FUNCTIONAL ANALYSIS AND PHYSIOLOGICAL CORRELATES OF THE MIN K PROTEIN

by

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George R.Wallis

(1913-1993)

Those friends thou hast and their adoption tried, grapple them unto thy soul...

William Shakespeare

ABSTRACT

The min K protein is structurally and functionally distinct from other potassium channel proteins. Expression in *Xenopus* oocytes or in HEK 293 cells results in voltage-dependent potassium currents which activate with a characteristic slow time course. The aim of work presented in this dissertation is to address three fundamental questions. First, does the min K protein underlie the slow component of the delayed rectifier current in guinea pig myocytes and thus have a physiological role in cardiac excitability and action potential repolarization? Isolation, expression, and functional characterization of a guinea pig min K clone permit testing of this hypothesis. Second, what is the molecular basis for the regulation of min K currents by stimulation of protein kinase C? Site-directed mutagenesis indicates that a serine residue in a cytoplasmic domain is necessary for inhibition of currents. Third, how does this structurally succinct protein form an ion channel? Biochemical tools are used to examine the gating mechanism for this unique channel.

A clone encoding the guinea pig (gp) min K potassium channel was isolated and expressed in *Xenopus* oocytes. The resulting currents, gp I_{sK}, exhibit many of the electrophysiological and pharmacological properties characteristic of guinea pig I_{Ks}, the slow component of the delayed rectifier potassium conductance in guinea pig cardiac myocytes. Depolarizing commands evoke outward potassium currents which activate slowly, with time constants on the order of seconds. The currents are blocked by the class III antiarrhythmic compound clofilium, but not by the sotalol derivative E4031 or

low concentrations of lanthanum. Like I_{Ks} in guinea pig myocytes, gp I_{sK} is modulated by stimulation of protein kinase A (PKA) and protein kinase C (PKC). In contrast to rat and mouse I_{sK}, which are decreased upon stimulation of PKC, myocyte I_K and gp min K currents in oocytes are increased after PKC stimulation. Substitution of an asparagine residue at position 102 by serine (N102S), the residue found in the analogous position of the mouse and rat min K proteins, results in decreased gp I_{sK} in response to PKC stimulation. These results support the hypothesis that the min K protein underlies the slow component of the delayed rectifier potassium current in ventricular myocytes, and account for the species-specific responses to stimulation of PKC.

To probe the mechanism by which min K induces channels, a membrane-impermeable chemical cross-linking agent was applied to oocytes expressing min K. This decreased the time-dependent current, increased its rate of activation, and induced persistently activated inward and outward potassium currents. These effects required membrane depolarization and demonstrated use dependence, suggesting that cross-linking modifies an open state of min K. Persistently activated channels retained potassium selectivity and sensitivity to block by clofilium and barium. Cross-linking effects were not mimicked by application of a related, monofunctional agent, nor were they seen in oocytes expressing the *Shaker*-like potassium channel, RBK1. The results suggest that a major conformational change occurs during min K channel gating which can be stabilized by chemical cross-linking, and they support a model in which min K channels activate by voltage-dependent subunit aggregation.

CHAPTER I

INTRODUCTION

Potassium channel diversity

Potassium channels are a ubiquitous class of membrane proteins integral to the signaling properties of virtually all eukaryotic cells. Their diversity underlies the heterogeneity and flexibility of cellular excitability and transport. The manifold physiological duties performed by K^+ channels depend on their selective permeability to potassium, the precise manner in which individual members of this group of channels open and close, and how they are modulated by various second messengers. This profound functional diversity has long been apparent from electrophysiological and pharmacological studies of native channels in many different cell types (for review, see Rudy, 1988). Among the broad family of voltage-sensitive K channels that are gated by changes in voltage across the membrane are delayed rectifiers, transient A channels, some calcium-dependent-type channels, and inwardly rectifying channels. These channels variously serve to shape action potentials and limit their duration, time the intervals between action potentials, repolarize or hyperpolarize the cell, counterbalance excitatory inputs to the cell, and/or contribute strongly to setting the resting potential (Hille, 1992).

In the past six years, the cloning of genes encoding the structural elements for a multitude of different K^+ channels has opened a portal into further biophysical observation and understanding of K^+ channel structure and function, reinforcing our appreciation for their huge diversity. Three structural patterns have emerged: (1) the Shaker-like family of

large polytopic proteins of approximately 500-1500 amino acids, with six predicted transmembrane domains and a conserved pore region; (2) the inward rectifiers, with two predicted transmembrane domains and a related pore region; and (3) the min K protein, consisting of approximately 130 amino acids with a single predicted transmembrane domain. This latter example is the subject of this dissertation.

Additional potassium channel variety is offered by multiple related genes (Jan and Jan, 1990), alternative splicing of mRNA for some of those genes (Papazian et al., 1987; Timpe et al., 1988; Adelman et al., 1992), heteropolymerization of channel forming subunits (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990), the addition of ancillary subunits (Scott et al., 1994), post-translational modifications such as phosphorylation (Levitan, 1985; Moran et al., 1991; Lotan et al, 1990), and alteration of biophysical properties by level of channel expression (Guillemare et al., 1992; Busch et al., 1993a).

Delayed rectifier potassium channels

One important type of K^+ channel is functionally classified as the delayed rectifier. This general type of current was originally described by Hodgkin and Huxley in their elucidation of the action potential mechanism of the *Loligo* giant axon. They noted the delayed increase in potassium conductance with depolarization, its maintenance at a high level during depolarization (in contrast to the decrease in sodium conductance), and the dependence of its reversal potential on external potassium concentration (Hodgkin and Katz, 1949; Hodgkin and Huxley, 1952a, 1952b, 1952c). Potassium channels of this sort have been termed "delayed rectifiers" because of their kinetics of activation and the outward rectification of their current-voltage relationship. These essential properties permit delayed rectifiers to participate in cell repolarization, thus limiting action potential

duration. It is important to note that delayed rectifiers are not really a discrete set of channels with a narrowly defined set of characteristics. They have been described in vast numbers of different cell types in various species and they encompass a myriad of divergent properties, including a wide range in kinetics of activation (Rudy, 1988).

Cloning of a unique protein with potassium channel activity

Historically, expression cloning has permitted the isolation of genes encoding novel channel proteins that lack structural or sequence similarity with known channels and thus evade screening on the basis of nucleotide homology. Boyle and her coworkers (Boyle et al., 1987a) first expressed a novel, slowly activating potassium current from rat uterine poly $(A)^+$ mRNA injected into *Xenopus* oocytes and assayed using the two electrode voltage clamp. They found that expression levels for this current were hormonally regulated (Boyle et al., 1987b) and hypothesized that estrogen induced an mRNA species encoding a K^+ channel. Shortly thereafter, Takumi et al. (1988) cloned a membrane protein from a rat kidney expression library that induced a similar slow voltage-dependent potassium current when heterologously expressed in *Xenopus* oocytes. In its general characteristics, this current behaved like a slower relative of the classical delayed rectifier. But the deduced amino acid sequence was only 130 amino acids long, with a single predicted alpha-helical transmembrane domain (Takumi et al., 1988). The amino acid sequence and putative structure demonstrated a complete lack of similarity to the contemporaneously cloned *Shaker* K^+ channels and indeed all K^+ channels that have been subsequently identified (Hoshi and Zagotta, 1993; Kaczmarek, 1991). This protein has been interchangeably termed the "IsK protein" (for slow K^+ current) or "min K" (for mini or minimal K^+ channel). Similarly, the currents induced by expression of this protein have been alternatively labeled "min K currents", "$I_{min\ K}$", or "I_{sK}". Min K has now been cloned from uterine tissue of estrogen-treated rats (Pragnell et al., 1990),

human genomic DNA (Murai et al., 1989), neonatal rat and mouse heart (Folander et al., 1990; Honore et al., 1991), and human T lymphocytes (Attali et al., 1992).

It has been suggested that min K may be related to primitive ion channels (Folander et al., 1990). Limited sequence homology exists between the carboxy-terminal portion of the putative transmembrane domain of min K and the channel-forming domain of colicin E1, a slowly activating, voltage-dependent prokaryotic ion channel (Folander et al, 1990; Liu et al., 1986). In addition, the channel forming peptide mellitin (Sansom, 1991) also exhibits some homology with this region of min K.

Min K may be part of a structurally cognate but functionally diverse superfamily of slowly activating ion channels formed by small proteins with single transmembrane domains, including the influenza virus M2 protein (Pinto et al., 1992) and phospholemman (Palmer et al., 1991; Moorman et al., 1992). M2 is a 97 amino acid integral membrane protein that induces slowly activating, monovalent-ion selective channel activity in *Xenopus* oocytes. Native M2 is minimally a homotetramer, but chemical cross-linking additionally revealed some higher molecular weight complexes that may represent 10-12 M2 subunits (Holsinger and Lamb, 1991). Mutations within the single transmembrane domain, which were associated with viral resistance to the anti-viral drug amantadine, induced currents that were insensitive to block by amantadine and exhibited altered channel activity and ion selectivity (Pinto et al., 1992). Phospholemman is a 72 amino acid cardiac membrane protein that induces a slowly activating, chloride-selective current when expressed in oocytes. Mutations within its single transmembrane region altered the rate of activation of the expressed currrents (Moorman et al., 1992).

Genomic organization of min K

Northern blot analysis of rat and mouse mRNA revealed the existence of multiple hybridizing species of min K transcripts (Iwai et al., 1990; Lesage et al., 1993). Further analysis of genomic and cDNA clones indicated that this heterogeneity arises from several processing mechanisms. There is a single, uninterrupted protein coding region, but mRNA is initiated at one of two alternatively spliced 5' untranslated exons, and polyadenylation occurs at multiple potential sites (Iwai et al., 1990; Lesage et al., 1992). These genetic details may relate to developmental and tissue-specific regulation of min K expression and reported variations in mRNA levels in response to hormonal changes.

Tissue distribution of min K

Unlike some K^+ channels, expression of min K is not ubiquitous but is instead limited to specific tissues. Northern blot analysis indicated that the rat min K message is present in kidney, stomach, duodenum, pancreas and uterus (Takumi et al, 1988; Pragnell et al., 1990). Min K gene expression in rat uterus is stimulated by estrogen and at the end of pregnancy (Boyle et al., 1987b; Pragnell et al., 1990). Min K mRNA is also expressed in neonatal rat and mouse heart (Folander et al., 1990; Honore et al., 1991). Furthermore, polyclonal antibodies directed against specific regions of the min K protein have been used to immunohistochemically localize this molecule in the periluminal membrane of endometrial cells, the apical membrane of renal proximal tubule cells, and striated and small excretory duct cells of the submandibular gland (Sugimoto et al., 1990). Recently, surface immunoreactivity for min K has been detected in adult guinea pig ventricular myocytes and sinoatrial nodal cells (Freeman and Kass, 1993).

Protein topology of min K

Min K is thought to represent a class III bitopic integral membrane protein: a single

predicted α-helical transmembrane domain, with its amino-terminus extracellular and its carboxy-terminus intracellular. Our previous work (Busch et al., 1992a) and work presented in this dissertation (Chapter III), which identify a regulatory domain in the C-terminus that is essential for modulation by the intracellular second messenger protein kinase C, supports this view. The identification of glycosylation sites in the amino terminal portion of min K suggests that its orientation is extracellular (Takumi et al., 1991). This contrasts with the topography proposed by Honore et al. (1991), which placed both the C-terminus and the N-terminus (with its presumptive Ca^{++}-calmodulin-dependent kinase II phosphorylation site) intracellular. There is no evidence to support this latter view.

Properties of min K currents

General

The currents induced by expression of min K in oocytes activate over a time course of tens of seconds, do not saturate and do not inactivate during voltage steps. They display a sigmoidal delay in activation that is sensitive to prepulse potential and to trains of pulses, suggesting a shift in equilibrium between multiple closed states toward the open state (Boyle et al., 1987b; Hausdorff et al., 1991). Miller and his coworkers have provided convincing evidence that min K currents are mediated by a true K^+ channel typical in its voltage dependence, its monovalent cation selectivity sequence, and its open channel blockade by cesium (Hausdorff et al., 1991).

Regulation

The regulation of min K currents has been the subject of much investigation. Min K currents expressed in Xenopus oocytes are sensitive to stimulation of protein kinases A and C (Blumenthal and Kaczmarek, 1992; Busch et al., 1992a, 1992b; Honore et al.,

1991). We have previously demonstrated that a serine residue (S103) within a region of min K that resembles a consensus site for protein kinase C phosphorylation (RXXSXR) is necessary for PKC-mediated down regulation of min K currents (Busch et al, 1992b). In addition, an increase in intracellular calcium increases min K currents (Honore et al., 1991; Busch et al., 1992a). Finally, min K currents are increased (in a calcium dependent fashion) by hypotonic solution and decreased by cytochalasin D, presumably through changes in the cytoskeletal network (Busch et al., 1992c). There remains some discrepancy in the literature as to the effect of protein kinase A stimulation on min K currents. Work presented in this thesis (Chapter III) partially addresses these reported differences.

Postulated physiological importance of min K

The unique properties of min K suggest that it may participate in several important physiological processes. In general, slow delayed rectifier K^+ currents can play vital roles in cell types that have comparatively long action potentials or demonstrate slow, prolonged burst firing patterns (Hille, 1991). Primarily on the basis of its tissue distribution, min K is postulated to function in smooth muscle cell excitability in the stomach, duodenum, and uterus . In the latter example, min K expression and activity may be regulated in concert with myometrial excitability during the estrous cycle and pregnancy (Boyle et al., 1987b; Pragnell et al., 1990; Toro et al., 1990). Min K channels may also serve in K^+ secretion and homeostasis in epithelial cell types, specifically in the apical membrane of renal proximal tubule cells, and striated and small excretory duct cells of the submandibular gland (Sugimoto et al., 1990). In addition, min K transcripts are expressed in T lymphocytes (Attali, et al., 1992), where they may be involved in K^+ conductance changes associated with volume regulation and mitogenic activation of T cells. Indeed, clofilium, but not charybdotoxin, blocked IL_2 mRNA

induction in activated Jurkat cells at concentrations parallel to those for block of min K currents in oocytes (Attali et al., 1992). Finally, min K is postulated to play a role in action potential repolarization in cardiac myocytes.

Cardiac delayed rectifier (I_K)

The delayed rectifier K^+ current, I_K, is vitally important for initiation of repolarization of cardiac action potentials (Carmeleit, 1977). Deactivation of I_K may also contribute to the complex balance of currents that determine cardiac pacemaking (Hille, 1992). I_K and its relationship to cardiac function have been extensively studied in guinea pig myocytes (Hume and Uehara, 1985; Doerr et al., 1990; Wettwer et al., 1991). This current exhibits complex kinetics with very slow activation rates, and does not inactivate (Matsuura et al., 1987; Bennett et al., 1985). Noble and Tsien (1969) proposed that in sheep Purkinje fibers, I_K consists of more than one component. It was argued, however, that the kinetics of I_K could be accounted for by a Hodgkin-Huxley type scheme in which the conductance variable **n** was raised to the second power (Matsuura et al., 1987), or by presuming the existence of multiple closed states and a complex kinetic pathway (Balser et al., 1990). The hypothesis of Noble and Tsien was later substantiated in guinea pig myocytes by use of the class III antiarrhythmic sotalol-derivative E4031, which demonstrated that I_K consists of a fast activating, inwardly rectifying current, I_{Kr}, and a slowly activating current, I_{Ks} (Sanguinetti and Jurkiewicz, 1990a; Sanguinetti and Jurkiewicz, 1991). In guinea pig myocytes, I_{Ks} comprises the major component of I_K and, due to its slow kinetics of deactivation, represents the predominant repolarizing current during increased heart rate (Sanguinetti, 1992).

Regulation of I_K

Neurotransmitter-mediated regulation of heart rate, action potential duration, conduction

velocity, and contractile force depends on the modulation of ion channels that are functional constituents contributing to myocardial contractility (Hartzell, 1988). In this regard, regulation of I_K by α_1 -adrenergic and β-adrenergic receptor stimulation is particularly important for the control of action potential duration. This regulation has been studied in greatest detail using the guinea pig model. Guinea pig ventricular I_K is enhanced by α_1-adrenergic receptor stimulation acting through activation of protein kinase C (Tohse et al., 1987; Toshe, 1990; Dirksen and Sheu, 1990; Tohse et al., 1992), and by β-adrenergic receptor stimulation acting through protein kinase A (Walsh and Kass, 1988; Walsh et al., 1991; Busch and Maylie, 1993). Protein kinase A stimulation increases I_{Ks} but not I_{Kr}; consequently, with high β-adrenergic tone I_{Ks} becomes the predominant repolarizing current (Jurkeiwicz and Sanguinetti, 1993). Furthermore, I_K is modulated by relative levels of intracellular calcium (Tohse, 1987). PKC has species-specific effects, decreasing mouse I_K but increasing guinea pig I_K (Toshe et al, 1987; Honore et al., 1991). These second messenger systems couple cardiac ion channels to the neurotransmitters that modulate heart rate and sympathetic tone.

