Annu. Rev. Pharmacol. Toxicol. 1995. 35:581–606

ADENOSINE RECEPTOR SUBTYPES: Characterization and Therapeutic Regulation

Mark E. Olah and Gary L. Stiles
Duke University Medical Center, Departments of Medicine and Pharmacology, Durham, North Carolina 27710

KEY WORDS: G protein, ligand binding, site-directed mutagenesis, xanthines, cloning

ABSTRACT

Adenosine receptors (ARs) are members of the G protein–coupled receptor family and mediate the multiple physiological effects of adenosine. Currently, four AR subtypes have been cloned: A_1AR, A_{2a}AR, A_{2b}AR, and A_3AR. All subtypes are distinctly distributed throughout the body and AR agonists and antagonists have potential therapeutic utility. Knowledge of AR amino acid structure has been utilized in mutagenesis studies to identify specific receptor regions that interact with distinct classes of ligands. Cloning of ARs has also permitted receptor regulatory processes such as desensitization to be studied in greater detail, in particular, the molecular mechanisms underlying this event. Cloning of the human A_1AR has revealed that alternate splicing generates distinct receptor transcripts. The existence of a particular transcript in a tissue or cell apparently regulates the level of A_1AR expression in the tissue. This review focuses on these aspects of AR structure and function and their therapeutic regulation.

INTRODUCTION

When acting upon cell surface receptors, adenosine elicits a large number of responses throughout several organ systems. The activation of adenosine receptors (ARs) can occur in response to endogenous adenosine, whose local levels may rise upon stress to the system—e.g. cardiac hypoxia (1), central

0362-1642/95/0415-0581$05.00

nervous system (CNS) ischemia (2), and seizure activity (3)—or upon administration of adenosine or its more metabolically stable analogues. Additionally, application of AR antagonists can induce a variety of physiological effects. The regulation of ARs is therefore recognized as having significant therapeutic potential (4). However, the extensive distribution of ARs and the variability of response upon activation of different AR subtypes requires agonists and antagonists that are selective in their action if they are to be useful therapeutically.

This review focuses on the knowledge of ARs that has been obtained over approximately the last five years. Many novel findings have stemmed from the cloning of these receptors, which was initiated in 1989, and much of this review is directed toward those aspects of ARs. Understanding of AR function in the intact animal is far from complete, and significant advances are currently being made in this area. More complete reviews of this nature have been published (5, 6). In this discussion, each AR subtype is described structurally, pharmacologically, and functionally, because these parameters define the subtypes and have physiological implications. Because the A_3AR has only been recognized and studied in the last few years, its physiological roles are addressed more extensively than those of the other subtypes. A review of studies correlating receptor structure to function and regulation is then provided. Finally, the current and potential therapeutic uses of adenosine and related compounds are briefly reviewed.

STRUCTURE AND CHARACTERIZATION OF AR SUBTYPES

General Structure

The cloning of ARs was initiated with the isolation of the "orphan" cDNAs, RDC7 and RDC8, by Libert and coworkers (7). A set of degenerate oligonucleotide primers based on the sequence of the third and sixth transmembrane domains of several G protein–coupled receptors (GPCRs) was employed to amplify human thyroid cDNAs (7). A group of these clones was then used to screen a dog thyroid cDNA library. Two of the isolated full-length cDNAs, termed RDC7 and RDC8, were eventually expressed in mammalian cells and, based on their ligand-binding properties and effects on adenylyl cyclase activity, were identified as coding for the canine A_1AR (8) and A_{2a}AR (9), respectively.

The cloning of species homologues of the A_1AR, A_{2a}AR, A_{2b}AR, and A_3AR shortly followed the identification of RDC7 and RDC8. Clones for all four of the currently identified AR subtypes have been isolated for rat and human. The overall and transmembrane domain amino acid identity among the four human AR subtypes is approximately 30 and 45%, respectively. ARs all fit

the structural motif typical of GPCRs, of which the adrenergic and muscarinic receptor families have been the most extensively studied and from which most generalities of receptor structure-function relationships are based (10, 11). Very briefly, this architecture features seven stretches of hydrophobic α-helical regions, each composed of ~22–26 amino acids that traverse the cell membrane. The regions connecting the membrane-spanning domains 2-3, 4-5, and 6-7 (typically referred to as extracellular loops 1, 2, and 3, respectively) and the amino terminus of the receptor are oriented into the extracellular space. Regions connecting transmembrane domains 1-2, 3-4, and 5-6 (intracellular loops 1, 2, and 3 respectively) and the carboxy-terminal tail of the receptor are directed cytoplasmically. Portions of these segments are believed to interact directly with the α subunits of G proteins in order to transmit the signal of receptor activation, and the segments also contain sites involved in receptor regulatory processes such as phosphorylation. Compared to many GPCRs, the A_1AR, A_{2b}AR, and A_3AR are small in size, and all ARs possess a rather short amino terminus. The A_1AR (12) and A_{2a}AR (13) in native tissues have been shown to be glycoproteins, and consensus sites for N-linked glycosylation exist on all ARs. For the A_1AR and A_2ARs, two sites are present on the putative second extracellular loop of the receptor but none on the amino-terminal tail, which is characteristic of several other GPCRs. Because endoglycosidase F treatment of native rat brain A_1AR (12) and bovine striatum A_{2a}AR (13) suggests that these receptors contain a single carbohydrate chain, site-directed mutagenesis of these asparagine residues is required in order to identify the specific site for posttranslational modification. When expressed in COS 6M cells, the deglycosylated form of the canine A_{2a}AR, as produced by tunicamycin treatment, binds agonists in a manner identical to that of fully processed receptor (14). Glycosylation has not been extensively studied in ARs, and the majority of studies with other neurotransmitter GPCRs have not identified a well-defined role for it (11). Other structural features common to many GPCRs that are present in ARs include an aspartate residue in transmembrane domain 2 that may be involved in receptor regulation by sodium ions, an Asp-Arg-Tyr sequence in the second intracellular loop, and cysteines in extracellular regions that may be involved in intrareceptor disulfide bond formation. A conserved cysteine residue that may be a site for receptor palmitoylation exists in the carboxy-terminal tail of all AR subtypes, with the exception of the A_{2a}AR. Site-directed mutagenesis studies that would determine if any of these structural features have a role in AR function have not yet been performed for any subtype.

A_1AR

The continued use of degenerate oligonucleotide probes and the availability of the canine A_1AR cDNA sequence resulted in the subsequent cloning of the

A_1AR from rat (15, 16), bovine (17, 18), human (19–21), and rabbit (22) cDNA libraries. The various clones encode a protein of 326 amino acids, with the exception of the rabbit A_1AR, which is 328 amino acids. The corresponding size of the encoded protein is ~36,700 Daltons. The amino acid identity of the species homologues of the A_1AR is approximately 87 and 92% overall and in the transmembrane domains, respectively. The isolated clones, when expressed in mammalian cells, bind antagonist radioligands with high affinity (K_d ~ 1 nM) and AR agonists with the potency order of (–)-N^6-(R-phenylisopropyl)adenosine (R-PIA) > 5′-*N*-ethylcarboxamidoadenosine (NECA) > (+)-N^6-phenylisopropyladenosine (S-PIA), which is the profile traditionally used to define the A_1AR (15, 19, 21). An exception is the bovine A_1AR (cloned and native), which is characterized by R-PIA > S-PIA > NECA (17, 18).

Analysis of rat tissue mRNA via Northern blotting and in situ hybridization show the A_1AR to be highly expressed in brain (particularly abundant in cortex, cerebellum, thalamus, and hippocampus), spinal cord, fat, and testis (15, 16). Reppert and coworkers (16) have presented an extensive characterization of rat brain A_1AR distribution. The A_1AR message can also be detected in rat (15, 16) and bovine (17) heart and kidney. In the rat kidney, in situ hybridization indicates that the receptor is most abundant in the medullary and papillary collecting ducts (23). Human A_1AR transcript distribution is described below (see section on human A_1AR expression). In general, the amount of the A_1AR message correlates well with the level of the expressed receptor protein.

The A_1AR has been classically associated with the inhibition of adenylyl cyclase (24, 25), and this intracellular signaling event has been used in the characterization of cloned A_1ARs. The coupling of the A_1AR to activation of phospholipase C (PLC), with subsequent elevations in inositol phosphate content and intracellular calcium levels, is less well defined. In native tissues or cells, A_1AR agonists have been reported to have no effect on enzyme activity (26), activate PLC (27, 28), or enhance (29, 30) or inhibit (31, 32) the PLC activation induced by other receptor agonists. The atypical features of R-PIA–stimulated inositol phosphate accumulation in guinea pig myometrium (33) would appear to warrant further characterization of the receptor mediating this effect. The direct stimulation of phospholipase C by A_1AR agonists has been reported for rabbit cortical collecting tubule cells (27). Interestingly, in rabbit renal blood vessels adenosine analogues have been shown to produce a vasoconstriction, whereas the application of adenosine is typically associated with vasodilation (34). Gerwins & Fredholm (28) characterized the elevation of inositol phosphate and intracellular calcium levels in the hamster smooth muscle (vas deferens) cell line DDT_1 MF-2. The pharmacological profile of the response strongly implies involvement of the A_1AR. The PLC activation was abolished by pertussis toxin and was shown to be independent of changes in cAMP levels. A marked synergism between the A_1AR-mediated response

and the bradykinin-induced PLC stimulation was also described, though the mechanism of this phenomenon is undefined (35). Divergent results have also recently been published regarding PLC activation in mammalian cells expressing the cloned human (36) and canine (37) A_1AR. Activation of the human A_1AR by agonists produced a modest increase in the intracellular calcium level, which was apparently dependent on intracellular (initial phase) and extracellular (sustained phase) calcium sources (36). Conversely, the canine A_1AR mediated no change in inositol phosphate content or intracellular calcium levels (37). However, application of AR agonist to cells cotransfected with the canine A_1AR and M_3 muscarinic receptor enhanced the stimulation of PLC elicited by muscarinic receptor agonists (37). Differences in cell type (CHO was employed in both studies), A_1AR density, and variations in amino acid sequence (eight nonconserved residues in intracellular regions of canine and human A_1AR) apparently do not account for these divergent results.

