactivation of *rSlo* in coexpression studies (Saito et al., 1997). Although the rapid inactivation of BK_i is trypsin-sensitive, it does not appear to occur directly at the cytosolic mouth or inner half of the ion permeation pathway and is likely mechanically distinct from that of inactivating Kv channels. It is possible that a second beta subunit may required in the inactivating of BKi. (Solaro et al., 1997; Solaro and Lingle, 1992).

In summary, *Slo* genes have been cloned from several species. Like *dSlo*, they have multiple alternative cassettes in the C-terminal domain of the subunits, generating variants with different functions. Two distinct types of BK channels are present in rat chromaffin cells, inactivating and non-inactivating channels, the cloned *rSlo* is likely the component of non-inactivating BK channel. Differences exist between the heterologously expressed and native BK channels. A beta subunit has been cloned from bovine peripheral tissue, and its expression overlaps only partially with expression of the alpha subunit. Coexpresssion of alpha and beta subunits results in channels with many properties of native BK channels.

Distribution and regulation of BK channels

The distribution, density and complexity of ion channels determine the electrical properties of a cell, and alterations of any of these parameters will affect excitability. Potassium channels, and particularly BK channels show remarkable structural and functional diversity, and are regulated on every biosynthetic level, transcription, mRNA splicing, translation, posttranslational modification, subcellular localization and association with beta subunits.

1) Transcriptional regulation

BK channel genes are expressed in a tissue specific manner. In *Drosophila* as mentioned above, the electrogenesis of I_{CF} (*dSlo*) is regulated during embryonic myogenesis (Salkoff, 1985). Using in situ hybridization and immunochytochemistry, the expression pattern of *dSlo* has been described throughout development, in such diverse tissues as muscle, mushroom bodies, some cells in embryonic and larval midgut, epithelial-derived tracheal cells and prominently in the central nervous system (CNS) and peripherial nerve system (PNS). Indeed, during muscle pupation and embryogenesis, *dSlo* mRNA is detected many hours prior to the appearance of functional channels during development (Becker et al., 1995). The expression of the *dSlo* gene is controlled by at least four promotors (C1, C1b, C1c and C2) distributed over about 4.5 kb of DNA 5' to the transcriptional start site and the *dSlo* gene itself extends over more than 50 kb. Transcripts derived from promotors C1, C1b, C1c differ in their leader sequence but share a common translation start site. C2 transcripts append another translation start site which adds 17 amino acid to the N-terminus of the protein and all heterologously expressed *dSlo* clones reflect the C2 start site. Several transcriptional enhancer elements have been identified and deletion analysis indicates that four specific sequences are required for expression in CNS, midgut, tracheal cells, and muscle (Brenner et al., 1996). A 3' intronic region (C2/C3) modulates promoters C1 and C2, the activity of which is responsible for neuronal and muscle expression, respectively. Deletion of this C2/C3 region silences Promotor C1 in adult but not larval CNS and causes a substantial reduction in Promotor C2 activity in adult but not larval muscle. The C2/C3 region also activates Promotor C1 in the animal's eye (Brenner and Atkinson, 1996).

In summary, BK gene transcription is regulated in a complex manner by a series of promotors and enhancer elements. In *Drosophila,* at least four promoters are involved. This likely reflects a variety of transacting transcriptional modulating proteins..

2) mRNA splicing

Molecular cloning of *dSlo* and *Slo* genes from other species revealed astonishing diversity via mRNA splicing. In general, *Slo* genes have at least two to five exon cassettes in the C-terminal domain (A/C/E/G/I in *dSlo*) each with several choices which may be individually replaced to potentially generate a great number of isoforms (Figure 1.3; (Adelman et al., 1992). Each of the isoforms likely has different functional profiles, such as the single channel conductance, gating and calcium sensitivity (Lagrutta et al., 1994; Saito et al., 1997; Tseng-Crank et al., 1994). Indeed, different *hSlo* mRNA isoforms are differentially expressed throughout human peripheral tissues and within different regions of the central nervous system (Tseng-Crank et al., 1994). The number and kinetic properties of BK channels in the receptor epithelium of the chick cochlea are shown to help determine the resonant frequency of electrically tuned hair cells. Variants of alternatively spliced cDNAs derived from *cSlo* are found differentially distributed among hair cells along the tonotopic axis of the chick cochlea. It has been observed that single hair cells express more than one splice variant at a given splice site (Navaratnam et al., 1997; Rosenblatt et al., 1997). Thus, mRNA alternative splicing provides an unique process for every single cell to fine tune its electrical transduction by the blend of *Slo* splicing isoforms.

3). Post transcriptional regulation

Post transcriptional regulation is critical to the development of an organism when functional requirements are dynamic. In mature chick ciliary gangion (CG) neurons, the sole source of parasympathetic motor output to the eye, the BK current is a large macroscopic current that is carried by several BK channel subtypes and plays an important role in regulating the late phases of spike repolarization and the temporal pattern of repetitive firing. Functional BK currents are undetectable before embryonic day 8 (E8) and peaking at E13, coinciding with the stages at which CG neurons form synapses with target tissues in the iris, ciliary body, and choroid (Dourado et al., 1994; Dourado and Dryer, 1992). The developmental

expression of macroscopic BK currents in chicken ciliary ganglion (CG) neurons is apparently dependent upon cell-cell interactions. BK channels have very low expression levels prior to synapse formation with target tissues or preganglionic innervation, and they are absence in developing CG neuron without presenting target tissues (iris) in vitro or in situ, though in all cases the voltage-activated calcium channel expresses normally and *cSlo* (BK) mRNA is readily detectable (Dourado et al., 1994; Subramony et al., 1996).

Subramony et al found that the emergence of BK currents in cultured CG neurons in the presence of iris does not require protein synthesis, as the presence of the transcriptional inhibitor actinomycin-D or the translational inhibitors cycloheximide and anisomycin had no effect on this process. The stimulation of BK currents in CG neurons is induced by an active component of iris extracts and neuregulin can mimic the effect of the extracts (Subramony and Dryer, 1997; Subramony et al., 1996). It is likely that BK channels are already present in CG and are released from intracellular stores and transferred to cell surface following induction by neuregulin.

4) Subcellular localization

After translation, BK channel subunits must fold correctly, assemble in the right order and combination with other subunits, and perhaps undergo posttranslational modifications such as glycosylation, as they are transported to the plasma membrane where they undergo the final subcellular distribution to the sites such as synapses, nodes of Ranvier, or axon hillock. Improperly folded or mis-assembled subunits will be degraded within the endoplasmic reticulum (ER) or lysosome (Hurtley and Helenius, 1989).

To functionally participate in regulation of nerve cell firing, BK channels must be localized close to calcium channels. In *Helix* pomatia neurons, BK channels are actively involved in spike repolarization. Gola and Crest found that submembrane calcium diffusion was

sufficiently limited to prevent the BK channels from being opened by remote calcium entry. Active BK channels were clustered together with calcium channels which forms calcium domains on neural cell body, and those BK channels located in areas devoid of calcium channels remained quiescent during cell firing (Gola and Crest, 1993). Studies of postnatal development of a BK channel in neocortical infragranular pyramidal neurons reveal that the BK channel density increases during development in somata and proximal apical dendrites. The clustering of BK channel in somata begins in late development phase (Kang et al., 1996).

At the neuromuscular junction (NMJ), BK channels have been shown to regulate calcium entry and transmitter release (Robitaille and Charlton, 1992) and calcium channels are clustered at the sites where transmitter release occurs (Cohen et al., 1991; Robitaille et al., 1990). Physiological and morphological evidence indicates that BK channels are colocalized with calcium channels in the presynaptic terminal, clustered at the release sites of the NMJ (Robitaille et al., 1993). BK channels were also found located at presynaptic terminal of goldfish retinal bipolar cells (Sakaba et al., 1997). Direct recording of presynaptic currents in cultured embryonic *Xenopus* NMJ supports other people's reports that calcium and BK channels are colocalized at presynaptic active zones (Yazejian et al., 1997).

BK channels are also distributed at specific subcellular sites in non-neuronal cell. The bullfrog taste bud cell membrane may be divided into four membrane parts: receptive area, apical process, cell body and proximal process. The BK channels with single channel conductance of 80pS are located exclusively on the receptive membrane and the apical process membrane (Fujiyama et al., 1994), another BK channel (210pS) are also identified at the apical membranes of both ciliated and nonciliated epithelial cells grown as monolayers from the primary culture of rabbit oviduct (James and Okada, 1994).

Although the mechanisms underlying the targeting of particular classes of BK channels to specific subcellular sites are not clear, it appears that proteins with PDZ domains are important for other channel clustering. PDZ domains are 90-100 amino acid repeats found in many proteins which mediate protein-protein interactions (Kornau et al., 1997; Sheng and Kim, 1996). The postsynaptic density-95 (PSD-95/SAP90) family of proteins (SAP97/hdlg, SAP102, PSD93) which contain several PDZ domains are capable of clustering potassium channels and GluR. In cerebellar basket cell, Kv1.1, Kv1.2 and SAP90 are predominantly colocalized to septate-like junctions, which connect the basket cell axonal branchlets, while Kv3.4 is uniformly distributed (Laube et al., 1996). In *Drosophila*, mutations in *discs-large*(dlg/SAP97) a PSD-95 family member prevents *Shaker* potassium channel clustering at postsynaptic neuromuscular junctions (Tejedor et al., 1997). The second PDZ domain of PSD-95 also interacts with C-terminal end of some NMDA receptors (NR2B).

Besides the proteins with PDZ domains described above, other proteins are also found to mediate the distribution and localization of channels. One of the beta subunits for voltage-gated potassium channels, beta2 acts like a molecular chaperone by promoting cell surface expression of coexpressed Kvα subunits (Shi et al., 1996). Recently, a new mouse beta subunit has been identified (mKvBeta$_4$) which may have a similar chaperone-like effect on Kv2.2 (Fink et al., 1996).

In summary, BK channel are distributed and localized at specific subcellular sites in neurons and other polar cells. The mechanism underlying the distribution and localization is not well understood. Proteins with PDZ domains cluster potassium channels and organize subcellular structure of different ion channels and signal transduction pathways. Besides the proteins with PDZ domain, the beta subunits of voltage-gated potassium channels have a unique chaperone function in facilitating the surface delivery of the channels.

5) Post-translational regulation

Regulation of ion channel activity is accomplished not only through biosynthesis and relative localization within the cell, but also by modification of channel activity by extrinsic molecules. In this regard, many different second message pathways converge on BK channels.

A) Cyclic AMP-dependent protein kinase (PKA)

Several examples of BK channel regulation by PKA have been described. In rat olfactory bulb neurons, chicken growth plate chondrocyte and in a cell line derived from term pregnant human myometrium, channel open probability of native BK channels is dramatically increased by application of PKA together with MgATP, and this effect can be prevented by a PKA antagonist. Phosphorylation alters the distribution of channel closed times but has little effect on open times. The treatment of alkaline phosphatase reverses PKA effect (Egan et al., 1993; Long and Walsh, 1994; Meera et al., 1995).

BK channels in rat brain have been categorized as being type I or type II based upon gating kinetics and toxin sensitivity. These two types of BK channel were incorporated into planar lipid bilayers via plasma membrane vesicles fusion and the effects of exogenous PKA were examined. Both types of BK channel were regulated by PKA, but with opposite effects. Type I channels showed a higher open probability following PKA treatment, while type II channels were down-regulated. The effect of PKA on both channel types were reversed by application of catalytic subunit of protein phosphatase 2A (PP2A), but not by protein phosphatase 1 (PP1; (Reinhart et al., 1991).

The regulation of type-I and type-II BK channels in rat brain via phosphorylation involves more than one site and the phosphorylations have different effects on channel activity. While

the activity of most type-I channels is up-regulated by exogenous PKA, type-II channels are consistently down-regulated by PKA. The effects of PKA on both channel types are reversed by the catalytic subunit of PP2A, but not by PP1 ((Reinhart et al., 1991).

PKA also stimulates the activity of *Drosophila Slo* channels expressed in *Xenopus* oocytes. The site of phosphorylation has been putatively identified, as the effect is blocked either by application PKA inhibitor or the mutation of a single serine residue in the intracellular C-terminal domain of *dSlo*. Further results indicate that *dSlo* channels in oocyte membrane patches can be modulated by an endogenous PKA-like protein kinase which remains functionally associated with the channels in the detached patch (Esguerra et al., 1994).

B) Protein kinase C (PKC)

PKC also affects rat brain BK channels. Following incorporation into lipid bilayers, addition of MgATP (but not nonhydrolyzable analogues) increases channel open probability, an effect which is reversed by addition of PP1. These results suggest that an endogenous protein kinase remains tightly associated with the channel. Further investigation reveals that the endogenous protein kinase activity is PKC-like and can be mimicked by exogenous PKC, and blocked by a specific peptide inhibitor of PKC. Moreover, not only the kinase, but a PP1-like activity is intimately associated with type-II BK channels. The results imply that type-II BK channels exist as part of a regulatory complex that includes a PKC-like protein kinase and a PP1-like protein phosphatase, both of which participate in the modulation of channel function (Reinhart and Levitan, 1995).

C) Cyclic GMP-dependent protein kinase (cGMP-kinase)

In smooth muscle BK channels are activated by phosphorylation through guanosine-3',5'-cyclic monophosphate (cGMP)-dependent protein kinase (cGMP-kinase). Direct application of cGMP-kinase at the cytoplasmic side of inside-out patches together with cGMP and

MgATP increases the channel mean open time four to eight-fold compared with controls (Robertson et al., 1993; Taniguchi et al., 1993). The activation of BK channel activity by cGMP-kinase has also been observed in Chinese Hamster Ovary cells and requires PP2A (Zhou et al., 1996). In neurons, the Alzheimer's beta-amyloid precursor protein (beta-APP) is widely expressed, and secreted forms of beta-APP (sAPPs) are released from membrane-spanning holo-beta APP in an activity-dependent manner. Furukawa, et al use whole-cell perforated patch and single-channel patch-clamp analysis of hippocampal neurons to demonstrate that sAPPs suppress action potentials and hyperpolarize neurons by activating BK channels. Activation of BK channels by sAPPs was mimicked by a cyclic GMP analogue and sodium nitroprusside and blocked by an antagonist of cGMP-dependent kinase and a phosphatase inhibitor, suggesting that the effect is mediated by cGMP kinase and protein dephosphorylation (Furukawa et al., 1996).

D) G-proteins

BK channel activity is also modulated by G-proteins subsequent to activation of neurotransmitter receptors. Studies in isolated canine colonic myocytes with whole cell voltage-clamp first showed that G-proteins mediate suppression of a Ca^{2+}-activated potassium (K_{Ca}) current evoked by acetylcholine (ACh). Extracellular application of ACh causes reversible depression of K_{Ca} current when GTP is in the pipette solution, and the effect can be abolished if the G protein inhibitor pertussis toxin is also included in pipette. The intracellular diffusion of non-hydrolyzable GTP analogs (GTPgammaS or GppNHp) irreversibly inhibits most of K_{Ca} current. These data suggest that coupling of muscarinic receptors to the inhibition of K_{Ca} channels is mediated by pertussis toxin-sensitive G-proteins (Gi/Go) in colonic smooth muscle cells (Cole and Sanders, 1989). However, in cultured bovine adrenal chromaffin cells, BK channel activity is upregulated by activating G-proteins either via GTPgammaS or AlF-4, or application of the mixture of Gi/Go alpha subunit(s) on the intracellular site of the excised, inside-out patches (Walsh et al., 1996).

Using BK channels reconstituted in planar lipid bilayers, Toro et al. demonstrated that BK channels in rat or pig myometrium are subject to G-protein regulation. They found that addition of GTP or GTPgammaS increased channel Po only in the presence of Mg^{2+}, characteristic of G-protein-mediated mechanisms, and even in the presence of AMP-PNP, a nonphosphorylating ATP analogue, suggesting a direct G protein gating of K_{Ca} channels. Norepinephrine (NE) or isoproterenol potentiated the GTP-mediated activation of K_{Ca} channels. The results indicate that myometrium possesses beta-adrenergic receptors coupled to a GTP-dependent protein that can directly gate K_{Ca} channels, possibly by forming a stable, functionally coupled, molecular complex (Toro et al., 1990). In coronary artery smooth muscle, BK channels are activated by a similar G-protein coupled to beta-adrenergic receptors, as well as the purified activated stimulatory G protein ($G_s$lapha; Scornik et al., 1993).

E) Other modulators

In rabbit vascular smooth muscle, BK channels can be directly activated by nitric oxide (NO) independent of the cGMP kinase-mediated activation pathway. The rapid activation of BK is due to increased open probability. NO has no effect on channels which have been modified by N-ethylmaleimide (NEM; (Bolotina et al., 1994). The lipid composition of the cell membrane influences channels activity (Ordway et al., 1991; Petrou et al., 1994). The BK channels in rabbit smooth muscle modified by NO may be more effectively activated by fatty acid myristic acid (Bolotina et al., 1994). In a separate study, BK channels recorded from human aortic smooth muscle have several fold elevation in open probability after application of 2-decenoic acid (DA;(Bregestovski et al., 1989). BK channel function is also under the influence of cellular redox potential. Macropatch and single-channel analysis of heterologously expressed *hSlo* indicates that intracellular application of the reducing agent dithiothreitol (DTT) increases single-channel open probability, shift V_{half} to more negative

potentials, speeds activation kinetics and slows the rate of run-down by 10-fold. In contrast to DTT treatment, oxidation with hydrogen peroxide has the opposite effect. On the other hand, *dSlo* is not modulated by addition of DTT (DiChiara and Reinhart, 1997).

