Physiological Substrates of cAMP-Dependent Protein Kinase

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Received April 27, 2001

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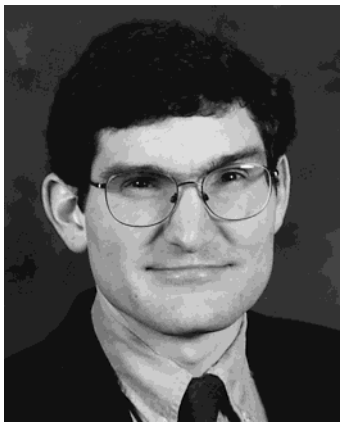

John B. Shabb earned his Ph.D. degree in Biochemistry under the mentorship of Michael R. Miller, Ph.D., at West Virginia University in 1984 studying cAMP-dependent protein kinase. He pursued postdoctoral training in the Department of Molecular Physiology and Biophysics at Vanderbilt University, first studying hormonal regulation of transcription with Darryl K. Granner, M.D., and then structure–function of cAMP-dependent protein kinase with Jackie D. Corbin, Ph.D. In 1992 Dr. Shabb joined the Department of Biochemistry and Molecular Biology at the University of North Dakota, where he continues to study the structure, function, and regulation of cAMP-dependent protein kinase.

I. Introduction

A. Scope and Perspective

The purpose of this review is to provide an account of the physiological substrates of cAMP-dependent protein kinase (PKA). Discussion is limited to just over 100 unique gene products for which one or more in situ or in vivo phosphorylation sites are known (Table 1). By necessity, treatment of each substrate is brief, focusing principally on its interaction with PKA and the functional consequences, if known, of the phosphorylation event. When possible, the reader is directed to in-depth reviews of individual proteins and their regulation by phosphorylation.

The list of PKA substrates discussed in this review is extensive but not exhaustive. This is in part because new substrates for PKA are continually being described. There are also many protein substrates not included here, for which there is substantial evidence of cAMP-dependent regulation but for which an in situ PKA site has yet to be defined. Though the compilation of substrates arises from a systematic search of the literature, it is very possible that some legitimate protein substrates have been overlooked, and for these omissions, apologies are extended in advance. Occasionally, however, the omissions have been intentional, and the reasons for some of these near misses or red herrings are mentioned in subsequent pages. Conversely, there is room for debate as to whether all the substrates included in this review merit status as "physiological" substrates. In such cases, further experimentation

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10.1021/cr000236l CCC: $36.00
Published on Web 07/17/2001

Table 1. Physiological Substrates of PKA

	substrate	in vivo/in situ site	sequence	accession no./species	ref
A	**Autophosphorylation**				
	cAMP-dependent protein kinase type regulatory subunit type IIα	Ser-95[a]	pgrfdrrvsvcaet	P00515 (bovine)	90
B1	**cAMP signaling**				
	β_2-adrenergic receptor	Ser-262[c]	tghglrrsskfclk	P07550 (human)	101,103
		Ser-345[c]	qellclrrsslkay		
		Ser-346[c]	ellclrrsslkayg		
	cAMP-specific phosphodiesterase PDE4D3	Ser-54[a,c]	fvhsqrresflyrs	P14270 (rat)	113,114
	cGMP inhibited phosphodiesterase PDE3B	Ser-302[a,c]	sgkmfrrpslpcis	Q63085 (rat)	111,112
	D1 dopamine receptor	Thr-268[c]	fkmsfkretkvlkt	P18901 (rat)	108
	Metabotropic glutamate receptor subunit mGluR2	Ser-843[a,c]	fgsaaprasanlgq	P31421 (rat)	109
B2	**cGMP signaling**				
	Phosducin	Ser-73[a]	kermsrkmsiqeye	P20942 (rat)	118
B3	**Phosphoinositide and calcium signaling**				
	Elongation factor-2 kinase	Ser-499[c]	srlhlprpsavale	P70531 (rat)	129
	Inositol 1,4,5-trisphosphate Type I receptor	Ser-1589[a]	arnaarrdsvlaas	P29994 (rat)	125-127
		Ser-1755[a]	irpsgrresltsfg		
	Phospholipase C-γ1	Ser-1248[a]	fhvraregsfeary	P10686 (rat)	124
	Phospholipase C-β3	Ser-1105[a,c]	ildrkrhnsiseak	Q01970 (human)	123
	Thromboxane A_2 receptor TPα	Ser-329[c]	prlstrprslslqp	NP 001051 (human)	121
B4	**Rho signaling**				
	RhoA small GTP-binding protein	Ser-188[a,c]	qarrgkkksgclvl	P06749 (human)	131,132
B5	**T cell receptor signaling**				
	COOH-terminal Src kinase (Csk)	Ser-364[a,c]	ealrekkfstksdv	P41240 (human)	133
B6	**Mitogen-activated protein kinase signaling**				
	Hematopoeietic protein tyrosine phosphatase (hePTP)	Ser-23[a,c]	vrlqerrgsnvalm	P35236 (human)	148
	Mammalian STE20-like kinase 3 b isoform (MST3b)	Thr-18[c]	lalnkrratlphpg	AAD42039 (human)	146
	v-Mos	Ser-56[a,c]	psvdsrscsiplva	P00538 (maloney	144
		Ser-102[a,c]	vclmhrlgsggfgs	murine sarcoma virus)	
		Ser-263[a,c]	qdlrgrqaspphig		
	GTPase activating protein specific for Rap1 (rap1GAP)	Ser-490[a,c]	gksptrkksgpfgs	P47736 (human)	153,154
		Ser-499[a,c]	gpfgsrrssaigie		
	Guanine nucleotide exchange factor Ras-GRF1	Ser-916[a,c]	nkevfrrmslantg	P27671 (mouse)	155
	Protein tyrosine phosphatase-SL (PTP-SL)	Ser-231[c]	iglqerrgsnvslt	NP 035347 (human)	149
	Raf-1 serine/threonine protein kinase	Ser-43[a,c]	qfgyqrrasddgkl	P11345 (rat)	136,138
	Rap1b low molecular weight GTP/GDP-binding protein	Ser-179[c]	vpgkarkksscqll	P09526 (human)	151
	Striatal-enriched protein tyrosine phosphatase, 61 kDa (STEP) $STEP_{61}$ and 46 kDa $STEP_{46}$ splice variant	Ser-160[a,c]	lppedrrqsvsrqp	P54830 (mouse)	150
		Ser-221[a,c,e]	mglqerrgsnvslt		
B7	**Modulators of protein phosphatase 1**				
	Glycogen binding (G) subunit of protein phosphatase 1	Ser-46[a]	spqpsrrgsdssed	NP 002702 (human)	169-171
		Ser-65[a]	psgtrrvsfadsf		
	Inhibitor-1of (I-1) protein phosphatase I	Thr-35[a]	eqirrrrptpatlv	P01099 (rabbit)	160,161
	Dopamine and cAMP-regulated phosphoprotein, 32 kDa (DARPP-32)	Thr-34[a,b]	emirrrrptpamlf	P07516 (bovine)	165,166
C	**Transcriptional regulation**				
	Cyclic AMP response element binding protein (CREB)	Ser-133[a,b,c]	reilsrrpsyrkil	P15337 (rat)	175-177
	Cyclic AMP responsive element modulator (CREMτ)	Ser-117[c]	reilsrrpsyrkil	P27699 (mouse)	178
	Nuclear factor of activated T cells 3 (NFAT3)	Ser-272[a,c]	spcgkrrysssgtp	Q14934 (human)	189
		Ser-289[a,c]	spalsrrgslgeeg		

Table 1. (Continued)

	substrate	in vivo/in situ site	sequence	accession no./species	ref
	Nuclear factor κB (NF-κB)	Ser-276[a,c]	vsmqlrrpsdrels	Q04207 (mouse)	186
	Retinoic acid receptor-α (RARα)	Ser-369[a,c]	vyvrkrrpsrphmf	P11416 (mouse)	196
	Retinoid X receptor-α (RXRα)	Ser-27[c]	ltsptgrgsmaaps	P19793 (human	197
	Sex determining region of Y gene product (SRY protein)	Ser-32[c]	nipalrrsssflct	Q05066 (human)	205
	SRY-box related transcription factor SOX9	Ser-64[c]	gepdlkkeseedkf	P48436 (human)	206
		Ser-181[b,c]	kyqprrrksvkngq		
	Steroidogenic factor-1 (SF-1)	Ser-430[c]	clvevralsmqake	P33242 (mouse)	203
	Thyroid hormone receptor α1	Ser-28[a,c]	ldgkrkrkssqclv	P04625 (chick)	198,199
		Ser-29[a,c]	dgkrkrkssqclvk		
	Vasoactive intestinal polypeptide receptor transcriptional repressor protein (VIPR-RP)	Ser-245[c]	ktkkarkdseeges	AAC40192 (rat)	204
		Ser-361[c]	kgsptkresvsped		
D	**Histones**				
	Histone H1c	Ser-37[a]	pagvrrkasgppvs	P15864 (mouse)	217,218
	Histone H3	Ser-10[a,b,c]	rtkqtarkstggka	P16106 (human)	214,215
E	**Apoptosis and cell survival**				
	Bcl-2/Bcl-X_L-antagonist, causing cell death (BAD)	Ser-155[a,b,c]	ygrelrrmsdefeg	Q61337 (mouse)	31,221,223
	Glycogen synthase kinase-3α (GSK-3α)	Ser-21[b]	gsgrartssfaepg	P18265 (rat)	228
	Glycogen synthase kinase-3β (GSK-3β)	Ser-9[b,c]	msgrprttsfaesc	P18266 (rat)	227,228
	Interleukin receptor-3 β_c chain	Ser-585[b,c]	ylgpphsrslpdil	NP_000386 (human)	225
F1	**Ligand-gated ion channels**				
	$GABA_A$ receptor β1 subunit	Ser-409[c]	kgrirrrasqlkvk	P50571 (mouse)	236
	$GABA_A$ receptor β3 subunit	Ser-408[c]	kthlrrrssqlkik	P15433 (mouse)	237
		Ser-409[c]	kkthlrrrssqlki		
	Glutamate receptor GluR1 subunit (AMPA receptor)	Ser-845[a,b,c]	rtstlprnsgagas	P19490 (rat)	240,241
	Glutamate receptor GluR4 subunit (AMPA receptor)	Ser-842[a,c]	airnkarlsitgsv	P48058 (human)	242
	Glutamate receptor GluR6 subunit (kainate receptor)	Ser-684[c]	afmssrrqsvlvks	P42260 (rat)	238,239
	Glutamate receptor NR1A subunit (NMDA receptor)	Ser-897[a,b,c]	ssfkrrrsskdtst	P35439 (rat)	243
	Nicotinic acetylcholine receptor δ subunit	Ser-361[a,c]	ndlklrrsssvgyi	P02718 (*T. californica*)	35,231,232,234
		Ser-362[a,c]	dlklrrsssvgyis		
	P_{2X2} purinoreceptor	Ser-431[c]	avqsprpcsisalt	P49653 (rat)	251
F2	**Sodium ion movement**				
	Na^+ H^+ exchanger 3 (NHE3)	Ser-552[a,c]	vaegerrgslafir	P26433 (rat)	34,257
		Ser-605[a,c]	qsleqrrrsirdte		
	Na^+,K^+ ATPase α1 subunit	Ser-943[a,b,c]	vicktrrnsvfqqg	P06685 (rat)	32,253
	Serum and glucocorticoid regulated kinase (Sgk)	Thr-369[c]	ddlinkkitppfnp	O00141 (human)	273
	Voltage sensitive Na^+ channel (Rat brain type IIA) α subunit	Ser-573[a,c]	slfsprrnsraslf	P04775 (rat)	266,267
		Ser-610[a,c]	ednds rrdslfvph		
		Ser-623[a,c]	hrhgerrpsnvsqa		
		Ser-687[a,c]	teirkrrsssyhvs		
	Voltage-sensitive Na^+ channel (cardiac type H1) α subunit	Ser-526[a,b,c]	rtsmrprssrgsif	P15389 (rat)	265
		Ser-529[a,b,c]	mrprssrgsiftfr		
F3	**Chloride conductance**				
	Cystic fibrosis transmembrane conductance regulator (CFTR)	Ser-660[a,c]	qfsaerrnsiltet	P13569 (human)	277,278
		Ser-700[a]	efgekrknsilnpi		
		Ser-737[c]	deplerrlslvpds		
		Ser-795[c]	ttastrkvslapqa		
		Ser-813[c]	idiysrrlsqetgl		

Table 1. (Continued)

	substrate	in vivo/in situ site	sequence	accession no./species	ref
	Phospholemman	Ser-68[a,c]	frssirrlstrrr	O08589 (rat)	286,288
F4	**Potassium channels**				
	Shaker K^+ channel	Ser-507[c]	tlgqhmkksslses	P08511 (*Drosophila*)	290
		Ser-508[c]	lgqhmkksslsess		
	Slo K_{Ca} channel splice variant A1C2E1G3I0	Ser-942[b,c]	pivlqrrgsvygan	JH0697[d] (*Drosophila*)	311,312
	h*Slo* BK_{Ca} α subunit of large conductance Ca^{2+}-dep. K^+ channel (maxi-K)	Ser-869[c]	vhgmlrqpsittgv	NP 002238 (human)	313
	Kv1.1 α subunit of the *Shaker* RCK1 Voltage-gated K^+ channel	Ser-446[c]	dsdlsrrssstisk	P10499 (rat)	291,292
	Kvβ1.3 subunit of the Kv1.5 K^+ channel Ik_{ur}	Ser-24[c]	entklrrqsgfsva	AAC41926.1 (human)	295
	Kv4.2 α subunit of the *Shal*-type K^+ channel	Thr-38[a,b]	pprqerkrtqdali	NP 062671 (mouse)	296
		Ser-552[a,b]	nvsgshrgsvqels		
	Kir 1.1 Renal outer medullary K^+ channel 1, 2 (ROMK1, ROMK2)	Ser-25[a,c]	srqrarlvskegrc	P35560 (rat)	300-302
		Ser-200[a,c]	irvanlrkslligs		
		Ser-294[a,c]	satcqvrtsyvpee		
	Kir2.1	Ser-425[c]	eprplrresei	Q64273 (rat)	306
	Kir2.3 Inward rectifier K^+ channel (IRK)	Ser-440[a,c]	dnisyrresai	P48050 (human)	305
	Kir6.2 subunit of the ATP-sensitive K^+ channel (K_{ATP})	Ser-372[c]	argplrkrsvpmak	Q14654 (human)	308,309
		Thr-224[c]	hmqvvrkttspege		
	SUR1 subunit of the ATP-sensitive K^+ channel (K_{ATP})	Ser-1571[c]	ekllsrkdsvfasf	Q09428 (human)	308
G	**Water homeostasis**				
	Aquaporin-2	Ser-256[b,c]	erevrrrqsvelhs	P34080 (rat)	316,317,320
H	**Other transporters**				
	P-glycoprotein mdr1b	Ser-665[a,c]	sksplirrsiyrsv	P06795 (mouse)	323
		Ser-681[a,c]	kqdqerrlsmkeav		
	Steroidogenic acute regulatory protein (StAR)	Ser-57[c]	inqvrrrssllgsr	P49675 (human)	322
		Ser-195[c]	vrcakrrgstcvla		
I	**Extracellular proteins**				
	Atrial natriuretic peptide	Ser-104[a]	gprslrrrsscfggr	P01161 (rat)	332
	Vitronectin	Ser-378[a]	rnqnsrrpsratwl	P04004 (human)	327-329
J	**Trafficking and motility**				
	Actin bundling protein L-plastin	Ser-5[a,c]	margsvsdee	P13796 (human)	353-355
	Low-density lipoprotein receptor-related protein (LRP)	Ser-4520[c]	mgghgsrhslastd	Q07954 (human	346
	Myosin light-chain kinase (MLCK) and telokin splice variant	Ser-1005[a,c,f]	sglsgrksstgspt	P29294 (rabbit)	359,360
	Protein tyrosine phosphatase-PEST (PTP-PEST)	Ser-39[a]	dfmrlrrlstkyrt	NP 002826 (human)	347
		Ser-435[a]	dkklernlsfeikk		
	Small heat shock-related protein HSP20	Ser-16[a,b]	qpswlrrraaplpg	O14558 (human)	356
	Synapsin I	Ser-9[a]	mnylrrrlsdsnfm	P09951 (rat)	340,341
	Vasodilator-stimulated phosphoprotein (VASP)	Ser-157[a,c]	sehierrvsnaggp	P50552 (human)	350,351
		Ser-239[a,b,c]	agaklrkvskqeea		
		Thr-278[a,c]	mlarrrkatqvgek		
K	**Striated muscle contraction**				
	Myosin-binding protein-C cardiac isoform	Ser-275[a,c]	llsafrrtslaggg	Q14896 (human)	390,391
		Ser-284[a,c]	lagggrrisdshed		
		Ser-304[a,c]	ssllkkrdsfrtpr		
	Phospholamban	Ser-16[a,b]	trsairrastiemp	P26678 (human)	377,378
	Ryanodine receptor type 2 (sarcoplasmic reticulum Ca^{2+} release channel)	Ser-2809[c]	lynrtrrisqtsqv	P30957 (rabbit)	375
	Troponin I	Ser-23[a]	apapirrrssnyra	P19429 (human)	383,384
		Ser-24[a]	papirrrssnyray		