Pharmacology of I_K

There has been considerable recent interest in the therapeutic potential of class III antiarrhythmic agents that target cardiac and smooth muscle potassium channels. Drugs which inhibit I_K extend action potential duration (APD) and are effective class III antiarrhythmic agents (Colatsky et al., 1990). Until recently, the most potent class III compounds have been specific for I_{Kr}, with the exception of clofilium which appears to block both I_{Kr} and I_{Ks} (Colatsky, et al., 1990). However, under conditions such as tachycardia or high sympathetic tone, class III agents which only block I_{Kr} are significantly less effective (Sanguinetti, 1992; Jurkiewicz and Sanguinetti, 1993). Recently, a novel class III agent (NE10064) has been described (Tatla et al., 1993); this

compound possesses potent antiarrhythmic activity, prolongs action potential duration, and blocks I_{Ks} and I_{Kr} (Busch et al., 1993c). We have also demonstrated that this agent blocks min K currents expressed in oocytes at concentrations similar to those which inhibit I_{Ks} in myocytes (Busch et al., 1993c).

The slow kinetics and voltage dependence of min K expressed in *Xenopus* oocytes was previously noted to be striking in its resemblance to that of the slow component of cardiac I_K in guinea pig myocytes. As stated above, some of the pharmacological properties of the two currents also compare favorably (Folander et al., 1990; Honore et al., 1991; Busch et al, 1993c). In addition, Freeman and Kass (1993) recently demonstrated surface immunoreactivity for min K in adult guinea pig ventricular myocytes and sinoatrial nodal cells.

Two major problems remain in relation to this analogy between min K channels and cardiac I_{Ks}. First, the species from which min K channels have been previously cloned (rat and mouse) demonstrate expression of min K mRNA in neonatal heart but little or no expression is observed in adult heart (Folander et al., 1990; Honore et al., 1991). Furthermore, the murine cardiac model has not been adequately characterized, and the rat cardiac "delayed rectifier" K^+ current lacks a slow component (Apkon and Nerbonne, 1988) and is thus quite different from the well described guinea pig model and from human atrial myocytes (Wang et al., 1993). Second, the comparative regulation of guinea pig cardiac I_{Ks} and min K currents by second messengers is not in precise agreement. There are discrepancies in the literature as to the effect of protein kinase A stimulation, a potent modulator of I_K in myocytes (Walsh, et al., 1991), on min K currents (Honore et al., 1991; Blumenthal and Kaczmarek, 1992). Moreover, stimulation of protein kinase C decreased rat (Busch et al., 1992a) and mouse (Honore et al., 1991)

min K currents, probably by direct phosphorylation of a serine residue at position 103 (Busch et al., 1992b), and decreased neonatal mouse I_K (Honore et al., 1991), but is known to increase guinea pig I_K (Walsh and Kass, 1988; Walsh, et al., 1991). Part of the work presented in this dissertation (Chapter III) is directed at addressing these two controversies and the previously unsubstantiated hypothesis that the min K protein underlies the cardiac K^+ current I_{Ks}.

Min K structure and function

An increasingly sophisticated paradigm has emerged to explain the structure and function of voltage-gated potassium channels of the *Shaker* superfamily (Jan and Jan, 1992; Hoshi and Zagotta, 1993). They are composed of four subunits (MacKinnon, 1991), each with multiple transmembrane domains, organized around a central ion-conducting pore (Guy and Conti, 1990; Durell and Guy, 1992). The sensitivity to channel blockers and the conductance properties have been localized within a highly conserved part of the channel molecule designated as the P (pore) region (MacKinnon and Miller, 1989; MacKinnon and Yellen, 1990; Yellen et al., 1991; Kavanaugh et al., 1991; Hartmann et al, 1991; Kavanaugh et al., 1992). Some of the determinants of potassium selectivity have been mapped to specific residues in this region (Kirsch et al., 1992; Heginbotham et al., 1992). Gating of voltage-dependent K^+ channels of the *Shaker* variety has also been explored in great depth (for review, see Hoshi and Zagotta, 1993). The domains important to the voltage-dependent activation process and to N- and C-type inactivation have been determined (Papazian et al., 1991; Hoshi et al., 1990a, 1990b; Zagotta et al., 1990; Ruppersberg et al., 1991). The measurement of gating currents has enabled study of the conformational changes associated with channel activation (Bezanilla et al., 1991; Schoppa et al., 1992).

In contrast, little is known about the mechanism by which min K functions as a channel. It is not understood how a change in membrane potential signals a conformational change in min K leading to channel opening, nor which domains are important to voltage sensing and gating. The behavior of currents induced by min K expression is consistent with a genuine K^+ channel rather than a carrier or transporter type protein (Hausdorff et al., 1991). However, the amino acid sequence and predicted subunit structure of min K is so radically different from Shaker-like K channels that it is impossible to extrapolate from experiments detailing Shaker channel structure and function to min K channels. One of the goals of work described in this dissertation (Chapter IV) is to contribute to our understanding of how this structurally succinct protein may function as a voltage-gated potassium channel.

Several theories have been proposed for how min K promotes potassium channel activity. Its minimalist structure and the reported difficulty in expressing min K currents in many cell types (Lesage et al., 1993) has led to the idea that the min K protein activates an otherwise silent potassium channel present in oocytes (Attali et al., 1993). Furthermore, Attila and his coworkers (1993) have suggested that min K may regulate both potassium and chloride conductances in the oocyte. However, Freeman and Kass (1993) have recently shown that expression of min K currents is not limited to *Xenopus* oocytes. Interestingly, they used transfection of a cell line (HEK 293) that was derived from the same tissue (kidney) from which min K was originally cloned (Takumi et al., 1988).

Alternatively, min K may form a channel as a homo-oligomeric bundle of subunits, or as a hetero-oligomer with some as yet unidentified protein. In this model, the hydrophobic faces of amphipathic transmembrane α-helices would interact with the lipid environment

and with each other, while the more hydrophilic faces would produce a conducting pore (Sansom, 1991). Goldstein and Miller (1991) have demonstrated that point mutations within the transmembrane domain alter both the ion selectivity of min K currents and their sensitivity to open channel block by cesium and tetraethylammonium. When Phe 55 was changed to Thr, min K was three times as permeable to NH^+ and Cs^+ compared to wild-type. This effect was largely reversed by a reciprocal mutation at position 59 (Thr to Phe), suggesting that these residues face the channel pore (Goldstein and Miller, 1991). Takumi and coworkers (1991) demonstrated that several residues within the putative transmembrane region of min K were essential for channel activity. In addition, one substitution (Leu 52 Iso) enhanced channel activity by altering the kinetics of channel activation (Takumi et al., 1991). We have shown that an amino acid residue in the carboxy-terminal domain is the probable phosphorylation site for protein kinase C-mediated regulation of min K currents (Busch et al., 1992b). Furthermore, a synthetic 32 amino acid peptide, containing the hypothesized transmembrane sequence of min K, formed voltage-dependent single channels in planar lipid membranes (Ben-Efraim et al. 1993). The currents induced by this peptide, however, lacked the appropriate ion selectivity and displayed a single channel conductance much larger than that expected for min K. Taken together, however, these results support the view that min K forms an integral part of the channel itself.

Because expression of min K currents is so far limited to oocytes and a human embryonic kidney cell line (Freeman and Kass, 1993), and because the effects of channel expression level and presumed channel density on the properties of min K currents are complicated (Busch et al., 1993a; Attila et al., 1993; Blumenthal and Kaczmarek, 1994), min K has been proposed to need an ancillary protein for activity. The identity of this protein remains unspecified, but it may include a cytoskeletal element, a regulatory protein, or

another subunit that contributes structurally to the pore of the channel (and that is present in oocytes and HEK 293 cells but not other cells).

Channel gating

It is not known how min K channels gate. Channel activation for min K is at least two orders of magnitude slower than that of polytopic K^+ channels. The activation kinetics of min K are sensitive to temperature (Maylie et al, 1994), state of expression and current density (Busch et al, 1993a), min K mRNA concentration (Blumenthal and Kazmarak, 1994; Varnum, Adelman and Maylie, unpublished observations), and species-specific variations in charged residues of the amino-terminal domain (Hice et al, 1994). In addition, a specific mutation (Leu 52 Iso) within the putative transmembrane domain also altered the rate of channel activation (Takumi, et al., 1991).

From studies of alamethicin gating, a simple peptide ion channel that induces slowly activating, voltage-dependent currents in lipid bilayers, Baumann and Mueller originally proposed a general model for channel activation that included aggregation of freely diffusing subunits to form a central pore (Baumann and Mueller, 1974). No "biological" channels are known to gate in this manner (Hille, 1992). Yet, the succinct structure of min K, the remarkably slow activation of min K currents, and the profound susceptibility of gating kinetics to temperature and to presumed channel density, all suggest a subunit aggregation model for min K channel activation. Experiments detailed in Chapter IV of this dissertation address this hypothesis.

Modifiers of channel gating

Biological and chemical modifiers of channel gating have provided important insights

regarding the structural and functional properties of voltage-gated ion channels (Hille, 1992). Among the tools that have been used are water-soluble scorpion α toxins that slow inactivation of Na^+ channels, scorpion β toxins that shift activation and cause prolonged opening of channels even at the normal resting potential, lipid-soluble toxins that both shift activation and slow inactivation of Na^+ channels, and enzymes and chemical agents that eliminate inactivation of Na^+ channels and Shaker -like K channels (for reviews, see Catterall, 1980; Hille et al, 1987; Strichartz et al., 1987; Narahashi and Herman, 1992). Treatment of myelinated nerve fibers with an amino-group modifiying agent decreased the current magnitude of a K^+-channel and slowed its closing rate, but had no effect on activation (Pappone and Cahalan, 1984). Furthermore, there is a rich history relating the use of chemical cross-linking agents to study the quaternary structure of proteins and the conformational changes associated with their activity (for review, see Gaffney, 1985). The rationale for experiments described in Chapter IV of this dissertation is to test a membrane impermeable, chemical cross-linking agent as a modifier of min K gating and thus a probe of the channel activation mechanism.

CHAPTER II

MATERIALS AND METHODS

General molecular biological procedures

Rat min K was originally provided by Dr. Michael P. Kavanaugh after PCR amplification using oligonucleotides derived from sequences flanking the coding region reported by Takumi et al. (1988). Common manipulations were performed as previously described (Busch et al, 1992a; Bond et al., 1989; Adelman, et al., 1992) or as is standard (Maniatas et al., 1989). Synthetic mRNA was prepared by in vitro transcription with T-7 RNA polymerase (BRL) (from rat min K cDNA) or SP6 RNA polymerase (BRL) (from guinea pig min K cDNA) in the presence of capping nucleotide G(5')ppp(5')G (Pharmacia).

Isolation of gp min K coding sequence

A guinea pig genomic DNA library constructed in λEMBL3 was purchased from Clontech (Palo Alto, CA). A PCR fragment encompassing the entire rat min K coding sequence (Takumi et al., 1988) was radiolabeled by random priming, and used as probe to screen 750,000 guinea pig genomic DNA clones (Colony/Plaque Screen; NEN, Boston, MA). Hybridization was carried out in 1 M NaCl, 1% SDS, 50% formamide, 100 µg/ml yeast tRNA at 37 °C; filters were washed in 0.2 X SSC, 0.1% SDS at 42 °C and exposed to X-ray film (Eastman-Kodak, Rochester, NY). Positively hybridizing phage were purified by repeated screenings at reduced density. Restriction analysis revealed a ~650 bp hybridizing Hind III-EcoR1 restriction fragment, which was subcloned into M13 phage and the nucleotide sequence determined using the

dideoxynucleotide termination method (Sanger et al., 1977) as specified in the Sequenase kit (Pharmacia). This same fragment was subcloned into the pSelect⁻ vector and used as substrate for *in vitro* mRNA synthesis from the SP6 RNA promoter.

RNA extraction and PCR

For tissue distribution studies, total RNA was isolated using the RNAgents Kit (Promega). Briefly, ~0.5 g of tissue from adult guinea pig heart, brain, skeletal muscle, uterus, and kidney were homogenized in denaturing solution containing guanidinium thiocyanate. The RNA was phenol-chloroform extracted, precipitated twice using isopropanol, washed with 75% ethanol and dried in a vacuum desiccator. The quality of the RNA was determined spectrophotometrically by the A_{260}/A_{280} ratio. Subsequently, 1 μg of total RNA was primed with oligo(dT) and reversed transcibed using MUMLV reverse transcriptase (BRL). A 1/50 dilution on this reaction was used for PCR amplification of gp min K sequence using the following oligonucleotides: 5' ACAGTTCAACCCAGTAGC 3' and 5' CACTCAGAAAGACTA 3'. Oligonucleotides for PCR were synthesized on an Applied Biosystems 391 DNA synthesizer; PCRs were performed with AmpliTaq DNA Polymerase on a Perkin-Elmer 9600 thermocycler (Perkin-Elmer-Cetus, Norwalk, CT).

For verification of the gp min K stop codon and C-terminal sequence, polyA+ mRNA from heart tissue was directly isolated using the FastTrack mRNA Isolation Kit (Invitrogen). Briefly, ~0.5 g of tissue from heart was homogenized in 5 M guanidinium isothiocyanate lysis buffer containing 5% β-mercaptoethanol. DNA was sheared by passage through a 21 gauge needle. PolyA+ mRNA was purified using oligo(dT)-cellulose, washed, eluted and precipitated with 2 N sodium acetate and ethanol. Reverse transcription was performed as described above, using ~0.1 μg of mRNA primed with an

oligonucleotide containing a random hexemeric sequence combined with the Hind III restriction site sequence. PCR amplification was carried out as described above using oligonucleotides representing the Hind III restriction site sequence and internal coding sequence (5' GGCAGGAGAAGGACAAAG 3'). PCR products were subcloned into M13 phage and the nucleotide sequence of the inserts determined, as described (Sanger et al., 1977).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the Altered Sites method (Promega, Madison, WI). Briefly, the channel sequence of interest was subcloned into the pSelect^{-} phagemid vector and single stranded DNA was isolated. The nucleotide sequence was altered by simultaneously annealing a mutagenic oligonucleotide with the required base changes and an oligonucleotide designed to repair the defective ampicillin resistance gene in the pSelect^{-} vector, followed by complete synthesis of the second DNA strand using T-4 DNA polymerase, ligation and transformation of a repair deficient stain (BMH) of *E. coli*.. DNA from ampicillin resistant bacteria were retransformed into JM 101 or JM 109 *E. coli*.. Plasmid DNA from these subsequent ampicillin resistant colonies was isolated and sequenced, as described (Sanger et al., 1977).

Oocyte expression and electrophysiology.

Oocyte injection and handling have been described (Christie et al., 1989; Christie, et al., 1990). Briefly, ovary were surgically removed from *Xenopus laevis*; oocytes were dissected apart in modified Barths solution and defolliculated by digestion in calcium-free solution containing collagenase A (2 mg/ml; Boehringer) (performed by Yan-na Wu, Wei-bin Zhang or Brooke Maylie). Oocytes were then injected within 36 hours with 0.5 ng of capped cRNA in 50 nl of sterile water, using a pressure injector, and incubated at

18 ^{o}C with rotary agitation in ND 96 (see below) supplemented with gentamicin (50 μg/ml), sodium pyruvate (2.5 mM) and theophyllin (500 μM).

Two electrode voltage clamp recordings were made from oocytes 2-7 days after RNA injection with a TEV-200 or CA-1 amplifier (Dagan Corporation, Minneapolis, MN) interfaced to a LSI 1173 computer. Microelectrodes were filled with 3 M KCl and electrode resistance ranged from 0.5 to 1.5 Mohm. Oocytes were continuously superfused (1-2 ml/min) with a solution (ND 96) containing (mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 5; pH 7.6 at room temperature (21-23 °C). Currents for long voltage commands were generally sampled at 50-100 Hz and filtered at 1 kHz. Experiments were performed with the oocyte membrane held at -80 mV or -100 mV; interpulse "resting" periods ≥ 30 s were routinely used to permit complete deactivation of min K currents. Oocyte input resistance and capacitance were also periodically monitored. For experiments with gp min K, the voltage dependence of the currents was determined from measurements of tail currents following repolarization to -60 mV; the baseline for the tail currents was obtained from a 1 s prepulse to -60 mV preceding each test pulse.

