Instituto Juan March
de Estudios e Investigaciones

22 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Molecular Bases of Ion Channel Function

Organized by

R. W. Aldrich and J. López-Barneo

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C. M. Armstrong
P. Ascher
K. G. Beam
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A. Castellano
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INTRODUCTION

J. López-Barneo

Ion channels are ubiquitously distributed membrane proteins involved in almost every cellular function. They have special importance in medicine because they participate in many pathophysiological conditions and are the target of drugs of common clinical use such as, for example, psychotropic, antiarrhythmic, and antihypertensive drugs. In the past few years the rate of progress in the knowledge of the structure and function of ion channels has been impressive. Experimental work has been boosted thanks to the development of the patch clamp technique and to the application to ion channel research of concepts and methodologies brought from the field of molecular biology. Several ion channels have been cloned and expressed as functional proteins and, as a consequence, a picture about the molecular mechanisms underlying channel gating, selectivity, and modulation is beginning to emerge.

The aim of this workshop on the "Molecular Bases of Ion Channel Function" was to contribute to international scientific communication in a rapidly growing field. The major goal was to favor the interchange of new ideas, experimental results, and concepts among scientists having a common interest in ion channels. The meeting is divided in six sections focusing on the structure-functional relationships of ligand- (section I) and voltage-gated (sections II-IV) channels, the participation of ion channels in specific cellular functions (section V), and the genetic regulation of ion channel expression and functional properties (section VI). Besides the lectures of invited speakers, the workshop also included a few oral communications and the exhibition of about 20 posters with the work of the selected applicants.

I. Ligand-Gated Channels

MOLECULAR DETERMINANTS OF Ca^{2+}INFLUX IN AMPA AND NMDA RECEPTOR CHANNELS. B. Sakmann

The lecture addresses the question of how Ca^{2+} is entering the postsynaptic cell through glutamate gated ion channels in the postsynaptic membrane of excitatory synapses in the CNS and the work is the result of a combined effort of physiologists and molecular biologists. Glutamate is the major excitatory transmitter and acts on at least two classes of receptor channels -designated as AMPAR and NMDAR channels -according to two specific agonists which activate selectively these receptors. Both receptors are probably colocated in the subsynaptic membrane in most CNS synapses. Both channels are thought to be Ca^{2+} permeant to different extents and presumably the Ca^{2+} inflow through these channels is a signal for cellular responses that occur on a much slower time scale -such as stabilisation of synapses or changes in synaptic efficacy of excitatory synapses. Native AMPA and NMDAR channels in membrane patches isolated from the soma and dendrites of neurones in rat brain slices show a large diversity in their Ca^{2+}/Cs^{+} reversal potentials ranging from -50mV to +20mV in biionic conditions. Experiments with recombinant AMPA and NMDAR channels have identified single amino acids in two GluR channel subtypes which regulate the inflow of Ca^{2+}. These amino acids are at homologous positions in the putative M2 transmembrane segments of the constitutive receptor subunits. For AMPAR channels the experiments based on Ca^{2+}/Cs^{+} *reversal potential* measurements in biionic conditions suggest that there are two mechanisms which may control Ca^{2+} inflow through AMPAR channels: 1. Abundance of GluR-B mRNA relative to the level of the other GluR subunit mRNAs. GluR channels with different Ca^{2+} permeability. 2. Editing of GluR-B mRNA. Measurements of *fractional* Ca^{2+} *currents* of recombinant channels show similar differences between AMPAR subunits as expected from reversal potential measurements. For recombinant NMDAR channels, based on reversal potential measurements, no difference in the Ca^{2+} permeability between channel subtypes was found. It is more likely that the amount of Ca^{2+} inflow through various channel subtypes is regulated by subunit specific differences in the Mg^{2+} block as particular subunit combinations show large differences in Mg^{2+} block. Amino acids responsible for Ca^{2+} selectivity are also responsible for Mg^{2+} block. Those amino acids responsible for differences between subtypes have not yet been

identified. Surprisingly fractional Ca^{2+} current measurements indicate that the N2A and N2C subunits confer differences in fractional Ca^{2+} currents. Mapping of the size of the cross-sectional area of the channels constrictions with organic cations reveals that the cross-sectional area of AMPAR channels is about twice as larger as that of NMDAR channels.

FUNCTIONAL GLUTAMATE RECEPTORS OF THE KAINATE TYPE IN HIPPOCAMPAL NEURONES

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Glutamate receptors mediate fast synaptic transmission at the majority of synapses in the vertebrate central nervous system. Different types of glutamate receptors coexist in the same synaptic contact and they have been classified from a pharmacological point of view as of the N-methyl-D-aspartate (NMDA) type and of non-NMDA type (1,2). The so-called non-NMDA receptors include AMPA receptors, which were initially characterized by rapid activation kinetics and fast desensitization, and kainate receptors which were differentiated from AMPA receptors in that they were non-desensitizing. However, molecular biology studies have provided recently new insight into the functional and structural diversity of NMDA and non-NMDA type of glutamate receptor channels. These studies have indicated that all glutamate receptor channels may be assemblies from structurally homologous subunits, which can be grouped into families according to sequence characteristics (3). On the basis of ligand affinity, the GluR-A to D (GluR-1 to 4) subunits are constituents of AMPA receptor channels, while the GluR-5, GluR-6, GluR-7 and KA subunits form recombinant receptors with high affinity for kainate. However, kainate gates AMPA receptors and AMPA can activate receptor channels formed by particular combinations of the high-affinity kainate receptor subunits. Thus, the functional distinction between AMPA and kainate receptors presently is unclear and the existence of pure kainate or AMPA receptors in the brain has been questioned. In autoradiographic experiments, the regional distribution of high affinity [^{3}H]-kainate binding sites does not match the AMPA receptors distribution, but corresponds well to the brain areas with high susceptibility to the neurotoxic actions of kainate (e.g. hippocampal CA3 field). However, patch-clamp recordings from adult hippocampal neurones have revealed that native glutamate receptors are similar to the AMPA type recombinant glutamate receptors expressed from cDNA clones but have failed so far to detect receptor-channels of the kainate type.

We have determined the existence of glutamate receptors in hippocampal neurones which are selectively activated by kainate. Kainate-evoked responses undergo rapid and total desensitization, the recovery from which is considerably slow. Other glutamate receptor agonists like glutamate, quisqualate or domoate are also able to gate kainate-selective receptors. AMPA, even at high concentrations, does not activate such a current. The kainate-selective receptor has a much higher affinity for kainate than has the AMPA type of receptor. The fast desensitization of kainate-selective receptors may induce to overestimate the value for half-maximal activation in dose-response curves. However, the EC_{50} value for kainate was 10-fold lower when calculated from data obtained in cells with desensitizing responses than when estimated from cells showing non-desensitizing responses to

kainate. Glutamate receptors with a similar affinity and with desensitizing properties when activated by kainate have been observed in dorsal root ganglion (DRG) cells in culture (4). However, unlike DRG cell receptors, the type of high-affinity kainate receptors we found in hippocampal cells: 1- are not activated by AMPA, 2- are totally desensitized in the presence of ligand, 3- exhibit fast and monoexponential desensitizing kinetics, 4- are less sensitive to CNQX than AMPA receptors, 5- coexist with AMPA receptors and 6- they probably represent GluR-6 homomeric assemblies (see below).

It is important to determine the molecular identity of kainate-selective receptor channels in native membranes. The pharmacological profile, as well as the fast and the total desensitization of kainate-selective receptors are consistent with properties observed in GluR-6 homomeric assemblies expressed in mammalian cells. GluR-6 subunits form homomeric channels which are the only receptor channels gated by kainate but not by AMPA. In addition, AMPA does not cross-desensitize homomeric GluR-6 receptors. Furthermore, recent data from the expression of GluR-6 subunits show that editing of TM1 may affect divalent ion permeability (5). While the Q/R site in TM2 determines the rectification properties, in the same way as for the AMPA receptors, the Ca^{2+} permeability of homomeric GluR-6 channels is changed in the opposite way. Expression of the GluR-6(Q) variant edited in the TM1 generates channels with very low Ca^{2+} permeability but with a prominent inward rectification. We have found cells expressing kainate-selective receptors with these same properties. This result strongly suggests that the glutamate receptors selective for kainate expressed by hippocamapal cells correspond to assemblies of GluR-6 subunits. In keeping with this suggestion we have found that about 20% of the cells in our cultures express the GluR-6 gene, as determined by *in situ* hybridization. In addition, reverse transcriptase-PCR analysis using RNA harvested from single cells exhibiting prominent peak responses to kainate, resulted in an intense hybridization signal with a GluR-6 probe.

In summary, our results provide evidence for the existence in hippocampal neurones of a functional glutamate receptor with high affinity for kainate, not sensitive to AMPA. Our data strongly suggest that homomeric GluR-6(Q) assemblies form functional kainate-selective receptors in neuronal membranes. The functional significance of this novel glutamate receptor is not known so far.

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Ca-permeability of neuronal glutamate receptor channels

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Ionotropic glutamate receptors represent the major class of excitatory postsynaptic receptors in neurons of the central nervous system. These receptors are coupled to non-specific cation channels permeable to monovalent Na^+ and K^+ ions, but also to divalent Ca^{2+}-ions. The Ca^{2+}-fraction of the total cation current is different for the various types and subtypes of ionotropic glutamate receptors. A particularly high Ca^{2+}-permeability was reported first for N-methyl-D-aspartate-receptor channels (NMDA-R) (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). More recent reports indicated that also a few subtypes of AMPA/kainate-receptor channels (AMPA/kainate-R) are highly permeable for Ca^{2+} (for example Iino et al. 1990; Keller et al., 1992). The Ca^{2+}-permeable AMPA/kainate-R are characterized in addition by a rectifying current-voltage relation (Hollmann et al., 1992, Burnashev et al., 1993) and a specific pharmacological profile (Blaschke et al., 1993).

A quantitative evaluation of the relative contribution of the Ca^{2+}-flux through ligand-gated channels is usually achieved by calculating permeability ratios for Ca^{2+} over monovalent ions from reversal potential measurements in ion substitution experiments. These measurements provide values of permeability ratios under conditions close to the current reversal, but they cannot readily be extrapolated to predict fluxes at substantial driving forces, which is the physiologically more interesting case. Therefore we introduced an alternative approach (Schneggenburger et al., 1993a, Schneggenburger et al., 1993b) by which the Ca^{2+}-fraction of the ion current flowing through glutamatergic NMDA- and AMPA/kainate-R is determined by overloading cells with the Ca^{2+}-indicator dye fura-2 (1

mM) via the recording patch-pipettes. By using this approach we found in forebrain neurons of the medial septum that at negative membrane potentials and at an physiological extracellular free Ca^{2+}-concentration (1.6 mM), the Ca^{2+}-fraction of the current through the NMDA-receptor channels is only 6.8 %. This value is about two-fold lower than that previously estimated from reversal potential measurements. The fractional Ca^{2+}-current through linearly-conducting AMPA/kainate-R channels was 1.4 %, and thus only 5-fold smaller than that through NMDA-R channels. Similar studies were carried out also in CA1 hippocampal pyramidal (Garaschuk, Tempia, Schneggenburger, Konnerth, manuscript in preparation) and in cerebellar Purkinje neurons (Tempia, Kano, Konnerth, submitted for publication). In the hippocampal neurons we found NMDA-R with a fractional Ca^{2+}-current similar to that seen in forebrain neurons. Interestingly, no Ca^{2+}-influx through AMPA/kainate-R was detected neither in hippocampal pyramidal nor in cerebellar Purkinje neurons.

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Patch-clamp studies of the NMDA receptor-channel
Methodological problems

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The NMDA receptors constitute one of the main families of glutamate receptors in the central nervous system of Vertebrates, where they are present at the majority of the excitatory synapses. Originally defined pharmacologically by their selective activation by N-methyl-D-aspartate, NMDA receptors have been found to possess a series of singular molecular properties. Some of these properties express peculiarities of the ionic channel opened by glutamate : the best known are the voltage dependence of the conductance (due to Mg block) and the high calcium permeability of the open channel. Other atypical properties concern the mechanism of activation and deactivation, and in particular the slow deactivation (due to the slow dissociation of glutamate) which accounts for the slowness of the decay of the NMDA component of synaptic currents. Finally the NMDA receptor is unusual by the fact that it is modulated by a variety of agents other than L-glutamate, such as glycine, external pH, Zn, reducing and oxidizing compounds, spermine, protein kinase C, to quote only a few (see Watkins and Collingridge, 1989).

A large part of the progress made in the last ten years in the study of the NMDA receptor has been linked to the application of patch-clamp techniques to individual neurons. The NMDA responses have been studied in all the classical patch-clamp configurations : classical whole-cell, perforated whole-cell, inside-out, outside-out, cell-attached. In 1990, we introduced a new configuration, called the "nucleated patch" (see Sather *et al.*, 1992) which we hoped would be able to explain some of the discrepancies between the data obtained with some of the other configurations.

To obtain a nucleated patch a pipette is first sealed on the cell surface. The membrane facing the pipette tip is ruptured by succion, and the pipette is then pulled away while the succion is maintained. During this phase, the nucleus of the cell usually sticks to the pipette tip. As the nucleus is progressively extracted from the neuron, it remains surrounded by plasma membrane. Eventually the link between this membrane and that of the rest of the cell will

break, leaving at the end of the pipette a structure which is a "giant" outside-out patch surrounding a nucleus.

In our first use of desensitization of NMDA receptors such nucleated patches, we were able to solve some of the contradictions surrounding two patterns ("glycine-sensitive" and "glycine insensitive"). We showed that, just after the patch has been formed, the kinetics of the responses resemble those observed in the whole cell mode. In the next 30 minutes, however, the kinetics evolve towards that observed in classical "outside-out" patches.

More recently, we have discovered that in nucleated patches the nucleus is less inert than one could initially suspect. In a study where external applications of ammonium chloride or sodium acetate were used to alter the "intracellular" pH, we observed major changes in the NMDA responses which could not be reproduced when similar applications were made in the whole cell recording mode. A detailed analysis of the results has led us to suspect that the effects of changes in internal pH are actually mediated through a volume change of the nucleus (see Oberleithner *et al.*, 1993) and that intracellular pH has no direct effect on NMDA response. This reevaluation led us to identify a new property of the NMDA receptor, namely its sensitivity to membrane stretch. The experimental data supporting this stretch sensitivity will be presented at the meeting with some speculations on their possible functional significance.

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MOLECULAR CLONING, FUNCTIONAL EXPRESSION AND ALTERNATIVE SPLICING OF THE α7 SUBUNIT OF THE NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR FROM CHROMAFFIN CELLS.

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Introduction

The nicotinic acetylcholine receptor (AChR) is a cation-specific ion channel whose activity is regulated by acetylcholine. Receptors of neuronal and muscular origin are both composed of related subunits that, expressed in various combinations, determine different channel properties. α-Bungarotoxin blocks specifically and with high affinity muscular nicotinic receptors, but not neuronal receptors, even though binding sites for the toxin exist in the central and perypheral nervous systems. Recently, the molecular cloning of neuronal subunits able to assemble channels activated by cholinergic agonists and blocked by α-bungarotoxin (termed α7 and α8; see *Schoepfer R., et al (1990) Neuron 5, 35-48; Couturier, S., et al (1990) Neuron 5, 847-856*), has opened the way for the study of the structure, function and localization of these α-bungarotoxin binding proteins. In an attempt to understand the function of what seems to be a particular subtype of neuronal AChRs, we have chosen the chromaffin cell of the adrenal medulla as a model system. In the adrenal gland these cells, which derive from the neural crest, are cholinergically innervated from the sympathetic nervous system through the splanchnic nerve. Acetylcholine is released upon stimulation of this nerve and activates AChRs in chromaffin cells. The subsequent depolarization trigger catecholamine secretion. However, this functional response is not blocked by α-bungarotoxin, although the existence of α-bungarotoxin binding sites in chromaffin cells is well established. It is evident that two receptor subtypes are expressed, one being involved in the activation step of the secretory process and the second, able to bind α-bungarotoxin, having a function not yet determined.

Molecular cloning of the α7 subunit

In order to characterize the α-bungarotoxin binding component from chromaffin cells, we first tried to clone the subunit(s) that may be composing this receptor. By using PCR and Northern blot analysis we were able to show that the α7 subunit was being expressed in these cells. Further use of PCR techniques allowed us to clone the complete protein coding region of this subunit. The bovine α7 polypeptide is composed of 499 amino acids and share 92 and 89% homology (excluded the leader peptide) with its rat and chicken counterparts, respectively. Sequence homology with other neuronal AChR subunits is drastically reduced.

Functional expression

Expression of the cloned subunit was attempted in two different systems: *Xenopus leavis* oocytes and transfected neuroblastoma cells.

α7 subunits expressed in oocytes after injection of the corresponding cRNA, form cation channels that are activated by nicotinic agonists, desensitize very fast and are completely blocked by α-bungarotoxin. d-Tubocurarine was also found to block channel activity. A significant proportion of the current through α7 channels is carried by Ca^{2+}, since Ca^{2+} influx activated a Ca^{2+}-dependent chloride channel endogenously expressed in Xenopus oocytes. Interestingly, there was no correlation between the number of α7 receptors present in the oocyte membrane, as measured by α-

bungarotoxin binding, and the current produced by them: toxin binding sites seem to outnumber functional channels. The reason for this discrepancy is not clear, given that the α-bungarotoxin binding proteins that reach the oocyte membrane appear to be assembled in the proper way and, on the other hand, the amount of current produced is similar to the one obtained by others with chicken and rat α7 AChRs.

Expression of α7 subunits was also tested in transfected neuroblastoma cells (neuro-2a cells). For this purpose the cDNA coding for the α7 subunit was cloned into an eukariotic expression vector under the control of a heat shock promoter and transfected. Cells were selected with geneticin and two clones were further characterized. After induction of α7 expression, the corresponding mRNA was produced but neither functional channels nor binding of α-bungarotoxin to the cells could be detected. However, if after α7 induction the cells were kept at 27°C instead of the usual 37°C, binding sites for α-bungarotoxin appeared at the cell membrane. This dependence on culture temperature for a proper expression has been previously observed with the *Torpedo* AChR subunits, and may be related to a decrease in the production of incorrectly assembled protein aggregates when the temperature is lowered.