Table 1. (Continued)

	substrate	in vivo/in situ site	sequence	accession no./species	ref
	Voltage-sensitive L-type Ca^{2+} channel (skeletal muscle) α1 subunit	Ser-1757[a,c]	pergqrrtsltgsl	P07293 (rabbit)	369
		Ser-1854[a,c]	pgslsrrsslgsld		
	Voltage-sensitive L-type Ca^{2+} channel (cardiac) α1 subunit	Ser-1928[a,b,c]	saslgrrasfhlec	P15381 (rabbit)	370,371
	Voltage-sensitive L-type Ca^{2+} channel (cardiac) β_{2a} subunit	Ser-459[a,c]	drsaprsasqaeee	A42044 (rat)	372
		Ser-478[a,c]	vkksqhrsssathq		
		Ser-479[a,c]	kksqhrsssathqn		
L	**Metabolic enzymes**				
	ATP citrate lyase	Ser-454[a]	tpapsrtasfsesr	P16638 (rat)	418
	Cytochrome P450 CYP2E1	Ser-129[a,c]	twkdvrrfslsilr	P05182 (rat)	429,430
	Glycogen synthase (muscle type)	Ser-7[a,c]	mplsrtlsvsslp	AAB69872 (rabbit)	400,402
		Ser-697[a,c]	apewprrasctsss		
		Ser-710[a,c]	sssggskrsnsvdt		
	Hormone-sensitive lipase	Ser-563[a,c]	rltesmrrsvseaa	P15304 (rat)	415-417
		Ser-659[a,c]	pdgfhprrssqgvl		
		Ser-660[a,c]	dgfhprrssqgvlh		
	Phenylalanine hydroxylase	Ser-16[a,b]	npglgrklsdfgqe	P00439 (human)	427,428
	Phosphorylase kinase α subunit (muscle type)	Ser-1018[a]	kqvefrrlsistes	P18688 (rabbit)	398
	Phosphorylase kinase β subunit (muscle type)	Ser-26[a]	rartkrsgsvyepl	P12798 (rabbit)	398
	6-phosphofructo-2-kinase-fructose-2,6-bisphosphatase liver isozyme 1	Ser-32[a]	svlqrrrgssipqf	P07953 (rat)	406,407
	6-phosphofructo-1-kinase, isozyme A (muscle type)	Ser-376[a]	eamklrgrsfmnnw	P00511 (rabbit)	409,410
	Pyruvate kinase (liver type)	Ser-43[a]	pagylrrasvaqlt	P12928 (rat)	411
	Tyrosine hydroxylase	Ser-40[a,b,c]	prfigrrqslieda	P04177 (rat)	422,424
M	**Proteins of unknown function**				
	cAMP-regulated phosphoprotein, 16/19 kDa (ARPP-16/19)	Ser-104[a,b,g]	qdlpqrkpslvask	P56211 (human)	435
	cAMP-regulated phosphoprotein, 21 kDa (ARPP-21)	Ser-55[a,b]	aqnqerrksksgag	A34957 (bovine)	437,438
	Serine/threonine protein kinase LKB1	Ser-431[b,c]	ssnkirrlsackqq	NP_035622 (mouse)	432,433

[a] Direct sequencing and/or phosphopeptide mapping. [b] Phospho/dephospho-specific antibodies. [c] Site-directed mutagenesis. [d] Ser-952 in this splice variant. [e] $STEP_{61}$ Ser-221 is equivalent to $STEP_{46}$ Ser-49. [f] MLCK Ser-1005 is equivalent to telokin Ser-13. [g] ARPP-19 Ser-104 is equivalent to ARPP-16 Ser-88.

may very well be needed for definitive evidence of direct physiological regulation by PKA.

This review does not focus on the structure and function of the cAMP-dependent protein kinase itself, and it only briefly touches on the rapidly expanding field of PKA localization via anchoring protein interactions. Both of these topics are the subject of other reviews in this issue by Taylor and colleagues. The reader is also referred to a sampling of other reviews on this extensively studied enzyme, both recent[1−5] and older.[6−12]

Though cAMP-dependent phosphorylation is a much-studied area with a long history, there are few comprehensive accounts of the physiological substrates of this enzyme. Perhaps the most definitive is by Beavo and Krebs,[13] which concisely summarizes the state of the field of protein phosphorylation/dephosphorylation in 1979. Subsequent lists of physiological PKA substrates are brief and without annotation[6,14] or are included in lists of protein and peptide substrates with no distinction made regarding physiological relevance.[15−17] The latter listings form the basis for the determination of the consensus phosphorylation sites for PKA which are Arg-Arg-X-Ser/Thr, Arg/Lys-X-X-Ser/Thr, and Arg/Lys-X-Ser/Thr.[15−18] All of these lists, except the web-based PhosphBase 2.0 database,[17] precede the common use of molecular biological techniques and improved sequencing techniques that have greatly accelerated the identification of physiologically relevant phosphorylation sites.

B. Criteria for Selection of Physiological Substrates of PKA

General criteria for selection of physiological substrates for protein kinases are set forth by Krebs and Beavo[13] and repeated here.

> (1) Demonstration in vitro that the enzyme can be phosphorylated stoichiometrically at a significant rate in a reaction(s) catalyzed by an appropriate protein kinase(s) and dephosphorylated by a phosphoprotein phosphatase(s).
>
> (2) Demonstration that functional properties of the enzyme undergo meaningful changes and correlate with the degree of phosphorylation.
>
> (3) Demonstration that the enzyme can be phosphorylated and dephosphorylated in vivo or in an intact cell system with accompanying functional changes.
>
> (4) Correlation of cellular levels of protein kinase and/or phosphoprotein phosphatase effectors and the extent of phosphorylation of the enzyme.

In the current review, the demonstration of in situ phosphorylation in response to elevation of cAMP (criteria 3 and 4) is the primary evidence upon which a substrate is considered, with the added stringency of the precise identification of the in situ site of phosphorylation. This takes ascendancy over identification and characterization of in vitro PKA sites since it is not uncommon for the in vitro and in situ sites to differ. Not every substrate included in the current list, however, may necessarily meet all four standards after rigorous inspection. Several substrates have been included for which no functional consequence of the phosphorylation has been attributed. Such "silent" phosphorylation events may actually turn out to affect functions that have yet to be considered, including modulation of subcellular localization or degradation rate in the intact cell. Some of the more recently identified substrates, on the other hand, may not yet have been carefully evaluated in terms of the in vitro characteristics of phosphorylation.

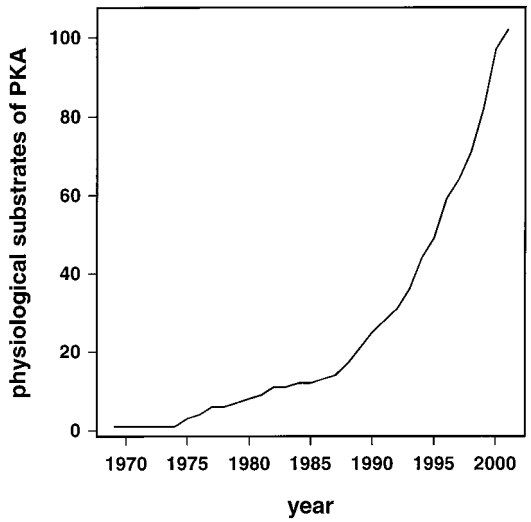

Figure 1. Progress in the identification of physiological PKA substrates. The increased rate of identification of physiological substrates of PKA that began in the late 1980s correlates with the introduction and increasingly common use of specialized reagents and molecular techniques for the study of PKA described in section I.B.

The number of identified physiological PKA substrates has quadrupled in the past decade (Figure 1). The reasons for the increased ease of identification of in situ phosphorylation sites are severalfold. There is now the potential of ectopically expressing a gene product of any cDNA in mammalian cells. These cDNAs are easily manipulated by site-directed mutagenesis to create phosphorylation site mutants (usually Ser/Thr-to-Ala). Phosphopeptide-specific antibodies have become increasingly common tools for probing in situ and/or in vivo phosphorylation states of target proteins.[19] Microsequencing by either Edman degradation or mass spectrometry has steadily improved to the point that very small amounts of proteins or peptide fragments are needed to obtain reliable sequence data. Also, commercially available cell-permeable PKA-selective inhibitors are available, such as H89, a competitive inhibitor of ATP binding to the PKA catalytic subunit,[20] and Rp-cAMPS, an antagonist that competes with cAMP for binding to the PKA regulatory subunit.[21] These join other established tools to elevate intracellular cAMP concentration, including G protein-coupled receptor agonists such as isoproterenol, G protein activators such as cholera toxin, the adenylyl cyclase activator forskolin, and cell-permeable cAMP analogues that activate PKA directly.

Table 2. Co-localization of PKA with Physiological Substrates

	anchoring protein	ref
C subunit-directed		
cAMP-dependent protein kinase type regulatory subunit type IIα		
nuclear Factor κB (NF-κB) (in association with IκB)		186
Slo K_{Ca} channel splice variant A1C2E1G3I0 (*Drosophila*)		312
SRY-box related transcription factor SOX9		206
R subunit-directed		
aquaporin-2	AKAPs	318
β_2-adrenergic receptor	NHERF?	105−107
	Gravin	
	AKAP79/150	
Bcl-2/Bcl-X_L-antagonist, causing cell death (BAD)	D-AKAP 1/S-AKAP84	30
cystic fibrosis transmembrane conductance regulator (CFTR)	Ezrin	281,282
	EBP50 (NHERF1)	
	E3KARP (NHERF2)	
glutamate receptor GluR1 subunit (AMPA receptor)	AKAP79/150	246,247
	PSD-95	
	SAP-97	
glutamate receptor NR1A subunit (NMDA receptor)	Yotiao	249,250
Kir 1.1 renal outer medullary potassium channel 1, 2 (ROMK1, ROMK2)	AKAPs	303
Kir2.1	PSD-95	305
Kir2.3 inward rectifier potassium channel (IRK)	PSD-95	305
ryanodine receptor type 2	mAKAP	375
	FKBP12.6	
sodium hydrogen exchanger 3 (NHE3)	EBP50 (NHERF1)	258,259
	Ezrin	
	E3KARP	
voltage-sensitive L-type calcium channel (skeletal muscle) α1 subunit	AKAP15/18	365,366
voltage-sensitive L-type calcium channel (cardiac) α1 subunit	AKAP15/18	367,368
	MAP2B	
voltage-sensitive sodium channel (rat brain type IIA α subunit)	AKAP-15/18	269,270

Even though there is a wealth of tools to investigate PKA phosphorylation events, potential pitfalls in their use exist. For example, forskolin may exert cAMP-independent effects on the activities of membrane proteins (reviewed in ref 22). Direct effects of forskolin on physiological PKA substrates such as the nicotinic acetylcholine receptor,[23,24] the L-type calcium channel,[25,26] or potassium channels[27] complicate the interpretation of experiments using this diterpene. Though H89 is a potent inhibitor of PKA, it is equally effective at inhibiting other protein kinases such as S6 kinase-1, MSK1, and Rho-dependent kinase II.[28] Recognition of this cross-reactivity aided in the identification of MSK1 as the protein kinase responsible for the physiological phosphorylation of histone H3 and HMG-14 even though the phosphorylation of both is inhibited by H89 and both are in vitro substrates for PKA.[29] Some of the phosphoserine antibodies directed against putative PKA sites may not be as specific as would be hoped. The misidentification of PKA phosphorylation of BAD Ser-112[30] is attributed to cross-reactivity of anti phospho-Ser-112 antibody with phospho-Ser-155.[31] Even though a phosphopeptide antibody has been used to demonstrate in situ phosphorylation of Na^+/K^+ ATPase,[32] its cross-reactivity with other phosphoproteins[33] potentially limits its usefulness in tracking the phosphorylation state of this transporter in cell lysates. Site-directed Ser-to-Ala mutants may also occasionally cause effects that are independent of PKA phosphorylation. Even though site-directed mutagenesis of the Na^+/H^+ exchanger NHE3 isoform at a putative PKA phosphorylation site, Ser-634, affects forskolin-dependent inhibition of the exchanger, this occurrs without altering PKA phosphorylation.[34] Conversely, mutation of a putative PKA phosphorlation site could allow for the phosphorylation of a secondary site that is mutation-dependent. Mutation of the PKA site (RRS*S*(P)S) in the δ-subunit of the nicotinic acetylcholine receptor to an Ala yields a δ-subunit that is phosphorylated by PKA at an adjacent Ser (RRS*AS*(P)).[35] The vagaries of individual techniques used to demonstrate PKA-dependent phosphorylation therefore make it prudent to use multiple independent means to confirm PKA's physiological involvement.

A unique tool to demonstrate the involvement of PKA scaffolding in phosphorylation reactions is the use of the A kinase anchoring protein (AKAP) peptide Ht31.[36] The peptide is designed to compete with AKAPs for binding to the dimerization domain of the PKA regulatory subunits. When perfused into a cell, Ht31 can block cAMP-dependent phosphorylation of the target protein if AKAPs are involved in co-localization of PKA and substrate. This technique has been most useful for demonstrating the cAMP-dependent phosphorylation of ion channels, mainly because functional changes can be measured at the single cell level by electrophysiological techniques. Table 2 summarizes the physiological PKA substrates that have been shown to be physically associated with PKA. In most cases, the association is AKAP-mediated. There are some notable exceptions, however, in which the PKA catalytic subunit interacts directly with a substrate.

The newer generation of reagents and techniques permit the reevaluation of a number of substrates that were characterized as physiological targets of PKA before their availability. It must be kept in mind that during the 1970s and early 1980s when many

Table 3. Frequency of Phosphorylation of Consensus Sites in Physiological Substrates of PKA[c]

consensus sequence	PKA-phosphorylated sites	total consensus sites[a]	probability of PKA phosphorylation
		Phospho-Ser	
R R X S	75	94	0.80
R X X S not R (R/K) X S	20	239	0.08
X R X S not (R/K) R X S	18	259	0.07
R K X S	14	30	0.47
K R X S	3	17	0.18
K K X S	4	22	0.16
TOTAL	136	661	
		Phospho-Thr	
R R X T	3	8	0.38
R X X T not R (R/K) X T	1	147	0.01
X R X T not (R/K) R X S	0	116	0
R K X T	3	11	0.30
K R X T	1	7	0.14
K K X T	1	14	0.07
TOTAL	9	303	
		Nonconforming sites	
M K K S	1	ND[b]	ND
H S R S	1	ND	ND

[a] Sites in extracellular loops of membrane proteins were excluded from the tabulation of total sites. [b] Not determined. [c] Decreasing probability: RRXS > RRXT,RKX(S/T) > KKX(S/T), KRX(S/T) > RXXS,RXS.

phosphorylation events were ascribed to cAMP-dependent protein kinase, only a handful of other protein kinases were known. Now hundreds of serine/threonine protein kinases have been identified, and quite a few have overlapping substrate specificities with PKA. Another level of complexity of signaling through PKA that has emerged has been the identification of several modulators of serine/threonine protein phosphatase PP1 as substrates of PKA. This has major implications in terms of whether the increase in phosphorylation of a protein in response to cAMP is due to direct phosphorylation of the site by PKA or to inhibition of PP1 through phosphorylation of a PP1 inhibitor. This, in fact, is the reason enzymes such as acetyl CoA carboxylase, which is readily phosphorylated in vitro by PKA, are no longer considered direct physiological PKA substrates.[37]

Occasionally the efficacy of PKA phosphorylation is altered by reversible posttranslational modification of physiological substrates by effectors other than PKA. For example, the brain voltage-gated sodium channel must be phosphorylated by protein kinase C before it can be subject to cAMP-dependent inhibition.[38] Agonist-dependent depalmitoylation of the β2-adrenergic receptor at Cys-341 must precede in situ phosphorylation of serines 345 and 346 by PKA.[39] Finally, reversible intracellular *o*-glycosylation of serine and threonine residues can directly block potential phosphorylation sites.[40] Though no specific examples are yet known for antagonism of a PKA phosphorylation event, activation of PKA in cultured cerebellar neurons can reduce the global level of *o*-glycosylation.[41]

C. PKA Consensus Phosphorylation Site

The phosphorylation sites listed in Table 1 permit the evaluation of consensus sequences for PKA in a physiological context. A tabulation of the occurrence of all permutations of these sites (Table 3) demonstrates that no single consensus sequence can satisfactorily predict actual sites of phosphorylation. Thus, experimental confirmation of precise PKA phosphorylation sites remains a necessity.