Several of the actions of adenosine on cardiac tissues or in the nervous system involve A_1AR-mediated potassium channel activation or modulation of calcium channels, and these topics have been reviewed (38, 39).

$A_{2a}AR$

The A_{2a}AR has been cloned from canine (9), rat (40), and human (41) cDNA libraries. The overall amino acid identity of the rat and human A_{2a}ARs to the canine subtype is 82 and 93%, respectively. The A_{2a}AR is a larger protein than the other AR subtypes; species homologues of the A_{2a}AR are 410–412 amino acids, corresponding to a protein of ~45,000 Daltons. The larger mass of the A_{2a}AR relative to the A_1AR had been demonstrated prior to their cloning via photoaffinity labeling of the receptors in native tissues followed by resolution on SDS-PAGE gels (12, 13). The additional 80–90 amino acids of the A_{2a}AR compared to other AR subtypes constitutes the carboxy-terminal tail of the receptor. The functional significance of this relatively large tail has not been determined. Canine A_{2a}ARs truncated at amino acid 309 (14) and 316 (T Palmer & G Stiles, unpublished results) via genetic engineering bind ligands (14) and stimulate adenylyl cyclase in response to agonists (T Palmer & G Stiles, unpublished results) in a manner identical to that of the full-length receptor. The appreciable number of serine and threonine residues present in this region represent potential phosphorylation sites for G protein–receptor kinases and could therefore be involved in desensitization processes. Structural differences of this nature between the AR subtypes may be significant because compared to the A_1AR, the A_{2a}AR displays a rapid desensitization response (42).

The A_{2a}AR is characterized pharmacologically by its high-affinity binding of the agonist radioligands [^{3}H]CGS21680 and [^{125}I]-2-[4-[2-[2-[phenylmethylcarbonylamino] ethylaminocarbonyl]ethyl]phenyl]ethylamino - 5′ - *N*-ethyl-

carboxamidoadenosine ([^{125}I]-PAPA-APEC), which are far superior to the previously employed [^{3}H]NECA. The binding of these two radioligands differentiates the A_{2a}AR and A_{2b}AR subtypes (see below). Traditional AR agonists display the potency order of NECA > R-PIA > S-PIA at the A_{2a}AR. Several xanthine derivatives that are high-affinity antagonists at the A_1AR display much lower affinity at the A_{2a}AR (43). This low affinity has mostly precluded the use of these compounds as antagonist radioligands at the A_{2a}AR, and furthermore, high-affinity antagonists displaying substantial selectivity for the A_{2a}AR over the A_1AR do not exist. Antagonists displaying moderate A_{2a}AR selectivity include 8-styryl–substituted 1,3,7-alkylxanthines (44, 45) and certain nonxanthine analogues such as the triazoloquinoxaline CP66,713 (46).

Unlike that of other AR subtypes that display a fairly wide distribution in brain, the A_{2a}AR message in rat brain, as analyzed by Northern blotting, is limited. Abundant expression is reported for the striatum, with much smaller amounts in cortex and midbrain and no detectable signal in hypothalamus or cerebellum (40). These findings are in agreement with those determining A_{2a}AR distribution via autoradiography with [^{3}H]CGS21680 (47). In situ hybridization has revealed site-specific colocalization of rat A_{2a}AR mRNA with D_2 dopamine receptor mRNA (40) and enkephalin mRNA (48) on subsets of striatal neurons. A_{2a}AR transcript is also present in human brain, heart, kidney, and lung (49). Pharmacological and functional studies have shown the presence of A_{2a}AR in liver (50) and platelets (51).

Activation of the A_{2a}AR has been classically associated with activation of adenylyl cyclase via G_s (24, 25). The resulting generation of cAMP is responsible for A_{2a}AR-mediated inhibition of platelet aggregation (51) and perhaps, in part, for the vasodilation of certain vascular beds (52). Vassart and coworkers examined the effects of A_{2a}AR-mediated adenylyl cyclase activation on cell growth via generation of transgenic mice (53). Transgenic animals expressing the A_{2a}AR under regulation of the thyroglobulin gene promoter were characterized as markedly hyperthyroid both histologically and functionally. Although the actual level was not quantitated, thyroid tissue from transgenic mice displayed high levels of [^{3}H]CGS21680 binding, whereas no specific binding was found in the organ of control animals. Whether the corresponding increase in basal cAMP levels of thyroid of transgenic animals relative to control was due to a constitutively active A_{2a}AR or to stimulation of the receptor by endogenous adenosine remains unclear. A constitutively active A_{2a}AR was also reported for the canine clone expressed in *Xenopus* oocytes, and this basal activity was not completely prevented by treatment with 2 U/ml adenosine deaminase (9). Obviously, greatly elevated levels of A_{2a}AR expression in the thyroid gland represent an artificial situation, and further study of AR-mediated changes in cell growth and morphology is required. However,

the previous study may represent a future direction of AR research in which transgenic, or knockout, animals or cell culture models may help delineate the specific physiological roles of AR subtypes.

$A_{2b}AR$

The discrimination of A_2ARs into two subtypes had been proposed based on differences observed for agonist binding (high affinity, A_{2a}AR; low affinity, A_{2b}AR) and variations in anatomical distribution (striatum, A_{2a}AR; other brain regions, A_{2b}AR) (54, 55). An adenosine receptor demonstrating the characteristics described for the A_{2b}AR has been cloned from rat (56, 57) and human (58) brain cDNA libraries. In the transmembrane domain regions, the rat and human A_{2b}ARs both display ~73% amino acid identity to the A_{2a}AR of the respective species. The A_{2b}AR consists of approximately 80 amino acid residues fewer than the A_{2a}AR and is similar to the A_1AR and A_3AR in size. The relative lack of information regarding the function or physiological significance of the A_{2b}AR, along with a rather imprecise means to identify the clone, arises from a lack of ligands truly suitable for studying this AR subtype. The A_{2b}AR displays low affinity for [^{3}H]NECA and does not bind [^{3}H]CGS21680 or [^{125}I]-PAPA-APEC. Thus, when expressed in mammalian cells, the rat and human clones were identified as A_{2b}ARs based on their lack of binding of the latter two radioligands and their ability to mediate a theophylline-sensitive, NECA-induced stimulation of adenylyl cyclase (56, 58). A more extensive characterization of the stimulation of adenylyl cyclase by the cloned A_{2b}AR by Rivkees & Reppert (57) demonstrated higher affinity of several xanthine antagonists at the rat A_{2b}AR as compared to the cloned rat A_{2a}AR.

Northern analysis of rat mRNA demonstrated the greatest expression of the A_{2b}AR transcript in cecum, large intestine, and urinary bladder, with lesser amounts in brain, spinal cord, and lung (56). Physiological roles for the A_{2b}AR may be postulated based on the demonstration that *Xenopus* oocytes injected with rat A_{2b}AR RNA responded to application of adenosine or NECA with an inward current typical of that elicited by receptors coupled to phospholipase C activation (59). However, examples of coupling of the A_{2b}AR to phospholipase C stimulation is limited to this experimental system, and receptor-mediated increases in inositol phosphate have not been reported in native tissues. An A_{2b}AR has recently been cloned from mouse bone marrow–derived mast cells, and mRNA for this receptor was shown to be present in the rat basophilic leukemia cell line, RBL-2H3 (60). Based on these findings and the description of A_{2b}AR-mediated stimulation of calcium channel activity in oocytes, Marquardt et al (60) have proposed that this receptor subtype mediates the secretory action of adenosine on mast cells. The role of adenosine in this response is more fully described below.

A_3AR

The screening of cDNA libraries with degenerate oligonucleotide probes resulted in the isolation of an AR subtype whose existence had not been postulated. In 1991 Meyerhof and coworkers (61) published the sequence of a clone isolated from a rat testis cDNA library. The clone encoded a protein of 320 amino acids that displayed 47 and 42% overall amino acid identity to the canine A_1AR and $A_{2a}AR$, respectively. However, pharmacological or biochemical properties of the expressed protein were not determined. The same clone was independently isolated from a rat brain cDNA library by Zhou and coworkers (62) and analyzed for binding of AR ligands as a result of its sequence similarity to existing cloned ARs. The clone, stably expressed in CHO cells, demonstrated a pharmacologic profile not typical of any characterized A_1AR or A_2ARs. N^6-2-(4-amino-3-[^{125}I]iodophenyl)-ethyladenosine ([^{125}I]-APNEA), a high-affinity A_1AR agonist radioligand, bound with a K_d of 15 nM, and this binding was displaced with the potency order of R-PIA = NECA > S-PIA (62). Affinities of agonist ligands were much lower for the cloned receptor than for the A_1AR. Perhaps most striking, xanthine derivatives bound very poorly to the cloned receptor (62). Stimulation of the receptor with NECA or R-PIA resulted in a pertussis toxin–sensitive inhibition of forskolin-stimulated adenylyl cyclase activity (62). Based on these properties and the sequence similarity, the clone was identified as coding for a unique AR subtype and termed the A_3AR (62).

