Ion channels

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Summary. Insulin secretion by the pancreatic Beta cell is dependent upon transmembrane ion fluxes gated by the ATP-regulated potassium channel and the voltage regulated, L-type calcium channel. This work group examined major recent advances in the structure and modulation of ion channels and how those advances may pertain to the physiology of insulin secretion and the pharmacological treatment of Type 2 (non-insulin-dependent) diabetes mellitus. Structural studies have revealed that voltage gated ion channels are related, complex, and comprised of multiple components: sodium channels consist of three distinct subunits. L-type calcium channels, crucial to the insulin secretory response are structurally related to the sodium channel but contain additional subunits. Potassium channels are less closely related and appear to function as homotetramers. Modulation of ion channel activity is similarly complex: site specific phosphorylation by multiple protein kinases under the control of several intracellular second messenger systems may increase or decrease conductance. Subunit composition and relatively stable changes in the modal state of ion channels also appear to be critical to ion channel gating properties. Functional studies of the Beta-cell ATP-regulated potassium channel suggest two distinct nucleotide binding sites which link this channel to the metabolic state of the Beta cell. The multiple paths of ion channel modulation provide multiple targets for therapeutic intervention. Where detailed characterisation of ion channel structure has been achieved, those targets are being used for specific drug design. Such complete characterisation has not yet been achieved for Beta-cell ion channels and this presents a major goal for diabetes research.

Key words: Ion channels, phosphorylation, insulin secretion, electrophysiology, second messenger systems.

Introduction

Ion channels control the flow of ions across cell membranes, maintain transmembrane potentials, mediate action potentials, and allow for rapid and selective changes in intracellular ion concentration. Thus, they serve to control many aspects of cellular activity in a broad range of cell types. In the pancreatic Beta cell, they serve to mediate insulin secretory effects of most, if not all, secretagogues. Moreover, the Beta-cell ion channels influence glucose usage and probably play an important role in many peripheral side effects of diabetes. A specific ATP-gated potassium channel of the Beta cell and peripheral tissues is the site of action of the principal class of drugs used to treat Type 2 (non-insulin-dependent) diabetes mellitus, the sulphonylureas. A variety of other drugs and toxins alter ion channel activity and it is anticipated that rational drug design based on further understanding of ion channel structure will produce more specific and more potent agents for the treatment of diabetes.

The goal of the work group on ion channels was to consider the most recent information available on structural and functional aspects of ion channels in the tissues where they have been studied most completely, and then to apply this new data to current issues in diabetes research, with particular attention to the role of ion channels in the regulation of pancreatic Beta-cell insulin secretion. Available evidence suggests that Beta-cell ion channels share many of the structural elements and regulatory processes found in channels of other excitable tissues. However, the precise combination of channel types and their modes of regulation are clearly not universal, and the Beta cell has its own distinct set of channels and regulatory mechanisms. The discussions of the work group were divided into four overlapping sections concerned with (1) ion channel structure and function, (2) modulation of ion channel function, (3) regulation of secretion by ion

channels, and (4) the ion channels of the pancreatic Beta cell and their modulation.

Structure and function of Na⁺, K⁺, and Ca²⁺ channels

Sodium channels. Sodium channels initiate action potentials in electrically excitable cells. Their regenerative activation in response to depolarising stimuli is responsible for the rapid depolarisation that is essential for conduction of electrical signals. They are structurally homologous to calcium and potassium channels, the other members of the voltage-gated ion channel gene family. Sodium channels consist of three glycoprotein subunits: a principal α subunit of 260 kDa which is non-covalently associated with a $\beta 1$ subunit of 36 kDa and disulphide-linked to a $\beta 2$ subunit of 33 kDa [1]. The α subunits have four internally homologous domains with six α -helical transmembrane segments in each. Sodium channel α subunits are functionally autonomous when expressed in *Xenopus* oocytes or mammalian cells, but their functional properties differ from those observed in neurons in situ. $\beta 1$ subunits have a single transmembrane segment and a single, glycosylated extracellular domain [2]. Co-expression with α subunits in *Xenopus* oocytes causes a five-fold acceleration of sodium channel inactivation, a 20 mV shift in inactivation to more negative membrane potentials, and a substantial increase in peak sodium current. In the presence of $\beta 1$ subunits, sodium channel function in oocytes more closely resembles that in rat brain. Thus, normal sodium channel function depends on both α and $\beta 1$ subunits.

Since α subunits alone can function as voltage-gated sodium channels, the structures required for voltage-dependent gating and ion conductance must be localised within this subunit [1]. The fourth transmembrane segment in each homologous domain, designated S4, consists of a motif of two hydrophobic residues followed by a positive charge repeated four to eight times. These segments are thought to serve as voltage sensors in the process of voltage-dependent activation. Changes in transmembrane potential exert a force on these gating charges, causing them to move outward, normal to the plane of the membrane and initiate the conformational changes in each domain that lead to activation. Mutagenesis of either charged or hydrophobic residues in these segments has strong effects on voltage-dependent activation consistent with the proposal that they serve as voltage sensors.

If sodium channels are depolarised for more than one millisecond or so, they rapidly enter an inactivated state from which they cannot be opened by further depolarisation. Inactivation can be prevented by anti-peptide antibodies directed against the conserved intracellular loop connecting homologous domains III and IV [1]. In the presence of these antibodies, sodium channels open repetitively or stay open continuously during long depolarising stimuli. Mutations of charged and non-polar amino acid residues in this loop can slow or

completely prevent sodium channel inactivation. It is hypothesised that this peptide segment serves as the inactivation gate of the sodium channel by folding in and occluding the ion conductance pathway of the activated sodium channel [1].

