PKA-Dependent and PKA-Independent Pathways for cAMP-Regulated Exocytosis

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I. Introduction 1304
II. Fundamental Features of Regulated Exocytosis 1304
   A. Neurons 1304
   B. Endocrine and neuroendocrine cells 1307
   C. Exocrine cells 1309
III. Intracellular cAMP Metabolism 1310
   A. Adenylyl cyclases 1310
   B. Phosphodiesterases 1311
IV. Effectors of cAMP and Their Properties 1311
   A. Structure of cAMP-binding proteins 1311
   B. cAMP-dependent protein kinase 1311
   C. Cyclic nucleotide-gated channels 1312
   D. cAMP-GEF/Epac 1314
   E. CRP/CAP 1314
V. Pharmacological Agents Targeting the cAMP Pathway 1315
   A. cAMP analogs 1315
   B. Inhibitors targeting catalytic subunits of PKA 1316
VI. PKA-Dependent Effects of cAMP on Regulated Exocytosis 1316
   A. Neurons 1316
   B. Pituitary cells 1319
   C. Adrenal chromaffin cells 1320
   D. Pancreatic β-cells 1320
   E. Exocrine acinar cells 1322
VII. PKA-Independent Effects of cAMP on Regulated Exocytosis 1322
   A. Pancreatic β-cells 1323
   B. Neurons 1324
VIII. A Model for cAMP-Regulated Exocytosis 1324
IX. Concluding Remarks 1327

Seino, Susumu, and Tadao Shibasaki. PKA-Dependent and PKA-Independent Pathways for cAMP-Regulated Exocytosis. Physiol Rev 85: 1303–1342, 2005; doi:10.1152/physrev.00001.2005.—Stimulus-secretion coupling is an essential process in secretory cells in which regulated exocytosis occurs, including neuronal, neuroendocrine, endocrine, and exocrine cells. While an increase in intracellular Ca2+ concentration ([Ca2+]i) is the principal signal, other intracellular signals also are important in regulated exocytosis. In particular, the cAMP signaling system is well known to regulate and modulate exocytosis in a variety of secretory cells. Until recently, it was generally thought that the effects of cAMP in regulated exocytosis are mediated by activation of cAMP-dependent protein kinase (PKA), a major cAMP target, followed by phosphorylation of the relevant proteins. Although the involvement of PKA-independent mechanisms has been suggested in cAMP-regulated exocytosis by pharmacological approaches, the molecular mechanisms are unknown. Newly discovered cAMP-GEF/Epac, which belongs to the cAMP-binding protein family, exhibits guanine nucleotide exchange factor activities and exerts diverse effects on cellular functions including hormone/transmitter secretion, cell adhesion, and intracellular Ca2+ mobilization. cAMP-GEF/Epac mediates the PKA-independent effects on cAMP-regulated exocytosis. Thus cAMP regulates and modulates exocytosis by coordinating both PKA-dependent and PKA-independent mechanisms. Localization of cAMP within intracellular compartments (cAMP compartmentation or compartmentalization) may be a key mechanism underlying the distinct effects of cAMP in different domains of the cell.
I. INTRODUCTION

The fusion of secretory vesicles to the plasma membrane in multicellular organisms is a crucial event in regulated exocytosis and is tightly controlled to release vesicle contents in response to specific signals, often in a specialized region of the plasma membrane (78). There are two major types of regulated exocytosis: secretory granule (large dense-core vesicle, LDCV) exocytosis, as in neuroendocrine, endocrine, and exocrine cells, and synaptic vesicle (SV) exocytosis, which occurs in neurons. In some neurons and endocrine cells, both LDCV and SV exocytosis are found (32, 205, 358, 362, 445, 476, 512, 562, 582, 620). They can be distinguished by morphological appearance of secretory vesicles and by kinetics of exocytosis, other intracellular signals including cAMP, diacylglycerol (DAG), phospholipids, and ATP also regulate or modulate Ca\(^{2+}\)--triggered exocytosis (85, 306, 433). Among these, cAMP is well known to regulate exocytosis in a variety of secretory cells (79, 85, 112, 177, 306, 322, 334, 353, 365, 393, 433, 469, 477, 553). In neurons, cAMP has been shown to induce long-term potentiation (LTP) (44, 329, 400) by increasing neurotransmitter release at mossy fiber synapses in the hippocampus of cerebrum (552, 581) and parallel fiber-Purkinje neuron synapses in the cerebellum (104, 472). cAMP increases transmitter release at many synapses in vertebrate peripheral ganglia and invertebrate nervous system, including sympathetic (64, 317) and parasympathetic ganglion neurons (55), neuromuscular junctions of crayfish (618), central synapses of Aplysia (73, 90, 279, 308, 505), and neuromuscular junctions of Drosophila melanogaster (Drosophila) (322, 604). cAMP also regulates release of various hormones in endocrine cells, including pancreatic hormones such as insulin from pancreatic β-cells (60, 226, 230, 365, 433, 496, 524), glucagon from pancreatic α-cells (140, 208), pituitary hormones such as adrenocorticotropic (ACTH) from pituitary corticotrophs (10, 357, 588), and catecholamines from adrenal chromaffin cells (107, 413, 434, 586). In exocrine parotid acinar cells, cAMP rather than Ca\(^{2+}\) is the primary signal in amylase release (177, 441).

PKA has been thought to be the major target of cAMP in cAMP-regulated exocytosis in multicellular organisms. However, cAMP is now known to have other targets as well, including cyclic nucleotide-gated (CNG) channels (294), hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (43), and cAMP-specific guanine nucleotide exchange factors (cAMP-GEF)/exchange proteins directly activated by cAMP (Epac) (hereafter cAMP-GEF/Epac) (53, 514). Although the PKA-dependent mechanisms of regulation and modulation of exocytosis by cAMP have been studied extensively (79, 85, 112, 177, 306, 322, 334, 353, 365, 393, 477, 553), the PKA-independent mechanisms are currently being unveiled. In this review, we discuss both the PKA-dependent and the PKA-independent pathways of cAMP-regulated exocytosis and suggest a mechanism of differential implementation of these two pathways within the cell.

II. FUNDAMENTAL FEATURES OF REGULATED EXOCYTOSIS

Regulated exocytosis has been well studied in both neurons and nonneuronal cells. In neurons, neurotransmitters are released by fusion of SVs with the presynaptic plasma membrane. Nonneuronal secretory cells possess LDCVs (dense-core granules or secretory granules) containing various bioactive substances such as peptide hormones, amines, and enzymes, the contents of which exert diverse biological effects on cellular functions. Despite differences in the time course, Ca\(^{2+}\) dependency, and signal input between SV and LDCV exocytosis, both involve common processes: vesicle recruitment to the plasma membrane, docking of vesicles at the plasma membrane, priming of fusion machinery, and fusion of vesicles with the plasma membrane. Although these processes are central to understanding exocytosis, they are difficult to distinguish by currently available methods. In neurons, release of neurotransmitters is triggered primarily by Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) in response to action potentials, while in secretory cells such as neuroendocrine, endocrine, and exocrine cells, various intracellular signals in addition to the Ca\(^{2+}\) signal also participate in stimulus-secretion coupling. Exocytosis has been extensively investigated in neurons (18, 86, 522), but the detailed molecular mechanism is still largely unknown. Although basic components of the regulated exocytic apparatus are highly conserved among different secretory cell types, the secretory processes are specialized and distinct (79, 235). Since the molecular mechanisms of regulated exocytosis have been extensively reviewed recently (79, 343, 346, 392, 452, 522), we describe only the principal components here (Fig. 1). We discuss characteristic features of stimulus-secretion coupling in various cell types.

A. Neurons

Action potential-induced Ca\(^{2+}\) influx is the principal signal that triggers synaptic vesicle exocytosis. In some neurons, SVs and LDCVs, which store low-molecular-weight transmitters and neuropeptides, respectively, are present together (32, 205, 358, 562, 620). For example, in certain neurons in which ACh and vasoactive intestinal polypeptide (VIP) coexist, stimulation of muscarinic cho-
linergic autoreceptors inhibits ACh and VIP release, while VIP enhances ACh release, suggesting that release of one of the coexisting transmitters modulates release of the other (32). Exocytosis of SVs and LDCVs occurs in response to distinct and specific signals, and the Ca\(^{2+}\) threshold for initiation, kinetic properties, and requirement for release sites differ in the two types of secretion, suggesting distinct regulatory mechanisms (562).

The conserved protein components of the exocytotic machinery in neurons include SNAREs [soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors], ATPase N-ethylmaleimide-sensitive factor (NSF), and its cofactor α-SNAP, Munc18/Sec1, Munc13, synaptotagmins, and Rab3 and its effectors. SNAREs are membrane proteins characterized by an α-helical coiled-coil domain consisting of ~60 amino acids that is called the SNARE motif (269, 459, 465, 580). The human genome contains 36 SNAREs (45, 269). SNAREs were initially classified as v-SNAREs and t-SNAREs, based on their localization on the vesicular and target plasma membrane, respectively (511). More recently, they were reclassified as arginine (R)-SNAREs and glutamine (Q)-SNAREs, depending on whether conserved arginine or glutamine is present in the center of the SNARE motif (161). Formation of the SNARE complex is proposed to mediate membrane fusion (38, 465). The SNARE complex comprises three proteins: vesicle-associated membrane protein (VAMP-2) (synaptobrevin), syntaxin 1, and the 25-kDa synaptosomal-associated protein (SNAP-25) (Fig. 1). VAMP-2 is an R-SNARE originally identified as a brain-specific neuronal membrane protein (414). VAMP-2 and syntaxin 1 each contains a single SNARE motif, while SNAP-25 contains two SNARE motifs. The four motifs from these proteins form an extremely stable ternary complex (SNARE complex). Neuronal SNAREs have been shown to interact with many other vesicle-associated proteins including Munc18/Sec1 (224), Munc13 (41), synaptotagmins (200), and complexin/synaphin (263, 377). Genetic disruption of neuronal SNAREs in Drosophila, Caenorhabditis elegans, and mice shows that SNARE is essential for evoked synaptic transmission but is not always involved in spontaneous synaptic events (67, 133, 403, 484, 487, 572), indicating that the exocytotic processes of evoked and spontaneous release differ in their requirement for the SNARE complex.

To recycle the individual proteins of the SNARE complexes, they must be disassembled after exocytosis. NSF and α-SNAP act to disassemble the complexes (29). NSF is a hexameric protein that belongs to the AAA+ ATPase superfamily of chaperone-like ATPases (221, 372, 558, 622). α-SNAP, an adaptor protein, interacts with the assembled SNARE motifs, allowing subsequent binding of NSF (221, 239, 583). Hydrolysis of ATP by NSF then dissociates the complex into its individual components. α-SNAP stimulates the ATPase activity of NSF as well as recruits NSF to the SNARE complex (29, 225, 371, 388, 515).

Munc18/Sec1 is a family of hydrophilic proteins with no recognizable motifs (217, 268). The first member of the family was discovered in C. elegans in the UNC-18 mutant (Munc18) (63, 247) and was later found in yeast in the first secretory mutant (Sec1) (405). The mammalian ho-
mologs, Sec1 and Munc18, were also found (217). The human genome contains seven UNC-18/Sec1 homologs, three of which are Munc18–1, 18–2, and 18–3. Munc18–1 is predominantly expressed in neuronal and endocrine tissues, while Munc18–2 and Munc18–3 are ubiquitously expressed (194, 196, 224, 427, 542). Disruption of Munc18–1 in mice completely abolishes neurotransmission without affecting SV docking, suggesting a role of Munc18 in a postdocking step (561). In contrast, in adrenal chromaffin cells of Munc18–1 knockout mice, LDCV exocytosis is reduced, with fewer docked vesicles, suggesting that Munc18 functions at the docking step in LDCV exocytosis (564). Munc18 specifically binds to the 

\[ H_{abc} \]

domain of syntaxin 1 in the closed conformation, which prevents formation of the SNARE complex (147, 384, 599). Although Munc18 was initially proposed to function as a negative regulator of membrane fusion by inhibiting SNARE complex assembly (426, 488), recent studies favor the possibility that Munc 18 regulates transition of syntaxin 1 from closed to open conformation, thereby facilitating SNARE complex assembly.

UNC-13 was originally identified in *C. elegans* (475). Munc13 (four isoforms, Munc13–1, 13–2, 13–3, and 13–4) is a mammalian homolog of *C. elegans* UNC-13 (70, 117, 311). Munc13 interacts with the 

\[ H_{abc} \]

domain of syntaxin 1 (41). Munc13 is thought to prime synaptic vesicles by hindering the interaction between syntaxin 1 and Munc18, thereby promoting activation of syntaxin 1 for the formation of the SNARE complex in the active zone at the presynaptic terminal membrane (16, 41, 203, 475). Synaptic transmission of *Drosophila* UNC-13 has also been studied, and found to be essential for synaptic transmission (13). Munc13 is also involved in DAG-mediated, PKC-independent neurotransmitter release through its C1 domain (325, 383, 406, 454). In addition to interacting with syntaxin 1, Munc13 interacts with several other proteins such as Rim1 (42) and Doc2 (412, 471).

Rab proteins are members of the Ras superfamily of small G proteins that function in vesicular transport (428, 490, 614). Rab proteins cycle between an inactive state (the GDP-bound form localized in the cytosol) and an active state (the GTP-bound form localized in the membrane). In the human, there are at least 60 Rab isoforms (45, 422, 614). Of these, Rab3 has been implicated both in synaptic and secretory granule exocytosis (124, 197, 522). In mammals, there are four structurally related Rab3 isoforms, Rab3A, B, C, and D, which are differentially expressed. Rab3A is expressed at high levels in the brain (166, 269) and is involved in the regulation of various steps in synaptic vesicle trafficking including targeting, docking, and postdocking processes (197, 335, 404). The amount of evoked transmitter release per stimulus is enhanced in Rab3A knockout mice (198), and LTP in mossy fiber synapses of the CA3 region in the hippocampus is abolished (91). Quadruple knockout mice (lacking all Rab3 isoforms) are born alive but exhibit respiratory failures (480). These knockout mice exhibit no apparent changes in synapse structure or expression levels of proteins associated with SV exocytosis, except for the loss of Rabphilin3, a Rab3-binding protein. Analysis of cultured hippocampal neurons from these knockout mice revealed that Rab3 is not essential for synaptic membrane trafficking, but modulates basic release machinery.