The strong preferences for arginines at P-2 and/or P-3 (relative to the phosphorylated residue at P0) confirm the prediction by Kennelly and Krebs that the substrate specificity of PKA is more restricted in a physiological context.[18] The canonical Arg-Arg-X-Ser is the most abundant consensus sequence, representing slightly more than one-half of all sites. This motif is highly efficient as a PKA substrate since 80% of all Arg-Arg-X-Ser sites within physiological substrates are subject to phosphorylation. Though arginines are the preferred basic residues at P-2 and P-3, one in six sites contains at least one Lys at these positions. Five sites are entirely absent of an Arg at P-2 and P-3. Forty seven percent of all Arg-Lys-X-Ser sites are actually phosphorylated in physiological substrates by PKA, whereas the phosphorylating efficiency is less than one-half that for Lys-Arg-X-Ser sites. The single base-containing motifs Arg-X-X-Ser and Arg-X-Ser may also serve as substrates, albeit with less than 10% efficiency. Their high representation among PKA substrates is due to the 3-fold higher abundance of these motifs compared to the double-base motifs.

Serine is by far preferred over threonine as the phosphate acceptor, occurring in 135 of the 145 sequences examined. This is mostly due to the 12-fold greater prevalence of Arg-Arg-X-Ser vs Arg-Arg-X-Thr in physiological substrates. Nevertheless, there is still a 2- to 3-fold more likelihood of a double-base Ser-containing consensus site to be phosphorylated, and there is only one physiologically relevant single-base Arg-X-X-Thr or Arg-X-Thr phosphorylation site out of 263 potential sites. It is interesting to note that eight substrates have adjacent serine residues in which both serve as phosphate acceptors for PKA. These serines are usually immediately preceded by at least two basic residues. Another 21 sites have adjacent Ser/Thr residues abutted by basic residues

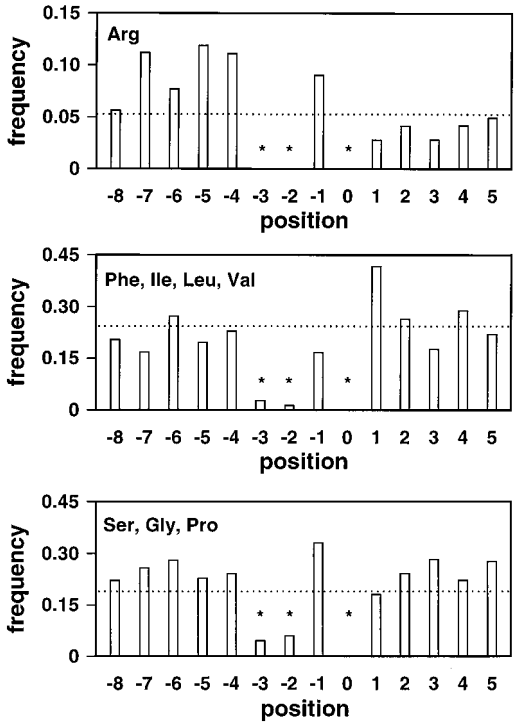

Figure 2. Frequency of occurrence of selected groups of amino acids at positions near the PKA consensus phosphorylation site. Asterisks represent the canonical Arg-Arg-X-Ser at position P-3 through P0. The dashed lines represent the frequency of occurrence of the indicated amino acids in a general protein population ($R = 0.051$; FILV = 0.249; SGP = 0.091).[439]

but are only phosphorylated at one serine by PKA.

Other more subtle preferences at positions outside of the P-2 and P-3 positions also emerge upon closer inspection (Figure 2). There is a slight preference for Arg at the P-4 to P-7 positions and an increased occurrence of the hydrophobic residues Phe, Ile, Leu, and Val at P+1. There is also an increased frequency of the small residues Ser, Gly, and Pro at P-1. These positional biases are consistent with numerous in vitro studies utilizing peptides based on well-characterized substrates such as the pyruvate kinase LRRASLG heptapeptide "kemptide" as well as through biochemical and crystallographic analysis of PKA−inhibitor peptide interactions[6,8,15] and confirmed through the characterization of PKA substrates in highly complex peptide libraries.[42,43]

D. Alternate Strategies for cAMP Regulation

Though cAMP-dependent protein kinase mediates most of the functions of cAMP, work in the past decade has shown that cAMP signaling can be mediated through other mechanisms. PKA is one of several proteins that are allosterically regulated by cyclic nucleotides.[44] These include cGMP-dependent protein kinases, cyclic nucleotide-gated cation channels, and the bacterial cAMP receptor protein, all of which share homologous cyclic nucleotide-binding domains. The most recent additions to this family are the cAMP-guanine nucleotide exchange factors. This family of cyclic nucleotide binding proteins will likely continue to diversify as a result of genome database mining. Two other distinct families of cyclic nucleotide-binding domains, the cGMP-regulated phosphodiesterases and the *Dictyostelium discoideum* extracellular cAMP receptor, provide yet further targets for cyclic nucleotide action.

1. cAMP Cross-Activation of cGMP-Dependent Protein Kinase

In certain physiological and pathophysiological circumstances, cAMP may act through cGMP-dependent protein kinase (PKG).[45] This is best illustrated with PKG-mediated smooth muscle relaxation. Though PKG has a 10- to 100-fold preference for cGMP, the concentration of cAMP is typically 5−6 times higher in cell types such as smooth muscle cells. Relaxant effects of elevated cAMP have been demonstrated to be due to "cross-activation" of PKG in a variety of intact vascular smooth muscle[46−50] and isolated vascular smooth muscle cell preparations.[51,52] These observations have yet to be demonstrated in an intact animal model. Mice deficient in the type I isoform of PKG exhibit defects in nitric oxide-dependent vascular and intestinal smooth muscle regulation while maintaining cAMP-responsiveness, indicating independent pathways of smooth muscle regulation by cAMP and cGMP.[53−57] This animal model does not permit the demonstration of a secondary cAMP effect mediated through PKG. Such a demonstration would require a comparable PKA-deficient animal model to conclusively determine if elevated cAMP might act in part through PKG to mediate smooth muscle relaxation. The possibility that cAMP may act as a physiological activator of a closely related protein kinase suggests that caution must be used in assigning a cAMP-dependent phosphorylation event to PKA in cell types in which PKG may also be present.

2. Cyclic Nucleotide-Gated Cation Channels

Though cyclic nucleotide-gated channels (CNGs) generally bind cGMP preferentially, several of these channels are probably physiologically modulated by cAMP.[58,59] The best studied of the cAMP-gated channels is the olfactory cyclic nucleotide-gated channel, which is activated with equal potency by cAMP and cGMP.[60] This nonselective cation channel is activated in response to odorant binding to a class of G protein-coupled olfactory receptors that in turn activate adenylyl cyclase.[61,62] Mice deficient in the olfactory channel are anosmic, indicating that olfaction is dependent on this cAMP-signaling pathway.[63] PKA-independent cAMP-gating of ion channels also occurs in cell types other than olfactory neurons, including sinoatrial node myocytes,[64] adrenocortical cells,[65] hippocampal pyramidal cells,[66] and gonadotropin releasing hormone-secreting neuronal cells.[67] The channels that are modulated directly by cAMP binding are either homologues of the olfactory cyclic nucleotide-gated channel or more closely related to the prototypical *Drosophila* cyclic nucleotide-regulated voltage-gated potassium channel *ether-á-go-go*.[68] The detection of cyclic nucleotide-gated channel isoforms in still other tissues suggests that more examples of cAMP regulation via direct interaction with channels will emerge soon.

3. Guanine Nucleotide Exchange Factors

The most recent additions to the cAMP receptor family are a group of cyclic nucleotide-regulated guanine nucleotide exchange factors (cAMP-GEFs), also referred to as Epac (exchange protein activated by cAMP).[69,70] This family of regulatory proteins provides an important PKA-independent link to the cross-talk between growth factor and cAMP signaling pathways (reviewed in refs 71 and 72). Two Epac isoforms, Epac1 and Epac2, activate the small G proteins Rap1 and Rap2 upon direct binding of cAMP.[69,70,73] Epac2 also interacts with the Rab3-interacting molecule Rim2, implicating this form of cAMP regulation in synaptic vesicle fusion.[74] A third cAMP-GEF, CNRasGEF, activates Ras upon direct binding of cAMP.[75] This latter cAMP-GEF is most likely responsible for the cAMP-mediated PKA-independent activation of the Ras/MAPK pathway in melanocytes and thyroid cells.[76,77] The integration of PKA-mediated cAMP signaling and growth factor signaling is discussed in a later section.

4. Other Cyclic Nucleotide Receptors

The cAMP receptor protein of *Esherichia coli* is yet another member of the PKA/CNG/cAMP-GEF family of cyclic nucleotide binding domain-containing proteins, indicating that this domain has ancient origins. This DNA-binding protein regulates the transcription a large number of genes including many involved in catabolite repression (reviewed in refs 78 and 79). No comparable eukaryotic cAMP-binding transcription factors are known.

The remaining characterized families of cyclic nucleotide receptors are structurally unrelated to the ones thus far discussed. Several cyclic nucleotide phosphodiesterases, including PDE2, PDE5, and PDE6, also contain distinct cGMP-binding domains that allosterically modulate their catalytic activity.[80,81] The PDE cGMP-binding domains are members of the GAF regulatory domain superfamily.[82] A recently cloned novel PDE isoform, PDE10A, may represent the first example of a GAF-containing protein that is allosterically regulated by cAMP.[83−85]

The extracellular cAMP receptor proteins of *Dictyostelium discoideum* are a family of G protein-coupled receptors that mediate the progression of slime mold differentiation.[86] Cyclic AMP has acquired the unusual role of chemoattractant in this organism in addition to its conventional function as intracellular second messenger. Other potentially important mediators of cyclic nucleotide action are the cyclic nucleotide exporters.[87] These transporters have yet to be fully characterized, but it is likely that they are important for helping to maintain intracellular cAMP and cGMP levels through ATP-dependent export of these nucleotides. The multidrug resistance protein 5 has recently been identified as a functional cyclic nucleotide exporter.[88] Since this transporter is selective for cGMP, it is very likely that other cAMP exporters remain to be identified.

II. Targets of PKA Phosphorylation

Though the modes of cAMP regulation are varied, the most pervasive means of mediating cAMP action remains through PKA. PKA is distinguished from all other cAMP receptors by virtue of its ubiquitous distribution in eukaryotic organisms and its pleiotropic effects. The description of targets of PKA regulation is therefore organized along functional lines. The organization is somewhat arbitrary. For instance, cAMP is intimately involved in dopaminergic neurotransmission, yet, in this review, the relevant neural substrates are scattered in topics ranging from cAMP signaling to protein phosphatases, to cellular trafficking and motility. In a few systems, an attempt is made to describe all the relevant components of a physiological process affected by PKA. It is for this reason that a discussion of cAMP regulation of calcium flux will be found along with contractile proteins in the context of striated muscle contraction instead of being grouped with sodium and potassium channels under the heading of ion conductance. Discussion will begin with a description of PKA as a substrate of itself.

A. Autophosphorylation

The type II regulatory subunit isoforms of PKA, RIIα and RIIβ, are subject to autophosphorylation (reviewed in ref 89).[1,9,11] Of these, RIIα has been the most carefully studied. Direct sequencing of this subunit, purified from bovine muscle, established the in vivo PKA site at Ser-95.[90] In the absence of magnesium and ATP, RIIα has subnanomolar affinity for the C subunit. RIIα is immediately phosphorylated in the presence of magnesium and ATP, and though its affinity for the C subunit decreases 100-fold, it still remains a very effective competitive inhibitor with respect to other substrates.[91,92] This inhibition is allosterically relieved by cAMP binding to RIIα. It is intriguing that autophosphorylation of RIIα differentially increases its affinity for certain AKAPs from as little as 2-fold for Ht31 to as much as 250-fold for mAKAP in vitro.[93]

Though the in vitro studies with RIIα suggest potential modulatory roles for autophosphorylation, it is unlikely that such a role exists in the cell since the presence of ATP would dictate that RIIα is in the phosphorylated state whenever it is complexed with the C subunit. Consistent with this, RIIα is predominantly phosphorylated in resting tissues. In the presence of cAMP, dissociated RIIα is exposed to phosphatases but the rate of dephosphorylation varies considerably among tissue types.[94−96] Under certain pathophysiological conditions, a change in RII autophosphorylation may occur. For example, human hearts with dilated cardiomyopathy exhibit lower steady-state RIIα autophosphorylation than do nonfailing hearts.[97] A possible consequence is the differential redistribution of PKA based on altered affinities to AKAPs and hence altered β-adrenergic responsiveness of failing myocardium.[93,97]

The type I regulatory subunit isoforms of PKA inactivate the C subunit in a manner similar to RIIα except that the autophosphorylation site is replaced by a high-affinity "pseudosubstrate" that contains an alanine in place of a phosphorylatable serine. The heat-stable protein kinase inhibitor (PKI) of PKA also contains a pseudosubstrate site, but unlike the

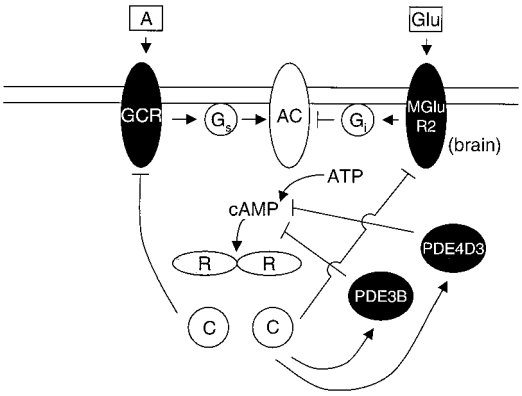

Figure 3. Generalized scheme of PKA-mediated regulation of cAMP signaling. Different cell types will have distinct complements of phosphodiesterases and receptors. PKA regulatory (R) and catalytic (C) subunits. Components shown are as follows: Agonists (A, Glu) for G_s-coupled receptors (GCR) such as β_2 adrenergic or D1 dopaminergic receptors or the G_i-coupled metabotropic glutamate receptor 2 (mGluR2), adenylyl cyclase (AC), heterotrimeric G proteins (G_s, G_i), cyclic nucleotide phosphodiesterases (PDE3B, PDE4D3). Black components are targets of PKA regulation. Gray components are cyclic nucleotide-binding proteins.

regulatory subunits, PKI inhibition of the C subunit is not relieved by cAMP.

B. Intracellular Signaling

Cyclic AMP action occurs in concert with multiple intracellular signaling pathways as cells respond to a complex array of extracellular stimuli. The response of an individual cell type to its environment is dependent on its complement of cellular receptors and of the isoform makeup of the various intracellular signaling cascades. Cyclic AMP can exert its influence on other signaling pathways not only through direct phosphorylation via PKA (and in a few instances PKG), but also through modulation of small G proteins by cAMP-GEFs and regulation of calcium flux by cyclic nucleotide-gated cation channels. Depending on the combination of cAMP effectors and downstream targets, cAMP can have opposing effects on other signaling cascades. This can explain, for example, why cAMP inhibits the growth of some cell types yet stimulates the growth of other cell types. Before discussing the signaling pathways with which cAMP is known to network, the autoregulation of cAMP will first be examined.

1. Cyclic AMP Signaling

An overview of PKA-mediated regulation of cAMP signaling is illustrated in Figure 3. In most cases, PKA acts as a feedback inhibitor to blunt cAMP elevation, either through desensitization of G protein-coupled receptors or stimulation of phosphodiesterases. PKA may also potentiate cAMP levels, as happens with phosphorylation of the metabotropic glutamate receptor 2 (mGluR2).