In addition to the rapid effects just described, voltage-gated ion channels are also modulated on a slower time scale via intracellular second messengers. Sodium channel α subunits are rapidly phosphorylated by cAMP-dependent protein kinase and protein kinase C in purified and reconstituted channel preparations. Activation of protein kinase C in neurons or in mammalian cells expressing type IIA sodium channels slows sodium channel inactivation and reduces peak sodium currents [3]. These two effects are prevented by mutation of a single serine residue in a protein kinase C consensus sequence in the inactivation gating loop connecting domains III and IV [4]. Evidently, phosphorylation of this site is required for both functional effects of protein kinase C. In contrast, cAMP-dependent protein kinase phosphorylates a cluster of five consensus sites in the large, hypervariable intracellular loop between domains I and II both in vitro and in vivo. It causes reduction in peak current without change in the time course or voltage dependence of the sodium current [5]. Convergent phosphorylation of sodium channels by these two distinct second messenger pathways may have an important regulatory influence on sodium channel function.

Potassium channels. Voltage-regulated K⁺ channels such as the channel encoded by the *Shaker* gene from *Drosophila* are structurally distinct in that they function as non-covalently linked homotetramers rather than as a single four-domain protein as is the case in voltage-gated Na⁺ and Ca²⁺ channels. However, they display many of the functional characteristics of the other voltage-gated channels [6]. The *Shaker* potassium channel has served as a model for studying the process of channel inactivation which is one of the primary mechanisms by which channel activity is determined. Experiments by Aldrich and colleagues [7] have shown that *Shaker* potassium channels exhibit two distinct inactivation mechanisms, termed N-type and C-type. Both mechanisms are coupled to activation and have little if any intrinsic voltage dependence. N-type inactivation can occur within a few milliseconds, is inhibited by intracellular tetraethylammonium, and occurs by a mechanism whereby residues at the amino terminal act as a tethered-inactivation particle [8, 9]. C-type inactivation can occur within a few tens of milliseconds. In contrast to N-type, C-type inactivation is inhibited by extracellular tetraethylammonium ion. C-type inactivation is also modulated by permeant extracellular monovalent ions, and involves amino acid residues in the sixth membrane spanning region rather than the amino terminal amino acids involved in N-type inactivation [7].

An important experiment suggesting that the amino terminal region could act as an inactivation particle was the

demonstration that synthetic peptides with the sequence of the amino terminal could restore inactivation in non-inactivating mutant channels. Synthetic peptides with altered amino acid composition have now been used to determine the important features that influence the ability of the inactivating peptide to bind to the internal mouth of the channel. The naturally occurring amino sequence can be divided into an uncharged region and a highly charged region. The charged amino acids interact with negative charges at or near the mouth of the channel to influence the rate of binding. The more positive charges in the charged region, the faster the binding rate. Negative charges in the charged region inhibit binding. A surprising result is that the net charge in this region seems to be the important factor for the binding rate, regardless of which of the amino acids are charged. The effects of charge on binding rates follows a simple exponential dependence, suggesting an interaction with a surface potential at or near the binding site influencing the binding rate by an electrostatic mechanism. The dissociation rate is not markedly influenced by the charge on the peptide. The uncharged region is important for determining the overall affinity and the dissociation rate, with more hydrophobic peptides binding tighter. The *Shaker* B inactivation gate peptide also blocks a large conductance calcium-activated potassium channel from mammalian brain. Alterations in the peptide sequence that render it inactive in blocking the *Shaker* channel also make it ineffective in blocking the calcium-activated channel. This peptide also blocks a non-inactivating potassium channel from rat brain. It seems that the receptor site for the inactivation-inducing peptide may be common in a wide range of different potassium channels.

Calcium channels. L-type calcium channels are closely related in primary structure to voltage-gated sodium channels and are related less closely to potassium channels. These channels are activated by strong depolarisations, are inhibited by the calcium channel blockers, and are modulated by cAMP-dependent protein kinase and G proteins. L-type channels are of particular significance to the insulin secretory response because they mediate the calcium entry that initiates insulin release. They also serve as molecular targets for calcium channel blocking drugs which are important in the therapy of cardiovascular disease. The drug binding protein is the $\alpha 1$ subunit of the calcium channel which has been purified from rabbit skeletal muscle [10]. The purified calcium channel contains five protein subunits: the 175 kDa $\alpha 1$ subunit, the 132/28 kDa $\alpha 2\delta$ disulphide linked subunits, the 55 kDa β subunit, and the 32 kDa γ subunit. The primary structure of these subunits has been determined by cloning their cDNAs. The structure of the $\alpha 1$ subunit is similar to that of voltage-dependent sodium channels and the $\alpha 1$ subunit forms the calcium-conducting pore. The $\alpha 2\delta$ and the γ subunits are membrane-spanning glycosylated proteins whereas the intracellularly oriented β subunit may be of cytoskeletal origin.

The $\alpha 1$ subunit binds the dihydropyridine and phenylalkylamine calcium channel blockers. Phenylalkylamines such as verapamil act from the intracellular side of the calcium channel and are thought to enter the intracellular mouth of the pore and block it. Dihydropyridines act from the extracellular side of the calcium channel and modulate its voltage-dependent gating. Covalent labelling of the phenylalkylamine receptor site with a verapamil derivative followed by proteolytic cleavage and identification of the labelled peptide fragments by immunoprecipitation with site-directed anti-peptide antibodies implicates the intracellular end of transmembrane segment S6 of domain IV in formation of the phenylalkylamine receptor site [11]. Since phenylalkylamines are intracellular pore blockers, this segment may also help form the intracellular mouth of the pore. The same experimental approach implicates the extracellular ends of both transmembrane segments IIIS6 and IVS6 in formation of the dihydropyridine receptor site [12]. Evidently, this receptor site is formed at the contact regions between domain III and IV; dihydropyridines bound at that site can act through an allosteric mechanism to modulate channel gating. In addition, there is evidence for a second site of dihydropyridine association with the calcium channel in the intracellular carboxyl terminal domain [13].