The diverse effects of Rab3 are mediated by interaction with its multiple effectors (81, 119, 124, 199, 503, 522). Rab3 has several potential effectors, including Rabphilin3 (502), Rim (Rim1 and Rim2) (415, 570, 571), Noc2 (369), and Granuphilin (118). Among these, Rabphilin3 and Rim1 are expressed predominantly in the brain. Rabphilin3, a SV-associated protein, is reversibly recruited to synaptic vesicles by Rab3 (504). Rabphilin3 knockout mice do not exhibit any of the obvious phenotypes of regulated exocytosis. (481). Rim (now called Rim1) was discovered originally as a Rab3-interacting molecule by yeast hybrid screen and is an active-zone scaffolding protein that binds to Rab3 when synaptic vesicles dock with GTP-bound Rab3 (570). Rim2, which was found later as an isoform of Rim (415, 571), is expressed in endocrine cells as well as in the brain (see sect. II for details). Rim contains a Zinc finger domain and two C2 domains. Rim1 knockout mice exhibit an altered short-term synaptic plasticity and an absence of LTP in mossy fiber synapses in the hippocampus (92). The Rim null mutation in *C. elegans* decreases the number of fusion-competent vesicles, suggesting a role in the postdocking process (315). Rim1 interacts with Munc13–1 (42). Disruption of this interaction causes a drastic decrease in the size of the readily releasable pool, suggesting that the interaction of Rim1 and Munc13–1 is involved in regulating synaptic vesicle priming (42). Rim1 and Rim2 interact with cAMP-GEFII/Epac2, a cAMP-binding protein exhibiting GEF activity toward Rap (415). In pancreatic β-cells, the interaction of Rim2 and cAMP-GEFII/Epac2 mediates cAMP-dependent, PKA-independent insulin granule exocytosis (see sect. VII). This mechanism involving cAMP-GEFII/Epac2 may also be present in certain neurons in which cAMP modulates transmitter release, since cAMP-GEFII/Epac2 binds to Rim1, which is expressed predominantly in the brain (see sect. VII).

An increase in [Ca2+], almost universally triggers the fusion of secretory vesicles to the plasma membrane. Among many Ca2+–binding proteins that might function as Ca2+ sensors for vesicle fusion, synaptotagmin is the best characterized candidate (17, 98, 521). Synaptotagmin was originally reported as p65, a 65-kDa synaptic vesicle protein, and renamed after its cloning (370, 424). Synaptotagmins include 13 isoforms in humans (521), and the presence of an additional 6 potential isoforms has been suggested by database search (120). While most synaptotagmins are localized on transport vesicles, some (synap-
Synaptotagmin III, VI, and VII) are present at the plasma membrane (23). Synaptotagmin has an NH$_2$-terminal transmembrane region followed by two C$_2$ domains (C$_2$A and C$_2$B) and binds phospholipids with considerable variations in Ca$^{2+}$ dependence. In addition, synaptotagmin I also binds to syntaxin 1, SNAP-25, Ca$^{2+}$ channels, and Rim1 (119). Studies of mutations in synaptotagmin I in mice, Drosophila, and C. elegans have shown that synaptotagmin I is essential only for fast, Ca$^{2+}$-triggered release, but not for other modes of the exocytotic process such as stimulus-induced asynchronous release or spontaneous release, which suggests that synaptotagmin I functions at the Ca$^{2+}$-sensing step for fast vesicle fusion (23, 125, 350). Synaptotagmin IX, which has close homology to synaptotagmin I, also binds to Ca$^{2+}$, but does not associate with the SNARE complex, suggesting a role distinct from that of synaptotagmin I (500). The presence of different synaptotagmin isoforms with distinct Ca$^{2+}$-binding affinities might account in part for variations in the effective concentration of Ca$^{2+}$ in exocytosis seen in various types of secretory vesicles (288, 305). Vesicular synaptotagmins with low Ca$^{2+}$ affinities may be more important for fast synaptic vesicle exocytosis, while plasma membrane synaptotagmins with a higher Ca$^{2+}$ affinity may be more important in slower, secretory granule exocytosis (521).

B. Endocrine and Neuroendocrine Cells

In endocrine cells there is a large, reserved pool of secretory granules and a readily releasable pool constituting only a small fraction of the secretory granules. Stimulus-secretion coupling has been characterized in many endocrine and neuroendocrine cells, particularly insulin-secreting pancreatic β-cells and catecholamine-secreting adrenal chromaffin cells. The pancreatic β-cell plays a central role in glucose homeostasis by regulating insulin secretion. The details of the mechanism of insulin secretion have been reviewed recently (3, 202, 273, 463, 587). Ca$^{2+}$, ATP, cAMP, phospholipids, and DAG are major intracellular signals in stimulus-secretion coupling in insulin granule exocytosis. The generally accepted model of glucose-induced insulin secretion is depicted in Figure 2. An increase in the ATP concentration (or ATP-to-ADP ratio) due to elevated glucose metabolism closes ATP-sensitive K$^+$ (K$_{ATP}$) channels and depolarizes the β-cell membrane, leading to opening of the VDCCs and resultant Ca$^{2+}$ influx. The rise in [Ca$^{2+}$]$_i$ triggers exocytosis of the insulin granules. Sulfonylureas, widely used for treatment of diabetes mellitus, stimulate insulin secretion by closing the K$_{ATP}$ channels directly. Thus the K$_{ATP}$ channels are critical in glucose- and sulfonylurea-induced insulin release by coupling metabolic changes to electrical activities (15, 116, 381, 382, 491). Opening of the VDCCs represents a common step in insulin secretion induced by glucose, sulfonylureas, and amino acids (14). Modulation of VDCC activities affects insulin secretion (35, 252, 336). Although Ca$^{2+}$ influx through VDCCs is indispensable in glucose-induced insulin granule exocytosis, it has been suggested that mobilization of intracellular Ca$^{2+}$ from ryanodine-sensitive Ca$^{2+}$ stores by cyclic ADP-ribose generated by glucose stimulation also contributes (292, 531). On the other hand, incretins such as gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) potentiate insulin secretion through cAMP-PKA signaling (see sect. VI D for details) (72, 243, 302). ACh, a major parasympathetic neurotransmitter, generates phospholipid-derived messengers. One of these, DAG, activates PKC (202). Activation of DAG-sensitive PKC by a DAG analog, phorbol.
ester, induces a prolonged insulin secretory response in pancreatic islets and insulin-secreting cell lines (344, 518, 555). Inositol 1,4,5-trisphosphate (IP3), which is also produced by ACh stimulation, mobilizes Ca^{2+} from intracellular stores (202, 344). Free fatty acids act as signaling molecules as well as an energy source in insulin secretion (216). Among free fatty acids, long-chain free fatty acids in insulin-secreting MIN6 cells potentiate glucose-induced insulin secretion through direct activation of GPR40 (264), an orphan G protein-coupled receptor (GPCR) (65, 264). Peptide agonists including somatostatin, norepinephrine, and galanin inhibit insulin secretion through the activation of pertussis toxin (PTX)-sensitive G protein G_{i,0} (3, 497, 595, 596).

The basic components of the exocytotic machinery present in neurons are also found in pancreatic β-cells and β-cell-derived cell lines. These include VAMP-2 (447); cellulbrevin (267, 447); syntaxin isoforms 1A, 4, and 5 (267, 367, 394, 468); SNAP-25 (267, 466); α-SNAP (304, 395); and NSF (304, 395). Synaptotagmins are expressed in pancreatic β-cells (synaptotagmin III, IV, V, VII, VIII, and IX) and β-cell-derived cell lines (synaptotagmin I, II, III, IX, V, VII, VIII, and IX) (192, 256, 386). Synaptotagmin III has been suggested to serve as a Ca^{2+} sensor in insulin granule exocytosis, as assessed by application of anti-synaptotagmin III antibodies to permeabilized pancreatic β-cells (386). In addition, a study of overexpression of recombinant C2 domains into pancreatic islets suggests that synaptotagmin V, VII, and VIII may also function as Ca^{2+} sensors (214). Thus various combinations of synaptotagmin isoforms may be responsible for the precise tuning of Ca^{2+} sensing.

Because the GTPase-deficient mutation of each of the four Rab3 isoforms inhibited nutrient-induced secretion in hamster β-cell line HIT-T15 cells (255), Rab3 was suggested to be involved in insulin granule exocytosis (255, 342, 446). This is confirmed by a finding in mice that knockout of Rab3A impairs glucose-induced insulin secretion (594). Noc2, a target of Rab3, suppresses the inhibitory effect of G_i protein signaling on insulin secretion, thereby playing an important role in the maintenance of normal insulin secretion (369). Disruption of the interaction between Rab3 and Noc2 in mice unmasks G_i protein signaling, resulting in reduced insulin secretion under conditions such as stress. Rab27, another member of the Rab family, also has been implicated in regulated exocytosis of LDCVs (602) and melanosomes (181). Overexpression of Rab27 increases insulin secretion in the insulin-secreting cell line MIN6 (602). Granuphilin, a recently identified protein associated with insulin granules (569), has been suggested to be a target of Rab27 (602). In addition to Rab27, Granuphilin also binds to syntaxin 1A (549) and Munc18 (118).

In pancreatic β-cells, the secretory granules are mainly LDCVs, but synaptic-like microvesicles (SLMVs), which contain GABA, are also present (445). There are two major components, fast and slow, in Ca^{2+}-triggered exocytosis in pancreatic β-cells (526). It is proposed that fast exocytosis is associated with release of GABA, while slow exocytosis is associated with secretion of insulin, as assessed by capacitance measurement and amperometric detection of vesicular contents (526). GABA released from SLMVs may act as a paracrine inhibitor on adjacent glucagon-secreting α-cells and somatostatin-secreting δ-cells, and serve as an autocrine regulator on pancreatic β-cells (380, 476, 512, 582). The inhibitory effect of GABA on insulin secretion in pancreatic β-cells occurs through G_{i,0} protein signaling (62). GABA release is regulated by glucose (362), suggesting that GABA-containing SLMVs also undergo regulated exocytosis. Overexpression of α-SNAP in rat pancreatic islets enhances glucose-induced insulin secretion (395). Overexpression of mutant α-SNAP lacking the binding region to syntaxin 1A in insulin-secreting MIN6 cells inhibits glucose-induced insulin release, but does not alter GABA release, indicating that the regulatory mechanism of exocytosis differs in insulin-containing LDCVs and GABA-containing SLMVs (395).

The adrenal gland plays an essential role under various stresses (19, 306). The chromaffin cells in the adrenal medulla secrete catecholamines (epinephrine and norepinephrine) and a number of neuropeptides, all of which are stored in dense-core vesicles called chromaffin granules. Chromaffin cells are excitable, generating action potentials in response to ACh (59) or electrical stimulation of the splanchnic nerve (26, 278). Innervation of adrenal chromaffin cells is principally cholinergic, by preganglionic sympathetic fibers in the splanchnic nerve, which originate in the intermediolateral cell column of the thoracic spinal cord (11, 241). Peptidergic nerves containing enkephalin, substance P, or VIP are also present (349, 486). Cholinergic fibers all seem to innervate both epinephrine- and norepinephrine-secreting cells, while enkephalin-containing fibers surround only epinephrine-secreting cells (242), thus regulating epinephrine secretion. In addition, pituitary adenyl cyclase activating polypeptide (PACAP)-containing nerve terminals, the cell bodies of which are located in the sensory neurons of dorsal root ganglia and nodose ganglia, are present in the adrenal medulla. Components of SNARE proteins are also present in chromaffin cells. VAMP is found on the membrane of chromaffin granules (238, 240). VAMP-2 mediates MgATP-dependent catecholamine exocytosis from permeabilized chromaffin cells (331). In addition, syntaxin 1A and 1B are expressed in these cells (238, 240). In permeabilized chromaffin cells, an anti-syntaxin antibody inhibits the Ca^{2+}-triggered catecholamine release, indicating that syntaxin also functions in exocytosis of chromaffin granules (215). SNAP-25 is expressed at high and low levels in noradrenergic and adrenergic chromaffin cells, respectively (283). The following mechanism of stimulus-secretion coupling...
in chromaffin cells is generally accepted. Upon activation of nicotinic ACh receptors, ACh opens the ionophore of the receptor, allowing influx of Na\(^+\) and, to a lesser extent, Ca\(^{2+}\), which results in a depolarization of the membrane. Depolarization opens the voltage-dependent, tetrodotoxin-sensitive Na\(^+\) channels (93), inducing further depolarization of the membrane and opening of the VDCCs (193).

Opening of both Na\(^+\) and Ca\(^{2+}\) channels enhances Ca\(^{2+}\) influx. The resultant rise in [Ca\(^{2+}\)]\(_i\) triggers exocytosis of catecholamine secretory granules (59, 300, 301). The time constant for exocytosis in chromaffin granules has been reported to be 150–1,000 ms, much longer than that for exocytosis of synaptic vesicles (286). cAMP has been reported to be 150 – 1,000 ms, much longer than that for exocytosis of synaptic vesicles (286). cAMP increases norepinephrine secretion in response to depolarization by high K\(^+\) or stimulation by ACh in a pathway distinct from that controlling internal Ca\(^{2+}\) levels in PC12 cells (368).

C. Exocrine Cells

Although many exocrine glands including salivary, gastric and intestinal glands, and exocrine pancreas are known to possess systems of regulated exocytosis, the molecular basis for stimulus-secretion coupling in exocrine cells has been characterized in only a few exocrine glands.