Data analysis

The variability of values from experiments with multiple data points is presented as mean ± SEM with *n* indicating the number of cells contributing to the mean. The significance of data was determined by paired or unpaired t-tests. Analysis was performed using Igor Pro (WaveMetrics) or Kalaidigraph (Abelbeck Software). Activation curves were fitted by a Boltzmann relation using a Levenberg-Marquardt algorithm to minimize the sum of squares. Inhibition curves for barium and clofilium were fit according to the Michaelis-

Menten equation. The voltage dependence of inhibition was determined using the Woodhull equation (Woodhull, 1973).

Chemicals and reagents

Modifying agents and drugs were applied externally in ND 96. Chemical modifying agents 3, 3-dithiobis(sulfosuccinimidyl proprionate) (DTSSP) and sulfo-NHS-acetate were obtained from Pierce (Rockford, IL). Neither chemical agent substantially altered the pH of the perfusing solution (pH = 7.6). Because the activity of these compounds varied with lot number, the general reactivity of the lots used in these experiments was confirmed by monitoring hydrolysis in ND 96 as indicated by the increase in absorbance at 260 nm caused by release of the N-hydroxysuccinimide group (Abdella et al., 1979). DTSSP is a membrane impermeable, homobifunctional cross-linking agent that principally targets primary amine groups by an irreversible acylation reaction, forming an amide bond (Staros, 1982). It is reactive at physiological pH and contains a thiol-cleavable disulfide bond in the middle of a 12 Å spacer arm. Other chemicals used were clofilium (Research Biochemicals Inc., Natick, MA), dithiothreitol (DTT), phorbol-12,13-didecanoate (PDD), 4-α-phorbol-12,13-didecanoate, staurosporine, and CPT-cyclic AMP (Sigma Chemical Co., St Louis, MI), isoproterenol (Winthrop Pharmaceuticals, New York, NY), E-4031 (Eisae Co., Tsukuba Research Laboratories, Japan), N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) and cherlerythrine (LC Laboratories, Woburn, MA).

CHAPTER III

THE MIN K CHANNEL UNDERLIES THE CARDIAC POTASSIUM CURRENT I_{Ks} AND MEDIATES SPECIES-SPECIFIC RESPONSES TO PROTEIN KINASE C

Summary

To investigate the postulated physiological role of the min K protein in cardiac function, a clone encoding the guinea pig (gp) min K potassium channel was isolated and expressed in *Xenopus* oocytes. The currents, gp I_{sK}, exhibit many of the electrophysiological and pharmacological properties characteristic of gp I_{Ks}, the slow component of the delayed rectifier potassium conductance in guinea pig cardiac myocytes. Furthermore, RT-PCR indicated that the mRNA encoding gp min K is present in heart. Like I_{Ks} in guinea pig myocytes, gp I_{sK} is modulated by stimulation of protein kinase A (PKA) and protein kinase C (PKC). In contrast to rat and mouse I_{sK}, which are decreased upon stimulation of PKC, myocyte I_K and gp I_{sK} in oocytes are increased after PKC stimulation. Substitution of an asparagine residue at position 102 by serine (N102S), the residue found in the analogous position of the mouse and rat min K proteins, results in decreased gp I_{sK} in response to PKC stimulation. These results support the hypothesis that the min K protein underlies the slow component of the delayed rectifier potassium current in ventricular myocytes, and account for the species-specific responses to stimulation of PKC.

Cloning of guinea pig min K

A guinea pig genomic DNA clone encoding the min K protein was isolated using the rat min K coding sequence as probe. The predicted amino acid sequence (Figure III-1) is 78% identical to rat and human min K sequences and 79% identical to mouse min K. Its general features include two potential N-glycosylation sites in the amino-terminal, putatively extracellular portion, and a single hydrophobic, putative transmembrane domain that is identical to that found in rat and mouse min K and differs at only one amino acid from human min K. Interestingly, three cysteine residues reside in a cluster in the carboxy-terminal portion, near the previously defined protein kinase C regulatory domain (Busch et al., 1992b). In this "regulatory domain", the residue in the guinea pig clone which aligns with the PKC phosphhorylation site in the rat clone (serine 103) is instead an asparagine residue. Although the predicted protein is highly homologous to min K proteins from other species, the open reading frame is five residues shorter than rat min K, and four shorter than mouse or human min K. To determine whether this C-terminal truncation reflects the sequence encoded in the guinea pig min K mRNA, or resulted from the presence of an intron in the genomic DNA, mRNA was isolated from guinea pig heart tissue and converted to single stranded cDNA by reverse transcription (R.T.). This cDNA was used as substrate for polymerase chain reactions (PCRs) using oligonucleotides which flank the predicted stop codon. The nucleotide and predicted amino acid sequences derived from the reaction products confirmed the presence of a stop codon at the position indicated in Figure III-1.

Tissue distribution of guinea pig min K mRNA

The tissue distribution of guinea pig min K mRNA was determined using RT-PCR. The results shown in Figure III-2 demonstrate expression in adult heart, skeletal muscle, uterus and kidney; a weak signal was consistently detected from brain. No signal was

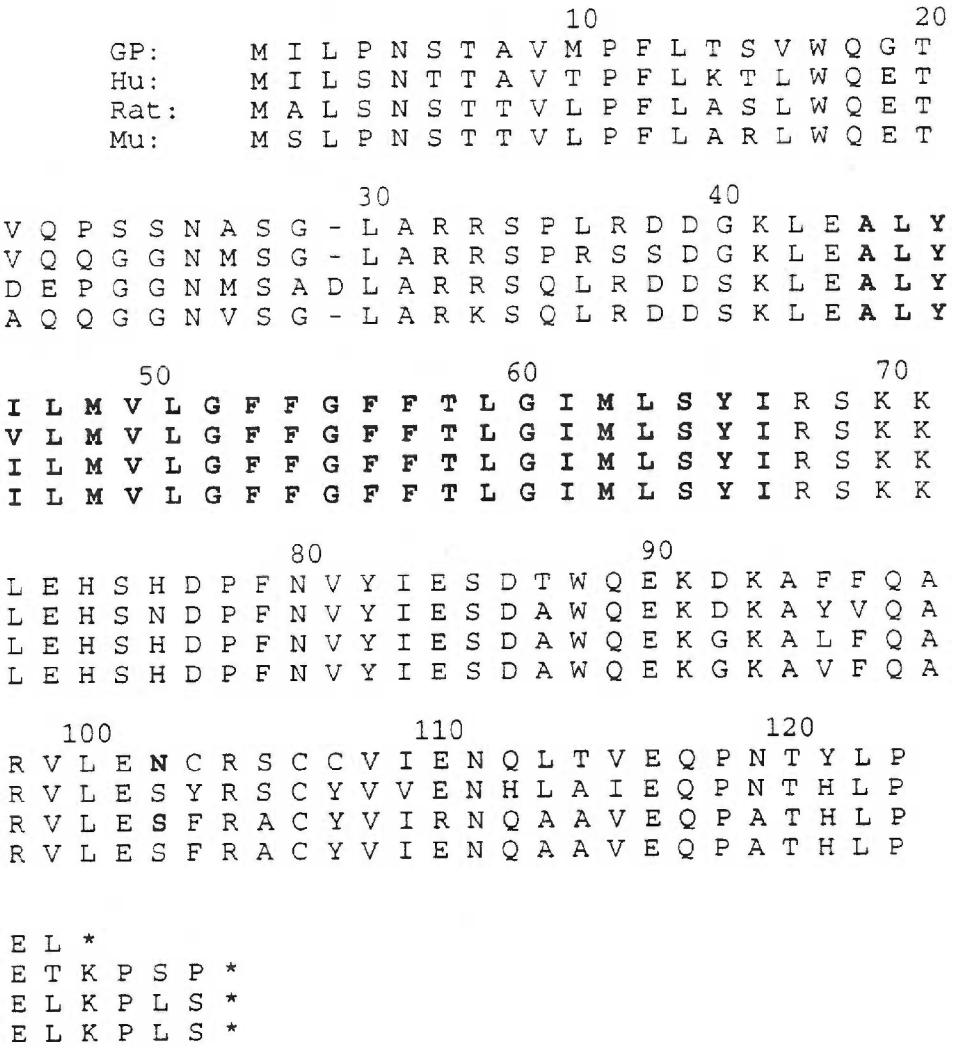


Figure III-1. Primary structure of gp min K. Amino acid sequences of cloned min K proteins. The predicted transmembrane domains are in bold, as are residues in the guinea pig and rat sequences which mediate species-specific responses to PKC (see text). GP, guinea pig; Hu, human; Mu, mouse.

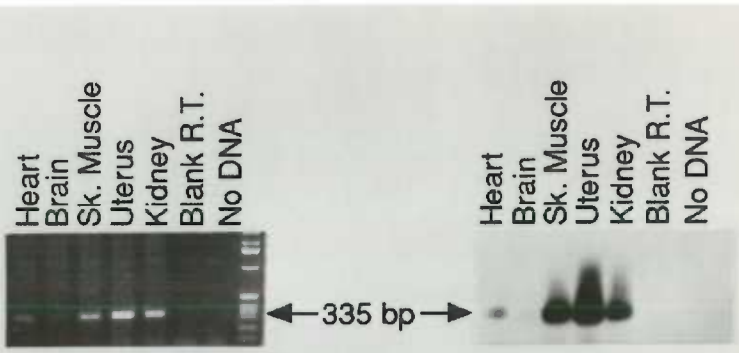


Figure III-2. Tissue distribution of guinea pig min K mRNA. Reverse transcribed RNA from the indicated tissues was used in the PCR with oligonucleotides specific for sequences within the coding region of the min K mRNA. Reaction products were separated through an agarose gel (left), prepared as a Southern blot and probed with a radiolabeled oligonucleotide directed to an internal sequence (right). A weak signal was consistently detected from brain. From left: heart, brain, skeletal muscle, uterus, kidney. Control lanes show PCRs which used a mock reverse transcription reaction, without added RNA, as substrate (blank RT), and in which no substrate was added (no DNA).

observed from PCR of a blank RT without added mRNA or from PCR in which no DNA substrate was added. To verify the specificity of the PCR, a southern blot of the reaction products was probed with a radiolabled oligonucleotide directed to sequence internal to the amplification primers (Figure III-2).

Expression of Guinea Pig I_{sK}.

In oocytes expressing the cloned gp min K, depolarizations to potentials positive to -50 mV evoked a slowly activating outward current, after an initial delay in onset, which failed to reach steady state during 30 s steps (Fig. III-3A). The kinetics of activation following the initial delay were described by a sum of 2 exponentials plus a constant, a fast component that decreased from 3.4 ± 1.2 s at -20 mV to 1.6 ± 0.4 s at 40 mV and a slow component that decreased from 32 ± 8 s at -20 mV to 12.8 ± 1.3 s at 40 mV (n = 3). The relative amplitude of the fast component was 0.25 at -20 mV and 0.31 at 40 mV. Applying a third exponential to I_{sK}, to account for the delay in onset, yields a time constant that decreases from approximately 1.5 s at -20 mV to 0.5 s at 40 mV. The delay in onset is more prominent at lower test pulse potentials and is exaggerated if the prepulse duration is made shorter or the prepulse potential more negative. This dependence on previous potential (Boyle et al., 1987b; Hausdorff et al., 1991) suggests that the delay is due to a shift in equilibrium between multiple closed states toward the open state.

The voltage dependence of I_{sK} can be approximated by the product of a linear current-voltage relation and a Boltzmann function representing the voltage dependence of channel activation (Fig. III-3B). However, since the open channel conductance may rectify, measurement of tail currents yields a better estimate of the voltage dependence of activation of I_{sK}. Because currents through min K channels fail to reach steady state even with long depolarizing commands, analysis of tail currents provides a 'quasi'

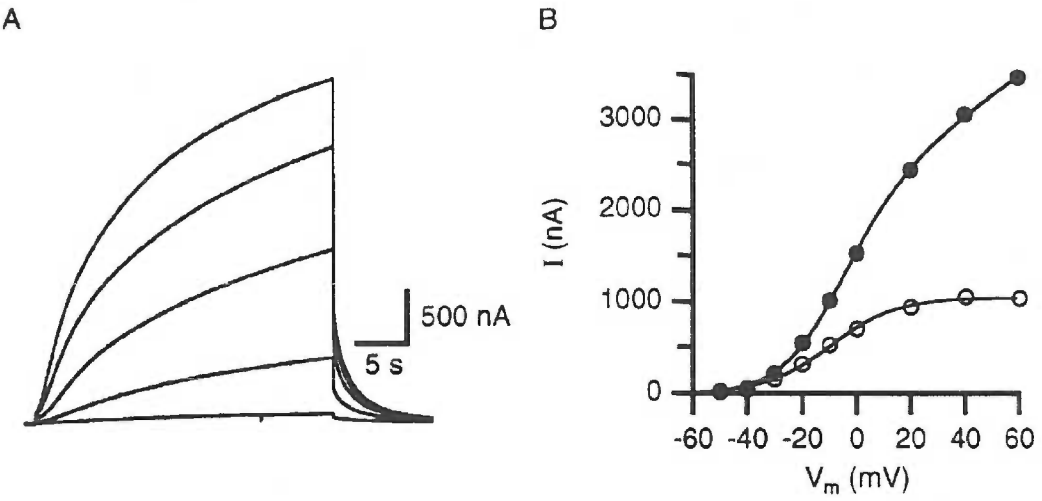


Figure III-3. Expression of gp min K clone in *Xenopus* oocytes. (A) Currents elicited by 30 s depolarizing pulses from -40 to 40 mV in 20 mV steps. (B) Outward current-voltage relation of I_{sK} (•) measured as difference between the final and initial current during 30 s depolarizing pulses. The continuous curve is drawn according to the product of a linear current-voltage relation and a Boltzmann function representing $g_{max}(V_m - E_{rev})/\left(1+e^{-(V_m-V_{1/2})/k}\right)$; g_{max} = 21.7 μS, E_{rev} = -101 mV, $V_{1/2}$ = -9.8 mV, k = 11.6 mV. Voltage dependence of activation of I_{sK} (o) determined from measurements of tail currents as described in methods. Continuous curve drawn according to Boltzmann function, $I_{max}/\left(1+e^{-(V_m-V_{1/2})/k}\right)$; I_{max} = 1037 nA, $V_{1/2}$ = -9.5 mV, k = 11.8 mV.

steady state voltage dependence profile. Figure III-3B shows tail current amplitudes with a fitted Boltzmann function. The voltage for half-maximal activation, $V_{1/2}$, and maximally activated tail current, I_{max}, varied between different batches of oocytes; the average values for $V_{1/2}$ and I_{max} were -4.3 ± 1.4 mV and 1327 ± 129 nA (n = 16), respectively. The effect of stimulation of PKA and PKC on $V_{1/2}$ and I_{max} are therefore expressed as changes relative to control values in the same oocyte. The slope factor was less variable and averaged 11.2 ± 0.1 mV (n = 16).

Potassuim selectivity

Consistent with min K channels cloned from other species, gp min K is selective for potassium ions. The reversal potential of gp I_{sK} in 2, 20, or 100 mM external K^+ (substituted for sodium) followed a slope of 58.4 mV per decade change in K^+, consistent with a channel selective for K^+ over Na^+ and Cl^- (n = 4, Fig. III-4A). In guinea pig myocytes, I_{Ks} exhibits only slight rectification in comparison to the strong rectification of I_{Kr} (Sanguinetti and Jurkiewicz, 1990). Measurement of "instantaneous" tail currents at various potentials following a fixed test pulse revealed a small degree of rectification that increased slightly when extracellular K^+ was increased from 2 to 100 mM (Fig. III-4B).