$\alpha 1$ -like cDNAs have been isolated from the heart, lung, and brain, and β -like cDNAs have been isolated from heart and brain on the basis of sequence homology with the skeletal muscle proteins [14]. The gene encoding the cardiac form of the L-type calcium channel is also expressed in brain and smooth muscle. Injection of the $\alpha 1$ cRNAs into *Xenopus* oocytes leads to the expression of high-voltage-activated calcium channels [15]. The channels expressed from cardiac and smooth muscle cDNAs are modulated by dihydropyridines while some brain-specific calcium channels are not affected by these compounds. The $\alpha 1$ subunits from heart and smooth muscle are over 95% identical, but differ due to tissue-specific alternative splicing. The cloned sequences demonstrate the existence of at least six genes for the $\alpha 1$ subunit.

Co-expression of the cardiac $\alpha 1$ subunit with the other subunits from skeletal muscle in *Xenopus* oocytes alters the electrophysiological properties of the channel including the time course of the calcium current, the voltage dependence of inactivation, and the level of expression of peak calcium current [14]. Physiologically correct activation and inactivation is only seen in the presence of the other subunits. Co-expression of the β subunit increases the density of the calcium channels in oocytes and Chinese hamster ovary cells 10-100 fold [16]. These results suggest that the $\alpha 1$ subunit does not specify all the properties of a cardiac L-type calcium channel. Other subunits of the calcium channel are necessary for normal electrophysiological and hormonal regulation of the channel.

Modulation of ion channel function

In addition to rapid voltage-dependent gating of open and closed states, voltage-gated channels undergo slower and more long-lasting regulatory influences which can appreciably alter their ion conductance activity. While all three classes of voltage-gated ion channels are modulated, this session focussed on calcium channels because of their importance in the control of secretion. Hille and Tsien described different mechanisms of N-type calcium channel modulation in rat and frog superior cervical ganglia, respectively, and Hofmann and Catterall detailed the structural parameters of the L-type calcium channel required for cAMP-dependent phosphorylation of the $\alpha 1$ subunit of this channel. These reports indicate both the complexity and flexibility of ion channel control of cellular function. Voltage-gated calcium channels of neurons can be modulated by a wide variety of neurotransmitters acting through G-protein coupled receptors. Most known modulatory actions in neurons decrease calcium currents and might serve in presynaptic membranes to depress transmitter release (presynaptic inhibition/autoreceptor inhibition) and in the soma to alter both excitability, the post-spike trajectory, and repetitive firing. The molecular mechanisms of the modulation might well share features with the calcium channel modulation that influences excitability and secretion in endocrine cells.

Multiple pathways of calcium channel modulation. Superior cervical ganglion (SCG) neurons of the rat are convenient because they express mostly one type of calcium channel, the omega-conotoxin-sensitive N-type which is primarily neuron-specific. Many laboratories have shown that the calcium currents of SCG cells can be depressed by muscarinic and alpha-adrenergic agonists, somatostatin, adenosine, substance P, dopamine and other agonists. Hille and his colleagues [17-19] set out to investigate in detail what intracellular signal pathway was used in this rich modulation and were surprised to discover that at least three pathways act in parallel on the N-type channels. Most previous investigators had come to the conclusion that the modulation by these agonists uses a pertussis toxin-sensitive G protein (i.e. G_i or G_o) and affects the channels by slowing their activation and shifting the voltage dependence of their gating to more positive voltages. In patch-clamp experiments these investigators used high concentrations of calcium buffers (EGTA or BAPTA) in their whole cell recording pipettes. Experiments under the same conditions (BAPTA in the pipette) have confirmed the above conclusions for muscarinic and alpha-adrenergic actions and have shown that this pathway acts rapidly (< 400 ms to completion) and is, in the terminology of Brown and colleagues, a membrane-delimited pathway that does not use a cytoplasmic diffusible second messenger [17]. With adrenergic agonists only half of the modulation was pertussis toxin-sensitive. The other half uses a different G

protein (G_q/G_{11} ?) that also has a fast, membrane-delimited effect on N-type channels. This action may simply reduce the probability that channels will open rather than affecting the voltage dependence and speed of gating.

Hille's group has also done experiments with a very small amount of calcium buffer (0.1 mmol/l BAPTA) in the pipette, which is assumed to be more physiological. This revealed an additional action of muscarinic agonists. The fast pertussis toxin-sensitive action that is membrane-delimited remained, but a slow, pertussis toxin-insensitive action using a second messenger appeared [18, 19]. Seen in cell-attached patches, this new pathway depressed the probability of opening of N-type channels with no effect on open time, single channel conductance or voltage dependence. Interestingly, the same slow, BAPTA-sensitive muscarinic action reduces the opening probability of the small number of L-type channels in SCG cells recorded in the presence of a dihydropyridine agonist to enhance their activation. In addition, the muscarinic depression of whole cell M-type K^+ currents is slow and BAPTA-sensitive. Thus, this intracellular modulatory pathway is pleiotropic, reducing current in N- and L-type channels as well as M-type K channels.