Pancreatic acinar cells synthesize, package, and release a variety of digestive enzymes and are the system in which the secretory pathway was first examined (416). The cells are highly polarized, with two distinct plasma membrane domains, an apical domain and a basolateral domain. Digestive enzymes are stored in secretory vesicles known as zymogen granules, the majority of which are present at the apical pole of the cell. Various secretagogues including a gastrointestinal hormone, cholecystokinin (CCK), neurotransmitters, ACh and VIP, and a mammalian bombesin homolog, neuromedin C, stimulate enzyme secretion by acting through the intracellular signals Ca\(^{2+}\), cAMP, and DAG (573, 585). A rise in [Ca\(^{2+}\)]\(_i\) is the primary signal triggering fusion of the zymogen granule membrane to the apical membrane. The mode of Ca\(^{2+}\) signaling seems to depend on the concentration of the secretagogue (573). Low concentrations of secretagogues evoke an oscillatory pattern of [Ca\(^{2+}\)]\(_i\), depending largely on Ca\(^{2+}\) release from intracellular stores (611). In contrast, high concentrations induce a completely different pattern of [Ca\(^{2+}\)]\(_i\) change, consisting of a rapid initial rise followed by a decline to a sustained plateau (87). This pattern requires initial Ca\(^{2+}\) release from intracellular stores followed by both Ca\(^{2+}\) extrusion from the cell and Ca\(^{2+}\) influx into the cell. Ca\(^{2+}\) extrusion is mediated by Ca\(^{2+}\)-ATPase localized to the apical membrane (37), while Ca\(^{2+}\) influx is thought to be through transient receptor potential (TRP) channels, most likely residing in the basolateral membrane (437), by a mechanism of capacitative or store-mediated Ca\(^{2+}\) entry. Cyclic ADP ribose (545), arachidonic acid (327), and NAADP also modulate Ca\(^{2+}\) signals (88). cAMP-increasing ligands such as VIP and PACAP have been shown to potentiate CCK-induced zymogen granule exocytosis in isolated pancreatic acinar cells, as assessed by enzyme secretion (80, 227) and membrane capacitance measurements (477).

SNARE proteins are also present in pancreatic acinar cells. VAMP-2 is present on the membrane of zymogen granules (186). Three syntaxin isoforms are localized in acinar cells: syntaxin 2 on the apical plasma membrane, syntaxin 4 on the basolateral membrane, and syntaxin 3 on the zymogen granule membrane (184). The finding that botulinum toxin C (BoNT/C) treatment of plasma membrane completely cleaves syntaxin 2 and blocks granule-plasma membrane fusion indicates that syntaxin 2 mediates granule-plasma membrane fusion in pancreatic acinar cells (220). On the other hand, syntaxin 3 is involved in both granule-granule and granule-plasma membrane fusion, as BoNT/C treatment of granules completely cleaves syntaxin 3 and blocks both granule-granule and granule-plasma membrane fusion (184). SNAP-23, a widely expressed homolog of SNAP-25, is found on the basolateral plasma membrane domain, where it has been proposed to play a role in granule-membrane fusion in pancreatitis (187). On the other hand, SNAP-25 is not found in pancreatic acinar cells. Two isoforms of Munc18 (b and c) are present in pancreatic acinar cells. Both Munc18-b and Munc18-c appear to be localized on the basolateral plasma membrane, while Munc18-b is also present on the granule membrane (185). Among Rab3 members, Rab3D is present on the zymogen granule membrane (408). It has been shown in transgenic mice overexpressing Rab3D in pancreatic acinar cells that Rab3D is important in the initial phase of amylase secretion induced by CCK (409). On the other hand, an experiment overexpressing a dominant-negative form of Rab3D in pancreatic acinar cells indicates that Rab3D regulates terminal steps in exocytosis of zymogen granules (105, 409). However, a study of Rab3D knockout mice has suggested that Rab3D is not required for exocytosis of zymogen granules, but rather plays a role in the maintenance of granule maturation (408). Genetic disruption in mice of Noc2, a target of Rab3, Rab27, and Rab8 (180), causes no amylase response to stimuli and a marked accumulation of zymogen granules, suggesting that Noc2 is essential in stimulus-secretion coupling in pancreatic acinar cells (369).

In parotid acinar cells, stimulation of \(\beta\)-adrenergic receptors results in intracellular cAMP accumulation, inducing exocytosis of amylase-containing granules without a rise in [Ca\(^{2+}\)]\(_i\), whereas a rise in [Ca\(^{2+}\)]\(_i\) by activation of \(\alpha\)-adrenergic, cholinergic, or substance P receptors has
only a mild effect on amylase release (25, 436, 532, 606). In perfusion experiments using isolated parotid acinar cells, amylase release induced by stimulation with isoproterenol reaches maximum at ~6 min, followed by a gradual decrease in release (606). On the other hand, both carbachol and substance P cause a transient increase in amylase release (30–60 s), after which release is maintained at a steady-state level (606). Both cAMP signaling and Ca\(^{2+}\) signaling are proposed to act at different steps in amylase release (605). The combination of isoproterenol and substance P evokes biphasic amylase release (a first, large peak followed by a sustained plateau), and the amount of released amylase is greater than that induced by each agonist alone (605).

The basic components involved in exocytosis (including SNARE proteins, Rab, and their related proteins) are also expressed in parotid acinar cells. Syntaxins, except for syntaxin 1, VAMPs (except for VAMP-7), SNAP-23, α-SNAP, and NSF are all expressed (259). The major t-SNARE proteins, syntaxin 1 and SNAP-25, both of which are involved in regulated exocytosis in neuronal, endocrine, and endocrine cells, are not expressed (179, 259). The SNARE-related proteins, synaptotagmins (III, IV, and XI) and Munc18s (1, 2, and 3), are also expressed (259). As cleavage of VAMP-2 by botulinum toxin B in parotid acinar cells inhibits isoproterenol-induced amylase release, SNARE proteins are involved in fusion of granules to plasma membrane in cAMP- as well as Ca\(^{2+}\)-triggered exocytosis (179).

Rab3D and Rab27B are present in parotid acinar cells (260, 443). Introduction of wild type or the dominant active form of Rab3D into permeabilized parotid acinar cells inhibits cAMP and Ca\(^{2+}\)-triggered amylase release (399). However, Rab3D knockout mice show that Rab3D is not essential for regulation of exocytosis in parotid acinar cells, but exerts regulatory effects on granule maturation. Rab27B regulates amylase release in parotid acinar cells through formation of a complex with its effector protein MyRIP/Slac-2, which was identified as a myosin Va/VIIa and actin-binding protein (151, 260). In Noc2 knockout mice, there is also a marked accumulation of secretory granules in salivary glands (571). Thus Rab3, Rab27, and their target proteins participate in exocytosis in parotid acinar cells.

III. INTRACELLULAR cAMP METABOLISM

Ligands such as hormones and neurotransmitters require at least four components to regulate the intracellular cAMP concentration: GPCR, heterotrimeric G protein, adenyl cyclase, and cyclic nucleotide specific phosphodiesterase (PDE). Intracellular cAMP metabolism is determined primarily by activities of adenyl cyclase and PDE. However, the intracellular cAMP concentration ([cAMP]) varies in amplitude, duration, and gradient within the cell in response to the different ligands. In addition, the presence of multiple isoforms of adenyl cyclase and PDE, distinct kinetic and regulatory properties of each isoform, and unique distribution of the various isoforms in the cells all contribute to cAMP compartmentation (see sect. VIII) as well as to great diversity in the cAMP synthesis and degradation processes (see Refs. 114, 117, 128, 157, 250, 536, for recent reviews).

A. Adenylyl Cyclases

Adenylyl cyclase, an enzyme that synthesizes cAMP from ATP (431, 432, 461, 462), comprises a large superfamily (117, 128, 219, 523). Activation of adenylyl cyclase is induced mainly by G\(_{\lambda}\alpha\), an α-subunit of heterotrimeric G proteins. In mammals, nine membrane-bound forms (type I-IX) and one soluble form expressed specifically in sperm have been identified (316, reviewed in Refs. 117, 128, 523). The membrane-bound forms of ~120 kDa share a common structure composed of two cytosolic domains (C1a and C2a), two transmembrane domains (TM1 and TM2). Both C1 and C2 domains have ~230 amino acid regions (designated C1α and C2α, respectively) that share more than 50% similarity and contribute to ATP binding and formation of the catalytic core (598). Types II, IV, VI, VII, and IX are expressed in tissues, while type V is expressed predominantly in heart. Ca\(^{2+}\)/calmodulin (CaM)-regulated isoforms (type I, III, and VIII) are expressed in neurons and nonneuronal secretory cells, including hippocampus, cerebellum, pancreatic acini, and pancreatic islets (546, 568, 574, 591). Ga protein stimulates adenylyl cyclase activities of all isoforms, while G\(_{\lambda}\alpha\), G\(_{\beta}\gamma\), Ca\(^{2+}\)/CaM, PKA, PKC, and Ca\(^{2+}\)/CaM-dependent kinase (CaMK) regulate the activities in an isoform-specific manner. Ga protein inhibits activities of types V, VI, and VIII (539). G\(_{\beta}\gamma\) subunits negatively regulate activity of type I and positively regulate activities of types IV and VII (188, 534, 610). Ca\(^{2+}\)/CaM stimulates activities of types I and VIII by binding to the CaM-binding site in the catalytic core (212, 339, 565). Mice lacking type I or VIII exhibit defects in synaptic plasticity, including LTP, and in long-term memory formation (1, 478, 574, 589, 590). Types I and VIII are activated by Ca\(^{2+}\)/CaM at the EC\(_{50}\) values of 150–200 and 800 nM, respectively (402). Ca\(^{2+}\)/CaM also inhibits activities of types I, III, and IX via CaMK IV (575), CaMK II (578), and calcineurin (421), respectively. At physiological submicromolar concentrations, Ca\(^{2+}\) directly inhibits activities of types V and VI (485, 608). Ca\(^{2+}\) also stimulates activities of type V via its phosphorylation by PKCα (295). Other intracellular messengers involved in regulation of adenyl cyclase activity include DAG and cAMP. A DAG analog, phorbol ester, stimulates activities of types I, II, III, and VII by phosphor-
ylation via PKC (110, 149, 266, 359, 507, 609). cAMP inhibits activities of types V and VI by phosphorylation via PKA (106, 265). Thus feedback inhibition of adenyl cyclase by PKA phosphorylation may be involved in ligand-induced desensitization.

IV. EFFECTORS OF cAMP AND THEIR PROPERTIES

cAMP has been shown to be a universal intracellular messenger that mediates a wide variety of biological responses in almost all tissues in mammals (34). An elevation of [cAMP]i activates several effectors of cAMP, leading to various cellular responses. PKA is the first effector that was characterized and has been studied extensively. cAMP is now known to have several other effectors, including cAMP receptor protein (CRP)/catabolite gene activator protein (CAP) (present in E. coli), CNG channels, HCN channels, and cAMP-GEF/Epac (135, 144, 155, 195, 294, 297, 356, 473, 538, 541, 624).

B. Phosphodiesterases

Cyclic nucleotide-specific PDEs are enzymes that hydrolyze the phosphodiester bond of cyclic nucleotides, thus playing an important role in the regulation of intracellular cyclic nucleotide levels (114, 157, 510). There are 25 PDE genes in mammals that encode a large number of isoenzymes, resulting in more than 50 different PDE proteins (114, 157, 439). This large superfamily of PDEs is subdivided into 11 distinct families based on structure, regulation, and kinetic properties. PDE families also are distinguished functionally by their unique pharmacological inhibitors (157, 298, 373, 556). They share common structural features, having targeting domains and regulatory domains in the NH2-terminal region and a conserved catalytic domain consisting of 270–300 amino acids, usually located toward the COOH-terminal half of the protein. Among PDE isoforms, PDE1C, PDE3A and B, PDE4 (A, B, C, and D), PDE7A and B, PDE8A and B, PDE10A, and PDE11A are specific for cAMP (167, 223, 249, 378, 379, 438, 439, 509, 550, 597). The Michaelis constant (Km) of these PDEs for cAMP is 0.06–4.00 μM. PDE4s, PDE7s, PDE8s, and PDE10A are expressed in the brain (167, 249, 379, 509, 550, 597). PDE1C, PDE3B, PDE4s, and PDE8A are expressed in a pancreatic β-cell line and islets (2, 439, 494, 597).

PDE1s contain a Ca2+/CaM-binding site. Activities of PDE1s are strongly enhanced by Ca2+/CaM (99, 597). Activities of PDE4 are enhanced by its PKA-mediated phosphorylation in TSH-responsive thyroid, vascular smooth muscle, and lymphocytic cell lines (5, 150, 352, 493). Phosphorylation of PDE4D3 and PDE4D5 is known to contribute to cAMP homeostasis and cAMP signaling at a specialized region in the cell through their interaction with the complex of the A-kinase anchoring protein (AKAP) and PKA (115, 250, 411). cAMP-specific PDEs are involved in regulation of central nervous system activities, including hormone secretion, inflammatory response, and fertility and growth of mice (270, 407, 439, 550).

IV. EFFECTORS OF cAMP AND THEIR PROPERTIES

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A. Structure of cAMP-Binding Proteins

cAMP-binding proteins share a common cyclic nucleotide monophosphate (cNMP)-binding domain (Fig. 3) consisting of a stretch of ~120 amino acids. The three-dimensional structure of CRP/CAP has been determined by X-ray crystallography (375) and may be a model of other cAMP-binding proteins. The cAMP-binding domain of CRP/CAP comprises three α-helices (A, B, and C) and an eight β-stranded anti-parallel β-barrel structure (β1 to β8). cNMP-binding occurs in a phosphate-binding cassette formed by the COOH-terminal α-helices and the β-barrel (375) (Fig. 4). Cyclic nucleotide binds to this domain through a network of polar and nonpolar interactions. In addition to the cAMP-binding domain, these cAMP-binding proteins have other functional domains (Fig. 4). Binding of cAMP induces activation of these functional domains in CRP/CAP and cAMP-GEF/Epac (420, 448), and also causes dissociation and activation of the catalytic subunit from the regulatory subunit and the catalytic subunit complex of PKA (271, 538, 541).

