Unifying concepts in stimulus-secretion coupling in endocrine cells and some implications for therapeutics

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Submitted 9 December 2008; accepted in final form 16 June 2009

Misler S. Unifying concepts in stimulus-secretion coupling in endocrine cells and some implications for therapeutics. Adv Physiol Educ 33: 175–186, 2009; doi:10.1152/advan.90213.2008.—Stimulus-secretion coupling (SSC) in endocrine cells remains underappreciated as a subject for the study/teaching of general physiology. In the present article, we review key new electrophysiological, electrochemical, and fluorescence optical techniques for the study of exocytosis in single cells that have made this a fertile area for recent research. Based on findings using these techniques, we developed a model of SSC for adrenal chromaffin cells that blends features of Ca2+-dependent SSC (characteristic of neurons) with G protein receptor-coupled, Ca2+-release-dependent and second messenger-dependent SSC (characteristic of epithelial exocrine cells and nucleated blood cells). This model requires two distinct pools of secretory granules with differing Ca2+-sensitivities. We extended this model to account for SSC in a wide variety of peripheral and hypothalamic/pituitary-based endocrine cells. These include osmosensitive magnocellular neurosecretory cells releasing antidiuretic hormone, stretch-sensitive atrial myocytes secreting atrial natriuretic peptide, K+-sensitive adrenal glomerulosa cells secreting aldosterone, Ca2+-sensitive parathyroid chief cells secreting parathyroid hormone, and glucose-sensitive β- and α-cells of pancreatic islets secreting insulin and glucagon, respectively. We conclude this article with implications of this approach for pathophysiology and therapeutics, including defects in chief cell Ca2+-sensitivity, resulting in the hyperparathyroidism of renal disease, and defects in biphasic insulin secretion, resulting in diabetes mellitus.

ENDOCRINE SECRETORY CELLS (ESCs), although small in number, are diverse and strategically located sensor cells entrusted with directly or indirectly controlling the critical functions of a host of other cells.1 They release into the circulation a variety of hormones that bind to receptors at distant target cells. Some ESCs maintain background levels of cell metabolism and the chemical “steady state” of the blood by sensing very small changes in serum K+, Ca2+, glucose, or osmolarity and then releasing hormones that activate target cells to secrete or absorb a solute or water. This attenuates concentration changes in the blood before they can compromise the function of other cells (e.g., nerve or muscle cells). Other ESCs respond uniquely to specific peptides and release potent, long-acting hormones, which sustain rhythms of tissue buildup and breakdown (e.g., bone and uterus).

Traditionally, endocrine physiologists have focused on hormones and their actions at target cells. However, over the past three decades, as techniques for studying single cell function have proliferated, increasing attention has been devoted to the study of stimulus-secretion coupling (SSC) in ESCs. These studies have been extended to examining how genetic/acquired defects and pharmacological agents modulate these processes, how the functions of ESCs can be preserved under conditions that cause these cells to malfunction, and how failing ESCs can be replaced by bioengineered stem cells.

In the past, each type of ESC was viewed as a unique entity with its own highly specialized mode of operation. However, over the past two decades, it has become increasingly clear that ESCs share common critical features. Many ESCs depolarize after exposure to their stimuli and then undergo depolarization-exocytosis coupling reminiscent of that present at synaptic terminals, with exocytosis being defined as the process of granule fusion with the plasma membrane and the release of granule content into the extracellular space. At synaptic terminals, Ca2+ entry, through voltage-gated Ca2+ channels opened by membrane depolarization, triggers the fusion of small-diameter (~50 nm) transmitter-containing vesicles, often docked near the voltage-gated Ca2+ channel, with the plasma membrane (22, 28, 48) (see Fig. 1A for additional details). As similar voltage-gated Ca2+ entry triggers the discharge of large-diameter (200–350 nm) hormone-containing vesicles in ESCs (11), this pattern might be considered a paraneuronal mode of SSC. In contrast, ESCs also respond to blood-borne stimuli, which bind to G protein-coupled plasma membrane receptors (GPCRs). Directly or indirectly, this pathway activates/generates second messengers (e.g., Ca2+ released from intracellular stores, cAMP, or monomeric GTPases), which then trigger the exocytotic release of small molecules or peptides from large-diameter hormone-containing granules (23, 38, 42, 43) (see Fig. 1B for additional details). This nonexcitable pathway is reminiscent of those used by exocrine epithelial cells of the gut or reproductive tract, by cells of the tracheobronchial-alveolar tree for the release of mucus, enzymes, or surfactants, or by circulating or tissue-fixed nucleated blood cells (e.g., mast cells, platelets, and leukocytes) for the release of vasoactive modulators, clotting protein, and bacteriostatic or bacteriocidal factors. Hence, it might be called a paracrine mode of SSC. To differing degrees, ESCs combine these two distinct secretory modes to optimize their ability to use cross-talk and additive effects of stimuli to become excellent long-term monitors of a wide variety of slowly changing internal stimuli.

1 Supplemental Material for this article is available at the Advance in Physiological Education website. The supplemental animation was created by Alon Friedman (Advanced Teacher Training Center, Shlomi, Israel).

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secretion is usually independent of depolarization and Ca\(^{2+}\) concentration-dependent secretion (displayed by epithelial and circulating blood cells), whereas secretion is often dependent on species-specific antibodies. Fortunately, over the past 25 years, several new tools have made possible the real-time, single cell study of hormone exocytosis, with nearly the ease, speed, and accuracy previously attainable only at synapses, where both presynaptic and postsynaptic synapses are sometimes assayable by simultaneously recording current and voltage (e.g., Ref. 28). The maintenance of long-term exocytosis requires endocytosis, or the retrieval of granule membrane from the surface, so as to preserve cell surface area, volume, and excitability as well as to recycle some granule components, although we shall not deal with this directly in this review.