Sheep (49) and human (63) A_3ARs were subsequently cloned, and both display a 72% overall amino acid identity to the rat A_3AR; the sheep and human subtypes are more similar to each other than to the rat homologue. The A_3AR is similar to the A_1AR in size; it is composed of 317–320 amino acids, depending on the species. Unlike other cloned ARs, the A_3AR possesses a consensus site for N-linked glycosylation on both the amino terminus and the second extracellular loop. The relevance of these sites is unknown because neither photoaffinity labeling nor Western blotting of A_3ARs has been reported, and the size of the fully processed receptor is unknown.

Since the initial isolation of the rat A_3AR cDNA, the cloning of additional A_3ARs and more extensive study of the rat A_3AR has permitted the pharmacological properties of this AR subtype to be more fully elucidated. In a study of ligand structure-activity relationships at the rat A_3AR, van Galen and coworkers (64) found that disubstitution of adenosine analogues at the N^6 position of the adenine ring and 5′ position of the ribose moiety resulted in the greatest enhancement of affinity at this receptor. As a consequence of this finding, the disubstituted analogue, N^6-(3-[^{125}I]iodo-4-aminobenzyl)-5′-*N*-methylcarboxamidoadenosine ([^{125}I]-AB-MECA), has been developed as a high-affinity (K_d = 1 nM), albeit nonselective, radioligand for the A_3AR (65). An extensive

analysis of antagonist binding (64) also supported the findings of the initial characterization (62) of the rat A_3AR because it demonstrated that several xanthine derivatives displayed little binding at this receptor, with 10 μM concentrations of traditional antagonists producing approximately 10–30% displacement of agonist radioligand binding. However, the cloned sheep (49) and human (63) A_3ARs do bind certain xanthine antagonists, e.g. 3-(3-iodo-4-aminobenzyl)-8-(4-oxyacetate) phenyl-1-propylxanthine (I-ABOPX), with relatively moderate affinity, although, for most compounds, not to the degree typical of the A_1AR. The species homologues of the A_3AR also differ substantially in the binding of various agonist ligands, though this binding is typically of lower affinity than at the A_1AR.

Significant differences also exist among species in regards to tissue distribution of mRNA for the A_3AR. The most abundant message for the rat A_3AR is found in testis (61, 62). In situ hybridization indicates that mature sperm and not testicular tissue are specifically labeled by an A_3AR probe, and the degree of expression correlates positively with sexual maturity (61). Rat tissues containing moderate amounts of A_3AR message include lung, kidney, and heart, while lower levels are detected in brain regions that include cortex, striatum, and olfactory bulb (62). Salvatore and coworkers (63) reported a profile of lung = liver >> brain = aorta > testis > heart for the tissue distribution of human A_3AR mRNA. For many tissues, the expression level of the A_3AR, as assessed by radioligand binding, is unknown, and its physiological role has not been clearly determined. However, the abundant level of A_3AR mRNA in lung in all species may be significant, for, as described below, findings suggest a role for the A_3AR in mediating allergic responses in the pulmonary system.

When expressed in CHO cells, all three species homologues of the A_3AR have been shown to mediate the inhibition of adenylyl cyclase with a maximal inhibition of ~40–50% (49, 62, 63). A demonstration of this response with the native A_3AR has not been reported, though this may largely result from the lack of tissue sources containing solely the A_3AR and from the unavailability of highly selective A_3AR agonists to study those tissues containing a mixed population of AR subtypes.

Now that the existence of the A_3AR is recognized, its physiological role is being explored. Currently, all data have been obtained using rat or mouse models or cell lines, and nothing is known about A_3AR function in other species, which is due to the relatively recent characterization of the A_3AR and the lack of specific agonist or antagonist ligands.

The initial identification of a physiological role for the A_3AR arose from work that described the novel pharmacological profile of the receptor mediating the action of adenosine on RBL-2H3 cells. Ali and coworkers (66), employing RBL-2H3 cells as a model system to study signaling pathways in mast cell secretion, had described the ability of NECA, which while having little

effect alone, enhanced antigen-induced secretion from the cells; this response could only be inhibited by very high concentrations of xanthines. Following the cloning of the rat A_3AR, the possibility that this receptor might be involved in mediating the responses elicited by NECA in RBL-2H3 cells was examined. RBL-2H3 cell membranes were shown to bind [^{125}I]-APNEA with an affinity much lower than that of the rat A_1AR and similar to that described for the cloned A_3AR (67). The binding of this radioligand was equipotently displaced by R-PIA and NECA but was relatively insensitive to the antagonist xanthine amine congener (XAC). More recently, nearly identical binding parameters have been obtained for the cloned rat A_3AR and RBL-2H3 cells using the high-affinity radioligand [^{125}I]-AB-MECA (65). Augmentation of antigen-induced secretion by AR agonists fits the potency order characteristic of the A_3AR. Northern blotting and reverse transcription–polymerase chain reaction (RT-PCR) analysis both demonstrated the presence of A_3AR transcript in RBL-2H3 cells; no signal was detected for the A_1AR or the A_{2a}AR (the existence of an A_{2b}AR was not examined) (67). A subsequent report (60) noted the presence of A_{2a}AR and A_{2b}AR transcripts in RBL-2H3 cells, and this discrepancy regarding the A_{2a}AR may be due to the use of canine A_{2a}AR sequence–based oligonucleotide primers in the earlier study (67). Nonetheless, the pharmacological and functional data strongly suggest that the A_3AR is responsible for the action of adenosine analogues on RBL-2H3 cells and perhaps on other mast cells. The second-messenger pathway underlying the augmentation of secretion has not been precisely defined, though activation of the A_3AR in RBL-2H3 cells results in elevation of cellular inositol phosphate levels, with an accompanying increase in intracellular calcium levels (66, 67). This response has been described as sensitive to both cholera and pertussis toxins (66). The rat A_3AR expressed in COS-7 cells can mediate a modest increase in total inositol phosphate levels in response to agonists that is quantitatively similar to that elicited by activation of receptors coupled to G_i (M Olah, unpublished observations).

Qian & McCloskey (68) recently reported the activation of an outwardly rectifying potassium current in RBL-2H3 cells upon application of NECA. However, the pharmacology, e.g. xanthine sensitivity or agonist-potency order, of the receptor mediating the channel activation was not examined. Qian & McCloskey (68) hypothesized that the augmentation by NECA of antigen-induced secretion may be due in part to potassium channel activation and the resulting membrane repolarization, which would promote an enhancement of antigen-stimulated calcium influx.

The unique pharmacology of the rat A_3AR has also served to identify cardiovascular actions of AR agonists in this species. Fozard & Carruthers (69) originally demonstrated that infusion of APNEA, R-PIA and NECA elicited hypotensive responses in the pithed rat. Involvement of the A_3AR in

this response was indicated by the inability of the antagonist, 8-sulfophenyltheophylline to block the hypotensive activity when infused at concentrations shown to antagonize A_1AR-mediated bradycardia and A_{2a}AR-mediated hypotension. The hypotensive response produced by APNEA could be inhibited by infusion of high doses of I-ABOPX, a xanthine derivative containing a para-acidic substitution that was shown in radioligand-binding studies to have high affinity at the sheep and human A_3ARs, though its rat A_3AR affinity has not been reported (70). This finding reiterates the requirement for selective agonists and antagonists for study of the physiological significance of the A_3AR, especially in the human where certain xanthines display moderate affinity. The apparently A_3AR-mediated hypotension is pertussis toxin sensitive (71); however, the mechanism responsible for this response is undefined.

An apparent A_3AR-mediated CNS response has recently been reported (72). The high-affinity A_3AR agonist N^6-(3-iodobenzyl)-5′-*N*-methylcarbixamidoadenosine (IB-MECA), when administered intraperitoneally to mice, produced a depression of locomotor activity (72). A similar locomotor-depressant activity occurs upon administration of A_1AR and A_{2a}AR agonists. However, the IB-MECA–induced depression was not affected by coadministration of antagonists at concentrations that reversed the behavioral effects of selective agonists of the A_1AR and A_{2a}AR. Furthermore, [^{125}I]-AB-MECA was able to detect low levels (10–15 fmol/mg) of A_3AR expression in cerebellum, striatum, hippocampus, and cortex (72). Much work is required to further define CNS roles of the A_3AR. In regards to the finding described above, it is unclear why the same physiological response (locomotor depression) occurs following activation of AR subtypes coupled to different second-messenger responses.