Alternative splicing

During PCR cloning of the α7 subunit from chromaffin cell mRNA, the presence of a minor DNA species, slightly smaller than the predominant α7 DNA, was repeatedly observed. The sequence of that DNA was determined and turned out to be identical to the predominant band except for a stretch of 87 nucleotides, that were missing and roughly coded for the M2 transmembrane region of the protein. Analysis of α7 genomic clones revealed that the missing stretch corresponded to a complete exon that was being skipped during mRNA splicing. Interestingly, this alternative transcript maintained the reading frame, so that a complete protein, not truncated, would be translated, At difference with the original α7 polypeptide, the alternative form would just lack the M2 transmembrane region. The corresponding cRNA was injected in oocytes and, as expected, neither functional channels nor α-bungarotoxin binding sites were detected at the oocyte membrane. Trying to mimic the situation that could be taking place "in vivo", we co-injected full-length and alternative RNAs. The result was a marked inhibition of α7 expression, again at the level of channel activity and toxin binding sites, suggesting that the alternative polypeptide was being translated and therefore, interfering with the assembly and surface expression of the complete α7 polypeptide. Our hypothesis is that the alternative polypeptide, though lacking the M2 transmembrane segment, contain the necessary structural information to be recognized by the full-length subunits, assembling with them and giving rise to "unproductive" complexes. "In vivo", this situation could lead to the regulation of the genetic expression of the α7 receptor, constituting a naturally occurring example of what has been called "dominat negative mutations" (*Herskowitz, I. (1987) Nature 329, 219-222).*

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II. Voltage-Gated Channels (Potassium Channels)

The Structure and Function of Voltage Dependent Ion Channels.
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In the last decade enormous progress has been made in understanding voltage dependent ion channels, which are found almost everywhere in the body, and are involved in activities as diverse as transmission of the nerve impulse, the timing of the heartbeat, and the control of secretion. Both Na^+ and K^+ channels have been cloned (Noda et al., 1984; Tempel et. al., 1987) from many sources. The sodium channel is formed of a single peptide, with four repeating 'domains'(Noda et al 1984; Guy and Seetharamulu, 1986; Catterall,1988) while the K^+ channel can be formed by the aggregation of four identical peptides, each about one-fourth the size of the Na^+ channel (Tempel et. al., 1987; MacKinnon, 1991) Within each K^+ channel peptide and each domain of the Na^+ channel, there are six membrane crossings, identifiable by their hydrophobicity. One of these is a positively charged helix, called S4, with arginine (R) or lysine (K) at every third position. The S4 helix is, so far, unique to voltage sensitive channels, and is almost certainly the major element in their voltage sensitivity. The S4 units presumably move outward when V_m goes positive. For energetic reasons there must be negative residues that are charge-paired to the R's and K's of S4 (Armstrong, 1981). These negative charges have not yet been clearly identified, although there are detailed folding models of the channels (Guy and Seetharamulu, 1986; Stuart and Guy, 1992).

The ion conduction path, the pore, has been almost certainly identified in both the K^+ (MacKinnon and Miller, 1989; MacKinnon and Yellen, 1990; Yellen et. al., 1991; Hartmann et. al., 1991) and the Na^+ channel (Noda et. al., 1989; Heinemann et. al., 1992). It lies in the residues linking S5 and S6, which dip into the membrane and reemerge, making two additional partial crossings called SS1 and SS2 (Guy and Seetharamulu, 1986). This region is well conserved within each channel type, but has differences among the various types that are presumably related to selectivity. Ca^{2+} channels, for example, have a positive (K1422,) or neutral (A1714) residue in SS2 of two domains where the Ca^{2+} channel has a glutamate. The point mutation K1422E in the Na^+ channel confers on it some of the characteristics of a Ca^{2+} channel (Heinemann et. al, 1992).

The molecular structure of the inactivation gate has been clearly identified in Shaker B channels. The first 20 residues at the N terminus are the inactivating particle or ball, and the next 60 residues form a flexible chain to hold the ball in place and allow it to diffuse into the mouth of the open channel (Hoshi et. al., 1990; Zagotta et. al. 1990). There is evidence that the ball, in inactivating position, binds to the S4-S5 linker (Isacoff et. al. This linker may be pulled out of channel-blocking position when S4 moves outward, opening the channel mouth (Durell and Guy, 1992).

What is missing from this picture? Some questions to be discussed are the following. 1) First, the identity of the negative residues that charge pair the to the arginines and lysines of S4 is not certain, as mentioned. A question that may be related is the ion occupancy of the channel, particularly in the closed state. Ion

occupancy, enters the question because any cation in the closed channel is part of the charge balance equation, and would likely be paired to a negative residue. 2) Second, it is not clear that the S4's alone can provide enough voltage sensitivity (that is enough 'equivalent charge movement', or Q); and even the target in this case is uncertain, with Q estimates varying from 4 to 12 e_o (Schoppa et. al., 1992; Logothetis et.al., 1992; Liman and Hess, 1992). 3) There are clear indications that the last step in activation of the channel is unlike the earlier steps. Perhaps the first hint of this came from gating current measurements, which showed the last step in Na channel activation to be slower and more V dependent. Experiments in Shaker B (Zagotta and Aldrich, 1990) also can best be fit with a slow step near the end of the activation chain. Finally, a mutant Shaker K channel, L370V shows a fascinating separation between the early and the late part of the activation sequence: the early steps, as judged from gating current records, occur in the normal range, while the last step occurs only when V_m is driven very positive Schoppa et.al.,1992). 4) Both Na and K channels can inactivate before they conduct. The significance of this 'closed state' inactivation is not clear. Also unclear are the mechanisms that allow Na channels to recover from inactivation without leaking Na ions. 5) The effects of divalent cations on channel gating are only partially known and partially understood.

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Activation of the Shaker Potassium channel.

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The voltage dependent activation of the *Shaker* potassium conductance was studied in channels expressed in *Xenopus* oocytes after injection of cRNA. We used the H4-IR variety of the *Shaker* B K channel that has a deletion of amino acids 6 to 46 to eliminate inactivation (2) and charge immobilization (1). In addition we used the W434F mutation that eliminates ionic conduction without altering gating (3). The cut-open oocyte (4) and the patch clamp techniques were used to measure macroscopic ionic currents, gating currents and gating current fluctuations. The gating currents recorded with the cut-open oocyte technique were virtually identical in their kinetics and steady-state properties to the currents recorded in macropatches as long as the macropatches were in the cell attached mode.

The charge vs. potential (Q-V) curve is shifted to the left with respect to the conductance vs potential (G-V) curve and it is not a simple Boltzmann distribution. Two Boltzmann distributions, Q_1 with a valence of about 2 and a center point of -63 mV and Q_2 with a valence of 5 and a center point of -44 fit the data quite well. A kinetic analysis of the gating currents also show two components which have the voltage dependence of the steady state components. Q_1 correlates with the effects of prepulses that give origin to the Cole-Moore shift of the ionic and gating currents. Q_2 is correlated with the events occurring between closed states just preceding, but not including, the final transition to the open state. When Q_2 develops, there is a pronounced rising phase in the onset of the gating current consistent with the movement of a large, very voltage dependent, charge movement following an initial less voltage dependent process. The reset of the gating current only shows a rising phase when Q_2 has been activated indicating that the transition from the open to the adjacent closed state is less voltage dependent.

The two components of gating cannot be considered as two separate charge systems because they do not behave independently. Models that assume the two charges moving independently but interacting in the last step do not fit the data. We have proposed a model that consists of several steps between closed states carrying less than 2 elementary charges in each step followed by a transition carrying about 3.5 charges and a final transition that opens the channel carrying only 0.5 charges. The series of early transitions account for Q_1 and the last few for Q_2.

Analysis of gating current noise reveals that there clear and resolvable fluctuations when the charge is moving between -50 and 20 mV with an estimated valence of about 2.7 elementary

charges. The fluctuations are undetectable during the peak of the gating current when pulsing from -110 mV to around -50 mV and the variance starts increasing later in the decaying phase of the gating current. These results confirm that in the activation sequence there are several steps carrying a small amount of charge (Q_1) followed by a transition that carry a large amount of charge (Q_2), just before the channel opens. The series of early steps may be the cooperative contributions of the channel subunits while the final steps may be interpreted as the large synchronous rearrangement of the channel molecule moving a large amount of charge that leads to the final step of channel opening.

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Mechanism of Voltage-Dependent Gating in Potassium Channels

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Voltage-dependent ion channels respond to changes in the electric field across the membrane by undergoing conformational changes that open an ion permeable pore. This activation process requires a number of conformational transitions in the channel protein. The molecular mechanism that underlies these conformational changes, however, remains unclear. Recently cDNA clones have been isolated and the primary structure determined for a number of different voltage-dependent ion channels (for reviews see Hoshi and Zagotta, 1993; Stuhmer and Parekh, 1992; Tsien et al., 1991). These channels all possess four subunits, or pseudo-subunits, each of which contains a positively charged S4 segment. This region has been proposed to span the membrane and represent the voltage sensor for the voltage-dependent transitions. The presence of multiple similar, or identical, subunits in each channel suggests a possible physical mechanism for the activation conformational changes whereby opening of the channel might require separate voltage-dependent conformational changes to occur in some or all of the subunits. We have studied the activation gating mechanism of voltage-dependent potassium channels by analyzing single-channel ionic currents, macroscopic ionic currents, and gating currents (Hoshi et al., 1993; Zagotta et al., 1993a; Zagotta et al., 1993b). The channels were expressed from the Shaker cDNA clone in ooctyes, and the inactivated process in these channels was selectively removed by a mutation in the channel gene that deletes a large part of the amino terminal domain (Hoshi et al., 1990).

The analysis of single-channel currents provided a direct estimate of the number of open and closed conformations entered after first opening and of the rate and voltage-dependence of the transitions between these conformations. Comparison of the single-channel first latency distribution and the time course of the ensemble average current showed that the activation time course and its voltage dependence are largely determined by the transitions before first opening. The open dwell time data were consistent with a single kinetically distinguishable open state. Once the channel opens, it can enter at least two closed states which are not traversed frequently during the activation process. The rate constants for the transitions among these closed states and the open state are nearly voltage-independent at depolarized voltages (>-30 mV). During the deactivation process at more negative voltages, the channel can close directly to a closed state in the activation pathway in a voltage-dependent fashion.

We have utilized steady-state and kinetic measurements of the ionic and gating currents to identify several general properties of the activation gating mechanism that must be accounted for by any kinetic model. The voltage dependence of the steady-state open probability indicated that the activation process involves the movement of the equivalent of 12 to 16 electronic charges across the membrane. The sigmoidal kinetics of the activation process, which is maintained at depolarized voltages up to at least +100 mV indicated the presence of at least 5 sequential conformational changes before opening. The voltage dependence of the forward transition rates was less than the voltage dependence of the reverse transition rates suggesting that most of the charge movement occurs after the transition state for each conformational change. These results were generally consistent with models involving a number of independent and identical

transitions with a major exception that the first closing transition is slower than expected.

Predictions of different classes of gating models involving identical conformational changes in each of four subunits were compared to the gating behavior of Shaker potassium channels without N-type inactivation. Each model was tested to see if it can simulate the voltage dependence of the steady-state open probability, and the kinetics of the single-channel currents, macroscopic ionic currents and macroscopic gating currents using a single set of parameters. Activation schemes based upon four identical single-step activation processes were found to be incompatible with the experimental results, as were those involving a concerted, opening transition. A model where the opening of the channel requires two conformational changes in each of the four subunits can adequately account for the steady-state and kinetic behavior of the channel. In this model, the gating in each subunit is independent except for a stabilization of the open state when all four subunits are activated, and an unstable closed conformation that the channel enters after opening.

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Conformational Changes Involved in Shaker K+ Channel Gating

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The fast inactivation of Shaker potassium channels is probably the best understood conformational change involved in ion channel gating. Previous studies have provided evidence for a mechanism whereby inactivation (N-type) of Shaker K channels occurs by an intracellular portion of the channel, consisting of amino acid residues near the amino terminal, occluding the internal mouth of the channel. A crucial piece of evidence for this mechanism was that a synthetic peptide with the sequence of the amino-terminal domain was able to restore inactivation in channels that did not inactivate because of mutations in the amino terminal region. We have used these inactivation peptides and site-directed mutagenesis of the the amino terminal inactivation domain to investigate the biophysical mechanisms of the interaction between the N-terminal inactivation domain and its receptor site at the inner mouth of the channel.

Synthetic peptides of the 5 alternative NH_2-terminal sequences of Shaker when applied to the cytoplasmic side of ShB channels that have an NH_2-terminal deletion (ShBD6-46) block the channel with potencies correlated with the rate of inactivation in the corresponding variant. These peptides share no sequence similarity and yet 3 out of the 5 have affinities between 2 and 15 μM, suggesting that the specificity requirements for binding are low. To identify the primary structural determinants required for effective block of ShBD6-46, we examined the effects of substitutions made to the 20 residue ShB peptide on association and dissociation rates. Non-polar residues within the peptide appear to be important in stabilizing the binding through hydrophobic interactions. Substitutions to leucine-7 showed there was a clear correlation between hydrophobicity and the dissociation rate (k_{off}) with little effect on the association rate constant (k_{on}). Substituting charged residues for hydrophobic residues within the region 4 - 8 disrupted binding. Within the COOH-terminal half of the peptide, substitutions that increased the net positive charge increased k_{on} with relatively small changes in k_{off}, suggesting the involvement of long-range electrostatic interactions in increasing the effective concentration of the peptide. Neutralizing charged residues produced small changes in k_{off}. Charges within the region 12-20 act equivalently; alterations which conserved net charge produced little effect on either k_{on} or k_{off}. The results are consistent with this region of the peptide having an extended conformation and suggest that when bound this region makes few contacts with the channel protein and remains relatively unconstrained. Analogous mutations within the NH_2-terminal domain of the intact ShB channel produced qualitatively similar effects on blocking and unblocking rates.

To investigate the role of electrostatic interactions in both peptide block and the inactivation process we measured the kinetics of block of macroscopic currents

recorded from the intact ShB channel, and from ShBD6-46 channels in the presence of peptides, at different ionic strengths. The rate of inactivation and the association rate constants (k_{on}) for the ShB peptides decreased with increasing ionic strength. k_{on} for a more positively charged peptide was more steeply dependent on ionic strength consistent with a simple electrostatic mechanism of enhanced diffusion. This suggests that a rate limiting step in the inactivation process is the diffusion of the NH_2-terminal domain towards the pore. The dissociation rates (k_{off}) were insensitive to ionic strength. The temperature dependence of k_{on} for the ShB peptide was very high, ($Q_{10} = 5.0 \pm 0.58$), whereas k_{off} was relatively temperature insensitive ($Q_{10} \approx 1.1$). The results suggest that at higher temperatures the proportion of time either the peptide or channel spends in the correct conformation for binding is increased. There were two components to the time course of recovery from block by the ShB peptide, indicating 2 distinct blocked states, one of which has similar kinetics and dependence on external K^+ concentration as the inactivated state of ShB. The other is voltage-dependent and at -120 mV is very unstable. Increasing the net charge on the peptide did not increase sensitivity to knockoff by external K^+. We propose that the free peptide, having fewer constraints than the tethered NH_2-terminal domain binds to a similar site on the channel in at least two different conformations.

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III. Potassium and Non Selective Channels

POTASSIUM CHANNELS ACTIVATED BY HYPERPOLARIZATION

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Inward-rectifier channels open in respone to hyperpolarization and they play important roles in maintaining normal resting potential. The KAT1 channel from *Arabidopsis* shows many inward-rectifier like properties. The deduced amino acid sequence shows that the structural organization of the KAT1 channels is similar to that of the *Shaker* channel activated by depolarization.

Although the KAT1 channel opens in response hyperpolarization, I find that the kinetic model developed for the Shaker channel well describes its gating properties. The KAT1 channel voltage dependence is not dependent on the intracellular Mg^{2+}. The structural organizational similarity between the KAT1 and Shaker channel manifests in the functional similarity as well.

Many experiments have shown that the ion channel activity can be regulated by various intracellular factors. Experimentally, this regulation of the channel activity by intracellular factors can be often observed as the difference in the channel behavior in the cell-attached configuration and in the excised patch-clamp configuration. In the cell-attached configuration, the intracellular factors are present to regulate the channels whereas in the excised configurations, these factors diffuse away and the regulation is lost, often resulting in the loss of the channel activity called "rundown". The KAT1 channel also shows this rundown phenomenon when the patch is excised. After the patch excision, the hyperpolarizing pulses elicit progressively smaller currents. The KAT1 channel activity can be restored by "cramming" the patch back into the oocyte interior, presumably exposing the channel to the required intracellular factors. I have identified the intracellular factors necessary for the KAT1 channel behavior. The results suggest that the forward kinetic transitions in the KAT1 channel are directly dependent on the agonist concentration. Therefore, the KAT1 channel can be considered as a voltage- and agonist activated inward rectifier channel.

INTERACTIONS OF IONS AND PEPTIDES WITH A Ca^{+2}-ACTIVATED K^+ CHANNEL.

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The following studies are an attempt to further increase our knowledge of the molecular characteristics of the conduction machinery of large conductance Ca^{2+}-activated K^+ channels.

The large conductance Ca^{2+}-activated K^+ (K_{Ca}) channel, is strongly blocked by internally added Ba^{2+}. By interacting with the conduction system of the channel, Ba^{2+} not only hindered the passage of ions, but also modified the channel gating (Miller et al. 1987); when a Ba^{2+} occupies the channel pore it takes more energy to close the channel. Interestingly, the modulatory effect of Ba^{2+} on channel gating is in turn regulated by external K^+. The Ba^{2+} blocked-open state is stabilized at K^+ concentrations larger than 5 mM (Miller et al. 1987) whereas in the absence of external permeant ions stabilization of the blocked-close configuration of the channel is found (Neyton and Pelleschi, 1991). These results strongly suggest the existence of a K^+-binding site located towards the external end of the K_{Ca} channel pore able to control the Ba^{2+} blocking reaction. We have restudied the effect of external K^+ on Ba^{2+} blockade and found that increasing the external monovalent ion concentration increases both the time the channel dwells in the blocked state *and* the time the channel remains in a burst of activity. We have interpreted these results assuming that an open channel (O) may acquire the following configurations: O, O-Ba (absence of K^+) or O-K, O-K-Ba (presence of external K^+). Once the channel binds the monovalent cation, state O-K, the rate of entry of Ba^{2+} to the channel becomes 25-fold smaller compared to the on rate when the channel is in the O state. Thus, external K^+, not only hinders the exit of Ba^{2+} from the channel as previously proposed, but also its entry. More important, using this kinetic scheme, selectivity sequences for the external K^+ site can be obtained when the channel is occupied by Ba^{2+} and when the channel only contains monovalent cations. In these two different occupancy states of the channel the seletivity sequence for monovalent cations of the external "K^+" site is not the same. We conclude that the presence of the divalent cation in the conduction system of a KCa channel not only alter the monovalent cation binding strength, but also distort the pore structure.