The exocytosis of a secretory granule consists of two sequential processes: the fusion of the granule membrane with the plasma membrane producing an increase in the surface area of the plasma membrane followed by the discharge of the packet of to-be-secreted substance contained within the granule. Conceptually, exocytosis can be tracked in real time in several different ways (see Fig. 2A). The increase in the surface area of the plasma membrane can be detected electrically as an increase in plasma membrane capacitance (C_m), where C_m is the amount of charge needed to change the membrane potential by a fixed amount and, hence, is directly proportional to the surface area to be charged. Measurement of a stepwise increase in C_m of magnitude predictable from the surface area of a secretory granule is highly suggestive of the fusion of the membrane of the granule with the plasma membrane. In exocrine or endocrine cells, the fusion of a 300-nm-diameter granule with the plasma membrane often yields an electrically detectable (2–3 EF) step in C_m (e.g., Refs. 26, 33, and 36). The packet-like (or quantal) release of the thousands of molecules of hormone contained within a granule [or quantal release event (QRE)] can be detected electrochemically as the synchronized oxidation of C-OH to C=O in the cases of epinephrine, norepinephrine, dopamine, or serotonin or the reduction of a disulfide S-S bond in the case of insulin (8, 54, 55). Here, the tip of a small-diameter (1–10 \(\mu m\)) carbon fiber electrode, positioned directly over the release site, is held at a fixed potential to achieve maximal oxidation/reduction, a technique known as amperometry. In some ESCs, even a single action potential (AP) simultaneously evokes a small step in C_m and one or more accompanying QRE(s). Also, the approach and fusion of secretory granules with the plasma membrane can be detected optically using total internal reflectance mi-

In this review, we examine some basic electrophysiological, electrochemical, and optical fluorescence approaches that have made the study of exocytosis in single cells a fertile area for recent cell physiology research. Based on findings using these techniques, we developed a “mixed-mode” model of SSC in the adrenal medullary chromaffin cell (ACC). It blends the paraneuronal mode of Ca\(^{2+}\) entry-dependent SSC with the paracrine mode of G protein receptor-coupled, internal Ca\(^{2+}\) release-dependent and other second messenger-dependent pathways of SSC. This model relies on two distinct pools of secretory granules with different sensitivities to Ca\(^{2+}\) and second messengers. We then extended this mixed-mode model to account for SSC in a wide variety of peripheral and hypothalamic/pituitary-based ESCs, focusing on glucose-sensitive \(\beta\)- and \(\alpha\)-cells of the pancreatic islets of Langerhans, which

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**Fig. 1.** Overview and comparison of two different modes of secretion, depolarization-induced, Ca\(^{2+}\) entry-dependent secretion (displayed by neurons) versus depolarization-independent, internal messenger generation-dependent secretion (displayed by epithelial and circulating blood cells). **Left:** In neuronal secretion, depolarization instigated by an action potential (AP) propagating into the nerve terminal (step 1) opens Ca\(^{2+}\) entry channels (step 2). Localized rises in cytosolic \([Ca^{2+}]_c\) into the range of tens of micromolar (step 3) promote the fusion of transmitter-containing, small-diameter secretory granules (usually called synaptic vesicles), clustered at hot spots or active zones, with the plasma membrane (step 4). The transmitter is released into the extracellular space (synaptic cleft), where it diffuses a short distance toward the postsynaptic cell (step 5), shown here as a muscle fiber. Binding of the transmitter to its receptor channels in the postsynaptic membrane gives rise to a postsynaptic current (step 6). The entire process of depolarization-secretion coupling followed by postsynaptic receptor often occurs over 1–2 ms. **Right:** In contrast, in exocrine epithelial cells and nucleated blood cells, where secretion is usually independent of depolarization and Ca\(^{2+}\) entry, different agonists (A and B) may bind to distinct receptors (R1 and R2), which are often G protein coupled, to release one or more intracellular mediators of secretion. Here, we show that the binding of agonist A to G protein-coupled receptor R1 (step A1) triggers the activation of enzyme E (PLC; step 2A), which releases diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP3) from the membrane bilayer. In turn, DAG stimulates PKC (step 3A), while IP3 releases Ca\(^{2+}\) from the endoplasmic reticulum (ER; step 4A). PKC phosphorylates granule proteins, promoting the docking of large-diameter secretory granules with the plasma membrane (step 5A), while the IP3-promoted rise in cytosolic \([Ca^{2+}]_c\), although only into the range of 1 \(\mu M\) to several micromolars, is sufficient to trigger Ca\(^{2+}\) release-dependent fusion of the granules with the plasma membrane (step 6A). In addition, binding of agonist B to a second receptor (R2; step 1B) may trigger the activation of a second internal messenger, for example, a G protein of the monomeric Rab variety (Gx) (38) (step 2B), which then promotes Ca\(^{2+}\)-independent fusion of the granules with the plasma membrane (step 3B). This entire process may take up to several seconds.
Single cell exocytosis can be stimulated in several ways. The application of a natural or pharmacological stimulus can result in cell depolarization (Fig. 2A, pathway 1a) or receptor-mediated release of Ca\(^{2+}\) or other second messengers into the cytoplasm (Fig. 2A, pathway 1b) (e.g., Ref. 55). These actions can be mimicked by second messengers introduced into the cytoplasm by diffusion from the pipette (Fig. 2A, pathway 1c) (e.g., Refs. 37 and 41–43) or membrane permeation (Fig. 2A, pathway 1d). Alternatively, direct depolarization of the patch-clamped ESC can produce measurable Ca\(^{2+}\) entry-dependent exocytosis (Fig. 2A, pathway 2) (35, 36). Finally, exposure of the cell to a flash of light can liberate Ca\(^{2+}\) or another second messenger compounds [e.g., inositol (1,4,5)-trisphosphate (IP\(_3\))] from a photolysis-sensitive caging compound previously introduced into the cytoplasm, whereupon the second messenger then widely distributes within the cytoplasm (Fig. 2A, pathway 3) (9, 51–53).

The processes of membrane fusion and release of granule contents are very complex. Once routinely referred to as “a miracle,” the basic steps have been slowly, although not totally, unraveled (see Fig. 2B). The fusion process itself involves at least the following steps (see Ref. 20 for an overview). Initially, the secretory granule is docked and primed at the plasma membrane by the interaction of sticky receptor for N-ethylmaleimide-sensitive attachment (SNARE) proteins, present on both membranes, which allows the close approach of the leaflets of the opposite bilayers. Later, the fusion pore is formed, which might be lipidic (i.e., formed by the contact of bilayer leaflets) or might be based on protein scaffolds (i.e., formed by the intramembranous domains of SNARE proteins), and provides the pathway of exit for granule contents. When exocytosis is triggered by a rise in cytosolic [Ca\(^{2+}\)], the fusion pore may be catalyzed by the rearrangement of the intramembranous domain of a Ca\(^{2+}\) sensor protein, synaptotagmin.