Other AR Subtypes

It is not unreasonable to speculate that additional AR subtypes may be identified in the future, with oligonucleotide probes based on the sequence of already defined receptors serving as the tools to isolate clones from cDNA libraries or as primers in RT-PCR. Several pharmacological observations have been reported that substantiate the existence of additional AR subtypes. Cornfield and coworkers (73) described a possibly novel AR in rat brain based on the unique binding properties of [^{3}H]CV1808. The binding of this adenosine analogue could only partially be displaced by several classical AR ligands. A canine atrial receptor with properties unlike the A_1AR or A_2ARs in regards to ligand selectivity has been described (74). Ameri & Jurna (75) reported an excitatory effect of adenosine analogues on rat hippocampal neurons following blockade of inhibitory A_1ARs with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX). The response was not mimicked by adenylyl cyclase activation by forskolin, suggesting that A_2ARs may not be involved. However, the charac-

terization of a radioligand-binding activity or an adenosine analogue–induced response as being mediated by an additional AR subtype requires the actual cloning of the receptor prior to its unequivocal acceptance.

STRUCTURE-FUNCTION RELATIONSHIPS OF ARs

Ligand Binding

Extensive study of structure-activity relationships of AR ligands has identified substitutions on the adenosine and xanthine parent compounds that produce selective, high-affinity agonists and antagonists, respectively. A_1AR research has particularly benefited from these substitutions, but as discussed in previous sections, selective A_{2a}AR, A_{2b}AR, and A_3AR ligands are still required. Knowledge of AR structure at the amino acid level may now be employed to study AR regions involved in ligand recognition, and this knowledge may further the development of subtype-selective AR ligands.

Cloning of ARs followed that of several other GPCRs, e.g. adrenergic and muscarinic receptors, by approximately 3–5 years. Therefore, initial analysis of structural requirements for AR-ligand binding could be based on results obtained with the previously cloned receptors. These findings include the demonstration of the importance of amino acids located specifically in putative membrane-spanning domains of the receptor and the likelihood of multiple, not necessarily contiguous, receptor regions involved in binding (10, 11). However, unlike the endogenous biogenic amine ligands for those receptors mentioned above, adenosine is uncharged at physiological pH and is bulkier than these molecules. Thus, precise binding features may differ substantially between ARs and these receptors. For instance, the aspartate residue that is conserved in transmembrane domain 3 of all cationic neurotransmitter receptors and is believed to form an ion pair with the endogenous ligand (10, 11) is absent in ARs.

The initial mutational analysis of structural requirements involved in AR-ligand binding examined the role of histidine residues in transmembrane domains of the A_1AR. These amino acids were selected based on a prior demonstration by Klotz and coworkers (76) that treatment of rat brain membranes with the histidyl-specific reagent diethylpyrocarbonate (DEPC) perturbed binding of both [^{3}H]PIA and [^{3}H]DPCPX. The selective, partial protection of [^{3}H]PIA and [^{3}H]DPCPX binding by treatment of membranes with agonists or antagonists, respectively, prior to DEPC exposure suggested separate binding sites for the two classes of ligands and perhaps involvement of multiple histidines in this binding. To more directly study this possibility, histidine 251 (transmembrane domain 6) and histidine 274 (transmembrane domain 7) of the bovine A_1AR were replaced with leucine residues, and the binding of the

constructs were studied, following transient expression in COS-7 cells (17). Substitution of histidine 274 resulted in a complete loss of both agonist and antagonist binding, and these losses suggest a critical role of this histidine in the binding of both classes of ligands. This mutation may possibly have produced an alteration in overall protein conformation, resulting in an unexpressed receptor. However, immunocytochemistry with a receptor-specific antibody reveals that a human A_1AR in which the analogous histidine was replaced with an alanine residue is expressed at the cell surface (A Townsend-Nicholson, personal communication). This would indicate that the receptor does obtain the conformation required for membrane insertion. Replacement of histidine 251 in transmembrane 6 resulted in a fourfold decrease in antagonist affinity, whereas agonist-binding affinities were unaffected (17). Thus, these findings demonstrate the involvement of two histidines in ligand binding by the A_1AR, and these residues may be differentially involved in agonist and antagonist recognition.

The importance of histidines in AR-ligand interactions is further suggested by the conservation of these residues in the analogous location in transmembrane domains 6 and 7 of all A_1ARs and A_2ARs thus far cloned. These are the only histidines present in membrane-spanning domains of these receptors. Site-directed mutagenesis of the histidines of A_2ARs has not been performed. However, experiments similar to those described for the A_1AR employing DEPC treatment of rabbit striatal membranes also suggest involvement of histidine residues in the binding of agonists and antagonists by the A_{2a}AR (77). The species homologues of the A_3AR also contain two histidine residues in the membrane-spanning regions, including the conserved site in transmembrane domain 7. However, histidine is absent in transmembrane domain 6 but is located in transmembrane domain 3. Site-directed mutagenesis of the rat A_3AR to place a histidine in transmembrane domain 6 or to substitute for the histidine in transmembrane domain 3 did not alter ligand-binding properties of this receptor (M Olah, unpublished results).

Several structural features of AR-ligand binding have been elucidated from study of chimeric A_1-A_3 receptors. Because these two subtypes differ markedly in their binding properties, the analysis of the effects of interchanging discrete receptor regions should identify key amino acids. Replacement of the entire second extracellular loop of the rat A_3AR with that of the bovine A_1AR resulted in a chimeric receptor displaying binding affinities for both agonists and antagonists intermediate between that of the wild-type parent receptors (78). Substitution of the region at the amino-terminal portion of this loop of the A_3AR with that of the A_1AR had no effect on binding as compared to wild-type A_3AR. However, substitution of eleven amino acids constituting the distal portion of the loop reproduced the pharmacological profile observed with the entire loop replacement: Affinities for agonists (~30-fold) and antagonists

(>500-fold) were markedly enhanced over those characteristic of the wild-type A_3AR (78). The ability to demonstrate a "gain of function," i.e. enhancement of ligand binding at the A_3AR, via mutation of the distal portion of the loop indicates that ligand-binding properties of the receptor are specifically being altered. However, in the analysis of chimeric receptors, it is not known if the identified amino acids directly interact with the ligand or if the mutation alters conformation of another region that is the specific contact point. Extracellular regions have been identified as forming the binding site in other GPCRs; however, the endogenous ligands for these receptors are typically relatively large molecules, such as glycoprotein hormones or peptides. Based on the proximity of the defined region of the loop to transmembrane domain 5 and the uncertainty of the exact location of the AR ligand-binding pocket, amino acids in this region may directly interact with the ligand. Future mutagenesis experiments analyzing single amino acids, perhaps in concert with study of ligands containing specific chemically reactive functional groups, may further define the precise role of this region.

A chimeric receptor consisting predominantly of A_3AR sequence with replacement of a region encompassing transmembrane domains 6 and 7 with that of the A_1AR also displayed agonist- and antagonist-binding affinities intermediate between those of the wild-type A_1AR and A_3AR (78). Therefore, based on the findings described above, multiple regions of ARs appear to be involved in the binding of agonists and antagonists, and there is considerable overlap in the binding pocket for both types of ligands. The individual contribution of both of the identified regions (distal portion of extracellular loop 2 and transmembrane domains 6 and 7) is demonstrated in a chimeric receptor consisting of A_3AR sequence with substitution of both of these regions with those of the A_1AR. Although composed primarily of A_3AR sequence, this chimera binds agonists and antagonists with affinities very close to those typical of the wild-type A_1AR (78). The importance of individual amino acids in transmembrane domains 6 and 7 of ARs in ligand recognition has been described (17, 79), but the role of extracellular loop 3 in the architecture of the ligand-binding pocket also warrants future study.

Despite the findings described above suggesting significant overlap in the agonist- and antagonist-binding pocket of ARs, other studies have differentiated not only between the structural requirements for agonist and antagonist recognition but also between distinct classes of agonists. Specifically, different receptor regions are involved in the binding of agonists containing a substitution at the N^6 position of the adenine ring, e.g. R-PIA, and those substituted at the 5′ position of the ribose group, e.g. NECA. Townsend-Nicholson & Schofield (79) showed that mutation of threonine 277 (transmembrane domain 7) of the human A_1AR to alanine produced a 400-fold decrease in NECA affinity, while that of [^{3}H]DPCPX, R-PIA, and S-PIA was relatively (<8-fold)

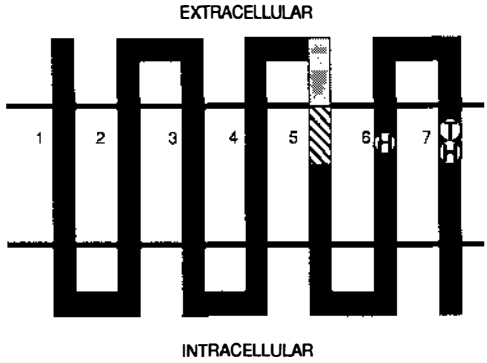

Figure 1 Schematic representation of regions of ARs shown via site-directed mutagenesis to be involved in ligand recognition. Transmembrane domains are numbered one to seven. H represents histidine residues at positions 254 (transmembrane domain 6) and 278 (transmembrane domain 7) of the bovine A_1AR (17). T represents a threonine residue at position 277 (transmembrane domain 7) identified in the human A_1AR (79). The stippled area represents an 11–amino acid segment of the second extracellular loop identified in a study of chimeric A_1 and A_3 receptors that is involved in agonist and antagonist recognition (78). The cross-hatched region represents a 6–amino acid segment of transmembrane domain 5 identified in chimeric A_1 and A_3 receptors that is involved in binding of 5′-substituted agonists (80). See text for details.

unaffected. Interestingly, this threonine is adjacent to the histidine residue in transmembrane domain 7 that was previously discussed. An analysis of chimeric A_1 and A_3 receptors has also revealed structural requirements specific for the binding of 5′-substituted AR agonists (80). Replacement of the fifth transmembrane domain of the A_1AR with the analogous region of the A_3AR resulted in binding parameters identical to the wild-type A_1AR in regards to antagonist recognition and binding of those agonists that contained solely a substitution at the N^6 position (80). However, the binding of 5′-substituted agonists was of significantly greater affinity at the chimeric receptor as compared to the wild-type A_1AR. An additional chimeric demonstrated that the enhancement of binding of 5′-substituted agonists could be localized to a six–amino acid segment located at the exofacial region of transmembrane domain 5 (80). These two studies imply that multiple regions of ARs, including portions of transmembrane domains 5 and 7, are involved in the formation of the ligand-binding pocket for 5′-substituted analogues. Regions of ARs identified by site-directed mutagenesis to be involved in ligand binding are shown schematically in Figure 1.