B. cAMP-Dependent Protein Kinase

In eukaryotic cells, PKA is the best-characterized protein kinase and has served as a model of the structure and regulation of cAMP-binding proteins as well as of protein kinases (541). Numerous endogenous and exogenous ligands activate PKA by binding to GPCR. PKA is a heterotetramer composed of two regulatory and two catalytic subunits. PKA isoforms were originally identified as type I and type II, which differ in the content of the regulatory subunits (62). cAMP binds cooperatively to the phosphate-binding cassette in the inactive holoenzyme, only the B site is exposed for cAMP binding. Upon stimulation, occupation of the B site by cAMP induces binding of cAMP to the A
site (172). Binding of four cAMP molecules to the inactive R₂C₂ tetramer (two cAMP molecules to each regulatory subunit) leads to a conformational change and dissociation into a regulatory subunit dimer (with four bound cAMP molecules) and two catalytic subunit monomers (314), which in turn become catalytically active (271, 508). PKA type I holoenzymes (RI/H₉₂₅₁₂C₂, RI/H₉₂₅₂₂C₂) have relatively higher affinities for cAMP, while PKA type II holoenzymes (RII/H₉₂₅₁₂C₂, RII/H₉₂₅₂₂C₂) have lower affinities. The presence of PKA isozymes having distinct biochemical properties and tissue distributions is the basis for the functional diversity and specificity of the effects of PKA.

PKA activities are modulated by the expression level and localization of the regulatory or catalytic subunit (94, 248). Intracellular targeting and compartmentation of PKA is determined mainly by association with AKAPs, a family of structurally related proteins consisting of more than 50 members (537). AKAPs target PKA to specific substrates and specialized subcellular compartments (31, 113, 537). AKAPs also serve as scaffolding proteins that assemble PKA with other PKA signaling regulatory proteins such as phosphatases and cAMP-specific PDE. Thus spatial and temporal integration of PKA signaling components as a complex in a particular compartment of a cell is required for the precise regulation of PKA signaling (31, 113, 537).

### C. Cyclic Nucleotide-Gated Channels

CNG channels were first discovered in the plasma membrane of the outer segment of rod photoreceptors in vertebrates, in which they are essential for generation of the primary electrical signal in photoreceptor response to light (163). CNG channels were found later in various tissues including kidney, testis, heart, and brain (68, 579, 601). CNG channels are nonselective cation channels that mediate Ca²⁺ and Na⁺ influx in response to direct binding of intracellular cyclic nucleotides (601). In vertebrates, six members of the CNG channel gene family have been identified (163). Based on sequence similarity, they are classified into two groups: CNGA (A1, A2, A3, and A4) and
CNGB (B1 and B3) (56, 294). The pore-forming CNGA subunits form functional channels by themselves, while the CNGB subunits do not (351, 560). However, CNGA4 does not form a functional channel by itself, and functions as a modulator of olfactory CNG channels (50, 57, 138, 430). CNGA1 and CNGB1 are subunits of rod channels (616, 617), while CNGA3 and CNGB3 are subunits of cone channels (49, 201). The cNMP-binding domain located in the COOH-terminal region regulates activities of these channels (169, 272). CNG channels exhibit a high degree of cyclic nucleotide specificity. CNGA1 channels have the highest, intermediate, and lowest affinity for cGMP, cIMP, and cAMP, respectively, while CNGA2 channels have higher and lower affinity for cGMP and cAMP, respectively. Native olfactory CNG channels and heteromeric channels composed of CNGA2 and CNGA4 have similar affinities for both cAMP and cGMP (9, 57, 397, 495). Olfactory sensory neurons express a variety of GPCRs that activate the G protein G_{olf}, which is similar to the stimulatory G_{s} protein. Activated G_{olf} then increases the activity of adenyl cyclase type III, leading to cAMP production. The resultant increase in cAMP concentration opens the CNG channels (174, 320, 397), allowing influx of Ca^{2+}/H^{+} and Na^{+}, which depolarizes the neuron and causes outward Cl^{-}/H^{+} flux (307, 320, 355). In addition to the electrical excitation of neurons, influx of Ca^{2+} through CNG channels induces hormone release (563) and protein phosphorylation in presynaptic terminals (389).

Recently, other members of the CNG channel superfamily, called HCN channels, have been identified. HCN channels (I_{h}) play a role in initiation and modulation of cardiac and neuronal pacemaker depolarization. In vertebrates, the HCN channel family comprises four members (HCN1–4) (195, 293, 356, 473, 474), all of which are expressed in the brain and three of which (HCN1, HCN2, and HCN4) are also expressed in the heart. HCN channels

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**Figure 4.** Alignment of cNMP-binding domains. A: secondary structure of PKA R1α and CRP/CAP are indicated according to their crystal structure: α, α-helix; β, β-barrel. Phosphate-binding cassette is indicated by red letter. [Modified from Su et al. (519).] B: amino acid sequence of phosphate-binding cassette and hinge region from various cNMP-binding domains. Conserved amino acid residues are shown with black background. Leucine (L) and phenylalanine (F) (indicated by dot) in the phosphate-binding cassette and hinge region, respectively, are proposed to play an important role in conformational change upon cNMP binding (448). cAMP-binding domain A of rat PKA R1α (accession no. M17068), cAMP-binding domain A of rat PKA R1α (J02934), cAMP-binding domain B of rat PKA R1α (M17068), cAMP-binding domain B of rat PKA R1α (J02934), cAMP-binding domain of rat cAMP-GEFII (AADD1279), cAMP-binding domain A of mouse cAMP-GEFII (AB021132), cAMP-binding domain B of mouse cAMP-GEFII (AB021132), cAMP-binding domain of rat CNGB1a (CA04133), cAMP-binding domain of rat HCN1 (AF028737), and cAMP-binding domain A of mouse PKG IB (AADD16044).
are similar to CNG channels in many respects. HCN channels have six transmembrane regions and a cyclic nucleotide-binding domain in the intracellular COOH-terminal region. HCN channels function as homotetramer or heterotetramer and are permeable to both Na\(^+\) and K\(^+\). Opening of HCN channels occurs in response to membrane hyperpolarization rather than depolarization. HCN channels are regulated by various neurotransmitters including norepinephrine and ACh (417). cAMP enhances the intrinsic rate of firing by a positive shift in the voltage dependence of \(I_h\) activation through direct binding (48). cGMP, which has 10–100 times lower affinity than cAMP for the cNMP-binding domain of HCN channels, modulates HCN channel activity only at very high concentrations (356). cAMP also modulates HCN channel activity via a PKA-dependent mechanism (97).

### D. cAMP-GEF/Epac

A family of novel cAMP-binding proteins, cAMP-GEF/Epac, has been discovered recently (134, 135, 297). cAMP-GEF/Epac consists of four functional domains: cAMP-binding domains, a DEP (Dishevelled, Egl-10, and Pleckstrin) domain responsible for its localization to the plasma membrane, a REM (Ras exchange motif) domain required for stabilizing GEF (guanine nucleotide exchange factor) activity, and the GEF domain, which exerts GEF activity toward the Ras-like small GTP-binding proteins Rap1 and Rap2 (134, 135, 297). The GTP-bound forms of Rap interact specifically with their effector proteins and activate downstream targets in various cells (54, 89, 517, 529, 566, 603). There are two isoforms of cAMP-GEF/Epac, cAMP-GEFII/Epac1 and cAMP-GEFII/Epac2, which are coded by different genes (557; http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l/H11005, http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l/H11001). cAMP-GEFII/Epac is present in a range of 5.0–24.6 nM (75, 319, 457). Accordingly, cAMP-GEFII/Epac1 mRNA is ubiquitously expressed, at high levels in adult tissues containing thyroid, kidney, ovary, skeletal muscle, and heart, and at low levels in the brain (135, 297, 415). cAMP-GEFII/Epac2 mRNA is predominant in the brain and neuroendocrine and endocrine tissues. A short form of cAMP-GEFII/Epac2 protein lacking the first cAMP-binding domain and DEP domain is expressed specifically in the liver (626). cAMP-GEFII/Epac1 mRNA is expressed widely but at low levels in the adult brain, while it is expressed at high levels in septum and thalamus of the neonatal brain (297). On the other hand, cAMP-GEFII/Epac2 mRNA is expressed at high levels in the cerebral cortex, hippocampus, cerebellum, olfactory bulb, thalamus, habenula, and pituitary (297, 415). The significance of signal transduction mediated by cAMP-GEFII/Epac has just begun to emerge. cAMP-GEF has been suggested to play roles in many PKA-independent processes in eukaryotes, including cell proliferation, cell adhesion, apoptosis, and secretion (111, 121, 160, 182, 324, 330, 340, 363, 444, 482, 547). The role of cAMP-GEF/Epac in exocytosis is discussed in section VII.

### E. CRP/CAP

CRP/CAP has been found in a variety of prokaryotes including both eubacteria and archaeabacteria and regulates transcription of >150 genes (82, 127, 453). CRP/CAP is a 45-kDa dimer composed of two identical subunits (313). The large NH\(_2\)-terminal domain (amino acid residues 1–139) is responsible for both dimerization of CRP/CAP and binding to cAMP. cAMP binding induces an allosteric conformational change of CRP/CAP, resulting in binding of the small COOH-terminal DNA-binding domain to a specific DNA sequence and transcriptional activation of RNA polymerase. Both the cAMP- and DNA-binding domains show similarities to the respective domains found in a variety of other proteins from prokaryotes to eukaryotes. The cAMP-binding domain of CRP/CAP has sequence and structural similarities to the regulatory subunit of PKA (519, 576, 577), the cNMP-binding domain of CNG (396), and the cAMP-binding domain of cAMP-GEF/Epac (134, 135, 297, 448). Thus the structure of CRP/CAP has been a useful model for studies of both cyclic nucleotide sensitivity and the mechanism of activation by cyclic nucleotides (206, 613).
V. PHARMACOLOGICAL AGENTS TARGETING THE cAMP PATHWAY

To clarify the physiological roles of the cAMP signal, cAMP-binding protein-specific pharmacological agents are useful. Based on analysis of the cAMP-binding properties of PKA regulatory subunits and CRP/CAP, various cAMP analogs have been developed (489). These cAMP analogs are generally cAMP-specific, PDE-resistant, and membrane-permeable compounds and bind to PKA and cAMP-GEF/Epac selectively or nonselectively and modulate their activities positively or negatively. Modulators of the catalytic subunits of PKA have also been developed (109, 132, 289, 540). Agents that modulate cAMP production and degradation are also useful for analysis of cAMP signaling. Readers are referred to recent reviews of these agents (136, 148, 157, 407, 535).

A. cAMP Analogs

On the basis of site-directed mutagenesis and structural analysis, cAMP has been shown to interact with amino acid residues in a highly conserved phosphate-binding cassette motif \[\text{GELAL(X)}_{3-5}\text{PR(A/T)A(T/S)}\] in the loop linking \(\beta 6\) and \(\beta 7\) of the PKA regulatory subunit and cAMP-GEF\text{II}/Epac2 (448, 519) (Fig. 4). The phosphate and ribose rings of cAMP interact with the binding cassette through a network of hydrogen bonds and electrostatic interactions. The interaction of the adenine ring of cAMP with the binding cassette occurs in and near \(\alpha\)-helix C through hydrophobic and stacking binding. Glu\textsuperscript{202} in domain A of the PKA regulatory subunit and Glu\textsuperscript{326} in domain B interact with 2'-OH of ribose. Glu in the phosphate-binding cassette is highly conserved among PKA, CNG, HCN, and CAP (294, 356, 519). Lys\textsuperscript{423} in domain B of cAMP-GEF\text{II}/Epac2 is a cAMP-GEF\text{II}/Epac2-specific residue and is thought not to participate in the interaction with 2'-OH of ribose (448). Arg and the first Ala in the PRAA(S/T) motif of the PKA regulatory subunit and the cAMP-GEF\text{II}/Epac2 domain B interact with cAMP (Fig. 5) (448, 519). Most cAMP analogs are modified at 6- and 8-positions in adenosine, 2'-position in the ribose ring, and the cyclophosphate ring (Fig. 5).

\(\text{Rp-cAMPS}\) and \(\text{Sp-cAMPS}\) are phosphorothioate analogs. Exocyclic oxygen in the equatorial (\(\text{Rp}\)) or the axial (\(\text{Sp}\)) position of the cyclophosphate ring is replaced by

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**FIG. 5.** Chemical structure of cAMP and its analogs. \(A\): structure of cAMP. Positions of adenine ring and ribose ring are numbered. Solid line indicates covalent bond. Dotted line indicates hydrogen bond. \(B\): various cAMP analogs. Modifications at side chains are indicated (see text for details).
exocyclic sulfur (Fig. 5). Rp-cAMPS competitively binds to both cAMP-binding domain A and B of the PKA regulatory subunit and inhibits dissociation of the catalytic subunit from the regulatory subunit. An apparent inhibition constant is found between 0.8 and 8.0 µM (559). In contrast, Sp-cAMPS binds to both domains and induces dissociation of the catalytic subunit. Half-maximal activation of PKA occurs at micromolar concentrations (0.8 –7.0 µM) (559). Both Rp-cAMPS and Sp-cAMPS have a high affinity for the PKA-regulatory subunit and for cAMP-GEF/Eapc (11). Rp-cAMPS inhibits cAMP-GEF/Eapc-induced GEF activity toward Rap1 (450). Sp-cAMPS stimulates GEF activity of cAMP-GEF/Eapc (450). These findings indicate that Rp-cAMPS is an inhibitor of both PKA and cAMP-GEF/Eapc, whereas Sp-cAMPS is an activator of both. The 8-position in adenosine of cAMP is often modified to increase membrane permeability of analogs. 8-Bromo-cAMP and 8-pCPT-cAMP, commonly used as PKA activators, bind to both cAMP-GEF/Eapc1 and the PKA-regulatory subunit with a higher affinity than cAMP. 8-pCPT-cAMP especially has a high affinity for cAMP-GEF/Eapc (111). Both 8-bromo-cAMP and 8-pCPT-cAMP activate kinase activity of PKA at 53–65 and 50 nM concentrations, respectively (489). cAMP analogs modified at 8-position activate GEF activity of cAMP-GEF/Eapc (450). Both 6-Bnz-cAMP and 6-Phe-cAMP, which are modified at 6-position, are selective activators of site A of PKA I and II and induce kinase activities. Although both analogs bind to cAMP-GEF/Eapc with higher affinities than cAMP, the effects on GEF activity of cAMP-GEF/Eapc are not known. 2′-O-Me-cAMP, which is modified at 2′-OH position of ribose, has a lower affinity than cAMP for both the PKA regulatory subunit and cAMP-GEF/Eapc (111). However, this analog cannot activate PKA or cAMP-GEF/Eapc1. Sp-p-cAMPS exhibits a high affinity for cAMP-GEF/Eapc1 (Kd 2.2 µM) and a low affinity for the PKA regulatory subunit (Kd 20–30 µM) (156, 450). The effect of 8-pCPT-2′-O-Me-cAMP (10 µM) on type I and II PKA activities is <25% of full activation in vitro (156, 450), while 10 µM 8-pCPT-2′-O-Me-cAMP fully activates cAMP-GEF/Eapc (156). 8-pCPT-2′-O-Me-cAMP also has more than 100-fold higher affinity for cAMP-GEF/Eapc1 than PKA in vivo (156). Thus 8-pCPT-2′-O-Me-cAMP is a useful analog to distinguish PKA and cAMP-GEF/Eapc-mediated effects. In fact, 8-pCPT-2′-O-Me-cAMP has been shown to activate cellular functions in even the presence of the PKA inhibitors H-89 or KT5720 in human pancreatic β-cells and rat clonal β-cells (282) and in the nerve terminal of the calyx of Held (280). Selective inhibitors for cAMP-GEF/Eapc have not yet been developed and would be useful for investigation of the physiological functions of cAMP-GEF/Eapc.