The fusion pore is a dynamic structure: it starts narrow, flickers open, closes several times to allow the exchange of granule Ca\(^{2+}\) for extracellular Na\(^+\), the influx of water, and a trickle of release of soluble contents, and then finally expands fully, coincident with granule swelling, to allow most of the granule contents to diffuse or ooze out (e.g., Ref. 56). In cells where the granule content is a tightly packed gel or matrix, which must expand and decondense before the dissociation of the hormone, the release of hormone into the extracellular space may lag behind a stable increase in C\(_{m}\) by up to seconds (30). Exocytosis is followed by endocytosis, where complex cytoskeletal machineries, consisting of actin, dynamin, myosin, and myosin light chain kinase, among other components, retrieve the granule membrane.

SSC in a “Model” Endocrine Cell: Evidence for Distinct Pools of Granules in the Catecholamine-Releasing ACC

The ACC, the most neuron-like of endocrine cells, has long been the ESC of choice for the study of SSC. As a neural crest-derived, cholinergically innervated, electrically excitable cell, long considered analogous to postganglionic sympathetic neurons, the ACC is triggered by ACh to fire APs (13) and secrete catecholamines by exocytosis (36). As shown in Fig. 3, left, early combined patch-clamp/exocytosis studies have shown that the binding of a cholinergic agent such as carbamylcholine (CCH) to a nicotinic ACh receptor channel (nAChR; step 1) produces membrane depolarization (step 2), resulting in...
Fig. 3. Two modes of stimulation of adrenal medullary chromaffin cells (ACCs) resulting in vigorous quanta secretory of catecholamines measured by amperometry ($I_{amp}$). Left: initial application of the cholinergic agonist carbamylcholine (CCH) evokes the depolarization-induced, Ca$^{2+}$ entry-dependent quanat release of catecholamines (as measured amperometrically, top). In the schematic immediately below, CCH activates nicotinic ACh receptors (nAChRs; step 1), producing membrane depolarization (step 2); trains of APs based on the opening of voltage-dependent Na$^+$ and Ca$^{2+}$ channels (steps 3 and 4), Ca$^{2+}$ entry (step 5), and Ca$^{2+}$ entry-dependent exocytosis (QREs of norepinephrine (NE) and epinephrine (E); step 6). $V_m$, memembrane potential. Right: after nAChRs have desensitized, subsequent larger applications of CCH result in a more slowly developing barrage of QREs by a depolarization-independent, Ca$^{2+}$-release-dependent process. Binding of CCH to G protein-coupled muscarinic ACh receptors (mAChRs; step 1) activates PLC (step 2), producing IP$_3$ (step 3), which triggers Ca$^{2+}$ release from ER stores (step 4) and ultimately results in exocytosis (step 5). Each amperometric spike (or QRE) consists of the release of 0.5–2 million molecules of epinephrine or norepinephrine. Ca(L), L-type Ca$^{2+}$ channels.

electrical activity (step 3), opening voltage-activated Ca$^{2+}$ entry (step 4), a rise of local cytosolic Ca$^{2+}$ (step 5), and ultimately Ca$^{2+}$ entry-induced exocytosis of catecholamines (step 6). Use of the $C_m$ assay to compare the rates and overall quantities of exocytosis evoked by depolarization-induced Ca$^{2+}$ entry versus flash photolysis-induced liberation of Ca$^{2+}$ suggested that during depolarization-induced exocytosis, local cytosolic [Ca$^{2+}$] rose as high as many micromolars in a small spatial domain, being maximal near the mouths of a cluster of Ca$^{2+}$ channels but extending toward nearby docked secretory granules (9). Hence, the depolarization-induced, Ca$^{2+}$ entry-dependent mode of SSC appeared to resemble that occurring in nerve terminals, where smaller-diameter vesicles are clustered at membrane hot spots known as active zones. However, it quickly became clear that chromaffin cells were quite distinct from nerve terminals: CCH can trigger chromaffin cells to secrete catecholamines in the depolarization-independent mode of exocytosis even after nAChRs are desensitized (55). As shown in Fig. 3, right, the binding of CCH to a G protein-coupled muscarinic ACh receptor (mAChR; step 1) produces the activation of PLC (step 2), resulting in the release of IP$_3$ from the plasma memerane (step 3). The binding of IP$_3$ to a Ca$^{2+}$-release channel in the Ca$^{2+}$-storing regions of the endoplasmic reticulum (ER) releases Ca$^{2+}$ into the bulk of the cytoplasm (Fig. 3, right, step 4). Although only raising local cytosolic [Ca$^{2+}$] near granules into the range of several micromolars (40) (Fig. 3, right, step 5), this internal Ca$^{2+}$ release evokes major exocytosis (Fig. 3, right, step 6). Hence, the release of ACh from innervating neurons results in subsequent chromaffin cell exocytosis via two separate receptors that are linked with two separate effector systems that ultimately raise cytosolic Ca$^{2+}$ in the vicinity of secretory granules over a wide range of Ca$^{2+}$ concentrations. While both modes of exocytosis are Ca$^{2+}$ dependent, the mAChR-evoked sequence is depolarization independent but internal Ca$^{2+}$ release dependent.

This brings us face to face with a paradox. How do both modes of SSC ultimately discharge chromaffin granules at high frequency, although at different kinetics of onset, even though each mode would be expected to evoke very different spatio-temporal profiles of cytosolic Ca$^{2+}$ increases? Ca$^{2+}$-releasing regions of ER are likely to be situated up to a micrometer from the granules and, even when fully emptied of their stores, raise “near-granule” cytosolic [Ca$^{2+}$] to only a few micromolars, whereas AP-provoked Ca$^{2+}$ entry raises cytosolic [Ca$^{2+}$] in the vicinity of Ca$^{2+}$ channels to tens of micromolars. Are the granules and Ca$^{2+}$ channels less well colocalized in chromaffin cells than in nerve terminals? Are there two distinct sets of granules with different localizations vis-a-vis Ca$^{2+}$ channels and with two very different Ca$^{2+}$ sensitivities?