The second messenger for this BAPTA-sensitive action has not yet been discovered. Ca^{2+} , cAMP, cGMP, nitric oxide, arachidonic acid, protein kinase C, and IP_3 have been ruled out, yet we know there is a diffusible messenger. This is the same position as other workers who are analysing the modulation of M current and Ca^{2+} current in frog. Perhaps there is a novel messenger that has not yet been described in other signal transduction systems.

Kinetic mechanism of calcium channel modulation. The recent work presented by Tsien focussed on modal changes in Ca^{2+} channel gating as a mechanism for regulation of Ca^{2+} entry and hormone or neurotransmitter release. This is relevant to general questions about ion channel modulation, but pertains most specifically to the fast, BAPTA-insensitive type of modulation as discussed by Hille.

In many neurons, N-type Ca^{2+} channels are a major Ca^{2+} entry pathway and strongly influence neurotransmitter release [20, 21]. Cell-attached patch recordings (110 mmol/l Ba^{2+} as charge carrier) were carried out to characterise the rapid open-closed gating kinetics of N-type Ca^{2+} channels in frog sympathetic neurons. Single channels display at least three distinct patterns of gating, characterised as low, medium, and high open probability (p_o) modes. This is more complex than the scheme of reluctant and willing gating modes proposed by Bean [22] because it includes a third mode of gating. The three modes were distinguished on the basis of channel open probability during depolarising pulses to -10 mV [23]. In the low p_o mode, $p_o \sim 0.05$ at -10 mV, and voltage-dependent activation occurs over a relatively depolarised range of voltage in this mode. Openings are sometimes sufficiently well resolved to show a larger unitary current amplitude than openings in gating modes with higher p_o . Two

additional patterns of gating with higher p_o can be distinguished by consideration of mean open and closed times ($\langle t_o \rangle$, $\langle t_c \rangle$) for individual sweeps: medium p_o sweeps ($p_o \sim 0.3$) show shorter openings and longer closings while high p_o sweeps ($p_o \sim 0.6$) display longer openings and briefer closings. The negative correlation between $\langle t_o \rangle$ and $\langle t_c \rangle$ points to the existence of two gating pathways.

Spontaneous transitions from one mode to another are infrequent, with an exponential distribution of dwell times and mean sojourns of ~ 10 s in each mode. Thus, a channel typically undergoes hundreds or thousands of open-closed transitions in one mode before switching to another. Transitions between modes during a depolarisation were occasionally detected, but were rare, as expected for infrequent modal switching. Within each mode, the activation kinetics were well described by a simple scheme (C2-C1-O), as previously reported for other types of Ca^{2+} channels, including the L-type Ca^{2+} channels found in pancreatic Beta cells. Rate constants are strikingly different from one mode to another, giving each mode its own characteristic kinetic signature. The gating behaviour at -10 mV ranges from brief openings (~ 0.3 ms) and long closures (10–20 ms) for low- p_o gating to long openings (3 ms) and brief closures (~ 1 ms) for high- p_o gating. Intermediate values for mean open durations (~ 1.5 ms) and mean closed durations (~ 3 ms) were found for medium- p_o gating. In addition to being kinetically distinct, channel openings in the low- p_o mode often exhibit a unitary current ~ 0.2 pA larger than openings in the medium or high- p_o modes. Each mode is characterised by its own voltage dependence: activation occurs at relatively negative potentials and is most steeply voltage-dependent in the high- p_o mode, while activation requires very strong depolarisations and is weakly voltage-dependent in the low- p_o mode. The proportion of time spent in the individual modes varies greatly from one patch to another, suggesting that modal gating may be subject to cellular control.

Cell-attached patch recordings show that the modes of N-type channel gating change under the influence of norepinephrine. With $100 \mu\text{mol/l}$ norepinephrine in the pipette, the pattern of gating shifts toward modes of lower p_o , but within each mode the open-closed kinetics remain essentially unchanged. The change in the mode of gating causes a several-fold decrease in the amount of divalent cation entry into the cell. Thus, alteration in the mode of gating represents an interesting and powerful mechanism for regulation of neurotransmitter or hormone release. It is interesting that L-type channels (such as those found in pancreatic Beta cells) also show multiple modes of gating. Perhaps modal changes in gating might also be used as a way of modifying insulin secretion.

Modulation of L-type calcium channels by protein phosphorylation. L-type calcium channels in muscle and endocrine tissues are strongly regulated by protein

phosphorylation. Hofmann described the physiological consequences of regulation of cardiac and smooth muscle calcium channels by protein phosphorylation. The same L-type calcium channel $\alpha 1$ subunit gene is expressed in both tissues, but in alternatively spliced forms [15]. Regulation of the calcium channel by beta-adrenergic agonists differs between the two cell types in that regulation in heart is primarily via protein phosphorylation while regulation in smooth muscle is primarily via a membrane-delimited G protein pathway. Analysis of the two alternately spliced forms by expression of cDNAs encoding them in mammalian cells and patch clamp recording does not reveal major differences in function or regulation. Thus, the difference between the regulation of the two proteins must be due to differences in the basic signal transduction pathways in the two cell types or in the other subunits of the calcium channel complex. The β subunit is phosphorylated by several protein kinases like the $\alpha 1$ subunit [16]. Three genes have been described which encode β subunits and one is present in the heart in two alternately spliced forms. The $\beta 2$ and $\beta 3$ genes are differentially expressed in heart and different smooth muscle tissues suggesting a possible role in determining tissue-specific properties.