A serine protease (SerP) domain is present in many proteins which may serve as a binding site for other proteins. Sequence analysis of cloned *dSlo* and mammalian *Slo* suggests that a region of 250 residues near C-terminal is homologous to the SerP domain. Intracellular application of bovine pancreatic trypsin inhibitor BPTI produces a unitary subconductance which exhibits strong inward rectification in the presence of symmetrical KCl, and corresponds to 15% of open channel current at +60 mV and 70% of open state at -40 mV (Lucchesi and Moczydlowski, 1991). Trypsin which has very high affinity with BPTI ($Kd=6X10^{-14}M$) is able to reverse the effect induced by BPTI. It's possible that the SerP domain of Slo mediates the interaction of BK channels with an intracellular protein homologous to BPTI (Moss et al., 1996; Moss and Moczydlowski, 1996).

In summary, BK channel activity is modified through protein phosphoralation, being up-regulated by PKA, PKC or cGMP-kinase, and down-regulated by PKA. The effects can be reversed by certain type of phosphatases. G-proteins can also modify BK channels to increase ($G_s$alpha, Gi/Goalpha) or decrease (Gi/Go) channel activity. Besides protein modifiers, nitric oxide(NO) can directly activate BK channels and the lipid composition and the intracellular redox potential modify BK channel activity as well.

Identification of BK modifiers

Cloned potassium channels facilitate structure and function studies. However, heterologous expression of cloned alpha subunits, though forming functional potassium channels, does not recapitulate all characteristics of native channels. Molecular identification of auxiliary

subunits and channel modifiers are essential for reconstituting the activity profile of native channels and for a complete understanding of the operations of channels in living cells.

For instance, voltage-dependent sodium and calcium channels are large heterooligomeric proteins. For both classes the alpha subunit is associated with heterologous beta subunits, frequently in a tissue specific and developmental specific manner. Association with these beta subunits alters channel function (Hullin et al., 1992; Isom et al., 1992; Neely et al., 1993; Patton et al., 1994; Perez-Reyes et al., 1992; Varadi et al., 1991) or assembly and expression level of the channel (Isom et al., 1992; Snyder et al., 1995; Tareilus et al., 1997). Auxiliary subunits of potassium channels have also been identified and coexpressions of the beta subunit with the alpha subunit changes the properties of the channel (see above beta subunits of BK channels). Generally, beta subunits were identified by biochemical purification, or genetic methods. However, the yeast-two-hybrid system can be used to screen for proteins which interact with alpha subunits, as well.

1) Biochemical purification

A) Radio ligand binding

Most beta subunits of voltage-gated ion channels were initially identified as the molecules which copurified with the alpha subunits (Garcia-Calvo et al., 1994; Hartshorne and Catterall, 1984; Rettig et al., 1994; Ruth et al., 1989; Witcher et al., 1993). Each native protein has a distinct purification process, however the purification is enhanced by the availability of a high affinity ligand. Starting with tissue enriched with the channel, purification may be monitored by following radioligand binding activity. If other proteins bind tightly to the purified channels, they may be copurified and identified. Micro-amino acid sequencing requires only several micrograms of protein and this information may lead to identification of cDNA clones. The beta subunit of BK channel mentioned above was identified via this approach (Garcia-Calvo et al., 1994; Knaus et al., 1994).

B) Immuno-affinity

If a high affinity antibody against the alpha subunit is available, immunoprecipetation may be performed to precipitate the alpha subunit and coprecipitate any proteins tightly bind to the alpha subunit. One example is the identification of a 115 KD protein which coimmunoprecipitates with the voltage-gated chloride channel (CLC-0). Kehne et al use a polyclonal antibody against CLC-0 to precipitate the channel complex solubilized from the electric organ of *Torpedo californica*. In their immunopurification experiments, beside CLC-0, a major polypeptide of M(r) approximately 115,000 copurified and was identified as the 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS)-binding protein (Jentsch et al., 1989; Kehne et al., 1996). SITS is an inhibitor of a variety of anion transport proteins, likely mediated via the SITS-binding protein. Similarly, coimmunoprecipitation was used to determine that calmodulin interacts with NMDA receptor NR1 subunits (Ehlers et al., 1996).

C) Cross-linking

If two macro-molecules are in close proximity, they may be crosslinked together by applying a small active chemical molecule. Because the linkage is through a stable chemical bond, it can endure harsh treatment during purification and examination. After purification, the crosslinked protein may be released for further analysis. In fast twitch skeletal muscle, the signal for excitation-contraction coupling is transferred from transverse tubule (T tubule) across the triad junction from the terminal cisternae of sarcoplasmic reticulum. The feet structures of terminal cisternae, which bridge the gap at the triad junction, have been identified as the ryanodine receptor and in turn with the calcium release channels of sarcoplasmic reticulum. To identify the component in T tubule which ligands with the foot structure to form the triad junction, Chadwick et al. used sulfosuccinimidyl-2-(p-azidosalicylimido)-1,3'-dithiopropionate (SASD), a thiol-cleavable, ^{125}I-iodinatable, and

photoactive probe, to show that ryanodine receptors selectively crosslink to a protein with Mr of 71,000 in isolated transverse tubules (Chadwick et al., 1988).

2) Yeast-two-hybrid system

Eukaryotic transcription requires the recruitment of basic transcriptional machinery containing multiprotein RNA polymerase and other proteins. The typical activator such as Gal4 has two functionally and physically separable modular domains: a specific DNA-binding domain which localizes the transcription factor to the regulatory sequences, and an acidic 'activating region' which interacts with other transcriptional proteins to initiate transcription. The genetic specificity of the activator is determined solely by the DNA-binding address of the activator. For example, the activator Gal4 normally activates genes required for galactose metabolism in yeast. But when any of genes is modified so as to bears Gal4 binding sites near the promoter, Gal4 activates that gene as well. Furthermore, although separation of the two domains of the activator disrupts its function, functional transcription activators may be reconstituted if two chimeric proteins (two-hybrid), one containing a DNA-binding domain and another containing a activation domain, interact and brings the two domains in proximity. These two domains, although on separate proteins are able to form a functional unit. Taking advantage of this, a genetic assay, the yeast-two-hybrid screening system was developed (Figure 1.4).

The yeast-two-hybrid system was first developed by Fields and Song to study the interactions between transcription factors, and soon thereafter was applied more generally to study protein-protein interaction (Chien et al., 1991; Fields and Song, 1989). Two-hybrid experiments are usually performed in a specific yeast strain with a) two specific auxotrophic markers (*leu-trp-*), b) two modified, normally silent genes (*his* and *lacZ*) which bear Gal4 binding sites at the upstream of the two genes, c) disruption of the *gal4* gene and its repressor *gal80* gene to prevent the interference of the system. The bait(s) is the Gal4 DNA-binding

domain fused with the protein of interest, while the prey may be a cDNA library with the inserts constructed as fusions with Gal4 activation domain. Yeast are transformed with bait and prey. Molecules from the cDNA library which interact with the bait will bring together the Gal4 DNA binding and activation functions, activating both *his* and *lacZ* genes and conferring histidine prototrophy and beta-galactosidase activity (Figure 1.4).

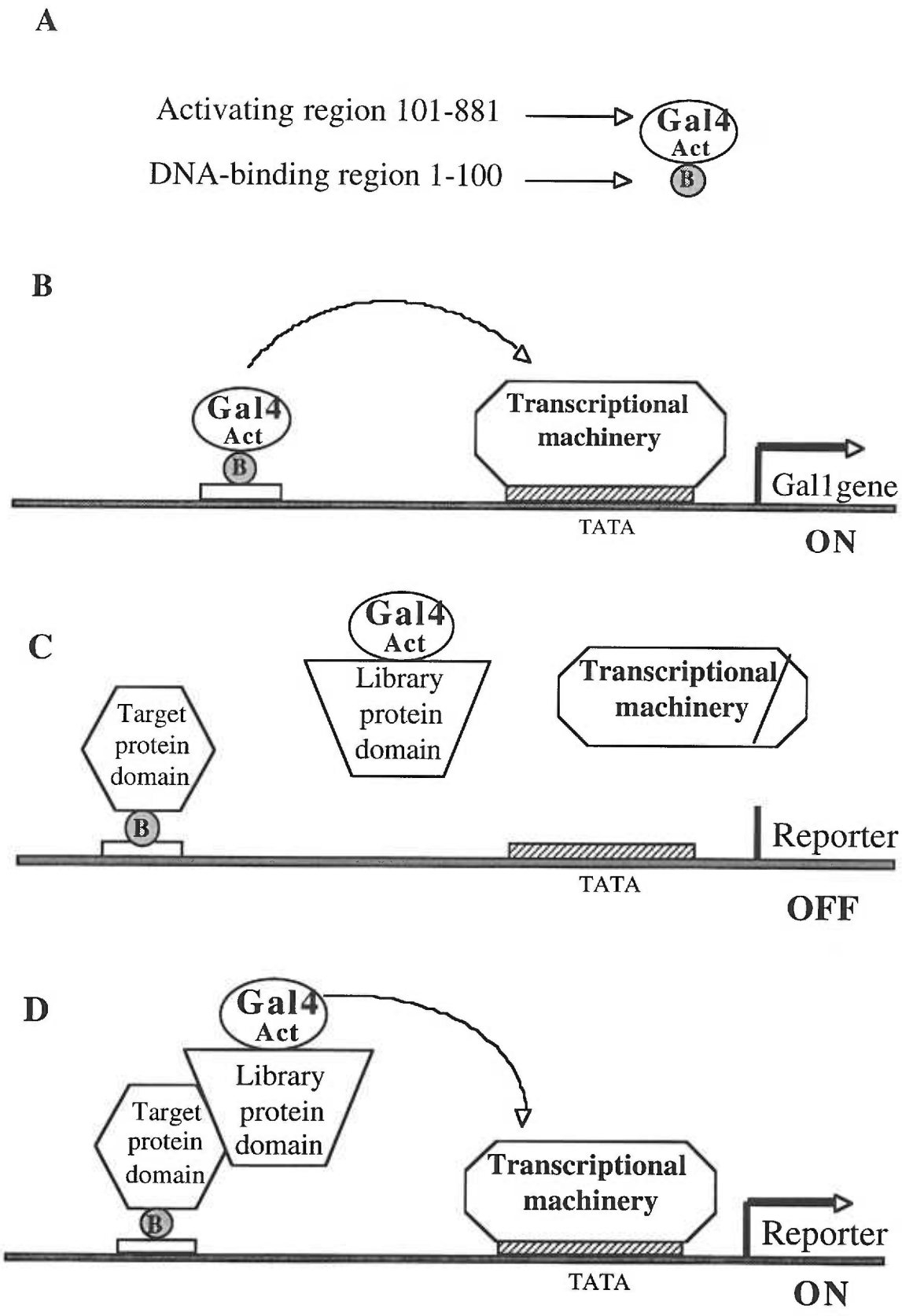
A
Activating region 101-881
DNA-binding region 1-100
Gal4
Act
B
B
Gal4
Act
B
Transcriptional
machinery
Gal1gene
TATA
ON
C
Gal4
Act
Library
protein
domain
Target
protein
domain
B
Transcriptional
machinery
Reporter
TATA
OFF
D
Gal4
Act
Library
protein
domain
Target
protein
domain
B
Transcriptional
machinery
Reporter
TATA
ON

Figure 1.4 Schematic representation of the modular transcription activator principle underlying the yeast two-hybrid system. A) The yeast activator Gal4 has two physically and functionally separable domains, an activating domain and a DNA-binding domain. The specificity of transcriptional activation resides in the binding of DNA via the DNA-binding domain. **B**) Activation of the Gal1 gene is triggered when Gal4 binds to specific DNA site(s) and contacts the transcriptional machinery. **C**) Shown are two separate fusion proteins in the nucleus. The DNA-binding domain of the Gal4 binds to the specific site of DNA but cannot initiate functional transcription. **D**) When a protein encoded by the activation-domain-library fusion (prey) binds to the target protein construct (bait), a functional transcription factor is assembled and transcription of the reporter gene is initiated.

The yeast-two-hybrid system is successful but also has limitations. First, the system depends on the physically and functionally separable domains of the proteins of interest. Second, the regeneration of Gal4 activation requires the interaction of the two hybrid molecules within the nucleus, so some protein interactions which require lipid membrane environment will not be detected. Third, because the yeast-two-hybrid assay depends on regeneration of functional transcription activators, even minor contacts between the two hybrid proteins will activate Gal4 function. Therefore, the system shows significant background, and the interaction of proteins indicated in the yeast-two-hybrid assay must be independently confirmed by other techniques.

Specific Aims of the Thesis

The aim of my work was to identify heterologous proteins which modify BK channel functions by using the yeast-two-hybrid system. BK channels have been shown to be modified by phosphatases, kinases, G-proteins and beta subunit. BK channels are found to distribute at certain subcellular sites, such as the colocalization with calcium channels presynaptically. During the subcellular distribution, many proteins are required and likely interact with BK channels. Because BK channel modifiers and subcellular distributions require the interaction of BK channel with the modifiers or other proteins via the specific domains of the two molecules, I hypothesized that the modules involved will retain their ability to interact even as chimeras with the DNA-binding domain and activation domain, and thus proteins interacting with BK channels could be identified by the yeast-two-hybrid assay. The experiments first identified molecules which physically interact with *dSlo*, and then examined the possible function of the molecules relative to BK channel function.

Chapter II: Material and Methods

The interaction trap

A modified yeast-two-hybrid system, the interaction trap, was used in our search of dSlo-interacting proteins. The interaction trap system, instead of using two distinct domains of Gal4 to construct fusions, uses bacterial LexA protein as the DNA binding motif (bait; Golemis and Brent, 1992) and B42 as an acidic activation domain (prey; Gyuris et al., 1993; Ma and Ptashne, 1987). The system incorporates several approaches to reduce or eliminate possible false positives: A) two independent genes, *leu2* on yeast chromosome and *lacZ* on pSH18-34 plasmid, are modified to be controlled by LexA operators which will be transcribed if bait and prey interact. pSH18-34 is a yeast/bacteria shuttle vector which contains an expression cassette for beta-galactosidase driven by Gal1 promoter under the control of four LexA operators, as well as the ura3 selectable marker. The Leu and beta-galactosidase double selection eliminates a large number of false positives. B) the system has three specific yeast strains EGY48 (MAT *alpha*, *his3*, *trp1*, *ura3*, *6LexAop-leu2*), EGY195 (MAT *alpha*, *his3*, *trp1*, *ura3*, *4LexAop-leu2*) and EGY191(MAT *alpha*, *his3*, *trp1*, *ura3*, *2LexAop-leu2*), which have the same phenotype otherwise 6, 4 and 2 LexA binding sites upstream of the *leu2* gene, respectively. As a result, the strain with more LexA binding sites is more sensitive while that with fewer LexA binding sites is less sensitive to transcription activation of the *leu2* reporter gene by the LexA fusions or by proteins interacting with them. Baits which generate many false positives in EGY48 may be used with a less sensitive strain (EGY195 or EGY191) to perform screening. C)the expression of cDNA fusion is under the control of the Gal1 promoter, so the transcription of cDNA fusions is activated with galactose and inhibited with glucose. This allows certain false positives selected for Leu^+ to be eliminated due to *cis* or *trans*-acting yeast mutations that activate the *leu2* promoter.

Four baits (N-terminal dSlo-N1-126, C-terminal dSlo-C665-1164, C-terminal dSlo-C340-1164, C-terminal dSlo-C211-1164) were constructed in the parent plasmid pEG202 by fusing *dSlo* to the C-terminal oligomerization region of LexA. PEG202 is a yeast/bacteria shuttle vector which has the *his3* as a selectable marker in yeast, and the *bla* gene (Amp^r) for selection in *E.coli*. The fusion protein (bait) is expressed constitutively from the yeast ADH promoter/terminator. Each bait is tested for whether it activates transcription of *leu2* and *lacZ* by patching EGY48 transformed with pSH18-34 and individual baits onto an Xgal indicator plate and a leucine minus plate. Baits that activate transcription cause expression of beta-galactosidase and allow growth of EGY48 in the absence of leucine. Baits that do not activate transcription are tested if they enter the nucleus and bind LexA operators in a repression assay which detects DNA binding by transcriptionally inert LexA fusion proteins (Brent and Ptashne, 1984). For this assay, yeast EGY48 are transformed with the plasmid pJK101, which contains the *lacZ* gene driven by the Gal1 promoter and a LexA operator between the Gal1 promoter and the Gal1 transcription start. Yeast harboring pJK101 will have significant beta-galactosidase activity when grown on galactose medium. Most LexA fusion baits that enter the nucleus and bind the Lex A operator but do not activate transcription repress this beta-galactosidase activity from 2 to 20 fold.

pJG4-5 was used to construct a *Drosophila* embryo cDNA library to make fusion proteins (generous gift of Dr. Russ Finley). It is also a yeast/bacteria shuttle vector containing *trp1* gene and *bla* gene as selectable marker for yeast and *E.coli*. The fusion protein coding sequence starts with the SV40 nuclear localization signal, followed by the B42 acid transcription activation domain, then a hemaglutinin epitope tag, and then the cDNA-coded proteins. Fusion protein expression is controlled by the GAL promoter, so expression is galactose-induced, glucose-repressed, and the transcription is terminated by the yeast AHD1 terminator.