Consideration of carefully performed experiments using flash photolysis of caged Ca$^{2+}$ as the secretory trigger has suggested a model consisting of two distinct pools of secretory granules individually or jointly activated by distinct stimulus patterns and giving rise to distinct exocytotic responses (55). This model has been examined in other ESCs as well (33, 52). As in neurons, there is a small immediately releasable pool of granules (IRP) with low Ca$^{2+}$ affinity situated near or docked at clusters of voltage-dependent Ca$^{2+}$ entry channels (9, 19). Granules in the IRP would likely be activated by individual APs, which transiently raise local Ca$^{2+}$ into the range of many micromolars over narrow spatial domains near the open Ca$^{2+}$ channels. However, these local domains of greatly enhanced [Ca$^{2+}$] collapse rather quickly and, at most, extend a distance of hardly more than tens of nanometers from the mouths of open Ca$^{2+}$ channels. That is, Ca$^{2+}$ is rapidly buffered by mobile and fixed buffers, extruded across the plasma membrane or sequestered into Ca$^{2+}$ stores (see the general model in Fig. 4A and the stimulus-response paradigm in Fig. 4B, top). Hence, in response to a single AP, release from the IRP begins within milliseconds and produces a nearly synchronous exocytosis, indicated by a step in $C_m$ and coincident amperometrically detectable QRE(s) (not shown here). However, more prominently than in most neurons, in ACCs there is a much larger pool of granules of higher Ca$^{2+}$ affinity, the so-called highly Ca$^{2+}$-sensitive pool (HCSP), located farther from clusters of voltage-dependent Ca$^{2+}$ entry channels (53). This pool is activated by repetitive or prolonged depolarization, producing long-term Ca$^{2+}$ entry, saturating local cytoplasmic Ca$^{2+}$ buffers, and allowing Ca$^{2+}$ to diffuse over a longer distance, and equilibrate, at low concentrations, over a portion of the cytoplasm, especially its outermost, immediately submembrane ring. Ultimately, this produces delayed asynchronous exocytosis last-
It is likely that both the IRP and HCSP should be activated by a train of APs (see the stimulus-response paradigm in Fig. 4B, middle). As with single widely spaced APs, individual depolarizations in the train should transiently raise “near-channel” Ca\(^{2+}\) domains, activate granules in the IRP, and produce a synchronous step of \(C_m\) during each depolarization. However, additionally, the clustering of the APs should result in a sufficient buildup of Ca\(^{2+}\) and give rise to a slow asynchronous component of \(C_m\) lasting for up to several seconds after the train has ended (see the stimulus-response paradigm in Fig. 4B, middle) (32, 33, 50, 51). Recently, using genetic knockout techniques, the two granule pools with differing Ca\(^{2+}\) affinities have been found to have appropriately distinct isoforms of the Ca\(^{2+}\) sensor synaptotagmin (46).

Evidence has suggested that the mACHr-induced Ca\(^{2+}\) release by ER stores raises cytosolic [Ca\(^{2+}\)] at discrete regions near the plasma membrane to values in the range of 1 \(\mu\)M to several micromolars. In our model, this increase should be sufficient to activate member granules of the HCSP and account for mACHr-induced exocytosis with the prolonged application of ACh (see the stimulus-response paradigm in Fig. 4B, bottom). In addition, pharmacologically, the slow sustained exocytosis evoked by \(\alpha\)-latrotoxin, a neurotoxin forming long-lived Ca\(^{2+}\)-permeable plasma membrane channels and producing a continuous rise in global cytosolic [Ca\(^{2+}\)], should also result from the activation of the HCSP (27).

In addition to nACHR- and mACH-activated pathways evoking release individually, it is likely that the two pathways work in concert under a wide variety of patterns of cell stimulation to increase the efficiency or extent of stimulus-evoked exocytosis, thus priming the cell for subsequent exocytosis in a manner analogous to the short-term facilitation or rapid recovery from post-use depression seen at many synapses. There is evidence that low-level stimulation via the mACHR or nACHR, or low-amplitude depolarizations, which in themselves do not provoke enough of a rise in cytosolic Ca\(^{2+}\) to trigger release, nonetheless increase both the synchronous and asynchronous release seen after a train of APs. Here, the IRP and HCSP may be increased directly, by the residual Ca\(^{2+}\) left in the wake of plasma membrane Ca\(^{2+}\) entry and/or Ca\(^{2+}\) release from cytoplasmic stores, or indirectly, by the activation of PKC by residual Ca\(^{2+}\) or diacylglycerol generated by PLC (47).

Two complicating features merit comment. First, as chromaffin cells contain a variety of Ca\(^{2+}\) channels, it is likely that some types of Ca\(^{2+}\) channels are more closely colocalized with granules than others, and their preferential opening would be more likely to trigger release from the IRP. In addition, it is likely that some types of Ca\(^{2+}\) channels may be differentially modulated by neurotransmitters (1). Second, as chromaffin granules contain neuropeptides as well as catecholamines, and the peptides are preferentially released by intense activity patterns, it is not unreasonable to anticipate that intense activity predisposes to wider pore expansions and differentially favors the discharge of peptide contents, perhaps by altering the tug of the cytoskeleton on the evolving fusion pore or on granule membrane endocytosis (10).
SSC in Many Endocrine Cells: Variations on the Chromaffin Cell Theme

Conceptually, SSC in a wide variety of endocrine cells may be viewed as specialized variations on the chromaffin cell’s theme of the mixed mode of exocytosis triggering. Here, we provide some key examples.

Cells may adapt the depolarization-induced, Ca\(^{2+}\) entry-dependent mode of exocytosis by exchanging the nAChR for another stimulus-receptor channel that provides the initial depolarization. A prime example of this is the magnocellular neurosecretory neuron (MNN; see Fig. 5A,1). MNNs, with cell bodies in the supraoptic nucleus and modified nerve terminals in the posterior pituitary, respond directly to increases in serum osmolality as small as 1%. Antidiuretic hormone (ADH) secretion by these cells results in the insertion of water channels, known as aquaporins, into the apical membrane of principal cells of the renal tubule collecting duct, hence completing the pathway for water reabsorption across the collecting duct and then into the capillaries that traverse the hypertonic renal medulla.

The cell bodies of MNNs, situated in a region of the hypothalamus locally devoid of the blood-brain barrier, serve as tiny osmoreceptors/stretch receptors (5). In response to increases in serum osmolality, the plasma membrane deforms (or crinkles) slightly (Fig. 5A, 1, step 1), opening specialized nonselective cation channels (NSCCs) usually deactivated at resting cell volume (Fig. 5A, 1, step 2); this depolarizes the cell body and increases its rate of intrinsic AP firing. Worth noting is that the NSCCs, here of the transient receptor potential, vanilloid-type variety, are situated on a region of membrane underlain by an actin-based cytoskeleton. Membrane deformation either activates the channel directly or sets off a mechano-sensitive enzyme that does this indirectly. APs propagating into the modified nerve terminal (Fig. 5A, 1, step 3) increase Ca\(^{2+}\) entry via voltage-activated Ca\(^{2+}\) channels (Fig. 5A, 1, step 4), thereby stimulating the exocytosis of ADH (Fig. 5A, 1, step 5). Interestingly, although the nerve terminals have large Ca\(^{2+}\) currents, significant exocytosis of ADH is “tuned” to the firing of trains of APs, suggesting that the initial Ca\(^{2+}\) entry primes the pool of granules available for release as well as immediately triggering release (24).