Pharmacology

The sensitivity of guinea pig I_{sK} to compounds known to block I_{Kr}, I_{Ks} and I_K in guinea pig myocytes was tested. The class III antiarrhythmic E4031, which blocks I_{Kr} (IC_{50} = 400 nM) but not I_{Ks} in myocytes (Sanguinetti and Jurkiewicz, 1990), had no effect on gp I_{sK} at concentrations as high as 5 μM (102.8 ± 2.6% of control, n = 6). Lanthanum, which at low concentrations (1 μM) also blocks I_{Kr} (Sanguinetti and Jurkiewicz, 1990b), did not reduce gp I_{sK} (1 μM La^{3+}, 102.9 ± 1.7 % of control, n =

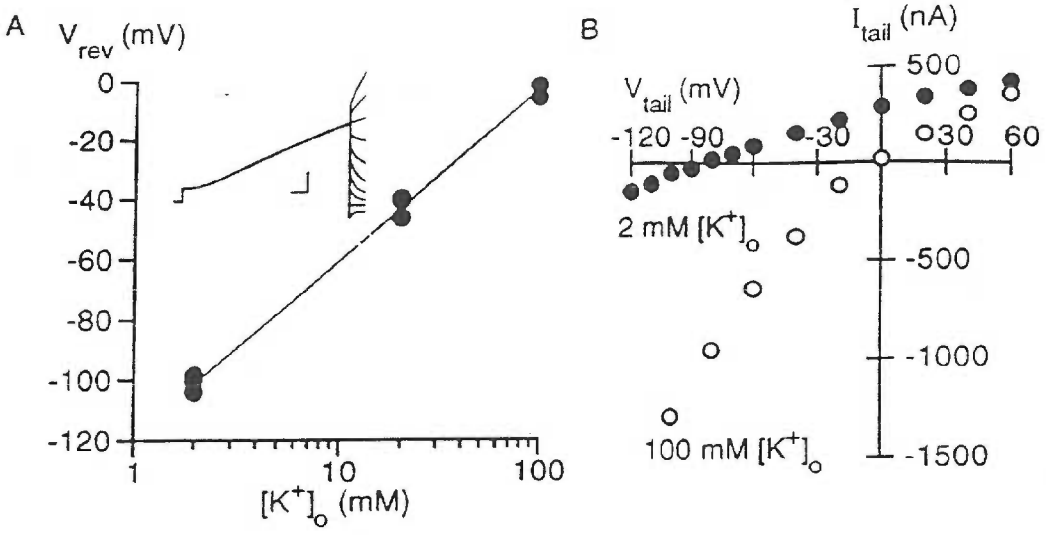


Figure III-4. (A) Reversal potential of gp min K tail currents in varying concentrations of external K^+. (*Inset*) The oocyte in 2 mM external K^+ was depolarized to 20 mV for 10 s and repolarized to test potentials from -120 to 60 mV in 20 mV steps; calibration bars, 1 s and 100 nA. The external solutions contained the indicated K^+ concentration (K^+ substituted for Na^+). Continuous line drawn according to 58.4 mV/decade. (B) The open channel current-voltage relation of guinea pig min K in 2 and 100 mM external K^+ obtained from the same oocyte. "Instantaneous" tail currents were determined after the capacity transient following repolarization to potentials between -120 and 60 mV from a fixed test potential of 20 mV (inset panel A).

4). Higher concentrations of La^{3+} (100 μM) did induce a slight reduction (not shown), consistent with results reported for myocytes (Sanguinetti and Jurkiewicz, 1990b). The class III antiarrhythmic clofilium, which blocks both components of myocyte I_K (Arena and Kass, 1988; Colatsky, et al., 1990), inhibited gpI_{sK} with a K_i of 92.7 μM (Fig. III-5). This is comparable to the reduction of myocyte I_K by clofilium, in which 100 μM blocked 56.7 % (Arena and Kass, 1988). Furthermore, NE10064 inhibited gp min K currents with a K_i of 3.6 μM, similar to block of myocyte I_{Ks} (Busch et al., 1993c). These results are summarized in Table III-1.

Regulation by PKA

Guinea pig ventricular I_K is increased following: 1) stimulation of ß-adrenergic receptors by isoproterenol, 2) addition of cAMP analogs to permeabilized guinea pig myocytes, or 3) application of PKA catalytic subunit to the intracellular face of excised membrane patches (Walsh and Kass, 1988; Walsh et al., 1989, 1991; Yazawa and Kameyama, 1990). In oocytes expressing gp min K, the membrane permeable cAMP analog CPT-cAMP increased gp I_{sK} 32.3 ± 3.0 % (n = 6) (Fig. III-6A, B). CPT-cAMP treatment slightly shifted the voltage dependence of activation to more negative potentials, and increased it's voltage sensitivity (Fig. III-6C; Table III-2). Comparable effects were seen when endogenous oocyte ß-adrenergic receptors (Kusano, Miledi, and Stinnakre, 1982) were stimulated by 2 μM isoproterenol (Fig. III-7, Table III-2). Oocyte membrane capacitance was unaffected by either CPT-cAMP or isoproterenol (-0.1 ± 0.5%, n = 6; -3.2% ± 1.2%, n = 5, respectively). The selective PKA inhibitor, H89 (Chijiwa et al., 1990), decreased gp I_{sK} when applied alone, and attenuated the effect of concomitantly applied CPT-cAMP (n = 3) (Fig. III-6A).

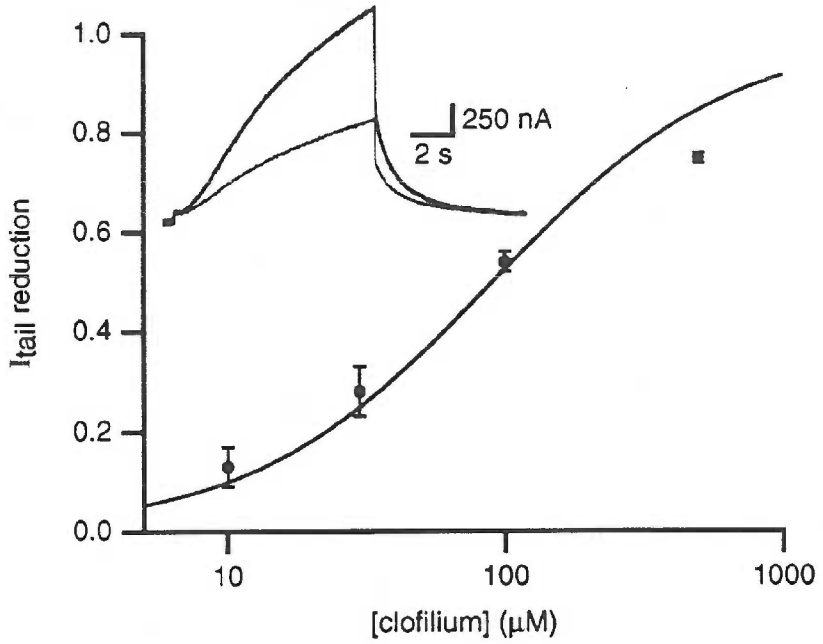


Figure III-5. Dose-response relationship for inhibition of gp min K tail currents by clofilium. Data represent mean ± SEM, n = 4. The continuous curve was drawn according to the Michaelis-Menten equation (K_i = 92.7 μM). (*Inset*) Current traces elicited by 10 s steps to +20 mV before (upper trace) and after application of 100 μM clofilium.

Table III-1 GP min K exhibits pharmacological properties similar to guinea pig I_{Ks}

blocker	gp min K	I_K	
		I_{Ks}	I_{Kr}
clofilium, 100 μM	57 ± 2% (n = 4)	56.7 % *	
E4031, 5 μM	No	No	Yes #
lanthanum, 1 μM	No	No	Yes †
NE 10064, 3 μM	47.8 ± 1.5% (n = 9)	58 ± 5% ‡	---

*Arena and Kass (1988)

#Bennett et al. (1985)

†Sanuinetti and Jurkiewicz (1990)

‡Busch et al. (1993c)

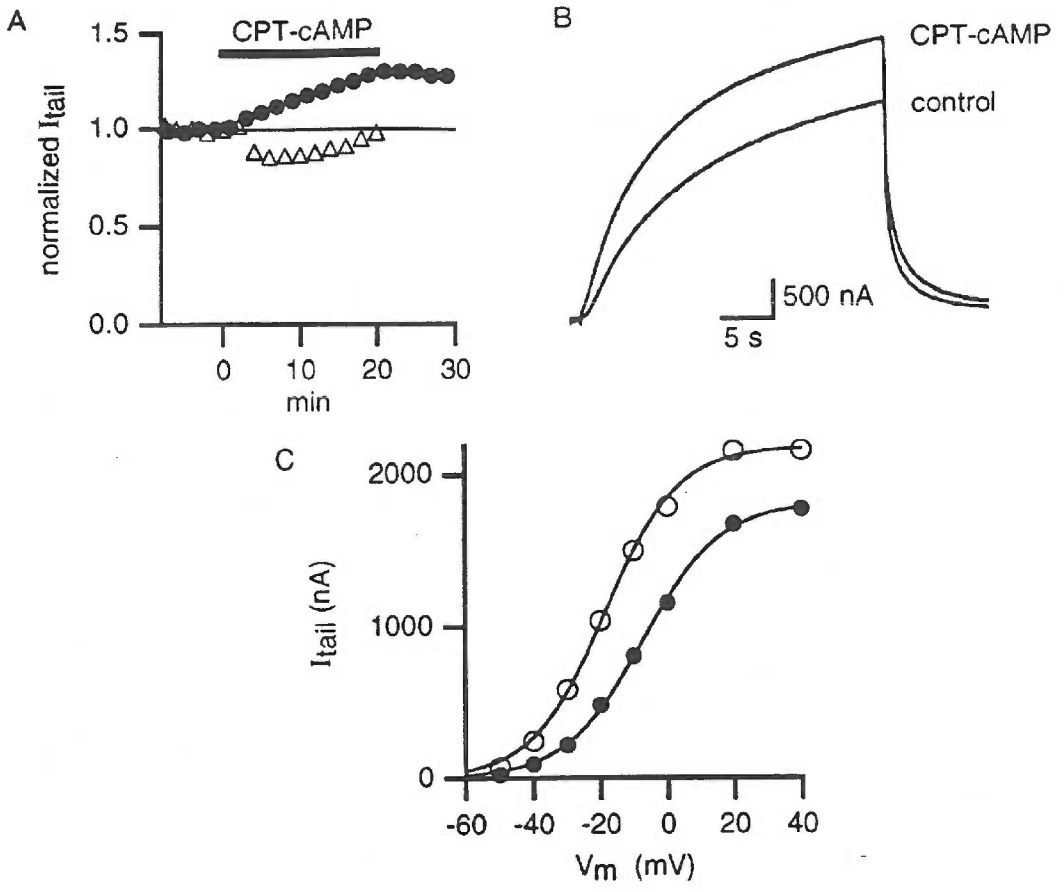


Figure III-6. PKA regulation of gp min K. (A) Time course of CPT-cAMP effect (1mM applied for 20 min) with (Δ) and without (•) prior and concomitant application of H89 (30 μM). 10 sec steps to +20 mV were made every 2 min; tail currents were measured at -60 mV and normalized to control values (before CPT-cAMP application). (B) Leak subtracted current traces elicited by 30 sec steps to +20 mV before and after application of CPT-cAMP. (C) Effects of PKA stimulation on activation of I_{sK}, determined from tail current measurements at -60 mV, following 30 sec depolarizations to test potentials shown, before (•) and after (o) application of CPT-cAMP. Continuous curves were drawn according to a Boltzmann function (as in Fig. III-3B). For control: I_{max} = 1813 nA, $V_{1/2}$ = -7.2 mV, k = 11.7 mV; treated: I_{max} = 2181 nA, $V_{1/2}$ = -18.5 mV, k = 11.0 mV.

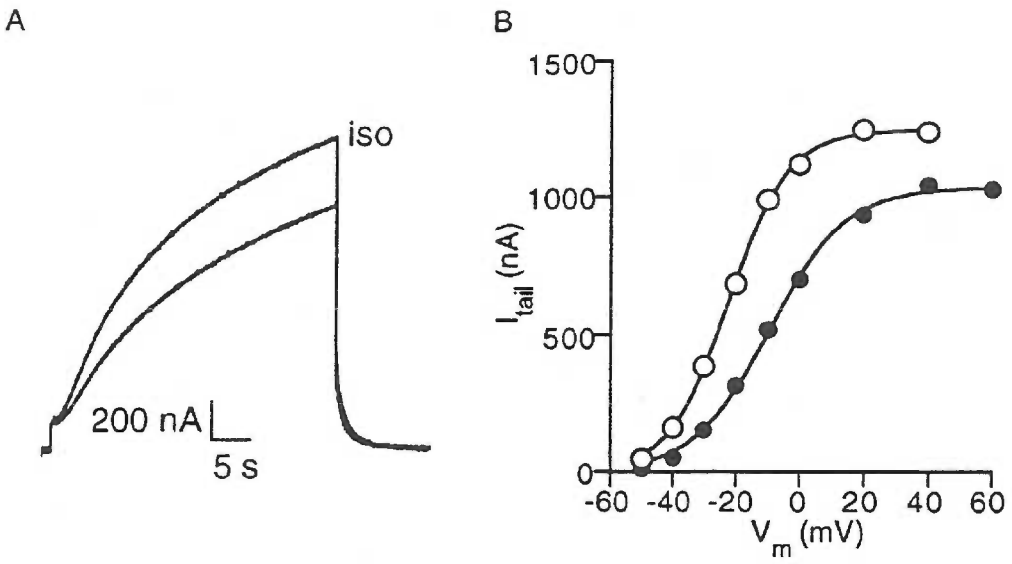


Figure III-7. β–adrenergic regulation of gp min K. (A) Current traces elicited by 30 sec steps to +20 mV before and after application of 2 μM isoproterenol. (C) Effects of β–adrenergic stimulation on activation of I_{SK}, determined from tail current measurements at -60 mV, following 30 sec depolarizations to test potentials shown, before (•) and after (o) application of CPT-cAMP.

Table III-2. Regulation of gp min K by PKA and PKC

Group	I_{tail} (% control)	n	Boltzmann parameters I_{max} (% control)	$\Delta V_{1/2}$ (mV)	k (mV)	n
W.T.						
control	100		100	---	11.2±0.1	16
CPT-cAMP	132±3*	6	110±2*	-4.1±1.9*	10.3±0.3*	6
ISO	147±7*	7	113±6*	-6.1±1.9*	10.5±0.3*	5
PDD	133±4*	11	118±5*	-0.7±1.1	11.2±0.2	5
N102S						
control	100		100	---	10.2±0.3#	7
PDD	72±6*	8	86±4*	9.9±0.9*	12.8±0.3*	5

Values represent mean ± SEM. I_{tail}, tail current at -60 mV following 10 s depolarization to 20 mV; n, number of oocytes; Boltzmann parameters determined from tail currents following a 30 sec test pulse; *, $p < 0.05$ for respective paired control values determined by a t-test; #, $p < 0.05$ for N102S control compared to W.T. control determined by an upaired t-test.

Regulation by PKC

Stimulation of PKC increases I_K in guinea pig myocytes (Tohse et al., 1987; Walsh and Kass, 1988). Application of phorbol-12, 13-didecanoate (PDD) to oocytes expressing gp min K induced an increase of the current (Fig. III-8A, inset B). After phorbol application, the tail current measured at the end of a 10 s depolarizing pulse to +20 mV was increased by 33.2 ±3.7% (n = 11). The inactive enantiomer, α-PDD, had no effect (Fig. III-9). The increase of I_{sK} by PDD was blocked in oocytes treated with the PKC inhibitor cherlerythrine (Herbert et al., 1990) (Fig. III-8A, Fig. III-9); inhibitor alone had no effect. Similar effects were seen with the less selective inhibitor staurosporine, but staurosporine alone slightly decreased current amplitude (not shown). The effects of PKC on the voltage dependence of gp min K (Fig. III-8B) appear to differ from those seen following PKA stimulation. While PKC stimulation increased I_{max}, the $V_{1/2}$ and the slope factor, k, were unchanged (Table III-2).