The group of Aldrich (Hoshi et al. 1990; Zagotta et al. 1990) has identified the inactivation gate in *Shaker* A-type K^+ channel as the cytoplasmic domain of the channel forming-protein located in the amino terminus. We have found that the synthesized *Shaker B* inactivating peptide (BP) is able to interact with a K_{Ca} channel (Toro et al. 1992). The inhibition of channel activity induced by BP can be most simple interpreted in terms of a voltage-dependent blockade that results from peptide binding to the internal mouth of the channel. One of our aims in studying the interaction of BP and K_{Ca} channels was to use this peptide as a probe of the internal mouth of the channel. Accordingly, we have modify both charge and hydrophobicity of the peptide in order to inquire about the structural parameters that regulate peptide binding to the channel. We found: a) increasing the peptide net charge increases peptide binding affinity. Since a kinetic analysis indicates that only the association rate constant is modified by alteration in BP net charge, this effect can be fully explained assuming that the internal channel vestibule contains a fixed negative charge; b) mutations that made the BP more hydrophobic induce a tighter peptide binding. However, in this case the effect of the mutations is on the dissociation rate constant. This last result can be rationalized assuming the existence of an hydrophobic pocket in the internal vestibule.

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High-Conductance Ca^{2+}-activated K^{+} channel from smooth muscle: molecular characterization and purification. Maria L. Garcia, Margarita Garcia-Calvo, Kathleen M. Giangiacomo, Gregory J . Kaczorowski, Hans-Günther Knaus and Owen B. McManus. Department of Membrane Biochemistry and Biophysics. Merck Research Laboratories. P.O. Box 2000. Rahway, N.J. 07065 U.S.A.

High-conductance Ca2+-activated K^{+} channels (maxi-K channels) represent a family of proteins that display high conductance and selectivity for K ions. The discovery and characterization of selective, high affinity, peptidyl probes for these channels has allowed not only the development of the molecular pharmacology of the maxi-K channel but also the study of the physiological role that these channels play in target tissues. In addition, with the use of [^{125}I]charybdotoxin (ChTX), receptor sites have been identified in different smooth muscle tissues and shown to be associated with maxi-K channels. The ChTX receptor from bovine tracheal smooth muscle has been purified to homogeneity by a combination of conventional chromatographic techniques and sucrose density gradient centrifugation. Purified ChTX receptor preparation consists of two subunits α and β of 62 and 31 kDa, respectively. The purified preparation has been reconstituted into liposomes and these fused with artificial lipid bilayers. This process leads to the appearance of single channels that display the same biophysical and pharmacological properties as the native channel. Thus, the two molecular components identified in the purified preparation are sufficient to form maxi-K channels.

Charybdotoxin as a Structural Probe of Shaker: Complementary Mutagenesis and Electrostatic Distance Geometry

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A new method is introduced for using charybdotoxin (CTX), a peptide of known structure, as a molecular caliper for estimating distances between residues near the CTX receptor in the external mouth of a Shaker K^+ channel. CTX block of K^+ channels is known to result from the binding of a single CTX molecule to the externally facing mouth of the channel (1,2), and the locations of the CTX receptor determinants in Shaker are known (3,4). Some of these residues act by purely through-space electrostatic energies, such that the charge, but not the chemical structure, of the residue influences the affinity of CTX binding. For such residues, it can be shown on general grounds (assuming that the conformation of CTX remains the same in the blocked state) that the "electrostatic compliance," σ_{ij}, is a direct measure of the distance, r_{ij}, between residue i on the toxin and residue j on the channel:

$$(1) \quad \sigma_{ij} = \partial^2 \Delta G^\circ / \partial q_j \partial z_i = f(r_{ij}),$$

where ΔG° is the standard-state free energy of toxin binding, and z_i, q_j are the charges of the particular residues on the toxin and channel, respectively.

The function $f(r_{ij})$ describes the distance dependence of the charge-charge interaction energy between the i and j residues. We argue that near the aqueous-protein interface, this distance-dependence can be validly approximated by:

$$(2a) \quad f(r) = \frac{Ke^{-r/k}}{r},$$

where K and k are known (or estimatable) constants.

Thus:

$$(2b) \quad r_{ij} = f^{-1}(\sigma_{ij}),$$

where f^{-1} is the inverse function of (2a).

The electrostatic compliance as defined by (1) is an experimentally measurable quantity. A single value of σ_{ij} is derived from $\Delta G°$ of toxin binding measured on a matrix of 9 channel-toxin mutant pairs, with charges of -1, 0 and +1 each at toxin residue i and channel residue j. We are currently road-testing this approach by focusing on a "cleanly electrostatic" residue of Shaker, Lys427, and several residues of CTX. We use as genetic background an inactivation-removed Shaker BN variant with an engineered high-affinity CTX receptor: ShB (Δ 6-46/F425G) expressed in Xenopus oocytes, and a fusion protein bearing a cleavable CTX sequence expressed in E. coli.

As measured from blocking efficacy, CTX binding to our Shaker channel displays a dissociation constant of 50 pM at 100 mM ionic strength. The binding affinity of CTX increases about 7-fold per unit charge, as position 427 is changed:

Lys ---> Gln ---> Asp.

Thus, the channel mutant K427D binds CTX with K_i=1-2 pM. This sensitivity of CTX binding to channel charge at position 427 is insensitive to the toxin charge at position 25, but it is strongly sensitive to the toxin charge at position 11, for example. From eq. (2), we interpret this result to mean that channel position 427 is physically far away from toxin positions 25 (σ_{ij} = 0), but physically close to toxin position 11 (substantial σ_{ij}).

When this approach is combined quantitatively with the usual hand-waving about dielectric constants and electrolyte screening, we estimate that CTX residue 25 is far away from (>18 Å) and residue 11 close to (<6 Å) Shaker position 427. In starting to triangulate the channel, we use this result to place Shaker residues Thr449 and Lys427 about 15 Å apart.

The "electrostatic distance geometry" method is by no means established definitively. Additional tests must be applied of electrostatically good behavior by the mutants constructed, and the serious problem of four fold mutation in homotetrameric channels needs to be addressed. Fortunately, the known structure of CTX places consistency checks on the deduced distances between channel and toxin residues, and so far none of these requirements has been transgressed. We are currently in the process of estimating distances between other pairs of residues and of subjecting the method to further experimental tests.

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STUDIES ON THE PORE OF CYCLIC NUCLEOTIDE-GATED CHANNELS

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Cyclic nucleotide-gated (CNG) channels produce the initial electrical response in the olfactory and visual sensory transduction systems. These channels conduct monovalent cations and are blocked efficiently by divalent cations from both the extracellular and intracellular solutions. The divalent blockade in phototransduction cells reduces the effective channel conductance and thus allows a smooth membrane voltage change in response to light. My presentation will focus on ion conduction and blockade in CNG channels expressed in Xenopus oocytes. External Ca^{2+} and Mg^{2+} blockade of a retinal CNG channel expressed in oocytes occurs at micromolar concentrations and is a strong function of voltage. At the single channel level, blockade is manifest as flickering interruptions of the open channel current. The pore-forming region (P-region) of CNG channels contains a highly conserved glutamate residue. When the glutamate residue is replaced by an amino acid with a neutral side chain (glutamine, asparagine, serine, glycine) the channel is no longer sensitive to external Mg^{2+} or Ca^{2+}. On the other hand, the charge-conservative glutamate → aspartate mutant channel is sensitive with properties similar to those of wild type. The ability to block the channel by divalent cations from the intracellular side is much less affected by mutations of the P-region glutamate residue. These results place the glutamate residue in the pore near the extracellular surface where it can bind divalent cations from the outside. The results also imply that internal divalent cations must inhibit by binding at a separate site.

Ether-à-go-go : a potassium and calcium permeable channel modulated by cAMP.

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The *Drosophila ether-à-go-go* (*eag*) mutants show behavioural abnormalities under ether anaesthesia. It has been shown that in larval muscle of *eag* mutants several K^+ currents are altered, including fast inactivating (I_A), delayed rectifier (I_K) and two Ca^{2+}-activated K^+ (I_{CF} and I_{CS}) currents[1]. The *eag* gene encodes a polypeptide[2] that shares sequence similarities with the family of voltage-gated ion channels as well as with the cyclic-nucleotide-gated (CNG) channels that contain a putative cyclic-nucleotide binding domain.

We have expressed *eag* cRNA in the *Xenopus* oocytes and found that it functionally expresses a channel permeable to both K^+ and Ca^{2+}. The sequence of permeabilities is $K^+ > Rb^+ > Cs^+ > NH_4^+ > Na^+ > Li^+$ which corresponds to Eisenman`s series IV.

The kinetics observed in inside-out patches show a fast inactivating component as well as a non-inactivating component. In addition, the current is directly modulated by cAMP and indirectly by cGMP. Taken together, these properties are able to explain the electrophysiological alterations seen in the mutant larval muscle. We think that *eag* is implicated in the presynaptic facilitation mechanism observed at the neuromuscular junction of *Drosophila melanogaster*.

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IV. Calcium and Sodium Channels

STRUCTURAL BASIS for CALCIUM CHANNEL FUNCTION. Kurt G. Beam, Dept. of Physiology, Colorado State Univ., Fort Collins, Colorado.

In collaboration with the laboratory of the late Professor Shosaku Numa, our laboratory has been studying the structural basis for calcium channel function in muscle. A principal experimental approach has been to inject expression plasmids, carrying cDNAs that encode calcium channels, into cultured skeletal muscle myotubes from mice with the muscular dysgenesis mutation. Because this mutation alters the gene encoding the skeletal muscle dihydropyridine (DHP) receptor, dysgenic myotubes lack both excitation-contraction (e-c) coupling and L-type calcium current that are characteristic of normal skeletal muscle. However, dysgenic myotubes injected with cDNA encoding the skeletal muscle DHP receptor (CAC6) display both slowly activating L-type calcium current and skeletal muscle-type e-c coupling (that is, the coupling results from the intracellular release of calcium from the sarcoplasmic reticulum and this release does not require the entry of extracellular calcium). By contrast, dysgenic myotubes injected with cDNA encoding the cardiac DHP receptor display rapidly activating L-type calcium current and cardiac-like e-c coupling which does require the entry of extracellular calcium. Expression of cDNAs encoding chimaeras of the skeletal muscle and cardiac DHP receptors has been used to identify specific portions that are critical for the differences in activation kinetics of L-type calcium current and in the type of e-c coupling. It was found that the putative cytoplasmic loop linking homology repeats II and III (the II-III loop) is critical for determining whether e-c coupling is skeletal- or cardiac-type and that homology repeat I is critical for determining whether channel activation kinetics are rapid or slow. Additionally, it was found that DHP receptors produce rapid intramembrane charge movements ("gating currents") with kinetics and voltage dependence that are very similar, independent of the type of e-c coupling or the rate of activation of L-type current. Measurements in which the calcium indicator fluo-3 was loaded into normal myotubes or CAC6-injected dysgenic myotubes indicate that intramembrane charge movement is followed closely in time by calcium release from the SR, whereas the activation of L-type calcium current occurs much more slowly than either of these processes. Freeze-fracture analysis, carried out in the lab of Dr. C.-F. Armstrong, indicates that clusters of particles in groups of four ("junctional tetrads") are present in normal myotubes but absent in dysgenic myotubes. Junctional tetrads are restored by transfection of dysgenic myotubes with CAC6, suggesting that these structures are composed of DHP receptors. The junctional tetrads are arrayed in such a fashion as to allow them to interact with the calcium release channels of the SR.

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PRIMARY STRUCTURE AND FUNCTIONAL EXPRESSION OF CALCIUM CHANNEL BETA SUBUNITS. Castellano, A., Wei, X., Bimbaumer, L. and Perez-Reyes, E. Dept. Molecular Physiology and Biophysics. Baylor College of Medicine. Houston, TX 77030, USA.

From the electrophysiological and pharmacological point of view, calcium channels have been classified in four major types: T, N, L, and P. The best studied calcium channel is the L-type or DHP receptor from skeletal muscle. This channel/receptor complex is comprised of five subunits: α_1, α_2, β, γ and δ. Expression studies have demonstrated that the α_1 subunit alone is sufficient to form a voltage-dependent calcium channel, and that it is the receptor for different types of drugs. All of these subunits have been cloned, and using their cDNAs as probes new homologous subunits have been identified. There are, at least, six different α_1 subunit genes, five of them expressed in brain. Some of these α_1 subunits correspond to L-type calcium channels, but others correspond to the N- and P-type channels. So far, only one gene encoding for the $\alpha_2\delta$ subunit and another for the γ subunit have been identified. Previously to our work only one gene encoding for a β subunit had been cloned. This gene is expressed in skeletal muscle and brain.

In order to probe for the existence of new β subunits, we used two different strategies: screening of a rat brain cDNA library, and amplification of related sequences using the polymerase chain reaction (PCR). Using these approaches, we and others have identified new homologous β subunits in different tissues. So far, four different β subunit genes have been identified. Comparison of the amino acid sequences of the β subunits shows that although there are some amino acid substitutions spread all over the sequences, the main differences are group in the amino and carboxi termini, and in a short region in the central part of the sequence. The central core regions of the proteins have an average homology of 78%. The tissue distribution of the β subunits was determined using Northern blot analysis. Each β subunit is expressed in several tissues, however, the four β subunit genes are expressed in brain.

In order to study the function of the β subunits we have used two different expression systems: we injected *Xenopus laevis* oocytes with cRNAs of the cardiac α_1 and the different β subunits, and measured the calcium channel currents with the two microelectrode voltage-clamp technique. Injection of any of the β cRNAs alone led to the appearance of barium currents that were considerably larger than those in uninjected oocytes. Those currents resembled endogenous calcium channel currents. Injection of the cardiac α_1 cRNA also led to the induction of barium currents. Those currents were different to the endogenous currents: they were DHP sensitive, they activated with a slower time course, and their activation threshold occurred at more depolarized potentials. Coexpression of any of the β cRNAs with the cardiac α_1 led to a dramatic increase (6-10 fold) in the peak currents measured. The currents induced by the different combinations of α_1 and β also activated and inactivated faster than the α_1 induced currents. Analysis of the current-voltage relationships indicates that the β subunits produce a shift in the I/V limb to more hyperpolarized potentials.

The second expression system used in our laboratory was the transfection of COS cells with combinations of different α_1s and βs cDNAs, and the subsequent measuring of DHP binding to the membranes. Binding of DHP to membranes from COS cells transfected with either skeletal muscle or cardiac α_1 alone was very low. Cotransfection with the β subunit cDNAs increased binding of DHP to the α_1 subunit.

Our results show that β subunits can interact with two L type calcium channel α_1 subunits and also with a DHP insensitive calcium channel endogenous to *Xenopus* oocytes. The effects of the β subunits on the activity of the α_1 can be divided in two categories: quantitative, such as the increase in functional activity measured as either increased barium currents in Xenopus oocytes or increased DHP binding activity in COS cells, and qualitative, such as its ability to shift the activation threshold to hyperpolarizing potentials and its ability to alter activation and inactivation kinetics.

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MOLECULAR DETERMINANTS OF CALCIUM SELECTIVITY AND ION PERMEATION IN L-TYPE CALCIUM CHANNELS.

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Ion selectivity in Ca^{2+} channels is believed to be achieved by high-affinity binding in the permeation pathway. Four highly conserved glutamate residues, one in each of the pore-lining H5 regions, stand out as likely participants in forming the high-affinity binding site (Heinemann et al., '92; Kim et al., '93) and are designated as E*1* (residue #393), E*2* (#736), E*3* (#1145) and E*4* (#1446)). We have made single E→Q mutations in each of the H5 regions of an L-type Ca^{2+} channel and studied the expressed mutants in Xenopus oocytes. The apparent binding affinities of the wild-type (WT) and mutant channels to Ca^{2+} and Cd^{2+} were assessed by studying block of Li^+ or Ba^{2+} current. The IC_{50}'s for Ca^{2+} block of I_{Li} were E*3* > E*2* > E*4* > E*1* > WT (18.6, 10.7, 7.7, 1.85, 0.97μM). The order of IC_{50}'s for Ca^{2+} block of I_{Ba} was E*3* > E*4* > E*2* > E*1* > WT. The order of IC_{50}'s for Cd^{2+} block of I_{Ba} or I_{Li} was E*3* > E*1* > E*2* > E*4* > WT (9.7, 3.3, 2.4, 0.53, 0.26 μM for block of I_{Ba}; 658, 132, 48, 18, 1.4 nM for block of I_{Li}).

Additionally, we have made E→K mutations to further characterize Ca^{2+} binding in the pore. All the E→K mutants carried I_{Li}, but only the E*4*K mutant carried significant I_{Ba}. Thus, the E→K mutation tended to switch the channel from divalent- to monovalent-selective. The IC_{50}'s for Ca^{2+} block of I_{Li} were: E*3* > E*2* > E*1* > E*4* > WT (1114, 819, 195, 109, 0.97 μM). For a double mutant in which E*3* and E*4* were replaced with their counterparts in Na^+ channels (E*3*K·E*4*A), I_{Li} was blocked by Ca^{2+} with an IC_{50} of 2.2 mM.

The findings for E→Q mutations indicate that all four glutamates participate in the binding of divalent cations, and that their contributions are not equal (conclusions reinforced by our data for single E→A mutations). The repeat 3 glutamate, E*3*, is especially important for both Ca^{2+} and Cd^{2+} binding. E*1* contributes more than E*4* to Cd^{2+} binding, whereas the reverse is true for Ca^{2+} binding. Thus, the binding configurations for Ca^{2+} and Cd^{2+} appear different. These configurations depend primarily on the nature of the blocking ion rather than the permeating ion. However, for either Ca^{2+} or Cd^{2+}, the apparent binding affinity is significantly less with Ba^{2+} as the charge carrier than with Li^+, reflecting ion-ligand and ion-ion interactions at the locus of high-affinity binding.