Inversely, a decrease in ambient osmolality, which causes mild swelling of the cell body, hyperpolarizes it by opening stretch-activated K\(^+\) channels, thereby decreasing the intrinsic AP frequency. Additionally, these cells respond to decreases in intravascular volume of >10% via a polysynaptic vasomotor command pathway. The release of the neurotransmitter glutamate, from the final neuron of this pathway, results in the opening of transmitter-operated NSCCs.

Cells may use the stimulus-receptor channel itself as the major source of Ca\(^{2+}\) entry for exocytosis, thereby bypassing the dependence on AP generation. In the atrial myocyte, an honorary ESC, membrane stretch, resulting from increased atrial volume due to renal salt and water retention, increases the secretion of atrial natriuretic peptide (ANP; see Fig. 5A, 2). ANP in turn enhances renal salt and water loss by a variety of mechanisms, including the enhancement of glomerular filtration, by vasodilation, and a reduction in Na\(^{+}\) reabsorption from the glomerular filtrate, by cGMP-induced closure of epithelial Na\(^{+}\) channels in the renal collecting duct. In the atrial myocyte, evidence is emerging (54) for the stretch activation (Fig. 5A, 2, step 1) of NSCCs that have high Ca\(^{2+}\) permeability (Fig. 5A, 2, step 2), hereby providing sufficient Ca\(^{2+}\) entry to trigger the exocytosis of ANP-containing secretory granules (Fig. 5A, 2, step 3). These granules appear to be located at regions of the plasma membrane free of T-tubule invaginations and far from voltage-dependent Ca\(^{2+}\) channels involved in AP-driven excitation-contraction coupling. Hence, in the same cell, the tonic release of ANP, induced by membrane stretch, can occur independently of propagating AP activity (and its resultant periodic cell contraction).

Cells may use the depolarization-induced, Ca\(^{2+}\) entry-dependent mode of stimulus-depolarization coupling even in the absence of exocytotic release of the hormone. A prime example is the aldosterone-secreting cells of the adrenal zona glomerulosa (see Fig. 5A, 3) (29). These cells respond to mildly elevated extracellular [K\(^{+}\)] (5.5–6 mM) (Fig. 5A, 3, step 1) with depolarizations to between −60 and −55 mV (Fig. 5A, 3, step 2). This depolarization is sufficient to open low voltage-activated (or T-type) Ca\(^{2+}\) channels (Fig. 5A, 3, step 3), trigger APs, and increase cytosolic [Ca\(^{2+}\)], which in turn contributes to the final enzymatic modification, in the cytoplasm, of the sterol precursor to aldosterone (Fig. 5A, 3, step 4). The lipid soluble sterol, which can now more easily dissociate from its carrier protein, rapidly diffuses from the cytoplasm, crossing the plasma membrane (Fig. 5A, 3, step 5) and then entering the extracellular space and adjacent capillary bed. The net effect of
K⁺-triggered aldosterone secretion is to stimulate, via genomic and nongenomic pathways, Na⁺/K⁺ exchange across the apical membrane of the principal cell of the collecting duct of the renal tubule and hence net K⁺ loss in the urine. Interestingly, angiotensin II, the other major stimulus for aldosterone secretion, likewise depolarizes these cells, here by receptor-mediated closure of the background K⁺ channels, thereby enhancing Ca²⁺ entry and increasing the final link in aldosterone synthesis.

Cells may combine an initial depolarization-independent, internal Ca²⁺ release-dependent mode of exocytosis with depolarization-induced Ca²⁺ entry used to refill Ca²⁺ stores. An excellent case in point is that of pituitary gonadotropes (51), which secrete luteinizing hormone and follicle-stimulating hormone to affect both male and gamete maturation (see Fig. 5B). In these cells, the binding of gonadotropin-releasing hormone (GnRH) to a GPCR (Fig. 5B, step 1) generates IP₃ (Fig. 5B, step 2), thereby leading to the release of Ca²⁺ from ER stores (Fig. 5B, step 3). The rise in cytosolic [Ca²⁺] triggers exocytosis (Fig. 5B, step 4) even as it opens Ca²⁺-activated K⁺ channels (Fig. 5B, step 5). The net result is membrane hyperpolarization that is synchronous with exocytosis. The subsequent waning of cytosolic Ca²⁺ results in the closure of Ca²⁺-activated K⁺ channels, rebound depolarization, and electrical activity (Fig. 5B, step 6), resulting in a round of Ca²⁺ entry and refilling of stores. After exposure to GnRH, the interplay of Ca²⁺ store release and refilling results in cyclical exocytosis and electrical activity. Interestingly, in these cells, there is evidence that exocytotic rates, comparable with those of granules of the HCSP of chromaffin cells.

Cells may ignore all of the paradigms thus far presented; their stimuli may provoke exocytosis that is independent of a rise in cytosolic [Ca²⁺] but instead is dependent on other second messengers. Such is the case with the chief cells of the parathyroid gland (see Fig. 5C). These secrete parathyroid hormone (PTH) in response to either a fall in ionized serum Ca²⁺ or massive stimulation by β-adrenergic agents. Both of these stimuli fail to increase, and may actually decrease, cytosolic [Ca²⁺]. The resultant rise in serum PTH increases Ca²⁺ entry into the circulation by stimulating the continuous reclamation of Ca²⁺ from bone by osteoclasts and reabsorption of Ca²⁺ from the glomerular filtrate by cells of the distal nephron. Over hours, this raises serum [Ca²⁺], thereby maintaining excitability and excitation-secretion coupling in neurons and neuroendocrine cells as well as excitation-contraction coupling in the heart and smooth muscle, with the latter insuring cardiac output and vascular tone during shock and other stress responses. Inversely, a rise in ionized serum [Ca²⁺] inhibits PTH granule exocytosis rather than augmenting granule exocytosis, as it does in most cells.

Although few patch-clamp and no single cell exocytosis studies have been done on chief cells, a review (6) of previous experiments, including those with membrane-permeabilized cells, suggested the following hypotheses.