Yeast (EGY48 or EGY195) harboring individual bait plasmid (pEG202-bait) and the reporter plasmid pSH18-34 were used to screen the *Drosophila* embryo cDNA library constructed in pJG4-5 by transforming the cDNA plasmid library into the yeast and selecting on media lacking histidine, uracil, tryptophan, and leucine, and containing 1% galactose and 0.5% raffinose. The transformation complexity (~1.5x 10^6) was determined by plating an aliquot on a plate containing leucine. The yeast colonies (~760 EGY195 harboring bait dSlo-C665-1164; ~1000 EGY48 harboring bait dSlo-C665-1164; no colony showed up on other strains/baits combinations) obtained from the selection plates were tested for beta-gal activity, and then after colony purification, tested for galactose-dependent growth on plates lacking leucine (-his, -ura, -trp, -leu, +gal/raff) and again for beta-gal activity. Ultimately, eight clones remained for yeast EGY48 harboring dSlo-C665-1164(X1 to X8) and eight clones remained for yeast EGY195 harboring dSlo-C665-1164(Y1 to Y8). The pJG4-5 cDNA plasmids (trp+) were then rescued from the yeast colonies by transforming yeast miniprep DNA into *E.coli* KC8 (*pyrF::Tn5*, *hsdR*, *leuB600*, ***trpC9830***, *lacD74*, *strA*, *galK*, *hisB436*) and selecting on minimal media lacking tryptophan. The four colonies were picked from each transformation; plasmid DNAs were isolated and analyzed by restriction enzyme digestion to determine if the four cDNA clones were the same. It turned out that one of yeast clones, Y3, contained two different cDNA plasmids(Y3-1, Y3-2) and each of the others had only one. Each of the rescued pJG4-5 cDNA plasmids was then re-introduced into yeast EGY195 containing reporter plasmids with or without bait plasmid, to confirm their galactose-dependent growth on media lacking leucine (-his, -ura, -trp, -leu, +gal/raff) and beta-gal activity, which required the present of bait plasmid. One of the cDNA clones, Y3-2, supported yeast galactose-dependent growth on media lacking leucine (-his, -ura, -trp, -leu, +gal/raff) and showed beta-gal activity in absent of bait plasmid; the clone was eliminated. The other clones grew on gal media lacking leucine only in the presence of bait plasmid and

showed strong beta-gal activity. These cDNA inserts were subcloned into M13 and their nucleotide sequence were determined.

Preparation and transformation of yeast cell

For yeast transformation, yeast were inoculated in 50ml of DOB/CSM selective medium (BIO 101 Inc, 1070 Joshua Way, Vista, CA92083 , 1-800-424-6101), grown at 30^{o}C for over 20 hours to reach OD_{600}=1 (10^{8} cells/ml), then spin down at 3000g for 3-5min at room temperature, resuspended in 200ml pre-warmed YPAD medium (BIO 101) and then grown for anther 4-5 hours. After pelleting cells at 3000g for 3-5min at room temperature, yeast were washed once with 40ml cold TE (10mM Tris, 1mM EDTA, pH7.5), once with 40ml cold TE/Li-acetate (10mM Tris, 1mM EDTA, 100mM LiAc, pH7.5), once with 40ml cold TE/Li-acetate/8%glycerol, and then resuspended in 2ml of cold TE/Li-acetate/8%glycerol (>2x10^{9} cells/ml). Cells were frozen immediately on dry ice and store at -80^{o}C in 300-1000 ul of aliquots. Transformation may be performed after thawing the competent yeast cells at room temperature, or by using freshly prepared competent cells, as described above but finally resuspending in 2ml of 0.5XTE/LiAc. 1ug plasmid DNA was mixed with 100ug denatured, sheared salmon sperm DNA and 100 ul of yeast competent cell suspension in an eppendorf tube, and then 700ul TE/LiAc/PEG3325 (10mM 100mM LiAc, 40%PEG3350, 10mM Tris, 1mM EDTA, pH7.5), was added and mixed well and then incubated at 30^{o}C for 30-45min. 88ul DMSO (<10% of original volume) was mixed and then the mixture was heat-shocked at 42^{o}C for 7-15min. Cells were collected and washed once with TE, and then plated on the selective media. For library transformation, cells were allowed to recover after transformation by growing them in the DOB/CSM minimal media for 4-5hr and then plated on the DOB/CSM selective media. The *Drosophila* embryo cDNA library in plasmid pJG4-5 (a generous gift of Dr. Russ Finley) was first amplified by electrotransforming *E.coli* DH5alpha. Bacteria cells were grown in 5ml LB broth at 37^{o}C overnight and 2ml was used

to inoculate a 200ml low-salt LB (5g/liter NaCl). The culture was grown to OD_{600}=0.6, the cells were pelleted, washed twice with ice-cold 100ml of mili-Q water and twice with 100ml of ice-cold 10% glycerol. In the final step, the cells were suspended in 150ul 10% glycerol and 40ul aliquots were made. Each aliquot may be used to electrotransform 40ng DNA yielding about 10^8 colonies (Cell-Porator^{TM} Electroporation System, BRL Life Technologies, Inc.). All experiments were performed on wet-ice.

Yeast plasmid miniprep and rescue of cDNA library plasmid

For the positive yeast clones identified by the yeast two-hybrid screen, the cDNA library plasmid may be rescued. Yeast cells were grown in 5ml of appropriate selective DOB/CSM medium (-Trp) at 30°C overnight, and collected by centrifuging at 3000g for 5 min, resuspended in 0.5 ml of 1 M sorbitol, 0.1 M Na_2EDTA, pH7.5, and then mixed with 20 ul of 2.5 mg/ml zymolyase 100,000. After incubating at 37°C for over one hour, yeast were pellected and resuspended in 0.5 ml of 50 mM Tris-HCl, 20 mM Na_2EDTA, pH 7.4, then mixed with 50 ul of 10% SDS and incubated at 65°C for 30 min. After mixing with 0.2 ml of 5 M KAc and incubating on ice for 60 min, supernatant was collected by centrifuging 5 min in a table centrfuger and mixed with one volume of isopropanol. DNA was pelleted, air dried and resuspended in 0.3 ml of TE, pH 7.4 containing 15 ul of 1mg/ml RNase A. The DNA solution was incubated at 37°C for 30min and mixed with 30 ul of 3 M NaOAc and 0.2 ml of isopropanol. DNA pellet, air dried and resuspended in 50 ul of TE contains cDNA plasmid. 5 ul was used to transform E.coli K-12 strain KC8 (*pyrF::Tn5*, *hsdR*, *leuB600*, ***trpC9830***, *lacD74*, *strA*, *galK*, *hisB436*) and bacteria were selected on M9 plates containing ampicillin, uracil, histidine, leucine, thiamine but lacking tryptophan to rescue pJG4-5 cDNA library plasmid (trp1+).

Beta-Galactosidase assays

To test if the bait molecules get into nucleus and bind LexA operators, a repression assay was performed. In the experiment, yeast strain EGY48 (Mat *alpha, his3, trp1, ura3, 6LexAop-leu2*) was cotransformed with plasmid pJK101 and an individual bait. pJK101 is a yeast/*E.coli* shuttle vector, similar to pSH18-34, except that it contains most of the GAL1-upstream activating sequence (UASG), and importantly, it also contains, between UASG and the GAL1 transcriptional start site, a colE1-derived LexA operator which provides high a affinity binding site for LexA-bait fusions. The Gal1 gene on pJK101 is constitutively activated when the yeast is grown on galactose medium. However, when LexA-bait fusions enter the nucleus and bind to the lexA operator, the activation of Gal1 is blocked. Baits which cannot enter the nucleus do not affect the beta-galactosidase activity.

Every bait tested was cotransformed with pJK101 in EGY48 and selected on Ura-His- plate. Several single colonies were picked and grown in 5 ml of Ura-His- Glucose medium at 30^{o}C to saturation. The culture was diluted 1:25 into fresh Ura-His- Gal/Raff medium and to grown to OD_{600}= 0.5-1.0. 50 ul was taken and mixed with 950 ul of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50mM beta-mercaptoethanol), and 50 ul of 0.1%SDS, 3 drops of Chloroform, vertexed and 200 ul of 4 mg/ml ONPG (o-nitrophenyl-beta-D-galactoside dissolve in 100 mM phosphate buffer, pH 7.0) was added. After incubating at 30^{o}C for 15 min to develop sufficient yellow color, the reaction was stopped by adding 500 ul of 1M Na_2CO_3. The optical density at both 420 mu and 550 mu was recorded for each sample.

The beta-galactosidase activity was calculated by formula:

$$\text{Units} = 1000 \times \frac{OD420 - 1.75 * OD_{550}}{t * v * OD_{600}}$$

OD_{420} and OD_{550} are read from reaction mixture,

1.75 is a approximate correction factor of light scattering by the cell debris,

OD_{600} reflects the cell density just before assay,

t= time of the reaction in minutes,

v= volume of yeast culture used in the assay, in ml

For testing beta-galactosidase activity in yeast colonies, a dry nitrocellulose filter was laid onto yeast colonies carefully, removed, and immersed in liquid nitrogen for 10 sec. The filter was then removed and placed at room temperature on a clean surface, colony side up, to thaw. The filter was placed on whatman paper soaked with 1mg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside) in Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl 1mM $MgSO_4$, 50 mM beta-mercaptoethanol) and incubated at 30°C in a humidified chamber. Visible blue color was seen between 30 min to 4 hours.

Preparation and transformation of *E.coli* DH5alpha cells

E.coli DH5alpha was innoculated directly from a frozen stock into LB media and grown at room temperature over night. The next day, a 1:10 dilution was made into LB broth and grown at room temperature to OD_{600} 0.3 - 0.4, chilled on ice for 10 min, and pelleted 1500g for 10 min. The supernatant was decanted and the pellet kept on ice. 1/10 original volume of freshly mixed cold 1:1 of TSS: FSB (For 100 ml of FSB: 1.0 ml of 1.0M KAC, 5.0 ml of 2.0M M KCl, 0.89 gm $MnCl_2*4H_2O$, 0.147 gm $CaCl_2*2H_2O$, 0.08 gm $Co(NH3)_6Cl_3$ Hexamminecobalt chloride, 10.0 ml Glycerol, pH 6.3. For 100 ml of TSS: 1.0 gm Bacto-tryptone, 0.5 gm Bacto-yeast extract, 0.5 gm NaCl, 0.48 gm $MgSO_4$, 10.0 gm PEG8000, pH 6.5) was added and the cells gently resuspend. A 5% volume of DMSO was added and the solution left on ice for 5-10 min. Aliquots of 200 ul were made into pre-cooled eppendorf tubes and the competent cells need frozen on dry ice and stored at -80°C. For plasmid

transformation, cells were thawed on ice, mixed with 1-5 ul of DNA (10ng) and incubated on ice for 30 min. After heat shock at 42°C for 45 seconds, cells were chilled on ice for 5 min and 1 ml of LB broth was added. The cells were grown at 37°C with shaking 30 min, gently pelleted and plated to selective media. The transformation efficiency may be $>10^8$ colonies/ug DNA ((Inoue et al., 1990; Sambrook et al., 1989).

cDNA Library screening and DNA sequencing

To search for the 5' sequence of dSLIP1, a *Drosophila* head cDNA library (lambda gt10, gift of Michael Forte) was screened. Roughly, $1X10^6$ lambda clones were plated and probed with a randomly labeled cDNA fragment of dSLIP1 in 1 M NaCl, 1% SDS and 50% formamide at 37°C overnight. The filters were then washed extensively in 0.5XSSC and 0.1% SDS at room temperature. The blots were exposed to X-ray film for 12-18 hours; four colonies were identified as positive. The DNA of the positive lambda clones was isolated and cDNA inserts were subcloned in pBlueScript and M13 vectors for sequencing (Sequenase^{TM}, United States Biochemical Corp., Cleveland, OH44128). For double strand sequencing, 30 ul of double strand DNA (5ug) was first denatured with 150 ul of 0.4N NaOH for 10 min at 65°C and neutralized with 60 ul of 5 M NH_4OAc (pH 7.4), and then precipitated. The sample was then sequenced in the same way as M13 single strand DNA. Similarly, the cDNA insert of an identified yeast-two-hybrid positive clone was excised by restriction digests with EcoRI and XhoI. The insert was subcloned into M13 phage vectors mp18 and mp19 for single strand sequencing. Sequence data was analyzed by using GCG/Blast program.

5' RACE(Rapid Ampification of cDNA Ends)

The original clone dSLIP1 contained an open reading frame fused to the B42 acid transcription activation domain (for details please see the section the yeast interaction trap), a

stop codon, 3' untranslated sequences and a poly(A) tail, but lacking a 5' initiator methionine. To obtain 5' sequence and search for the translational start site, 5' RACE was performed. 1ug *Drosophila* embryo poly(A)+ RNA was reverse transcribed (RVT; Superscript, BRL) using two synthetic primers. The 3' primer used at downstream of mRNA comprised the 18 nucleotides dSLIP1 specific sequence (RVT 3' primer); the 5' primer used at upstream of mRNA is a bipartite primer, comprised a six random position at 3' domain and a 14 nucleotides containing HindIII restriction site at 5'(RVT 5' primer). This unambiguous 14 nucleotide region of RVT 5' primer was used as the target of 5' primer of all PCRs described bellow. 1ul of a 1:100 dilution of the reverse transcription reaction was used in 50 ul of first PCR reaction. In the first PCR reaction, the 3' primer was a 20 nucleotide dSLIP1 specific sequence (outer 3' primer) which is at 5' of the RVT 3' primer. The PCR reaction was performed in 50 ul using 1 unit of Vent polymerase (NEB) and the first PCR reaction was used as the template in the second PCR reaction but with a different 3' primer (inner 3' primer) which comprised, at the 3' domain, an 18 nucleotide dSLIP1-specific sequence over lapping with 5' of the outer 3' primer, and at the 5' domain, 12 nucleotide containing a SalI restriction site. The final PCR products were purified and cleaved with HindIII and SalI then subcloned into M13 vectors and sequenced (Bond et al., 1994).

Purification of His-tag proteins

Polyhistidine has a very high affinity for Ni ($Kd=10^{-13}$, pH8.0). Recombinant proteins which have 6-8 histidine tag at the N- or C-terminal may be affinity-purified using a Ni-column (Qiagen, Chatsworth, CA). The pET16b vector (Novagen, Madison, WI) was used to make dSlo (C665-1164) and dSLIP1 (full length) his-tag constructs. The two constructs were transformed into *E.coli* BL21(DE3) (Novagen, Madison, WI), and the His-tag proteins were induced with 0.2 mM isopropyl thiogalactoside (IPTG). Bacteria were collected and lysed with lysozyme and sonication, and the inclusion bodies were then pelleted, and washed once with PBS. To purify the His-tag proteins, the inclusion bodies were solubilized with

Buffer A (8 M Urea, 100 mM NaPhosphate, 10 mM Tris, pH 8.0) at room temperature for 45 min. Following centrifugation at 10,000g for 20 min, the supernatant was collected and mixed with Ni-NTA resin, previously equilibrated in Buffer A, stirred at room temperature for 45 min, then the resin was loaded a column and washed with 10 column volumes of Buffer A, 10 volumes of Buffer B (8 M Urea, 100 mM NaPhosphate, 10 mM Tris, pH6.3). The His-tag protein was eluted with Buffer D (8 M Urea, 100 mM NaPhosphate, 10 mM Tris, pH 4.5). The eluate was collected in small fractions and analyzed by SDS-PAGE, dialyzed in PBS, and the recombinant protein was collected.

Antibody affinity purification

To generate antibodies against *dSlo* and dSLIP1, the His-tag proteins (C665-1164 of *dSlo* and full-length dSLIP1) were used as immunogens to immunize rabbits (20ug, subcutaneous, Biodesign International, Kennebunk). Bleeds were taken regularly to be assessed by probing immunoblots of either dSlo or dSLIP1 GST-fusion proteins. The antibodies against *dSlo* and dSLIP1 were purified through an affinity column. To make to the affinity column, 5 mg of purified GST-fusions of *dSlo* and dSLIP1 (dSlo C1032-1164 and dSLIP C1305-396, see GST-fusion proteins) were individually cross-linked to the pre-treated AffiGel (BioRad, 2 ml of slurry of AffiGel 10 and 15 washed with cold 10 mM Sodium acetate, pH 4.5 and then cold water) by shaking at 4^{o}C overnight in 4 ml of PBS. The free esters on the Affigel were blocked with 5 ml of 100 mM Ethanolamine in 40 mM Hepes pH 7.4, at 4^{o}C for 1 hr. The gel was transferred to a column, washed extensively with water, PBS, 100 mM Glycine pH 2.5, 100 mM Diethylamine, and finally PBS. To affinity purify antibodies, 2-10 ml antiserum was loaded onto the prepared column and the flow-through was reapplied for a total of 3 times. The column was washed with PBS and the antibodies were eluted off the column first with 10 ml of 100 mM Glycine, pH 2.5 into a tube containing 2 ml 1M Tris, pH 8.0 to neutralize the pH, and then after washing with 10 ml PBS, the second elution was performed with 10 ml 100 mM Diethylamine, pH 11.5 into a

tube containing 2 ml 1M Tris, pH 8.0. The acid and base eluates were combined and dialyzed against PBS overnight at 4°C, then concentrated by centricon concentrators (Amicon) to final volume of 0.5 ml.