One mechanism by which PKA-mediated feedback inhibition of cAMP signaling occurs is through heterologous desensitization of selected G protein-coupled receptors. By far the most extensively studied of these receptors is the β_2-adrenergic receptor (reviewed in ref 98).[99] Upon agonist binding, the β_2-receptor activates the heterotrimeric G protein G_s, which in turn stimulates adenylyl cyclase, resulting in the elevation of intracellular cAMP and activation of PKA. PKA-dependent phosphorylation causes a switching of receptor coupling from G_s to the adenylyl cyclase inhibitory G protein G_i, thus lowering intracellular production of cAMP.[100] The β_2-receptor is phosphorylated by PKA at up to two sites in situ. The primary site involved in desensitization is Ser-262, a site that is readily phosphorylated in situ.[101,102] Secondary sites at Ser-345 and Ser-346 are located close to a cysteine that can be reversibly palmitoylated. Phosphorylation at this site occurs only when the β_2-receptor is in the depalmitoylated state.[103] Since depalmitoylation is agonist-induced,[104] phosphorylation of Ser-345 and Ser-346 can be seen in agonist-induced receptors but not in receptors phosphorylated by direct activation of PKA with agents such as dibutyryl cAMP.[39] Though phosphorylation of Ser-345 and Ser-346 is necessary for rapid onset of β2-receptor desensitization, it is one step in a sequential mechanism that also includes receptor phosphorylation by the β-adrenergic receptor kinase.[39] Recent evidence suggests that cAMP-dependent phosphorylation of the β_2-receptor also requires co-localization of PKA with the receptor mediated by AKAP79/150[105] or gravin.[106,107]

At least one other G_s protein-coupled receptor has been demonstrated to be a physiological substrate for PKA. Coupling of the D1 dopamine receptor to G_s is reduced upon cAMP-dependent phosphorylation, resulting in attenuation of dopamine-induced adenylyl cyclase activity in C6 glioma cells.[108]

Whereas the β_2-adrenergic receptor and D1-dopamine receptors mediate their own desensitization through stimulation of PKA, there are at least two examples of G protein-coupled receptors not linked to G_s that are heterologously desensitized in response to signaling through G_s-coupled receptors in the same cell. One such receptor, the G_q-coupled thromboxane A_2 receptor, is described in section II.B.3. The metabotropic glutamate receptor mGluR2 also undergoes heterologous desensitization by cAMP-dependent phosphorylation.[109] This neural receptor is coupled to G_i/G_o, which inhibits adenylyl cyclase and gates several cation channels. PKA can potentially antagonize mGluR2-induced inhibition of adenylyl cyclase in neurons. In this case, a physiologically relevant G_s-coupled receptor has not been identified yet. PKA-mediated heterologous desensitization is therefore a generalized phenomenon that not only desensitizes G protein-coupled receptors that directly elevate cAMP but can also attenuate coupling between receptors and other classes of G proteins.

A second mechanism in which PKA mediates feedback inhibition of cAMP production is through activation of at least two cAMP phosphodiesterases (reviewed in ref 80).[110] In adipocytes, isoproterenol-induced increases in cAMP lead to PKA-dependent activation of the cGMP-inhibited phosphodiesterase PDE3B through phosphorylation of Ser-302.[111,112] This phosphodiesterase nonselectively hydrolyzes both cAMP and cGMP. Similarly, the cAMP-specific

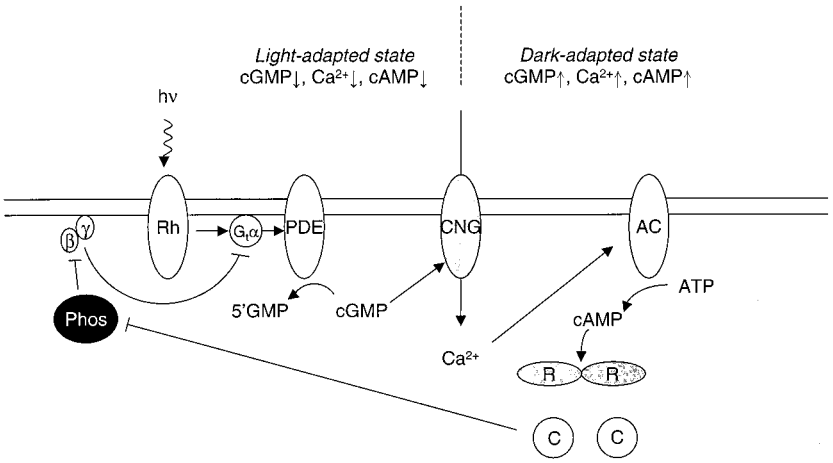

Figure 4. PKA feedback inhibition of light-induced phosphodiesterase in the retina. R, C, and AC are defined in Figure 3. Other components are rhodopsin (Rh), the α, *β*, and *γ* subunits of the heterotrimeric G protein transducin ($G_t\alpha$, *β*, *γ*), cGMP phosphodiesterase (PDE), cyclic nucleotide-gated cation channel (CNG), and phosducin (Phos). Shaded components are described in Figure 3.

phosphodiesterase PDE4D3 is activated by PKA-dependent phosphorylation at Ser-54[113,114] in thyroid-stimulating hormone-induced rat thyroid cells or chorionic gonadotropin-induced mouse leydig tumor cells.[113,115] The general effect of the PKA-dependent activation of phosphodiesterases is to dampen the initial burst of cAMP that is produced in response to hormonal challenge, possibly producing a spike of cAMP intensity that may be important for controlled acute regulation through this second messenger.

2. cGMP Signaling

Protein kinase A can affect cGMP signaling in at least two ways. As described in section II.B.1, cGMP signaling is impacted by direct PKA phosphorylation of the cGMP-inhibited phosphodiesterase PDE3B. PKA can also indirectly inhibit light-induced cGMP phosphodiesterase activity in the retina (Figure 4). Cyclic GMP levels are modulated in the retina in part through PKA-dependent phosphorylation of the retinal protein phosducin. This is an indirect adaptive feedback mechanism in response to changing light levels that involves an integration of cGMP, cAMP, and calcium signaling.[116] In dark-adapted retina, elevated cGMP levels keep cGMP-gated cation channels open, maintaining rod cells in a depolarized state. In the light-adapted state, rhodopsin stimulates a membrane-bound cGMP phosphodiesterase through activation of the heterotrimeric G protein transducin (G_t). As cGMP levels decrease, the cGMP-gated channels close and the rod hyperpolarizes. Elevated calcium in the dark-adapted state activates a Ca^{2+}/calmodulin-dependent adenylyl cyclase, which leads to increased cAMP and activation of PKA.[117] The lower calcium in the light-adapted state results in decreased cAMP and inactivation of PKA. Thus, phosducin is phosphorylated in the dark-adapted retina and dephosphorylated in the light-adapted retina.[118] Dephospho-phosducin competes with $G_t\alpha$ for the binding of $G_t\beta\gamma$,[119,120] effectively reducing the level of activated $G_t\alpha$ over time. The net effect is a dampening of light-induced cGMP phosphodiesterase activity. When the retina returns to the dark-adapted state, phosducin is phosphorylated and no longer able to interact with $G_t\beta\gamma$,[119,120] permitting reassociation of $G_t\alpha$ with $G_t\beta\gamma$ making it available for reactivation.

3. Phosphoinositide and Calcium Signaling

Cyclic AMP-dependent phosphorylation affects phosphoinositide signaling at multiple levels (Figure 5). Like certain G protein-coupled receptors linked to cAMP metabolism (section II.B.1), G protein-coupled receptors linked to phosphoinositide metabolism can also undergo heterologous desensitization. The thromboxane A_2 receptor TPα, which is coupled to phosphoinositide signaling through a G_q-related G protein, is the target of PKA phosphorylation and desensitization mediated through G_s-coupled prostacyclin receptor activation.[121] It is postulated that this type of cross-talk may occur in platelets or smooth muscle. Phospholipase C isoforms are also targets of PKA regulation.[122] The coupling of ectopically expressed $G_q\alpha$ and phospholipase C*β*3 is attenuated in COSM6 cells by PKA phosphorylation of Ser-1105.[123] Phospholipase C*γ*1 is also a substrate for PKA. Forskolin-treatment of Jurkat T cells results in phosphorylation of phospholipase C*γ*1 at Ser-1248, preventing its T cell receptor-mediated tyrosine phosphorylation-induced phosphoinositide hydolysis.[124] The type I inositol 1,4,5 trisphosphate (IP_3) receptor is also a physiological substrate for PKA in neuronal and kidney cells.[125−127] The effect of cAMP-dependent phosphorylation of this calcium channel of the endoplasmic reticulum is uncertain, however, and may depend on the cell type and physiological context in which it is being examined.[128]

Protein kinase A interacts with calcium signaling primarily at the level of calcium mobilization as described in the context of striated muscle contraction (see section II.K). Less common is the direct action of PKA on a calcium/calmodulin-dependent enzyme. Elongation factor-2 kinase (eEF-2K), a calcium/calmodulin-dependent protein kinase, inhibits protein synthesis through phosphorylation and inactivation of elongation factor-2. Calcium/calmodulin-independent activity of eEF-2K is enhanced in vitro and in situ by PKA phosphorylation at Ser-499.[129] It is proposed that this is the likely mechanism of

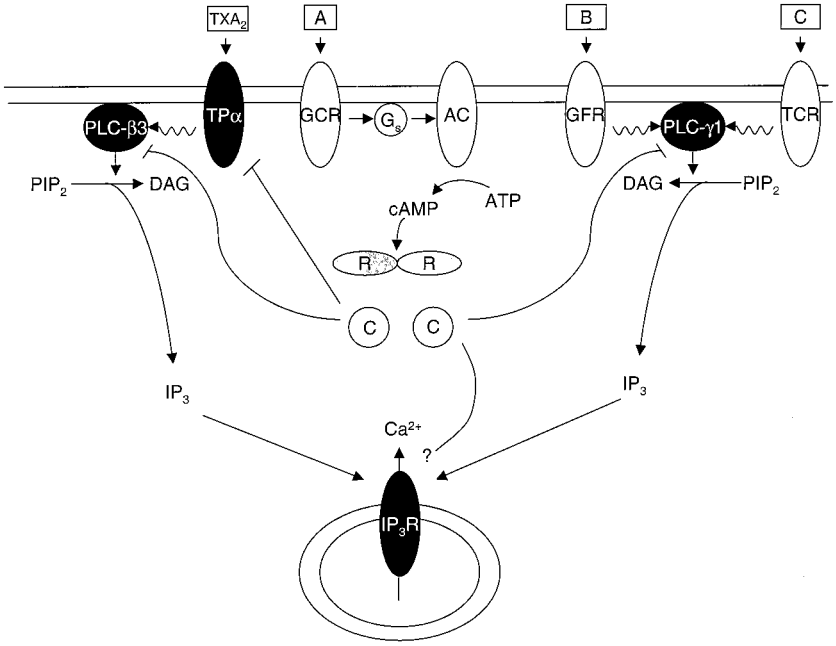

Figure 5. PKA regulation of phosphoinositide signaling. Different cell types will have distinct complements of receptors and phospholipases. The components AC, G_s, R, and C are defined in Figure 3. Other components shown are agonists (A, B, C, TXA_2) for G_s-coupled receptors (GCR) such as the prostacyclin receptor, growth factor receptors (GFR), T cell receptor (TCR), and the thromboxane A_2 receptor (TPα), phospholipases (PLC-β3, PLC-γ1), and the type I inositol 1,4,5 trisphosphate receptor (IP_3R) found in the endoplasmic reticulum. Shaded components are described in Figure 3.

protein synthesis inhibition by catabolic hormones such as glucagon.

4. Rho Signaling

The small G proteins of the Rho subfamily transduce signals from G protein-coupled receptors via activation of heterotrimeric G proteins and Rho modulators, including guanine nucleotide exchange factors (GEFs), GDP dissociation inhibitors (GDIs), and guanine nucleotide activating proteins (GAPs) (reviewed in ref 130). GTP-bound Rho A activates Rho kinases, which in turn modulate an array of physiological responses including actin cytoskeleton rearrangements. RhoA can also be directly modulated by PKA phosphorylation, which can affect its interaction with upstream and downstream components of the Rho signaling pathway. Phosphorylation enhances translocation of RhoA to the cytosol through increased interaction of GTP-bound RhoA with Rho-GDI, possibly terminating its activity.[131] RhoA phosphorylation by PKA also reduces RhoA-mediated actin stress fiber formation, possibly through inhibition of interaction with the Rho kinase ROKα.[132]

5. T Cell Receptor Signaling

Activation of T cells through the T cell receptor involves a complex series of events that includes the activation of the Src family tyrosine kinases Lck and Fyn. Cyclic AMP exerts an inhibitory effect on this pathway through PKA phosphorylation and activation of the COOH-terminal Src kinase (Csk), which in turn phosphorylates and inhibits Lck.[133] Mutation of Ser-364 yields poorly expressed inactive Csk, whereas a Ser-to-Cys mutation yields a fully active Csk that is refractory to cAMP and PKA activation. Since mutagenesis of Ser-364 can affect the structural integrity of Csk, there may be some question as to whether this residue is indeed a phosphate acceptor site for PKA or if mutation of this site leads to structural perturbations that prevent Csk phosphorylation. The inhibition of T cell receptor signaling by cAMP is one of the best prospects for defining a physiological role for PKA type I isozyme-specific co-localization with a putative target.[133,134]

6. Mitogen-Activated Protein Kinase Signaling

The interplay between cAMP and mitogen-activated protein kinase (MAPK) cascade is complex (summarized in Figure 6). Stimulation or inhibition of cell growth by cAMP is dependent on the complement of MAPK cascade components in a given cell type that are substrates of PKA or are targets of regulation by cAMP-GEFs (reviewed in refs 71 and 135). Targets of cAMP regulation include protein kinases, tyrosine phosphatases, and small G proteins and their modulators.

The cAMP-dependent inhibition of the MEK kinase Raf is thought to be a major point of cross-talk between the cAMP and MAPK pathways (reviewed in ref 71). Raf-1 is phosphorylated at Ser-43 in response to elevated cAMP in intact cells.[136,137] Mutation of Ser-43 to alanine prevents in situ phosphorylation in response to forskolin.[138] Phosphorylation at this site reduces the capacity for Raf-1 to interact with Ras in vitro.[136,139] Since the EGF-activated forms of both wild-type and the Ser-43-Ala mutant forms of Raf-1 are inhibited by forskolin treatment in HEK293 cells,[138] this suggests that cAMP inhibition of Raf-1 is independent of Ser-43 phosphorylation. Further work is needed to define a functional role for Ser-43 phosphorylation. Another Raf-1 residue, Ser-621, is also phosphorylated in COS-1 cells overexpressing the PKA catalytic subunit.[140] The in vitro

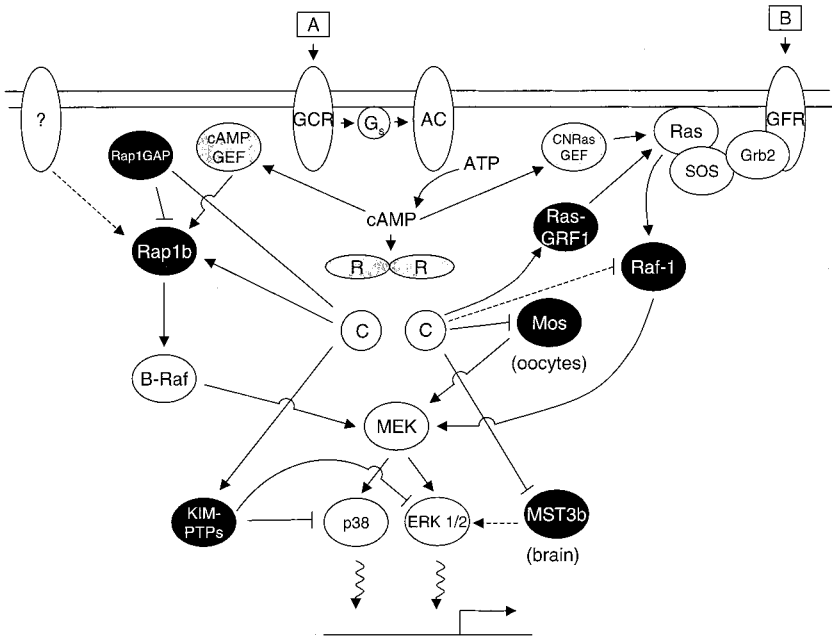

Figure 6. Cyclic AMP regulation of the mitogen-activated protein kinase signaling pathway. Different cell types will have distinct complements of signaling proteins. A, B, GCR, G_s, AC, GFR, R, and C are defined in Figures 3 and 5. Other components shown are defined in the text (see section II.B.6).

phosphorylation of Ser-621 inhibits Raf-1 kinase activity.[140] Though Ser-621, like Ser-43, is an in vivo site of phosphorylation,[136,141] its phosphorylation state is not increased in response to cAMP[136,138] and is therefore not listed in Table 1. Instead, Ser-621 may be either a site of autophosphorylation[142] or of another protein kinase such as the AMP-activated protein kinase.[143] Although Raf-1 is a physiological substrate of PKA, the mechanism by which cAMP inhibits Raf-1 function in cells is still unresolved.