**PATH A.** In **path A**, dissociation of Ca²⁺ from a G protein-coupled Ca²⁺-sensing receptor (CaSR; Fig. 5C, step 1A) activates an unknown intermediate (Fig. 5C, step 2A), which in turn stimulates a G protein associated with exocytosis (originally called Gₛ; Fig. 5C, step 3A), promoting the recruitment of granules into a readily releasable pool that slowly undergoes exocytosis at low cytosolic Ca²⁺ (25–100 nM; Fig. 5C, step 4).

**PATH B.** In **path B**, binding of β-adrenergic agents to a GPCR (Fig. 5C, step 1B) activates adenylyl cyclase (Fig. 5C, step 2B) and increases cytosolic [cAMP] (Fig. 5C, step 3B), again leading to the increased phosphorylation of granules and their recruitment into a highly Ca²⁺-sensitive, readily releasable pool from which they slowly exocytose in the face of low background cytosolic [Ca²⁺] (Fig. 5C, step 4). It is unclear whether both pathways activate the same pool of granules. In contrast, a rise in ionized serum [Ca²⁺], resulting in the increased binding of CaSRs, activates PLC to generate IP₃ and release Ca²⁺ from intracellular stores and inhibits adenylyl cyclase, decreasing cytosolic [cAMP] and producing a net inhibitory effect on exocytosis. Curiously though, the elevation of cytosolic [Ca²⁺] to much higher levels (several to tens of micromolars) will also trigger PTH release, suggesting that while these cells have the capacity for Ca²⁺-triggered release, physiologically they bypass it.

The CaSR of the chief cell has become a target of pharmacological modulation in patients with chronic renal disease (7). In these individuals, chronically low ionized serum [Ca²⁺], elevated serum [phosphate], and depressed [vitamin D] all alter chief cell function. By reducing the expression of CaSRs, these factors shift the dose-response characteristics for PTH secretion as a function of ionized [Ca²⁺] and induce massive cell hypertrophy and ongoing PTH secretion; PTH continues to be secreted at high levels even after ionized [Ca²⁺] has been acutely restored. The debilitating reality of continuous bone resorption, in >0.5 million people worldwide with end-stage renal disease, has prompted the development of now widely used calcimimetic drugs, which appear to sensitize CaSRs to Ca²⁺, thereby reducing their chronic multifaceted stimulation.

**SSC in the Most Clinically Explored Endocrine Cells, the β- and α-Cells of the Pancreatic Islet of Langerhans: Complexity, Ability to Be Modulated, and Possible Ability to Be Replaced**

Pancreatic islets cells as metabolic sensor cells. With the worldwide epidemic of type II DM, in large part due to increased caloric intake and decreased daily energy expenditure in both developed and developing nations, the normal function and decline of function of endocrine cells of the pancreatic islets of Langerhans has become a public health issue (4). Interspersed in a huge branching exocrine gland, these small islands, of ~1,000 ESCs, regulate metabolite uptake, storage, and release by depot store tissue such as hepatocytes, adipocytes, and skeletal myocytes (44). Sixty to sixty-five percent of islet cells are insulin-producing β-cells. Easily identified by their large diameter, autofluorescence, and distinctive (almost cuboidal) crystals in their secretory granules, β-cells are triggered to secrete by an increase in serum glucose. Twenty to twenty-five percent of islet cells are glucagon-producing α-cells; they are largely triggered to secrete by a decrease in serum glucose. The combination of β-cells...
with α-cells forms a “push-pull system” with respect to glucose metabolism and general energy homeostasis. The remaining 15% of islet cells are δ-cells or pancreatic polypeptide (PP) cells, which secrete the modulatory peptides somatostatin or PP, which inhibit SSC of both α-cells and β-cells. In its simplest form, β-cell insulin secretion, induced by high serum [glucose], stimulates glucose transport into, while inhibiting its release from, storage cells (hepatocytes and muscle), thereby rapidly reducing circulating glucose levels. In contrast, α-cell glucagon, induced by low serum [glucose], stimulates glycogenolysis in hepatocytes and muscle, thereby releasing glucose and increasing its increasing circulating serum concentration. In DM, glucose-induced insulin secretion is either reduced because β-cells have developed a reduced sensitivity to glucose as a result of their accumulation of lipids (in the case of type II DM) or is abolished because β-cells have been destroyed by an immunological attack (in the case of type I DM) (4). In type I DM, while insulin must be administered exogenously to maintain a semblance of glucose homeostasis, this treatment is often tricky because α-cells may no longer secrete glucagon in response to low glucose, thereby prolonging the recovery from episodes of hypoglycemia (low plasma [glucose]).

What makes α-, β-, and δ-cells of the islet cells so special is that they are all metabolic sensor cells. However, rather than sensing glucose using a specific GPCR linked to an effector cascade, these cells import glucose, aerobically metabolize it, and then use the resultant changes in cytosolic [ATP] and [ADP] to control their respective secretion. In addition, although individual islet cells secrete in response to the appropriate stimuli when in isolation, these cells secrete most potently when they are in clusters containing representatives of each cell type. In the latter case, β-cells are electrically coupled, while α-, β-, and δ-cells communicate via local paracrine secretory interactions. Glucagon enhances stimulus-induced insulin secretion, whereas somatostatin inhibits it. However, insulin, glucagon, and somatostatin all inhibit glucagon secretion.

An overall consensus scheme for SSC in β-cells (e.g., Ref. 32) is shown in Fig. 6A. A rise in serum glucose from a baseline level of 3 mM to stimulatory levels of 5–6 mM enhances glucose importation via a moderate-affinity transporter [glucose transporter (GLUT)] as well as phosphorylation by glucokinase (Fig. 6A, step 1) to permit its intracellular metabolism. Glycolysis and mitochondrial oxidation of 3-carbon glucose fragments results in a small rise in cytosolic ATP metabolism. Glycolysis and mitochondrial oxidation of 3-carbon glucose fragments results in a small rise in cytosolic ATP concentration. In DM, glucose-induced insulin secretion is either reduced because β-cells have developed a reduced sensitivity to glucose as a result of their accumulation of lipids (in the case of type II DM) or is abolished because β-cells have been destroyed by an immunological attack (in the case of type I DM) (4). In type I DM, while insulin must be administered exogenously to maintain a semblance of glucose homeostasis, this treatment is often tricky because α-cells may no longer secrete glucagon in response to low glucose, thereby prolonging the recovery from episodes of hypoglycemia (low plasma [glucose]).