Since Ca^{2+} binding is greatly reduced by any of the E→K mutations, each of these four glutamates appear important for high-affinity binding. Abolition of high-affinity binding in the double mutant suggests that no other high-affinity site exists independently of the four glutamates. We propose that the unequal contributions of the glutamates reflect asymmetry in the relative positions of the side chains, which may arise from either dissimilar interactions with neighboring residues or an asymmetric placement of glutamate α-carbons along the axis of the pore. Since the E*4*K mutant was the only one to allow significant I_{Ba}, it probably affected the outer energy barrier for cation permeation more than the inner energy barrier, whereas the reverse may be true for the other E→K mutants. We thus hypothesize that E*4* may be positioned closest to the external mouth of the pore. Conversely, E*1* may be placed closest to the cytoplasm since the effects of mutations at this residue differ the most from those at E*4*. The dominance of E*3*, shown by the potency of mutations to either Q, A or K, would be consistent with a central position. Such axial asymmetry might allow the four glutamates to form a distorted tetrahedral coordination site.

Sodium Channel Defects in Myotonia and Periodic Paralysis

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The electrical excitability of muscle and nerve is mediated by voltage-gated ion channels. Several inherited neuromuscular disorders in man and animals are caused by altered excitability of the muscle fiber (1), and the underlying ion channel defects are now being elucidated at the molecular and functional levels. Two clinically delineated disorders of muscle excitability are caused by mutations of the voltage-gated Na channel.

Hyperkalemic periodic paralysis (HPP) is inherited autosomal dominantly and is characterized by recurrent attacks of weakness in association with elevated serum K^+ (5-7 mM; normal 3.5-4.5). An equine form of HPP occurs in American Quarter Horses. For man and horse, weakness lasts from minutes to hours. During attacks the muscle fibers are depolarized and refractory from firing action potentials in response to neuronal or direct electrical stimulation. Voltage-clamp recordings from biopsied fibers showed that a persistent TTX-sensitive inward current causes the depolarization (2). A related disorder, paramyotonia congenita (PC), presents as cold-induced stiffness. The membrane is hyperexcitable and repetitive after-discharges cause a delay in relaxation (myotonia). For some patients, transient weakness may also occur during a severe attack. Conversely in some families with HPP, patients also have myotonia.

Genetic analysis has established that HPP, equine periodic paralysis, and PC are all caused by mutations in the α subunit of the skeletal muscle Na channel ((3) for review). In all families tested to date, both HPP and PMC are linked to SCN4A, the locus of the gene coding for the α subunit on chromosome 17q. Four mutations in SCN4A have been identified in families with HPP and 9 others have been found in association with PMC. In every case the mutations cause a substitution at one highly conserved amino acid residue. Most of the substitutions interchange one neutral residue for another. They are located either at the cytoplasmic face of transmembrane segments - near the putative pore-forming region between S5 and S6, or in the cytoplasmic loop between domains III and IV - a region involved in the inactivation of the Na channel. Equine HPP is genetically homogenous. All cases can be traced to a common ancestor and are caused by a Phe to Leu substitution in S3 of domain IV (4).

We have investigated the functional consequences of the HPP mutations. Sodium currents were measured in human myotubes cultured from a patient with HPP (5). The functional defect is a disruption of inactivation. Single-channel conductance and activation are not affected. At low [K] normal and mutant channels are indistinguishable. In 10 mM [K] a small proportion of mutant channels have prolonged open times and open repetitively throughout the depolarization. The steady-state open probability is only about 0.02 to 0.05, but this is 10 to 20 times larger than that observed for normal Na channels. These HPP myotubes contained the Met1592→Val

mutation. We showed that this mutant and the Thr704→Met mutation are sufficient to disrupt inactivation by using site-directed mutagenesis in the normal rat α subunit and expression in HEK cells (6). Unitary Na currents recorded from affected equine myotubes also revealed an impairment of inactivation.

A toxin-based animal model (7) and computer simulation (8) demonstrated that a small proportion of non-inactivating Na current (P_{open} of 0.015 to 0.05) is sufficient to produce the myotonic and paralytic phenotypes. When applied to rat muscle, anemone toxin (ATX II, 10 μM) selectively disrupts Na channel inactivation and causes a steady-state P_{open} of 0.02 at -10 mV (*vs* 0.001 for controls). In fibers exposed to ATX II, relaxation of twitch tension was delayed by an order of magnitude and trains of repetitive action potentials were elicited by injection of depolarizing current pulses. After-discharges were abolished by detubulating the fiber with glycerol which suggests that the myotonic discharges are initiated by the depolarizing influence of activity-driven K accumulation in the T-tubule. Thus there are two possible contributions from raised extracellular [K] in the pathogenesis of HPP: activity-dependent K accumulation in the T-tubule depolarizes the fiber and promotes myotonia, and high $[K]_o$ increaes the proportion of mutant Na channels failing to inactivate. With a computer simulation, it is possible to separate the effects of these two influences on the production of myotonia or paralysis. If $[K]_o$ is fixed at 4 mM, then myotonia cannot be produced regardless of how large P_{open} is for Na channels in steady-state. Depolarized resting potentials of -40 to -30 mV occur when P_{open} exceeds 0.06. This causes paralysis and explains the dominant pattern of inheritance: the depolarization inactivates a large proportion of the normally-gated Na channels and the fiber is refractory from firing discharges. In 10 mM $[K]_o$, values of P_{open} larger than 0.02 produce paralysis. Furthermore, in 10 mM $[K]_o$ there is no stable resting potential when $0.01 < Popen < 0.02$; the only possible behavior is myotonia. These simulations demonstrate a synergistic effect of raised $[K]_o$ and higher steady-state P_{open} of Na channels in the production of myotonia and paralysis.

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Calcium entry pathways and the role of passive calcium influx on calcium waves

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Receptor-mediated increases $[Ca^{2+}]_c$ result from mobilization of Ca^{2+} from intracellular stores as well as transmembrane Ca^{2+} influx, in most mammalian cells. IP_3 releases calcium from intracellular stores by opening a Ca^{2+} -permeable channel in the endoplasmic reticulum. However, the mechanism and regulation of Ca^{2+} entry into non-excitable cells has remained elusive because the entry pathway has not been defined. We characterized a novel inositol 1,3,4,5-tetrakisphosphate (IP_4) and Ca^{2+}-sensitive Ca^{2+} channel in endothelial cells. IP_4, previously shown to induce Ca^{2+} influx into acinar cells, enhanced the activity of the Ca^{2+}-permeable channel when exposed to the intracellular surface of endothelial cell inside-out patches. Our results provided a molecular mechanism likely to play an important role for receptor-mediated Ca^{2+} entry.

Ca^{2+} entry into non-excitable cells occurs through multiple pathways, but one major route is gated by Ca^{2+} release from internal stores. This mechanism, present in a variety of cells including A431 cells, has not been linked to any known second messenger but is controlled by the filling state of the calcium stores, presumably endoplasmic reticulum. An inward Ca^{2+} current, named I_{CRAC} (calcium-release activated calcium current), has been demonstrated recently in mast cells during dialysis with inositol 1,4,5 trisphosphate (InsP3) and/or high capacity Ca^{2+} buffer, used to deplete stores. We characterized another Ca^{2+} entry pathway, a Ca^{2+} and Ba^{2+} permeable ion channel in A431 cells in which calcium stores were depleted by dialysis with the Ca^{2+} chelator bis(o-aminophenoxy)ethane-N,N,N',N', tetra-acetic acid (BAPTA). The channels were stimulated by thapsigargin (blocking Ca^{2+} uptake into the stores) and by low external Ca^{2+} but not by 1,4,5 $InsP_3$ or 1,3,4,5 $InsP_4$, distinguishing them from another Ca^{2+} entry pathway in endothelial cells5. The channels described here represent a pathway for Ca^{2+} influx activated by depletion of internal Ca^{2+} stores.

As noted above, activation of the PI cycle induces Ca^{2+} influx, which refills the intracellular Ca^{2+} stores. Confocal microscopy was used to show that receptor-activated Ca^{2+} influx, enhanced by hyperpolarization, modulated the frequency and velocity of IP_3-dependent Ca^{2+} waves in *Xenopus* oocytes. These results demonstrated that transmembrane voltage and calcium influx pathways may regulate spatial and temporal patterns of IP_3-dependent Ca^{2+} release. Most importantly, the results show that even though many receptors converge to activate an identical PI pathway, enormous diversity in calcium signalling results from the particular receptor's interaction with Ca^{2+} entry pathways and with ion channels that control membrane potential.

V. Modulation of Ion Channels and Cellular Functions

Effect of postsynaptic calcium on the efficacy of inhibitory synapses of the cerebellar cortex

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Intracellular calcium ions are known to play a role both pre- and post-synaptically in the regulation of synaptic efficacy. In cerebellar Purkinje cells, for example, the long term depression of synaptic efficacy of the parallel fibre input elicited by conjunctive stimulation of parallel fibre and climbing fibre inputs depends on a calcium rise in the postsynaptic cell (Sakurai, 1990). Here we examine the consequence of a calcium rise in Purkinje cells on the synaptic efficacy of the GABAergic synapses formed by stellate and basket cells onto Purkinje cells.

Cerebella were removed from 9d-20d old rats. Slices (120-150 µm) were cut in the vermis parallel to the sagittal plane. For recording, slices were examined with an upright microscope equipped with a 40 x immersion objective (Edwards et al., 1989). Patch-clamp pipetttes were filled with a CsCl solution including a calcium buffer, MgATP and GTP. They had a resistance of 2-3 MΩ. During whole cell recording (Marty & Neher, 1983), the pipette resistance rose to about 5-8 MΩ. Part of this resistance was compensated electronically. If this was carefully done, Purkinje cells could be entirely voltage clamped with a time constant as low as 2 ms (Llano et al., 1991a). Interneurones of the molecular layer (stellate and basket cells) were stimulated extracellularly by delivering short (0.2 ms) voltage pulses to a saline-filled pipette positioned just above the slice.

Following a train of depolarizations of the recorded Purkinje cell, evoked GABAergic currents were depressed during 1 min and then potentiated during 10-40 min (Llano *et al.*, 1991b ; Vincent *et al.*, 1992). Both effects were abolished by perfusing the Purkinje cell with a high calcium buffer (30 mM BAPTA), and were therefore calcium-mediated.

The potentiation was paralleled by an equivalent potentiation of current responses induced by local applications of exogenous GABA, indicating that it was generated postsynaptically.

The short-lasting depression, however, was generated presynaptically. This is indicated by the following lines of evidence : (1) When using low stimulus intensity such that occasional failures were observed in the control run, depolarizing the Purkinje cell resulted in a marked increase in the

number of failures. (2) The frequency of miniature IPSCs was decreased during the phase of depression. (3) The amplitude of miniature IPSCs or of GABA-induced currents was increased, not decreased, during the phase of depresssion.

To study further the mechanisms by which activity in one Purkinje cell influences that of the presynaptic interneurones, simultaneous recordings were performed from two neighbouring Purkinje cells. It was first observed that a large proportion of the spontaneous IPSCs recorded were synchronous, probably because they originated from the firing of common interneurones. Upon depolarization of one Purkinje cell, the spontaneous IPSCs were reduced not only in that cell, but also in its neighbour. These observations confirm that a retrograde signal is sent from the Purkinje cell to the presynaptic interneurone, and further show that this signal can lead to a lateral spread of information in the Purkinje cell layer.

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MODULATION OF SINGLE K^+ CHANNELS BY OXYGEN AND THE REDOX STATUS IN GLOMUS CELLS.

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O_2-sensing by glomus cells of the carotid body is mediated by oxygen-sensitive K^+ channels expressed in the cell membrane (1,2). Low P_{O_2} induces a reversible decrease in channel open probability withouth altering the single-channel conductance. The O_2-K^+ channel interaction occurs either directly or through an O_2 sensor intrinsic to the plasma membrane closely associated with the channel molecule (3). We have studied the kinetic properties of the O_2-sensitive channels and examined the specific changes of channel gating induced by low P_{O_2}. Exposure to hypoxia (switching between solutions equilibrated with a P_{O_2} of either 150 or 10-40 mm Hg) produced an increase in the first latency, a decrease in the number of bursts per trace, and a higher occurrence of closed-state inactivation. The open state and the transitions to adjacent closed or inactivated states seemed to be unaltered by hypoxia. Thus, at low P_{O_2} the number of K^+ channels that open on depolarization decreases and those channels that follow the activation pathway open more slowly and inactivate faster. At the macroscopic level, these changes are paralleled by a reduction in the peak current amplitude, slowing down of the activation kinetics, and acceleration of the inactivation time course (4). The fact that low P_{O_2} modifies kinetically defined conformational states of the K^+ channels suggests that O_2 tension could influence the structure of specific domains of the K^+ channel molecule by inducing modifications in accessible aminoacids. P_{O_2} could regulate the redox state of residues, such as cysteine, important for determining some of the channel's kinetic properties. Along with this idea it has been shown that in some cloned voltage-gated K^+ channels the redox state of sulfhydryl groups in the amino terminal regulates the time course of inactivation (5). We have also observed that in excised membrane patches the O_2-sensitive channels are reversibly inhibited by the thiol-reducing agents glutathione (GSH) and dithiothreitol (DTT) at concentrations between 0.5 to 5 mM added to the internal solution. These reagents produce specific alterations in the K^+ channels gating

properties but leave unaltered their single-channel conductance. On the average, GSH (1 mM) or DTT (1 mM) decrease single channel open probability to about 40% of the control value. During exposure to GSH the number of channels that open on depolarization decreases and those that follow the normal activation pathway open more slowly. However, the antioxidants do not alter the mean burst duration which is interpreted as an indication that they do not modify transitions near the open state. These results, demonstrating a close parallelism between the effects of hypoxia and the sulfhydryl reagents, suggest that the redox status of thiol groups in the channel molecule may be part of the mechanisms whereby O_2 tension regulates channel activity. In a broader sense, our data support the concept that the redox status can be an important variable regulating ion channel function.

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THREE TYPES OF Ca CHANNEL TRIGGER SECRETION WITH DIFFERENT EFFICACIES IN CHROMAFFIN CELLS. Cristina R. Artalejo. Dept. of Neurobiology and Physiology, Northwestern University, Evanston. Dept. de Farmacologia y Terapeutica, Fac. de Medicina, U.A.M. Madrid.

The relationship between various classes of Ca channel and secretion is currently a subject of much debate. We have used chromaffin cells as a system to directly analyse the coupling between three distinct types of Ca channel and exocytosis. These are the dihydropyridine-sensitive "facilitation" Ca channels, that are normally quiescent, but are activated by depolarizing pre-pulses, repetitive depolarizations to physiological potentials or agents that raise cAMP; ω-Conotoxin-GVIA-sensitive "non-classical" N (NCN)-type channels and ω-Agatoxin-IVA-sensitive P-type Ca channels. Using the patch-clamp technique we have simultaneously monitored changes in capacitance, as an assay of catecholamine secretion, and Ca currents. While all three types of Ca channel are able to trigger secretion individually, the facilitation channel is coupled much more efficiently to exocytosis. These results suggest that facilitation Ca channels may be physically nearer vesicle release sites and may underlie the massive catecholamine release that occurs during the "fight or flight" response. They also demonstrate for the first time, how different types of Ca channel preferentially regulate low and high levels of transmitter release.

The combination of nisoldipine, ω-Conotoxin-GVIA (ω-CgTx) and ω-Agatoxin-IVA (ω-Aga-IVA) completely suppressed Ca currents in chromaffin cells Together with previous data these results indicate that chromaffin cells have three pharmacologically-distinct types of Ca channels and that individual components can be selectively blocked using the appropriate antagonist. With this information it became possible to test the role of each Ca channel in catecholamine secretion.

Combinations of antagonists were used to measure the secretion produced by individual Ca channels. Fig. 1 confirms that all three types of Ca channel found in chromaffin cells promote secretion, but that facilitation Ca channels do so more efficiently. Fig. $1A_1$ shows the contribution of pharmacologically-isolated facilitation currents to catecholamine secretion. The combination of ω-CgTx and ω-Aga-IVA was used to suppress non-facilitation components. A train of 10

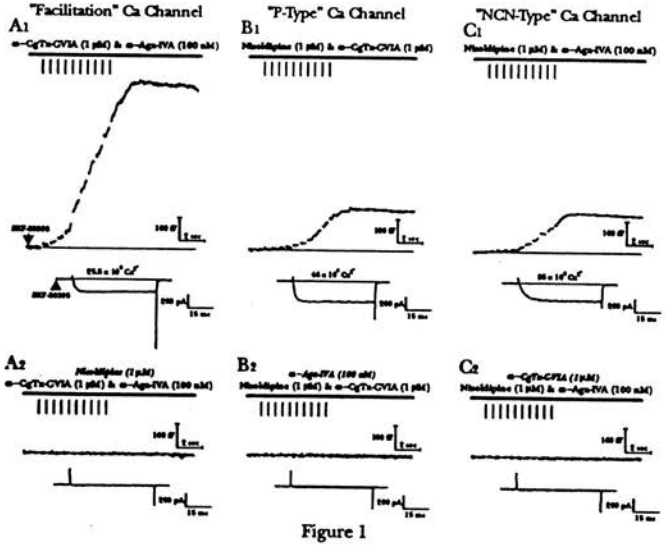

Figure 1

depolarizations to +10 mV was applied after treatment with a D_1 agonist to recruit facilitation current. Plotted below are the Ca currents obtained during the first depolarization of the train. Addition of nisoldipine (1 μM) completely inhibited facilitation current and the associated increase in capacitance (Fig. $1A_2$). Recruiting facilitation current by pre-pulses to +120 mV gave similar results indicating that the influence of D_1 agonists on secretion is due to an effect on the channels and not to a cAMP/ protein kinase A effect on the secretory process downstream from Ca channel activation. Moreover, D_1 agonists or membrane permeant cAMP analogues had no effect on capacitance in cells where facilitation channels were blocked by nisoldipine. P-type Ca currents also promoted catecholamine secretion, but did so less efficiently than facilitation Ca currents (Fig. $1B_1$). Nisoldipine and ω-CgTx were used to isolate P-type Ca currents. ω-Aga-IVA blocked the P-type current and the associated secretion (Fig. $1B_2$). NCN-type Ca currents, isolated by using nisoldipine and ω-Aga-IVA, were also found to be coupled to secretion with lower efficacy than facilitation Ca currents (Fig. $1C_1$). Adding ω-CgTx blocked the NCN current and capacitance changes (Fig. $1C_2$). From a summary of 54 similar experiments, all three types of Ca channels contribute to catecholamine release but for equivalent Ca current amplitudes (facilitation: 190 pA (25.5 x 10^6 Ca^{2+} ions); P-type: 320 pA (46 x 10^6 Ca^{2+} ions); NCN-type: 260 pA (35 x 10^6 Ca^{2+} ions)), the recruitment of facilitation Ca channels resulted in the exocytosis of ~306 vesicles while activation of P- or NCN-type Ca channels only resulted in the exocytosis of ~88 or ~83 vesicles respectively.