The skeletal muscle calcium channel has provided a molecular model for studies of the molecular basis of calcium channel modulation, because it can be purified in good yield from transverse tubule membranes and has been successfully cloned and expressed in heterologous cells. Phosphorylation of the purified and reconstituted skeletal muscle Ca^{2+} channel increases its open probability [24, 25]. Catterall and Hofmann described the distinctive phosphorylation patterns of truncated and full-length versions of the L-type calcium channel $\alpha 1$ subunit. The activation of purified skeletal muscle L-type calcium channels is enhanced by phosphorylation of the $\alpha 1$ and β subunits by cAMP-dependent protein kinase. The $\alpha 1$ subunit is present in muscle cells and in purified calcium channel preparations in two size forms: a full-length 212 kDa form and a truncated 190 kDa form. The full-length form comprises less than 5% of the total protein, but can be clearly detected by site-directed anti-peptide antibodies against its unique C-terminal domain [26]. The full-length form of the $\alpha 1$ subunit in intact skeletal muscle cells is phosphorylated on three phosphopeptides that are unique to it. The truncated form of the $\alpha 1$ subunit is phosphorylated in vitro by cAMP kinase at serine 687 in minutes and at serine 1617 more slowly [27]. In contrast, serine 1854 of the full-length $\alpha 1$ subunit is phosphorylated in seconds in vitro [28]. Quantitative comparison of phosphorylation of different phosphopeptides of full-length $\alpha 1$ subunits of purified calcium channels showed that serine 1854 near the carboxyl terminal was phosphorylated 100 times more rapidly than other sites when the calcium channel was in its native conformation. Treatment with harsh detergents accelerates phosphorylation of other sites. These results

indicate that serine 1854 and the carboxyl terminal domain of the $\alpha 1$ subunit are likely to play important roles in calcium channel regulation. Consensus sequences for phosphorylation by cAMP-dependent protein kinase are clustered in the carboxyl terminal domains of other calcium channel $\alpha 1$ subunits, suggesting that there may be shared regulatory mechanisms among different calcium channels.

Perspectives for ion channel modulation in the Beta cell.

The results discussed in this session emphasised the multiplicity and complexity of regulatory pathways that modulate calcium channels in excitable cells. These evidently include at least protein phosphorylation by cAMP-dependent protein kinase and other protein kinases, membrane-delimited modulation by multiple G proteins, and modulation by additional pathways involving unidentified intracellular messengers. Every aspect of calcium channel function – activation, gating mode, inactivation, ion conductance – is subject to modulation. The calcium that triggers insulin release enters the Beta cell through L-type calcium channels that are closely related to calcium channels in nerve and muscle cells and may share similar regulatory properties. Initiation or enhancement of insulin release by secretagogues that act through G proteins and intracellular second messengers, including norepinephrine, galanin, and somatostatin, likely act through modulation of calcium channels by the pathways described above. Pharmacological interventions to enhance insulin secretion through these second messenger pathways may serve to amplify normal glucose-induced insulin release in patients with depressed Beta-cell number or function.

Regulation of secretion by ion channels

Most, if not all, secretory cells utilise ion channels to control the secretory response. Intracellular calcium is critical to the exocytotic process of all secretory cells including the pancreatic Beta cell. For example, like pancreatic Beta cells and other endocrine cells, anterior pituitary cells are stimulated to secrete their peptide hormone when the intracellular calcium rises sufficiently. These pituitary cells are typically regulated by peptide releasing hormones (TRH, GnRH, CRH, GHRH, etc.) delivered via a portal circulation from neurons in the hypothalamus. In this session, control mechanisms used by the gonadotrophs and somatomammotrophs of the anterior pituitary to regulate secretion of luteinising hormone (LH), follicle stimulating hormone (FSH), and prolactin, and mechanisms used by lacrimal acinar cells to regulate salt secretion were discussed as examples of the range of regulatory mechanisms that have been extensively studied.

Regulation of prolactin secretion in somatomammotrophs.

The role of ion channels, in particular calcium and potassium channels, in the response of anterior pituitary

cells to agents that either stimulate or inhibit secretion of various peptide hormones has been an area of increasing research emphasis in endocrinology. The advent of the patch clamp technique has allowed investigations at the single cell and single channel level. Equally important, the whole-cell and excised inside-out patch clamp recording configurations have provided experimental access to the intracellular surface of the plasma membrane and have permitted direct experimental intervention in the cytoplasmic and membrane signal transduction mechanisms that couple secretagogue receptors to ion channels in single cells.

Much of the work in this field has focussed on the clonal GH lines of pituitary cells which secrete prolactin and growth hormone. Unfortunately, these lines do not exhibit high affinity dopamine receptors, and dopamine (DA) is the major physiological regulator of prolactin secretion, exerting a tonic inhibitory control which is mediated by D2 receptors. Thus, Oxford and his colleagues [29] have used primary dissociated pituitary cells that have been identified as lactotrophs in vitro after dissociation using a reverse haemolytic plaque assay [29, 30]. Patch clamp techniques record the actions of DA on membrane potential and associated DA-activated whole-cell current, as well as single K^+ channels which are believed to underlie the inhibition of prolactin secretion. DA (5 nmol/l–1 μ mol/l) or D2 selective agonists (RU24213 and quinpirole) evoke a hyperpolarisation which is blocked by D2 antagonists and associated with an increased K^+ conductance. Whole-cell current responses to ramp voltage commands revealed a voltage-independent, DA-activated current whose reversal potential was near the calculated E_K , varied as a function of K^+ concentration, exhibited some inward rectification, and was independent of both cAMP and Ca^{2+} . Activation of this current is not voltage dependent. The current was insensitive to TEA (10 mmol/l), partially blocked by 4-aminopyridine (5 mmol/l), and almost completely inhibited by quinine (100 μ mol/l). Analysis of DA-induced single channel currents in cell-attached or outside-out patches showed a linear current-voltage relationship that yielded a unitary conductance of 40.2 pS in the presence of 150 mmol/l KCl and a pharmacology identical with the macroscopic currents.