The increase in gp I_{sK} following stimulation of PKC is similar to the effects of PKC stimulation in guinea pig myocytes, but contrasts with results in mouse myocytes (Honoer et al., 1991) and in oocytes expressing cloned mouse or rat min K (Honore et al., 1991; Busch et al., 1992a) where the currents are decreased after PKC stimulation. In the latter case, we have previously demonstrated that substitution of the serine residue at position 103 by alanine (S103A) eliminated the current decrease by PKC stimulation (Busch et al., 1992b). The analogous residue in gp min K is an asparagine (N102; see Fig. III-1). To determine whether this difference underlies the species-specific response to PKC, N102 was altered by site-directed mutagenesis to serine (N102S). Expression of gp min K (N102S) in oocytes resulted in voltage dependent potassium channels virtually indistinguishable from wild type, except in the response to PKC stimulation. In

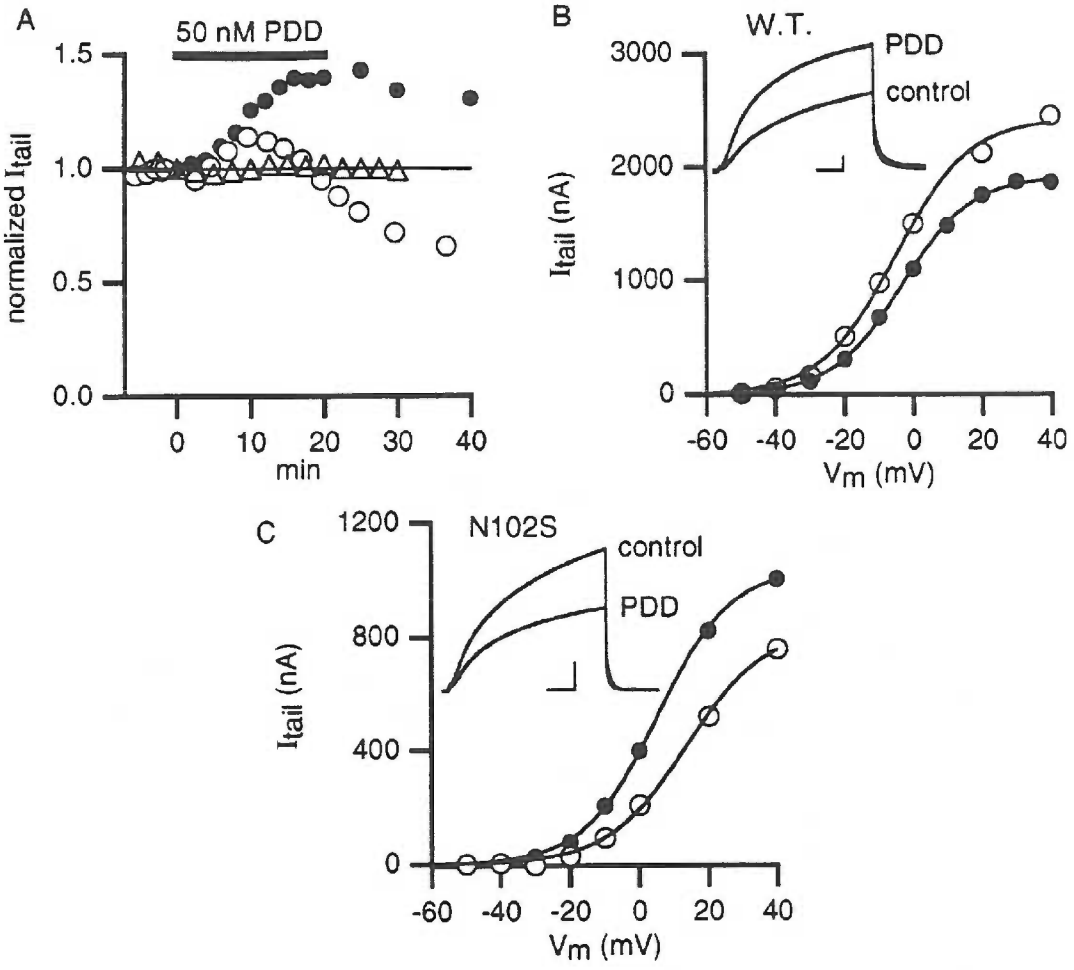


Figure III-8. PKC regulation of gp min K. (A) Time course of PDD effect (50 nM, 20 min) on tail currents for oocytes expressing wild type (wt) gp min K (•), wt gp min K with prior and concomitant application of 5 μM cherlerythrine (Δ), and gp min K N102S (o). (B) Voltage dependence of activation of wt I_{sK}: before (•) and after (o) 20 min application of 50 nM PDD. Continuous curves were drawn according to a Boltzmann function (as in Fig. III-3B). For control: I_{max} = 1917 nA, $V_{1/2}$ = -3.3 mV, k = 10.1 mV; treated: I_{max} = 2429 nA, $V_{1/2}$ = -5.1 mV, k = 10.9 mV. (*Inset*) Current traces elicited by 30 sec steps to +20 mV (scale bars: 5 sec, 500 nA). (C) GP min K N102S. For control: I_{max} = 1036 nA, $V_{1/2}$ = 5.3 mV, k = 10.6 mV; treated: I_{max} = 900 nA, $V_{1/2}$ = 16.5 mV, k = 12.6 mV.

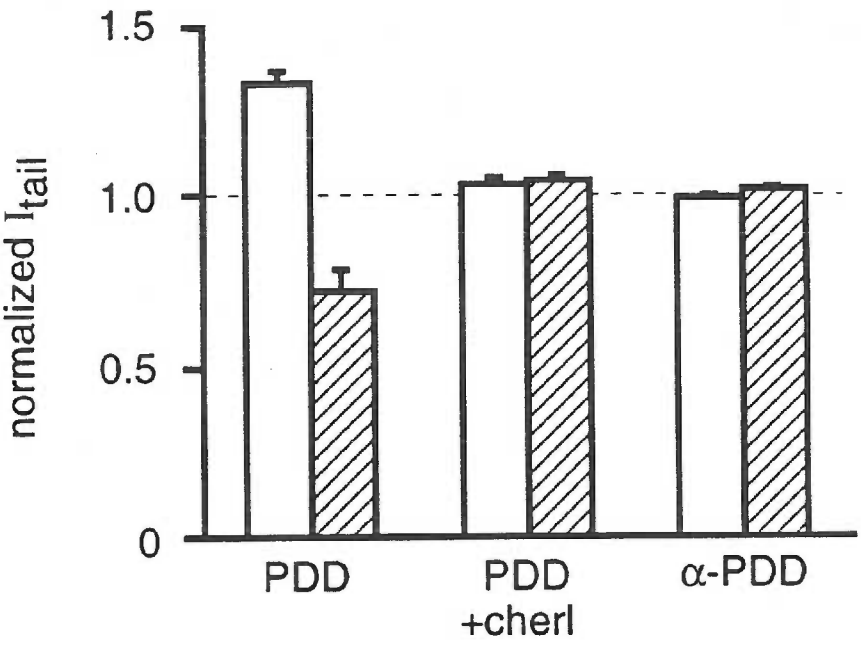


Figure III-9. The effect of PKC stimulation on wild type (open bars) and mutant N102S (shaded bars) gp min K. Conditions were: 50 nM PDD, 20 min (n = 11, 8); 50 nM PDD in the presence of 5 μM cherlerythrine (n = 3, 3) ; 100 nM α-PDD, 20 min (n = 4, 4). Values represent maximum changes in tail currents observed during and 20 min after PDD application. Tail currents were measured at -60 mV after 10 sec depolarizations to +20 mV. Cherlerythrine prevented the PDD-mediated increase of wild type gpminK currents ($p < 0.005$) and the decrease of N102S gpmin K currents ($p < 0.02$).

this case, application of phorbol ester induced a significant decrease in the current amplitude (at 40 min, -28.0 ± 6.3%; n = 8) (Fig. III-8A, inset C), comparable to the reduction in current following phorbol treatment that is seen with rat I_{sK} expressed in *Xenopus* oocytes (Busch et al., 1992b). Regulation of a double mutant (N102S, S105A) was not different from that seen with the single mutant (not shown). The voltage dependence of activation of gp min K(N102S) (Fig. III-8C, Table III-2) demonstrated that the phorbol-mediated reduction in current amplitude was accompanied by a positive shift in $V_{1/2}$ and a reduction in voltage sensitivity (Table III-2).

CHAPTER IV

PERSISTENT ACTIVATION OF MIN K CHANNELS BY CHEMICAL CROSS-LINKING

Summary

Expression of the structurally and functionally distinct min K protein in *Xenopus* oocytes results in voltage- and time-dependent potassium currents which activate with a characteristic slow time course. Application of a membrane-impermeable chemical cross-linking agent to oocytes expressing min K decreased the time-dependent current, increased its rate of activation, and induced persistently activated inward and outward potassium currents. These effects required membrane depolarization and demonstrated use dependence. Persistently activated channels retained potassium selectivity and sensitivity to block by clofilium and barium. Cross-linking effects were not mimicked by application of a related, monofunctional agent, nor were they seen in oocytes expressing the *Shaker*-like potassium channel, RBK1. The results suggest that a major conformational change occurs during min K channel gating which can be stabilized by chemical cross-linking, and support a model in which min K channels activate by voltage-dependent subunit aggregation.

Chemical cross-linking evoked persitently activated currents

To probe the mechanism by which min K induces channel activity, a membrane-impermeable chemical cross-linking agent was applied to oocytes expressing min K. Rat min K, engineered to include the FLAG epitope at the C-terminus and expressed in

Xenopus oocytes, generated the characteristic, slowly-activating outward current at potentials positive to -60 mV. 3, 3-Dithiobis(sulfosuccinimidyl proprionate) (DTSSP) is a membrane impermeable, homobifunctional cross-linking agent that principally targets primary amine groups by an irreversible acylation reaction, forming an amide bond (Staros, 1982). It is reactive at physiological pH and contains a thiol-cleavable disulfide bond in the middle of a 12 Å spacer arm. Application of 1.5 mM DTSSP for 15-20 min., with concurrent 20 s membrane depolarizations every minute, progressively: (1) reduced deactivation, producing persistent tail currents at -60 mV, (2) increased the instantaneous current observed at the beginning of the test pulse, and (3) decreased the time-dependent component of the outward current and altered its activation kinetics (Figure IV-1A and B). These changes were accompanied by a decrease in the oocyte input resistance (Figure IV-1B) and an increase in the holding current at -80 mV (not shown). The effect of DTSSP persisted even after prolonged washout (Figure IV-1A and B, trace "d"). In addition, the modification of min K currents by DTSSP was dose-dependent over concentrations up to 1.5 mM (not shown).

The current-voltage relation and the kinetics of activation and deactivation of the modified currents were examined. The family of current records demonstrated that chemical cross-linking induced currents at potentials negative to the activation threshold for untreated min K channels (Figure IV-2A and B). Little or no time-dependent current was observed at potentials between -120 and -80 mV, while at more depolarized potentials an additional time-dependent component remained. However, the voltage dependence of this component was altered by DTSSP (Figure IV-2C); applying the Boltzmann function indicated that the $V_{1/2}$ shifted -29.0 ± 1.1 mV and the slope factor, k, increased from 12.9 ± 0.3 to 16.2 ± 0.7 (n = 11). In addition, the time course for activation of the time-dependent component was faster following DTSSP treatment and lacked the characteristic

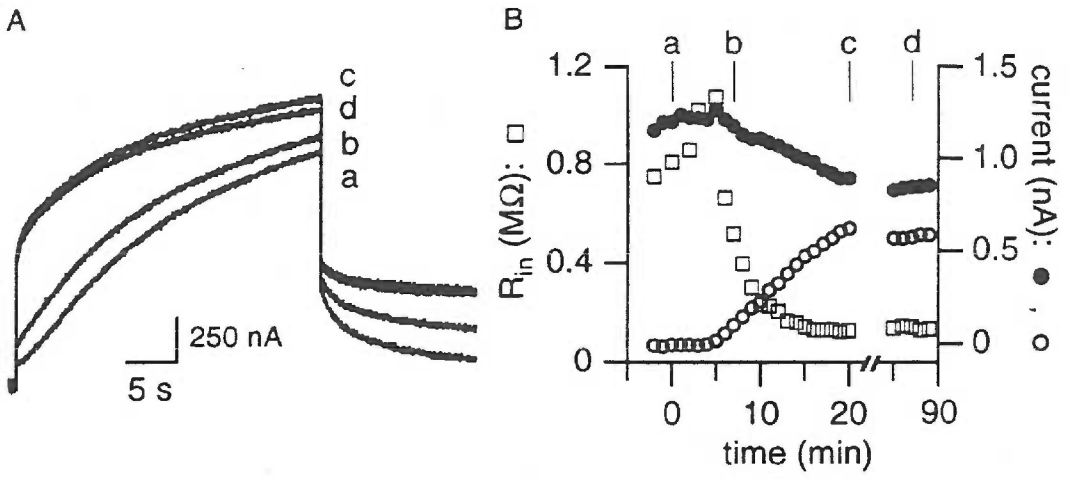


Figure IV-1. Chemical modification of min K currents by DTSSP. (A) DTSSP (1.5 mM) was applied for 20 min in the bath solution during repetitive 20 s depolarizing pulses to 20 mV made every minute. Shown are representative current traces elicited before, during, and after application and washout of DTSSP; a, time 0; b, 7 min; c, 20 min; d, 87 min. A 500 ms prepulse to -100 mV was used; tail currents were recorded at -60 mV. (B) Time course of the DTSSP effect on oocyte input resistance (□), and instantaneous (o) and time-dependent (•) min K currents for 20 minute application of cross-linking agent and subsequent washout. Twenty second depolarizations to 20 mV were made every minute.

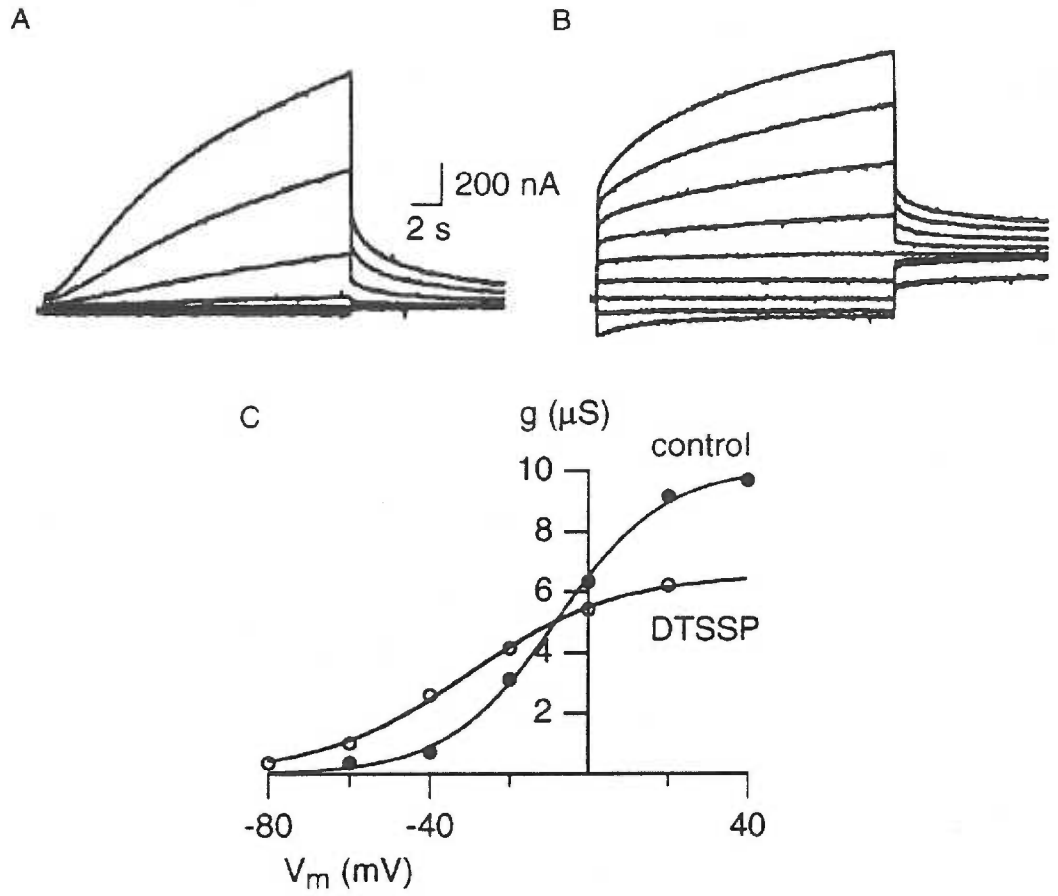


Figure IV-2. Representative family of current traces elicited by 20 s voltage commands from a prepulse potential of -100 mV to potentials from -140 to 20 mV in 20 mV steps, before (A) and after (B) 20 min application and washout of 1.5 mM DTSSP. (C) Representative conductance-voltage relationship for the time-dependent component of min K currents (from A and B above) before (•) and after (o) modification by DTSSP. Continuous curves were drawn according to a Boltzmann function.

sigmoidal activation kinetics (Figure IV-2B). A sum of two exponentials fitted to the delayed increase in current at 20 mV showed that τ_{fast} decreased from 7.73 ± 0.48 s to 1.94 ± 0.10 s, τ_{slow} decreased from 24.6 ± 1.3 s to 13.6 ± 0.6 s and the fractional amplitude of the fast component, $A_{fast}/(A_{fast}+A_{slow})$, decreased from 0.38 ± 0.02 to 0.23 ± 0.01 after DTSSP treatment (n = 28). In contrast, the kinetics of the deactivating tail currents, described by a single exponential fit, increased only slightly with DTSSP application, from 2.15 ± 0.08 to 2.41 ± 0.04 s.