From these results it is possible to speculate that the Ca channels found in chromaffin cells are not randomly distributed; facilitation Ca channels may be physically nearer the docking and release sites than either of the other two channels. Indeed, physical association of Ca channels with components of the exocytotic apparatus has already been posited. If all three channel types were randomly distributed, they might be expected to support secretion with equivalent efficiences. The fact that this is not the case suggests that the precise localization of Ca^{2+} entry may be as important as its magnitude in determining its role in secretion. Other explanations for the difference in efficiency between distinct Ca channel types and secretion, such as clustering of channels or differential buffering of entering Ca^{2+} ions, seem unlikely. Our previous single-channel experiments gave no evidence for significant differences in clustering among the three types of channels. Activation of any of the three Ca channels produced similarly sized $[Ca^{2+}]_i$-transients, as assayed by fura-2, indicating equivalent buffering of Ca^{2+} loads no matter which route of Ca entry is employed. A further consideration arising from the present results is that the stoichiometry between Ca^{2+} entry and secretion need to be evaluated for each type of Ca channel; this can only be done when a pharmacological dissection such as that performed here is feasible.

The present data suggest that different Ca channels in chromaffin cells regulate secretion depending on the level of electrical activity. Under conditions when few action potentials are generated only NCN- and P-type channels would be expected to activate. Because these channels are inefficiently coupled to exocytosis, little secretion occurs and may be regulated on a vesicle by vesicle basis. Under conditions that elevate electrical activity facilitation Ca channels become activated. Because facilitation Ca channels are coupled more efficiently to secretion, catecholamine release can increase dramatically. The novel gating properties of facilitation Ca channels and their possible proximity to release sites suggests that these channels may be responsible for the dramatically augmented catecholamine secretion from the adrenal medulla that occurs during conditions of stress or danger. As most neurons and other secretory cells have more than one type of Ca channel, the concept of differential coupling of specific channels to different rates of transmitter or hormone release may be generally applicable.

ROLE OF IONIC CHANNELS IN THE STIMULUS-SECRETION COUPLING IN THE PANCREATIC B-CELL.

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The major physiological stimulus for insulin release is an increase in the plasma glucose concentrations. Glucose stimulation elicits within B-cells a complex cascade of ionic, metabolic and effector events [1,2]. Briefly, when a resting pancreatic B-cell is challenged with glucose, this interacts with a tissue-specific glucose transporter [3], enters the cell, and is rapidly metabolized. The ATP generated by glucose metabolism blocks the K^+ channels controlling resting membrane potential (K^+_{ATP}) [4]. As these channels close, the cell depolarize causing the opening of voltage-activated Ca^{2+} channels [5] which in turn leads to Ca^{2+} entry [6]. This Ca^{2+} raises the intracellular concentration of the cation ($[Ca^{2+}]_i$) to the level required for activating exocytosis of secretory granules.

An increase in cytosolic free calcium concentration is generally accepted as the major triggering event that induces insulin secretion [7]. In the mouse pancreatic B-cell an elevated $[Ca^{2+}]_i$ level is sufficient stimulus to induce secretion, as wittnessed by an increase in cell membrane capacitance [8].

Glucose-induced $[Ca^{2+}]_i$ increases have been demonstrated in isolated pancreatic B-cells from rat [9], mouse [6,10,11] and in insulin secreting cell lines [12]. However, when the pancreatic B-cell is in normal environment (the intact islet), glucose (above 5-7 mM) induces oscillations of $[Ca^{2+}]_i$ which are modulated by changes in glucose concentration [13,14,15].

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Protein kinase C-mediated inhibition of volume regulated P-glycoprotein associated chloride channel

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Multidrug resistance in eukaryotic cells can be conferred by expression of the 170kD P-glycoprotein, a member of the ABC family of transporters. In addition to its transport properties, we have shown that expression of P-glycoprotein confers a volume-regulated, ATP-dependent chloride channel activity in various cell lines (Valverde et al, 1992). Studies on the relationship between channel and transport functions of P-glycoprotein have shown that drug transport requires ATP hydrolysis whereas ATP binding is sufficient for channel activation. In addition, transport and channel activity have been separated using direct mutagenesis of the nucleotide binding domains (Gill et al, 1992). These data indicate that the channel and transport activities of P-glycoprotein reflect two distinct functional states of the protein. However, the biochemical mechanism(s) which might regulate the dual functioning of P-glycoprotein is unknown. MDR phenotype has been associated with changes in the protein kinase C (PKC) activity (Chambers et al, 1990) and more recently the sites of phosphorylation of P-glycoprotein by protein kinase C (PKC) have been identified (Chambers et al, 1993).

To examine the involvement of PKC phosphorylation in the volume activated chloride current associated with P-glycoprotein we have used the whole cell patch clamp technique to study the effect of 12-O-tetradecanonoil phorbol-13-O-acetate (TPA), a recognised activator of PKC, on hypotonicity activated chloride currents in 3T3 fibroblasts overexpressing P-glycoprotein (MDR1 gene).

3T3 fibroblasts permanently transfected with the human MDR1 gene, bathed in isotonic 140mM N-methyl-D- glucamine chloride (300 ± 15mOsm) using an intracellular (pipette) solution containing symmetrical NMDG-Cl plus 2mM ATP and 150nM free calcium, gave negligible whole-cell currents in response to voltage pulse protocol from -80 to +80 mV. On transferring the cells into hypotonic bathing solution (210 ± 10mOsm), large outwardly rectifying currents appeared within 2 minutes as previously described (Valverde et al, 1992). However, after bathing the cells with isotonic solution containing 30nM TPA for 2 minutes, exposure to hypotonic solutions failed to elicit significant increases in currents in the 3T3MDR cells ($n=5$, for up to 2 minutes), whereas cells exposed to the hypotonicity and vehicle (DMSO) generated a marked increase in volume activated chloride currents ($n=22$). This inhibition of chloride current activation was also observed in 3T3MDR cells dialysed with high concentrations of free calcium ($1.7\mu M$) in the pipette solution ($n=5$). In addition, similar results were also obtained in 3T3 fibroblasts transiently transfected with the human MDR1 gene but not in the untransfected 3T3 cells. A proportion of wild type 3T3 fibroblasts showed volume induced chloride currents, but these were unaffected by pretreatment with TPA.

TPA was only effective in preventing the activation of the chloride currents as exposure to 30nM TPA after hypotonicity induced chloride currents had developed had no effect (n=4).

The specificity of the PKC activation was examined using the PKC pseudosubstrate peptide inhibitor (House & Kemp, 1987). Significant activation of volume activated chloride currents occurred in all 3T3MDR cells dialysed with 1μM PKC-inhibitor peptide despite 2 minutes incubation with 30nM TPA (n=11) in contrast to those cells treated with the control peptide in which no significant currents were seen after treatment with TPA and hypotonicity (n=6).

In summary, activation of protein kinase C either by phorbol esters or high concentrations of intracellular calcium prevents the activation of volume activated chloride currents only in 3T3 fibroblasts overexpressing P-glycoprotein. No such effect was seen in the wild type 3T3 fibroblasts. These results indicate that the phosphorylation state of the P-glycoprotein modulates its role in channel and transporter activity.

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VI. Genetic Regulation of Ion Channels

Control of Excitability Through Genetic Silencing: A Plug for Sodium Channels

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The alpha sub unit of the voltage-gated sodium channel is encoded by a large family of genes. The family members are regulated in a tissue-specific manner, with different excitable tissues expressing a distinctive repertoire of alpha subunit genes (reviewed in Mandel, 1992). In brain, anatomically distinct populations of neurons express different alpha subunit genes, and neurons of the central and peripheral nervous systems express distinct alpha sub unit types. The mechanisms mediating differential expression of alpha sub unit genes are not well resolved. As a first step toward addressing this problem, we have studied the genetic basis of tissue-specific expression of the rat brain type II alpha subunit gene.

The type II alpha subunit gene is expressed at very high levels in rat brain, and at extremely low levels in skeletal muscle or non-excitable tissues. This cell type specificity has been recapitulated *in vitro* by introducing a cloned 5' flanking region of the type II gene, fused to a bacterial reporter gene, into a variety of transformed cell lines. Mutational analysis has shown that a 23 basepair (bp) silencer element, located within 1000 bp of the type II promoter, is required for neural-specific expression of the type II promoter. In the absence of this silencer sequence, the type II promoter is active in all cell types, excitable or non-excitable, which have been tested. In the presence of the silencer element, type II promoter activity is repressed in non-excitable cell types, as well as in neuronal cell types which express exclusively other members of the sodium channel gene family (Kraner et al, 1992). DNA elements sharing sequence homology with the type II silencer have now been shown to mediate neural-specific expression of other genes, suggesting a common strategy for restricting gene expression to the nervous system. Experiments are in progress to identify the transcription factors which recognize the silencer element and thus mediate neural-specific expression of the type II alpha subunit gene.

A new sodium channel type, termed Peripheral Nerve type I (PN1), has been identified recently in our lab through molecular cloning experiments. The predicted

primary structure is highly related to the other rat brain sodium channel alpha subunits. Although expressed at very low levels in brain and spinal cord, PN1 mRNA is found at high levels in peripheral nervous tissue such as dorsal root ganglion (DRG) and superior cervical ganglion. It is likely that the encoded sodium channel represents a predominant sodium channel type in the PNS. We have determined, using RNA hybridization analyses, that the rat brain type I and PN1 mRNAs are expressed differentially in developing rat DRG. Type I mRNA predominates in embryonic ganglia. At birth, there is a switch in relative amounts of expression, with PN1 predominating within one week after birth. Two distinct types of action potentials have been detected in developing DRG. We hypothesize that the relative expression of type I and PN1 sodium channels are important in shaping these action potentials. Experiments combining molecular and electrophysiological approaches should help delineate the relative contributions of different sodium channel types to excitability in the PNS.

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Frequenin: A Novel Calcium Binding Protein that Modulates Synapse Efficacy

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The behavior of an organism is largely determined by the connectivity of its nervous system and the efficacy of its synapses. Thus, the study of the mechanisms involved on these aspects of the neural function will shed ligth on many different levels of animal biology. The proper convergence of technical abilities on *Drosophila* has allowed the isolation and study of mollecular species that were unsuccesfully sought for a long time. Studies on the *Shaker* gene led to the isolation of the first voltage gated K^+ channel from which the corresponding homologs in vertebrates including humans were soon identified. This trend is repeating with other genes whose proteins subserve basic processes common to many species.

As the number of known mechanisms grows it becomes more demanding to address the issue of their coordinated regulation. It is increasingly astounding the exquisite tunning that these mechanisms show, for instance, at the synapse. How is this coordination ensured?. The genetic analysis of *Shaker* has shown that this gene is part of a larger cluster of functions possibly related to each other: the *Shaker gene complex (ShC)*.

The analysis of one gene within *ShC* revealed that the structural product is a novel type of a neural Ca^{++} binding protein that we named **frequenin** (Frq) after its role on synapse firing frequency (see below). This gene is located 120 Kb proximal to the locus encoding the voltage dependent K^+ channels and represents the proximal limit of *ShC* on the X chromosome. Frequenin exhibits two active sites for Ca^{++} binding and two degenerated ones. This feature and the overall sequence comparison indicate a possible pedigree among other members of this novel family of Ca^{++} binding proteins: recoverin, visinin, VILIP and NVP. Furthermore, the structural similarity reflects on a functional equivalence since *Drosophila* Frq con substitute for vertebrate recoverin in its ability to activate guanylyl cyclase from bovine retina in a Ca^{++} dependent manner.

The *in vivo* function of Frq can be deduced from the corresponding mutant phenotypes. The mutation V7 yields an overexpression of Frq. Studies on facilitation of neurotransmitter release at the neuromuscular junction of V7 larvae show that the mutant condition causes a usage dependent enhanced release. In other terms, it seems that the V7 synapses are chronically facilitated. This finding is most likely the functional correlate of a behavioral trait also found in this mutant: the learning defect in the olfactory paradigm. V7 flies are unable to associate an

odour with an electric shock. Aside from the usual tremor of appendages of all mutants in the *ShC*, the V7 flies do not exhibit other so far detectable abnormalities.

A further inquiry on the ionic bases of the abnormal enhancement of neurotransmitter release lead us to study individual currents in the muscles of V7 larvae. Under voltage clamp conditions, only the I_A current is affected. The analysis of I_A in V7 served to discover the regulation of this current by Ca^{++} in the wild type. The overexpression of Frq in V7 or in transformant constructs causes the loss of this regulation. The selective effect of Frq upon the ionic current whose channels are encoded also within the *ShC* represents the demonstration that these two functions, Frq and Sh, are intimately related and, presumably, coordinated in their expression and activity. Current studies try to unravel the functional organization of *ShC* that regulates the expression of its components.

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POSTERS

Characterization of a Swelling-Induced Chloride Current and its Regulatory Protein, pI_{Cln}, in *Xenopus* Oocytes. Ackerman MJ, Krapivinsky GB, Gordon EA, Clapham DC. Mayo Foundation, Rochester MN USA 55905

The ability to precisely regulate cell volume is a fundamental property of most cells. Although the phenomenon of regulatory volume decrease (RVD), whereby a swollen cell loses salt and water to restore its original volume, has been appreciated for decades, the molecular identities of the proteins responsible for the volume control machinery and their regulation are essentially unknown. It appears that the rate-determining step in gaining volume control involves the activation of potassium and chloride conductance pathways. Using the two-electrode voltage clamp technique, we have identified a novel chloride current ($I_{Cl.swell}$) responsive to cell swelling in *Xenopus* oocytes. Reducing the extracellular osmolarity by 50% elicited a calcium-independent chloride current having an anion conductivity sequence identical with swelling-induced chloride currents observed in epithelial cells. The hypotonicity-activated current was blocked by chloride channel blockers, trivalent lanthanides, and nucleotides. G-protein, cAMP-PKA, and arachidonic acid signalling cascades were not involved in $I_{Cl.swell}$ activation. $I_{Cl.swell}$ is distinct from both stretch-activated nonselective cation channels and the calcium-activated chloride current in oocytes. The properties of this native $I_{Cl.swell}$ conductance were virtually indistinguishable from the heterologously expressed chloride current (I_{Cln}) resulting from the expression of cRNA encoding a 235 amino acid protein (pI_{Cln}) cloned from MDCK cells.

Antibodies specific for pI_{Cln} recognized a single 40 kDalton polypeptide in oocytes. Using these polyclonal affinity purified anti-I_{Cln} antibodies, we immunoscreened a *Xenopus* ovarian library and found a highly conserved homologue (74% identical with MDCK clone) of pI_{Cln}. In vitro translation of the oocyte pI_{Cln} clone yielded a 40 kDalton product similar to that detected natively. The predicted protein shares no similarities with any known proteins. One striking peculiarity is the abundance of negatively charged residues (24%) in this molecule. There are two concentrated acidic acid domains found in oocyte pI_{Cln}. Regions of clustered acidic residues in transcription factors have been suggested to mediate protein-protein interactions. pI_{Cln} is not found in the plasma membrane but is predominantly a cytosolic protein existing in molecular complexes with several distinct proteins, one of which appears to be soluble actin.

Given that oocytes possess not only pI_{Cln} but also a native swelling-induced chloride current ($I_{Cl.swell}$) indistinguishable from the heterologously expressed MDCK-I_{Cln} current, it was crucial to establish if endogenous pI_{Cln} and the endogenous hypotonicity-activated chloride current are in fact related. To explore this putative relationship, we injected monoclonal anti-I_{Cln} antibody, which specifically recognized a single pI_{Cln} polypeptide, into oocytes.The injection of monoclonal anti-I_{Cln} antibody completely suppressed the swelling-induced chloride current ($I_{Cl.swell}$). 5 ng of monoclonal antibody attenuated $I_{Cl.swell}$ by 90% (n=8) and 25 ng of anti-I_{Cln} antibody (n=4) completely eliminated $I_{Cl.swell}$ in comparison to $I_{Cl.swell}$ elicited in oocytes injected with the vehicle (100 mM KCl, n=9). This action of anti-I_{Cln} antibody seems to be specific for antibody-pI_{Cln} interaction because 5 ng of injected antibody (33 nM final oocyte concentration) approximates the affinity constant for this antibody-antigen complex (20 nM according to ELISA). In addition, an unrelated IgG_1 at a 25-fold higher concentration did not inhibit $I_{Cl.swell}$. The half-maximal suppression occurred approximately 12 hours following antibody injection. Increasing the concentration of injected antibody by 25-fold resulted in only a modest increase (≈2-fold) in the rate of $I_{Cl.swell}$ inhibition. This suggests that the interaction between antibody-pI_{Cln} is not the rate-limiting step for the attenuation of $I_{Cl.swell}$.

We conclude that endogenous oocyte pI_{Cln} is critical for the activation of the endogenous swelling-induced chloride current, $I_{Cl.swell}$. The molecular properties of pI_{Cln} are distinct from known properties of channel-forming proteins. Thus, we believe that pI_{Cln} functions as a regulator of swelling-induced chloride channels whose molecular identity is unknown. This new understanding of pI_{Cln} should provide the basis to not only extend our knowledge of the underlying regulatory mechanisms involved in volume control but should enable the discovery of other proteins which comprise a cell's volume control machinery. It has been suggested that targeting alternative chloride conducting pathways such as swelling-induced and calcium-activated chloride currents in airway epithelial might provide a therapeutic modality for cystic fibrosis circumventing the chloride conduction defect through CFTR. The identification of a cloned protein (pI_{Cln}) involved in one such pathway ($I_{Cl.swell}$) is a critical first step towards this objective.

Cd^{2+}-BLOCKADE OF HYPERPOLARIZATION-ACTIVATED CURRENT I_{AB} IN CRAYFISH MUSCLE.

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A novel hyperpolarization-activated current mediating inward rectification in opener crayfish muscle has been recently described (Araque and Buño, *J. Neurosci.*, 1993). This voltage- and time-dependent current, called I_{AB}, is selectively mediated by K^+, its activation curve depends on the $Vm-E_K$ (assuming a fixed $[K^+]_i$) and its underlying conductance (G_{AB}) is unaffected by $[K^+]_o$. It is insensitive to Cs^+, Rb^+ and Ba^{2+}, which are potent blockers of other currents mediating inward rectification, but is blocked by extracellular Zn^{2+} or Cd^{2+}.