### B. Inhibitors Targeting Catalytic Subunits of PKA

Protein kinase inhibitor (PKI) is a heat-stable PKA specific inhibitor (615). PKI contains a dibasic consensus sequence RRX(S/T)φ (X and φ are any amino acid and hydrophobic amino acid, respectively) that is recognized by the catalytic subunit of PKA. A sequence similar to this pentapeptide is contained in substrates for PKA and an autophosphorylation site in the PKA regulatory subunit (222, 540). PKI binds to both catalytic and regulatory subunits of PKA with similar affinities (<1 nM). Binding of PKI to a free catalytic subunit inhibits kinase activity in a cAMP-independent manner (76, 310). PKI inhibits cAMP-regulated, PKA-dependent exocytosis at 1–20 µM concentrations in adrenal chromaffin cells (312), pancreatic β-cells (451), and parotid acinar cells (533). H-89 and KT5720 bind to the catalytic subunit of PKA in a competitive fashion against ATP and inhibit the kinase activity with Ki values of 48 and 60 nM, respectively (109, 289). Both compounds inhibit cAMP-regulated, PKA-dependent exocytosis at 1.0–10.0 and 1.0–12.5 µM concentrations, respectively (280, 290, 398, 415, 469, 525).

### VI. PKA-DEPENDENT EFFECTS OF cAMP ON REGULATED EXOCYTOSIS

Among the intracellular signals, cAMP serves as an almost universal signal that modulates or regulates exocytosis in various secretory systems. For example, in neurons, cAMP modulates transmitter release in many preparations. In endocrine and neuroendocrine cells, cAMP potentiates hormone secretion. In certain exocrine cells, cAMP is the primary signal for enzyme secretion. PKA phosphorylation of the proteins associated with regulated exocytosis is thought to be a major event in the process of cAMP-regulated exocytosis.

#### A. Neurons

In synapses of invertebrates (such as *Aplysia* and *Drosophila*) to mammals, cAMP plays a critical role in facilitating synaptic transmission and regulating synaptic plasticity associated with learning and memory (102, 104, 141, 472, 619). At the central synapse of *Aplysia*, cAMP generated by the 5-hydroxytryptamine (5-HT)-adenylyl cyclase cascade closes various types of K+ channels, inducing membrane depolarization and opening of the VDCCs, allowing the Ca2+ influx that triggers neurotransmitter release (73, 90, 279, 308). This cAMP effect results in short-term facilitation at central synapses. Protein phosphorylation by PKA is required for 5-HT-induced facilitation in *Aplysia* sensory neurons (85). At the neuromuscular junction of *Drosophila*, cAMP-PKA signaling pro-
duced by tetanic stimulation induces recruitment of synaptic vesicles from the reserve pool, resulting in increased size of the exo/endocycling pool (corresponding to the readily releasable pool in other preparations, Ref. 321). At the Drosophila neuromuscular junction, two modes of vesicle fusion, nerve evoked and spontaneous, are both modulated by cAMP-PKA (604). The former requires neuronal-synaptobrevin (Drosophila homolog of synaptobrevin) and is modulated by cAMP/PKA independent of Ca\(^{2+}\), while in the latter mode, vesicle fusion is facilitated by Ca\(^{2+}\) influx enhanced by cAMP-PKA independently of neuronal-synaptobrevin. Thus cAMP-PKA signaling participates in the enhancement of vesicle fusion at multiple levels including recruitment of synaptic vesicles from the reserve pool to the exo/endocycling pool, enhancement of Ca\(^{2+}\) influx, and modulation of vesicle fusion (322, 604). In cerebellar and hippocampal synapses, cAMP-PKA signaling enhances synaptic transmission by increasing release probability independently of Ca\(^{2+}\) influx (103, 104, 552). In cultured hippocampal neurons, activation of PKA causes synaptic facilitation by directly elevating the release probability of individual vesicles during Ca\(^{2+}\)-induced exocytosis (552). At the mossy fiber synapse in the hippocampal CA3 region, PKA phosphorylates Rab3A effectors, which then mediate glutamate release (353). cAMP-PKA signaling is required in LTP at the hippocampal mossy fibers (254, 581), the cerebellar parallel fibers (472), and the cerebellar granule cell-Purkinje neuron pairs (348). cAMP-PKA signaling is also required in long-term depression (LTD) at the Schaffer collateral in the CA1 region in the hippocampus (58). The importance of PKA in LTP and LTD has been shown directly in studies using various PKA subunit knockout mice (58, 253, 440).

Although a number of the proteins associated with synaptic vesicle exocytosis in neurons can be phosphorylated by PKA in vitro (Table 1), the physiological roles of PKA phosphorylation on neurotransmitter release are largely unknown, so far being clarified in only several proteins including cysteine string proteins (CSP) (159), Snapin (108), Rim1 (354), SNAP-25 (396), syntaphilin (46), and synapsin (7). Phosphorylation of the proteins in these studies was usually performed in synaptosome preparations. In adrenal chromaffin cells and PC12 cells, however, the functional roles of PKA phosphorylation in exocytosis have been examined (46, 159, 396). Because catecholamine-secreting adrenal chromaffin cells and PC12 cells are models of LDCV exocytosis, the findings obtained in these cells might not always reflect SV exocytosis in neurons.

CSP is a ubiquitously expressed vesicle membrane protein originally discovered in Drosophila (623) and contains a “cysteine string,” a motif of 14 cysteine residues within a 20-amino acid stretch, which is palmitoylated and targets CSP to secretory vesicles (96). CSP is a member of the Dnaj family of cochaperones that binds Hsc (heat-shock cognate protein of 70 kDa) (61, 95). In vivo, CSP is an important component of the exocytotic machinery, as surviving CSP-null mutants in Drosophila, although the mutation is generally lethal (548), show reduced Ca\(^{2+}\)-triggered neurotransmitter release (126, 401). CSP has been shown to interact with both syntaxin 1 (401) and synaptotagmin I (158). Phosphorylation of serine (Ser10) of CSP by PKA reduces its binding to both syntaxin (159) and synaptotagmin I (158) and is required for prolonged fusion pore opening, as assessed by overexpression of wild-type CSP or nonphosphorylatable mutant CSP in adrenal chromaffin cells (159). PKA phosphorylation of CSP probably modulates the exocytotic machinery by altering its availability for protein-protein interactions (158).

Snapin, identified as a SNAP-25-binding protein in the yeast two-hybrid screen (258), is localized to the synaptic vesicle membrane. The interaction of Snapin with SNAP-25 occurs simultaneously with that of the SNARE complex (258). The PKA-phosphorylation of serine of Snapin (Ser50) significantly increases its binding to SNAP-25 (108). Constitutively phosphorylated Snapin (S50D mutant) mimics this effect and enhances the association of synaptotagmin with the SNARE complex. Overexpression of the S50D mutant of Snapin increases the number of release-ready vesicles in adrenal chromaffin cells, whereas overexpression of the unphosphorylated form (S50A mutant) reduces it. Treatment of rat hippocampal slices with the nonhydrolyzable cAMP analog Sp-5,6-DCI-cBiMPS (BIMPS) induces in vivo phosphorylation of Snapin and enhances interaction of Snapin and synaptotagmin with the SNARE complex. Phosphorylation of Snapin by PKA stabilizes the formed complex during priming, leading to stabilization of the release-ready vesicles (108).

Rim (now called Rim1), an active zone protein, was discovered originally as a putative effector of Rab3A by the yeast two-hybrid screen (570). Rim2, an isoform of Rim1, was later identified in the brain and in neuroendocrine and endocrine tissues (415, 571). In addition to Rab3, Rim1 interacts with Munc13–1 (42), SNAP-25 (119), synaptotagmin (119, 483), α-liprins (483), Rim-binding proteins (233, 571), Ca\(^{2+}\)-liprins (119), and cAMP-GEFII/Epac2 (415). Rim1α plays an important role as a scaffold protein in integrating both active zone proteins and synaptic vesicles into the sites of neurotransmitter release (483). The Rim1-null mutation in C. elegans reduces the number of fusion-competent vesicles despite the normal level of docked vesicles, suggesting a postdocking role in the regulation of synaptic vesicle priming (315). Mossy fiber LTP at the hippocampus and LTP at cerebellar parallel fiber synapses are both abolished in Rim1α knockout mice.
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Site(s)</th>
<th>Effects of PKA Phosphorylation In Vitro</th>
<th>PKA Phosphorylation of the Substrate In Vivo</th>
<th>Effects of PKA Phosphorylation In Vivo</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSP</td>
<td>Ser&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Decrease of the binding to syntaxin 1A and synaptotagmin I and snapin</td>
<td>Positive in 8-Br-cAMP or nicotine-stimulated chromaffin cells</td>
<td>Modification of exocytosis kinetics in chromaffin cells</td>
<td>158, 159</td>
</tr>
<tr>
<td>Synapsin</td>
<td>Ser&lt;sup&gt;164&lt;/sup&gt;</td>
<td>Inhibition of the binding to phospholipid</td>
<td>Positive in norepinephrine and isoproterenol-stimulated dentate slices</td>
<td>No effect on the transmitter release potentiated by PKA</td>
<td>12, 22, 246, 257, 285, 374, 418</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibition of the binding to calspectin</td>
<td>Positive in glycolipid-stimulated brain membrane fraction</td>
<td>Inhibition of granule movement in squid axoplasm</td>
<td></td>
</tr>
<tr>
<td>Aplysia synapsin</td>
<td>Ser&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Decrease of actin bundling activity</td>
<td>Positive in serotonin-stimulated pleural-pedal ganglia</td>
<td>Promotion of neurite outgrowth in Xenopus laevis embryonic neurons</td>
<td>7, 48</td>
</tr>
<tr>
<td>Syntaphilin</td>
<td>Ser&lt;sup&gt;43&lt;/sup&gt;</td>
<td>Decrease of binding to syntaxin 1A</td>
<td>Positive in BIMPS-stimulated synaptosome or syntaphilin-transfected HEK293 cells</td>
<td>Decrease of binding to syntaxin 1A in syntaphilin-transfected HEK293 cells</td>
<td>46</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Thr&lt;sup&gt;138&lt;/sup&gt;</td>
<td>No effect on the binding to syntaxin 1A, VAMP-2, synaptotagmin, and snapin</td>
<td>Positive in KCl-stimulated PC12 cells</td>
<td>Increase of slowly releasable vesicle pool size in PC12 cells</td>
<td>108, 232, 396, 458</td>
</tr>
<tr>
<td>Snapin</td>
<td>Ser&lt;sup&gt;50&lt;/sup&gt;</td>
<td>Increase of the binding to SNAP-25</td>
<td>Positive in BIMPS-stimulated hippocampal slice</td>
<td>Increase of the initial exocytotic burst in chromaffin cells</td>
<td>108</td>
</tr>
<tr>
<td>Rabphilin3</td>
<td>Ser&lt;sup&gt;234&lt;/sup&gt;</td>
<td>Decrease of the affinity for membrane</td>
<td>Positive in forskolin-stimulated synaptosome from hippocampus</td>
<td>ND</td>
<td>170, 183</td>
</tr>
<tr>
<td>Rim1α</td>
<td>Ser&lt;sup&gt;413&lt;/sup&gt;</td>
<td>No effect on known protein-protein interactions</td>
<td>Positive in forskolin-stimulated synaptosome from hippocampal CA3 region</td>
<td>Induction of LTP in cerebellar granule and Purkinje neurons</td>
<td>92, 354</td>
</tr>
<tr>
<td>Syntaxin 4</td>
<td>ND</td>
<td>Disruption of the binding to SNAP-23</td>
<td>ND</td>
<td>ND</td>
<td>171</td>
</tr>
<tr>
<td>α-SNAP</td>
<td>ND</td>
<td>Decrease of the binding to complex formed by syntaxin 1A, VAMP-2, and SNAP-25</td>
<td>ND</td>
<td>ND</td>
<td>159, 236</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Ser&lt;sup&gt;180&lt;/sup&gt;</td>
<td>ND</td>
<td>Positive in forskolin or GLP-1-stimulated, purified β-cells</td>
<td>Decreases of glucose transport activity by GLP-1 in purified β-cells</td>
<td>544</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt; channel</td>
<td>Kir6.2</td>
<td>Ser&lt;sup&gt;272&lt;/sup&gt;</td>
<td>ND</td>
<td>Positive in forskolin/IBMX or dibutyl-cAMP-stimulated Kir6.2-transfected COS-1 cells</td>
<td>Increase of channel activity</td>
</tr>
<tr>
<td>SURI</td>
<td>Ser&lt;sup&gt;1571&lt;/sup&gt;</td>
<td>ND</td>
<td>Positive in forskolin/IBMX or dibutyl-cAMP-stimulated SURI-transfected COS-1 cells</td>
<td>Decrease of burst duration, interburst interval, and open probability</td>
<td>36</td>
</tr>
<tr>
<td>VDCC α&lt;sub&gt;1,2&lt;/sub&gt; Subunit</td>
<td>ND</td>
<td>ND</td>
<td>Positive in BIMPS-induced hippocampal slice</td>
<td>Increase of the number of channel at the cell surface</td>
<td>228</td>
</tr>
<tr>
<td>VDCC α&lt;sub&gt;2,2&lt;/sub&gt; Subunit</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDCC α&lt;sub&gt;2,3&lt;/sub&gt; Subunit</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
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</tr>
</tbody>
</table>
mice (92, 354). Rim1α is phosphorylated by PKA at two sites, one between the NH2-terminal zinc finger and PDZ domain (Ser413) and the other at the COOH terminus (Ser1548). The defect in LTP in Rim1α knockout mice can be rescued by transfection of wild-type Rim1α, whereas mutant Rim1α containing either an Ala or Asp substitution at Ser413 is unable to rescue LTP in Rim1α-deficient neurons (354). Thus phosphorylation of Ser413 of Rim1α by PKA is critical for induction of LTP at cerebellar parallel fiber synapses. PKA phosphorylation of Rim1α is proposed to trigger an overall restructuring of the active zone by recruiting additional components (354).