Some relaxation of the persistently activated min K currents was observed during long pulses to very hyperpolarized potentials (Figure IV-2B, bottom trace). Associated with this relaxation was a small decrease in the instantaneous current apparent in the following depolarizing pulse and an increase in the input resistance (not shown). Both the instantaneous current and the input resistance recovered fully during subsequent depolarizations, without additional contributions to the size or kinetics of the time dependent outward current. The relaxation of inward currents at -140 mV was not dependent on external Mg^{2+} or Ca^{2+} (not shown).

The initial time course of DTSSP-modified "instantaneous" currents were examined at a faster sampling rate during 50 ms voltage steps. Only negligible currents were observed in untreated min K-expressing oocytes, as expected for its slow rate of activation (Figure IV-3A). In contrast, DTSSP treatment induced significant outward as well as inward currents during 50 ms pulses, and revealed no kinetics following the capacitive transient (Figure IV-3B and C). The nearly linear current-voltage relationship reflected the apparent loss of voltage-dependent gating for the persistently activated current. Some rectification, however, was present at more extreme potentials. In addition, persistently activated currents reversed at -91 ± 2.5 mV (n = 8), near the expected potassium reversal

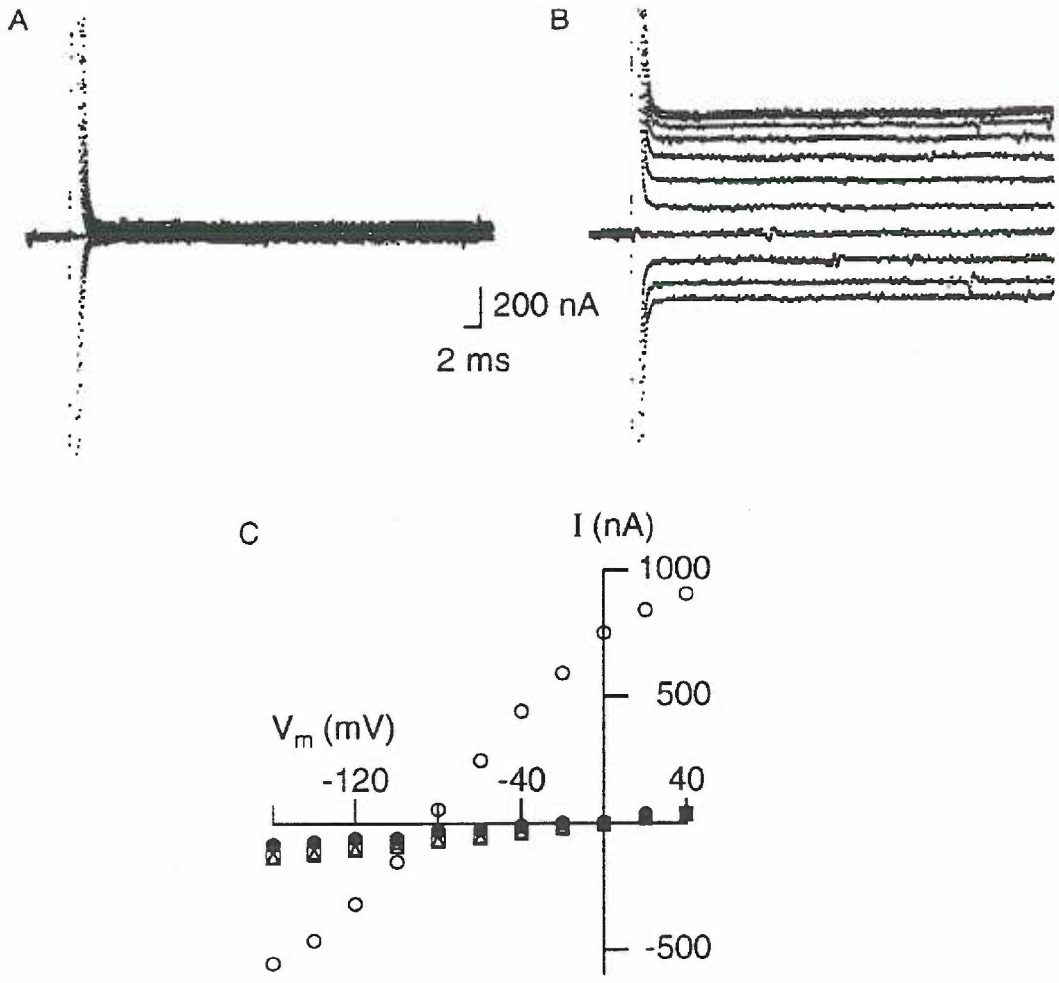


Figure IV-3. Examination of (A) control and (B) DTSSP-modified "instantaneous" currents evoked by 50 ms voltage commands to potentials from -160 and 40 mV in 20 mV steps. (C) Current-voltage relationship of 50 ms traces (as in A and B) for a representative min K-expressing oocyte before (•) and after (o) DTSSP treatment, and for a representative non-injected oocyte before (Δ) and after (□) DTSSP. In both cases, DTSSP was applied during repetitive 20 s depolarizations to 20 mV made every min.

potential for oocytes under these ionic conditions (Lotan et al., 1982). These results suggest that cross-linking of min K holds the channel in an open state.

The effects of DTSSP were specific for oocytes expressing min K.

Non-injected oocytes displayed small currents that were reduced or unchanged by DTSSP (Figure IV-3C). Application of DTSSP to oocytes expressing the *Shaker*-like potassium channel RBK1 resulted in only a small decrease of the time-dependent outward current (-8.7 ± 0.8 %, n = 3) and no increase in the instantaneous current (Figure IV-4A). There was no obvious change in the kinetics of activation. Furthermore, the threshold of activation and the voltage dependence of RBK1 currents was not altered by DTSSP (Figure IV-4B). These results demonstrate that the profound cross-linking effect described above was observed only in oocytes expressing the min K protein.

Cross-linked channels retained ion selectivity

The DTSSP-modified currents retained many of the essential attributes of min K channels. Like most potassium channels, min K channels are highly selective for potassium ions over sodium ions (Takumi et al., 1988). Changing the external K^+ concentration from 2 to 20 mM shifted the reversal potential of the persistently activated currents +56.2 ± 1.6 mV (n = 8), in agreement with that predicted by the Nernst equation for potassium (Figure IV-5). In contrast, removal of external Cl^- had little effect on the reversal potential of persistently activated currents, shifting V_{rev} only -0.84 ± 4.6 mV (n = 4).

Cross-linked channels remained sensitive to min K blockers

To determine whether the cross-linked channels retained other pore properties of min K channels, the effects of channel blockers were also examined. Min K currents are

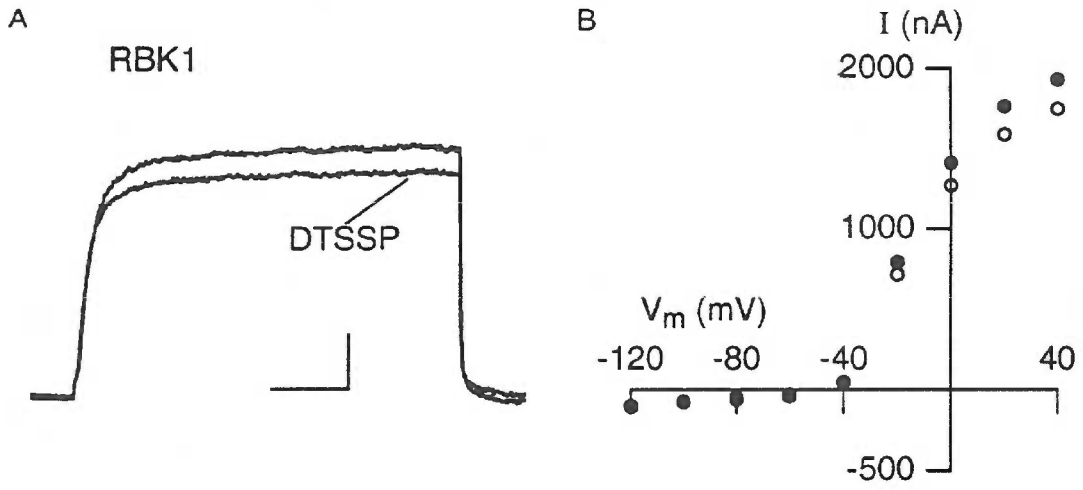


Figure IV-4. Chemical modification by DTSSP was specific for min K currents. DTSSP had little effect on RBK1 currents. (A) Current traces evoked by 100 ms depolarizations to 20 mV from a prepulse potential of -80 mV before and after 20 min. application of 1.5 mM DTSSP. Repetitive 5 s depolarizations to 20 mV made every 30 s during application and washout of DTSSP. Tail currents were measured at -80 mV. Scale bars represent 400 nA and 20 ms. The current amplitude for RBK1 currents under these conditions was reduced 8.7 ± 0.8 % (n = 3). (B) Current-voltage relationship for 100 ms steps in oocytes expressing RBK1, before (•) and after (o) DTSSP.

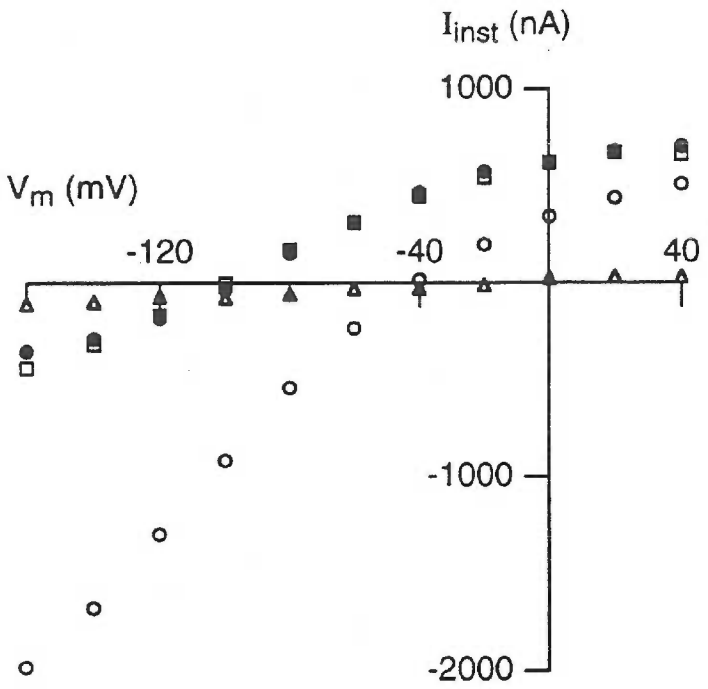


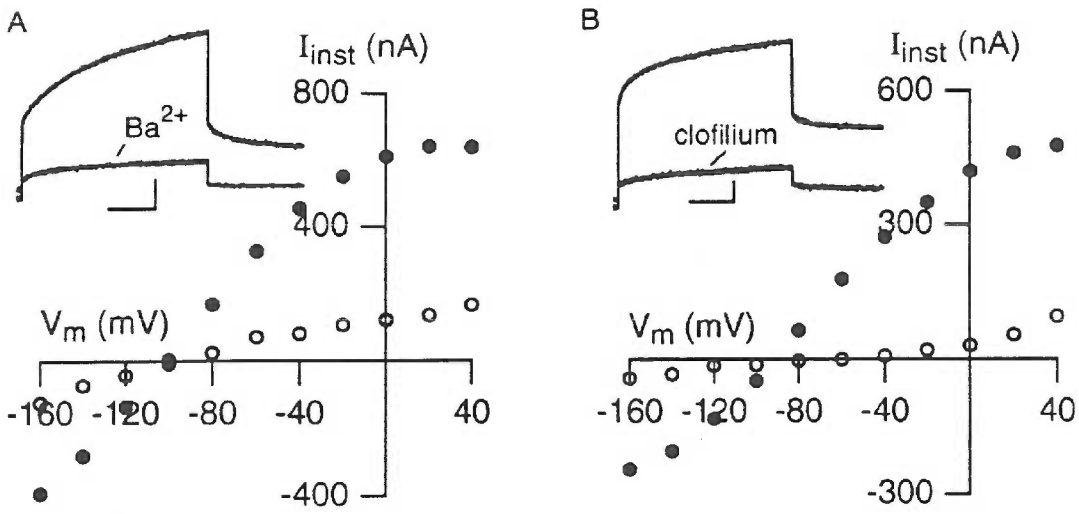
Figure IV-5. DTSSP-modified min K channels retained potassium selectivity. Current-voltage relation of 50 ms voltage steps for a representative min K-expressing oocyte before (Δ) and after DTSSP treatment (•) in 2 mM external potassium, 20 mM $[K^+]_o$ (o), and 2 mM $[K^+]_o$ in chloride free solution (□). K^+ was increased by substituting for Na^+, and Cl^- was removed by replacement with gluconate.

inhibited in a concentration dependent fashion by external barium ions (Hausdorff et al., 1991; Goldstein and Miller, 1991). External 20 mM barium reduced both instantaneous and time-dependent cross-linked currents by 92 ± 3 and 81 ± 2 % (n = 6), respectively (Figure IV-6A, inset); both inward and outward currents were blocked (Figure IV-6A). The dose-response for inhibition of currents during 50 ms steps to 0 mV gave a K_D of 0.33 ± 0.07 mM (n = 4); from the voltage-dependence of the K_D a δ value of 0.17 was obtained (Woodhull, 1973; Hausdorff et al., 1991).

Clofilium, a class III antiarrythmic drug known to inhibit min K currents (Folander et al., 1990; Honore et al., 1991, Varnum et al., 1993) also reduced DTSSP-modified currents (Figure IV-6B, inset); the instantaneous component was inhibited 78 ± 4.5 % and the time-dependent component 63 ± 3.0% (n = 9). Clofilium blocked inward currents nearly as effectively as outward currents (Figure IV-6B). These results indicate that while gating was dramatically altered by DTSSP, the fundamental pore properties of min K channels (Hausdorff et al., 1991; Goldstein and Miller, 1991) were not significantly changed.

DTSSP effect was dependent on channel opening

Persistent activation of min K currents by DTSSP required concomitant membrane depolarization. When the membrane potential was held at -100 mV during application of DTSSP, the first depolarization to 20 mV after washout revealed no persistently activated current compared to control (Figure IV-7A, inset). However, continued depolarizations at 1 minute intervals produced some increase in the instantaneous current (+200 ± 24 nA, n = 5). This increase was inhibited by addition of 20 mM glycine in the wash (+73.8 ± 36 nA, n = 5; $P < 0.05$), which provides a primary amine group that competes for DTSSP (Figure IV-7A) . The use-dependence of the cross-linking effect was further



FigureIV-6. The basic pharmacology of chemically modified currents was examined. (A)*(inset)* After DTSSP treatment and washout, external application of barium (20mM) inhibited both instantaneous and time-dependent min K currents. Current traces are shown for 20 s depolarizations to 20 mV. Scale bars represent 150 nA and 5 s. Current-voltage relation is shown for 50 ms voltage commands from -160 to 40 mV, before (•) and after (o) 20 mM barium. (B) Clofilium inhibited DTSSP-modified min K currents. *(inset)* Current traces are shown for 20 s depolarizations to 20 mV before and after external application of 100 μM clofilium. Scale bars represent 150 nA and 5 s. Current-voltage relation is also shown for 50 ms voltage commands from -160 to 40 mV, before (•) and after (o) clofilium.