The effects of Cd^{2+} on I_{AB} were studied under two-electrode voltage-clamp in opener muscle fibres.

The presence of 10 mM Cd^{2+} totally and reversibly reduced I_{AB}, without modifying the I_{AB} equilibrium potential (E_{AB}).

Lower extracellular Cd^{2+} concentration ($[Cd^{2+}]_o$) decreased the maximal conductance of I_{AB} ($G_{AB,max}$) and shifted its activation curve towards more hyperpolarized potentials without affecting the slope of the activation curve. While the I_{AB} activation time constant increased, the I_{AB} deactivation time constant was not modified by Cd^{2+}.

These effects of Cd^{2+} on I_{AB} were dose-dependent, obeying the Hill equation with $IC_{50} = 0.452 \pm 0.045$ mM and a Hill coefficient of 1.

The effects of Cd^{2+} on I_{AB} were independent on $[K^+]_o$, and the effects of $[K^+]_o$ on I_{AB} were unchanged in presence of Cd^{2+}.

We conclude that: *a*) the block of I_{AB} by Cd^{2+} was exerted in a dose-dependent manner, obeying the Hill equation and suggesting that one Cd^{2+} ion binds reversibly to one receptor with a dissociation constant of 0.452 mM; *b*) the selective permeability of I_{AB} channels was not modified by Cd^{2+}; *c*) the binding site for Cd^{2+} to block I_{AB} was different to the binding site for K^+ to permeate; *d*) the Cd^{2+}-blockade of I_{AB} was not voltage-dependent; *e*) Cd^{2+} blocked the I_{AB} channel gate interfering with its opening but not with its closing mechanism.

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THE SURFACE CHARGE OF DIFFERENT K CHANNELS: A COMPARISON BETWEEN ESTIMATIONS FROM ELECTROPHYSIOLOGY AND PRIMARY STRUCTURES

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The ionic strength of the intracellular or extracellular solution has been shown to affect the effective membrane potential felt by the voltage sensor of ion channels. This has been explained by a combination of binding and screening (according to the Gouy-Chapman-Stern theory) of soluted ions to fixed surface charges.

In a previous study[4] we have compared the surface charge density estimated from experiments with Gd^{3+} ions and that estimated from published primary structures. The value for the Na channel of the amphibian node of Ranvier was estimated to -0.6 e nm^{-2} and that of RBNa II (assumed to be the Na channel type of the nodal membrane) to -0.81 e nm^{-2}, thus in good agreement.

For the K channels the situation is more complicated, since several types of K_{DR} channels exist in the amphibian node of Ranvier. The two most abundant K_{DR} channels have been suggested[5] to be $K_v1.1$ (f_1 or I in the node literature) and $K_v3.4$ (or $K_v3.1$; f_2 or F in the node literature). From the published primary structures we estimated -1.13 (-1.04±0.21 (mean±S.D.) for five channels in the subfamily K_v1) and +0.21 (or -0.07; -0.11±0.32 for three channels in the subfamily K_v3) e nm^{-2} respectively[4].

The aim of the present study was to estimate the surface charge densities of the two K_{DR} channel types from experiments with polyvalent cations. The two populations were separated by fitting the sum of two Boltzmann expressions to the conductance (or permeability) vs. potential (G-V) curve[1]. Isolated single fibres from *Xenopus laevis* were voltage clamped by the method of Frankenhaeuser[2]. The conductance was calculated from a steady-state current vs. potential curve assuming E_K=-90 mV.

60 μM Gd^{3+} was found to shift the G-V curve for $K_v1.1$ +18 mV, and for $K_v3.4$ +6 mV respectively. The same marked difference between the shift values (a factor of about 3) was found at three other concentrations in the range 10-100 μM for two other axons. However, due to the binding to the channel and a direct effect on the gating of Gd^{3+}, the surface charge densities of the two channel types are difficult to estimate[3]. We therefore analysed the effect of Ca^{2+}, which is expected to bind less to the channels than Gd^{3+}. A change of the extracellular Ca^{2+} concentration from 0.7 to 5.4 mM was found to shift the G-V curves 16 mV ($K_v1.1$) and 1 mV ($K_v3.4$) respectively. These shifts correspond to (with frog Ringer solution) surface charge densities of -1.28 ($K_v1.1$) and -0.02 ($K_v3.4$) e nm^{-2} respectively. This is in good agreement with the estimations based on primary structures.

In conclusion we found (i) that the polyvalent cations shifted the G-V curves of the two populations of K channels in the node of Ranvier unequally much, and (ii) that the surface charge estimated from the experiments was close to that predicted from the primary structure.

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Regulation of Ca^{2+}-activated maxi K^+ channel from rabbit distal colon by phosphorylation/dephosphorylation and ATP.

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Ca^{2+}-activated K^+ channels have been identified in a number of mammalian tissues and can be distinguished by their single channel conductance, calcium sensitivity, voltage dependence and pharmacological properties (1). Ca^{2+}-activated K^+ channels are particularly interesting since they may provide a link between second messenger systems and membrane conductance. However the physiological significance of these channels is uncertain, because in many tissues they have been found to be stimulated by Ca^{2+}-concentrations far above the physiological range. In contrast to others (2), we have found that the Ca^{2+}-activation of maxi K^+ channels in the basolateral plasma membrane of surface cells from rabbit distal colon are regulated by Ca^{2+} in intracellular concentrations (3,4), and therefore could play an important role in the regulation of the aldosterone stimulated Na^+ reabsorption in this tissue (Fig. 1). The purpose of the present work is to examine the properties of the maxi K^+ channels from distal colon with particular emphasis on modulation of the channel activity by intracellular factors, such as Ca^{2+}, pH, phosphorylation/dephosphorylation and ATP.

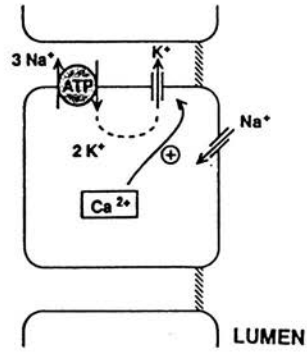

Fig. 1. Distal colon surface cell.

Basolateral plasma membrane vesicles are fused with planar lipid bilayers to obtain single channel recordings and the most predominant channel is a maxi K^+ channel (275 pS), whereas only on a few occations small conductance K^+ channels or Cl^- channels are seen. The maxi K^+ channel is activated by intracellular concentrations of Ca^{2+} in a voltage dependent manner; the $K_{0.5}$ for Ca^{2+} ranges from 20 nM at 0 mV to 300 nM at -40 mV. An increase in the intracellular H^+-concentration on the intracellular face leads to a decrease in the Ca^{2+}-sensitivity of the K^+ channel, consistent with at competition between H^+ and Ca^{2+} for binding to the Ca^{2+}-activation site.

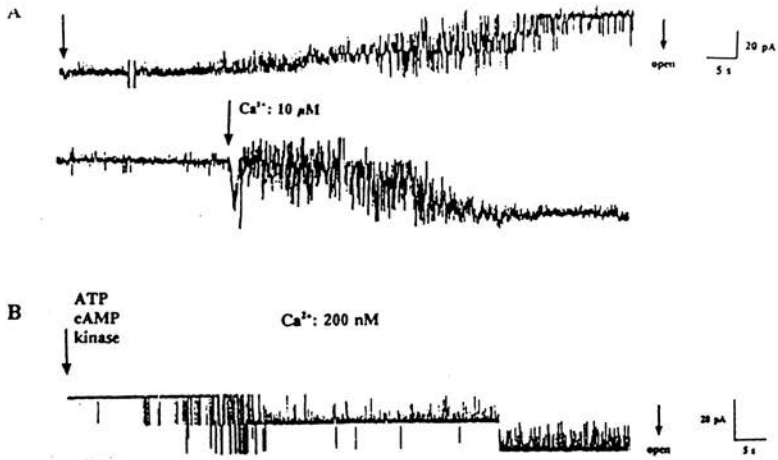

Fig. 2. A. Several maxi K^+ channels are incorporated into the bilayer at a low Ca^{2+}-concentration. Addition of a phosphatase inactivates the channels, but the channels can still be reactivated by addition of high concentrations of Ca^{2+}. B. Two dephosphorylated maxi K^+ channels are incorporated into the bilayer. They are inactive at the low Ca^{2+}-concentration (200 nM), but can be activated by phosphorylation.

After addition of protein phosphatase or activation of endogenous phosphatases by incubation with 1 mM Mg^{2+}, the K^+ channels loses their high sensitivity to Ca^{2+}, but they can still be activated by high concentrations of Ca^{2+} (10 μM). A dephosphorylated channel can regain its Ca^{2+}-sensitivity by phosphorylation from a cAMP-dependent protein kinase in the presence of 0.5 mM ATP (Fig. 2). There is no effect on the single channel conductance of phosphorylation or dephosphorylation.

Surprisingly we find, that ATP at higher concentrations inhibits the K^+ channel from the intracellular face; the $K_{0.5}$ for inhibition is 2.5 mM. Since the ATP-inhibition can take place in the absence of protein kinase, it is probably a direct effect of ATP. The ATP-inhibition cannot be reversed by addition of ADP as it is the case for e.g. ATP-sensitive K^+ channels from beta-cells. Also high concentrations of Ca^{2+} cannot reactivate the channel once it is inhibited by ATP, indicating that ATP does not interfere with the Ca^{2+}-activation of the channel.

In conclusion, the high sensitivity to Ca^{2+} of the maxi K^+ channel from rabbit distal colon is dependent on the protein being in a phosphorylated state. The variety in Ca^{2+} sensitivities for maxi K^+ channels from tissue to tissue and from different studies on the same tissue could be due to modification of the channels by second messenger systems. Although K^+ channels traditionally have been classified as either ATP-sensitive or Ca^{2+}- activated, our study shows inhibition of a Ca^{2+}-activated K^+ channel by ATP in physiological concentrations. This opens the possibility that the activity of the Ca^{2+}-activated K^+ channel is linked to the metabolic state of the cell.

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Cell volume-regulated Cl^- channel associated with the expression of P-glycoprotein: dual function of a single molecule?

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P-glycoprotein (Pgp) is an active transporter protein structurally related to the ATP-binding cassette (ABC) transporter family. Its ability to actively extrude a number of anticancer drugs has been known for over a decade, and much effort has been directed to characterize the multidrug resistance (MDR) associated to Pgp expression. It has been shown in cultured cells that multidrug resistance is associated with amplification of the *mdr1* gene, which encodes Pgp, and with overexpression of Pgp. Multidrug resistant cells are able to decrease the intracellular concentration of a number of cytotoxic drugs by an active (ATP-dependent) transport mechanism. Substrates for Pgp include several chemically unrelated cytotoxic drugs, such as colchicine, *Vinca* alkaloids and anthracyclines, and other compounds. Another heterogeneous group of chemicals, known as sensitizers or MDR-reversers, antagonize Pgp-mediated drug transport through different mechanisms, some competitive and others, apparently, by direct blockade of Pgp function. This group includes calcium channel blockers (verapamil, nifedipine), cyclosporins, forskolin and antioestrogens among others.

The structure of Pgp is that of a typical ABC transporter: four domains organized into two transmembrane domains, each consisting of six membrane-spanning segments, and two cytoplasmic nucleotide-binding domains. Most of the components of the ABC transporter family have been identified in prokaryotic organisms. Members of this protein family in superior organisms include an endoplasmic reticulum peptide transporter (RING4-RING11), the cystic fibrosis transmembrane conductance regulator (CFTR), and P-glycoprotein (1).

Because of the structural similaritiy between CFTR and Pgp, and the fact that both proteins are normally expressed in epthelia, we decided to explore the possibility that Pgp expression would be associated to any of the three different Cl^- conductances that had been identified electrophysiologically in epithelial cells. The three types of currents include a) outwardly rectifying currents regulated by intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$); b) outwardly rectifiying currents regulated by cell volume; and c) non-rectifying currents regulated by intracellular cyclic AMP concentration. Hence, initial experiments consisted of assaying the effects of the corresponding current-activating manoeuvres on cells expressing Pgp, using the whole-cell mode of the patch-clamp technique. We used two different cell lines (S1 and NIH 3T3) known to have no detectable Pgp expression, and for each cell line, a subclone selected for Pgp expression (S1 1.1 and MDR 3T3, respectively) was also available. Altering $[Ca^{2+}]i$ or intracellular cAMP levels did not generate the corresponding Cl^- currents in the parental nor the Pgp-expressing cell lines. When cells were forced to swell by exposing them to a hypotonic shock (220 mOsmol/L), parental cell lines showed only negligibly low current levels. However, cells expressing Pgp developed sizeable outwardly rectifying Cl^- currents over a 1 min period. To confirm that these currents were indeed associated to Pgp expression, we used antisense nucleotides directed against Pgp mRNA to inhibit expression of Pgp in S1 1.1 cells. Cells grown in the presence of the antisense nucleotides did not develop the hypotonicity-activated Cl^- currents and Western blots showed that their Pgp expression had been suppressed. On the other hand, cells of the same type that had been exposed to sense oligonucleotides expressed Pgp at normal levels and did develop Cl^- currents when challenged with hypotonicity. In a second group of experiments either the human *mdr*1 gene or a control sequence were transiently transfected into parental (not expressing Pgp) S1 cells. After transfection, cells that had received the *mdr*1 gene expressed large amounts of Pgp and responded to hypotonicty by developing Cl^- currents, while transfection of the vector alone did not modify the behavior of S1 cells. Thus, it appeared that a Cl^- conductance very similar to the cell volume-regulated conductance described in epithelial cells was indeed associated to the expression of Pgp (2). Experiments in which intracellular ATP was either removed or replaced by non-hydrolysable analogs showed that hypotonicity could not evoke the Cl^- currents when ATP was not included in the pipette solution. Replacement with non-hydrolysable analogs, however, still allowed the activation of volume-regulated currents, thus indicating that only

binding, but not hydrolysis of ATP, was necessary for the activation of the currents associated to Pgp expression.

We then investigated the interaction between the drug transport and ionic channel functions. In a first group of experiments, different drugs, known to be substrates of Pgp transport, were included in the intracellular solution either before or after exposure to hypotonicity. Our results showed that the currents could not be activated in the presence of intracellular drugs, while when the drugs were added intracellularly after eliciting the volume-regulated currents, no effect on them was detected. On the other hand, extracellular addition of the drugs did not modify the activation or development of the Cl^- currents. Our interpretation of these results was that in the presence of its substrates, Pgp engaged in pumping them and was unable to support the channel function while this process was on. To test this hypothesis, we repeated the experiments in conditions that did not allow ATP hydrolysis, and hence active transport, and under these conditions the presence of intracellular drugs no longer prevented the development of Cl^- currents. Further suppport for these results was provided by directed mutagenesis of the nucleotide-binding domain of Pgp. The mutant protein had been shown to be unable of hydrolysing ATP and transporting drugs while retaining the ability to bind ATP. Confirming our prediction, transient expression of the mutated protein conferred the channel activity to cells previously lacking it, and activation of the currents was not prevented by the presence of drugs in the intracellular solution (3).

The pharmacology of the Cl^- currents associated to Pgp expression is also a source of information about the relationship between the channel and the transporter functions. We looked at the effects of three groups of substances: MDR substrates, Pgp transport inhibitors (MDR reversers), and known Cl^- channel blockers, adding them both from the intracellular and extracellular sides of the plasma membrane (4). The fact that transport inhibitors also can block the volume-activated currents in a manner similar to conventional Cl^- channel blockers strengthens the hypothesis that Pgp itself can act as a Cl^- channel.

Based on these results, we have put forward a model to account for Pgp's dual function as a transporter and as a Cl^- channel. In this model, Pgp would reversibly bind ATP and enter into an undefined "ready" state. The Pgp-ATP complex could be recruited to a channel configuration by cell swelling, and it would be unable to act as a transporter while in the channel state. On the other hand, the binding of transport substrates would induce the undefined Pgp-ATP complex to start ATP hydrolysis and active translocation of the substrate. Activation of the channel mode would not be possible during active transport. Thus, the first stimulus to act on the Pgp-ATP complex would shift the protein to serve exclusively either the transporter or the channel functions until removal of the corresponding stimulus. This "first come, first served" model leaves open the question of which is the physiological, or the most frequent mode of action of Pgp. Although researchers studying the role of Pgp in multidrug resistance suggested that the normal function of Pgp might be the detoxification of harmful substances contained in food, no physiological transport substrate has been yet identified. On the other hand, Cl^- currents which are very similar electrophysiologically to those associated with Pgp expression have been shown to be involved in cell volume regulation in epithelia, and this finding can be taken as indirect support for the contention that Pgp is normally acting as a Cl^- channel. The final direct proof that Pgp itself forms the ionic channel would require the reconstitution of the purified protein into artificial membranes or the demonstration that Pgp mutations modify the channel properties. While this evidence is lacking, the hypothesis of the identity of Pgp and the volume-regulated Cl^- channel remains the explanation for the data requiring less complicated assumptions.

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Selective activation of large-conductance Ca^{2+}-dependent K^+ channels in vascular smooth muscle cells by NS 1619

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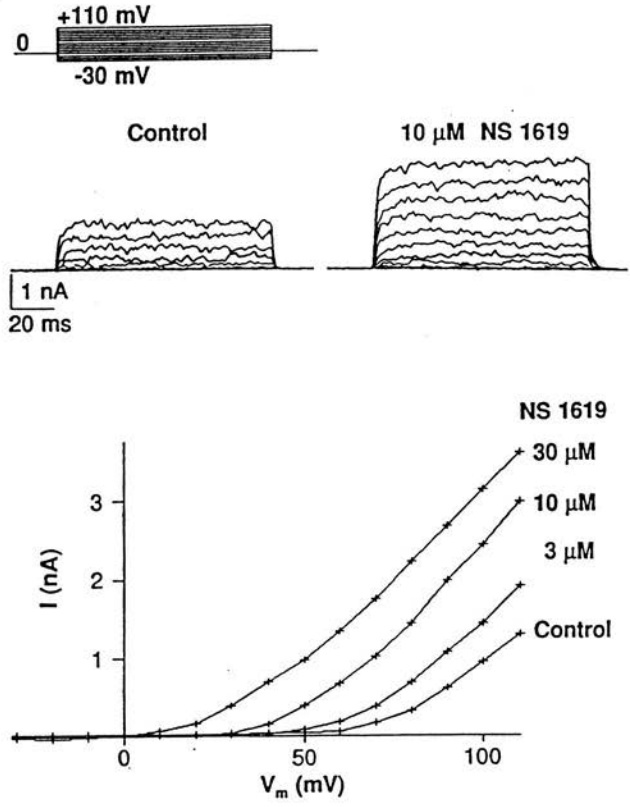
Large-conductance Ca^{2+}-dependent K^+ channels or BK channels are present in most smooth muscle cells. The probability of the channel being open is very low in resting cells. However, the opening is increased by depolarization and by an increase in the internal Ca^{2+} concentration. At positive membrane potentials the BK current often dominates the membrane conductance due to the large single channel conductance and the dense distribution of the channel. BK channels could potentially have a large impact on cell function, if the open probability curve was shifted towards more negative membrane potentials. We report here patch-clamp experiments on the activation of BK channels in aortic smooth muscle by a novel compound, NS 1619 (1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2(3H)benzimidazolone).