Currently, site-directed mutagenesis studies of A_{2a}AR-ligand binding have not been reported; however, the approach taken by Piersen and coworkers (81) suggests a role for transmembrane domain 5 of the A_{2a}AR in agonist binding. Peptide mapping of canine A_{2a}AR expressed in COS M6 cells following

photoaffinity cross-linking with [^{125}I]-azido-PAPA-APEC resulted in the generation of a specifically labeled fragment (81). This fragment, identified by its mass, sensitivity to endoglycosidase F, and recognition by specific antibodies appears to encompass transmembrane domain 5 of the receptor (81). Interestingly, the mutational analysis previously described strongly suggests that binding of 5′-substituted agonists specifically involves transmembrane domain 5, and [^{125}I]-azido-PAPA-APEC belongs to this category. However, in peptide mapping studies the possibility of incorporation of probe into receptor regions separate from the actual ligand-binding pocket exists.

Desensitization

A well-studied regulatory phenomenon of GPCR that has physiological and clinical relevance is that of desensitization. Desensitization typically refers to the lessened functional response of a receptor upon repeated exposure to the agonist. Desensitization of ARs has been studied at several levels: the intact animal, cell lines endogenously expressing ARs, and mammalian cells transfected with AR cDNAs. Considering the tremendous differences in these model systems, variability in results may be expected. It is hoped that mechanisms delineated at the molecular level in transfected cells may serve to better explain the processes observed in whole animals.

The effects of chronic R-PIA treatment on adipocyte (82, 83) and, more recently, cardiac (84) A_1AR function has been examined, and for the most part the results are in agreement. Multi-day infusion of rats with R-PIA resulted in a decreased ability of the adipocyte A_1AR to mediate the inhibition of adenylyl cyclase (82, 83) and the atrial A_1AR to mediate negative chronotropic and inotropic effects (84) in response to A_1AR agonists. This functional desensitization was accompanied by a decrease in membrane A_1AR expression and impaired coupling of the A_1AR to associated G proteins (assessed by agonist competition binding) (82–84). In response to R-PIA treatment, cellular changes are not restricted to the receptor. Western blotting with G protein α-subunit–specific antibodies revealed that in adipocytes from R-PIA–treated rats $G_{i\alpha 1+2}$ levels declined by ~60%, with no change in $G_{i\alpha 3}$ (83). $G_{s\alpha}$ levels were elevated by ~50% compared to control (83). Similar results were obtained for cardiac tissues, although R-PIA treatment did not alter $G_{s\alpha}$ levels (84). Northern blotting indicated that G protein mRNA levels were not altered in desensitized tissues, suggesting involvement of posttranslational events in altering protein quantities (83). Changes in G protein content are most likely to be responsible for the observation in both systems that R-PIA infusion produced a heterologous desensitization, i.e. diminished functional effects were not limited to those mediated by the A_1AR; responses to other receptor agonists were also decreased (82–84).

To eliminate some of the complicating factors present in the whole animal,

investigators have studied mechanisms involved in A_1AR desensitization in cultured cells. A_1AR desensitization in cultured adipocytes is very similar to that described above for the intact animal (85). Ramkumar et al (42) began to explore the cellular mechanisms responsible for the alterations in A_1AR function associated with desensitization in the smooth muscle cell line DDT_1 MF-2. In these cells, R-PIA treatment resulted in a loss of A_1AR-mediated inhibition of adenylyl cyclase, with accompanying receptor down-regulation and a decline in high-affinity agonist binding (42). Significant findings regarding underlying events that may be at least partially responsible for these effects include the demonstration of a R-PIA–induced 37% increase in the A_1AR population in a light vesicle membrane fraction indicative of receptor sequestration (42). Furthermore, partial purification of A_1ARs from control and desensitized cells revealed that R-PIA treatment induced a three- to fourfold increase in receptor phosphorylation (42). It was subsequently shown that the A_1AR purified from bovine brain can serve as a substrate for G protein receptor kinase–2 (GRK-2) (bovine βARK) and this phosphorylation resulted in impaired receptor–G protein coupling in in vitro assays (86).

The chronic effects of AR agonist treatment of whole animals on A_{2a}AR function have not been extensively studied. Makujina & Mustafa (87) have shown that 30-min treatment of isolated porcine coronary rings with NECA substantially reduces the relaxation of these vessels in response to an A_{2a}AR agonist but that this effect is apparently species dependent. Conversely, in cultured cells, a relatively rapid desensitization of the A_{2a}AR in regards to adenylyl cyclase stimulation has been consistently demonstrated (42, 88, 89). In DDT_1 MF-2 cells that endogenously express both receptor subtypes, the $t_{1/2}$ for A_1AR and A_{2a}AR desensitization was 16 h and 45 min, respectively (42). Events that accompany A_{2a}AR desensitization have recently been examined in CHO cells transfected with canine A_{2a}AR cDNA (88) and rat PC12 cells endogenously expressing the receptor (89). These studies report somewhat divergent results but are in agreement in regards to multiple, temporally dependent mechanisms underlying the observed functional responses. In CHO cells, stimulation of adenylyl cyclase by AR agonists is markedly reduced after 30-min treatment, and this reduction occurs with a 2-fold reduction in agonist affinity but no change in receptor number (88). Immunoblotting indicated that agonist exposure (30 min) induced A_{2a}AR sequestration into a light membrane fraction; however, blockade of this event did not prevent desensitization but rather attenuated the recovery response of the receptor (88). Short-term agonist exposure of CHO cells also induced phosphorylation of the A_{2a}AR, which was apparently not mediated by protein kinase A, thus suggesting the involvement of a GRK(s) (88). After 24 h of agonist treatment, additional regulatory processes include the loss of the membrane population of A_{2a}AR (down-regulation) and increased levels of $G_{i\alpha 2}$ and $G_{i\alpha 3}$, though the expression of $G_{s\alpha}$

is not altered (88). Conversely, in PC12 cells, long-term (14–15 h) CGS21680 treatment was reported to diminish $G_{s\alpha}$ content and up-regulate phosphodiesterase activity (89). These changes may be responsible for the observed reduction of forskolin and GTPγS stimulation of adenylyl cyclase in CGS21680-treated PC12 cells (89). The divergent results between studies may be due to variations in the cell type employed and differences in their regulatory mechanisms. For ARs, future experiments may be directed at identifying kinases and the structural elements of the receptors involved in the desensitization.

Human A_1AR Expression

The isolation of the human A_1AR genomic clone has served as the basis for the identification of a regulatory process in A_1AR expression that has not been reported for other GPCRs. The human A_1AR gene consists of at least six exons and five introns (21). Exons 1–4 and a portion of exon 5 constitute the 5′-untranslated region of the sequence and the 3′ portion of exon 5 with exon 6 represent the coding sequence and 3′-untranslated region (Figure 2). The coding sequence is interrupted by an intron of at least 5 kb in a region coding for the second intracellular loop of the receptor (21). The presence of a single intron in the coding sequence would not alter amino acid sequence of the expressed receptor. However, the presence of introns in the 5′-untranslated region results in the occurrence of alternative splicing events, which apparently affect the level of A_1AR expression in a tissue-specific manner. Analysis of multiple human tissues via RT-PCR revealed a transcript containing exons 4, 5, and 6 in all samples, while a separate, distinct transcript containing exons 3, 5, and 6 was detected in brain frontal cortex, cerebellum, testis, and kidney (21). A transcript containing exons 1 and 2 or a species consisting of both exons 3 and 4 was not detected in any tissue (Figure 2) (21). Interestingly, those regions found to possess the exon 3, 5, and 6 transcript are known to express relatively high amounts of the A_1AR, as judged by radioligand binding.