In vitro translation of dSLIP1 and Western blots

A coupled transcription-translation reticulocyte lysate (TnT; Promega) was used to translate the dSLIP1 protein *in vitro*. 1 ug of full length dSLIP1 cloned in pBF was mixed with 12.5 ul of TnT Lysate, 1 ul of TnT Reaction buffer, 1 ul of 1 mM complete amino acids mixture, 0.5 ul of 40 u/ul Rnasin, and 0.5 ul of Sp6 RNA Polymerase in a final of 25 ul volume. The reaction mixture was incubated at 30°C for two hours and the product was mixed with 75 ul of SDS-PAGE loading buffer and briefly centrifuged. 10 ul of the supernatant was used for a Western blot. For Western blots, *Drosophila* embryo (1g, 0-24 hours) were thoroughly homogenized and sonicated in 500 ul of 2xSDS-PAGE loading buffer and the insoluble material was pelleted in a table-top centrifuge; 15 ul of the supernatant was then subjected to 10% SDS PAGE. The samples from bacteria expressing His-tag dSLIP1 and the *in vitro* translated dSLIP1 were prepared as described above. After SDS PAGE, the proteins were transferred to a nitrocellulose membrane via semi-dry electrophoresis (Bio-Rad). The filter was preabsorbed with 2.5% dry milk in PBS and 0.1% Triton X-100 and probed at room temperature for 2 hr with a 1:5000 dilution of either *dSlo* or dSLIP1 antiserum (0.5% dry milk in PBS and 0.1 Triton X-100). Then the blot was washed three to five times with PBS and 0.1% Triton X-100, incubated at room temperature for 1 hr with secondary antibody (1:5000 dilution of HRP-conjugated anti-rabbit IgG antibody; Santa Cruz Biotechnology, Santa Cruz, CA), and washed three to five times with PBS and 0.1% Triton X-100. ECL detection reagent (Amersham, Arlington Heights, IL) was added and incubated for 1 min before exposure to X-ray film for 5-30 min. Finally, the image was scanned by an ARCUS II AGFA scanner (AGFA, Mortel, Belgium).

GST-pull down

dSlo and dSLIP1 were subcloned into the pGEX-KG vector to make GST fusion proteins (Hakes and Dixon, 1992). After transforming into DH5alpha, the expression of GST fusion proteins was induced by adding IPTG in bacteria culture when OD_{600}=0.5.

Two soluble GST-fusion proteins (dSlo-C1032-1164 and dSLIP-C1305-396) were isolated for affinity purification of antibodies (See Affinity Purification of Antibody). 500 ml culture of bacteria expressing GST fusion proteins were pelleted and resuspended in PBS containing 0.2 mM PMSF and 0.5 mM DTT, and then lysed with lysozyme, 1% Triton X100 and sonication. The supernatant, after centrifuging 15 min at 10,000 g, was applied to glutathione-agrose beads (Sigma, St Louis, MO) which were pre-equilibrated with PBS. The beads were then washed with a excess of PBS, and GST-fusion proteins were eluted with 10mM reduced glutathione, 50 mM Tris pH 8.0. Eluted proteins were dialyzed overnight at 4^{o}C in PBS and then concentrated with centricon concentrators (Amicon).

For GST-pull down experiments, the indicated GST fusion proteins were expressed in E.coli DH5alpha, and His-tag fusion proteins were expressed in BL21 (DE3) or NovaBlue (DE3). Following IPTG induction, bacteria were lysed and inclusion bodies were solubilized in 1.5% sarkosyl, 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF (Frangioni and Neel, 1993). GST fusion proteins (10 ug) were batch-bound to glutathione beads in the same buffer, rocked at 4^{o}C overnight, and washed 5 times with PBS. Solubilized His-tag fusion proteins (100 ug) were mixed with the glutathione-agrose beads bound with GST fusion proteins, in 1ml of binding buffer (10 mM HEPES pH 7.5, 0.5 mM DTT, 0.5 mM EDTA, 0.1% NP-40, 150 mM NaCl, 5 mg/ml BSA, 0.2 mM PMSF) and incubated for 12 hours at 4^{o}C, washed 3-5 times with excess binding buffer. The bound proteins were batch eluted with 30 ul of 10 mM reduced glutathione, 50 mM Tris pH 8.0 and

mixed with 2XSDS-PAGE loading buffer before subjecting them to 8% SDS-PAGE. Immunoblots from these gels were then probed with primary antibodies of either anti-*dSlo* or anti-dSLIP1, as described in the Western blot section.

Immunoprecipitation

COS7 cells were transiently transfected using the calcium phosphate method (Sambrook et al., 1989) with dSLIP1, *dSlo*, and dSLIP1+*dSlo*, each of which was subcloned in pcDNA3 (InVitrogen, San Diego, CA). 24 hours before transfection, COS7 cells were plated on 15 cm dishes at 60-80% confluency. 30 ug of plasmid DNA was mixed with 0.5 ml of 50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4 pH 6.95 and then 0.5 ml of 0.25 M $CaCl_2$ was added drop-wise. After incubating at room temperature for 20 min, the DNA/$CaCl_2$/BES mixture was added circularly into a COS7 plate which was rinsed with DMEM and then fed with 25 ml of prewarmed DMEM + 10% fetal calf serum (FCS) medium. The cells were then incubated at 37^{o}C up to 24 hours in a 5% CO_2 incubator, refed with DMEM + 10% FCS, and harvested 48 hours post-transfection. Cells transfected with the lacZ gene subcloned in pcDNA3 were used to determine transfection efficiency. The cells were washed with PBS and fixed with 2 ml of 2% paraformaldehyde and 0.2% glutaraaldehyde in PBS, rinsed with PBS 1X, PBS+0.1% Triton 1X, PBS 2X, and then stained with 2 ml of staining buffer (20 uM $MgCl_2$, 50 uM Potassium Ferricyanide, 50 uM Potassium Ferrocyanide Sigma, St. Louis, MI, plus 0.025% X-Gal in PBS) at 37^{o}C for 1-6 hours. Transfection-positive cells were distinguished by their blue color, with an average transfection efficiency of 60-80%. For coimmunoprecipitation experiments, the transfected cells were lysed with 50 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 1% Triton X 100, and 0.2 mM PMSF. Following sonication, insoluble debris was removed by a brief centrifugation, and the supernatant was incubated with preimmune serum (5 ul) for 1 hour at 4^{o}C. Protein A Sepharose CL-4B (Pharmacia, Piscataway, NJ) was added and incubated for an additional 3

hours at 4°C prior to centrifugation in a table-top centrifuge. The supernatant was used for coimmunoprecipitations by incubating with 3 ul of anti-*dSlo* antiserum at 4°C for 1 hour, after which 30 ul of Protein A Sepharose CL-4B was added and the mixture was rocked overnight at 4°C. The Protein A Sepharose beads were pelleted and washed 5 times with washing buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% Triton X 100, and 0.2 mM PMSF). The antibodies bound to Protein A were eluted with 50 ul of 0.1 M Glycine pH 2.9, and 50 ul of 2XSDS-PAGE loading buffer was mixed with the elution prior to loading the mixture onto an 8% SDS-PAGE followed by a Western blot.

Immunohistochemistry

COS7 cells were plated onto microscope slide cover slips at about 15% confluency and transiently transfected as described above, with d*Slo*+dSLIP1, each of which was subcloned in pcDNA3. Forty eight hours after transfection, cells was washed twice with PBS and then fixed with 4% parafix solution (4% paraformaldehyde, 20 uM $CaCl_2$, 20 uM $MgCl_2$ in PBS) for 15 min at room temperature. The fixed cells were washed once with PBS and 3 times with 0.1% Triton X 100, 0.2% BSA in PBS, blocked with 2% horse serum, 0.5% Triton X 100 in PBS for 30 min at 4°C, and then washed three times with 0.1% Triton X 100, 0.2% BSA in PBS. The primary antibody (Rabbit anti-Slo, 1:500 dilution) was added and incubated overnight at 4°C. The next day, cells were washed five times with 0.1% Triton X 100, 0.2% BSA in PBS, and then incubated with secondary antibody (1:200 dilution, biotinylated anti-mouse or rabbit IgG H+L, Vector, CA) in 0.1% Triton X 100, 0.2% BSA in PBS for 1-2 hour at room temperature. The cells were then washed 3 times with 0.1% Triton X 100, 0.2% BSA in PBS, and incubated with fluorescein avidin D (FITC; 1:500) for about 1 hour. Following washing with PBS 3 times, the coverslips were mounted on glass slides with 15 ul of mounting media (0.5 mg/ml P-pheneylenediamine, 100 mM Tris pH 7.5, 50% glycerol), and the edges were sealed with fingernail polish. The results were

observed and photographed using a fluorescence microscope (ERNST, Dialux 22EB, Germany).

Chromosomal localization on *Drosophila* polytene chromosomes

dSLIP1 cDNA was labeled by sulfonation and *Drosophila* polytene chromosome spreads were prepared on slides from salivary glands derived from third-instar larvae (Pardue and Gall, 1975).

In situ hybridization was performed as described (Quan et al., 1993). The hybridized probe of dSLIP1 was detected by an immunological method using a primary antibody against sulfonated cystine (made in rabbit), followed by a goat anti-rabbit IgG conjugated to horseradish peroxidase. The signal was then visualized (FMC Bioproducts ChemiProbe kit) and observed with a fluorescence microscope (Leitz Dialux 22EB, Germany).

***In situ* hybridization and Northern blot**

dSlo and dSLIP1 were subcloned into pBlueScript (SK) and the sense and anti-sense RNA probes of dSlo and dSLIP1 were synthesized in digoxigenin-U NTP mixture (Boehringer Mannheim, Indianapolis, IN). *Drosophila* embryos were prepared and hybridized with the probes as described (JW and Bier, 1994). The hybridized probes were detected by an anti-digoxigenin-alkaline phosphatase antibody (Boehringer) and visualized as blue precipitates after incubating with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tretrazolium). The results were observed and photographed with a light microscope.

For Northern blot, *Drosophila* embryos (about 1g; 0-24 hours) were homogenized with a Dounce homogenizer and Poly(A)+ RNA was isolated using the Fast Track RNA Isolation kit (Invitrogen). 3 ug Poly(A)+ RNA was denatured and electrophoresed on a denaturing RNA gel (1% agarose, 12% formaldehyde, 20 mM Mops pH 7.0, 5 mM NaOAc, 1 mM EDTA) and prepared for a Northern blot. The efficiency of RNA transfer was checked by

first staining the nitrocellulose membrane with 0.02% methylene blue (Sigma), 0.5M NaOAc for 10min and after a brief rinse in milli-Q water, followed by destaining with 20% EtOH. Complete destaining was achieved by washing with 0.2% SSC, 1% SDS. The hybridization was performed in 5% SDS, 400 mM Na_2HPO_4, 1 mM EDTA, 1 mg/ml BSA, 50% formamide, at 65°C overnight with radiolabeled full length dSLIP1 antisense RNA as probe, and then washed extensively in 1% SDS, 0.5 XSSC at 50°C. The blot was exposed to X-ray film for 18 hours and scanned using an ARCUS II AGFA scanner (AGFA, Mortel, Belgium).

In vitro cRNA synthesis and *Xenopus* oocyte injection

The full length clone of dSLIP1 with a 5' transcriptional initiation site and a 3' stop codon was subcloned into pBF vector (generous gift of Dr. Bernd Fakler) and linearized with BglI. 3 ug of DNA was mixed with 5 ul of 5xSP6 Buffer, 2.5 ul of 10mM DTT, 1 ul of 25 mM rNTP, 10 ul of 5mM CAP (^{m7}GppGTP), 0.5 ul 40 u/ul RNasin (Promega), 2 ul of 50 u/ul Sp6 RNA polymerase (BRL), and incubated 40°C for 1 hour. The reaction was then supplemented with additional 0.5 ul of 25 mM rNTP and 0.5 ul of 50 u/ul Sp6 RNA polymerase, and incubated for further 45 min to let the elongation finish. The DNA was separated from RNA by acid phenol extraction (75 ul of DEPC water + 200 ul water saturated phenol), and the RNA was precipitated by adding 40 ul of 5 M RNAse-free NH_4OAC, 350 ul of ethanol to the aqueous phase of the acid-phenol extraction. The RNA pellet was washed twice with 500 ul of 75% ethanol, air dried and dissolved in 20 ul of RNAse-free TE and 0.5 ul of RNasin. The quality of the RNA was regularly checked by running a 1% denaturing RNA gel (12% formaldehyde, 20 mM Mops pH 7.0, 5 mM NaOAc, 1 mM EDTA). The other RNAs used in the experiment were synthesized in a similar way as described above, except that *dSlo* was expressed from pS$^-$ (A1E1G3; Adelman, et al. 1992), dSlo-C665-1164 from pBF, and the noninactivating version of *Shaker* from pSK

(generous gift from Dr. Ligia Toro). Oocytes (Dumont stage V-VI) were harvested from adult *Xenopus laevis* under anesthesia (0.1% 3-aminobenzoic acid ethyl ester) as previously described (Christie et al., 1990). Theca and follicular layers were removed by incubation for 3 hours in calcium-free solution containing 2mg/ml collagenase (Boehringer). Denuded oocytes were maintained in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES pH7.6) containing 0.5 mM of theophylline, 2.5 mM of sodium pyruvate, 50 ug/ml of gentamycine for up to 1 week. Oocytes were injected using a micro-injection instrument within 10 hours of harvest with various concentrations and mixture of RNA encoding dSLIP1, *dSlo*, dSlo665-1164 and *Shaker*, each in 50 nl of DEPC- (Diethyl Pyrocarbonate, Sigma) treated water. *Xenopus* care and handling were in accordance with the highest standards of the institutional guidelines. Frogs underwent no more than two surgeries, separated by at least three weeks, and surgeries were performed using well established techniques by an experienced expert.

Electrophysiology

Oocytes were studied 3-7 days' after RNA injection. Whole cell currents were measured using a two electode voltage clamp with a Geneclamp 500 amplifier (Axon Instrument) interfaced to a Macintosh Quadra 800 computer. Data were acquired through Pulse (HEKA Elektronik, Germany) at 500 Hz. During recording, oocytes were continuously superfused with DN96 solution at room temperature.

Inside-out macropatches were excised into an intracellular solution containing 116 mM K-gluconate, 4 mM KCl, 10 mM HEPES (pH 7.25, adjusted with KOH) supplemented with $CaCl_2$ or EGTA or both. To obtain nominally Ca-free solution, 1 mM EGTA was added. Alternatively, $CaCl_2$ was added to the cytoplasmic solution to give free calcium concentrations of 10-100 um. In this case, the proportion of calcium binding to gluconate was determined by a computer program (CaBuf) assuming a stability constant for calcium

gluconate of 15.9 M-1 (Dawson et al., 1969). Electrode were pulled from thin-walled, filamented borosilicate glass (World Precision Instruments) and filled with 116 mM K-gluconate, 4 mM KCl, 10 mM HEPES pH 7.25. Electrode resistance was typically 2-5 M ohm. Membrane patches were voltage clamped using an Axopatch 200A amplifier (Axon Instruments). The data were low-pass Bessel filtered at 1 kHz and acquired using Pulse software (HEKA Elektronik, Germany). Analysis was performed using Pulse and Kaleidagraph (Abelbeck) software. Macropatch currents were measured during 500 ms voltage steps from a holding potential of 0 mV. All experiments were performed at room temperature.

To examine single channel properties, solutions used were the same as for macropatch recordings. Electrodes were pulled from Corning 7052 glass (Garner) and had resistances of 9-13 M ohm. Data were filtered at 1 kHz (Bessel), acquired at 10 kHz using Pulse (HEKA Elektronik) and stored directly on a Macintosh Quadra 650. Recordings were analyzed using MacTac (Bruxton Corporation, Seattle, WA). The "50% threshold" technique was used to detect openings which were visually inspected and adjusted for their amplitude before being accepted. Amplitude histograms were constructed using MacTacfit (Bruxton Corporation) and fit by a single Gaussian distribution. NP(o), the product of the single channel open probability multiplied by the number of channels, was calculated as the sum of the (dwell time x level number) divided by the total time. To calculate P(o), N was estimated as the maximum number of simultaneously open channels at 100 mV in 10 uM calcium.

Chapter III: dSLIP1, a Novel Protein which Interacts with Large-conductance Calcium-activated Potassium Channels

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Abstract

Large-conductance calcium-activated potassium channels (BK channels) are activated by depolarized membrane potential and elevated levels of intracellular calcium. BK channel activity underlies the fast afterhyperpolarization following an action potential, and attenuates neurotransmitter and hormone secretion. Using a modified 2-hybrid approach, the interaction trap, we have identified a novel protein from *Drosophila*, dSLIP1, which specifically interacts with *Drosophila* and human BK channels and has partial homology to the PDZ domain of α1 syntrophin. The dSLIP1 and *dSlo* mRNAs are coincidentally expressed throughout the *Drosophila* nervous system, the two proteins interact in vitro, and they may be coimmunoprecipitated from transfected cells. Coexpression of dSLIP1 with *dSlo* or *hSlo* BK channels in *Xenopus* oocytes results in reduced currents compared to expression of BK channels alone; current amplitudes may be rescued by coexpression with the channel domain which interacts with dSLIP1. Single channel recordings and immunostaining of transfected tissue culture cells suggest that dSLIP1 selectively reduces *Slo* BK currents by reducing the number of BK channels in the plasma membrane.