Two other protein kinases that modulate the activity of the MAPK pathway are also targets of cAMP regulation. Mos is an oocyte-specific MEK kinase required for oocyte maturation. PKA phosphorylates v-Mos at two sites in intact cells. Phosphorylation at Ser-263 inhibits its protein kinase activity,[144] whereas phosphorylation of Ser-56 prevents autophosphorylation of Ser-34, which is required for v-Mos activation.[145] The viral-encoded v-Mos is identical to the cellular homologue c-Mos, except for a 31 amino acid N-terminal extension, suggesting that c-Mos is subject to similar PKA-dependent regulation. The mammalian STE20-like kinase-3 (MST3b) is a brain-specific protein kinase that is an upstream activator of the MAPK pathway. Phosphorylation of Thr-18 negatively regulates MST3b kinase activity as inferred by increased phosphorylation of ERK 1/2 in HEK293 cells transfected with a Thr-18-Ala mutant.[146] The mechanism of this inhibition is not yet resolved.

A group of protein tyrosine phosphatases (PTPs) that share a so-called "kinase inhibitory motif" (KIM)[147] dock with Erk1/2 and p38 members of the MAPK family via their KIMs and prevent their translocation into the nucleus by dephosphorylating them. These KIM PTPs, including the neural phosphatases PTP−SL, the striatal-enriched tyrosine phosphatase STEP, and the hematopoietic PTP hePTP, have recently emerged as substrates for PKA in intact cells. PKA phosphorylation of a site within the KIM-containing PTPs does not affect PTP catalytic activity but rather disrupts the interaction between the PTP and its MAPK. Thus, the PKA-dependent phosphorylation of hePTP may explain how elevated cAMP can activate MAPK in Jurkat T cells.[148] It is also expected that cAMP will activate MAPK via PTP−SL in the specialized brain cells where it is expressed.[149] Finally, STEP phosphorylation by PKA may play an important role in mediating dopamine responses in D1 receptor-containing neurons in the striatum.[150]

Cyclic AMP may also stimulate cell proliferation via members of the Ras subfamily of small G proteins either through direct phosphorylation of a small G protein or through phosphorylation of one of the modulators of small G protein function. The Ras-related small G protein Rap1 is a substrate for PKA in intact cells.[151] Elevation of intracellular cAMP activates Rap1b by increasing its bound GTP/GDP ratio in situ, resulting in activation.[152] Phosphorylation, however, accounts for only part of the cAMP response.[152] Rap1b can still be activated by cAMP in a PKA-deficient cell line[70] or in the presence of the PKA inhibitor H89.[69,70] These observations are explained by the cAMP-GEFs described in section I.D.3.[70] Rap1b may connect to the MAP kinase pathway via B−Raf activation. The GTPase activating protein Rap1GAP is also a substrate for PKA in SK-MEL cells.[153,154] There is no evidence as yet, however, of the functional consequences of this phosphorylation. The Ras guanine nucleotide exchange factor Ras-GRF1 is phosphorylated by PKA in situ. This phosphorylation is necessary but not sufficient for full stimulation of RAS-GEF activity.[155]

7. Modulators of Protein Phosphatase 1

The regulation of phosphoprotein phosphatase 1 (PP1) activity by cAMP-dependent protein kinase is

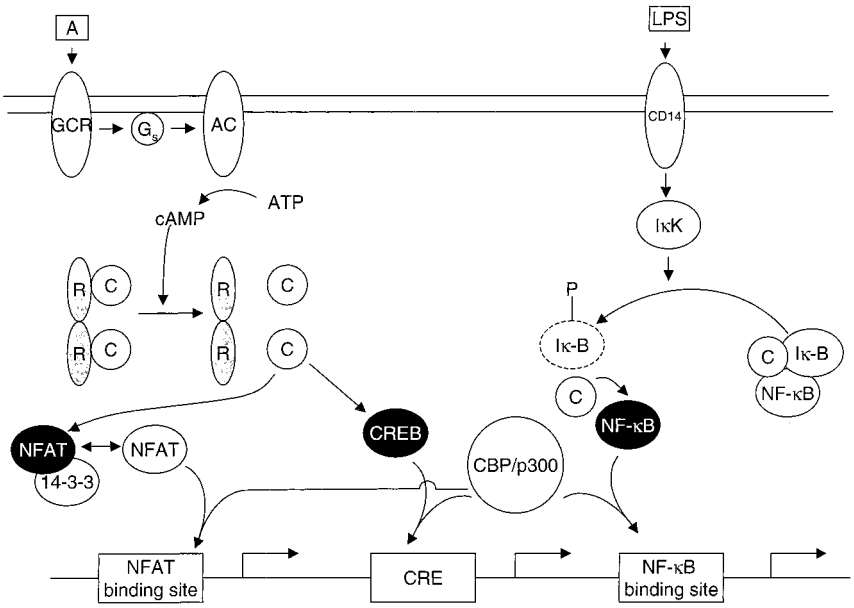

Figure 7. PKA regulation of transcription factors that interact with CBP/p300. Most components are defined in Figure 3 or in the text (see section II.C). Other components are the agonist lipopolysaccharide (LPA) and its receptor (CD14). The broken line around the phosphorylated form of Iκ-B represents its ubiquitin-dependent degradation. Shaded components are described in Figure 3.

fundamental to the understanding of an increasing number of cAMP-regulated events that cannot be explained by direct PKA phosphorylation of a target substrate (reviewed in refs 156−159). This broad-specificity ubiquitous serine/threonine phosphatase interacts with regulatory proteins or subunits that control its proximity to physiological substrates or its catalytic activity. Cyclic AMP-dependent phosphorylation modulates these interactions.

The inhibitor-1 peptide (I-1) of PP1 is phosphorylated at Thr-35[160] in skeletal muscle of epinephrine-treated rabbits[161] or isoproterenol-treated rat adipose tissue.[162] In its phosphorylated state, I-1 inhibits PP1 at nanomolar concentrations.[163] There is substantial evidence to suggest that cAMP-dependent phosphorylation of I-1 is an important step in the regulation of several metabolic enzymes, including cAMP-mediated inhibition of glycogen synthase and acetyl CoA carboxylase.[37] The 32 kDa dopamine and cAMP-regulated phosphoprotein (DARPP-32), a homologue of I-1, is enriched in dopamine receptor-containing nerve terminals in the brain. It is also converted into a potent inhibitor of PP1[164] when phosphorylated at Thr-34.[165,166] Dopamine can stimulate either the phosphorylation or the dephosphorylation of this site depending on whether it activates the G_s-coupled D1 or the G_i-coupled D2 dopamine receptor in appropriate neurons in mouse striatal slices.[167] The central role that DARPP-32 plays in mediating dopaminergic responses has been demonstrated in mice lacking this protein (reviewed in ref 158). The nuclear inhibitor of PP1A, NIPP-1, is yet a third member of this family of proteins that is activated by PKA, at least in vitro.[168] It remains to be seen if NIPP-1 is also phosphorylated directly by PKA in the intact cell.

Another class of PP1 regulatory proteins includes the glycogen-binding subunit G_M. This subunit anchors active PP1 to glycogen particles where it co-localizes with glycogen synthase and glycogen phosphorylase in striated muscle. Upon stimulation with epinephrine, rabbit skeletal muscle G_M is phosphorylated at two sites by PKA.[169−171] One of these sites, Ser-65, causes the release of the PP1 catalytic subunit.[170] In the resting state, the co-localized PP1 is thought to keep glycogen phosphorylase and glycogen synthase in the dephosphorylated state, thus favoring glycogen synthesis, whereas epinephrine stimulation leads to cAMP-dependent dissociation of PP1 from the glycogen particle and a shift toward phosphorylated glycogen phosphorylase and glycogen synthase and net glycogenolysis (reviewed in refs 37 and 157). The role of cAMP-dependent regulation of PP1 in cardiac muscle contraction is described in section II.K.

C. Transcriptional Regulation

Cyclic AMP, through PKA, exerts long-term effects on different tissues by modifying the functions of a large variety of nuclear proteins including ubiquitous transcription factors, nuclear hormone receptors, other transcriptional activators/repressors, and chromatin-associated proteins. As with effectors of the MAPK pathway, the tissue-specificity of PKA-phosphorylated transcription factors explains many of the variations in long-term responses to cAMP that are observed among cell types. Much like the modulation of protein kinases and phosphatases, PKA phosphorylation of multifunctional transcription factors results in pleiotropic responses of cells to extracellular stimuli.

The cAMP responsive element (CRE) is the most common cis-acting promoter element through which the transcriptional effects of cAMP are mediated (Figure 7). The trans-acting elements that specifically bind to these elements are the homologous and ubiquitous CRE binding protein (CREB) and the CRE modulator (CREM) (reviewed in refs 172−174). The role of PKA-dependent CREB phosphorylation in

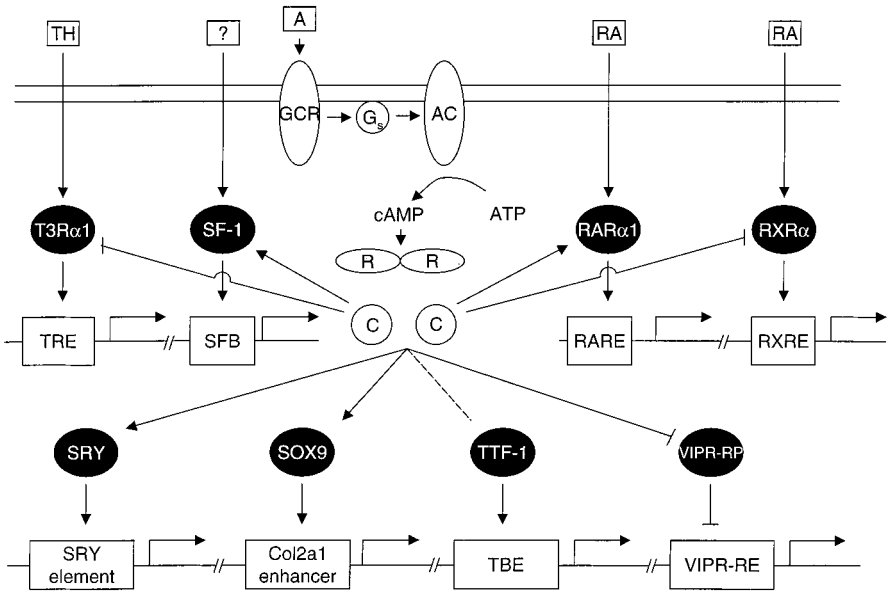

Figure 8. Multiplicity of tissue-specific nuclear receptors and other transcription factors regulated by PKA. Different cell types express distinct complements of transcription factors. Most components are defined in Figure 3 or in the text (see section II.C). Other components are thyroid hormone (TH) and retinoic acid (RA). Boxes are promoter or enhancer elements of the respective transcription factors. Shaded components are described in Figure 3.

activating transcription has been studied more extensively than that of CREM. Phosphorylation occurs in the "kinase inducible domain" at Ser-133 in CREB[175−177] and Ser-117 in CREMτ.[178] In addition to PKA, this and other sites on CREB are also the targets of physiological phosphorylation by other protein kinases, including calcium-calmodulin-dependent protein kinase IV, RSK2, and Protein kinase B. Phosphorylation of Ser-133 by PKA stimulates its transcriptional activity.[175] The exact mechanism by which this enhancement occurs is a subject of debate. Phosphorylation of CREB leads to increased interaction with the transcriptional coactivator CREB-binding protein or the closely related p300 (CBP/p300).[179,180] The phospho-CREB/CBP complex may then recruit and stabilize RNA polymerase to the CRE-containing promoter.[181,182] Alternatively, CREB may be able to recruit RNA polymerase to the CRE-containing promoter independent of its phosphorylation state and of CPB/p300,[183] suggesting that phosphorylation of CREB induces transcription from the preassembled transcription complex.

Another ubiquitous transcription factor, NF-κB, is also a physiological PKA substrate (reviewed in refs 173, 184, and 185). PKA regulates NF-κB through a highly unusual cAMP-independent mechanism[186] (Figure 7). In its inactive state, NF-κB is sequestered in the cytosol by its association with either the α or β isoform of the inhibitor protein I-κB. The I-κB also binds directly with and inactivates the PKA C subunit. Agents that induce NF-κB do so by targeting I-κB for degradation in a phosphorylation-dependent manner through the ubiquitin-26S proteasome pathway. NF-κB is then free to translocate to the nucleus. Protein kinase A is also released upon I-κB degradation and phosphorylates NF-κB. Phosphorylation by PKA stimulates NF-κB transcriptional activity[186] through enhancement of its interaction with the transcriptional coactivator CBP/p300.[187] The potential competition between CREB and NF-κB for binding to CBP/p300 leads to interesting and complex possibilities for cAMP-mediated transcriptional events.[184,187]

The nuclear factor of activated T cells (NFAT) is yet a third CBP/p300-interacting transcription factor. Calcium activation of the phosphatase calcineurin results in the dephosphorylation and nuclear localization of NFAT. Several protein kinases turn off NFAT activity by signaling its nuclear export (reviewed in ref 188). Rather than enhance nuclear export, PKA phosphorylation of the NFAT3 isoform inhibits transcriptional activity by increasing the interaction of NFAT with 14−3−3[189] (Figure 7). The 14−3−3 proteins are one group of a growing number of regulatory proteins that bind phosphoserine moieties on protein surfaces.[190−192] Though NFAT interacts with CBP/p300,[193] the effect of PKA phosphorylation on this interaction has not been examined.

PKA has been implicated in the regulation of at least six different nuclear hormone receptors including receptors for sex steroids, retinoids, and thyroid hormone (listed in ref 194), several of which are described here (Figure 8). Evidence for a physiological role for PKA phosphorylation is strongest for the retinoic acid receptor and the thyroid hormone receptor. Retinoid receptors are important regulators of development and differentiation in vertebrates. Retinoic acid receptors (RARα, β, and γ) are activated by all-trans retinoic acid and 9-cis retinoic acid, whereas retinoid X receptors (RXRα, β, and γ) recognize only the 9-cis isomer. Phosphorylation of RARα1, which is required for cAMP-induced parietal endodermal differentiation of F9 cells,[195] enhances its in vitro DNA-binding activity and its in situ transactivation of retinoic acid-inducible promoters.[196] In contrast, RXRα transcriptional activity is repressed upon in situ PKA phosphorylation.[197] The cAMP-dependent phosphorylation of Ser-28 and Ser-29 on the thyroid hormone receptor T3Rα-1[198,199] inhibits binding of its monomeric form to thyroid hormone

response elements.[199] Phosphorylation of analogous sites (Ser-16,17) on v-ERB A,[198,200] the viral oncogene homologue of the thyroid hormone receptor, is required for oncogenic transformation.[200] The orphan nuclear receptor steroidogenic factor-1 (SF-1) is critical for adrenal and gonadal development and for proper functioning of the hypothalamus−pituitary−gonadal axis. There is substantial evidence that SF-1 is subject to cAMP regulation through direct phosphorylation by PKA (reviewed in ref 201).[202] Loss of cAMP responsiveness of HDL receptor gene tanscription in human HTB9 bladder carcinoma cells upon mutagenesis of the PKA consensus site Ser-430 in SF-1 supports this model.[203]

Transcription factors other than those that interact with CBP/p300 are also modulated by cAMP-dependent phosphorylation, mediating their effects through promoter elements that are distinct from the canonical CRE. Furthermore, they tend to have a restricted tissue distribution and serve specialized functions. The vasoactive intestinal polypeptide receptor transcriptional repressor protein (VIPR−RP) is a transcription factor for the vasoactive intestinal polypeptide receptor-1 (VIPR-1) that shares identity with the differentiation-specific element binding protein. Cyclic AMP-dependent phosphorylation of VIPR−RP results in derepression of VIPR-1 transcription.[204] The sex-determining region of Y protein (SRY) is a Y chromosome-encoded HMG-box-containing transcription factor that is important for testis determination in mammals. Its phosphorylation by PKA increases transcriptional activity in COS7 cells and its in vitro DNA binding to a SRY DNA-binding consensus site.[205] SOX9 (SRY-box-related 9), another HMG-box-containing transcription factor, is expressed in chondrocytes during development as well as in other cell types. Cyclic AMP-dependent phosphorylation of this protein increases its in vitro DNA binding to the chondrocyte-specific enhancer Col2a1 as well as its transcriptional activity in rat chondrosarcoma cells.[206] The Ser-181 phosphorylated form of Sox9 is restricted to chondrocytes in the prehypertrophic zone of the growth plate in mouse embryo hind legs.[30] There is evidence that the PKA C subunit interacts directly with Sox9.[206] A cAMP-independent mechanism of SOX9 phosphorylation by PKA similar to that of NF-κB has not been investigated.