This tissue-specific splicing event regulates A_1AR levels, apparently resulting from the ability of ATG codons in exon 4 to repress receptor expression in those tissues containing solely the exon 4,5,6 transcript. Specifically, it was demonstrated that the lowest amounts of A_1AR expression in COS-7 cells consistently occurred in those cells transfected with constructs (either natural or genetically engineered) containing exon 4 (90). Furthermore, mutation of one or both of the two ATG codons in exon 4 that exist in favorable Kozak sequences was found to relieve translational repression such that expression of the A_1AR in the construct containing simultaneous mutation of both ATG codons was equal to the level obtained with the exon 3, 5, and 6 construct (90). Thus, it is the presence of the transcript containing exons 3, 5, and 6 in certain tissues that results in high A_1AR expression. This relatively unexplored

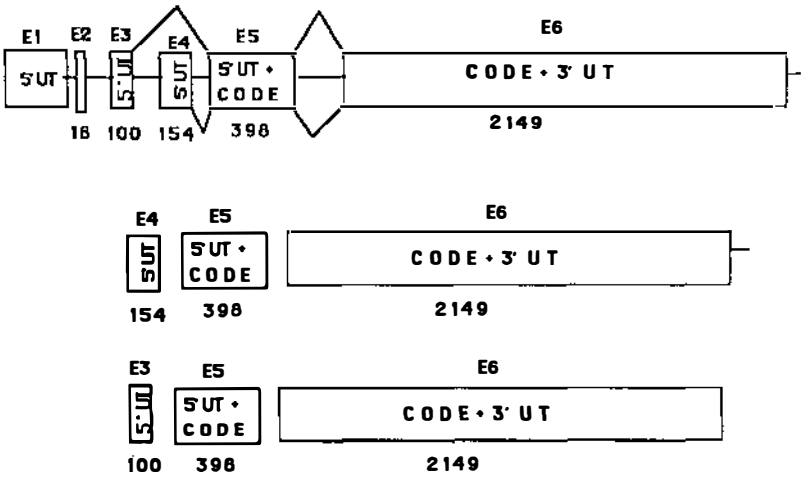

Figure 2 Genomic organization and mRNA splicing of human A_1AR. The top schematic represents the complete genomic structure; lines connect the various alternatively spliced messages that have been detected (21). Exons 3 and 4 appear mutually exclusive, and exons 1 and 2 have not been found in any mature message. The lower two schematics illustrate the two alternative prespliced transcripts found in human tissues. See text for details.

area of receptor regulation possesses many directions for future research. These include identification of the mechanisms and cell machinery involved in the alternative splicing events and possible alterations in A_1AR transcript expression in development and pathophysiological conditions.

CLINICAL UTILITY

Although adenosine was administered to humans on an experimental basis in 1930 and was found to induce transient sinus bradycardia and AV nodal block (91), its clinical usefulness in the United States was not recognized until the 1980s (92). Adenosine is now one of the most commonly used drugs in the diagnosis and treatment of supraventricular arrhythmias. It has now been demonstrated to be highly effective in terminating supraventricular tachycardias of the AV reciprocating type regardless of whether it is a consequence of a bypass tract or an AV nodal reentrant type. Adenosine is also useful in the diagnosis of narrow complex tachycardias. Patients with tachycardias involving the AV node are terminated in greater than 90% of the cases, whereas supraventricular arrhythmias not involving the AV node or atrial flutter or fibrillation or intraatrial reentry are not terminated. In addition, the slowing of the conduction across the AV node frequently allows flutter or fibrillation

waves to be discerned, thus confirming the correct diagnosis. There are several excellent reviews on the electrophysiological properties of adenosine to which the readers are referred (93, 94).

Another use of adenosine is to produce coronary vasodilation, which in combination with myocardial perfusion scintigraphy allows assessment of whether perfusion defects occur as a consequence of coronary stenosis (95). This technique can be extremely useful in patients who are physically unable to exercise as a result of arthritis or other physical handicaps. The pharmacologically induced vasodilatation increases coronary flow by 3- to 6-fold in normal coronaries, whereas a stenotic artery with > 50–75% luminal narrowing allows a much lower-fold increase in coronary flow. If a radioactive tracer such as thallium-201 is injected during maximal vasodilatation, the amount of radioactivity in a given area of the heart will be directly related to the level of coronary flow, which in turn will be inversely related to the extent of stenosis of a given coronary artery. Since specific coronary arteries subserve distinct regions of the heart, cardiologists can obtain important information on if and how much myocardium may be at risk for ischemic events, and document if functional coronary artery stenosis exists (95).

A potential therapeutic use for adenosine or a variety of synthetic analogues is to provide protection to the heart during ischemia or infarction. The approach used is known as preconditioning [first proposed by Ely et al (96)], wherein a sublethal period of ischemia with decreased coronary flow followed by release of the stenosis leads to a resistance of the myocardium to infarction with subsequent decreases in coronary flow. After much research, it has become evident that adenosine may be an important mediator of this cardioprotective effect (97). Adenosine is released in large quantities during ischemia and is important in helping to stabilize tissues and cells under stressful conditions.

Downey and coworkers have documented that a coronary occlusion of five minutes could establish this cardioprotective effect and that this antiinfarct state could last for as long as one hour (97, 98). Subsequent studies have documented that A_1AR-selective agonists could likewise produce this protective effect (99). Although these compounds are A_1 selective, it is now known that these agonists can activate A_3ARs, making it impossible to rule out some component of A_3AR-mediated effects.

Further evidence for an A_1AR-mediated event includes the ability to block the effect of A_1AR agonists by methylxanthine antagonists (which are largely ineffective at A_3ARs). The finding that prior treatment of animals with pertussis toxin [which inactivates the G_i proteins that couple ARs to their effector systems—adenylyl cyclase and K^+ channels (100)] blocks this cardioprotective effect directly implicates a G protein–coupled pathway in this important phenomenon.

This cardioprotective effect has been documented to occur in a variety of animal models, suggesting that it may be transferrable to the human heart (97). At present, new approaches will need to be taken to apply this technology to the patient. It will be impossible to infuse adenosine or an analogue just prior to a coronary occlusion because we simply do not know when an occlusion will occur. However, approaches to unstable patients or patients about to undergo complex angioplasty or coronary bypass surgery could be undertaken with current technology. Clinical trials will be needed to determine whether the approach will be successful.

A major question remains as to what mechanism(s) is responsible for this protective effect. Activation of protein kinase C or generation of heat shock proteins have been suggested as possible mediators. To date, there is no clear answer.

A completely different approach would be to manipulate the level or state of activation of the A_1ARs in the heart. With the cloning of the human A_1AR gene and the finding that the level of A_1ARs expressed in a given tissue is regulated by the type of mRNA produced (21, 90), there is the potential to manipulate directly how much A_1AR is expressed in the heart. The heart typically has very low levels of A_1AR; other tissues have 100 times more A_1AR. Tissues with low levels of A_1AR have different mRNAs than those with high levels. By discerning the mechanisms that the cell uses to produce the different transcripts, we could make the heart a producer of higher levels of A_1AR, which may provide additional cardioprotection. Much work will be required to see if this approach would be useful.

Similar to the situation in heart, cerebral ischemia induces the elevation of adenosine levels, which may serve a neuroprotective role. The mechanisms underlying this protection may include inhibition of excitatory neurotransmitter release and regulation of intracellular calcium levels (101). A review of the therapeutic potential of AR ligands as neuroprotective agents and a summary of studies in this area has been provided by Rudolphi et al (101). Williams (4) has recently analyzed the status of the potential therapeutic use of purinergic compounds in other disorders.

CONCLUSIONS

The cloning of AR subtypes has provided the basis for the study of AR function and regulation at a very basic level. Future AR research may be directed at further identifying amino acids involved in ligand binding and the receptor regions accounting for the differences in agonist and antagonist activity. The mechanisms involved in AR desensitization can also be further explored. Though not discussed extensively in this review, much work remains to be done to determine which specific regions of ARs interact with G proteins and

which features determine the selective activation of a particular G protein α subunit. This work will increase understanding of the coupling of ARs to various second-messenger systems. As discussed, AR genomic structure has a role in regulating receptor expression in a tissue-specific fashion. The mechanisms underlying this control and the regulation of transcript expression in various conditions represent targets of future research. The positive identification of additional AR subtypes is possible and would help explain the many physiological effects of adenosine. The use of transgenic animals or cell lines to define physiological roles for ARs may also be exploited. The current therapeutic use of adenosine was described; however, an understanding of AR function at the basic levels described above may be required for the therapeutic potential of AR regulation to be fully realized.

Literature Cited

1. Fenton RA, Dobson JG. 1987. Measurement by fluorescence of interstitial adenosine levels in normoxic, hypoxic and ischemic perfused rat hearts. *Circ. Res.* 60:177–84
2. Hagberg H, Andersson P, Lacarewicz J, Jacobson I, Butcher S, Sandberg M. 1987. Extracellular adenosine, inosine, hypoxanthine, and xanthine in relation to tissue nucleotides and purines in rat striatum during transient ischemia. *J. Neurochem.* 49:227–31
3. During MJ, Spencer DD. 1992. Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann. Neurol.* 32:618–24
4. Williams M. 1993. Purinergic drugs: opportunities in the 1990s. *Drug Dev. Res.* 28:438–44
5. Olsson RA, Pearson JD. 1990. Cardiovascular purinoceptors. *Physiol. Rev.* 70: 761–845
6. Daval J-L, Nehlig A, Nicolas F. 1991. Physiological and pharmacological properties of adenosine: therapeutic implications. *Life Sci.* 49:1435–53
7. Libert F, Parmentier M, Lefort A, Dinsart C, Van Sande J, et al. 1989. Selective amplification and cloning of four new members of the G protein-coupled receptor family. *Science* 244:569–72
8. Libert F, Schiffman SN, Lefort A, Parmentier M, Gerard C, et al. 1991. The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor. *EMBO J.* 10:1677–82
9. Maenhaut C, Van Sande J, Libert F, Abramowicz M, Parmentier M, et al. 1990. RDC8 codes for an adenosine A2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* 173:1169–78
10. Hulme EC, Birdsall NJM, Buckley NJ. 1990. Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* 30:633–73
11. Ostrowski J, Kjelsberg MA, Caron MG, Lefkowitz RJ. 1992. Mutagenesis of the β_2-adrenergic receptor: how structure elucidates function. *Annu. Rev. Pharmacol. Toxicol.* 32:167–83
12. Stiles GL. 1986. Photoaffinity cross-linked A_1 adenosine receptor-binding subunits: homologous glycoprotein expression by different tissues. *J. Biol. Chem.* 261:10839–43
13. Barrington WW, Jacobson KA, Stiles GL. 1990. Glycoprotein nature of the A_2-receptor binding subunit. *Mol. Pharmacol.* 38:177–83
14. Piersen CE, True CD, Wells JN. 1994. A carboxyl-terminally truncated mutant and nonglycosylated A_{2a} adenosine receptors retain ligand binding. *Mol. Pharmacol.* 45:861–70
15. Mahan LC, McVittie LD, Smyk-Randall