Smooth muscle cells were isolated and cultured from calf aorta and single BK channels were studied. The channels had an average unit conductance of 280 pS (symmetrical 146 mM K^+), were activated by internal Ca^{2+} (30-1000 nM), and were blocked by external charybdotoxin (20-100 nM) and TEA^+ (0.3-1 mM). NS 1619, administered to the bath, reversibly activated the channels and increased the mean open time as well as the opening frequency. The effect was dose-dependent, and 30 μM NS 1619 increased the open state probability, p_o, by more than 120 fold. In plots of ln $(p_o/1-p_o)$ as a function of V_m it was seen, that the action of the compound was to parallel shift the curves towards negative membrane potentials in a way similar to that of increasing $[Ca^{2+}]_i$. The average effects induced by 3, 10, and 30 μM NS 1619 were leftward shifts of -10, -22, and -45 mV, respectively. The compound did not influence the slope of the Bolzmann curves. The BK channel activating effect was independent of internal ATP or GTP, but the compound was ineffective in the absence of internal Ca^{2+}.

In whole-cell recordings the cells expressed a large, outward BK current at positive membrane potentials. NS 1619 significantly increased this current as shown in the figure and shifted the I-V curve towards negative membrane potentials. TEA^+ (1 mM) and charybdotoxin (50 nM) blocked the NS 1619-induced current, whereas glibenclamide (1 μM) failed to influence the current. 30 μM NS 1619 hyperpolarized the cells by an average -20 mV.

In cultured mouse cortical neurons and in cerebellar granule cells NS 1619 strongly activated BK currents without influencing Na^+ currents or voltage-activated K^+ currents. In rat β-cells the compound activated BK channels but did not modulate the activity of K_{ATP} channels.

In conclusion, NS 1619 is an effective activator of BK currents in arterial smooth muscle cells and may be an important tool for studying the physiological and pathological significance of BK channels.



Whole-cell currents activated by NS 1619. 3, 10 and 30 μM NS 1619 shifted the I-V curve by -12, -32, and -52 mV, respectively (V_h = 0 mV; C = 22 pF).

Permeability properties and modulation of*eag* potassium channels

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Ether à go-go (*eag*) was first isolated as a *Drosophila* mutant with altered behavior under ether anesthesia. The electrophysiology of the neuromuscular junction in the mutant larvae is also altered. All the K^+ currents are reduced, including the fast inactivating (I_A), the non-inactivating (I_K) and both fast and slow Ca^{2+}-dependent K^+ currents (I_{CF} and I_{CS}). The primary sequence of *eag* locus suggested that it encoded a novel K^+ channel subunit. These data led to the conclusion that *eag* might contribute to the heterooligomeric structure of different K^+-channels, being a general regulatory element.

We have expressed *eag* channels in *Xenopus* oocytes, and found that injection of*eag* cRNA induces the expression of voltage-activated K^+ currents with novel properties. First,*eag* channels show a distinct permeability profile, since they are quite permeable to Cs^+. Moreover, together with the efflux of K^+-ions in intact oocytes depolarization induces a chloride influx, resulting in a two-phase current. The fast initial component is due to K^+, while the slow second component is due to Cl^-. This biphasic behavior is minimized in excised patches. Inhibition of Ca^{2+}-activated Cl^- channels in whole oocytes by Niflumic acid, Mefenamic acid or chelation of internal Ca^{2+} with BAPTA injections reduces the amplitude of the chloride current. The kinetics of the resulting current match those of the current in patches. These data, together with the evidence of Ca^{2+} influx upon depolarization obtained in oocytes expressing *eag* channels and injected with the Ca^{2+}-sensitive fluorescent dye Fluo-3, indicate that*eag* encodes a voltage-activated K^+ channel with an unexpectedly high permeability to Ca^{2+}. Moreover, the Ca^{2+} influx trough*eag* channels is bigger than that of monovalents, such as Na^+ or Li^+. The permeability series of *eag* channels is $K^+ > Rb^+ > Cs^+ > Ca^{2+} > Na^+ > Li^+$.

The calcium permeability together with the kinetic properties of*eag* currents in excised patches offers a simple explanation for the electrophysiological alterations found in larval muscle.

On the other hand,*eag* channels show a consensus sequence for cyclic nucleotide binding near the carboxy terminus. We have tested whether cyclic nucleotides affect the properties of *eag* using both membrane-permeable analogs and inside-out patches. 8-Br-cGMP

induces an increase in current amplitude together with a shift in the activation to more negative potentials. The effect of cGMP depends on phosphorylation, and does not happen in cell-free conditions. On the other hand, as predicted from the sequence analysis, cAMP induces a transient increase in amplitude in the whole oocyte that is mimicked upon addition of cAMP to the cytoplasmic side of excised patches.

A Peptide Segment Critical for Sodium Channel Inactivation Functions as an Inactivation Gate in a Potassium Channel

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The short cytoplasmic segment connecting homologous domains III and IV of voltage-gated sodium channels (III-IV linker) is essential for fast inactivation (Patton et al., 1992, West et al., 1992). Armstrong and Bezanilla (1977) proposed that fast inactivation in sodium channels occurs via a tethered polypeptide "inactivation particle" occluding the pore by binding to the cytoplasmic face of the channel. Aldrich and co-workers have shown that this model can account for the fast inactivation process in *Shaker* potassium channels, with the amino terminus of the channel functioning as the cytoplasmic inactivation particle (Hoshi et al., 1990, Zagotta et al., 1990). We constructed chimeric ion channels in which the sodium channel III-IV linker was attached to the amino terminus of the non-inactivating MK1 (mKv1.1) potassium channel to test the functional similarity between the sodium channel III-IV linker and the *Shaker* H4 amino terminal inactivation particle.

To demonstrate that MK1 has a functional receptor for the *Shaker* H4 inactivation particle, we first constructed a chimeric channel in which the first 57 amino acid residues of the *Shaker* H4 potassium channel was attached to the amino terminus of MK1. Macroscopic currents recorded from oocytes expressing this chimeric channel decayed faster than currents recorded from oocytes expressing the wild-type Shaker H4 potassium channel. This indicates that MK1 posseses a functional receptor for the *Shaker* H4 amino terminal inactivation particle.

To test if the sodium channel III-IV linker can function as an inactivation gate in a potassium channel, we attached the III-IV linker to the amino terminus of MK1. This chimeric channel inactivated rapidly and displayed biophysical properties similar to *Shaker* A-type potassium channels. Recovery from inactivation in this chimeric channel was accelerated by high external potassium, indicating that the III-IV linker mediates inactivation in this channel by occluding the pore on the cytoplasmic face of the channel. We then introduced a mutation in the sodium channel III-IV linker portion of this chimeric channel which completely abolishes inactivation in the sodium channel, and this mutation also completely abolished inactivation in this chimera. These results demonstrate that the sodium channel III-IV linker can function as an inactivation gate, and suggest a functional relationship between the fast inactivation processes of sodium and potassium channels.

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Fractional Ca^{2+}-current through AMPA-gated glutamate-receptor channels in forebrain medial septal neurons.

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Simultaneous whole-cell recordings of ionic currents and fluorometric Ca^{2+}-measurements were performed in large forebrain neurons of medial septal slices. These neurons are part of the septo-hippocampal formation and provide an important contribution to the cholinergic input to the hippocampus. They are characterized by an almost complete lack of dendrites, by glutamatergic synaptic inputs to the soma [1] and by good voltage control in whole-cell voltage-clamp experiments. Therefore, these cells are a useful model system for quantifying the Ca^{2+}-influx through glutamate receptor channels in a central neuron.

Ca^{2+}-influx was measured by loading the neurons with high concentrations of the Ca^{2+}-indicator dye fura-2 via the recording patch-pipette. Voltage-gated Ca^{2+}-currents (pulses from V_h -80 mV to -10 mV, 50-1000 ms duration) were used to inject a defined electrical charge of Ca^{2+} into the cytoplasm. The decrement of the Ca^{2+}-sensitive fluorescence at 380 nm excitation (F_{380}) was devided by the Ca^{2+}-charge, giving the F/Q-ratio [2]. During the loading of medial septal neurons with 1 mM fura-2, the F/Q-ratio reached a saturating value (f_{max}), indicating that fura-2 had become the dominating intracellular Ca^{2+}-buffer [3]. Under these conditions, changes in F_{380} can be used as a direct measurement of Ca^{2+}-influx. At a V_h of -80 mV, local ionophoretic applications of AMPA or kainate to the soma of medial septal neurons produced inward currents, which were accompanied by clear changes in F_{380}. These Ca^{2+}-signals were interpreted as a Ca^{2+}-influx through the open AMPA-R channels for the following reasons: 1) they were not reduced in size by 100 µM D-600 (excluding a participation of voltage-gated Ca^{2+}-channels); 2) they were blocked by

CNQX (50 μM), and their amplitude was linearly related to the charge of the ionic currents; 3) they were abolished by a positive V_h or by omitting the extracellular Ca^{2+} from the bath solution (excluding a Ca^{2+}-release from internal stores).

In order to quantify the fraction of Ca^{2+} to the total charge flowing through the AMPA-R channels, the F/Q-ratio calculated from the AMPA- and kainate induced ionic currents and F_{380} changes was divided by f_{max}, yielding a fractional Ca^{2+}-current of 1.4 ± 0.6 % (n = 10 cells, V_h = -80 mV, 1.6 mM extracellular [Ca^{2+}]). No significant difference between AMPA- and kainate-induced currents was observed [4]. The reported fractional Ca^{2+}-current of 1.4 % is about 4-5 times larger than that observed through linearly conducting AMPA-R channels with a "low" Ca^{2+}-permeability (Tempia, Kano & Konnerth, unpublished observations). These results are, to our knowledge, the first report of a significant Ca^{2+}-influx through AMPA-gated glutamate-R channels in neurons of the central nervous system.

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MOLECULAR RECOGNITION BETWEEN MEMBRANE-EMBEDDED SYNTHETIC SEGMENTS OF ION CHANNELS.

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Current models of voltage-activated K+ channels predict that the channels are formed by the coassembly of four polypeptide monomers, each of which consists of six transmembrane segments (S1 to S6) and long terminal domains. Combined molecular-biology and electrophysiological approaches indicated that the H-5 segment, a highly conserved sequence that connects the S5 and S6 segments of K+ channels, is involved in the formation of the ion-conducting pore, thus, suggesting its location in the lumen of the channel[1-7]. Both protein/lipid and protein/protein interactions are thought to determine the correct assembly of the channel. However, the structural components involved in these two types of interactions are not yet clear. Li and coworkers[8] have proposed that the N-terminal domains of the Shaker K+ channel monomers and the core region, which include the transmembrane domains, are involved in mediating the appropriate assembly of the channels. The existence, within the core region, of sites for monomers recognition was also proposed by McCormack and coworkers[9].

In the frame work of our general interest in protein-membrane interactions, we are utilizing spectrofluorometric approaches to study the interaction of synthetic peptides, resembling putative membrane-embedded segments of ion channels and pore forming toxins, with phospholipid membranes.

Segments resembling different portion of the H-5 (12-23 amino acid long) and the S-2 regions of the *Shaker* K+ channel were synthesized and then structurally and functionally characterized. Circular Dichroism spectroscopy revealed that, in hydrophobic environment, S-2 adopts high α-helical structure (78%), and that H-5 can partially adopt α–helical structure, contributed by its N-part. Functional characterization demonstrated that the segments bind to zwitterionic phospholipids, with partition coefficients in the order of $10^4 M^{-1}$. However, a truncated H-5 derived peptide, without 4 amino acids within the most conserved region (amino acids 432-435), did not bind to the membranes at all. Moreover, the

single substitution of a conserved tryptophan at position 435 to serine, reduced the partition coefficient of the peptide ~5 fold, which may account for a mutated K+ channel with this substitution not producing functional channels[6]. Resonance energy transfer measurements, between donor/acceptor-labeled pairs of peptides, revealed that both H-5 and S-2 derived peptides self-associate in the membrane-bound states. Furthermore, membrane-embedded H-5 derived segments associates with membrane-bound S-2, but do not associate with unrelated, membrane-bound α-helical peptides. These results demonstrate molecular recognition between transmembrane segments of the *Shaker* K+ channel that might contribute to the oligomerization and correct assembly of the monomers which result in the formation of a functional channel.

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POTASSIUM CHANNEL OPENERS INDUCE MITOCHONDRIAL MATRIX VOLUME CHANGES VIA ACTIVATION OF ATP-SENSITIVE K^+ CHANNEL

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ATP-sensitive potassium (K_{ATP}) channel has been identified in rat liver mitochondria (Inoue et al., 1991). Recently, its activity has been extracted and successfully reconstituted into liposomes (Paucek et al., 1992). The channel was shown to be blocked not only by ATP, but also by antidiabetic sulfonylurea, glibenclamide (Inoue et al., 1991), providing an evidence that mitochondrial K_{ATP} channel may belong to the well known family of ATP-dependent potassium channels found in plasma membranes of cardiac, smooth and skeletal muscle cells, pancreatic B-cells, and in nervous system (Quast & Cook, 1989). Despite of blockage by sulfonylureas (Schmid-Antomarchi et al., 1987), these channels are activated by hormones (De Weille et al., 1988, 1989), free fatty acids (Müller et al., 1992), and by various K^+ channel openers (KCOs) (Weston and Edwards, 1992).

It is still a mater of question how the mitochondrial K_{ATP} channel may be engaged in the function of liver mitochondria. On the other hand, it is widely accepted that as a result of K^+ influx into mitochondrial matrix, water content of mitochondria changes, evoking mitochondrial swelling. Some of these changes are related to the action of hormones which cause an increase of cAMP (glucagon) or of calcium concentration (vasopressin and α-adrenergic agonists), and to the activity of mitochondrial respiratory chain. Also, it is known that an increase in intramitochondrial volume is responsible for significant activation of mitochondrial metabolism (for review see Halestrap, 1989).

It has been postulated that rat liver mitochondrial K_{ATP} channel may play an important role in mitochondrial swelling (Inoue et al., 1991). Therefore, the so-called passive swelling in KSCN medium and the energy-dependent swelling in CH_3COOK medium of mitochondria energized by the addition of respiratory chain substrate (succinate) were measured. The kinetics of light scattering changes, reflecting mitochondrial volume changes linked to solute transport, were measured at 540 nm with spectrophotometer. Under such conditions, e.g. in the presence of permeable anion (SCN^-) or CH_3COOH, swelling of mitochondria reflects influx of K^+ into the mitochondrial matrix. Two KCOs, namely diazoxide (700 μM) and RP 66471 (250 μM), are able to stimulate passive mitochondrial swelling. Other K^+ channel openers, i.e. pinacidil, KRN 2391, P1060, and minoxidil sulfate, at concentration of 700 μM, were also found effective. Ro 31-6930 and nicorandil, at the same concentration, were observed to have no effect. The most potent opener (fold of stimulation 5.48 $\pm$ 1.11) turned out to be RP 66471, with $K_{0.5}$ of 50 μM. Similar results were obtained in the acetate medium, e.g. for the energy-dependent swelling.

The stimulatory effect of RP 66471 (250 μM) and minoxidil sulfate (700 μM) was almost completely abolished by 100 μM glibenclamide, both in respiring and non-respiring mitochondria. Moreover, activation of mitochondrial swelling caused by 700 μM KRN 2391 and pinacidil was also observed to be blocked by 100 μM glibenclamide.

It is in agreement with observation published by others that glibenclamide is able to completely inhibit the activity of the reconstituted ATP-sensitive K^+ channel of rat liver

mitochondria. However, that inhibition by glibenclamide in the reconstituted system occured at a much lower concentration range, with IC_{50} of 62 nM (Paucek et al., 1992). The discrepancy in the concentration range between our results and that of Garlid's group could be explained as follows. As was shown by Paucek et al. (1992) magnesium ions reduce inhibitory potency of glibenclamide in the reconstituted system. Thus, relatively high concentration of glibenclamide required to abolish the effect of potassium channel openers on mitochondrial swelling could be ascribed to the presence of Mg^{2+} in mitochondrial preparation. In addition, the potency of glibenclamide, a highly hydrophobic compound, in inhibiting potassium channel opener-induced swelling must be limited by its surface concentration (compare to Hannun et al., 1991). Indeed, by lowering protein concentration we observed that the effectiveness of glibenclamide to reverse the effect of potassium channel openers was increased (data not shown). Last but not least, mitochondria are known to maintain high concentration of adenine nucleotides which can by itself lower the affinity of glibenclamide to its putative receptor (Ashcroft and Ashcroft, 1992).

Summarizing, our results may suggest that K_{ATP} channel of the mitochondrial inner membrane is involved in regulation of swelling of liver mitochondria. Since changes of mitochondrial volume are sufficient to modulate metabolic processes such as citruline synthesis, pyruvate carboxylation and/or fatty acid oxidation, it seems to be of particular importance to establish whether mitochondrial K_{ATP} channel activity could be, at least partially, responsible for the regulation of mitochondrial metabolism.

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IMMUNOCHEMICAL, MOLECULAR AND DEVELOPMENTAL CHARACTERIZATION OF POTASSIUM CHANNELS PROTEINS FROM THE *SHAKER* LOCUS OF *Drosophila*.