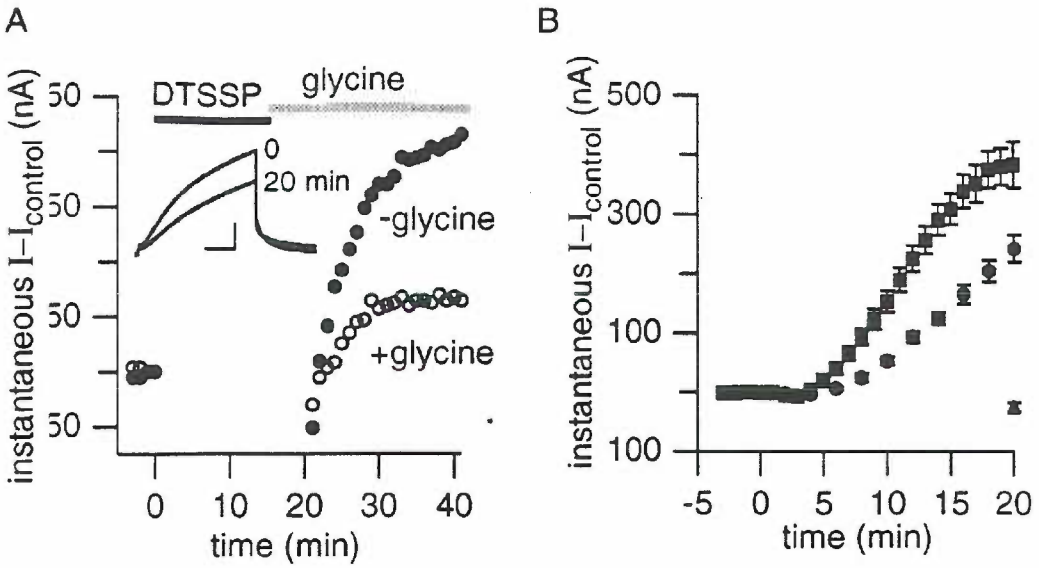


Figure IV-7. Chemical modification of min K currents required channel opening. (A)*(inset)* Current traces for a representative oocyte before (0) and after 15 min. application and 5 min. washout (20) of 1.5 mM DTSSP without concomitant membrane depolarizations. The membrane potential was held at -100 mV during DTSSP treatment and 5 min. washout. Scale bars represent 500 nA and 5 s. Time course of the subsequent increase in the instantaneous current with repetitive membrane depolarizations (•). The mean increase in the instantaneous current under these conditions was 200 ± 24 nA (n = 5); this increase was attenuated by 20 mM glycine in the wash (o) (+73.8 ± 36 nA, n = 5; $P < 0.05$). (B) Use dependence of the effect of DTSSP on the instantaneous min K current. Membrane depolarizations for 20 s to 20 mV were made every 1 min (■, n = 33-45), every 2 min (•, n = 4) or after 20 min (▲, n = 11) during application of 1.5 mM DTSSP. The slope of a line fit to the average increase over control in the instantaneous current was 29 nA/min. (r = 0.997) or 17 nA/min. (r = 0.997) for depolarizations every one or two minutes, respectively.

demonstrated by varying the frequency of depolarizing commands during DTSSP application. As shown in Figure IV-7B, the rate of change in the instantaneous current was proportional to the frequency of membrane depolarizations. These results demonstrate that min K channels need to be activated for DTSSP to exert its effects. The use dependence of the persistent activation of min K currents suggests that cross-linking modifies an open state of min K.

Cross-linking of min K currents requires bifunctional acylation

Sulfo-NHS-acetate (sulfosuccinimidyl acetate) provides an identical reactive group as DTSSP, but is monofunctional and thus incapable of cross-linking. No cross-linking effect was observed when sulfo-NHS-acetate was applied under conditions identical to those used for DTSSP (Figure IV-8A and inset; 8B). The time-dependent outward current was increased 11.1 ± 2.3 % (n = 7) but its voltage dependence was not significantly altered (Figure IV-8A). In contrast to the use-dependency of the DTSSP effect, there was little difference seen between application of sulfo-NHS-acetate during repetitive depolarizations or while channels were held closed at hyperpolarized potentials (n = 5).

DTSSP contains a disulfide bond centered within its symmetrical 12 Å spacer arm. The reducing agent DTT (5-10 mM) was briefly applied subsequent to DTSSP application and washout. After DTT, depolarizing commands evoked currents which were reduced in amplitude compared to currents before DTT application (Figure IV-8C). The instantaneous current was reduced by 35 ± 6 % (n = 5) but the time dependent component was only slightly altered (-7.7 ± 5 %). Thus, cross-linked channels may be partially sensitive to disruption of the cross-linking agent. However, it was not possible to fully reverse the effect of DTSSP due to the harsh conditions required for complete

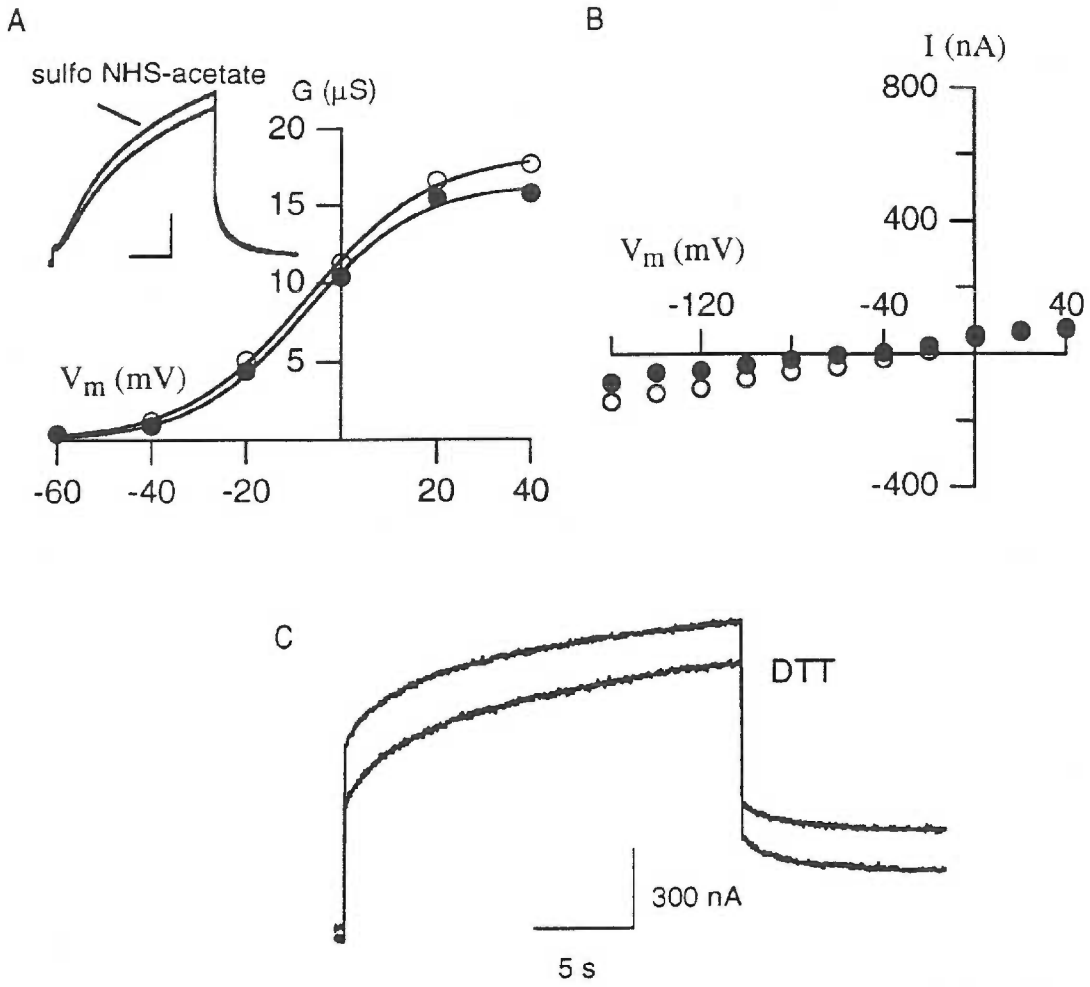


Figure IV-8. Modification of currents required bifunctional acylation. (A) Effect of the monofuctional acylating agent, sulfo-NHS acetate, applied under conditions identical to those used for DTSSP. *(inset)* Current traces for a representative oocyte before and after 20 min. application and washout of 1.5 mM sulfo-NHS acetate. Scale bars represent 500 nA and 5 s. Conductance-voltage relation for 50 ms currents before (•) and after (o) treatment with the monofunctional compound. (B) Current-voltage relation for 50 ms currents before (•) and after (o) treatment with the monofunctional agent. (C) Chemical reduction of cross-linked channels: 5 mM dithiothreitol (DTT) was applied for 15 min. to DTSSP-treated oocytes during repetitive 20 s depolarizations to 20 mV.

reduction of its disulfide bonds. These results, summarized in Figure IV-9, confirm that bifunctional conjugation is necessary for the cross-linking effect on min K currents.

Cross-linked channels can be regulated by protein kinase C

To investigate whether chemically modified min K channels were subject to regulation by second messengers, DTSSP-treated channels were subsequently exposed to the protein kinase C activator, phorbol 12, 13 didecanoate (PDD). Application of 50 nM PDD for 15 min. reduced both instantaneous and time-depedent currents, 42 ± 12 % and 20 ± 4 %, respectively (n = 3) (Figure IV-10). This result suggests that persistently activated channels can still be subject to phosphorylation by PKC (Busch et al., 1992b), and that the fundamental mechanism for inhibition of min K currents by PKC must involve more than inhibition of subunit oligomerization.

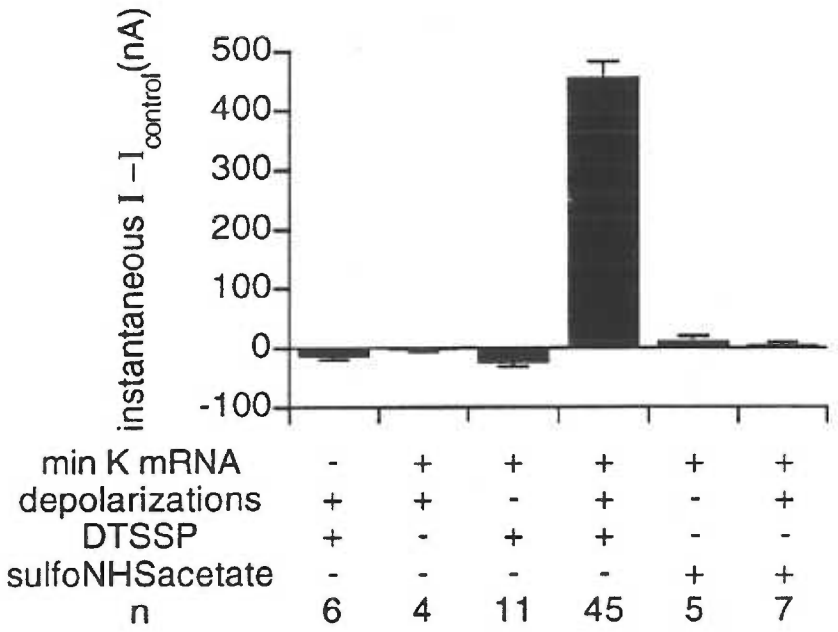


Figure IV-9 Summary of the average effect of chemical modification on the instantaneous current, and the dependence of that change on membrane depolarization and the bifunctional character of the modifying agent. Error bars indicate the SEM.

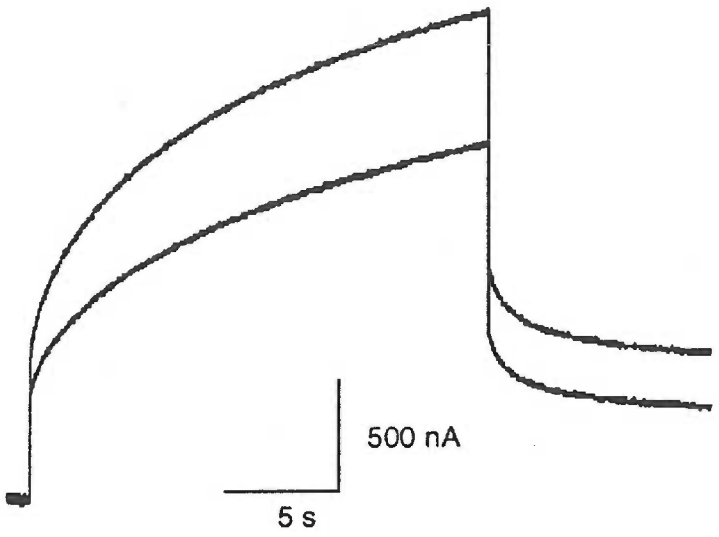


Figure IV-10. Effect of protein kinase C stimulation on cross-linked min K channels. After application and washout of 1.5 mM DTSSP, the PKC activator phorbol 12,13 didecanoate (PDD) was appilied at a concentration of 50 nM for 15 minutes. PDD reduced both instantaneous (42 ± 12 %, n = 3) and time-dependent currents (20 ± 4 %, n = 3).

CHAPTER V

DISCUSSION

Physiological correlates

Several pieces of evidence presented in this dissertation (Chapter III) support the hypothesis that the min K potassium channel underlies the slow component of the delayed rectifier potassium conductance in cardiac myocytes. First, I_{Ks} and guinea pig I_{sK} demonstrate similar kinetic characteristics, distinct from other potassium currents. Both show a prolonged lag following membrane depolarization, slow activation with time constants on the order of seconds, and similar voltage dependence. Neither inactivate, and both are increased in amplitude with trains of pulses (Sanguinetti and Jurdiewicz, 1990; Tohse et al., 1987; Takumi et al., 1988; Busch et al., 1992a). Second, I_{Ks} and I_{sK} share pharmacological profiles, being blocked with similar potency by clofilium, but not by the sotalol derivative E4031. Low concentrations of La^{3+}, which block I_{Kr}, had no effect. In addition, I_{Ks} and I_{sK} are blocked with equal potency by NE10064 (Busch et al., 1993b). Third, I_{Ks} and I_{sK} show similar responses to changes in $[Ca^{2+}]_{in}$ (Tohse, 1987; Busch and Maylie, 1993; Busch et al., 1992a) and stimulation of PKA and PKC (Walsh et al., 1991; Tohse et al., 1987). Indeed, the species-specific responses to PKC are now understood at the structural level, being due to amino acid differences between min K proteins . Fourth, the mRNA encoding the min K protein is expressed in heart.

Although application of PKA catalytic subunit to excised membrane patches increases myocyte I_K (Walsh et al., 1991), reports of the effects of PKA stimulation on cloned min K channels expressed in oocytes have differed. Honore et. al. (1991) reported that 1 mM 8-Br-cAMP had no effect on mouse I_{sK} in oocytes. In contrast, Blumenthal and Kaczmarek (1992) reported that elevated levels of cAMP increased rat I_{sK} in oocytes, although the increased current amplitudes correlated with increased membrane capacitance, suggesting that regulation involves selective insertion and deletion of channels from the plasma membrane. In addition, these authors reported that increased I_{sK} was not due to changes in voltage dependence or kinetics (Blumenthal and Kaczmarek, 1992). We found no change in membrane capacitance as a result of either CPT-cAMP or isoproterenol application. Furthermore, these agents increased current amplitude, shifted the $V_{1/2}$ to more negative potentials, and steepened the response to voltage, consistent with the effects of PKA stimulation on I_K and I_{Ks} in guinea pig ventricular myocytes (Walsh and Kass, 1988; Hartzell, 1988; Sanguinetti and Jurkiewicz, 1991).

In contrast to the previously reported temperature dependence of I_K regulation by protein kinases A and C (Walsh and Kass, 1988; Walsh et al., 1991), we observed no requirement of high temperature for the increase in gp min K current amplitude after PKA or PKC stimulation. All oocyte experiments shown were performed at 21 to 23 ^{o}C. In addition, preliminary results at higher temperatures demonstrated no substantial difference for regulation of min K currents by stimulation of PKC (Varnum, Maylie and Adelman, unpublished). Perhaps the basis for these discrepancies resides in some fundamental difference between oocytes and myocytes (such as specific second messenger proteins or membrane fluidity), or in the use of high divalent cation chelator concentrations in the patch pipet during myocyte recordings (Busch and Maylie, 1993).

The exact molecular basis for PKA-mediated up-regulation of min K currents is not known. It may involve phosphorylation of some intermediate protein that indirectly promotes min K channel activity. Candidates for this possibility include cytoskeletal elements or proteins involved in regulating intracellular calcium levels. Conversely, direct phosphorylation of serine 105 may be responsible for the increase in gp I_{sK}, but this remains an improbable scenario.