Introduction

Neuronal action potentials are followed by an afterhyperpolarization (AHP) that has several kinetic components and may have profound consequences for the firing pattern of the neuron. During an action potential, the concerted influences of increased intracellular calcium and depolarized membrane potential activates two classes of calcium-activated potassium channels, large conductance calcium- and voltage-dependent channels (BK channels), and small conductance (SK) channels activated only by calcium. Together, these distinct classes of calcium-activated potassium channels are responsible for the different kinetic components of the afterhyperpolarization (AHP). Application of BK channel blockers such as charybdotoxin (CTX) or tetraethylammonium (TEA) has shown that BK channels contribute to action potential repolarization and underlie the fast component of the AHP (fAHP), which develops rapidly (rise time 1-2 ms) and decays within 10's of milliseconds (Lancaster and Nicoll, 1987; Storm, 1987), while the subsequent slow components (sAHP) are due to SK channels and underlie spike-frequency adaptation (Hotson and Prince, 1980; Madison and Nicoll, 1984; Yarom et al., 1985).

Regulation of BK channel activity exerts a powerful modulation on neuronal excitability. Electrophysiological studies have shown that native BK channels are regulated by a wide range of second messengers including several protein kinases and protein phosphatases (Chung et al., 1991; Ewald et al., 1985; Reinhart et al., 1991) and G-proteins (Cole and Sanders, 1989; Scornik et al., 1993; Toro et al., 1990). In addition, mammalian BK channels have a closely associated b subunit which modifies the calcium sensitivity of the channel, and may itself be the target for regulatory second messengers (Dworetzky et al., 1996; Hanner et al., 1997; McManus et al., 1995).

Other mechanisms may influence the fAHP through indirect effects on BK channels. There is evidence that at least in some neuronal cell types, BK and voltage-dependent calcium channels (VDCCs) are closely associated, and may be physically coupled (Gola and Crest, 1993; Issa and Hudspeth, 1994; Robitaille et al., 1993). Other posttranslational modulatory effects on BK channels have been described, but the underlying molecular mechanisms have not yet been established (Subramony and Dryer, 1997; Subramony et al., 1996). For other voltage-gated potassium channels, a distinct β subunit, Kvβ2, associates with the α subunits early in channel biosynthesis and exerts dramatic, chaperone-like effects on the α subunits, including stabilization and increased cell surface expression (Rettig et al., 1994; Shi et al., 1996). These results suggest that the subcellular distribution and density of BK channels will affect the kinetics of the fAHP and neuronal excitability.

Both classes of calcium-activated potassium channels have now been cloned. Heterologous expression studies have demonstrated that the cloned channels faithfully recapitulate the biophysical characteristics of their native counterparts (Adelman et al., 1992; Atkinson et al., 1991; Butler et al., 1993; Kohler et al., 1996). To identify other proteins, such as β subunits, interact with BK channels and influence BK expression, the C-terminal domain of a cloned BK channel was employed in a 2-hybrid screen. One of the clones identified as interacting with the dSlo BK channel, dSLIP1, appears to regulate the number of BK channels in the plasma membrane.

Results

The nuclear localization of the bait molecules

The interaction trap (Gyuris et al., 1993), a modified yeast-two-hybrid system was used to search dSlo interacting proteins (see material and method). Four baits were constructed which fuse 1) N-terminal dSlo-N1-126, 2) C-terminal dSlo-C665-1164, 3) C-terminal dSlo-C340-1164, 4) C-terminal dSlo-C211-1164, to C-terminal oligomerization region of LexA in parent vector pEG202. These baits had no transcription activity and were tested if they enter the nucleus and bind LexA operators in a repression assay (Brent and Ptashne, 1984). Table 3.1 shows the assay results for the baits. Taken the beta-galactosidase activity of the yeast with pJK101 which had no repression as 100, the relative beta-gal activities of yeast with pJK101 and pRFM (positive control), and yeast with pJK101 and every four baits were calculated. The results showed that bait dSlo-C211-1164 may not enter nucleus, and baits dSlo-N1-126, dSlo-C665-1164, dSlo-C340-1164 entered nucleus and bound to LexA operator, and thus can be used for yeast-two-hybrid screening (Table 3.1).

Isolation of dSLIP1

Two yeast strains EGY48 and EGY195 were used as the hosts to harbor each of the three baits in the interaction trap screen of a *Drosophila* embryo cDNA library. However, only the yeast containing dSlo-C665-1164 were able to show positive clones in the screening. The sequence encoding the C-terminal 500 amino acids of *dSlo*, a region which does not vary by alternative exon choices among the known *dSlo* splice variants (Adelman et al., 1992; Atkinson et al., 1991). From approximately 10^6 transformed cDNAs, several hundred colonies were obtained on media lacking leucine from both EGY48/pEG202-dSlo-C665-1164 and EGY195/pEG202-dSlo-C665-1164. These were individually assayed on galactose media for growth in the absence of leucine and b-galactosidase activity, which eliminated all but 8 of the clones from EGY48(X1-X8) and 8 of the clones from EGY195(Y1-Y8). The remaining candidates of the cDNA plasmids were rescued and the nucleotide sequences of the inserts were determined. All the eight clones from yeast EGY48 were discarded; three

encoded RNA binding proteins, one encoded rRNA, one encoded ribosome protein, one encoded a transcription factor, one encode cytochrome oxidation C, one encoded mitochondrion ATP-syntheses subunit A. Among the eight clone from EGY195, four of the clones were discarded; one encoded a RNA binding protein, one encoded a transcription factor, and two contained mitochondrial DNA sequences. The remaining four clones fulfill the interaction trap requirements for interactions with the C-terminal domain of dSlo, and one clone, dSLIP1 (dSLo Interacting Protein), was chosen for further study (Figure 3.1 and Table 3.2; methods).

The original dSLIP1 clone contained an open reading frame fused to the B42 transcriptional activation domain, a stop codon, 3' untranslated sequences and a poly (A) tail. However, this clone did not contain an initiator methionine. To isolate a full length coding sequence, 5' RACE reactions were performed using *Drosophila* head cDNA (Bond et al., 1994). Analysis of the RACE products extended the N-terminal coding sequence and identified a putative initiator codon. The full length coding sequence of dSLIP1 predicts a protein of 396 amino acids with 5 potential substrate sequences for serine/threonine protein kinases (R/KXXT/S; Figure 3.2). Hydropathy analysis did not identify hydrophobic domains which may span the membrane, suggesting that dSLIP1 is a cytoplasmic protein(Figure 3.3). The dSLIP1 sequence does not show overall homology to any other known protein, but the N-terminus contains a region with homology to the PDZ domain of a1 syntrophin (Figure 3.4; (Adams et al., 1995; Gibson et al., 1994). To confirm that the clone encoding dSLIP1 contained the full length coding sequence, bacterially expressed dSLIP1, *in vitro* translated dSLIP1, and extracts from whole *Drosophila* embryos were prepared as a Western blot. Antisera specific for dSLIP1 recognized bands of the same size (Figure 3.5) only in these samples.

dSlo and dSLIP1 mRNAs are coexpressed in Drosophila

The expression patterns of *dSlo* and dSLIP1 mRNAs were compared using *in situ* hybridization on embryo whole mounts. *dSlo* mRNA is heavily expressed throughout the central nervous system (CNS) as well as in several peripheral locations (Figure 3.6, A,B). dSLIP1 mRNA is also expressed throughout the CNS (Figure 3.6, C,D); *dSlo* and dSLIP1 mRNAs are coincidentally expressed in virtually all CNS neurons. dSLIP1 mRNA is not expressed, however, in peripheral cell types which express *dSlo* mRNA. Northern blot analysis using a dSLIP1 probe detected a single band of 3.6 kb in whole embryo mRNA (Figure 3.7). The genomic location of the dSLIP1 gene was determined by probing polytene chromosome squashes with dSLIP1 sequences. Cytological examination unambiguously identified the dSLIP1 gene as residing on the fourth chromosome between bands 102C-D (not shown). This region does not contain a high density of genetic markers and no known mutations in this area appear to involve the dSLIP1 gene (FlyBase).

Domains mediating the interaction between *dSlo* and dSLIP1

The interaction between the C-terminal domain of *dSlo* and dSLIP1 was examined *in vitro*. Each protein was expressed in bacteria and isolated as either a GST- or pHis-fusion protein. Pull-down experiments were performed by binding either GST-dSlo or GST-dSLIP1 proteins to a glutathione-agarose column and passing the other protein as a polyHis-fusion over the column. Bound proteins were eluted by applying reduced glutathione, separated by polyacrylamide gel electrophoresis, and visualized by Western blot analyses. The results show that GST-dSLIP1 specifically retained pHis-dSlo-C665-1164 (Figure 3.8, left). The region of *dSlo* which mediates the interaction with dSLIP1 was defined by similar experiments in which different C-terminal domain fragments of dSlo were produced as GST-fusion proteins and bound to glutathione agarose beads prior to the application of pHis-dSLIP1 protein. The results demonstrate that inclusion of the region of *dSlo* between amino acids 1032-1164 retained dSLIP1 protein, while further deletion abolished the interaction

(Figure 3.8, right). Similar results were obtained by complementary 2-hybrid analyses (Table 3.3 and 3.4). To more precisely localize the domain of dSLIP1 which mediates the interaction with dSlo, 2-hybrid experiments with dSlo-C665-1164 and different regions of dSLIP1 were performed. These experiments localized the interacting domain to the C-terminal 100 residues of dSLIP1; further deletions from either end resulted in loss of the interaction (Table 3.3).

dSlo antibody coimmunoprecipitates dSLIP1

The pull-down experiments indicate a stable interaction in vitro between dSlo-C665-1164 and dSLIP1. Therefore, *dSlo* and dSLIP1 were transiently cotransfected into COS7 cells and total cellular proteins were used for immunoprecipitations with antibodies directed against either *dSlo*, dSLIP1, or FLAG (control). Immunoprecipitated proteins were prepared as a Western blot and probed with dSLIP1 antibodies. Figure 3.9 shows that immunoprecipitations using *dSlo* antibodies coprecipitated dSLIP1.

dSLIP1 decreases *dSlo* current amplitudes

Functional interactions between *dSlo* and dSLIP1 were examined in inside-out patches from *Xenopus* oocytes expressing either *dSlo* alone or *dSlo* and dSLIP1. Prior to injection, *in vitro* synthesized dSLIP and dSlo-C665-1164 mRNAs were adjusted to approximately equal molar ratios based on spectrophotometric and agarose gel analyses, while *dSlo* mRNA was injected at 3-5-fold lower concentrations. Current amplitudes were measured at 100 mV in the presence of 10 μM internal Ca^{2+}, using electrodes of similar resistances (~3 MW). In a representative experiment (Figure 3.10A), patches from *dSlo*-expressing oocytes had current amplitudes of 3.0 ± 2.5 nA (n=21), while similar patches from oocytes coexpressing *dSlo* and dSLIP1 showed current amplitudes of 0.8 ± 1.9 nA (n=21; $p<0.005$). The reduction in

patch current was specific for the interaction between *dSlo* and dSLIP1 as the effect was reversed by coexpression with dSlo-C665-1164, the fragment used to screen for dSLIP1. In the experiment shown, current amplitudes in patches from oocytes expressing *dSlo*, dSLIP1, and dSlo-C665-1164 were not different than for dSlo alone, 3.6 ± 1.9 nA (n=18; p>0.1). *dSlo* current reduction by coexpression with dSLIP1 gave similar results in 12 out of 15 experiments using different batches of oocytes; the average reduction was 71 ± 18 %. In 7 batches of oocytes, inhibition of this effect by dSlo-C665-1164 was examined; the current was at least partially rescued in 6 batches resulting in a 3.4 ± 1.1-fold increase in the average patch current compared to oocytes only expressing *dSlo* and dSLIP1. There was no effect of dSlo-C665-1164 on *dSlo* current amplitudes (1.7 ± 0.8 nA, n=9 versus 2.0 ± 1.2, n=11; p>0.5). In 3 out of 5 experiments in which the human BK channel, *hSlo*, was substituted for *dSlo* the same specific inhibitory effect of dSLIP1 was observed (Figure 3.10B). On average, the current seen in oocytes expressing *hSlo* and dSLIP1 was reduced by 69 ± 10 % compared to oocytes expressing only *hSlo*. Oocytes expressing *hSlo*, dSLIP1 and dSlo-C665-1164 displayed a 2.4 ± 1.1-fold increase in current compared to oocytes expressing *hSlo* and dSLIP1. In contrast, dSLIP1 did not reduce currents in oocytes expressing *Shaker* channels (Figure 3.10C). The effect of dSLIP1 on *Slo* currents was sensitive to the amount of *Slo* mRNA injected. When *Slo* mRNA was injected in excess to dSLIP1, the reduction in current amplitude was reduced or eliminated.

To investigate whether the reduction of patch current at a given voltage and calcium concentration was due to a reduced calcium-sensitivity of *dSlo* channels in the presence of dSLIP1, the patch conductance holding at -100 to +100 mV was measured in three concentrations of intracellular Ca^{2+} for patches expressing *dSlo* alone (Figure 3.11A) or together with dSLIP1 (Figure 3.11B). Data were fit to a Boltzman relationship and the resulting values for the voltage of half-activation ($V_{0.5}$) were averaged for 3-5 patches and

plotted as a function of Ca^{2+} concentration in Figure 3.11C; no significant differences were observed at any of the Ca^{2+} concentrations examined.

To further investigate the mechanism underlying the reduction in *Slo* currents mediated by dSLIP1, single *hSlo* channels were examined. Channels from oocytes injected either with *hSlo* alone or with *hSlo* and dSLIP1 were exposed to 100 uM Ca^{2+} and showed the same single channel amplitude as a function of voltage (Figure 3.12A, B). The average single channel conductance was 242 ± 16 pS in 3 patches with *hSlo* alone and 224 ± 5 pS in 4 patches with *hSlo* and dSLIP1 ($p>0.05$). The open probability (P_o) as a function of voltage was also not affected by coexpression with dSLIP1 (Figure 3.12C), further confirming that dSLIP1 did not effect the calcium sensitivity of *hSlo* channels.

Since the gating and conduction properties of *Slo* channels were not obviously affected by dSLIP1, the reduction in macroscopic current amplitudes probably resulted from a reduced number of channels in the plasma membrane. This possibility was further examined by immunocytochemistry with *dSlo* antiserum on COS7 cells which had been transfected with *dSlo* alone or together with dSLIP1. In cells expressing only *dSlo*, a reticular, punctate staining pattern was obtained with *dSlo* immunoreactivity clearly seen on the outer membranes (Figure 3.13, left). In contrast, cotransfected cells showed diffuse, internal staining with little if any *dSlo* immunoreactivity on the cell surface (Figure 3.13, right).

Chapter IV: Discussion

The primary goal of this dissertation is to identify *dSlo* channel accessory proteins that are important for channel function. The experiments focused on three aspects: a) identify *dSlo* interacting proteins; b) confirm the interaction between *dSlo* and dSLIP1, and define the interacting domains of the two proteins; and c) study dSLIP1 function related to dSlo. In order to identify *dSlo* accessory proteins, we used a modified yeast-two-hybrid system, the interaction trap, to search for *dSlo* interacting proteins (Xia et al., 1998). Four baits were constructed with N or C-terminal regions of *dSlo*, and three of them which had no self activation and showed nuclear localization were used to screen a *Drosophila* embryo cDNA library. One of these, dSlo-C665-1164 identified three novel genes and one clone, dSLIP1, was chosen for further study. Several lines of evidence indicate that dSLIP1 specifically interacts with the C-terminal domain of *dSlo*. First, deletion analysis in yeast-two-hybrid assays demonstrate that the C-terminal domain of dSLIP1 specifically interacts with the C-terminal domain of *dSlo*. Second, GST pull-down experiments confirm that dSLIP1 can form a complex with C-terminal domain of *dSlo*, and finally, dSLIP1 and *dSlo* can be co-immunoprecipitated from co-transfected COS7 cells. The mRNAs of *dSlo* and dSLIP1 are colocalized throughout the *Drosophila* central nervous system. When coexpressed in *Xenopus* oocytes, dSLIP1 reduces macroscopic BK currents compared to the expression of BK channels alone, without affecting unit current amplitude (i) or open probability (P_o). In transfected COS7 cells, *dSlo* exhibits a reticular punctate distribution with clean membrane expression. However, cotransfection of dSLIP1 with *dSlo* changes the subcellular distribution of *dSlo* which now shows a dense perinuclear and diffused intracellular pattern with little membrane expression. Taken together with the electrophysiological studies, it is likely that dSLIP1 limits the number of functional BK channels in the plasma membrane.