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The *Shaker* locus of *Drosophila* harbors in its Viable region (Ferrús et al 1991) a very large transcription unit that by mean of alternative splicing can produce homologous proteins sharing a common central core region and having different N-tem and C-term regions which confers differential electrical properties (Pongs et al 1988, Schwarz et al.1988, Iverson et al 1988; Timpe et al 1988; Kamb et al1988, Iverson & Rudy, 1990) providing in this way a molecular basis for potassium channel diversity in the nervous system of this organism. Although previous attempts in other laboratories succeeded in preparing antisera against Sh proteins (Barbas et al 1989; Schwarz et al.,1990) they could not detect the expected variety of protein products in spite of the diversity of mRNAs detected and cDNAs isolated. Homology cloning in different species and tissues together with site directed mutagenesis and "in vitro" expression have unraveled the existence of various voltage-gated K-channels gene subfamilies and yielded a great advance in the knowledge of these molecules (see Jan & Jan, 1992 Drewe et al 1992 for recent reviews). However, we do not presently know very much about the biochemical properties, tissular and cellular distribution, and other relevant information of the proteins forming the K-channels.

In order to approach the biochemical study of those proteins, we have raised antisera against recombinant Sh proteins obtained by expression of cDNA fragments of the central core region common to all Sh transcripts. Two of these antisera succeeded in recognizing multiple bands in the range of 65-85 Kd when total proteins from adult *wt* flies were analyzed by Western-blot. Additionally, one antiserum raised against the whole core region recognizes a protein of 40Kd that seems to correspond to short Sh transcripts. All these bands are surely specific since they are detected by affinity purified antibodies and they are absent in preparations of deficient flies (double chromosomal translocation that precludes transcription of Sh). At least 90% of the signal is detected in the head of adults and the rest in thorax. No K-channel proteins are found in abdomen. Interestingly, the electrophoretic patterns of Sh proteins expressed in head and thorax are different. Parallelly, the K-channel proteins are greatly concentrated in the nervous system of third instar larvae.

Developmental experiments show that some of the proteins are first detected in LI stage increasing its intensity in further steps up to the adult fly. The pattern of Sh splice variant changes dramatically during puparation and the first day after hatching of flies. Our data also indicates the huge increase in the expression of Sh K-channel proteins that happen along puparation (more than ten times). We have also isolated membrane preparations and successfully solubilized the Sh K-channels with various detergents and carried out deglycosylation with specific enzymes. We have also made phosphorylation, and dephosphorylation experiments of Sh K-channels in order to further characterize these proteins.

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Triggered activation of a peripheral nerve-specific sodium channel gene by nerve growth factor

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The firing patterns of neurons change during development and as a result of synaptic plasticity in the adult animal. We have been examining molecular mechanisms underlying long-term modulation of specific sodium channel types. Our studies have exploited the rat pheochromocytoma cell line, PC12, which differentiates into sympathetic-like neurons after treatment with neuronal growth factors such as Nerve Growth Factor (NGF). PC12 cells extend neuritic processes and acquire the ability to generate sodium-based action potentials within 48 hours of treatment with NGF. We have determined previously that the acquisition of electrical excitability correlates with the induction of mRNAs encoding two distinct sodium channel alpha subunit types. One of the mRNAs appeared to encode a new sodium channel alpha subunit, which is predominant in tissues of the peripheral nervous system.

The structure and regulation of the new alpha subunit, termed peripheral nerve type 1 (PN1), was examined by obtaining a molecular probe specific for the PN1 transcript. The polymerase chain reaction (PCR) was used to amplify cDNA prepared from PC12 mRNA. A 400 bp fragment, encoding amino acids corresponding to # 1356-1469 of the brain type II subunit, and containing the putative pore-forming region, was obtained. The predicted primary structure of the peptide encoded by the cloned cDNA is 70% identical to that of the other rat brain alpha subunits. When the PCR product was used as a probe in Northern blot analysis with RNA purified from PC12 cells, the PN1 transcript was the only one

detected. Moreover, Northern blot and RNAase protection analyses indicated that PN1 mRNA was found at high levels in the peripheral nervous system (dorsal root, superior cervical, and trigeminal ganglia) and is barely detectable in brain. No PN1 transcripts were detected in heart, skeletal muscle or inexcitable tissues such as liver. Thus, PN1 represents a new sodium channel alpha subunit type which may underlie fast electrical signaling in the peripheral nervous system.

Previous electrophysiological studies have suggested the importance of NGF in influencing sodium channel excitability during peripheral neuron development. To investigate the mechanism of this regulation, we examined PN1 gene expression during PC12 differentiation by NGF. The induction of PN1 mRNA was found to be very rapid, peaking at five hours after exposure to growth factor. Induction of the PN1 transcript was irreversible, dependent upon only a brief (within one minute) exposure to NGF. Induction was independent of the established NGF signaling pathways, including the src, ras and raf proto-oncoprotein pathways, and the protein kinases A and C pathways, known to be important for NGF-induction of other neuronal genes. Experiments using translational inhibitors indicated that the induction of PN1 mRNA by NGF required protein synthesis only during the first hour of exposure to growth factor, suggesting the involvement of immediate early genes in this process. PN1 induction is also blocked by inhibitors of RNA synthesis, suggesting regulation at the level of transcription.

There are two major findings from this work . First, we have identified a new sodium channel alpha subunit expressed at high levels specifically in the peripheral nervous system. Characterization of the functional properties of this sodium channel type may contribute ultimately to an understanding of firing properties of specific neurons in the peripheral nervous system. Second, we have revealed a novel mechanism by which NGF triggers activation of genes, and which does not require the continued presence of the growth factor. This mechanism is distinct from that described for any other gene induced by NGF.

Stereoselective block of a cloned human cardiac potassium channel (hKv 1.5) by bupivacaine

Carmen Valenzuela

Bupivacaine is a widely used local anesthetic which has been described to inhibit several types of cardiac ion channels. In fact, bupivacaine inhibits the delayed rectifier outward K^+ current (I_K), as well as the transient outward current (I_{TO}). Several studies have demonstrated that S(-)-bupivacaine is less toxic than R(+)-bupivacaine, being the duration of its effects equal or longer than that of the racemic form.

The effects of the optical stereoisomers of the local anesthetic bupivacaine on a cloned human cardiac potassium channel (hKv 1.5) expressed in a stable mouse L cell line was studied using the whole-cell configuration of the patch-clamp technique. Neither S(-)-bupivacaine or R(+)-bupivacaine affected the initial sigmoidal activation time course of the current. However, S(-)-bupivacaine and R(+)-bupivacaine at 20 μM, reduced the peak current, being its more prominent effect the induction of a subsequent decline which appears with a time constant of 18.7±1.1 ms and 10.0±0.9 ms. After 250 ms-depolarizing pulses this decline induced an averaged block of 30.8±2.5% (n=6) and 79.5±3.2% (n=6) ($p<0.001$), in the presence of S(-)-bupivacaine and R(+)-bupivacaine (at +60 mV), respectively. The concentration dependence of hKv 1.5 block induced by both enantiomers of bupivacaine came up with a K_D values of 27.3 μM and 4.1 μM for S(-)-bupivacaine and for R(+)-bupivacaine, respectively. In both cases, the Hill coefficients obtained were close to unity: 1.31 and 0.83, respectively; thus suggesting that binding of one enantiomer molecule/channel is sufficient to block potassium permeation. The degree of block induced by both bupivacaine enantiomers resulted to be voltage dependent. Therefore, block increased from 0.53±0.02 at 0 mV to 0.62±0.07 at +60 mV in the presence of 50 μM S(-)-bupivacaine and from 0.75±0.01 at 0 mV to 0.82±0.01 at +60 mV in the presence of 20 μM R(+)-bupivacaine. This voltage dependence was described in both cases by an equivalent electrical distance δ of 0.16±0.01, which suggested that at the binding site, both enantiomers of bupivacaine experienced 16% of the applied transmembrane electrical field, referenced to the inner surface. Both bupivacaine enantiomers reduced the tail currents amplitude recorded on return to -40 mV and slowed their time course relative to control, resulting in a "crossover" phenomenon. These data indicate: 1) The charged form of both bupivacaine enantiomers

block the hKv 1.5 channel after it opens, 2) binding occurs within the transmembrane electrical field, 3) unbinding is required before the channel can close and 4) block of hKv 1.5 channels by bupivacaine is highly stereoselective. These results agree with those previously on cardiac Na^+ current (I_{Na}), where bupivacaine block resulted to be also stereoselective. However, experiments performed in rat ventricular myocytes recording the transient outward current (I_{TO}) failed to observe a stereoselective block of this current by bupivacaine. Very recently, Boyle & Nerbonne (1990) and Wang et al. (1993) have reported the existence of a K^+ current with kinetic and voltage-dependent properties very close to those of hKv 1.5 in rat atrial myocytes and human atrial myocytes. Therefore, this K^+ outward current would be very important in controlling the action potential duration, and, thus, a potential target of class III antiarrhythmic drugs.

CONCLUSIONS

R. W. Aldrich

The rapid pace and explosive growth of ion channel research in the last few years has been clearly demonstrated by the superlative presentations in the workshop. The invention and adaptation of new techniques, such as single-channel recording, whole cell patch clamp in tissue slices, and recombinant DNA techniques, has facilitated many advances in two general areas of ion channel research. On the one hand are studies of the molecular biophysics of ion channel function, on the other are studies of channel function in the context of cellular physiology and pathophysiology. The research presented at this workshop has nicely illustrated the rapid advances being made in both areas, and in several cases has illustrated the fascinating dependence of the details of cellular physiology on the precise molecular mechanisms of channel function. That these two areas are really interdependent was beautifully emphasized by the mixture of presentations on molecular function and cellular function.

The international nature of the workshop nicely emphasized the important contributions being made from researchers in several different countries. The exchange of data and ideas between different groups, and the international collaborations that preexisted or resulted from the meeting, are signs of a healthy and energetic discipline.

The pace of research in the field shows no sign of lessening, and we can expect many other important advances in the near future, some of which were hinted at in the preliminary results presented. It is clear that similar workshops in future years will continue to be a source of excitement and inspiration for future research.

List of Invited Speakers

Workshop on

MOLECULAR BASES OF ION CHANNEL FUNCTION

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246 **Workshop on Tolerance: Mechanisms and implications.**
Organized by P. Marrack and C. Martínez-A. Lectures by H. von Boehmer, J. W. Kappler, C. Martínez-A., H. Waldmann, N. Le Douarin, J. Sprent, P. Matzinger, R. H. Schwartz, M. Weigert, A. Coutinho, C. C. Goodnow, A. L. DeFranco and P. Marrack.

247 **Workshop on Pathogenesis-related Proteins in Plants.**
Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
Course on DNA - Protein Interaction.

249 **Workshop on Molecular Diagnosis of Cancer.**
Organized by M. Perucho and P. García Barreno. Lectures by F. McCormick, A. Pellicer, J. L. Bos, M. Perucho, R. A. Weinberg, E. Harlow, E. R. Fearon, M. Schwab, F. W. Alt, R. Dalla Favera, P. E. Reddy, E. M. de Villiers, D. Slamon, I. B. Roninson, J. Groffen and M. Barbacid.

251 **Lecture Course on Approaches to Plant Development.**
Organized by P. Puigdoménech and T. Nelson. Lectures by I. Sussex, R. S. Poethig, M. Delseny, M. Freeling, S. C. de Vries, J. H. Rothman, J. Modolell, F. Salamini, M. A. Estelle, J. M. Martínez Zapater, A. Spena, P. J. J. Hooykaas, T. Nelson, P. Puigdoménech and M. Pagès.

252 **Curso Experimental de Electroforesis Bidimensional de Alta Resolución.**
Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorín, M. Beato and A. A. Travers. Lectures by F. Azorín, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
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264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
Organized by F. X. Avilés. Lectures by T. L. Blundell, W. Bode, P. Carbonero, R. W.Carrell, C. S. Craik, T. E. Creighton, E. W. Davie, L. D. Fricker, H. Fritz, R. Huber, J. Kenny, H. Neurath, A. Puigserver, C. A. Ryan, J. J. Sánchez-Serrano, S. Shaltiel, R. L. Stevens, K. Suzuki, V. Turk, J. Vendrell and K. Wüthrich.

267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
Organized by R. Serrano and J. A. Pintor-

Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
Organized by R. Baker and J. M. Delgado-García. Lectures by C. Acuña, R. Baker, A. H. Bass, A. Berthoz, A. L. Bianchi, J. R. Bloedel, W. Buño, R. E. Burke, R. Caminiti, G. Cheron, J. M. Delgado-García, E. E. Fetz, R. Gallego, S. Grillner, D. Guitton, S. M. Highstein, F. Mora, F. J. Rubia Vila, Y. Shinoda, M. Steriade and P. L. Strick.

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2 **Workshop on DNA Structure and Protein Recognition.**
Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

3 **Lecture Course on Palaeobiology: Preparing for the Twenty-First Century.**
Organized by F. Álvarez and S. Conway Morris. Lectures by F. Álvarez, S. Conway Morris, B. Runnegar, A. Seilacher and R. A. Spicer.

4 **Workshop on The Past and the Future of Zea Mays.**
Organized by B. Burr, L. Herrera-Estrella and P. Puigdoménech. Lectures by P. Arruda, J. L. Bennetzen, S. P. Briggs, B. Burr, J. Doebley, H. K. Dooner, M. Fromm, G. Gavazzi, C. Gigot, S. Hake, L. Herrera-Estrella, D. A. Hoisington, J. Kermicle, M. Motto, T. Nelson, G. Neuhaus, P. Puigdomenech, H. Saedler, V. Szabo and A. Viotti.

5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
Organized by C. Gancedo and J. M. Gancedo. Lectures by T. G. Cooper, T. F. Donahue, K.-D. Entian, J. M. Gancedo, C. P. Hollenberg, S. Holmberg, W. Hörz, M. Johnston, J. Mellor, F. Messenguy, F. Moreno, B. Piña, A. Sentenac, K. Struhl, G. Thireos and R. S. Zitomer.

10 **Workshop on Engineering Plants Against Pests and Pathogens.**
Organized by G. Bruening, F. García-Olmedo and F. Ponz. Lectures by R. N. Beachy, J. F. Bol, T. Boller, G. Bruening, P. Carbonero, J. Dangl, R. de Feyter, F. García-Olmedo, L. Herrera-Estrella, V. A. Hilder, J. M. Jaynes, F. Meins, F. Ponz, J. Ryals, J. Schell, J. van Rie, P. Zabel and M. Zaitlin.

11 **Lecture Course on Conservation and Use of Genetic Resources.**
Organized by N. Jouve and M. Pérez de la Vega. Lectures by R. P. Adams, R. W. Allard, T. Benítez, J. I. Cubero, J. T. Esquinas-Alcázar, G. Fedak, B. V. Ford-Lloyd, C. Gómez-Campo, V. H. Heywood, T. Hodgkin, L. Navarro, F. Orozco, M. Pérez de la Vega, C. O. Qualset, J. W. Snape and D. Zohary.

12 **Workshop on Reverse Genetics of Negative Stranded RNA Viruses.**
Organized by G. W. Wertz and J. A. Melero. Lectures by G. M. Air, L. A. Ball, G. G. Brownlee, R. Cattaneo, P. Collins, R. W. Compans, R. M. Elliott, H.-D. Klenk, D. Kolakofsky, J. A. Melero, J. Ortín, P. Palese, R. F. Pettersson, A. Portela, C. R. Pringle, J. K. Rose and G. W. Wertz.

13 **Workshop on Approaches to Plant Hormone Action.**
Organized by J. Carbonell and R. L. Jones. Lectures by J. P. Beltrán, A. B. Bleecker, J. Carbonell, R. Fischer, D. Grierson, T. Guilfoyle, A. Jones, R. L. Jones, M. Koornneef, J. Mundy, M. Pagès, R. S. Quatrano, J. I. Schroeder, A. Spena, D. Van Der Straeten and M. A. Venis.

14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Avila. Lectures by J. Avila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
Organized by J. M. Mato and A. Ullrich. Lectures by M. Barbacid, A. Bernstein, J. B. Bolen, J. Cooper, J. E. Dixon, H. U. Häring, C.- H. Heldin, H. R. Horvitz, T. Hunter, J. Martín-Pérez, D. Martín-Zanca, J. M. Mato, T. Pawson, A. Rodríguez-Tébar, J. Schlessinger, S. I. Taylor, A. Ullrich, M. D. Waterfield and M. F. White.

16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

17 **Workshop on Cell Recognition During Neuronal Development.**
Organized by C. S. Goodman and F. Jiménez. Lectures by J. Bolz, P. Bovolenta, H. Fujisawa, C. S. Goodman, M. E. Hatten, E. Hedgecock, F. Jiménez, H. Keshishian, J. Y. Kuwada, L. Landmesser, D. D. M. O'Leary, D. Pulido, J. Raper, L. F. Reichardt, M. Schachner, M. E. Schwab, C. J. Shatz, M. Tessier-Lavigne, F. S. Walsh and J. Walter.

18 **Workshop on Molecular Mechanisms of Macrophage Activation.**
Organized by C. Nathan and A. Celada. Lectures by D. O. Adams, M. Aguet, C. Bogdan, A. Celada, J. R. David, A. Ding, R. M. Fauve, D. Gemsa, S. Gordon, J. A. M. Langermans, B. Mach, R. Maki, J. Mauël, C. Nathan, M. Röllinghoff, F. Sánchez-Madrid, C. Schindler, A. Sher and Q.-w. Xie.

19 Workshop on Viral Evasion of Host Defense Mechanisms.
Organized by M. B. Mathews and M. Esteban. Lectures by E. Domingo, L. Enjuanes, M. Esteban, B. N. Fields, B. Fleckenstein, L. Gooding, M. S. Horwitz, A. G. Hovanessian, M. G. Katze, I. M. Kerr, E. Kieff, M. B. Mathews, G. McFadden, B. Moss, J. Pavlovic, P. M. Pitha, J. F. Rodríguez, R. P. Ricciardi, R. H. Silverman, J. J. Skehel, G. L. Smith, P. Staeheli, A. J. van der Eb, S. Wain-Hobson, B. R. G. Williams and W. Wold.

20 Workshop on Genomic Fingerprinting.
Organized by M. McClelland and X. Estivill. Lectures by G. Baranton, D. E. Berg, X. Estivill, J. L. Guénet, K. Hayashi, H. S. Kwan, P. Liang, M. McClelland, J. H. Nadeau, D. L. Nelson, M. Perucho, W. Powell, J. A. Rafalski, O. A. Ryder, R. Sederoff, A. J. G. Simpson, J. Welsh and H. Zischler.

21 Workshop on DNA-Drug Interactions.
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

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