Stimulation of PKC also increased gp I_{sK}; however voltage dependent parameters were not changed. This is consistent with results obtained for PKC stimulation of guinea pig myocyte I_K (Toshe et al., 1987; Walsh and Kass, 1988; Tohse et al., 1990b), and suggests that PKA and PKC affect min K channels through different mechanisms. Site-directed mutagenesis has identified residues responsible for the species-specific effects of PKC stimulation on I_{sK}. It is possible that the min K channel activates in response to voltage by subunit aggregation. PKC-mediated phosphorylation of S103 in rat min K might present an electrostatic hinderance to subunit interactions, effectively limiting the number of available channels. However, results presented in Figure IV-10 indicate that stimulation of PKC after chemical crosslinking decreased both time-dependent and persistently activated (instantaneous) min K currents.

In the absence of a serine residue at the analogous position in gp min K, PKC stimulation increases the current amplitude. In this case, PKC may affect an intermediary protein which in turn acts to modulate the channel. We have previously shown that rat I_{sK} is increased by elevation of $[Ca^{2+}]_{in}$ and decreased by cytochalasin D, presumably through inhibiting changes in the cytoskeletal actin network (Busch et al., 1992c). Either of these processes may be affected by PKC-mediated phosphorylation. In this regard, it is

interesting to note that Toshe et al. (1990b) recently found that the concentration-response curve of guinea pig ventricular I_K for intracellular calcium was shifted by stimulation of PKC, suggesting that the increase in currrent amplitude may be mediated by calcium or that the sensitivity of I_K channels to calcium is somehow altered.

The rate at which gp min K N102S mutant currents decreased with stimulation of PKC was slower than that previously observed for rat min K currents (Busch et al., 1992; Varnum et al., 1993). This slower rate may reflect a different phosphorylation site geometry surrounding the serine residue. Other conspicuous amino acid differences exist; in particular, there are two additional cysteine residues in the guinea pig clone, C103 and C107 (Fig. III-1). Indeed, a very recent report confirms that mutation of these additional amino acid residues in this regulatory domain, to those present in the rat clone, enhances down-regulation by PKC stimulation--giving a rate and degree of inhibition closely paralleling that of the rat clone (Zhang et al., 1994).

Apart from min K, little correlation has been made between particular cloned potassium channels and their specific functions within native cells. Work presented in this dissertation supporting the hypothesis for a correspondence between min K channels and cardiac I_{Ks} is one of the first instances defining a physiological role for a specific cloned mammalian K^+ channel. Other spectacular examples have predominately come from Drosophila genetics, such as the *Shaker*, *Shab*, *Shal*, *Shaw*, *Slo*, and *ether-a-go-go* mutations (for review, see Jan and Jan, 1990). The min K protein, which is expressed in human atrium (Swanson et al., 1991), is potentially an important target for class III antiarrhythmic agents.

It is interesting that a protein with an architecture similar to min K, also expressed in cardiac cells, has recently been shown to function as a chloride channel (Moorman et al., 1992). This 72 amino acid protein, phospholemman, was originally characterized for its propensity to serve as a substrate for phosphorylation (Palmer et al., 1991). Indeed, currents through phospholemman channels share the distinctive slow activation and sigmoidal delay properties with I_{Ks} and I_{sK}; they are also increased by trains of pulses (Moorman et al., 1992). Thus, it appears that cardiac cells express at least two members of a structurally and functionally distinct class of voltage-dependent ion channels which operate on a relatively slow time scale and are modulated by a variety of intracellular second messengers.

Structure and function

The second half of this dissertation utilized a chemical modifying agent to target min K gating and address the problem of how min K forms a potassium channel. DTSSP, a membrane impermeable cross-linking agent, dramatically altered min K currents such that some channels lost voltage-dependent gating and remained persistently activated even at potentials negative to the threshold of activation for min K channels in untreated oocytes. For the remaining time-dependent currents, the amplitude was reduced and the kinetics of activation speeded. These effects were not reversed by prolonged washout, consistent with a covalent modification of the channel protein. The effect of DTSSP was entirely dependent upon membrane depolarization. This suggests that a major conformational change may occur during channel opening which can be stabilized by chemical crosslinking. A related but monofunctional acylating agent, sulfo-NHS acetate, did not mimic the effect of DTSSP, indicating that the bifunctional structure of DTSSP was crucial to its modification of min K currents. Persistent activation of min K currents by chemical cross-linking was specific for this channel. The persistently activated current

was inhibited by clofilium, a relatively selective agent reported to block min K currents, and by barium. Furthermore, DTSSP had no effect on oocytes that were not previously injected with min K mRNA, and had little effect on oocytes expressing a divergent potassium channel, RBK1. In this regard, it is interesting to note that expression of concatenated constructs of Shaker channels induced no substantial change in their gating kinetics (Tygat and Hess, 1992; Hurst et al., 1992). Activation of min K may thus involve a fundamentally different mechanism than that of Shaker-like channels.

The potential significance of these findings is that they may provide important clues regarding the gating mechanism for this channel. Differences seen between the effect of the cross-linking agent on expressed channels *with* versus *without* depolarization may reflect (a) dissimilar exposure of reactive sites in the extracellular domain of min K or (b) varying distances separating reactive sites in open versus closed configurations. In the first case, intra-subunit cross-linking of open channels may hold an activation "gate" open. In the latter case, channel activation may involve subunit oligomerization, and the proximity of subunits then permits DTSSP to grapple channel subunits together, thus favoring the conducting conformation.

These data are most consistent with the latter model for min K gating, in which channels activate by voltage-dependent aggregation of multiple, diffusing subunits and deactivate by the reverse process (Figure IV-11). We interpret the use-dependent, bifunctional modification of min K channels as cross-linking of proximal (within 12 Å) channel subunits, eliminating their ability to diffuse apart and thus favoring the conducting conformation (Figure IV-12). Changes in both the instantaneous and time-dependent currents after cross-linking suggest a mixed population of modified channel proteins, perhaps reflecting various linked multimers of min K. Functional complexes in which all

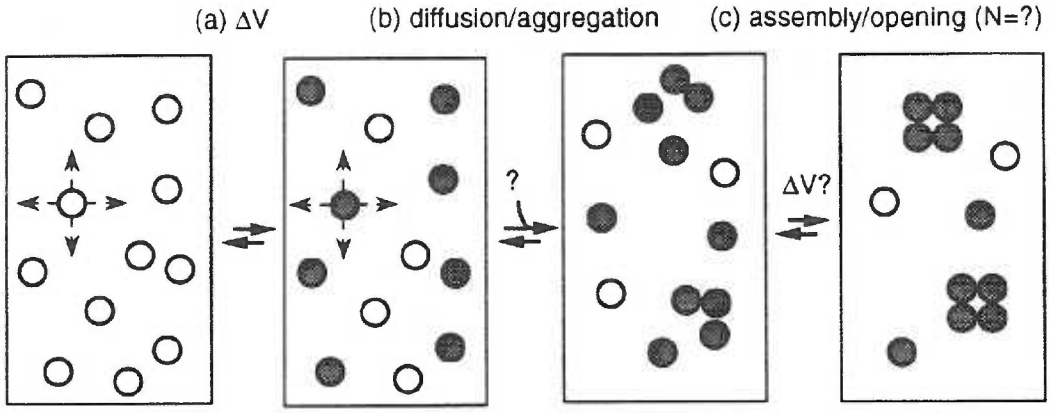


Figure V-1. Model for min K channel gating by subunit oligomerization. Open circles represent non-active min K subunits freely diffusing in the plain of the membrane. Filled circles represent min K subunits whose conformation has been altered by depolarization and are therefore able to aggregate and assemble into functional pores of unknown stochiometry (N). Complete channel complexes that have been tethered together by DTSSP would remain persistently activated. Dimers, trimers, etc. would activate with addition of other subunits, at a more rapid rate than untreated subunits alone; these would deactivate at a rate similar to untreated channels, with the loss of only one untethered subunit.

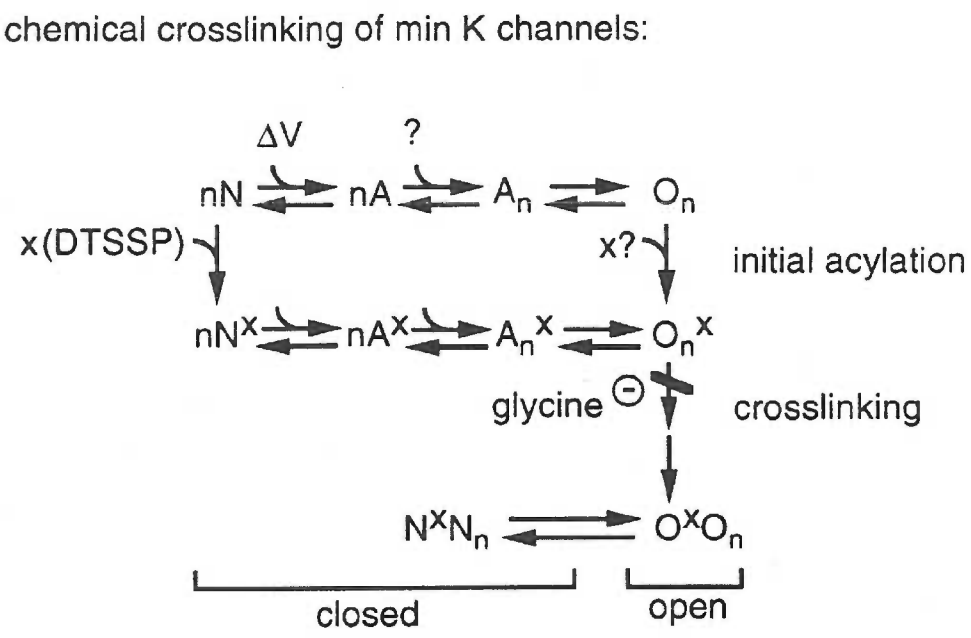


Figure V-2. State diagram model describing modification of min K channel gating by chemical cross-linking. Initially, DTSSP covalently binds (acylation) to "closed" and (perhaps) "open" channels. After depolarization, subsequent binding (cross-linking) of the adjacent subunits of "open" channels is permitted. This grapples these proximal subunits together, holding them in the open conformation (O^xO_n). The existence of a modified closed state (N^xN_n) is implied from experiments demonstrating that extreme hyperpolarizations reduced the persistently activated current (Figure 2B, bottom trace); the instantaneous current recoved fully, however, without subsequent contributions to the time-dependent current.

(n) subunits are cross-linked comprise persistently activated channels. Other complexes, in which fewer than n subunits have been linked, are still activated in a time-dependent manner, but open more rapidly than control channels with the addition of one or more subunits. Such channels, however, deactivate similar to unmodified channels, as deactivation would result from loss of only one subunit.

The aggregation model for min K gating is similar to one proposed for peptide ion channels such as alamethicin (Baumann and Mueller, 1974; Boheim, 1974) and may explain the slow activation kinetics of this structurally and functionally distinct ion channel. This model could account for the susceptibility of the activation kinetics to changes in presumed channel density (Busch et al., 1993c; Blumenthal and Kaczmarek,1993; Varnum, Adelman and Maylie, unpublished) and temperature (Busch and Lang, 1993; Maylie et. al., 1994). The aggregation model offers a reasonable explanation for how a simple protein with only one putative transmembrane domain could form an ion conducting pathway: by voltage- and time-dependent aggregation of multiple subunits. It should also be noted that the possibility exists for endogenous "crosslinking" proteins, such as cytoskeletal elements, to physiologically modulate min K channels.

Alternative models may be proposed to account for these results. DTSSP may chemically modify channel surface electrostatics as it would be expected to abolish the charges of participating amino acid side chains. This is unlikely to explain persistent activation of min K currents since sulfo-NHS acetate was unable to enhance channel activity. Modification of surface charge could, however, be responsible for the change in amplitude of time-dependent currents seen following simple acylation by the monofunctional agent or after application of the crosslinking agent while channels were

held closed. Alternatively, these conditions could affect ion permeation by subtly altering properties of the pore. An indirect effect of DTSSP on min K currents, acting via an endogenous membrane protein in the oocyte, cannot be ruled out at this time. Likewise, the possibility that the min K protein allosterically regulates an endogenous channel (Attila et al., 1993) cannot be excluded by these experiments, as such an interaction might also be stabilized by chemical crosslinking. The persistently activated currents, however, remained selective for potassium ions over chloride ions and were inhibited by clofilium and barium at all potentials tested.

One possible structural model for the min K channel is a parallel bundle of amphipathic α-helices with a pore centered between them. The hydrophobic domain is predicted to be an α-helix (Takumi et al., 1988). Calculation of the mean hydrophobic moment for the membrane-spanning domain, using the Eisenberg scale (Sansom, 1991), indicates that it is only mildly amphipathic ($\mu = 0.105$). The pore region of Shaker channels is also highly hydrophobic; conserved aromatic residues therein are vital to ion selectivity (Heginbotham et al., 1992) via postulated cation-π interactions (Kumpf and Dougherty, 1993). Min K has a conserved sequence containing aromatic residues (...G F F G...) that is vaguely similar to the selectivity sequence of Shaker K^+ channels (...G $^{Y}/_{F}$ G D...) (Heginbotham et al., 1992). One of these residues in min K (F55) has been shown to be important for ion selectivity (Goldstein and Miller, 1991). Thus, one can speculate that min K forms a channel as a homo-oligomeric bundle of subunits, or as a hetero-oligomer with some as yet unidentified protein.

The subunit stochiometry of min K remains unknown. If the effective radius of contributing α-helices (R) is assumed to be between 5 and 15 Å (Chothia et al., 1981),

then the radius of the central pore (r) for a bundle of N helices can be approximated by the formula (Sansom, 1991):

$$r = R[1/(\sin(\pi/N)-1]$$

Estimates obtained in this manner can be compared to the Pauling diameter for K^+ ions (2.66 Å). These calculations suggest that a minimum of four subunits is needed. The upper limit for subunit number depends on several factors, including ion selectivity and the hydration state of permeating ions. Preliminary results suggest that chemical cross-linking can form high molecular weight aggregates of min K which can be visualized by western blotting (Varnum, Maylie and Adelman, unpublished). We hope that work initiated and described in this dissertation (Chapter IV) will offer an effective pathway toward understanding the structural makeup and stochiometry of the min K channel.

It is relevant at this point to speculate as to the molecular basis for the voltage sensitivity of min K channel activation. The S-4 "voltage sensor" domain of Shaker-like channels, a repeated motif of positively charged amino acids spaced every third residue amoung hydrophobic amino acids, is thought to be essential to the voltage dependent conformational changes involved in opening of these channels (Jan and Jan, 1992). Min K lacks the conventional S-4 domain of other K^+ channels. However, min K does present a cluster of positively charged amino acids at the junction betweeen the putative transmembrane domain and the cytoplasmic region (Fig. III-1). This general feature is often associated with determining the topology and orientation of membrane proteins (Dalbey, 1990). Interestingly, neutralization of two of these positively charged residues (R68, K71) significantly attenuates channel activity (Takumi et al., 1991). Unfortunately, these mutations have not been characterized in detail. We have also shown that mutation of Ser 69 within this part of the molecule radically shifted the voltage necessary for channel activation, to more depolarized potentials (Busch et al.,

1992b). This region may constitute part of the voltage sensor of min K. In this regard, conformational charge movement during depolarization and channel activation may involve movement of these positive charges (further) into the membrane, as the inside of the cell becomes less negative, and tilting of the transmembrane domain α-helices during oligomerization and packing of subunits. It is also possible that some conditions or mutations that affect the rate of min K channel activation may do so by altering these charge movements or by affecting the stability of the aggregated/open state. These possibilities merit future investigation into the structural features responsible for the voltage dependence of min K activation and the nature of potential gating charge movements.

CONCLUSIONS

In summary, this dissertation offers two major findings contributing to our understanding of (1) the potential physiological role of the min K protein in cardiac function, and (2) the mechanism by which min K may form slowly-activating potassium channels. First, isolation and expression of a clone encoding the guinea pig min K protein, and the characterization of its pharmacological and regulatory properties, supports the hypothesis that the min K channel underlies the slow component of the delayed rectifier potassium conductance in cardiac myocytes. The molecular basis for the species-specific responses to protein kinase C are now understood at the structural level, being due to amino acid differences between min K proteins. Second, chemical cross-linking induced persistent activation of min K currents. These results suggest that a major conformational change occurs during min K channel activation which can be stabilized by cross-linking, and support a model in which min K channels activate by voltage-dependent subunit aggregation.

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COMPILATION OF THESIS PUBLICATIONS AND MANUSCRIPTS

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