Syntaphilin was identified as a molecular clamp that controls free syntaxin 1 and dynamin 1 availability, thereby regulating synaptic vesicle exocytosis (328). PKA phosphorylation of Ser43 decreases syntaphilin binding to syntaxin 1A both in vitro and in vivo (46). Mutation of Ser43 to Asp (S43D) in syntaphilin exerts similar effects on the binding. Treatment of rat brain synaptosomes or syntaphilin-transfected HEK293 cells with the cAMP analog BIMPS induces in vivo phosphorylation of Ser43. Overexpression of wild-type syntaphilin in human growth hormone-transfected PC12 cells reduces Ca2⁺-triggered secretion, whereas overexpression of the S43D mutant does not. Thus PKA phosphorylation of syntaphilin might function as an “off-switch” for syntaphilin, blocking its inhibitory effect in a PKA-dependent manner (46).

Other synaptic vesicle-associated proteins such as synapsins and Rabphilin3 can also be substrates for PKA-dependent phosphorylation (183, 234). Synapsins are a family of phosphoproteins specifically associated with the cytoplasmic surface of the synaptic vesicle membrane and regulate neuronal development and synaptic transmission (294). Although synapsins are the most abundant substrates for PKA in adult neurons, they can also be phosphorylated by other protein kinases, including CaMK II (123, 207) and mitogen-activated protein kinase (274). PKA phosphorylation of synapsins recently has been shown to be important for neurite outgrowth (285). In *Aplysia* neurons stimulated with 5-HT, *Aplysia* synapsin is involved in the recruitment of synaptic vesicles in short-term synaptic plasticity through its PKA phosphorylation (7). Phosphorylation of *Aplysia* synapsin is also required for synapsin-induced potentiation of neurotransmitter release (168). Rabphilin3 was identified by its GTP-binding to Rab3A (503) and was initially proposed to be a potential Ca2⁺ sensor on synaptic vesicles in addition to syntaptotagmin (341). However, because Rabphilin3 knockout mice do not exhibit a distinct phenotype, its physiological role in exocytosis is unknown at present (481).

### B. Pituitary Cells

Melanotrophs (pituitary par intermedia cells) secrete a number of peptides derived from posttranslational processing of proopiomelanocortin (POMC), including β-endorphin, α-melanocyte stimulating hormone (α-MSH), and ACTH (364). The Ca2⁺-dependent secretory activity in rat melanotrophs is enhanced by cAMP (506). The cAMP-mediated effect on the secretory activity is not mediated by modulation of [Ca2⁺]i or by increase in the frequency of exocytotic events (506). However, cAMP increases the size of the secretory granules, as assessed by capacitance measurement. In rat melanotrophs, dopamine regulates secretion by inhibiting adenyl cyclase activity (391). Bromocriptine, a dopamine D2 agonist, reduces the size of secretory granules in melanotrophs (290), while haloperidol, a dopamine antagonist, increases the size (21). Thus cAMP might promote hormone secretion by enlarging the size of the secretory granules. However, whether or not PKA activation accounts for all of the

### TABLE 1—Continued

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Site(s)</th>
<th>Effects of PKA Phosphorylation In Vitro</th>
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</tr>
</thead>
<tbody>
<tr>
<td>α1,2-Subunit</td>
<td>Ser478</td>
<td>ND</td>
<td>Positive in PKA catalytic subunit-treated RINm5F cell lysate</td>
<td>ND</td>
<td>190</td>
</tr>
<tr>
<td>α1,2-Subunit</td>
<td>Ser428</td>
<td>ND</td>
<td>Positive in forskolin and okadaic acid-stimulated HEK293 cells expressing α1,2-subunit</td>
<td>Loss of channels regulation in α1,2-subunit lacking phosphorylation site-transfected HEK293 cells</td>
<td>77</td>
</tr>
<tr>
<td>β2α-Subunit</td>
<td>Ser478</td>
<td>ND</td>
<td>ND</td>
<td>Requirement for PKA-mediated stimulation of Ca2⁺ current</td>
<td>467</td>
</tr>
<tr>
<td>β2α-Subunit</td>
<td>Ser479</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

* In mammals, synapsin I and III are phosphorylated at Ser9, while synapsin II is phosphorylated at Ser10. CSP, cysteine string protein; SNAP-25, 25-kDa synaptosomal-associated protein; YAP-2, vesicle-associated membrane protein-2; 8-Br-cAMP, 8-bromo-cAMP; IBMX, 3-isobutyl-1-methylxanthine; HEK293 cell, human embryonic kidney 293 cell; GH, growth hormone; α-SNAP, α-soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; LTP, long-term potentiation; Rim, Rab3-interacting molecule; VDCC, voltage-dependent Ca2⁺ channel; GLUT2, glucose transporter 2; BIMPS, Sp-5,6-DCI-cBIMPS; GLP-1, glucagon-like peptide-1; KATP channel, ATP-sensitive K⁺ channel; SUR, sulfonylurea receptor; ND, not determined.
effect of cAMP on secretory granule size remains to be clarified.

In corticotrophs, corticotropin-releasing hormone (CRH) is a major stimulatory regulator of ACTH secretion. CRH causes membrane depolarization, increases cell firing frequency, and evokes a large oscillatory increase in $[Ca^{2+}]_i$. The CRH-induced oscillatory increase in $[Ca^{2+}]_i$ is critically dependent on PKA activation, whereas the CRH-evoked membrane depolarization is only slightly blocked by a PKA inhibitor, H-89 (323), suggesting that the effect of CRH on membrane depolarization is mediated independently of cAMP-PKA signaling. The signaling process responsible for the cAMP-independent component of the CRH response is not known. In a corticotroph, cAMP signaling is proposed to open the VDCCs, probably through activation of nonselective cation channels and subsequent membrane depolarization, with resultant Ca$^{2+}$ entry that triggers ACTH secretion (357, 530). The direct targets of PKA in this process are not known, however.

In prolactin-secreting lactotrophs, secretory granule-to-granule (homotypic) fusion and granule-to-plasma membrane (heterotypic) fusion events both occur (compound exocytosis) during Ca$^{2+}$-triggered exocytosis (8, 176). Compound exocytosis is a relatively common mode of exocytosis found in hematopoietic cells such as eosinophils and mast cells (229, 332, 376). Both VIP activation of exocytosis found in hematopoetic cells such as eosinophils and mast cells (229, 332, 376). Both VIP activation of exocytosis found in hematopoetic cells such as eosinophils and mast cells (229, 332, 376).

C. Adrenal Chromaffin Cells

In adrenal chromaffin cells, agonists that elevate the intracellular cAMP concentration, including adenosine receptors (107), VIP receptors (586), GABA, receptors (413), and PACAP receptors (434), stimulate catecholamine secretion (see Ref. 534 for review). As forskolin and a nonhydrolyzable cAMP analog, pClp-cAMP, increase $[Ca^{2+}]_i$, in a dose-dependent manner, cAMP-stimulated catecholamine secretion is Ca$^{2+}$ dependent. This increase is mainly due to Ca$^{2+}$ entry through VDCCs, as treatment of the cells with cAMP in the absence of extracellular Ca$^{2+}$ significantly reduces increases in $[Ca^{2+}]_i$, and nifedipine, an L-type VDCC blocker, also inhibits increases in $[Ca^{2+}]_i$ (419). Inhibition of PKA markedly blocks PACAP-induced exocytosis (434). However, high concentrations of cAMP do not produce a significant increase in catecholamine secretion (419). Infusion of cAMP or an adenylyl cyclase activator, forskolin, into chromaffin cells also has no effect on secretion in capacitance or amperometric current measurement (396), while infusion of PKA inhibitors reduces the size of the releasable vesicle pools in chromaffin cells (396). Accordingly, constitutively activated PKA in the basal state is essential for maintaining the size of the releasable vesicle pools in chromaffin cells.

The molecular basis of modulation of catecholamine secretion by cAMP is still unknown. Among the secretory vesicle-associated proteins present in adrenal chromaffin cells, SNAP-25 has been well characterized in terms of PKA phosphorylation (108, 232, 396, 458). SNAP-25 is phosphorylated by PKA at Thr$^{138}$ in vitro (232) and is phosphorylated in native chromaffin cells (396). Constitutive phosphorylation of SNAP-25 at Thr$^{138}$ by PKA is required in the regulation of the size of the two primed vesicle pools, the slowly releasable pool and the readily releasable pool (396).

D. Pancreatic $\beta$-Cells

In pancreatic $\beta$-cells, intracellular cAMP signals generated by nutrient secretagogues are critical in regulating insulin secretion (226, 230, 231, 365, 496, 524). The role of the cAMP signaling system in exocytosis has been best characterized in pancreatic $\beta$-cells. Various hormones and neurotransmitters, including GLP-1 (145, 162, 303, 543), GIP (71, 145, 162), VIP, and PACAP, potentiate insulin secretion by generating cAMP in pancreatic $\beta$-cells. Eight adenyl cyclase isoforms (type I–VIII) are expressed in pancreatic islets and $\beta$-cell lines (131, 213). PDE1C, PDE3B, PDE4A, PDE4D, and PDE10A are also expressed in pancreatic islets and $\beta$-cell lines. Treatment of pancreatic islets with inhibitors specific for PDE1C enhances the potentiation of insulin secretion by cAMP (218). cAMP-regulated potentiation of insulin secretion is affected by treatment with an adenyl cyclase activator, forskolin, a PKA inhibitor, MDL-12330 [cis-N-(2-phenylcyclopentil)-azacyclotridec-1en-2-amine monohydrochloride], and trimetric G protein regulators such as cholera toxin (CTX) and PTX (143, 291, 366, 584). Potentiation is also regulated by PDE inhibitors and PDE-resistant cAMP analogs that change the cAMP levels in pancreatic islets (143, 439).

CAMP-increasing ligands potentiate both the first and second phases of glucose-induced insulin secretion (527, 584). In low glucose concentrations (1.0–2.8 mM) that do not induce a rise in Ca$^{2+}$, treatment of pancreatic $\beta$-cells with cAMP alone has little or no effect on insulin secretion (100, 101). However, a combination of cAMP and high glucose induces the potentiation of insulin secretion (4, 209, 251, 287). This indicates that the potentiating effect of cAMP on insulin secretion requires interaction be-
tween cAMP and the Ca\(^{2+}\) signal. The cAMP levels in β-cells are regulated partly by Ca\(^{2+}\) signals in synthesis and degradation steps. For example, adenyl cyclase type I and type VIII, which are activated by Ca\(^{2+}\)/CaM upon Ca\(^{2+}\) entry induced by glucose, increase cAMP production in pancreatic islets (131). On the other hand, PDE1C hydrolyzes cAMP in a Ca\(^{2+}\)/CaM-dependent manner in pancreatic islets (218). Thus under high Ca\(^{2+}\) concentrations, these enzymes might contribute to the regulation of insulin secretion by controlling cAMP levels in pancreatic β-cells. cAMP is now known to potentiate insulin secretion by both PKA-dependent and PKA-independent mechanisms (see sects. vi and vii).

Plasma insulin levels are higher when glucose is administrated orally rather than intravenously (124, 425). Gastrointestinal hormones mediating this effect are called incretins and include GLP-1 and GIP. GLP-1 and GIP are released into the bloodstream in response to ingestion of nutrients (153), from gastrointestinal endocrine L-cells and K-cells, respectively. Among the natural ligands that increase cAMP via GPCR in pancreatic β-cells, GLP-1 is well characterized (211). Glucose-induced insulin secretion is potentiated by GLP-1, the process involving multiple events including inhibition of K\(_{ATP}\) channels (245, 398, 501), Ca\(^{2+}\) influx through VDCCs (6, 66, 592), Ca\(^{2+}\) mobilization from ryanodine-sensitive Ca\(^{2+}\) stores (210, 244), and activation of Ca\(^{2+}\)-activated nonslective cation channels (333). cAMP enhances translocation of granules and increases the size of the readily releasable pool and the rate of its replenishment (237, 533). The effects of GLP-1 on exocytosis may be associated with these steps (211, 302, 361).