It is well established that thyroid transcription factor-1 (TTF-1) transcriptional activity is regulated by cAMP.[207−211] The two residues that have been proposed as sites of in situ PKA phosphorylation are not part of conventional consensus PKA phosphorylation motifs (KHTT and HAAS), however. Though Thr-9 can be phosphorylated in vitro by PKA, the only evidence for its in situ phosphorylation is the unresponsiveness of a mutated Thr-9-Ala TTF-1 to overexpression of the PKA C subunit in lung adenocarcinoma cells.[209] In contrast, endogenous TTF-1 is phosphorylated at Ser-337 in response to forskolin in differentiated thyroid cells, but mutation of this site does not affect the responsiveness of TTF-1 to overxpression of the PKA C subunit, and there is no direct evidence that PKA can phosphorylate this site in vitro.[211] Further studies are needed before either Thr-9 or Ser-337 can be confirmed as physiological PKA phosphorylation sites on TTF-1.

D. Histones

Histones are targets of a wide variety of posttranslational modifications, including phosphorylation (reviewed in ref 212). Phosphorylation of histone H3 on Ser-10 is an immediate-early response of mammalian cells to mitogens. It is also thought to play a role in regulation of transcription through chromatin remodeling. Mutation of Ser-10 to Ala results in aberrant chromosome condensation and segregation in *Tetrahymena* micronuclei.[213] Phosphorylation of this site is mediated by PKA in isolated FSH-stimulated granulosa cells.[214] PKA phosphorylation of H3 also occurs in gliotoxin-treated thymocytes.[215] Interpretation of the role of PKA modulation of histone H3 function is complicated by the observations that the mitogen-stimulated protein kinases RSK[216] and MSK1[29] can also phosphorylate Ser-10 in situ. It is especially instructive to note that MSK1 is potently inhibited by H89, a commonly used inhibitor of PKA.[29]

Histone H1 holds a place of honor as the first PKA substrate for which the in vivo phosphorylation site was determined. Its phosphorylation is induced by glucagon in perfused rat liver.[217] Phosphorylation in situ of histone H1c has also been observed in mouse neuroblastoma cells induced to differentiate with cAMP elevating agents.[218] The PKA site in Histone H1c is not conserved among subtypes.[219] The functional significance of cAMP-dependent phosphorylation of this chromatin component remains an enigma.

E. Apoptosis and Cell Survival

Programmed cell death, or apoptosis, is a mechanism by which cells are systematically destroyed in response to stress or as part of the normal course of development and differentiation. The pro-apoptotic factor BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) is a part of the complex signaling network that impacts apoptosis. It initiates apoptosis by binding to anti-apoptotic factor Bcl-2 or Bcl-X_L, thus permitting the permeabilization of mitochondrial membranes. BAD is the target of regulation by several protein kinases, which suppress its apoptotic activity (reviewed in ref 220). Recent evidence from a number of laboratories shows that BAD is phosphorylated by PKA in situ. Initially, the site of PKA phosphorylation was thought to be Ser-112,[30] but subsequent independent determinations show that Ser-155 is the preferred physiological site for PKA.[31,221−223] The discrepancy is attributed to the use of antibodies against phospho-Ser-112 that happen to cross-react with phospho-Ser-155.[31] The mitogen-activated protein kinase-activated kinase (MAPKAP−K1) is proposed to be a more likely Ser-112 kinase.[31] To make matters more complicated, both Ser-112 and Ser-155 are also subject to phosphorylation by RSK1.[223] Cyclic AMP-dependent phosphorylation is correlated with suppression of apoptosis,[30,221] probably by preventing BAD interaction with Bcl-2[31,222,223] and enhancing

BAD interaction with the cytosolic 14−3−3 protein.[31] This is the same mode of apoptotic suppression mediated by protein kinase B phosphorylation of Ser-136.[220] The PKA activity that phosphorylates BAD in situ is localized to the mitochondria through an AKAP association.[30]

In hematopoietic cells interleukin-3 (IL-3) promotes cell survival by activating the IL-3 receptor, which leads to phosphatidylinositol-3-kinase (PI-3-K) signaling, protein kinase B activation, and inhibition of BAD.[224] There is also evidence that IL-3 treatment leads to PKA activation and BAD phosphorylation.[30] The PKA-mediated antiapoptotic effects of IL-3 also include enhancement of IL-3 receptor-initiated PI-3-K signaling. Phosphorylation of the IL-3 receptor β_c chain at Ser-585 promotes the association of 14−3−3, which in turn recruits PI-3-K.[225] Though the in situ phosphorylation state of Ser-585 is stimulated by forskolin and inhibited by H89, the phosphorylation site (HSRS) is a poor in vitro PKA substrate, suggesting that there may be another more physiologically relevant but as yet unidentified Ser-585 kinase.

In certain contexts, glycogen synthase kinase-3 (GSK-3) can also be considered a pro-apoptotic factor. Named for the substrate it was first shown to phosphorylate, the physiological targets of GSK-3 now include a range of proteins involved in cell fate determination and tumorigenesis (reviewed in ref 226). In mammals, insulin and other growth factors inhibit the activity of this enzyme through activation of the PI-3 kinase/Protein kinase B pathway, promoting cell survival. In rat cerebellar granule neurons, cAMP also appears to exert some of its anti-apoptotic effects through inhibition of the GSK-3β isoform by direct PKA phosphorylation.[227] The PKA in situ phosphorylation of both GSK-3α and GSK-3β occur at the same sites[227,228] as those determined for protein kinase B,[229] making GSK-3 a point of convergence of cAMP and insulin signaling.

F. Ion Conductance

More than a third of all the PKA substrates listed in Table 1 are proteins involved in ion conductance. In this section, cation and anion channels gated by extracellular chemical ligands are discussed first. This will be followed by a discussion of PKA-regulated sodium, chloride, and potassium channels and transporters that are either voltage-gated or involved in maintaining electrochemical gradients. The role of PKA in modulating calcium ion conductance in striated muscle contraction is discussed in section II.K. The IP_3 receptor, another calcium channel, is discussed in section II.B.3. When cyclic nucleotide-gated cation channels, described in sections I.D.2 and II.B.3, are added to the milieu of PKA-regulated ion channels and transporters, it is clear that cAMP is a pervasive modulator of the electrical and ionic status of eukaryotic cells.

1. Ligand-Gated Channels

Electrical signals are propagated across synapses by neurotransmitters that bind to a variety of receptors on the postsynaptic neuron. Ligand-gated ion channels represent a class of neurotransmitter receptors that can rapidly change the electrical potential of the postsynaptic neuron by opening or closing its cation or anion pore upon neurotransmitter binding. This is in contrast to the slower acting G protein-coupled neurotransmitters, which must work through second messenger systems. The ligand-gated ion channels are regulated, principally by protein phosphorylation, to modulate their responsiveness to neurotransmitter stimulation (reviewed in ref 230). This section will outline the PKA regulation of six such channels.

Neuronal signals are transmitted to skeletal muscle through acetylcholine binding to the nicotinic acetylcholine receptor at the neuromuscular junction. The acetylcholine-dependent opening of the channel causes an influx of cations and depolarization of the muscle cell. The acetylcholine receptor is a pentamer with a subunit composition of $\alpha_2\beta\gamma\delta$. Though there is evidence for the phosphorylation of the α and γ subunits, the δ subunit has been best characterized in terms of physiologically relevant PKA phosphorylation. The δ subunit is phosphorylated in forskolin-stimulated rat myotubes,[231] most likely at Ser-361 based on comparative peptide mapping of the in situ-phosphorylated rat receptor and the in vitro-phosphorylated *Torpedo californica* receptor.[232] Mutagenesis of this site does not eliminate cAMP-dependent phosphorylation of the *Torpedo* receptor expressed in *Xenopus* oocytes, however, because an adjacent serine (Ser-362) is phosphorylated by PKA in the mutated proteins.[35] PKA phosphorylation at these two sites could therefore be artifacts of mutagenesis, though this has yet to be definitively shown. Analysis of contributions of phosphorylation at these sites using site-directed mutagenesis is therefore problematic.[35,233] It is interesting to note that both δ-Ser-361 and δ-Ser-362 are stably phosphorylated in vivo in *Torpedo* electric tissue and that both of these sites can be phosphorylated by PKA in vitro.[234] Most studies show cAMP-dependent desensitization of the nicotinic acetylcholine receptor,[230] though there are exceptions probably due to species and cell-type differences. For example, forskolin does not induce phosphorylation of the receptor in innervated chick embryonic skeletal muscle cells,[235] and cyclic AMP-dependent phosphorylation of the embryonic frog receptor results in an increased rate of recovery from desensitization.[233]

The γ-amino butyric acid type A ($GABA_A$) receptor is a gated chloride channel that causes hyperpolarization and synaptic inhibition. PKA phosphorylation of this channel can have no effect, inhibit, or enhance the function depending on its subunit composition (reviewed in ref 230). Like the acetylcholine receptor, the $GABA_A$ receptor is probably a pentamer composed of combinations of α, β,γ, and δ subunits, each represented by multiple isoforms. It is this diversity that explains its varied regulation. PKA phosphorylates the β1 and β3 subunits in situ but not the β2 subunit.[236,237] All three β subunits contain the consensus PKA site RRRXSQLK, where X is Ala in β1 and β2 and Ser in β3. Phosphorylation of β1 coexpressed in HEK293 cells with α1 and γ2 subunits

decreases peak amplitude of GABA-induced current.[236] In contrast, phosphorylation of β3 coexpressed in HEK293 cells with α1 and γ2S subunits enhances the peak and steady-state GABA-induced current. Both serines in the β3 consensus site, Ser-408 and Ser-409, are phosphorylated. Mutation of β3 Ser-408 to Ala converts a β3-type response to a β1-type response.[237] Through a similar mutagenesis strategy, the β1 subunit could be converted to a β-3-like subunit. Though Ser-408 phosphorylation is essential for a β-3-type response, phosphorylation of only Ser-408 gave no effect. The subtle differences in PKA phosphorylation sites of the β1, β2, and β3 subunits are sufficient to explain cell-type-specific $GABA_A$ responses to elevated intracellular cAMP.

Glutamate receptors mediate fast excitatory neurotransmission through the gating of cations. The multiple subunits that have been identified most likely form pentamers and are classed pharmacologically as AMPA, kainate, or NMDA receptors. Members of each glutamate receptor class are subject to regulation by phosphorylation (reviewed in ref 230). In general, in situ cAMP-dependent phosphorylation occurs at a single site and potentiates the amplitude of the glutamate-gated current of the targeted receptor ectopically expressed in cell culture or endogenously expressed in neurons. This is the case for kainate receptor GluR6[238,239]and the AMPA receptors GluR1[240,241] and GluR4.[242] Though the NMDA receptor NR1A is phosphorylated in vivo at Ser-897 by PKA[243] and NMDA current is potentiated in a cAMP-dependent manner,[244] there is no direct evidence that the potentiation is mediated through direct phosphorylation of the receptor by PKA. Dopaminergic potentiation of NMDA receptors in medium spiny neurons of the nucleus acumbens is mediated not only by the activation of PKA, but also by the PKA-mediated inhibition of DARPP-32.[245] Thus, PKA could enhance NMDA activity by preventing the dephosphorylation of a site phosphorylated by a different protein kinase. Association of PKA with AKAPs is required for cAMP responsiveness of AMPA/kainate receptors[246] and represents the first example of the physiological importance of AKAPs in mediating PKA function. The AKAP that is responsible for this interaction, AKAP79/150, is associated indirectly with GluR1 through its interaction with a membrane-associated guanylate kinase (MAGUK) such as PSD-95 or SAP97.[247] The observation that AKAP/PKA complexes can associate with MAGUKs is of general importance to cAMP signaling since a wide array of membrane proteins, some of which are already known to be PKA substrates, are clustered at membrane surfaces by this multifunctional scaffolding protein (reviewed in ref 248). Similarly, the cAMP-dependent phosphorylation of the NMDA receptor is also dependent on association with a PKA/AKAP complex. In this case, association of the receptor and kinase is mediated by the AKAP yotiao.[249,250]

One last ligand-gated ion channel merits discussion. The purinoreceptor P_{2X2} is an ATP-gated excitatory cation channel found in neurons and smooth muscle cells. This channel, when ectopically expressed in HEK293 cells, is inhibited by cAMP-dependent phosphorylation of Ser-431.[251]

2. Sodium Ion Movement

Sodium homeostasis is regulated by cAMP at multiple points. Energy is required to generate and maintain electrochemical gradients across the plasma membrane for Na^+ and K^+ ions. The Na^+/K^+ ATPase, which pumps Na^+ out of the cell and K^+ into the cell, is central to this process. Consequently, its activity is highly regulated (reviewed in ref 252). Forskolin or β-adrenergic-mediated phosphorylation of Ser-943 in the α1 subunit inhibits enzyme activity.[32,253] PKA phosphorylation at Ser-943 also enhances the PKC-mediated phosphorylation of an independent site on the α1 subunit, which leads to a further inhibition of enzyme activity.[254] On the basis of the structure of the homologous sarcoplasmic reticulum Ca^{2+} ATPase, the site of the phosphorylation in native membrane-bound Na^+/K^+ ATPase is predicted to be inaccessible as a substrate for PKA. This suggests that phosphorylation of Ser-943 in intact cells may only occur in improperly folded protein or that an accessory protein may be needed to facilitate the exposure of the site in the native protein.[255] There are likely other indirect means by which cAMP modulates its activity in addition to regulation by direct PKA phosphorylation of the Na^+/K^+ ATPase.[252]

Intracellular Na^+ is used in a neutrogenic antiport mechanism by the Na^+/H^+ exchanger to import protons and thus modulate intracellular pH. The activity of the Na^+/H^+ exchanger is dependent on intracellular Na^+ concentration, which is determined by the Na^+/K^+ ATPase. The Na^+/H^+ exchanger 3 (NHE3) isoform in renal and intestinal epithelial brush border cells is regulated by cAMP-dependent phosphorylation (reviewed in ref 256). Direct phosphorylation by PKA of Ser-552 and Ser-605 contribute to the cAMP-dependent inhibition of NHE3 activity, though other regions of NHE3 are probably also involved.[34,257] NHE3 phosphorylation is dependent on its association with the NHE3 regulatory factor NHERF,[258] which is linked to PKA through the adapter protein ezrin.[259] NHERF is itself phosphorylated in vivo, though by a G protein-coupled receptor kinase[260] rather than by PKA.[261]

The initiation of action potentials in excitable cells such as muscle cells and neurons is due to depolarization of the membrane potential through opening of voltage-sensitive sodium channels. The activities of the different sodium channel isoforms are modulated by direct phosphorylation (reviewed in refs 262−264). The in situ sites of cAMP-dependent phosphorylation have been determined for both the rat cardiac type H1[265] and rat brain type IIA[266,267] α subunit isoforms. Of these, the role of phosphorylation of the brain isoform has been the best characterized. Four PKA sites have been identified in the brain α subunit. Phosphorylation at Ser-573 is necessary and sufficient to attenuate sodium current amplitude.[267,268] Ser-610 and Ser-623 contribute to a lesser extent. No function has been assigned to Ser-687.[267] An odd feature of the cAMP-dependent inhibition of the sodium channel is the requirement for prior

phosphorylation of the channel at Ser-1506 by protein kinase C.[38] PKA is associated with the brain sodium channel through its interaction with AKAP-15. Cyclic AMP-dependent phosphorylation of the channel is dependent on this association.[269,270] There is direct evidence that PKA-mediated phosphorylation of Ser-573 is a consequence of dopaminergic stimulation in hippocampal neurons.[268]

The ameloride-sensitive epithelial sodium channel (ENAC) may be indirectly regulated by cAMP. In kidney apical epithelium, aldosterone enhances ENAC through rapid transcriptional activation of the serum- and glucocorticoid-regulated kinase Sgk.[271,272] Other hormones activate Sgk through direct phosphorylation. Though the primary pathway to posttranslational activation of Sgk is through PI-3-K, some hormones, like vasopressin and follicle-stimulating hormone, also activate Sgk through elevation of cAMP. There is evidence that PKA can activate Sgk by direct in situ phosphorylation at a site distinct from those of the PI-3-dependent kinase PDK-2 in COS7 cells overexpressing Sgk.[273] There may also be an additional cAMP-mediated, PKA-independent pathway that leads to phosphorylation and activation of Sgk,[274] possibly mediated by a cAMP GEF (reviewed in ref 72).