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Current, much attention is being focused on mutations of the KATP channel as the loci of origin of two neonatal diseases: neonatal diabetes (ND) and its converse, congenital hyperinsulinemia of infancy (CHI) (2, 37). In CHI, KATP channels show loss of function; in the case of ND, there is gain of function. In CHI, SUR 1 receptors are often poorly synthesized, assembled, or trafficked to the membrane or else have lost the ability to appropriately respond to MgADP. With functional KATP channels absent or largely closed, the depolarizing effect of NSCCs is poorly opposed, and the β-cell remains chronically depolarized and stimulated to secrete insulin, resulting in systemic hypoglycemia. In the case of ND, the best-explored mutations of Kir6.2 show reduced KATP...
channel closure in response to MgATP, presumably leading to inability of the β-cell to depolarize in response to glucose. It is likely that defective K_{ATP} channels are heteromultimers containing different ratios of wild-type to defective subunits; these ratios may even change with the progression of the disease. Interestingly, both diseases are associated with neurological deficits. In CHI, this is likely due to cytotoxicity produced by repeated episodes of hypoglycemia. In ND, which is often associated with delayed motor and cognitive development and epilepsy, this may be due to reduced excitability of central inhibitory (GABAergic) neurons, skeletal muscle, and terminals of motor neurons, where K_{ATP} channels make a sizeable contribution to resting membrane permeability.

While α-cells appear to be the inverse of β-cells, in that they cease to secrete as [glucose] rises, in detail their basic working parts are quite similar to those of β-cells… with some interesting twists (see Ref. 14). Although α-cells exhibit similar K_{ATP} channels as found in β-cells, even at low [glucose] (2–3 mM), α-cells metabolize sufficient glucose to close most of the channels. Having voltage-activated Na^+ channels and low voltage-activated (T-type) Ca^{2+} channels, in the face of largely closed K_{ATP} channels, α-cells fire “spontaneous” APs, which trigger Ca^{2+} entry through HVA Ca^{2+} channels and the exocytosis of glucagon-containing granules. As shown in Fig. 6B, an increase of extracellular [glucose] above 4–5 mM closes down the remaining K_{ATP} channels (steps 1–3) and depolarizes the α-cells into a voltage range (~40 to ~35 mV) where Na^+ and T-type Ca^{2+} channels are largely inactivated (steps 4 and 5) and the AP amplitude is decreased. This reduces the probability that an AP will open HVA Ca^{2+} channels, hence reducing Ca^{2+} entry (Fig. 6B, step 6) and inhibiting glucagon secretion (Fig. 6B, step 7). In addition, α-cells of some species have a high density of GABA receptor channels that carry Cl^-.

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Staying Current

Fig. 7. Biphasic insulin secretion: its timing, physiological significance, and possible underlying cellular mechanisms. A: in vivo timing of the first (FPIS) and second phase of insulin secretion (SPIS) in normal individuals and those with early type II diabetes mellitus (DM II). B: models for the cell biological/molecular bases of biphasic insulin secretion based on the concept of two pools of insulin secretory granules. These pools include one immediately available for release and another requiring one of the following: refilling of membrane-docked granules from a cytoplasmic pool, via movement along the cytoskeleton (i); a distinct glucose-derived metabolic stimulus to recruit granules into the second pool (ii); a second variety of voltage-activated Ca^{2+} channel, providing an alternative source of Ca^{2+} entry to help refill vesicles of the initial pool (iii); a set of granules docked by different SNAREs at distinct fusion sites (iv); and granules from an HCSP triggered by plateau depolarization (PD) rather than cluster of AP activity (v).
only after SPIS is greatly reduced and blood [glucose] remains persistently elevated for long periods after a meal.

Biphasic insulin secretion is also seen in isolated islets and in individual β-cells, where it has been attributed to insulin release from two different pools of granules: 1) an immediately available but easy-to-deplete pool and 2) a late-to-release but rapidly refillable backup pool (see Fig. 7B). Shortly after learning to isolate and study islets in vitro, the islet pioneer Paul Lacy obtained evidence that SPIS could be inhibited by cytoskeleton-disrupting agents (24) and postulated that the backup pool consisted of granules transported by motors on microtubules from their site of manufacture in the ER/Golgi complex to their site of exocytosis at the plasma membrane (Fig. 7B, i).

Although the mechanism(s) underlying biphasic insulin secretion remain elusive, several newer hypotheses, originating from single cell studies of exocytosis, merit consideration. As shown in Fig. 7B, ii, there is evidence that glucose may generate intracellular signals other than the increased [ATP]/decreased [ADP] that closes KATP channels and triggers initial insulin secretion. These signals may help enhance SPIS. Alternatively, there is evidence from gene knockout studies in mice that the activation of R-type (Cav2.3) voltage-dependent Ca2+ channels, distinct from the L- or N-type channels usually described, recruits granules into a backup pool and is responsible for SPIS (21) (see Fig. 7B, iii). Also, from another knockout mouse model, there is optical evidence for differential docking of granules at distinct sites, with granules involved in FPIS docked at sites rich in the SNARE protein syntaxin A, whereas those involved in SPIS docked at syntaxin A-poor sites (39) (see Fig. 7B, iv). Finally, there is evidence for differential docking of granules of varying Ca2+ sensitivity vis-à-vis Ca2+ channels. While a body of evidence from mouse β-cells has suggested that the IRP underlies FPIS (3), emerging evidence from other species is consistent with the HCSP, discharged during prolonged depolarization in several species, underlying much of SPIS (Fig. 7B, v). Unlike mouse β-cells, which display persistent bursts of electrical activity on exposure to secretagogues concentrations of glucose, single canine β-cells display biphasic electrical activity temporally and pharmacologically correlated with the two phases of insulin secretion (34). Trains of APs, ideal to activate the IRP, occur during FPIS; the sustained phase of plateau depolarization, ideal to activate the HCSP, occurs during the transition to SPIS. Both IRP and HCSP are enhanced by concentrations of glucose that trigger biphasic secretion, perhaps by its ability to increase transport or docking of granules, as Lacy suggested 35 years ago.