In pancreatic β-cells, glucose is transported through a high-\(K_m\) (17–18 mM) glucose transporter, GLUT2. GLUT2 is maximally phosphorylated in pancreatic islets in the basal state, but can be phosphorylated by GLP-1 (Ser\(^{189}\), Ser\(^{501/503}\), and Thr\(^{510}\)). Phosphorylation of GLUT2 by GLP-1 decreases glucose transport activity in purified β-cells (544). GLP-1 inhibits K\(_{ATP}\) channel activity, which is restored by a PKA specific blocker, Rp-cAMPS, indicating that PKA phosphorylation decreases K\(_{ATP}\) channel activity (209, 245). Phosphorylation of K\(_{ATP}\) channels by PKA was originally shown in RINm5F and HIT cells (455). Pancreatic β-cell K\(_{ATP}\) channels are composed of the pore-forming subunit Kir6.2 and the regulatory subunit SUR1 (491). Both subunits are phosphorylated by PKA, at Ser\(^{272}\) and Ser\(^{1571}\), respectively (36). Using a T224D Kir6.2 mutant, it has also been suggested that phosphorylation of Thr\(^{234}\) by PKA increases the activity of K\(_{ATP}\) channels (347). On the other hand, phosphorylation of SUR1 is responsible for basal channel properties including burst and cluster durations, interburst intervals, and open probabilities, and also increases the number of functional channels at the cell surface (36). The physiological significance of PKA phosphorylation of the K\(_{ATP}\) channel is less certain. In theory, phosphorylation of the channels by PKA inhibits insulin secretion, while activation of the cAMP-PKA signal enhances insulin secretion. The K\(_{ATP}\) channel could be maximally phosphorylated at the basal state so that PKA-mediated phosphorylation of the K\(_{ATP}\) channel cannot participate in the process of insulin secretion.

GLP-1 and other cAMP-increasing agents increase activity of L-type VDCCs via PKA (66, 284), enhancing Ca\(^{2+}\) influx and potentiating insulin granule exocytosis (6). The α\(_1.2\)-subunit of VDCCs is phosphorylated by PKA in β-cells lines (336, 467). Both the α\(_1.2\)-subunit and the β\(_{2a}\)-subunit are substrates of PKA phosphorylation (130, 320, 423). Phosphorylation of Ser\(^{1928}\) of the α\(_1.2\)-subunit and both Ser\(^{478}\) and Ser\(^{479}\) of the β\(_{2a}\)-subunit by PKA activates the VDCCs (77, 190). Thus, in pancreatic β-cells, the activity of the VDCCs, which include an α\(_1.2\)-subunit, may be regulated by cAMP-PKA signaling. Although an α\(_1.3\)-subunit also is expressed in pancreatic β-cells (492), whether or not this α\(_1.3\)-subunit can be phosphorylated by PKA is not known. GLP-1 also increases membrane Na\(^+\) permeability by elevating cAMP in hamster β-cells (385). A PKA inhibitor, H-89, partially inhibits the GLP-1-induced rise in [Na\(^+\)]\(_i\) and the 8-pCPT-2′-O-Me-cAMP analog increases [Na\(^+\)]\(_i\) and insulin secretion, suggesting that membrane Na\(^+\) permeability is modulated by both PKA-dependent and PKA-independent mechanisms (385) (see sects. vi and vii).

VIP and PACAP released at parasympathetic nerve terminals in pancreatic islets are known to modulate insulin secretion by cAMP-PKA. PAC1 and VPAC2 are receptors for PACAP and VIP in pancreatic islets and pancreatic β-cell lines (165, 261, 593). PAC1 is specific for PACAP, while VPAC2 binds both VIP and PACAP (164). Both PACAP and VIP potentiate insulin secretion in a glucose-dependent manner (296, 304, 479). Similarly to GLP-1, PACAP potentiates Ca\(^{2+}\) influx and exocytosis through cAMP-PKA signaling (309, 464). The effects of PACAP have also been suggested to increase Na\(^+\) influx (165). Both PACAP and VIP potentiate the acute phase of insulin secretion through cAMP-PKA signaling, while they potentiate the sustained phase of insulin secretion through phosphoinositide 3-kinase-dependent signaling (567).

In addition to modulation of ion channel and transporter activities by cAMP, direct effects of cAMP on exocytotic processes have been shown in cAMP-regulated insulin secretion. With the use of capacitance measurements, cAMP has been shown to increase the size of the readily releasable pool, which contains a small fraction of total insulin granules at the release sites of pancreatic β-cells (4–8%) (463). The PKA-independent mechanism is involved in this effect (152, 451) (see sect. vii). cAMP also promotes insulin granule mobilization (237). Because granule mobilization is enhanced by treatment with oka-
daic acid, an inhibitor of protein phosphatase 1 and 2A, in β-cell line HIT cells, phosphorylation of the exocytotic machinery by kinases such as PKA and CaMK may participate in the enhancement of insulin granule mobilization.

With the use of amperometric measurements in isolated pancreatic β-cells, high concentrations of ATP without cAMP-increasing agents have been shown to directly enhance the fast mode (mode 1, ~2.5 s after initiation of the exocytotic event) (526) of exocytosis of insulin granules (287, 525). The direct effect of ATP on exocytosis is blocked by PKA inhibitors. In addition, an adenylyl cyclase inhibitor, MDL-12330, blocked the effect of ATP. This effect was overcome by addition of exogenous cAMP. Thus the action of ATP in mode 1 depends on PKA-mediated phosphorylation of the proteins associated with exocytosis.

In pancreatic β-cells, both type I and type II forms of PKA are expressed. Association of type II (RIIα and RIIβ) with AKAP has been shown in pancreatic islets (338). Inhibition of AKAP activities by AKAP peptides, which block interaction of AKAP with PKA, disrupts subcellular localization of PKA and reduces insulin secretion (338). Subcellular localization of PKA is required for GLP-1-potentiated insulin secretion, although specific localization does not affect glucose-induced insulin secretion. An AKAP isoform, AKAP18, augments cAMP-dependent modulation of L-type VDCC currents (173). For this effect, localization of AKAP18 in the plasma membrane is required. Overexpression of wild-type AKAP18 in an insulin-secreting cell line, RINm5F, enhances GLP-1-potentiated insulin secretion, whereas a mutant AKAP18 that is not targeted to the plasma membrane does not enhance GLP-1 potentiation. Thus recruitment of PKA to the plasma membrane may induce phosphorylation of proteins associated with stimulus-secretion coupling and thereby promote insulin granule exocytosis. Because PKA and protein phosphatase 2B (PP2B) are involved in the maintenance of the phosphorylation/dephosphorylation process in pancreatic β-cell proteins associated with insulin secretion, insulin secretion may be regulated by PKA and PP2B targeted to AKAP (337).

E. Exocrine Acinar Cells

In pancreatic acinar cells, both VIP and secretin potentiate CCK-induced amylase release in a dose-dependent manner (80). The effects of these peptides are mimicked by cAMP-increasing agents including forskolin, 3-isobutyl-1-methylxanthine (IBMX), and 8-bromo-cAMP. VIP or secretin alone, either of which is a β-adrenergic agonist that increases [cAMP], does not stimulate amylase release, but in combination with CCK, carbachol, or the Ca²⁺ ionophore A23187, all of which increase [Ca²⁺]i, amylase release is stimulated, suggesting that VIP and secretin potentiate amylase release in concert with an elevation of [Ca²⁺]i (80, 227). With the use of capacitance measurements in pancreatic acinar cells, it has recently been suggested that cAMP alone promotes fusion of granules independently of Ca²⁺ and that this process can be controlled by a small G protein, presumably Rab3 (477). In pancreatic duct cells, ATP at high concentrations modulates exocytosis of secretory granules containing mucin, as assessed by carbon fiber amperometry. This modulation is mediated by purinergic (P2Y) receptors, which increase cAMP and [Ca²⁺]i (275). In general, mucin secretion is regulated by cAMP acting through PKA, by DAG acting through PKC, and by Ca²⁺ (235).

In most secretory cells, the amount of substance released by cAMP alone is less than that released by [Ca²⁺]i, but this is not the case in parotid acinar cells (177, 605). In the acinar cells of parotid glands, cAMP is the principal signal for exocytosis of amylase granules, which occurs without an elevation of Ca²⁺ (25, 533). β-Adrenergic agonists stimulate amylase secretion from parotid acinar cells by accumulation of intracellular cAMP (83, 436, 607). The amount of amylase secretion induced by an increase in [Ca²⁺]i is less than that induced by cAMP (442), although interaction between the cAMP and Ca²⁺ signaling systems potentiates amylase secretion (605). In parotid acinar cells, VAMP-2 is not detected in the immunoprecipitate from solubilized granule fraction in the absence of cAMP, but incubation of the cytosolic fraction with cAMP causes immunoprecipitation of VAMP-2, the effect of which is reduced by addition of a PKA inhibitor, H-89 (178). Thus it is proposed that upon PKA activation, an unidentified protein (protein X) that interacts with VAMP-2 may dissociate from VAMP-2, and thereby induce formation of the SNARE complex required for exocytosis in parotid acinar cells (178). However, whether or not cAMP-induced amylase secretion in parotid acinar cells depends solely on PKA activation is still unknown. A β-adrenergic agonist, isoproterenol, translocates Rap1 to cytoplasm in parotid acinar cells, and this translocation of Rap1 parallels amylase release (146). It is possible that cAMP-GEF/Epac, which has GEF activities toward Rap1, is involved in the PKA-independent mechanism of amylase release in parotid acinar cells (see sect. vii).

VII. PKA-INDEPENDENT EFFECTS OF cAMP ON REGULATED EXOCYTOSIS

To investigate the involvement of PKA-dependent effects in cAMP-regulated exocytosis, various PKA specific inhibitors are widely used. However, PKA inhibitors seem to be only partially effective in most secretory cells and are ineffective in some types of cells and preparations. cAMP-gated cation channels have been proposed to
participate in PKA-independent neurotransmission in certain neurons and endocrine cells (618). Accumulating data suggest that the newly discovered cAMP-GEF/Epac mediates cAMP-regulated, PKA-independent exocytosis (152, 175, 282, 290, 415). Studies of insulin granule exocytosis in pancreatic β-cells have led the way in clarifying the molecular mechanism of cAMP-GEF/Epac-mediated exocytosis, and the involvement of cAMP-GEF/Epac in cAMP-regulated, PKA-independent exocytosis in neurons has been revealed (280, 470).

A. Pancreatic β-Cells

As described in section vi, cAMP-increasing ligands potentiate glucose-induced insulin secretion. Previously, cAMP had been thought to act through PKA (387). However, in 1997, using capacitance measurements of pancreatic β-cells, it was found that cAMP stimulates exocytosis of insulin granules from a readily releasable pool and that this effect is unaffected by PKA inhibition (451), suggesting for the first time that cAMP potentiates Ca$^{2+}$-triggered exocytosis in a PKA-independent manner (451). The direct target of cAMP in the exocytotic machinery and the PKA-independent mechanism by which cAMP potentiates insulin granule exocytosis were not known at that time. In the search for a molecule coupling directly to the sulfonylurea receptor SUR1, the regulatory subunit of the pancreatic β-cell K$_{ATP}$ channel, the cAMP-binding protein CAMPS (cAMP sensor) was identified in insulin-secreting MIN6 cells by the yeast two-hybrid screen (451). CAMPS was found incidentally to be a mouse homolog of rat cAMP-GEFII/Epac2, an isoform of cAMP-GEFII/Epac1, which was identified by the sequence database search (135, 297). Although cAMP-GEFII/Epac2 was shown to have GEF activities toward Rap1 (see sect. iv for details), the physiological role of the protein was not known. Interestingly, cAMP-GEFII/Epac2 binds to Rim2 (415), an isoform of Rim, a putative target of the small G protein Rab3-interacting molecule that participates in exocytosis (451). The interaction of cAMP-GEFII/Epac2 with Rim2 indicates that cAMP-GEFII/Epac2 is involved in regulated exocytosis. In PC12 cells transfected with human growth hormone cDNA alone, forskolin-potentiated secretion of the hormone was completely blocked by a PKA inhibitor, H-89, whereas in PC12 cells transfected with human growth hormone and cAMP-GEFII/Epac2 cDNA, 42% of forskolin-potentiated secretion was not blocked by H-89, indicating that cAMP-GEFII/Epac2 mediates cAMP-dependent, PKA-independent exocytosis (415). In mouse pancreatic islets, H-89 partially but significantly reduced incretin (GIP and GLP-1)-potentiating insulin secretion, while combined treatment with H-89 and antisense oligodeoxynucleotides (ODNs) against cAMP-GEFII/Epac2, which suppresses endogenous cAMP-GEFII/Epac2 expression, caused a further reduction of incretin-potentiated insulin secretion (290). These findings suggest that potentiation of insulin secretion by incretins is mediated by both PKA-dependent and PKA-independent mechanisms, the latter involving cAMP-GEFII/Epac2. The cAMP-GEFII/Epac2-mediated potentiation of insulin secretion requires both Ca$^{2+}$ and cAMP signals.