It is intriguing to propose that dSLIP1 is involved in *dSlo* channel processing, targeting, distributing, and/or degradation. The electrical excitability of a neuron is determined not only by the types of channels it expresses, but also the number and the specific subcellular localization of these channels (Roeper and Pongs, 1996). Therefore, mechanisms which regulate channel density will have significant effects on neurons and other excitable cells. The several auxiliary subunits of voltage-gated channels facilitated the expression or assembly of major pore forming alpha subunits. The major form of the sodium channel in rat brain is a heterotrimeric complex of an alpha subunit, a noncovalently bound beta1 subunit, and a disulfide-linked beta2 subunit (Sutkowski and Catterall, 1990). Coexpression of both beta1 and beta2 subunits with alpha subunits in *Xenopus* oocytes increases the expression efficiency of alpha subunits and the functional properties of the channel, and moreover, beta2 can also cause expansion of the cell surface membrane which may be important for channel localization in neurons (Isom et al., 1992; Isom et al., 1995). In rat forebrain, a large intracellular pool of the alpha subunit appears several days prior to the emergence of functional sodium channel complexes on the cell surface, and it is likely that maturation, translocation, and plasmalemmal insertion are accompanied by association with beta subunits (Scheinman et al., 1989). Cardiac sodium channels are heterodimeric, composed of an alpha and only beta1 subunit. The kinetics and properties of the channels with either alpha or alpha plus beta show no obvious differences when expressed in *Xenopus* oocytes. However, coexpression of beta1 with alpha increases sodium channel currents up to 6-fold in a concentration-dependent manner. So, by controlling the number of sodium channel in the plasma membrane, beta1 subunits determine the level of excitability of cardiac myocytes (Qu et al., 1995). Voltage-gated calcium channels are also heteromultimeric. While alpha subunit can be expressed without coinjection of other subunit cRNA in oocytes, alpha subunit only yield minimal currents in the absence of additional subunits (Singer et al., 1991). However, coexpression of alpha plus beta subunits dramatically increase the current amplitude. Furthermore, even the small current generated by alpha subunit cRNA injection is probably potentiated by the basal level expression of oocyte endogenous beta

subunit, since coinjection of 20 uM of antisense oligo of beta subunit virtually abolished current in oocyte expressing only the alpha subunit (Tareilus et al., 1997), suggesting that beta subunit plays a critical role in assembly and expression of voltage-gated calcium channels. Other experiments also demonstrated that coexpression of calcium channel alpha and beta subunits increases the amplitudes of the currents, and the number of high affinity drug and toxin binding sites compared to expression of alpha subunits alone (Mori et al., 1991; Stea et al., 1993; Williams et al., 1992). Voltage-gated potassium channels may associate with one or more beta subunits. The beta2 subunit acts as a chaperone, regulating channel density and current amplitude by increasing the number of channels in the plasma membrane (Rettig et al., 1994; Shi et al., 1996). For BK channels, a beta subunit has been identified in muscle but not in neurons where BK channels are highly expressed. Therefore, one likely role for dSLIP1-like beta subunits is to regulate BK channel assembly and expression.

During normal development BK channels are regulated not only at the transcriptional level (Muller et al., 1998), but also at posttranslational levels (Subramony et al., 1996). The regulation of current density is well documented in developing parasympathetic neurons such as chick ciliary ganglia (CG). In developing CG, or in CG cultured in the absence of interactions with target tissues, BK channel mRNA is present but no BK currents are detected (Dourado et al., 1994). However, when CG neurons are cultured in the presence of target tissue (iris) extract, BK channel activity is induced and this regulation is independent of both mRNA and protein synthesis. In both conditions, voltage-activated calcium currents are expressed at normal levels (Subramony and Dryer, 1997; Subramony et al., 1996). The stimulation of BK currents in CG neurons is induced by an active component in iris extracts whose effect can be mimicked by neuregulins (Subramony and Dryer, 1997; Subramony et al., 1996). Although the underlying mechanisms have not yet been elucidated, the results are consistent with a regulated interaction, in the absence of target tissue, between intracellular BK channels and a dSLIP1 homolog required for storage of BK channel. In such a

posttranslational paradigm for the regulated appearance of functional BK channels in the plasma membrane, a dSLIP1 homolog may well be a major component, retaining BK channel alpha subunits in an intracellular pool until a physiological cue induces the release of alpha subunits translocating them into the plasma membrane.

Neuregulins are a large class of growth factors which bind to type 1 tyrosine kinase receptors (Fischbach and Rosen, 1997). Neuregulins are known to stimulate expression of AChR, voltage-activated sodium channels (Falls et al., 1993), regulate neurogenesis in some sensory and autonomic ganglia (Meyer and Birchmeier, 1995), regulate proliferation and differentiation of developing and mature glial cells and Schwann cells (Dong et al., 1995). Beta-neuregulin transcripts are expressed in the preganglionic neurons that innervate the chicken CG (Corfas et al., 1995), and it is possible that developing CG neurons are exposed to a factor such as neuregulins secreted from preganglionic nerve terminals and thus trigger the intracellular signal transduction pathway leading to the release of BK channels from dSLIP1. There are five potential phosphorylation sites for serine/threonine (S/T) protein kinase. The release of the BK channel alpha subunits of BK from dSLIP1 might be triggered via phosphorylation of any of these sites. Phosphorylation may either abolish the interaction between dSLIP1 and *dSlo*, or enhance degradation of dSLIP1. In our studies, a 1:5 molar ratio of *dSlo* to dSLIP1 cRNA injection was needed to see the macroscopic current reduction of *dSlo* by dSLIP1, and the effects of dSLIP1 may be overcome by additional expression of BK alpha subunits suggesting the possibility that one molecule of *dSlo* binds to several (four?) molecules of dSLIP1, or the turnover rate of dSLIP1 is much higher than that of *dSlo* in oocytes which could be accelerated upon cell signaling for release of *dSlo* alpha subunits. To test the first hypothesis, that phosphorylation of dSLIP1 will reduce the binding affinity of dSLIP1 to *dSlo*, mutants with S/T to D at the five potential phosphorylation sites, either individually or in combination, may be tested in the yeast-two-hybrid system to see if any of the mutants will not interact with *dSlo*. To test the second hypothesis, that phosphorylation of dSLIP1 will increase the turnover rate

of dSLIP1, dSLIP1 cRNA of the wild type and the same mutants mentioned above may be injected into oocytes and the dSLIP1 protein turnover rate may be measured by immunoprecipitation after "pulse-chase" experiments.

In *Drosophila,* mutations in *dSlo* and *Shaker* display increased excitability in presynaptic terminals of larval motor neurons. The mutant terminals respond to stimulation with an altered spiking pattern, which ultimately leads to an abnormal increase in neutrasmitter released from the presynaptic terminals (Roeper and Pongs, 1996). In the mammalian central nervous system, *Slo* immuoreactivity, as for of Kv1.2, Kv1.4 and Kv3.4, is especially enriched in terminal areas of the major projection tracts in rat brain (Knaus et al., 1996). While Kv1.2 and Kv1.4 which assembly into heteromutimeric channels are likely targeted into presynaptic terminal via their interaction with SAP-90 (synapse-associated protein-90, with PDZ domains, Laube et al., 1996; Sheng et al., 1993), it is not yet clear how BK channels which do not interact directly with PDZ domains are clustered at presynaptic terminals.

The C-terminal domain of dSLIP1 is required for the interactions with BK alpha subunits, while the N-terminal domain of dSLIP1 has limited homology with the PDZ domain of alpha1 syntrophin, which has been shown to bind nitric oxide synthase (NOS) and sodium channels (Brenman et al., 1996; Gee et al., 1998). At the neuromuscular junction , alpha1 syntrophin anchors NOS and sodium channels to the postsynaptic site through interaction of these molecules with dystrophin complex. In presynaptic sites, clustering of BK channels may also recruit an anchoring complex containing dSLIP1, being a linker between *dSlo* and other protein(s). Our observation of *dSlo* current reduction by dSLIP1 may result from the lack of other dSLIP1 interacting component(s) in our heterologous expression system, which in turn causes retention of *dSlo* at intracellular sites. This hypothesis may be tested: 1) study the presynaptic distribution of dSLIP1 and *dSlo* in wild type flies, and of *dSlo* in dSLIP1-knockout flies, 2) test via the yeast-two-hybrid system if dSLIP1 interacts with syntrophins,

SAP90 or other proteins with PDZ domains, 3) identify other component(s) which interact with dSLIP1 via the yeast-two-hybrid screening with dSLIP1, and determine localization, distribution and functions of these identified component(s).

The N-terminal domain of syntrophin has been implicated in calcium-binding (Newbell et al., 1997). Similarly, this domain of dSLIP1 may mediate interactions with additional regulatory components, perhaps in a calcium-dependent manner. In neurons, BK channels are functionally colocalized with calcium channels at presynaptic sites in controlling transmitter release (Robitaille et al., 1993). During *Drosophila* development, calcium currents appear prior to I_{FC} (*Slo*). Therefore, dSLIP1 may coordinate the colocalization of BK channels with calcium channels by interacting with the calcium channel complex. The yeast-two-hybrid system may be used to test if dSLIP1 directly interacts with calcium channel(s). It may also be possible that BK channels are delivered to the cluster sites of calcium channel via interaction of dSLIP1 with a component which requires high calcium concentration generated by opening of calcium channels. This hypothesis may be tested via identification of other protein(s) which interact with dSLIP1 and determining the calcium dependent binding of these proteins to dSLIP1 *in vitro* , such as through GST-pulldown experiments.

Our experiments suggest that dSLIP1 may participate in transporting, distributing and subcellular localization of BK channels. To understand the *in vivo* effect of dSLIP1, dSLIP1 over-expressing and knockout transgenic flies can be generated and studied. We speculate that dSLIP1 and *dSlo* proteins are colocalized at specific subcellular sites. In dSLIP1 knockout flies, *dSlo* channel proteins would not be delivered to subcellular sites. The surface expression of *dSlo* may become diffuse and overall expression level of *dSlo* may be lower than that of wild type. Thus, the dSLIP1 knockout flies would behave similar to *Slowpoke* flies, showing motor dysfunction. In dSLIP1 over-expressing transgenic flies, *dSlo* level may be up-regulated at those subcellular sites. Since BK channels hyperpolarize membrane potential, one possible

behavior phenotype of dSLIP1 over-expressing flies is that, they may also show motor dysfunction.

As dSLIP1 also reduces *hSlo* currents, mammalian counterpart of dSLIP1 (mSLIP) may exist. By performing genomic Northern blot with various stringency, multiple mSLIP homolog may be identified. These mSLIP homologs may be colocalized with BK alpha subunits in a tissue specific manner and their expression well be developmentally regulated. Some of them may function like dSLIP1 to fly, as BK channel delivering or anchoring protein, while and others may have different functions.

Beside dSLIP1, two other novel genes identified in this thesis (Y5/7, Y8) bind to different C-terminal regions of *dSlo*. Our preliminary experiments indicate that these mRNA are highly expressed in the digestive systems of *Drosophila* embryos. In *Drosophila* flight muscle which has a prominent I_{CF} (*dSlo*) and I_A (*Shaker*), neither *in situ* hybridization nor immunohistochemistry detected the presence of *dSlo* and *Shaker* products (Becker et al., 1995; Tseng-Crank et al., 1991). Similarly, *dSlo* mRNA can not be detected in the digestive systems via *in situ* hybridization. Although no electrophysiological studies was performed on, BK currents were recorded in mammalian intestines, and it is possible that *dSlo* is also expressed in the fly digestive systems. The two novel genes identified may well not be an artifacts of the yeast-two-hybrid screening and deserve further investigation.

In conclusion, BK channels are distributed in many cell types where they link membrane potential with intracellular calcium signals. BK channels are modified by phosphotases, kinases, G proteins and a beta subunit. In search of *dSlo*-interacting proteins, we identified a novel gene, dSLIP1, from a *Drosophila* embryo cDNA library by applying the yeast-two-hybrid system. dSLIP1 showed a specific interaction with C-terminal of *dSlo*. Electrophysiology and immunocytochemistry studies indicated that coexpression of dSLIP1

with *dSlo* specifically reduced macroscopic currents of *dSlo* compared with expression of *dSlo* alone by limiting number of *dSlo* channels in the plasma membrane. In neuron, the distribution, localization and density of BK channels determine the functional performance of BK channels, and thus the excitability of the cells. By interacting with *dSlo*, dSLIP1 may participate the transport and distribution of BK channels.

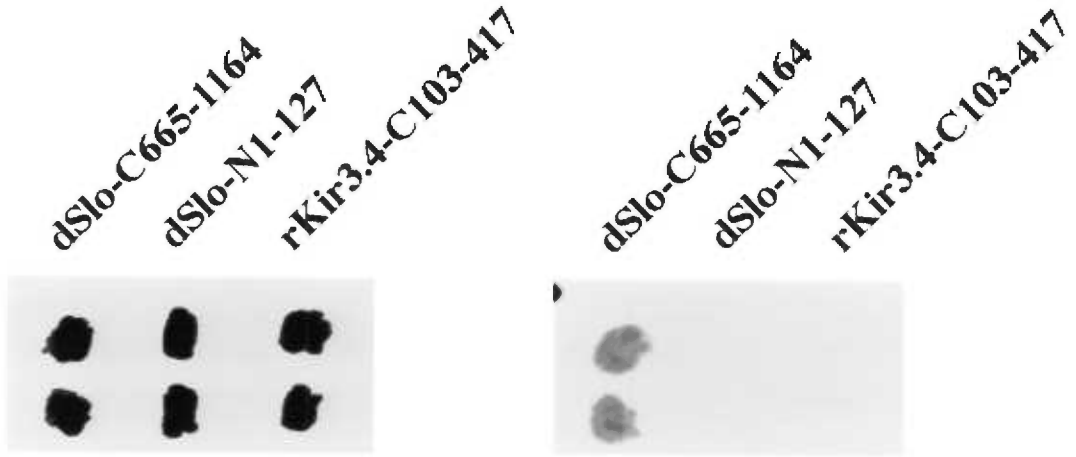

Figure 3.1. Interaction trap selection for dSLIP1

Figure 3.1 Interaction trap selection for dSLIP1.

dSLIP1 was introduced into EGY195 containing the indicated bait plasmids. The plate on the left shows that in the presence of glucose and leucine all combinations permit growth, while the plate on the right shows that only the combination of dSLIP1 with dSlo-C665-1164 survives in the presence of galactose and the absence of leucine. dSlo-C665-1164, the C-terminal 499 amino acids of *dSlo*; dSlo-N1-127, the N-terminal 127 amino acids of dSlo; $rK_{ir}3.4$-C103-417, the C-terminal 314 amino acids of $K_{ir}3.4$ (Krapivinsky et al., 1995).

```
   1  MSIADVEYEY VVLKINGYDI SHLSRYEAVQ KFLQSKETLV VEIRRQKHNA
                *
  51  LDLELKHGSN AKISKVDNPG ELSVLTDKSA EGTITAASAS QQINCPSSTS
                          *       *
 101  LKEIETKTPV VLTLRARSHE DRLGSLQAAS KETQTQSVVG TDVLKDNDLV
 151  NTITDNFIEH EHHLFEQCLE PEIDIEEVTL VKGVEQSSSN QIGLIVTSSG
 201  IQQSSTDTNK GDILGNAAAP GEEVDNSSSA YNTGDSNNSA SPHQNTTNPD
                *
 251  EAIATGRKLD STVIDSPNDH LDATGVSTML LLPFGKSGRI GLCSSNLPTA
 301  YVSERYTNVG SENEIHPLKS DIEILRVKPT DDSYSHCPQF NAPNLSSYHF
                           *
 351  VSSQEVANRC HISTSLQKNA TLLNGESAEE IPMVWKVKRR PDGTRT
```

Figure 3.2 Full length coding sequence of dSLIP1.

The domain recovered in the original interaction trap screen started from amino acid 101 and extended through the coding region; potential substrate sites for serine/threonine protein kinases are denoted by asterisks.

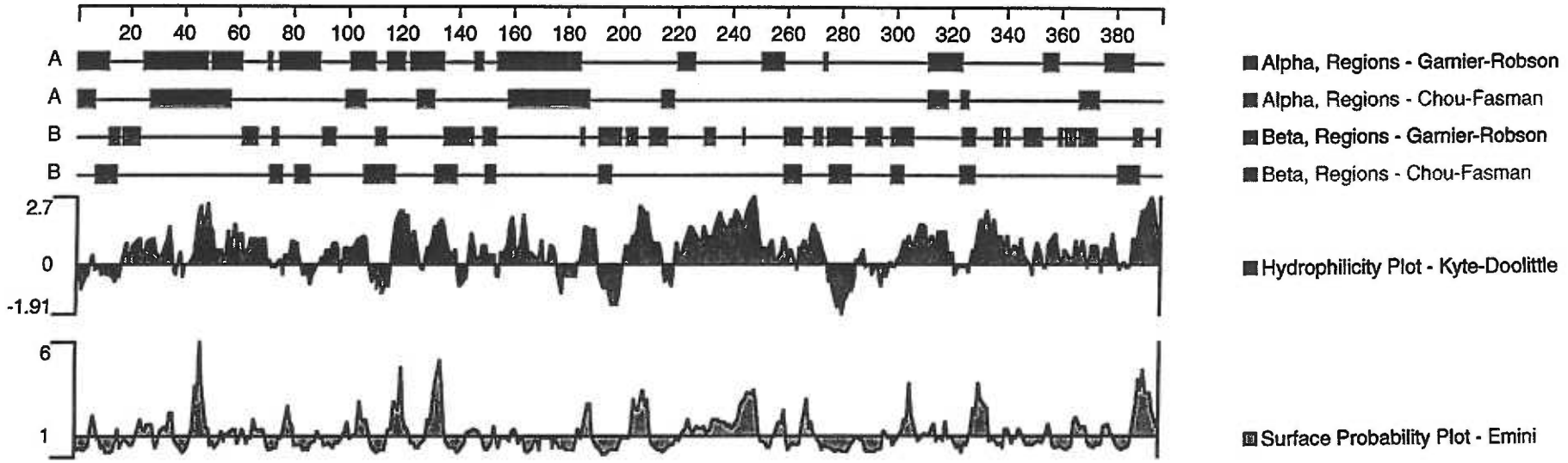

Figure 3.3 Hydrophilicity plot and alpha, beta regions analysis of dSLIP1

Hydropathy profile computed according to Kyte and Doolittle (Kyte and Doolittle, 1982). Window size is 9 and profile is plotted at one-amino acid residue intervals.

```
Identities = 12/36 (33%), Positives = 23/36 (63%)

dSLIP1:          12  VLKINGYDISHLSRYEAVQKFLQSKETLVVEIRRQK  47
                     +L +NG D+S  +  EAVQ   ++ + +V+E++  K
Syntrophin:     129  ILSVNGEDLSSATHDEAVQALKKTGKEVVLEVKYMK  164


Identities = 13/45 (28%), Positives = 24/45 (53%)

dSLIP1:          50  ALDLELKHGSNAKISKVDNPGELSVLTDKSAEGTITAASASQQIN  94
                     AL    +HG +  +  V++P EL+  T +  +G   AA   Q+++
Syntrophin:     365  ALRTGTRHGVDTHLFSVESPQELAAWTRQLVDGCHRAAEGIQEVS  409
```


Figure 3.4 Homology between dSLIP1 and the PDZ domain of α1 syntrophin.