The D2 receptors are linked to the activation of single K^+ channels via guanine nucleotide binding proteins (G proteins). Dialysis of cells with GDP- β -S inhibits DA responses, GTP- γ -S renders responses irreversible, and pertussis toxin treatment blocks DA responses. Attempts to define the exact G protein involved by reconstitution of pertussis toxin-treated cells with purified α subunits of G proteins have not yet been successful. Present experiments attempt to identify the G proteins involved through hybrid arrest studies with anti-sense oligonucleotides complementary to specific G protein subtypes or through rescue of DA responses in pertussis toxin-treated cell lines co-expressing cloned D2 receptors and pertussis toxin-resistant G proteins.

As mentioned above, GH cells do not express high affinity D2 receptors. Oxford and colleagues have taken advantage of this and expressed both long and short isoforms of the rat brain D2 receptor (resulting from alternative mRNA splicing) in these cells and have compared their ability to couple to K^+ channels. Both receptor isoforms couple to the K^+ channels, and both isoforms function equally well when normalised to the response of the transfected cells to somatostatin through endogenous receptors. Just as expression of D2 receptors restores electrical responsiveness, DA inhibition of prolactin release is also restored as determined by the reverse haemolytic plaque assay.

Recently, Lledo and co-workers [31, 32] reported a stimulation of voltage-dependent K^+ channels by DA and an inhibition of two classes of voltage-dependent calcium channels. Both of these effects could contribute to the inhibition of secretion. Using both whole-cell and nystatin-perforated patch recording methods, Oxford and colleagues consistently failed to replicate these results in cells from either normal cycling or lactating females. Nonetheless, decreases in intracellular calcium in resting cells challenged with DA can be measured spectrofluorometrically with fluo-3. However, no changes in calcium levels were seen in response to DA in K^+ -depolarised cells, again suggesting that calcium channels are not directly inhibited by D2 receptor activation. Similar observations have been made in cells transfected with cDNA for D2 receptors. Thus, as the only consistent ion channel target for regulation by DA appears to be a voltage-independent K^+ channel in cells in which DA inhibits prolactin secretion, the correlation may be causal. A similar conclusion has recently been reached by Stack and Suprenant [33] for DA responses in melanotrophs.

The possibility that subpopulations of DA responsive cells might contribute to these discrepancies is important to examine. Oxford and colleagues have recently begun to employ a new method, the sequential cell immunoblot assay which permits quantification of hormone secretion from individual cells, assay of their responsiveness to a secretagogue, and subsequent electrophysiological study. This assay may have broad use in studies of secretion from individual cells in vitro. It is hoped that this method will help address whether subpopulations of lactotrophs may have different regulatory mechanisms.

Regulation of release of hormones in the gonadotroph. For the reproductive axis, the relevant hypothalamic releasing hormone is GnRH (gonadotropin releasing hormone) and the pituitary target is the gonadotroph, which releases LH and FSH. Like the laboratories of Miller and Catt, Hille and colleagues found that GnRH induces regular oscillations of intracellular free calcium [34]. Each peak of the intracellular calcium induces exocytosis (secretion) as indicated by an increase of membrane surface area measured as membrane electrical capacitance. These oscillations can be studied by both direct calcium

measurement from indo-1 ratio fluorescence and from ionic currents in an apamin-sensitive calcium-activated K^+ channel that opens in synchrony. The GnRH receptor acts through a pertussis toxin-insensitive G protein to stimulate phosphatidylinositol turnover and release of IP_3 . The calcium oscillations, current oscillations, and exocytosis are mimicked by putting IP_3 in the whole-cell pipette and they can be blocked by including heparin (an IP_3 -receptor antagonist) in the pipette. All three events are blocked if the pipette contains 20 mmol/l of the calcium buffer BAPTA. All three continue to occur for several minutes after all extracellular calcium is removed, indicating that the calcium is coming from intracellular stores.

Like other pituitary cells, the gonadotroph is an excitable cell richly endowed with voltage-gated ion channels. At rest, the membrane potential is depolarised and many channels are inactivated, but during GnRH stimulation, the apamin-sensitive K^+ -activated calcium channels open and close periodically, hyperpolarising the cell and then letting it depolarise. This depolarisation causes the cell to fire a few action potentials. These action potentials probably let in enough calcium to keep the internal stores from being depleted during prolonged stimulation of secretion. This alternation of hyperpolarisation and plateau is reminiscent of the behaviour of pancreatic Beta cells during their glucose-controlled secretory activity. Interestingly, the gonadotroph calcium oscillation is stimulated by galanin, endothelin, and several other peptides that are co-localised in the hypothalamic cells that contain GnRH. Therefore, there may be significant parallels in the mechanisms.

Regulation of salt secretion by calcium-dependent ion channels in luminal and basolateral domains of clustered acinar cells. Ion channel mechanisms that regulate secretion in exocrine cells appear to be distinct from those in most endocrine cells. Calcium-dependent channels are thought to play an essential role in the transfer of electrolytes across the basolateral and luminal membranes of secreting acinar cells [35]. These channels are activated by inositoltrisphosphate-dependent Ca^{2+} release following stimulation by secretagogues such as acetylcholine (ACh). A recent study by Kasai and Augustine [36] examined Ca^{2+} imaging responses to ACh in partially dissociated clusters of murine exocrine pancreas. In such aggregates, some of the original polarity of acinar cells is maintained, as indicated by the packing of secreting granules next to the area of contact between adjacent cells. This contact area thus corresponds to the luminal domain of the acinar cells. It was found that the initiation of the ACh-induced Ca^{2+} rise is located in this luminal domain, and that the Ca^{2+} signal spreads later to the entire cell. Marty and colleagues performed simultaneous tight seal whole cell recording and Ca^{2+} imaging in such clusters of acinar cells, with the aim to study the basolateral/luminal distribution of the Ca^{2+} -dependent channels that regulate secretion.