3. Chloride Ion Conductance

The cystic fibrosis conductance regulator protein (CFTR) is an ATP-binding cassette transporter that exhibits chloride ion conductance. Defects in its gene are responsible for cystic fibrosis. The CFTR is subject to complex regulation involving cAMP-dependent phosphorylation at multiple sites (reviewed in refs 275 and 276). Five in situ PKA sites, all within the CFTR regulatory domain, are known,[277,278] though it is possible that other physiologically relevant sites may exist under the appropriate conditions.[275] The large number of PKA sites makes it a challenge to determine the individual contributions to CFTR function. In general, PKA phosphorylation leads to an increase in the rate of ATP hydrolysis, which is coupled to channel gating.[279] Phosphorylation at most of the sites (Ser-660, Ser-700, Ser-795, and Ser-813) contributes to the stimulation of chloride channel activity. At least one site though (Ser-737) is inhibitory.[280] Recent evidence shows that cyclic AMP-dependent phosphorylation of the CFTR requires an association with AKAPs.[281,282] As with the Na^+/H^+ exchanger, CFTR interacts with a NHERF,[283−285] which possibly co-localizes PKA through ezrin.[281]

Phospholemman, the major substrate for cAMP-dependent phosphorylation in the cardiac myocyte sarcolemma, exhibits weak chloride channel activity. It is phosphorylated at a single site in vivo in the β-adrenergic-stimulated rat diaphragm[286] and guinea pig heart.[287] Phosphorylation enhances the expression and chloride channel activity of phospholemman when expressed in *Xenopus* oocytes.[288]

4. Potassium Channels

The voltage-gated potassium channels (Kv) are a diverse family of proteins that are predominantly involved in neurotransmission, though these channels can also be found in other cell types. They are activated by membrane depolarization. A variety of protein kinases regulate Kv channels by phosphorylation (reviewed in ref 289). These channels are homotetramers of α subunits but may also have accessory β subunits. Both subunits may be targets of phosphorylation. Of the six subfamilies of Kv channels, Kv1−Kv6, there is good evidence that members from at least two are physiological substrates for PKA. The prototypical *Drosophila* K^+ *Shaker* channel is a homologue of the mammalian Kv1 family. Phosphorylation of a C-terminal PKA consensus site at Ser-508 and/or Ser-509 in this protein increases its rate of N-type inactivation.[290] Because identification of this site is based on a double Ser-to-Ala mutation at positions 508 and 509, it is not known if one or two phosphates are incorporated by PKA. A similar site in the mammalian homologue (Kv1.1) is also phosphorylated in situ,[291,292] but the functional consequences are more complex. Long-term elevation of cAMP causes an increase in Kv1.1 synthesis, most likely through PKA-mediated gene transcription, but direct phosphorylation of the channel also enhances its trafficking to the plasma membrane.[292] Short-term effects on Kv1.1 activity require the coexpression of its α and β subunits. Under such conditions, cAMP-dependent phosphorylation of the α subunit increases the extent of inactivation of the $\alpha\beta$ channel that is related to impairment of the interaction of the channel with microfilaments.[293,294] The function of another *Shaker*-related potassium channel, Kv1.5, is also modulated by PKA when coexpressed with the cytosolic accessory β subunit Kvβ1.3, both of which are expressed in the heart. In this case, however, the site of phosphorylation is on the β subunit rather than the α subunit and the effect is to reduce the Kvβ1.3-mediated fast inactivation of Kv1.5.[295] A *Shal*-type potassium channel abundant in hippocampal neurons, Kv4.2, is also a target of PKA phosphorylation.[296] Forskolin-dependent modulation of its phosphorylation state in the CA1 region of the hippocampus[296] suggests that earlier studies showing cAMP-dependent decreases in voltage-dependent activation of transient potassium currents in hippocampal CA1 dendrites[297] are due to direct phosphorylation of Kv4.2.

The inward rectifying potassium channels (Kir) are also diverse. These channels are hetero- or homomeric tetramers that may also be associated with subunits that confer ATP-dependence. In general, Kir channels maintain the membrane potential of resting cells near the equilibrium potential for potassium and are important for potassium recycling (reviewed in refs 298 and 299). Several Kir channels are subject to cAMP-dependent phosphorylation.

The renal outer medullary potassium channel Kir1.1 is phosphorylated in situ by PKA at multiple sites.[300−302] The two C-terminal sites, Ser-200 and Ser-294, increase the probability of channel opening.[301] These sites also appear to enhance the interaction of Kir1.1 with PIP_2.[302] In contrast, the N-terminal site may be involved in recruitment of the channel to the plasma membrane.[301] Cyclic AMP-

dependent phosphorylation of Kir1.1 is dependent upon PKA interaction with AKAPs.[303] Though the identity of the physiologically relevant AKAP has not been made, it is speculated that AKAP−KL, localized to the inner side of the apical membrane of nephron tubule epithelial cells,[304] is a likely candidate.[299]

Two strong inward rectifying potassium channels, Kir2.1 and Kir2.3, are phosphorylated at their extreme C-termini by PKA.[305,306] The phosphorylation sites reside in consensus PDZ domain binding sites, and both Kir2.1 and Kir2.3 can interact with the PDZ domain-containing PSD-95 scaffolding protein.[305] Phosphorylation of the Kir2.3 C-terminus disrupts this interaction.[305] Furthermore, cAMP-dependent phosphorylation of Kir2.1 inhibits channel activity.[306] Together, these observations suggest that disruption of Kir2 channel-PSD-95 interactions leads to channel inactivation.

The ATP-sensitive potassium channel Kir6.2 becomes more active as intracellular ATP levels fall. It provides a link between membrane excitability and the metabolic state within the cell. A variety of cell types express this channel including muscle, brain, and pancreatic β-cells, and it is subject to regulation by phosphorylation by multiple protein kinases (reviewed in ref 307). Cyclic AMP-dependent phosphorylation leads to activation of the channel, but the precise mechanism is a subject of debate. The channel is directly phosphorylated by PKA, but two different laboratories identify unique sites to the exclusion of the other. In one case, Ser-372 phosphorylation leads to an increase in channel activity.[308] In contrast, phosphorylation of Thr-224 leads to a reduction in the inhibitory interaction of ATP.[309] The discrepancies point to the pitfalls that can be encountered when relying solely on site-directed mutagenesis and heterologous expression systems to identify physiologically relevant phosphorylation events.

In addition to direct phosphorylation of Kir6.2, PKA may modify the activity of this channel through phosphorylation of its regulatory subunit, the sulfonylurea receptor 1, or SUR1. This subunit is a member of the ABC cassette superfamily, which includes the P-glycoprotein and the CFTR (both of which are also PKA substrates) and confers ATP sensitivity to Kir6.2. Cyclic AMP-dependent phosphorylation of SUR1 decreases Kir6.2 burst duration, interburst interval, and open probability and increases channel number.[308]

The calcium-activated potassium channels (K_{Ca}) provide a link between intracellular calcium levels and membrane excitability. This is a very diverse group of channels that is regulated by a number of protein kinases (reviewed in ref 310). Cyclic AMP-dependent phosphorylation of the prototypical *Drosophila Slo* channel stimulates its activity.[311] The colocalization of this channel with PKA is through direct interaction rather than through intermediary scaffolding proteins.[312] This opens the possibility that *Slo*, like NF-κB and SOX9, may also be regulated by PKA independent of cAMP and the PKA R subunit. The human homologue to *Slo* is the large conductance calcium-dependent potassium channel α subunit BK_{Ca}. Like its *Drosophila* counterpart, BK_{Ca} is phosphorylated at a single site in cells, stimulating its maxi-K current.[313] When coexpressed with β_2 adrenergic receptors in *Xenopus* oocytes, receptor-mediated activation of the channel is totally accounted for by cAMP-dependent phosphorylation of Ser-869, though forskolin stimulation can still elicit modest activation independent of this site.[313] This is just one example of how forskolin treatment can sometimes lead to effects that are not always consistent with other means of manipulating the cAMP-signaling cascade.

G. Water Homeostasis

Many membrane proteins are targets for PKA phosphorylation, including various transporters found in a variety of membrane compartments. A cAMP-regulated transporter that has generated considerable interest recently is aquaporin-2. This hormonally controlled water channel is important for mediating water resorption in the kidney in response to the antidiuretic hormone arginine-vasopressin (reviewed in refs 314 and 315). Aquaporin-2 is phosphorylated by PKA in rat renal collecting duct principal cells.[316−318] It induces trafficking of the channel to the plasma membrane in transfected LLC−PK1 cells.[319] Phosphorylation of aquaporin-2 by PKA at Ser-256 was first demonstrated in *Xenopus* oocytes expressing a recombinant form of the channel.[320] In rat renal collecting duct principal cells, vasopressin-induced phosphorylation of this residue stimulates translocation of aquaporin-2 to the apical membrane[317] in an AKAP-dependent manner.[318]

H. Other Transporters

One of the genes induced by the cAMP-responsive nuclear receptor steroidogenic factor-1 encodes the steroidogenic acute regulatory protein (StAR). The transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane by StAR is a rate-limiting step of steroidogenesis (reviewed in ref 321). This transporter may be directly regulated by PKA since mutagenesis of a PKA consensus phosphorylation site decreases steroidogenic activity in COS cells cotransfected with cholesterol side-chain cleavage system.[322] P-glycoprotein, or the multidrug resistance protein mdr1b, is phosphorylated in situ at Ser-665 and Ser-681 by PKA.[323] Though the phosphorylation does not affect the multidrug resistance properties of this transporter,[324] it does inhibit its swelling-activated chloride currents.[325]

I. Extracellular Proteins

Under certain circumstances, PKA may phosphorylate proteins in an extracellular environment that may be physiologically relevant, although proving it can be problematic. Two such cases illustrate this point. Vitronectin is a serum glycoprotein that can interact with numerous other serum proteins. Its phosphorylation in vitro stabilizes the inhibitory conformation of plasminogen activator inhibitor-1 (PAI-1), a protein involved in the termination of fibrinolysis (reviewed in ref 326). This protein is

phosphorylated at Ser-378 in serum[327−329] by PKA, which is apparently released from activated platelets.[330]

Atrial natriuretic peptide (ANP) can be phosphorylated in situ.[331,332] Intracellular pro-ANP is phosphorylated to a low extent in rat atria in response to cAMP elevating agents at Ser-104, which is the same site that PKA phosphorylates in vitro.[332] Though pro-ANP is apparently a poor intracellular substrate for PKA, processed extracellular ANP (ANP96−126) is phosphorylated by an "ecto" PKA associated with HeLa cells and other cell lines when the cells are treated with isoproterenol.[333] There are conflicting reports on the effect of phosphorylation on its bioactivity.[334,335] The physiological relevance of ANP phosphorylation remains obscure. Likewise, the physiological relevance of a mammalian ecto-PKA is unresolved, though recent descriptions of ecto-PKA activity on the surface of a variety of other cell types[336−338] suggests that cAMP-dependent phosphorylation of other extracellular proteins may be a general phenomenon.

The cAMP that activates ecto-PKA activity comes from the export of intracellular cAMP.[333,337] Cyclic nucleotide export from mammalian cells has been studied extensively for four decades (reviewed in refs 87 and 339). In conjunction with phosphodiesterases, this energy-dependent transport of cyclic nucleotides out of the cell may aid in the rapid reversal of hormone-stimulated intracellular cAMP and cGMP signaling. The recently characterized multidrug resistance protein mrp5 exports cyclic nucleotides and may represent the first of a family of such exporters.[88]

J. Trafficking and Motility

The loose collection of PKA substrates described in this section are involved in membrane trafficking, cytoskeletal rearrangement, adhesion, and smooth muscle contraction. In most cases, the physiological roles of these proteins, let alone the functional consequences of PKA phosphorylation, are poorly understood. Phosphorylation of synapsin I by PKA is perhaps the best understood.

Synapsin I was identified early as a major physiological target of PKA that was associated with synaptic vesicles in neuronal tissue.[340,341] Along with other synapsins, it sequesters synaptic vesicles in the presynapse, possibly through anchoring to the actin cytoskeleton. Vesicles are released in response to neuronal activity to sustain neurotransmission (reviewed in refs 342 and 343). Interaction of synapsin I with synaptic vesicles is cAMP-dependent. PKA phosphorylation dissociates synapsinI from synaptic vesicles in vitro through inhibition of phospholipid binding.[344]

The low-density lipoprotein receptor-related protein LRP is a single-transmembrane protein that interacts with and internalizes diverse ligands. Its properties suggest that it is a signaling receptor that interacts with the heterotrimeric G protein G_s and elevates cAMP in an agonist-dependent manner.[345] This unorthodox G protein-coupled receptor is subject to in vitro phosphorylation by PKA in its C-terminal cytoplasmic tail. Mutagenesis of the phosphorylation site prevents PKA-dependent phosphorylation and slows LRP internalization in human glioblastoma U87 cells.[346] It is interesting to note that a Ser-to-Thr mutant is also refractory to in situ PKA phosphorylation, suggesting that threonine is an unacceptable substitute as a phosphate acceptor for PKA in this substrate.

The first protein tyrosine phosphatase (PTP) to be identified as an in situ substrate for PKA was PTP−PEST. Phosphorylation of this ubiquitous cytosolic phosphatase at Ser-39 inhibits PTP−PEST activity in vitro and in intact HeLa cells.[347] The best candidates for physiological substrates of this phosphatase are components of integrin signaling, including $p130^{CAS}$ and paxillin, both of which help to mediate cell motility and spreading.[348,349]

The vasodilator-stimulated phosphoprotein (VASP) is abundant in platelets. Its cAMP-dependent phosphorylation at three distinct sites[350,351] correlates with inhibition of platelet aggregation, possibly through reduction of its binding to F-actin and suppression of actin nucleation.[352]

The actin bundling protein L-plastin is phosphorylated by PKA at Ser-5 in immune complex-induced polymorphonuclear neutrophils.[353] This same residue may also be phosphorylated by protein kinases other than PKA in response to inflammatory stimuli.[353−355] Phosphorylation of L-plastin correlates with activation of leukocyte-specific integrin-mediated adhesion.

HSP20, a heat-shock-related protein, is a major target of phosphorylation in response to cyclic nucleotide-dependent vascular smooth muscle relaxation. Treatment of permeabilized carotid arterial strips with a synthetic phosphopeptide of the PKA site in HSP20 inhibits agonist-induced contractile response.[356] This is consistent with a role of HSP20 phosphorylation in smooth muscle relaxation. The role of HSP20 in smooth muscle function is not yet known.

Another target of PKA phosphorylation in smooth muscle is myosin light-chain kinase. This calcium/calmodulin-dependent protein kinase phosphorylates the regulatory light chain of myosin II, initiating contraction.[357,358] Isoproterenol treatment of tracheal smooth muscle results in phosphorylation of a site that is also an in vitro site of PKA phosphorylation.[359] Telokin, a small C-terminal splice variant of MLCK, is also phosphorylated in forskolin-stimulated rabbit ileum at the same site.[360] The functional consequences of PKA-dependent phosphorylation of MLCK and telokin are not known.

K. Striated Muscle Contraction

Cyclic AMP-dependent phosphorylation is intimately involved in the hormonal control of striated muscle contraction. The role of PKA in controlling the strength of contraction and rate of relaxation of the heart has been a subject of particularly intensive investigation (summarized in Figure 9). One site of PKA regulation in this process is the voltage-gated L-type calcium channel. The distinct skeletal muscle and cardiac isoforms of this channel have somewhat different functions. Whereas the calcium channel activity of the skeletal muscle isoform is primarily to provide intracellular calcium for the eventual

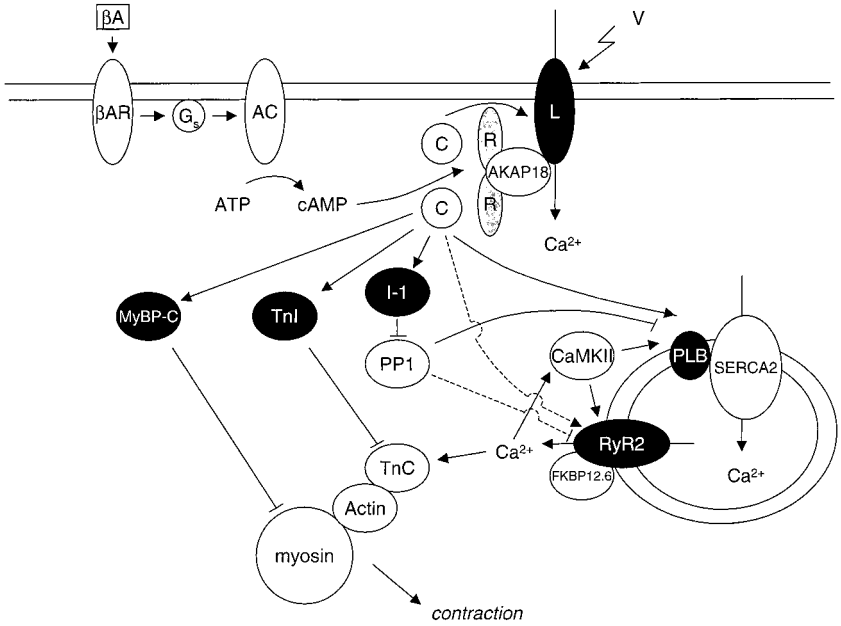

Figure 9. PKA regulation of cardiac muscle contraction. Most components are defined in Figure 3 or in the text (section II.K). Other components are voltage (V), β-adrenergic agonists (βA) and their receptor (βAR), the L-type calcium channel (L), protein phosphatase 1 (PP1), inhibitor-1 (I-1), troponin I (TnI), and troponin C (TnC). Phospholamban (PLB), SERCA, and RyR2 are found in the sarcoplasmic reticulum. Shaded components are described in Figure 3.

restoration of sarcoplasmic reticulum calcium stores, the calcium channel activity of the cardiac isoform is important for the initiation of muscle contraction. Phosphorylation of either channel increases its probability of opening (reviewed in refs 262 and 361). Both isoforms require AKAP-mediated co-localization of PKA for cAMP-dependent regulation.[362−364] The physiologically relevant AKAP is probably AKAP18 in the skeletal muscle[365,366] and heart.[367] In contrast, the neuronal L-type calcium channel, which is identical to the cardiac isoform, is associated with the AKAP MAP2B.[366−368] The α1 subunit is a target for PKA in both isoforms.[369−371] The cardiac L-type Ca^{2+} channel is distinguished by having additional phosphorylation sites in its β2 subunit.[372] All of the physiologically relevant phosphorylation sites have been shown to alter L-type Ca^{2+} channel activity.[362,363,371,373]

During excitation−contraction coupling in the heart, Ca^{2+} initially enters the myocyte through L-type Ca^{2+} channels, which in turn activate the intracellular Ca^{2+}-release channels in sarcoplasmic reticulum. The intracellular channels, also referred to as ryanodine receptors, are targets of cAMP-dependent phosphorylation (reviewed in ref 374). In response to phosphorylation at Ser-2809, the type 2 ryanodine receptor has an increased sensitivity to Ca^{2+}-induced activation. This is due to the phosphorylation-induced dissociation of the ryanodine receptor accessory protein FKBP12.6.[375] Though cAMP-dependent phosphorylation of the type 2 ryanodine receptor has been demonstrated in situ,[375] this site is also phosphorylated in situ by Ca^{2+}/calmodulin-dependent protein kinase II.[376] Further experiments will be needed to distinguish between direct phosphorylation by PKA or enhancement of Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation via inhibition of protein phosphatase 1 activity.