The domain of dSLIP1 with homology to the PDZ domain of α1 syntrophin is compared at amino acid level.

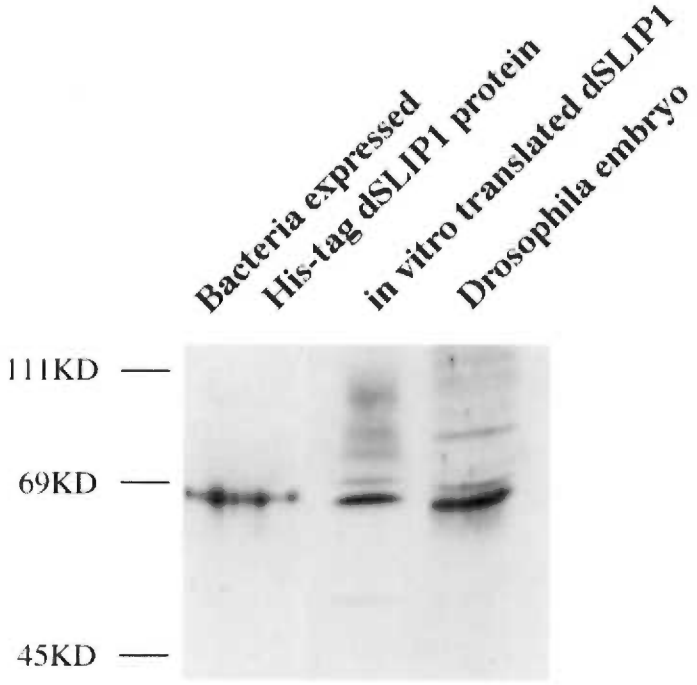

Figure 3.5 The dSLIP1 cDNA encodes the full length protein

Figure 3.5 The dSLIP1 cDNA encodes the full length protein.

Bacterially expressed dSLIP1 (His-tag fusion protein; lane 1), in vitro translated dSLIP1 (lane 2), and *Drosophila* embryo proteins (lane 3) were prepared as a Western blot and probed with a polyclonal antiserum directed against recombinant dSLIP1. The dSLIP1 antiserum detected bands of similar molecular weights.

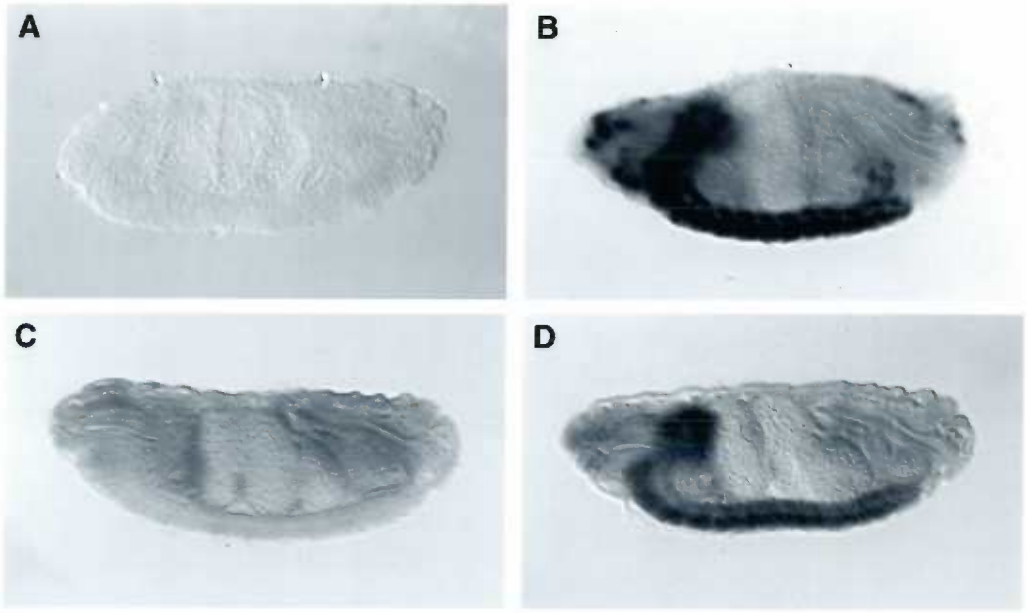

Figure 3.6. In situ hybridization of dSLIP1 and dSlo

Figure 3.6 In situ hybridization of dSLIP1 and *dSlo*.

dSlo and dSLIP1 mRNAs are expressed in a coincident pattern throughout the *Drosophila* CNS. Lateral views of whole mount *in situ* hybridization in late stage embryos of sense (A, C) and antisense (B, D) digoxigenin-labeled riboprobes generated from *dSlo* (A, B) and dSLIP1 (C, D) cDNAs. Strong hybridization of both antisense probes is present in the brain and ventral ganglion. In contrast to *dSlo*, hybridization of dSLIP1 is not present in anterior sensory cells. anterior, left; posterior, right.

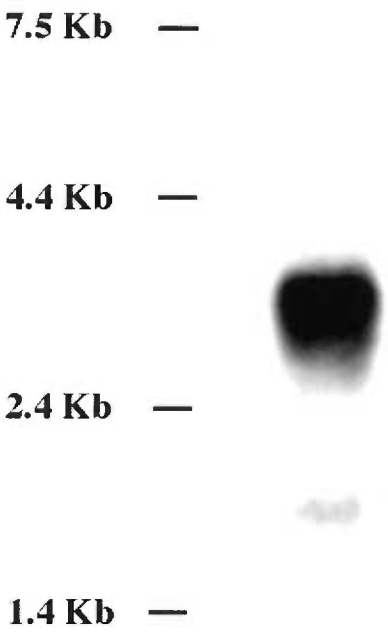

Figure 3.7 Northern blot analysis of dSLIP1 mRNA

Figure 3.7 Northern blot analysis of dSLIP1 mRNA.

3 μg of poly(A)$^+$ mRNA extracted from *Drosophila* embryos was prepared as a Northern blot and probed with a full length radiolabeled dSLIP1 riboprobe, detecting a single band of ~3.6 kb.

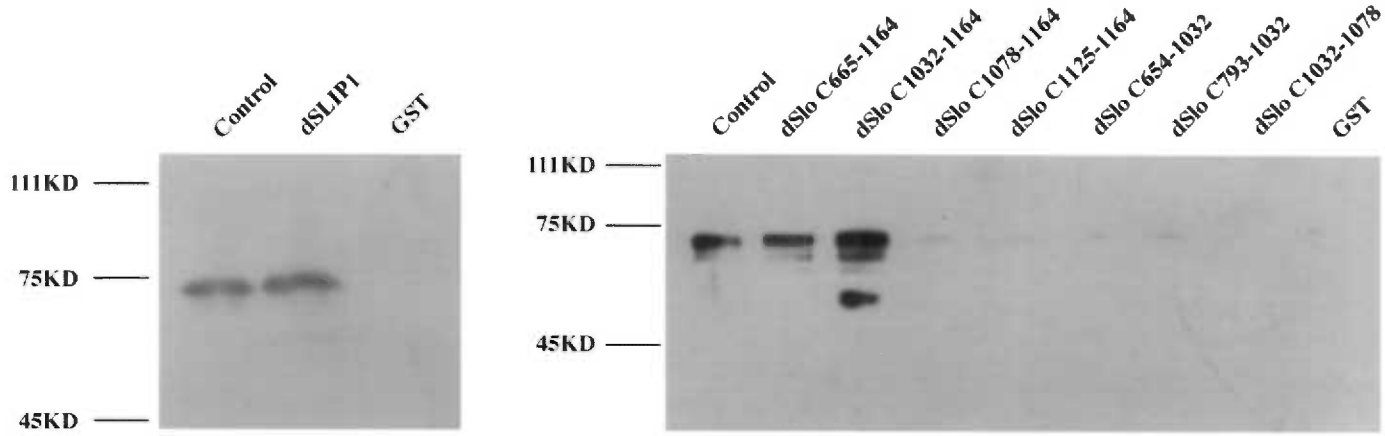

Figure 3.8 GST pull-down experiments show the interaction between dSlo and dSLIP1

Figure 3.8 GST pull-down experiments show the interaction between *dSlo* and dSLIP1.

GST-fusion proteins, indicated above, were bound to glutathione agarose beads and pHis-dSlo (top, left) or pHis-dSLIP1 (top, right) protein was applied. Following several washes, retained proteins were eluted with reduced glutathione and prepared as a Western blot.

Left: Western blot probed with dSlo antibodies. Lane 1 (control), bacterially expressed pHis-dSlo-C665-1164 protein, as marker. pHis-dSlo-C665-1164 protein was retained by GST-dSLIP1 (lane 2) but not by GST alone (lane 3). Right: Western blot probed with dSLIP1 antibodies. Lane 1, bacterially expressed pHis-dSLIP1 alone as marker; lanes 2-9, pHis-dSLIP1 was retained by GST- dSlo-C654-1164 or GST-dSlo-C-1032-1164, but not by other C-terminal fragments of dSlo, or by GST alone.

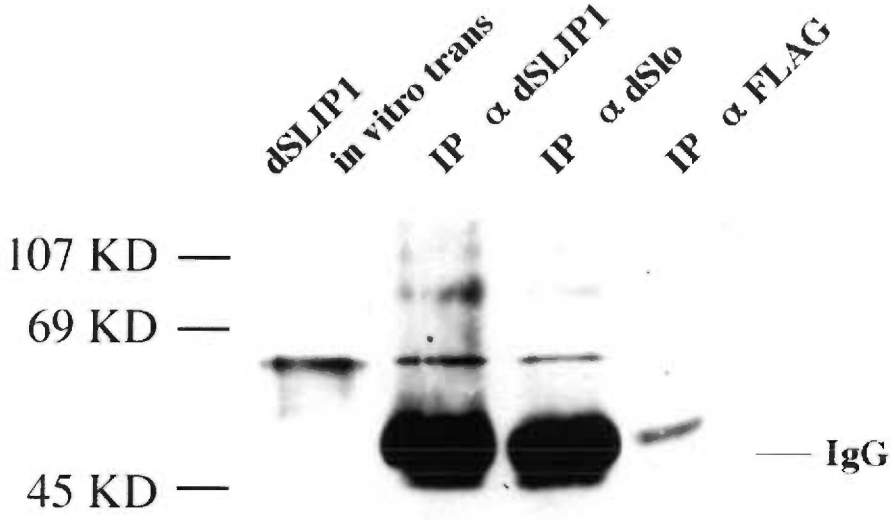

Figure 3.9 Antibodies to dSlo coimmunoprecipitate dSLIP1

Figure 3.9 Antibodies to *dSlo* coimmunoprecipitate dSLIP1 from cotransfected COS7 cells.

Western blot probed with dSLIP1 antibodies. Lane 1, *in vitro* translated dSLIP1; lane 2, proteins immunoprecipitated with dSLIP1 antibodies; lane 3, proteins immunoprecipitated with dSlo antibodies; lane 4, proteins immunoprecipitated with FLAG antibodies.

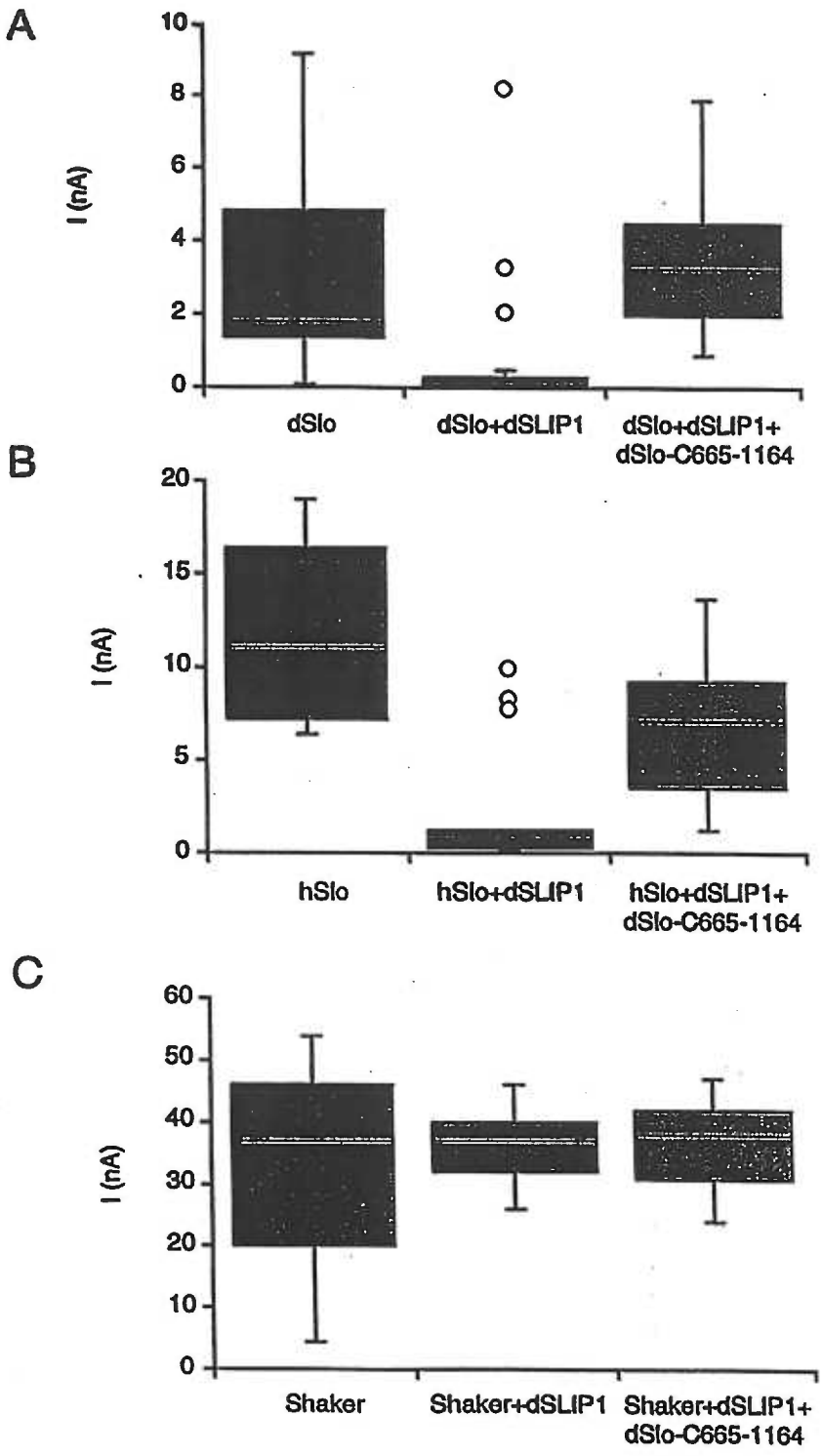

Figure 3.10 Coexpression with dSLIP1 reduces BK current amplitudes

Figure 3.10 Coexpression with dSLIP1 reduces BK current amplitudes.

Current amplitudes at 100 mV were determined in inside-out patches from oocytes excised into 10 mM Ca^{2+} (A and B) or using the 2-electrode voltage clamp (C). Oocytes expressed *dSlo* (A), *hSlo* (B), or *Shaker* (C) channels either alone (left columns) or in combination with dSLIP1 (middle columns) or dSLIP1 and dSlo-C665-1164 (right columns). Patch currents are shown as box plots where the median is represented by a line separating the upper and lower quartiles (UQ, LQ), the box (interquartile distance, IQD) contains ±25% of the data points and the error bars mark the minimum and maximum values that fall within UQ+1.5 x IQD and LQ+1.5 x IQD. The outliers in panels A and B were recorded from a single oocyte in each case. The number of patches in each plot were 21 for *dSlo* and *dSlo* + dSLIP1, 18 for *dSlo* + dSLIP1 + *dSlo*-C665-1164, and 15 for *hSlo*, *hSlo* + dSLIP1, and *hSlo* + dSLIP1 + dSlo-C665-1164. 11 oocytes expressing *Shaker* or *Shaker* + dSLIP1 and 6 oocytes expressing *Shaker* + dSLIP1 + *dSlo*-C665-1164 were examined.