Experiments were performed on partially dissociated acinar cells from rat lacrimal glands. The glands were

treated sequentially with trypsin, EGTA and collagenase to release clusters of acinar cells. Ca^{2+} imaging was performed using a system having an intensified CCD camera, and provides one Ca^{2+} image (128 x 128 pixels) every 120 ms. Cells were loaded with fura-2 AM. For tight-seal whole-cell recordings, pipettes were filled with a solution containing (in mmol/l): 140 KCl, 2 MgCl_2 , 0.5 EGTA, 10 HEPES, pH 7.2. ACh (0.20-0.25 $\mu\text{mol/l}$) was applied by a local perfusion system. The recording pipette was in contact with one cell in a cluster. Gap junctions allowed voltage control and current recording to be extended to all cells of the cluster. The presence of effective coupling was ascertained by examining capacitive current responses to 10 mV hyperpolarising pulses. It is known from previous work on this preparation that ACh applications activate both K^+ -selective and Cl^- -selective currents [37]. Each response was studied in isolation by setting the clamp potential either at 0 mV (the Nernst potential for Cl^- ions) to examine K^+ currents, or at -85 mV (the Nernst potential for K^+ ions) to examine Cl^- currents.

In response to ACh application both K^+ - and Cl^- -selective currents rose in a biphasic manner after a latency of 1-3 s. The first part of the response ("hump") corresponded to a small Ca^{2+} increase in the luminal domain. At 0 mV, the amplitude of the hump was, on the average, 50% of the total current. To quantify the current densities in the luminal and basolateral domains, the number of pixels where the Ca^{2+} increase exceeded a threshold set near two times the resting concentration was evaluated for each image. Plots of ACh-induced current as a function of these pixel numbers gave two linear regions. One linear component corresponded to the luminal membrane, and the other to the basolateral membrane. Current densities were calculated from each slope, giving (at 0 mV) 1.75 pA/pixel (luminal) and 0.10 pA/pixel (basolateral). Corresponding channel densities were 0.35/ μm^2 and 0.02/ μm^2 , respectively. Performing a similar analysis on isolated cells suggested that a significant redistribution of Ca^{2+} -dependent K^+ channels occurred upon cell isolation, but that individual cells nevertheless retain a residual luminal/basolateral density gradient. Current responses at -85 mV were similarly analysed to study the distribution of Ca^{2+} -dependent Cl^- channels. Again a higher channel density was found in the luminal domain than in the basolateral domain.

The results indicate that acinar lacrimal cells have a high density of Ca^{2+} -dependent K^+ and Cl^- channels in the luminal membrane and that this differential distribution of ion channels plays a crucial role in salt secretion.

Ion channels in Beta cells

Regulation of ATP-gated potassium channels in the Beta cell. ATP-regulated potassium channels (K-ATP channels) play a central role in stimulus-secretion coupling in the pancreatic Beta cell [38-40]. Both glucose metabolism (the

primary physiological stimulus) and sulphonylureas (the most important pharmacological secretagogue) close K-ATP channels leading to depolarisation of the Beta-cell membrane, initiation of repetitive action potentials and activation of voltage-gated Ca^{2+} channels, Ca^{2+} influx, increased intracellular Ca^{2+} and finally insulin secretion. Although the K-ATP channel plays an important role in the insulin secretory process, we still understand very little about how this channel is regulated by cytosolic constituents. A primary focus of this session was the functional significance of the K-ATP channel, its regulation, and determination of the channel protein structure through purification and analysis of the channel protein and cloning of its gene. In addition, Cook and Lazdunski presented new data suggesting that there are multiple voltage-gated calcium channels in the Beta cell and that these may be functionally distinguished from similar channels found in other cell types.

It is currently accepted that the conductance state of the Beta-cell K-ATP channel is regulated by ATP, ADP, and other products generated by glucose metabolism [38-40]. However, as was demonstrated for other ion channels discussed in this work group, the regulation of the Beta-cell K-ATP channel is complex: protein kinases, protein phosphatases, G proteins, and intracellular ligands in addition to ATP contribute to channel regulation. F. Ashcroft presented evidence that phosphorylation of the channel or a closely associated membrane protein was required to prevent channel rundown, the rapid loss of channel activity observed when membrane patches are removed from the cell. Mg-ATP, but not non-hydrolysable ATP analogues, inhibited rundown, and very low Mg^{2+} concentrations which may inhibit protein phosphatase activity also inhibited rundown. S. Ashcroft further demonstrated that Mg-ATP was required for the activity of diazoxide to open the K-ATP channel [41].

It is known that ADP promotes channel opening while ATP promotes channel closing. It was previously presumed that these actions were a function of competition for a single nucleotide binding site on the channel. However, Cook and Lazdunski provided independent lines of evidence which alter our view of K-ATP channel structure by demonstrating two sites for nucleotide binding: one site binds Mg-ADP and opens the channel, the other site binds either ATP or ADP and closes the channel [42]. This model conforms to the biphasic dose response curves seen for channel activity in the presence of increasing ratios of ATP to ADP. Lazdunski demonstrated that fluorescein derivatives, which have high affinity for certain nucleotide binding sites, could be used to distinguish and affinity label these two functional nucleotide binding sites of the K-ATP channel [43]. Binding of the activation site by fluorescein derivatives was shown to have a negative allosteric effect on sulphonylurea binding, implying physical coupling between these different domains.