Let us return to the issue of incretins and their ability to enhance biphasic insulin secretion. The two most likely incretins are ACh, which is released, as a reflex by the vagus nerve, upon stretching of the gut, and glucagon-like intestinal peptide (GLP)-1, which is released by enterochromaffin-like cells of the gut in response to newly digested nutrients. Both work through GPCRs and ultimately exert a pair of similar effects, although through different second messengers (15, 16). Both enhance the closure of KATP channels initiated by glucose metabolism, likely by increasing their phosphorylation; both enhance depolarization-exocytosis coupling, likely by stimulating the recruitment of granules into both the IRP and HCSP (see Fig. 8). Specifically, ACh, via its mAChR, stimulates the activity of CaMK II (12), GLP-1, via its GPCR, activates adenylate cyclase, increasing cytosolic [cAMP], thereby stimulating either classical PKA or the cAMP-regulated guanidine nucleotide exchange factor known as Epac (18). GLP-1 has received the bulk of the attention for several reasons: its effect is more targeted to β-cells than ubiquitously acting ACh, its secretion appears to be blunted in type II DM, and, additionally, it may reduce apoptosis in β-cells exposed to high [glucose]. Efforts to enhance circulating [GLP-1] has entered mainstream type II DM therapy as a key to normalizing insulin secretion and potentially delaying the progression of β-cell damage. Two classes of drugs now in development/early use are 1) GLP-1 mimetics, or long-acting recombinant GLP-1 analogs; and 2) GLP-1 enhancers, or inhibitors of the dipeptide peptidases that breakdown circulating GLP-1. The advantages of these drugs over earlier insulin secretion enhancers, such as sulfonylureas (which bind to and close KATP channels even in

Fig. 8. Cellular basis for a role of incretins in enhancing biphasic insulin secretion. A: outlines of how glucagon-like peptide (GLP)-1 and ACh are released by gut enterochromaffin-like cells (ECLs) and the vagus nerve reflex, respectively (each likely activated by some combination of gut stretch, increase in luminal [glucose], and osmolality). Each of these secretions enhances glucose-induced closure of KATP channels and accelerates insulin granule recruitment into release-ready pools. CNS, central nervous system. B: traces obtained from canine islet β-cells showing the effects of GLP-1 on stimulus-depolarization coupling (i) and depolarization-exocytosis coupling (2). GLP-1 speeds cell depolarization and the initiation of a moderate frequency of AP activity after a transition from 3 to 15 mM extracellular glucose (3). GLP-1 also enhances the exocytotic response (Cm increases) in response to a train of five 200-ms depolarizations from −70 mV to +10 mV applied at 1 Hz without increasing Ca2+ currents. Note that GLP-1 enhances the slow continuous Cm increases seen after the third and fourth depolarizations, at high gain seen to consist of small asynch Cm steps, likely indicating greater recruitment and utilization of the HCSP, as well as the HCSP, as well as enhancing the synchronous Cm step increases seen after the first and second depolarizations, likely indicating greater recruitment and utilization of the IRP. [S. Misler and D. W. Barnett, unpublished observation.]
the absence of glucose), are both predictable and novel (17). As GLP-1 mimetics/enhancers modulate glucose-induced K$_{ATP}$ channel closure rather than bypassing it, they are less likely to cause hypoglycemia. As the latter agents work to recruit granules into the readily releasable pools, they are less likely to cause pool exhaustion. As these agents appear to extend the life of ß-cells, they may retard the intrinsic progression of the disease. Finally, as GLP-1 mimetics/enhancers may act in the central nervous system to suppress appetite, they may ultimately help bring about a better caloric balance. The critical and as-yet-unresolved issue is whether GLP-1 receptors themselves become severely downregulated in type II DM.

Returning to our theme of SSC, evidence from clonal cell lines has suggested that GLP-1-secreting enterochromaffin-like cells, sparsely distributed endocrine cells of the gut mucosa, respond to glucose not by metabolizing it but by transporting it. Na+-glucose cotransport provides both a source of direct electrogenic depolarization and glucose entry for the closure of K$_{ATP}$ channels. Depolarization sets off electrical activity and Ca$^{2+}$ entry-dependent GLP-1 granule exocytosis (50). Perhaps gut stretch and the increasing osmolality occurring with progressive digestion also activate stretch-modulated channels in these cells and serve as backup sources of depolarization.

**Implications for Translating Basic Physiology to the Clinics**

In this review, we have demonstrated that fundamental mechanisms of SSC are shared by a variety of ESCs with widely differing sensory apparatus and secretory products. Cells appear to adapt basic modular elements of design to achieve specific functions. Additionally, we have demonstrated how understanding the specifics of these mechanisms has contributed to the development of new pharmacotherapies for several wide-ranging clinical problems, the two prominent examples being calcimimetics, used to treat the osteodystrophy of chronic kidney disease, and GLP-1 mimetics/enhancers, used to treat type II DM. Furthermore, in single-gene defect diseases of ESCs, such as ND and CHI, it is likely that an intricate understanding of mechanisms of SSC should help to uniquely target therapy (e.g., sulfonylurea therapy in ND).

It is intriguing to speculate that the basic understanding of SSC in endocrine cells might bring to fruition a “holy Grail search” that has motivated endocrinology since its beginnings: cell replacement therapy for failing hormone-secreting organs. The latest incarnation of this approach is islet transplantation for brittle type I DM patients, for whom insulin replacement therapy, even by infusion pumps and coupled with frequent blood sugar checks to close the feedback loop, still results in frequent episodes of debilitating hypoglycemia. Unfortunately, even in this carefully chosen population, the current approaches for intrahepatic transplantation of islets have largely transitory therapeutic effects while carrying the attendant risks of immunosuppression and perhaps even reawakening the inciting autoimmune response (45). Against the background of our increased understanding of SSC, the challenge remains for the identification and controlled proliferation of endocrine stem cells or the genetic engineering of cells to “turn on” of key elements of the secretory apparatus expressed in SSCs. Realizing the similarities of basic modular processes in SSC, a general advance in the guidance of development or the genetic engineering of any one type of secretory cell may be widely applicable to several others.

In conclusion, the study and understanding of the cell physiology and biophysics of SSC in endocrine cells, which in the past has been a side avenue of experimental endocrinology, may have an illustrious future with wide-ranging therapeutic potential.

**ACKNOWLEDGMENTS**

This text is based on a minicourse presented at the Universidade de Tras-os-Montes e Alto Douro (Vila Real, Portugal), kindly hosted by Prof. Amelia Silva. The ongoing superb help of Phillip Blevin (Office of Medical Education, Washington University School of Medicine) and Alon Friedman (Advanced Teacher Training Institute, Shlomi, Israel) in the design and execution of the figures is gratefully acknowledged.

This article is dedicated to Prof. Linda H. Mantel, my undergraduate professor of physiology at The City College of The City University of New York, in appreciation of her efforts in teaching many students that physiology, whether cellular, comparative, or integrative/adaptive, is best understood as variations on unifying concepts.

**GRANTS**

The original data traces presented in Figs. 3 and 8 were obtained by the author under funding from National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-37380.

**REFERENCES**