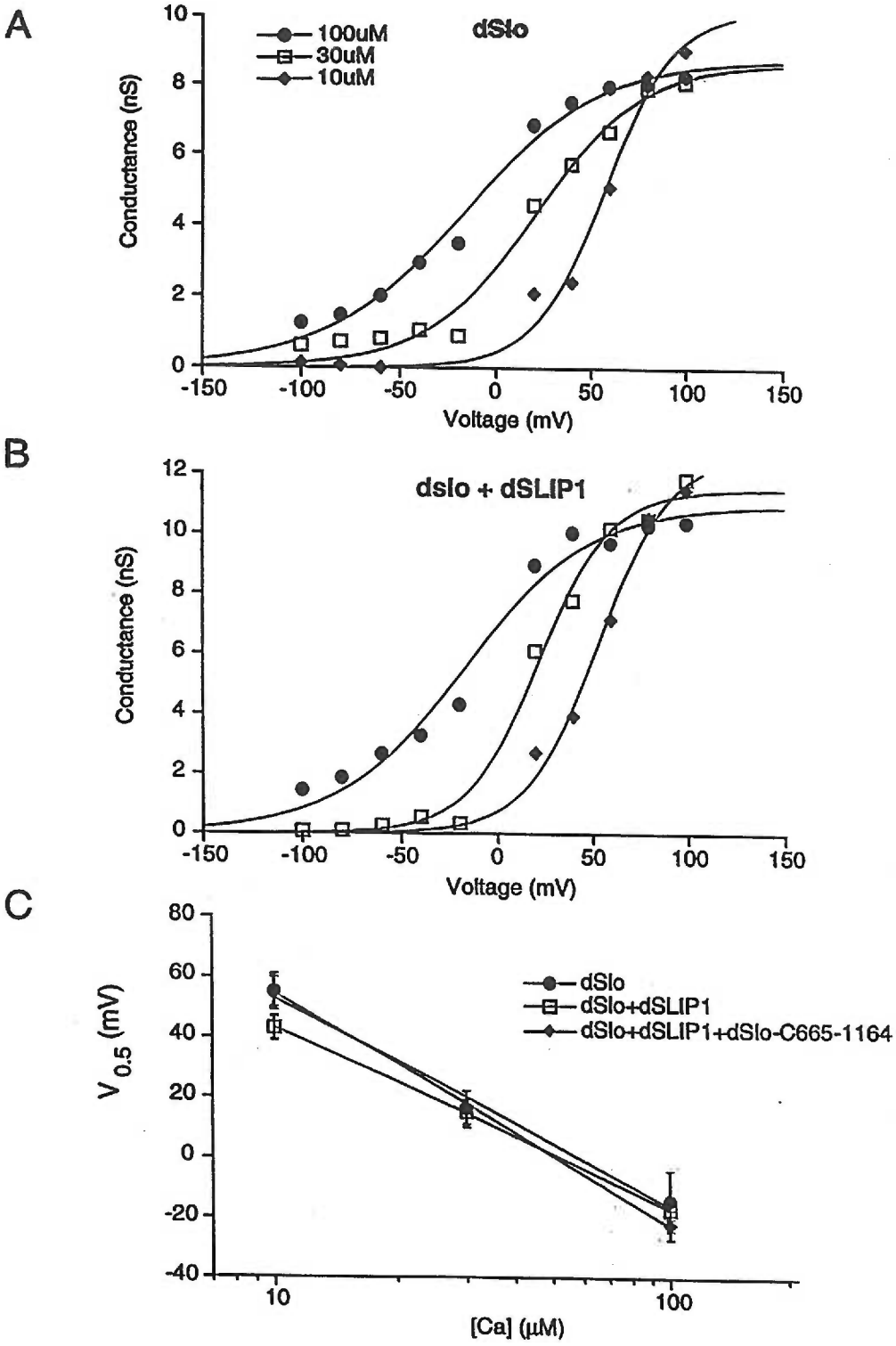

Figure 3.11 dSLIP1 does not change the calcium-dependence of *dSlo*

Figure 3.11 dSLIP1 does not change the calcium-dependence of *dSlo*.

Excised patches from oocytes expressing either *dSlo* (A), *dSlo* and dSLIP1 (B), or *dSlo*, dSLIP1 and dSlo-C665-1164 were exposed to 10, 30 and 100 mM free Ca^{2+}. Patch conductance was determined by dividing the steady-state current during a 500 ms voltage step by the holding potential. Data were fit to a Boltzman equation of the form $I = I_{max}/(1+\exp k^*(V-V_{0.5}))$. C) The resulting potentials of half-activation ($V_{0.5}$) were averaged for 3-5 patches and plotted as a function of Ca^{2+} concentration; error bars represent standard errors.

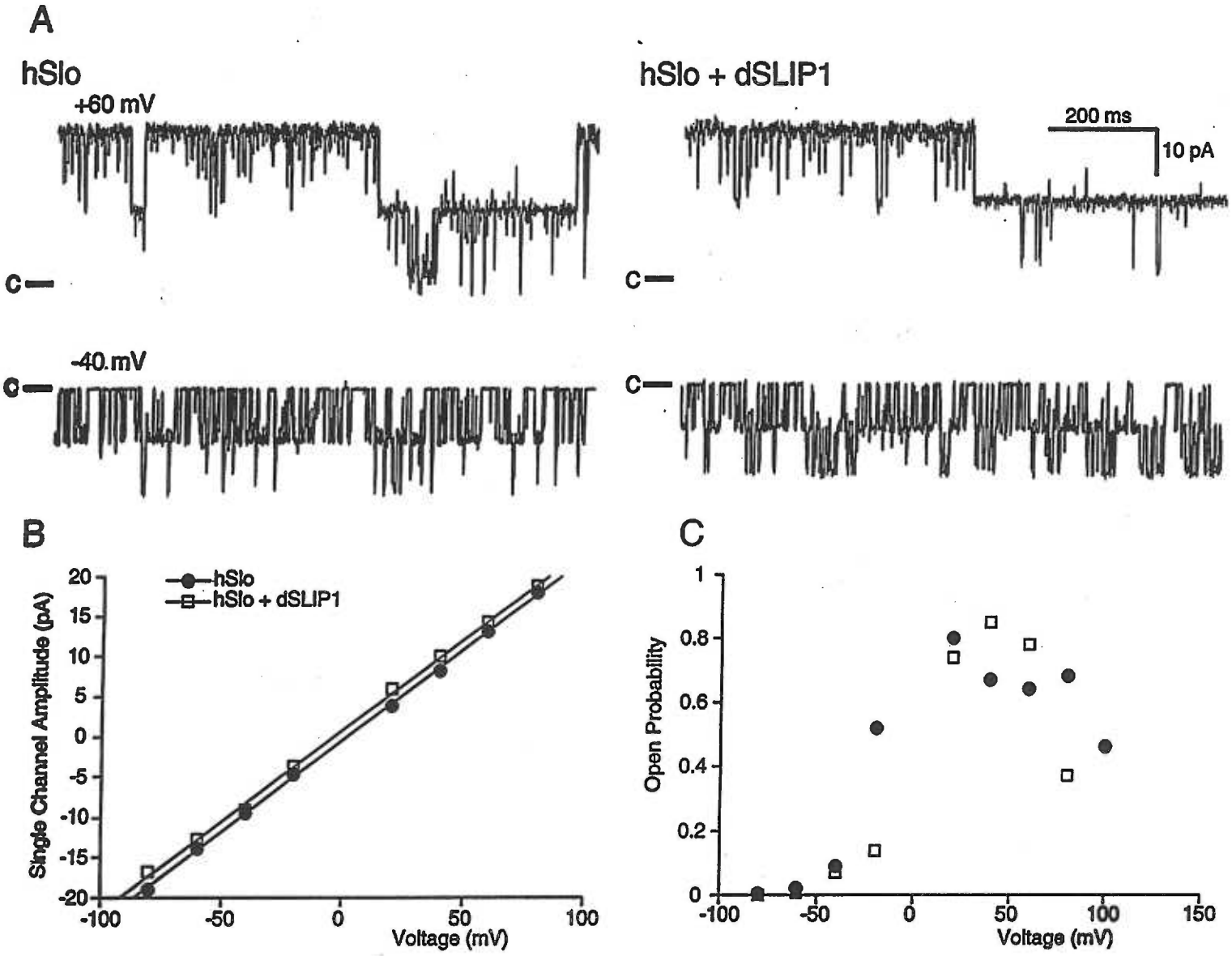

Figure 3.12 Coexpression with dSLIP1 does not affect the single channel conductance or open probability of *hSlo* channels

Figure 3.12 Coexpression with dSLIP1 does not affect the single channel conductance or the open probability of *hSlo* channels.

A. 1 second traces of steady-state recordings from patches excised from an oocyte expressing hSlo (left) or hSlo and dSLIP1 (right). Patches were exposed to 100 uM free Ca^{2+} and held at +60 mV (top) or -40 mV (bottom). Both patches contained 2 channels and the closed levels are indicated on the left.

B. Single channel amplitudes as a function of voltage in two patches containing 2 channels (filled circles, same patch as A) or a single channel (open squares) from an oocytes expressing hSlo or hSlo and dSLIP1, respectively. Amplitudes were determined by fitting Gaussian distributions to amplitude histograms representing at least 1000 events. Linear regression analysis yielded single channel conductances of 227 pS for hSlo and 226 pS for hSlo and dSLIP1.

C. Single channel open probability as a function of voltage for the two patches in B.

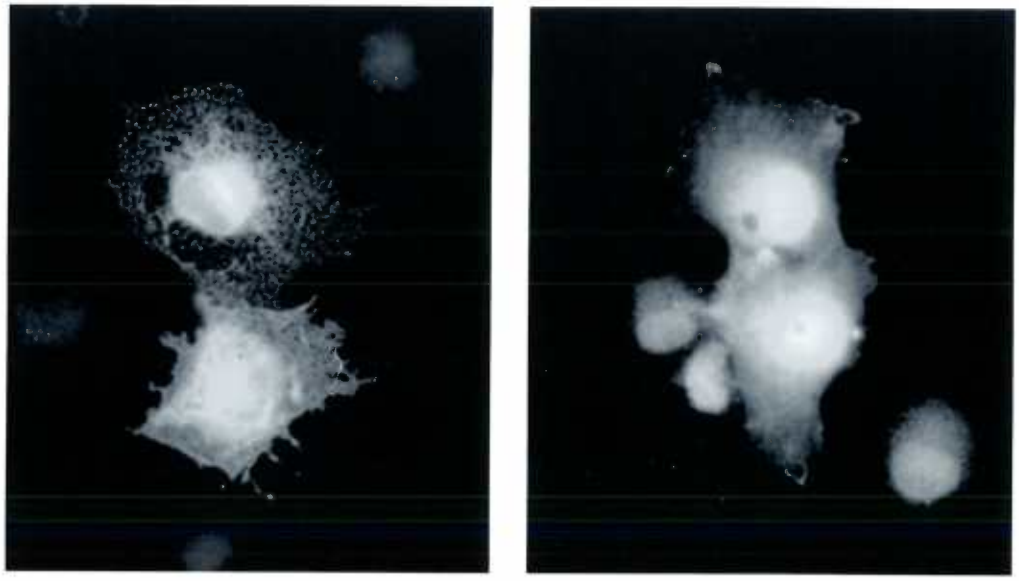

Figure 3.13 Immunostaining of transfected COS7 cells

Figure 3.13 Immunostaining of COS7 cells.

COS7 cells transiently transfected with dSlo (left) or cotransfected with dSlo and dSLIP1 (right) were immunostained with dSlo antiserum. Cells expressing dSlo only show a reticular, punctate staining pattern with clear cell surface staining, while cells transfected with dSlo and dSLIP1 show a diffuse, intracellular staining pattern.

Table 3.1. Nuclear localization of baits

Yeast with plasmid(s)	Relative beta-gal activity
EGY48/pJK101	100
EGY48/pJK101/pRFM	33
EGY48/pJK101/pEG202-dSlo-N1-120	13.7
EGY48/pJK101/pEG202-dSlo-C665-1164	7.8
EGY48/pJK101/pEG202-dSlo-C600-1164	5.4
EGY48/pJK101/pEG202-dSlo-C500-1164	130

Table 3.1 Shown are baits and testing results of nuclear localization and Lex A binding in a repression assay.

Table 3.2. Interaction verification of dSLIP1 and *dSlo*

	dSLIP1 Growth and Color		Y3-2 Growth and Color	
	Glu media	Gal media	Glu media	Gal media
EGY48 /pSH18-34/pEG202-dSlo-C665-1164	No	Yes/ Blue	No	Yes /Blue
EGY195/pSH18-34/pEG202-dSlo-C665-1164	No	Yes/ Blue	No	Yes /Blue
EGY191/pSH18-34/pEG202-dSlo-C665-1164	No	Yes/ Blue	No	Yes /Blue
EGY195/pSH18-34/pEG202-dSlo-N1-120	No	No	No	No
EGY195/pSH18-34/pEG202-rKir3.4-C103-417	No	No	No	No
EGY48 /pSH18-34 (add His)	No	No	No	Yes/Blue
EGY195/pSH18-34 (add His)	No	No	No	Yes/Blue

Table 3.2 Shown are interaction testing results of yeast-two-hybrid assay (growth in the absence of leucine and beta-galactosidase activity) for two cDNA clones, dSLIP1 and Y3-2, under different genetic background indicated. The results indicate that only the combination of dSLIP1 with the presence of specific bait(dSlo-C665-1164) permits growth in the media lacking leucine. Y3-2 permits growth in the absence of bait plasmids and disqualified the test.

Table 3.3 Regions of interaction between *dSlo* and dSLIP1

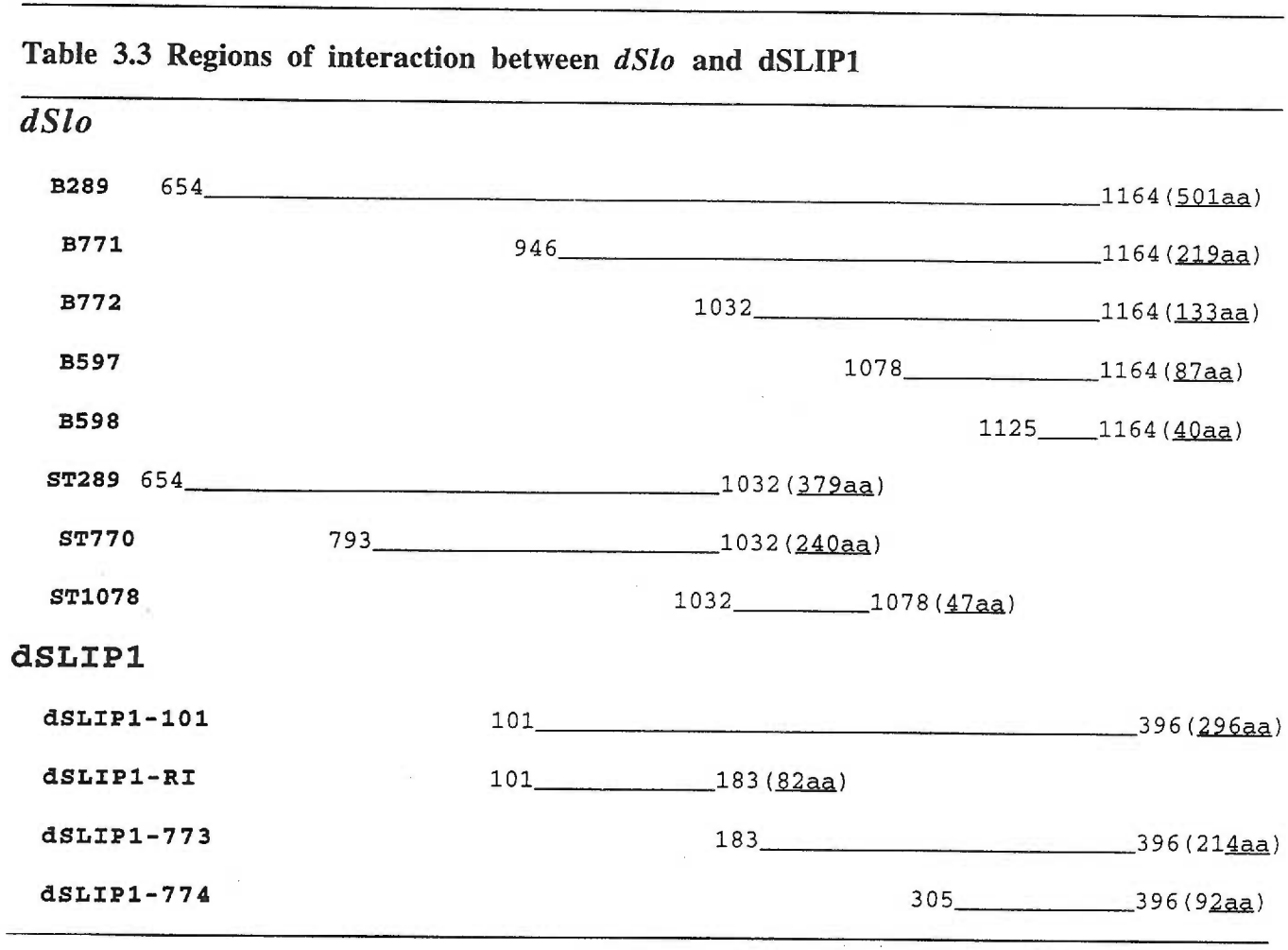

Table 3.3 Panel shows a diagrammatic representation of the regions from *dSlo* and dSLIP1 which were tested for complementation in the interaction trap.

Table 3.4 Assay results(on Gal+ Leu- Trp- Ura- His-)

	dSLIP1-101		dSLIP1-RI		dSLIP1-773		dSLIP1-774	
	Growth	beta-gal	Growth	beta-gal	Growth	beta-gal	Growth	beta-gal
B289	+++	Blue	-		+++	Blue	+++	Blue
B771	+++	Blue	-		+++	Blue	+++	Blue
B772	+++	Blue	-		+++	Blue	+++	Blue
B597	+	Blue	+/--		+	Blue	+	Blue
B598	+	Blue	+/--		+	Blue	+	Blue
ST289	+/-		+/--		+/-		+/-	
ST770	+/-		+/--		+/-		+/-	
ST1078	+/-		+/-		+/-		+/-	

Table 3.4 Yeast two-hybrid assay results of growth and b-galactosidase activity for indicated bait-prey combinations.

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