K-ATP channels can also be modulated by extracellular signalling molecules. An extracellular membrane ADP

binding site apparently distinct from the intracellular nucleotide binding sites of the K-ATP channel discussed above was shown by S. Ashcroft to close the channel, increase intracellular calcium, and increase insulin release [44]. The physiological significance of this extracellular site is uncertain but could be related to binding or hydrolysis of ATP released from cholinergic neurons innervating the islet. Both galanin and somatostatin, hormones of major physiological significance to insulin secretion, appear to act through a pertussis toxin-sensitive G protein on the K-ATP channel. These hormones open the channel via a G protein-mediated pathway and thus inhibit insulin secretion. Lazdunski pointed out that, in contrast to galanin or somatostatin, protein kinase C activators such as diacylglycerol and arachidonic acid increase insulin secretion but surprisingly enhance opening of the K-ATP channel [45, 46]. The mechanism by which these protein kinase C activators stimulate insulin secretion is clearly distinct from the classical mechanism for glucose.

Molecular properties of K-ATP channels. K-ATP channels are abundant in tissues other than the Beta cell where they display similar, though not identical, characteristics as observed in the Beta cell. These channels are also likely to be affected by sulphonylureas during their use in therapy. The functional significance of non-Beta-cell K-ATP channels is only recently appreciated. K-ATP channels have been identified in the brain by electrophysiological techniques and by autoradiographic localisation of sulphonylurea receptors using radiolabelled ligands [47, 48]. K-ATP channels are located in both cell bodies and synaptic terminals and are present at high levels in substantia nigra, globus pallidus, cerebellum, and hippocampus. They are involved in regulation of transmitters in ischaemic or anoxic conditions, and their response to glucose variations in substantia nigra might be responsible for the well-known nervous disorders associated with hypo- or hyperglycaemia in diabetic patients. Lazdunski also proposed that opening of cardiac K-ATP channels may provide protection against ischaemic cardiac damage and that sulphonylureas, by maintaining cardiac K-ATP channels in the closed state, may inactivate this physiological defense system. If this is correct, more specific drugs for closing the Beta-cell K-ATP channel are highly desirable to prevent cardiac damage.

The complexity of K-ATP channel regulation resembles that seen for other channel types discussed at the meeting, such as the voltage-regulated K⁺ channel, Ca²⁺ channel and Na⁺ channel. In each of these instances, isolation of the channels and determination of the channel structure via cDNA cloning has revealed a great deal about the structural basis of channel function. Structural similarities between the Beta-cell K-ATP channel and these other channels were suggested by F. Ashcroft's results which indicate that trypsin digestion can eliminate K-ATP channel gating. This may be analogous to the proteolytic cleavage of the inactivation gating region of the *Shaker* K⁺ channel. Efforts

to purify the protein components of the K-ATP channel, determine part of its amino acid sequence, and clone cDNA encoding its subunits are underway in several laboratories including those of both S. Ashcroft and Lazdunski [49]. Achievement of this goal may reveal the structural basis for the complexities of Beta-cell insulin secretion as mediated by this channel and may provide insight for the development of new drugs to act more specifically and with greater efficacy.

Calcium channels in the Beta cells. Ca²⁺ entry into the Beta cell and subsequent insulin secretion is ultimately controlled by voltage-dependent calcium channels. Cook proposed that secretagogue-induced electrophysiological activity in Beta cells, consisting of slow bursting activity and superimposed rapid spiking, is a function of two types of voltage-dependent calcium channel events. The slow bursting pattern is due to a slowly inactivating current and the spikes are due to rapidly inactivating currents. These two currents can be differentiated on the basis of activation threshold, dihydropyridine sensitivity, and inactivation mechanism. Since different secretagogues such as glucose and acetylcholine produce different patterns of spiking and bursting, it may be inferred that these two currents are physiologically regulated by different mechanisms. Recently cloning results have revealed at least two different Beta-cell calcium channels which may underlie these two different calcium currents. Their sequences did not reveal marked differences from non-Beta-cell channels. However, Lazdunski presented evidence utilising the L-type calcium channel blockers calciseptine and indolizinsulphone which indicated that Beta-cell L-type channels or associated proteins may not be identical to the corresponding proteins of other tissues. This differential sensitivity of Beta-cell calcium channels may present a target for Beta-cell specific drugs.

Conclusions and recommendations

The ion channels of the Beta cell are a crucial focus for future experimental work and therapeutic development in diabetes. A fundamental understanding of the structure and the complex modulation of the ATP-gated potassium channel will be an important advance providing new opportunities for selective modulation of channel function and insulin release. The modulation of this channel through intracellular second messenger systems suggests that selective modulation through these pathways may be possible in addition to direct modulation through the receptor sites for the sulphonylureas and related secretagogues. In addition, the complex modulation of voltage-gated calcium channels in the Beta cells and in other cell types suggests that interventions that may enhance activation of the L-type calcium channels of the Beta cell through protein phosphorylation pathways or G-protein coupling may also be valuable and highly specific

approaches to regulate insulin release pharmacologically. Enhancement of the activation of the Beta-cell calcium channels by second messenger regulation in response to normal changes in glucose concentration may provide increased insulin secretion with more physiologically normal regulation than can be obtained from persistent inhibition of the ATP-gated potassium channel by sulphonylureas. This therapeutic approach may increase insulin secretion with correct regulation in response to blood glucose concentration changes.

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