EM, Nakata H, Monsma FJ, et al. 1991. Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.* 40:1–7

16. Reppert SM, Weaver DR, Stehle JH, Rivkees SA. 1991. Molecular cloning and characterization of a rat A_1-adenosine receptor that is widely expressed in brain and spinal cord. *Mol. Endocrinol.* 5:1037–48
17. Olah ME, Ren H, Ostrowski J, Jacobson KA, Stiles GL. 1992. Cloning, expression, and characterization of the unique bovine A_1 adenosine receptor: studies on the ligand binding site by site-directed mutagenesis. *J. Biol. Chem.* 267: 10764–70
18. Tucker AL, Linden J, Robeva AS, D'Angelo DD, Lynch KR. 1992. Cloning and expression of a bovine adenosine A_1 receptor cDNA. *FEBS Lett.* 297:107–11
19. Libert F, Van Sande J, Lefort A, Czernilofsky A, Dumont JE, et al. 1992. Cloning and functional characterization of a human A_1 adenosine receptor. *Biochem. Biophys. Res. Commun.* 187: 919–26
20. Townsend-Nicholson A, Shine J. 1992. Molecular cloning and characterisation of a human brain A_1 adenosine receptor cDNA. *Mol. Brain Res.* 16:365–70
21. Ren H, Stiles GL. 1994. Characterization of the human A_1 adenosine receptor gene: evidence for alternative splicing. *J. Biol. Chem.* 269:3104–10
22. Bhattacharya S, Dewitt DL, Burnatowska-Hledin M, Smith WL, Spielman WS. 1993. Cloning of an adenosine A1 receptor-encoding gene from rabbit. *Gene* 128:285–88
23. Weaver DR, Reppert SM. 1992. Adenosine receptor gene expression in rat kidney. *Am. J. Physiol.* 263:F991–95
24. Londos C, Cooper DMF, Wolff J. 1980. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* 77: 2551–54
25. Van Calker D, Muller M, Hamprecht B. 1979. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J. Neurochem.* 33:999–1005
26. Nanoff C, Freissmuth M, Tuisl E, Schutz W. 1990. P_{2Y}-, but not P_1-purinoceptors mediate formation of 1,4,5-inositol trisphosphate and its metabolites via a pertussis toxin-insensitive pathway in the rat renal cortex. *Br. J. Pharmacol.* 100: 63–68
27. Arend LJ, Handler JS, Rhim JS, Gusovsky F, Spielman WS. Adenosine-sensitive phosphoinositide turnover in a newly established renal cell line. *Am. J. Physiol.* 256:F1067–74
28. Gerwins P, Fredholm BB. 1992. ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-trisphosphate in a smooth muscle cell line. *J. Biol. Chem.* 267:16081–87
29. Hollingswoth EB, De La Cruz RA, Daly JW. 1986. Accumulations of inositol phosphates and cyclic AMP in brain slices: synergistic interactions of histamine and 2-chloroadenosine. *Eur. J. Pharmacol.* 122:45–50
30. Hill SJ, Kendall DA. 1987. Studies on the adenosine-receptor mediating the augmentation of histamine-induced inositol phospholipid hydrolysis in guinea-pig cerebral cortex. *Br. J. Pharmacol.* 91:661–69
31. Long CJ, Stone TW. 1987. Adenosine reduces agonist-induced production of inositol phosphates in rat aorta. *J. Pharm. Pharmacol.* 39:1010–14
32. Delahunty TM, Cronin MJ, Linden J. 1988. Regulation of GH_3-cell function via adenosine A_1 receptors: inhibition of prolactin release, cyclic AMP production and inositol phosphate generation. *Biochem. J.* 255:69–77
33. Scheimann WP, Buxton IL. 1991. Adenosine A_1-receptor coupling to phosphoinositide metabolism in pregnant guinea pig myometrium. *Am. J. Physiol.* 261:E665–72
34. Rossi N, Churchill P, Ellis V, Amore B. 1988. Mechanism of adenosine receptor-induced renal vasoconstriction in rats. *Am. J. Physiol.* 255:H885–90
35. Gerwins P, Fredholm BB. 1992. Stimulation of adenosine A_1 receptors and bradykinin receptors, which act via different G proteins, synergistically raises inositol 1,4,5-trisphosphate and intracellular free calcium in DDT_1 MF-2 smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 89:7330–34
36. Iredale PA, Alexander SPH, Hill SJ. 1994. Coupling of a transfected human brain A_1 adenosine receptor in CHO-K1 cells to calcium mobilisation via a pertussis toxin-sensitive mechanism. *Br. J. Pharmacol.* 111:1252–56
37. Akbar M, Okajima F, Tomura H, Shimegi S, Kondo Y. 1994. A single species of A_1 adenosine receptor expressed in Chinese hamster ovary cells not only inhibits cAMP accumulation but also stimulates phospholipase C and arachidonate release. *Mol. Pharmacol.* 45:1036–42
38. Belardinelli L, Shyrock JC, Pelleg A. 1992. Cardiac electrophysiologic prop-

erties of adenosine. *Coron. Artery Dis.* 12:1122–26
39. Stone TW, Bartrup JT. 1991. Electropharmacology of adenosine. In *Adenosine in the Nervous System*, ed. T Stone, 8:173–92. London/San Diego: Academic. 278 pp.
40. Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, et al. 1992. Molecular cloning of the rat A_2 adenosine receptor: selective co-expression with D_2 dopamine receptors in rat striatum. *Mol. Brain Res.* 14:186–95
41. Furlong TJ, Pierce KD, Selbie LA, Shine J. 1992. Molecular characterization of a human brain adenosine A_2 receptor. *Mol. Brain Res.* 15:62–66
42. Ramkumar V, Olah ME, Jacobson KA, Stiles GL. 1991. Distinct pathways of desensitization of A_1 and A_2 adenosine receptors in DDT_1 MF-2 cells. *Mol. Pharmacol.* 40:639–47
43. Linden J. 1991. Structure and function of A_1 adenosine receptors. *FASEB J.* 5:2668–76
44. Jacobson KA, Gallo-Rodriguez C, Melman N, Fischer B, Maillard M, et al. 1993. Structure-activity relationships of 8-styrylxanthines as A_2 selective adenosine antagonists. *J. Med. Chem.* 36: 1333–42
45. Shimada J, Suzuki F, Nonaka H, Ishii A, Ichikawa S. 1992. (E)-1,3-Dialkyl-7-methyl-8-(3,4,5-trimethoxystyryl)xanthines: potent and selective adenosine A_2 antagonists. *J. Med. Chem.* 35:2342–45
46. Sarges R, Howard HR, Browne RG, Lebel LA, Seymour PA, Koe BK. 1990. 4-amino[1l,2,4]triazolo[4,3a]-quinoxalines. A novel class of potent adenosine antagonists and potential rapid-onset antidepressants. *J. Med. Chem.* 33:2240–54
47. Jarvis MF, Williams M. 1989. Direct autoradiographic localization of adenosine A_2 receptors in the rat brain using the A_2-selective agonist, [^{3}H]CGS21680. *Eur. J. Pharmacol.* 168:243–46
48. Schiffmann SN, Vanderhaeghen JJ. 1993. Adenosine A2 receptors regulate the gene expression of striatopallidal and striatonigral neurons. *J. Neurosci.* 13: 1080–87
49. Linden J, Taylor HE, Robeva AS, Tucker AL, Stehle JH, et al. 1993. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* 44:524–32
50. Johnson RA. 1982. Mn^{2+} does not uncouple adenosine "Rs" receptors from the liver adenylate cyclase. *Biochem. Biophys. Res. Commun.* 105:347–53
51. Huttemann E, Ukena D, Lenschow V, Schawbe U. 1984. R_a adenosine receptors in human platelets: characterization by 5′-*N*-ethylcarboxamido[^{3}H]-adenosine binding in relation to adenylate cyclase activity. *Naunyn Schmiedebergs Arch. Pharmacol.* 325:226–33
52. Sabouni MH, Cushing DJ, Makujina SR, Mustafa SJ. 1991. Inhibition of adenylate cyclase attenuates adenosine receptor-mediated relaxation in coronary artery. *J. Pharmacol. Exp. Ther.* 259: 508–12
53. Ledent C, Dumont JE, Vassart G, Parmentier M. 1992. Thyroid expression of an A_2 adenosine receptor transgene induces thyroid hyperplasia and hyperthyroidism. *EMBO J.* 11:537–42
54. Daly JW, Butts-Lamb P, Padgett W. 1983. Subclasses of adenosine receptors in the central nervous system: interaction with caffeine and related methylxanthines. *Cell Mol. Neurobiol.* 3:69–80
55. Bruns RF, Lu GH, Pugsley TA. 1986. Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol. Pharmacol.* 29:331–46
56. Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD, Reppert SM. 1992. Molecular cloning and expression of the cDNA for a novel A_2-adenosine receptor subtype. *Mol. Endocrinol.* 6:384–93
57. Rivkees SA, Reppert SM. 1992. RFL9 encodes an A_{2b}-adenosine receptor. *Mol. Endocrinol.* 6:1598–1604
58. Pierce KD, Furlong TJ, Selbie LA, Shine J. 1992. Molecular cloning and expression of an adenosine A_{2b} receptor from human brain. *Biochem. Biophys. Res. Commun.* 187:86–93
59. Yakel JL, Warren RA, Reppert SM, North RA. 1993. Functional expression of adenosine A_{2b} receptor in *Xenopus* oocytes. *Mol. Pharmacol.* 43:277–80
60. Marquardt DL, Walker LL, Heinemann S. 1994. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J. Immunol.* 152: 4508–15
61. Meyerhof W, Muller-Brechlin R, Richter D. 1991. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* 284:155–60
62. Zhou Q-Y, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O. 1992. Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* 89:7432–36
63. Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG. 1993. Molecular

cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* 90:10365–69

64. van Galen PJM, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, et al. 1994. A binding site model and structure-activity relationships for the rat A_3 adenosine receptor. *Mol. Pharmacol.* 45: 1101–11
65. Olah ME, Gallo-Rodriguez C, Jacobson KA, Stiles GL. 1994. ^{125}I-4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.* 45: 978–82
66. Ali H, Cunha-Melo JR, Saul WF, Beaven MA. 1990. Activation of phospholipase C via adenosine receptors provides synergistic signals for secretion in antigen-stimulated RBL-2H3 cells: evidence for a novel adenosine receptor. *J. Biol. Chem.* 265:745–53
67. Ramkumar V, Stiles GL, Beaven MA, Ali H. 1993. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* 268:16667–90
68. Qian Y-X, McCloskey MA. 1993. Activation of mast cell K^+ channels through multiple G protein-linked receptors. *Proc. Natl. Acad. Sci. USA* 90: 7844–48
69. Fozard JR, Carruthers AM. 1993. Adenosine A_3 receptors mediate hypotension in the angiotensin II-supported circulation of the pithed rat. *Br. J. Pharmacol.* 109:3–5
70. Fozard JR, Hannon JP. 1994. BW-A522 blocks adenosine A_3 receptor-mediated hypotensive responses in the rat. *Eur. J. Pharmacol.* 252:R5–6
71. Carruthers AM, Fozard JR. 1993. Effect of pertussis toxin treatment on the putative adenosine A_3 receptor-mediated hypotensive response in the rat. *Eur. J. Pharmacol.* 250:185–88
72. Jacobson KA, Nikodijevic O, Shi D, Gallo-Rodriguez C, Olah ME, et al. 1993. A role for central A_3-adenosine receptors. *FEBS Lett.* 336:57–60
73. Cornfield LJ, Hu S, Hurt SD, Sills MA. 1992. [^{3}H]2-phenylaminoadenosine ([^{3}H]CV 1808) labels a novel adenosine receptor in rat brain. *J. Pharmacol. Exp. Ther.* 263:552–61
74. Martin PL. 1992. Evidence that adenosine receptors in the dog left atrium are not of the typical A_1 or A_2 subtypes. *Eur. J. Pharmacol.* 214:199–205
75. Ameri A, Jurna I. 1991. Adenosine A_1 and non-A_1 receptors: intracellular analysis of the actions of adenosine agonists and antagonists in rat hippocampal neurones. *Brain Res.* 546:69–78
76. Klotz K-N, Lohse MJ, Schwabe U. 1988. Chemical modification of A_1 adenosine receptors in rat brain membranes: evidence for histidine in different domains of the ligand binding site. *J. Biol. Chem.* 263:17522–26
77. Jacobson KA, Stiles GL, Ji X-D. 1992. Chemical modification and irreversible inhibition of striatal A_{2a} adenosine receptors. *Mol. Pharmacol.* 42:123–33
78. Olah ME, Jacobson KA, Stiles GL. 1994. Role of the second extracellular loop of adenosine receptors in agonist and antagonist binding: analysis of chimeric A_1/A_3 adenosine receptors. *J. Biol. Chem.* 269:24692–98
79. Townsend-Nicholson A, Schofield PR. 1994. A threonine residue in the seventh transmembrane domain of the human A_1 adenosine receptor mediates specific agonist binding. *J. Biol. Chem.* 269: 2373–76
80. Olah ME, Jacobson KA, Stiles GL. 1994. Identification of an adenosine receptor domain specifically involved in binding of 5′-substituted adenosine agonists. *J. Biol. Chem.* 269:18016–20
81. Piersen CE, True CD, Wells JN. 1994. ^{125}I-2-[4-[2-[2-[(4-azidophenyl)-methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′ -*N*-ethylcarboxamidoadenosine labels transmembrane span V of the A_{2a} adenosine receptor. *Mol. Pharmacol.* 45:871–77
82. Parsons WJ, Stiles GL. 1987. Heterologous desensitization of the inhibitory A_1 adenosine receptor-adenylate cyclase system in rat adipocytes: regulation of both N_s and N_i. *J. Biol. Chem.* 262:841–47
83. Longabaugh JP, Didsbury J, Spiegel A, Stiles GL. 1989. Modification of the rat adipocyte A_1 adenosine receptor-adenylate cyclase system during chronic exposure to an A_1 adenosine receptor agonist: alterations in the quantity of $G_{s\alpha}$ and $G_i\alpha$ are not associated with changes in their mRNAs. *Mol. Pharmacol.* 36:681–88
84. Lee HT, Thompson Cl, Hernandez A, Lewy JI, Belloni FL. 1993. Cardiac desensitization to adenosine analogues after prolonged R-PIA infusion *in vivo*. *Am. J. Physiol.* 265:H1916–27
85. Green A, Johnson JL, Milligan G. 1990. Down-regulation of G_i sub-types by prolonged incubation of adipocytes with an A_1 adenosine receptor agonist. *J. Biol. Chem.* 265:5206–10
86. Ramkumar V, Kwatra M, Benovic JL, Stiles GL. 1993. Functional conse-

quences of A_1 adenosine-receptor phosphorylation by the β-adrenergic receptor kinase. *Biochim. Biophys. Acta* 1179: 89–97
87. Makujina SR, Mustafa SJ. 1993. Adenosine-5′-uronamides rapidly desensitize the adenosine A_2 receptor in coronary artery. *J. Cardiovasc. Pharmacol.* 22: 506–9
88. Palmer TM, Gettys TW, Jacobson KA, Stiles GL. 1994. Desensitization of the canine A_{2a} adenosine receptor: delineation of multiple processes. *Mol. Pharmacol.* 45:1082–94
89. Chern Y, Lai H-L, Fong JC, Liang Y. 1993. Multiple mechanisms for desensitization of A_{2a} adenosine receptor-mediated cAMP elevation in rat pheochromocytoma PC12 cells. *Mol. Pharmacol.* 44:950–58
90. Ren H, Stiles GL. 1994. Posttranscriptional mRNA processing as a mechanism for regulation of human A_1 adenosine receptor expression. *Proc. Natl. Acad. Sci. USA* 91:4864–66
91. Honey RM, Ritchie WT, Thomson WAR. 1930. The action of adenosine upon the human heart. *Q. J. Med.* 23: 485–90
92. DiMarco JP, Selers TD, Berne RM, West GA, Belardinelli L. 1983. Adenosine: electrophysiologic effects and therapeutic use for terminating paroxysmal supraventricular tachycardia. *Circulation* 68:1254–63
93. Camm AJ, Garratt CJ. 1991. Adenosine and supraventricular tachycardia. *Drug Ther.* 325:1621–27
94. Barber MJ. 1992. Clinical use of adenosine for arrhythmias. *Coron. Artery Dis.* 3:1127–34
95. Verani MS. 1992. Adenosine stress imaging. *Coron. Artery Dis.* 3:1145–51
96. Ely SW, Mentzer RM, Lasley RD, Lee BK, Berne RM. 1985. Functional and metabolic evidence of enhanced myocardial tolerance to ischemia and reperfusion with adenosine. *J. Thorac. Cardiovasc. Surg.* 590:549–56
97. Downey JM, Liu GS, Thornton JD. 1993. Adenosine and the anti-infarct effects of preconditioning. *Cardiovasc. Res.* 27:3–8
98. Van Winkle DM, Thornton JD, Downey DM, Downey JM. 1991. The natural history of preconditioning: cardioprotection depends on duration of transient ischemia and time to subsequent ischemia. *Coron. Artery Dis.* 2:613–19
99. Thornton JD, Liu GS, Olsson RA, Downey JM. 1992. Intravenous A_1 selective adenosine agonists limit infarct size in the rabbit heart. *Circulation* 85:659–65
100. Lasley RD, Mentzer RM. 1993. Pertussis toxin blocks adenosine A_1 receptor mediated protection of the ischemic rat heart. *J. Mol. Cell Cardiol.* 25:815–21
101. Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. 1992. Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol. Sci.* 13: 439–45