Phospholamban, an accessory protein for the sarcoplasmic/endoplasmic reticulum Ca^{2+}/ATPase (SERCA) in cardiac, slow-twitch skeletal muscle, and smooth muscle, is the most abundant PKA substrate in the heart. This protein is phosphorylated at Ser-16[377] in response to β-adrenergic stimulation of the heart.[378] Ser-17 is also phosphorylated in response to elevated cAMP. The protein kinase responsible for this phosphorylation is calcium/calmodulin-dependent protein kinase II and the cAMP-dependent increase in Ser-17 phosphorylation is most likely due to PKA inhibition of protein phosphatase 1.[378] The role of phospholamban phosphorylation in controlling heart function has been extensively studied (reviewed in ref 379). PKA phosphorylation causes an increased rate of calcium transport into the sarcoplasmic reticulum by increasing the apparent affinity of the sarcoplasmic reticulum calcium pump (SERCA2) for calcium. The net effect is an increase in re-uptake of calcium into the sarcoplasmic reticulum during the relaxation phase of the cardiac contraction/relaxation cycle. The attenuated β-adrenergic responsiveness of heart function in phospholamban-deficient mice indicates that phospholamban is the major target of cAMP-mediated regulation of cardiac contractility.[380,381]

Cardiac troponin I is unique among troponin I isoforms in that it has an N-terminal extension that contains a PKA site(s) that is phosphorylated in vivo (reviewed in ref 382). Like phospholamban, two adjacent residues in cardiac troponin I, Ser-23 and Ser-24, are phosphorylated in a cAMP-dependent manner.[383,384] Phosphorylation of Ser-24 precedes phosphorylation of Ser-23.[385] There is some question, however, as to whether Ser-23 is phosphorylated directly by PKA in situ.[382] Hyperphosphorylated troponin I may increase the rate of cardiac muscle relaxation by interacting with troponin C such that

it decreases its affinity for Ca^{2+}.[386,387] This translates into an increased rate of relaxation. The contribution of troponin I phosphorylation to β-adrenergic responsiveness of cardiac muscle has been demonstrated in a transgenic mouse model. Cardiac-specific overexpression of the slow skeletal muscle form of troponin I, which lacks the cardiac-type N-terminal PKA phosphorylation sites, impairs β-adrenergic responsiveness.[388] In the phospholamban-deficient mouse model, troponin I phosphorylation contributes up to one-fifth of the β-adrenergic induced increase in the rate of cardiac relaxation during maximal isometric contractions.[389]

Myosin-binding protein C (MyBP−C) is another contractile protein subject to cAMP-dependent phosphorylation. Like troponin I, only the cardiac isoform is subject to this type of regulation. Though in situ confirmation of the in vitro PKA sites[390,391] has yet to be done, a large amount of evidence points to this accessory protein as a physiological substrate for PKA (reviewed in ref 392).[393] The role of this protein in modulating heart contraction is somewhat enigmatic. Recent evidence, however, shows that addition of exogenously phosphorylated cardiac MyBP−C to skinned skeletal muscle fibers increases the force of contraction,[394] most likely by inhibiting its interaction with myosin-S2.[395]

L. Metabolic Enzymes

The study of cAMP-dependent phosphorylation has its roots in the control of metabolic enzymes. Earl Sutherland's discovery of cAMP in the 1950s was based on his studies of glucose mobilization in the liver in response to epinephrine (reviewed in ref 396). The discovery of cAMP-dependent phosphorylation is based on its role in stimulating glycogenolysis. Shortly after Sutherland's discovery, Edwin Krebs and Edmund Fischer detected cAMP-dependent protein kinase activity as a contaminating activator of phosphorylase kinase (reviewed in ref 397). Epinephrine and glucagon work to maintain blood glucose levels in times of stress or starvation by inhibiting glycogenesis and glycolysis and stimulating glycogenolysis and gluconeogenesis. The activities of several key regulatory and rate-limiting enzymes in these pathways are subject to direct in situ phosphorylation by PKA. Depending on the metabolic needs of a given tissue, the isozymes expressed in the tissue may or may not be subject to hormonal control through PKA phosphorylation. Each of these enzymes has been the subject of extensive investigation by multiple investigators.

As already mentioned, muscle phosphorylase kinase was the first physiological substrate for PKA to be identified. It took 20 years before the in situ phosphorylation site of this enzyme was eventually identified.[398] Cyclic AMP-dependent phosphorylation of phosphorylase kinase increases its activity (reviewed in ref 399). Compared to the relatively straightforward phosphorylation of phosphorylase kinase by PKA, the role of PKA phosphorylation in the inhibition of muscle glycogen synthase is labyrinthine (reviewed in refs 400 and 401). This enzyme is phosphorylated at multiple sites in vivo by PKA. The functions of Ser-697 and Ser-710 are not known, whereas phosphorylation of Ser-7 contributes to the inhibition of glycogen synthase by permitting hierarchical phosphorylation of Ser-10 by casein kinase I.[402] However, Ser-7 is phosphorylated by several other protein kinases in addition to PKA.[400,401] The relative contribution of in situ PKA phosphorylation of Ser-7 is not known. A major factor in PKA inhibition of glycogen synthase is the inhibition of protein phosphatase 1 through cAMP-dependent phosphorylation of Inhibitor-1 (reviewed in ref 37). The impact of PKA-dependent inactivation of glycogen synthase kinase-3 (see section II.E) on glycogen synthase function has yet to be addressed.

Key to the cAMP-dependent control of glycolysis and gluconeogenesis in the liver is the modulation of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, which contains distinct catalytic sites for the synthesis and hydrolysis of fructose-2,6-bisphosphate, a potent allosteric inhibitor of the glycolytic 6-phosphofructo-1-kinase and an allosteric activator of the gluconeogenic fructose-1,6-bisphosphatase (reviewed in refs 403−405). The liver isoform of the bifunctional enzyme undergoes cAMP-dependent phosphorylation in situ at Ser-32.[406,407] The net result is inhibition of its kinase activity and activation of its phosphatase activity to give a net decrease in fructose-2,6-bisphosphate, thus favoring glucose production.

Before the role of fructose-2,6-bisphosphate in liver carbohydrate metabolism was appreciated, investigators were perplexed by the inability to convincingly explain the control of phosphofructokinase and fructose-1,6-bisphosphatase activities in terms of direct phosphorylation by PKA. Though the liver isoforms of these enzymes apparently are not physiological substrates, the muscle isoform of 6-phospho-fructo-1-kinase is phosphorylated in vivo[408] at a site that is also phosphorylated by PKA in vitro in the presence of troponin C.[409] This same site undergoes cAMP-dependent phosphorylation in the heart.[410] The functional consequence of this physiological phosphorylation event remains unresolved.

Another important PKA-regulated liver glycolytic enzyme is pyruvate kinase. Direct phosphorylation by PKA of liver pyruvate kinase[411] inhibits its activity (reviewed in ref 412). Liver pyruvate kinase is an excellent in vitro PKA substrate, and the sequence surrounding Ser-43 is the template for numerous peptide studies designed to determine the in vitro substrate requirements for PKA.[15] The commonly used PKA heptapeptide substrate "kemptide" (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is also derived from this site.[413]

Both fatty acid synthesis and degradation are regulated by cAMP-dependent phosphorylation. Hormone-sensitive lipase is the hormonally regulated rate-limiting step in lipolysis (reviewed in ref 414). It is phosphorylated in adipocytes by PKA at multiple sites in response to isoproterenol or norepinephrine treatment.[415,416] Site-directed mutagenesis of each site indicates that the adjacent phosphorylated PKA sites, Ser-659 and Ser-660, are responsible for the enhancement of hormone-sensitive lipase activity,

whereas the function of the remaining Ser-563 remains unresolved. It is interesting to note that the more recent mutagenesis studies[415] amend a long-standing view that Ser-563 is the regulatory site based on phosphopeptide sequencing of the in vitro-phosphorylated enzyme.[417] The lipogenic enzyme ATP citrate lyase is phosphorylated at Ser-454 in the liver in response to glucagon treatment as well as in vitro by PKA.[418] This same site is also phosphorylated in response to insulin, most likely by another protein kinase,[419] even though glucagon and insulin exert opposing effects on enzyme activity. The ramification of this paradox remains unresolved.

The first step in catecholamine biosynthesis is catalyzed by tyrosine hydroxylase. This enzyme is subject to multiple forms of regulation, including protein phosphorylation. In particular, the activation of tyrosine hydroxylase by PKA phosphorylation at Ser-40 is well documented (reviewed in refs 420 and 421). Several labs have demonstrated the physiological relevance of cAMP-dependent phosphorylation for this enzyme. Tyrosine hydroxylase is phosphorylated by PKA at a single site in response to forskolin treatment in rat pheochromocytoma cells[422] and corpus striatum synaptosomes and in electrically stimulated rat corpus striatum.[423] Furthermore, Ser-40 phosphorylation mediates forskolin-induced L-DOPA synthesis in stably transfected AtT-20 cells.[424] Antagonism of dopaminergic neuronal activity in the striatum may also induce Ser-40 phosphorylation and tyrosine hydroxylase activity.[425] Phenylalanine hydroxylase converts the essential amino acid phenylalanine to tyrosine, primarily in the liver. Like tyrosine hydroxylase, this enzyme is activated by cAMP-dependent phosphorylation (reviewed in ref 426). The single site on phenylalanine that is phosphorylated by PKA in vitro[427] is also phosphorylated in response to glucagon treatment of isolated liver cells and in response to dibutyryl cAMP treatment of isolated kidney tubules.[428]

At least one member of the cytochrome P450 family is subject to physiological regulation by PKA. The P450 family of enzymes is often involved in the first step of metabolic activation of xenobiotics. The enzyme CYP2E1 is phosphorylated at Ser-129 in response to glucagon treatment of isolated hepatocytes.[429] It is also a PKA substrate when ectopically expressed in Chinese hamster ovary cells.[430] Though the end result is the reduction in CYP2E1 activity, there is contradictory evidence for how inhibition is achieved. In one case, phosphorylation enhances the denaturation and degradation of CYP2E1.[429,431] Alternatively, phosphorylation may directly inhibit its catalytic activity.[430]

M. Proteins of Unknown Function

Defects in the gene encoding the protein kinase LKB1 are responsible for the autosomal dominant cancers of Peutz−Jehgers syndrome. Since disruption of LKB1 leads to cell proliferation, it is considered a tumor suppressor. The downstream targets of this protein kinase are not known. Forskolin stimulation of cells expressing either a C-terminal portion of LKB or the endogenous wild-type form of the enzyme results in PKA-dependent phosphorylation of Ser-431.[432,433] This same site is also a physiological substrate for $p90^{RSK}$.[433] The functional consequences of phosphorylation are not known.

The dopamine receptor-enriched neostriatum in rat brain expresses a group of proteins that are phosphorylated in response to cyclic AMP.[434] The in situ PKA phosphorylation site is known for a few of these proteins. The most extensively characterized is the phosphoprotein phosphatase 1 inhibitor, DARPP-32 (see section II.B.6). A subset of these cAMP-regulated phosphoproteins (ARPPs) includes the ARPP-16/19 family of gene products. The 16 kDa isoform is enriched in the neostriatum, whereas the alternatively spliced 19 kDa isoform is ubiquitously expressed. These proteins are phosphorylated by PKA at a single site in striatal slices treated with forskolin or dopamine.[435] Though the function(s) of ARRP16/19 are unknown, they may share certain properties with the closely related α-endosulfine, a protein that displaces binding of sulfonylurea to the ATP-sensitive potassium channel Kir6.2/SUR1[436] (see section II.F.4). Another cAMP-regulated phosphoprotein enriched in dopamine receptor-containing neurons in the striatum, ARPP-21, is phosphorylated by PKA at Ser-55[437] and, like DARPP-32, is differentially phosphorylated/dephosphorylated in response to D1 or D2 dopamine receptor activation in mouse neostriatum.[438] The physiological role of ARPP-21 in dopaminergic neurotransmission is not known.

III. Summary and Conclusions

The cAMP/PKA signaling field is a mature one, with roots extending back to the 1950s. A remarkable aspect of the study of this venerable second messenger system is its resiliency. Though PKA is just one of hundreds of protein kinases, its ubiquitous distribution, the diversity of components that modulate its allosteric effector, and its simple yet flexible substrate recognition properties help to keep it as a central element in many of the regulatory processes in living cells. It is expected that many more PKA substrates remain to be characterized. It is also probable that yet other cAMP-dependent phosphorylation events will be attributed to indirect action of PKA or even to PKA-independent pathways. Drawing from the extensive literature on the subject, several general statements about cAMP and PKA signaling can be made. (1) Cyclic AMP mediates both acute and long-term responses to environmental changes through the direct PKA-dependent phosphorylation of a wide range of enzymes, ion channels, structural and regulatory proteins, and transcription factors. (2) Protein kinase A efficiently phosphorylates the consensus sequence Arg-Arg-X-Ser in physiological substrates, but this motif represents only one-half of all physiological PKA phosphorylation sites. The less-frequent phosphorylation of degenerate/variant consensus sequences in physiological substrates makes it difficult to predict likely phosphorylation sites solely on the basis of the substrate's primary structure. (3) Phosphorylation of physiological substrates usually results in altered function of the substrate. This may take the form of

altered enzyme or channel activity, altered protein−protein, protein−lipid, or protein−DNA interactions, or altered protein stability. (4) The PKA-dependent regulation of selected protein phosphatases permits cAMP-mediated enhancement of the phosphorylation state of sites on substrates targeted by protein kinases other than PKA. Alternatively, PKA-dependent regulation of other protein kinases may result in cAMP-dependent modulation of the phosphorylation state of arrays of substrates distinct from those phosphorylated directly by PKA. (5) Co-localization of PKA with physiological substrates permits the cAMP-dependent regulation of many substrates, particularly membrane proteins. Most commonly, PKA interacts with AKAP scaffolding proteins through one of its regulatory subunit isoforms. Occasionally, the PKA catalytic subunit may interact directly with a substrate or associated regulatory protein permitting cAMP-independent phosphorylation by PKA. (6) Cyclic AMP may elicit cellular responses independent of PKA activation through interaction with alternative intracellular receptors. These receptors include cyclic nucleotide-gated cation channels, cAMP-guanine nucleotide exchange factors, and cyclic nucleotide phosphodiesterases.

IV. Acknowledgments

The author thanks Clay Comstock and Robert Branco for helpful suggestions and critical reading of the manuscript. This work was supported by grants from the University of North Dakota, American Heart Association, and North Dakota EPSCoR (NSF EPS-9874802).

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CR000236L