The insulin secretory response to high concentrations of glucose is biphasic (122), the first phase and second phase being produced by exocytosis of insulin granules in the readily releasable pool and the reserve pool, respectively (27, 60, 231). Although the precise mechanism of biphasic secretion is still unclear, the first phase is known to be evoked primarily by Ca$^{2+}$ entry, while the second phase is sustained by Ca$^{2+}$ and metabolic signals generated by glucose (299, 433). cAMP potentiates both phases of glucose-induced insulin secretion (433). Treatment of pancreatic islets with antisense ODNs against cAMP-GEFII/Epac2 reduced both phases of cAMP-potentiated insulin secretion, indicating the involvement of cAMP-GEFII/Epac2 in both phases. Capacitance measurement of pancreatic β-cells demonstrates that GLP-1 enlarges the readily releasable pool as well as facilitates rapid Ca$^{2+}$-dependent exocytosis independently of PKA, which occurs within 200 ms of membrane depolarization (152). With the use of antisense ODNs against cAMP-GEFII/Epac2 and cAMP analogs, this rapid exocytosis of insulin granules was shown to be mediated by cAMP-GEFII/Epac2, while the slow exocytosis occurring during longer depolarization was shown to be PKA dependent (152). The PKA-insensitive capacitance is not affected by Rp-cAMPS (152, 451).

cAMP-GEFII/Epac2-mediated exocytosis of insulin granules requires direct interaction of cAMP-GEFII/Epac2 with Rim2 (290, 415). Rim2 is localized in both plasma membrane and insulin granules (498). Rim2 lacking the Rab3-binding region changes localization predominantly to the cytoplasm and does not participate in cAMP-GEFII/Epac2-mediated secretion in MIN6 cells (498). cAMP-GEFII/Epac2 also interacts with a CAZ (cytomatrix at the active zone) protein, Piccolo (175), which is structurally related to Rim, with a zinc finger, PDZ, and C2 domains. Piccolo forms a homodimer and a heterodimer with Rim2 through its C2A domain in a Ca$^{2+}$-dependent manner, whereas Rim2 does not form a homodimer (175). Thus Piccolo may function as a Ca$^{2+}$ sensor in this process. Interactions among cAMP-GEFII/Epac2, Rim2, Piccolo, and Rab3 participate in cAMP-regulated insulin granule exocytosis (498).

cAMP-GEFII/Epac2 interacts specifically with nucleotide-binding fold (NBF)-1 of the SUR1 subunit (415, 498) of K$_{ATP}$ channels, but does not affect K$_{ATP}$ channel activities (498). The interaction between cAMP-GEFII/Epac2 and SUR1 is not inhibited in the presence of high ATP concentrations. Thus cAMP-GEFII may be anchored to
SUR1 even when the ATP concentration has been increased by glucose metabolism. Because binding of cAMP to cAMP-GEFII/Epac2 induces its conformational change, cAMP-GEFII/Epac2 may dissociate from the SUR1-cAMP-GEFII/Epac2 complex upon cAMP stimulation. Thus interaction between SUR1 and cAMP-GEFII/Epac2 may be required in cAMP-dependent, PKA-independent exocytosis of insulin granules. This is supported by the findings that incretin-induced, PKA-independent insulin secretion is impaired in SUR1 knockout islets (398, 501) and that early PKA-independent exocytosis is lost in SUR1 knockout β-cells (152). On the other hand, it has been proposed that a putative SUR on insulin granules (gSUR) is coupled with the CIC-3 channel (CIC-3) and interacts with cAMP-GEFII/Epac2 (152). Opening of the CIC-3 channels allows Cl⁻ entry into insulin granules, thereby promoting the granular acidification required to prime the secretory granules and refill the readily releasable pool (28). It has been proposed that binding of cAMP to cAMP-GEFII/Epac2, which interacts with gSUR, induces opening of the CIC-3 channels and thereby increases the size of the readily releasable pool of granules (152).

In pancreatic β-cells, cAMP-GEFII/Epac2 mediates induction of glucagon secretion in a PKA-independent manner. This effect is impaired in α-cells from SUR1 knockout mice (360), indicating that cAMP-regulated, cAMP-GEFII/Epac-dependent glucagon secretion in α-cells requires interaction of SUR1 with cAMP-GEFII/Epac2. GIP-1 mobilizes Ca²⁺ from endoplasmic reticulum in pancreatic β-cells (210, 244), and Ca²⁺ is mobilized through IP₃ receptors and/or ryanodine-sensitive receptors (RYRs) (39, 40, 51). cAMP-GEFII/Epac2 mediates RYR-regulated Ca²⁺ mobilization independently of PKA (47, 281, 282). Glucose-dependent ATP production in mitochondria is a key event in insulin secretion from pancreatic β-cells. Ca²⁺ mobilization by GIP-1 is known to increase mitochondrial ATP production in MIN6 cells (554), and the effect of GIP-1 requires activation of RYRs through cAMP-GEFII/Epac2. GIP also potentiates insulin secretion through cAMP signaling by both PKA-dependent and PKA-independent mechanisms (290). While GIP and GLP-1 have a synergistic effect in potentiating exocytosis of insulin granules (139), the synergy occurs only when GIP treatment of the β-cells precedes GLP-1 treatment, and does not occur when the treatments are reversed, suggesting that GLP-1 activates all of the effectors for GIP signaling in exocytosis while GIP does not.

B. Neurons

cAMP/PKA signaling presynaptically induces synaptic potentiation at many synapses. The calyx of Held allows simultaneous presynaptic and postsynaptic whole cell recordings (52, 528). While specific inhibitors of PKA including Rp-cAMPS and KT-5720 effectively block potentiation of exocytosis in hippocampal neurons (552) and pancreatic β-cells (451), they are ineffective at the calyx synapse (469). Both forskolin and 8-bromo-cAMP potentiate excitatory postsynaptic currents (EPSCs) at the calyx of Held (469). Rp-cAMPS, KT-5720, and H-89, all of which are blockers of PKA, do not antagonize the potentiating effect of cAMP on release (469), suggesting the presence of a cAMP target other than PKA. Application of 8-pCPT-2′-O-Me-cAMP, a specific activator of cAMP-GEF/Epac, into presynaptic terminals potentiates EPSCs (280, 470), and effectively reverses the effect of guanosine 5′-O-(2-thiodiphosphate) (GDPβS) that blocks endogenous cAMP signals (470). Thus cAMP-GEF/Epac has been considered to be a target of cAMP-regulated synaptic potentiation (175, 280). An increase in the cAMP concentration at the nerve terminal probably facilitates transmitter release by increasing both release probability and the number of releasable vesicles through activation of the cAMP-GEF/Epac pathway in the calyx of Held (280). The interaction of cAMP-GEFII/Epac2 with Rim1, a Rab3 effector, supports such cAMP-GEF/Epac participation in cAMP-regulated exocytosis in neurons (415). At crayfish neuromuscular junctions, cAMP generation in response to 5-HT enhances glutamate release. This cAMP-dependent enhancement of transmission apparently involves direct activation of two targets: the HCN channels and cAMP-GEF/Epac (33). Activation of the HCN channels promotes integrity of the actin cytoskeleton, facilitating neurotransmission by cAMP through activation of cAMP-GEF/Epac (618). In mammalian cells, including retinal bipolar cells (390) and cerebellar inhibitory interneurons (513), HCN channels also regulate neurotransmitter transmission.

VIII. A MODEL FOR cAMP-REGULATED EXOCYTOSIS

Although cAMP is freely diffusible in the cell and theoretically equilibrates throughout the cell very rapidly, its diffusion is limited by various factors such as structural hindrances and degradation by PDEs. cAMP signals are now known to be localized in distinct microdomains or functional compartments within the cell (cAMP compartmentation), whereby they mediate distinct physiological effects (see recent reviews in Refs. 34, 117, 516, 537). cAMP compartmentation was proposed originally about 20 years ago based on the finding that prostaglandin E1 and a β-adrenergic receptor agonist, isoprenaline, both elevate cAMP and activate PKA in cardiomyocytes while eliciting very different effects on activation of glycogen phosphorylase (74, 84). Jurevicius and Fischmeister (276) showed, using whole cell patch-clamp recordings simultaneously at two different sites in single isolated cardiac
myocytes of the frog, that local application of β-adrenergic agonist preferentially stimulated local L-type VDCC activities but not those more distant, while local activation of adenyl cyclase by forskolin resulted in VDCC currents throughout the cell. They also found that local inhibition of PDE activity strongly reduced nonuniformity of the β-adrenergic response (276, 277). These findings were further confirmed using CNG channels in rat cardiac myocytes (460). These studies indicate that diffusion of cAMP depends on PDE activities and suggest that the effects of cAMP are compartmentally localized within cardiac myocytes. With the use of the CNG channel engineered as a cAMP biosensor, distinct cAMP compartments were found: transient cAMP signals, a rise and fall in the cAMP concentration near the plasma membrane (membrane-localized cAMP microdomain), and an increase to a steady level of the cAMP concentration throughout the cell (bulk cytosol) (456). It has been reported that β-adrenergic stimulation generates multiple cAMP microdomains in which the cAMP concentration increases with a range of action as small as 1 μm and that free diffusion of cAMP is limited by PDE activities in neonatal rat cardiac myocytes (612). Such cAMP microdomains have been proposed to selectively activate a subset of PKA anchored to t tubules through AKAPs, suggesting a mechanism of β-adrenergic signaling selectivity (612). However, one might argue against this proposal, since cardiac myocytes of neonatal rat do not possess t tubules. In addition, using FlCrhR as a fluorescence resonance energy transfer (FRET) cAMP probe, it was found that striations in the FRET signal occur in frog ventricular cells, in which there are no t tubules (204). GPCRs, Gs proteins, adenylyl cyclase isoforms, PKA isoforms, PDE isoforms, phosphoprotein phosphatases, AKAP isoforms, PKA substrates, and possibly other unidentified factors all contribute to cAMP compartmentation (24, 142, 180, 326, 460, 600). Thus cAMP compartmentation might well underlie the various cAMP-regulated physiological responses seen upon stimulation by the different cAMP-increasing ligands (137, 139, 456). However, the spatial organization of cAMP microdomains is unclear in most cells.

Until recently, PKA has been thought to be the major target of cAMP in cAMP-regulated exocytosis. However, accumulating evidence suggests that cAMP-GEF/Epac also is an important target of cAMP in cAMP-regulated exocytosis. cAMP-GEF/Epac is expressed in neuronal, neuroendocrine, endocrine, and exocrine cells, all of which manifest regulated exocytosis, suggesting that cAMP-GEF/Epac may be the common molecule in cAMP-regulated, PKA-independent exocytosis in secretory cells. Although there is no direct evidence, it is tempting to speculate that cAMP-GEF/Epac resides in a cAMP compartment distinct from those containing PKA. A cAMP-GEF/Epac-containing compartment would be expected to be located close to the exocytic machinery, as the effects of cAMP-GEF/Epac on exocytosis require direct interaction with Rab3-interacting Rim molecules (Rim1 and Rim2) and Piccolo, to participate in the exocytotic process (175, 290, 415). Because cAMP-GEF/Epac has a much lower affinity for cAMP than for PKA (75, 134, 319, 457), higher concentrations of cAMP might well accumulate a cAMP compartment in which cAMP-GEF/Epac-mediated signaling occurs than in a compartment in which PKA signaling occurs. In pancreatic β-cells, GLUT2, a glucose transporter, and SUR1, a regulatory subunit of the KATP channel, both of which are important in the process of glucose-induced insulin secretion, are already phosphorylated by PKA in the basal state in intact pancreatic islets. FIG. 6. Model of the potentiation of Ca2+-triggered exocytosis by cAMP. A: nonstimulatory (basal) state. Certain proteins associated with exocytosis are maximally phosphorylated by PKA in the basal state. Such tonically phosphorylated proteins may be required in Ca2+-triggered exocytosis. B: stimulatory state. cAMP potentiates Ca2+-triggered exocytosis through PKA-dependent and PKA-independent pathways. Upon stimulation, PKA also induces phosphorylation of proteins associated with the exocytic process (PKA-dependent pathway). In the PKA-independent pathway, cAMP directly potentiates Ca2+-triggered exocytosis through cAMP-GEF/Epac.
β-cells and β-cell lines (36, 544), which suggests that tonic phosphorylation by PKA is required for their function. On the other hand, Kir6.2, the pore-forming subunit of the $K_{ATP}$ channel, and the α-subunit of VDCC can be phosphorylated by PKA upon stimulation (36). These findings indicate that while some of the substrates for PKA are maximally phosphorylated by PKA in the basal state (Fig. 6A), others are phosphorylated in a regulated manner (Fig. 6B), the former most likely occurring independently of cAMP compartmentation (the bulk effect) and the latter being associated with cAMP compartmentation (see Fig. 7). Upon stimulation, cAMP modulates exocytosis by coordinating the PKA phosphorylation of the proteins associated with exocytosis and the PKA-independent mechanism mediated by cAMP-GEF/Epac (HCN channels in certain neurons) (33, 390, 513).

FIG. 7. Model of cAMP compartmentation associated with exocytosis. Upon stimulation by ligands, G protein-coupled receptor (GPCR) induces activation of adenyl cyclase (AC) through $G_s$ protein. A pool of cAMP is generated by AC in a localized region in a cell. Such a cAMP pool does not diffuse, primarily due to limitation by phosphodiesterase (PDE). Yellow circles indicate cAMP. A: cAMP microdomain. PKA, interacting with A kinase anchoring protein (AKAP), phosphorylates proteins associated with the exocytotic machinery in a regulated manner and potentiates $Ca^{2+}$-triggered exocytosis in a cAMP microdomain such as those originally proposed in cardiac myocytes (84). B: model of cAMP-GEF/Epac-containing compartment in a pancreatic β-cell. Specific interaction of cAMP-GEFII/Epac2 with the $K_{ATP}$ channel subunit SUR1 occurs in a specialized region of the cell. The increased cAMP concentration likely dissociates cAMP-GEFII/Epac2 from SUR1 (499), which then couples to the exocytotic machinery (see text for details). Whether or not cAMP-GEFII/Epac2 actually functions in a specific and localized cAMP compartment or microdomain has not been established.
IX. CONCLUDING REMARKS

Ca\textsuperscript{2+} is the principal signal in regulated exocytosis, but other intracellular signals are also involved. The cAMP signaling pathway that modulates exocytosis has been studied extensively in a variety of secretory cells, but the effects of PKA in cAMP signaling have been investigated mostly by pharmacological approaches using various PKA inhibitors. Although the PKA inhibitors are often partially effective in cAMP-regulated exocytosis, the cAMP action not blocked by PKA inhibitors was not investigated in detail. It is now clear that there are targets of cAMP other than PKA in cAMP-regulated exocytosis. New tools such as FRET-based probes and cAMP-effector specific agonists have been developed recently that are useful in analyses of cAMP, PKA, and cAMP-GEF/Epac signals, so the mechanism of cAMP-regulated exocytosis must be revisited. Answers to questions regarding 1) the downstream signaling pathway of cAMP-GEF/Epac in cAMP-regulated exocytosis; 2) the spatial and temporal regulation of the interaction between cAMP-GEF/Epac and its effectors associated with exocytosis; 3) the role of Rap1 (activated by cAMP-GEF/Epac in a cAMP-dependent manner) in exocytosis; 4) the spatial organization of cAMP-GEF/Epac containing cAMP microdomains; 5) the interaction of such microdomains with those containing PKA, AKAP, and PDE; and 6) the interaction between cAMP and Ca\textsuperscript{2+} microdomains should reveal the integrative processes of the PKA-dependent and PKA-independent signaling pathways in cAMP-regulated exocytosis.

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