Chapter 45
Proteinase-Activated Receptors (PARs) and Calcium Signaling in Cancer

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Abstract Proteinase activated receptors (PARs), a small subfamily of G protein-coupled receptors with four members, PAR_1, PAR_2, PAR_3 and PAR_4, are expressed in various tumours from epithelial origin and can play an important role in tumour progression and metastasis. Within the complex intracellular PAR signaling networks triggered by PARs, an elevation in intracellular free calcium ion concentrations represents a key second messenger system. In this review, we summarize current information about the mechanisms whereby PARs can signal via intracellular calcium in the setting of cancer and we discuss possibilities for using the PAR-$[Ca^{2+}]_i$ signaling pathway as a target for the therapy of epithelial cancer.

Keywords Proteinase activated receptors • PARs • Thrombin receptor • PAR_1 • PAR_2 • PAR_3 • PAR_4 • Signal transduction • Calcium signaling • Intracellular free calcium ion • Carcinogenesis • Cancer progression

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Proteinase Activated Receptors: A Specialized Subfamily of G Protein Coupled Receptors with Complex Intracellular Signal Transduction Pathways

Proteinase activated receptors (PARs) comprise a unique subfamily of G protein-coupled receptors (GPCRs) with four subtypes, PAR_1, PAR_2, PAR_3 and PAR_4 [for reviews see: [1–4]]. Although PARs share basic structural features of all GPCRs, including seven putative hydrophobic transmembrane-spanning alpha helices, they exhibit a novel mechanism of activation that distinguishes them from all other GPCRs. While most GPCRs are activated reversibly by small hydrophilic molecules to elicit cellular responses [5], PAR activation occurs through an irreversible proteolytic mechanism that involves the recognition and cleavage of the receptor by a proteinase at a specific 'cleavage-activation' site located at the extracellular amino-terminus (Fig. 45.1).

This cleavage exposes a cryptic N-terminal domain that acts as a 'tethered ligand' that binds to the receptor extracellular domains to trigger receptor signaling [3, 6–8]. Remarkably, short synthetic peptides modelled on the sequences of the proteolytically-exposed tethered ligand sequences are capable of binding to PARs 1, 2 and 4, mimicking the actions of agonist proteinases [right-hand portion, Fig. 45.1; [9, 10]]. However, the proteolytically exposed N-terminal sequence of PAR_3 and its corresponding synthetic peptides appear to be incapable of causing PAR_3 signaling and instead are able to activate PAR_1 and PAR_2 [11, 12]. As an alternative, a proteinase may cleave a PAR downstream of the 'tethered ligand sequence' (e.g. red arrow,

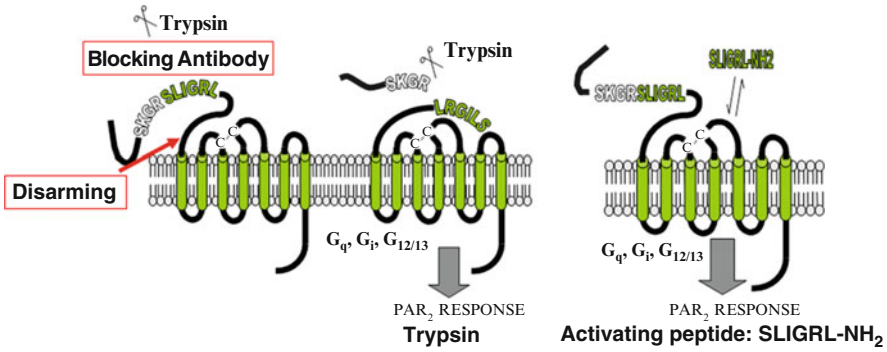

Fig. 45.1 Model for activation and dis-arming of PAR_2. The scheme illustrates activation of the intact receptor (*left-hand panel*) by two distinct mechanisms: either (I) by proteolysis and unmasking of the tethered ligand sequence (*middle Panel*: green sequence, SLIGRL, also seen in the intact receptor) or (II) by a receptor-derived peptide (SLIGRL-NH_2: *right-hand panel*) that activates signaling without the need for receptor proteolysis. The scheme also shows the 'disarming' site for the receptor, where cleavage removes the tethered ligand sequence and the 'cleavage-activation' site', where a 'blocking antibody' can prevent proteolytic activation of the receptor (Redrawn from Hollenberg and Compton, Ref. [6])

left-hand portion, Fig. 45.1), to as to 'dis-arm' and prevent activation of the PAR by an enzyme that would otherwise expose the tethered ligand. Thus, PARs have a variety of both endogenous 'tethered ligand-exposing' proteinase agonists as well as a number of endogenous proteolytic 'antagonists' that can 'silence' receptor activation by other proteinases. Therefore, in the setting of a tumour, both tumour-derived and non-tumour-derived proteinases in the microenvironment can play roles as either PAR agonists or antagonists.

During the last few years it has become evident that PARs, which are triggered by endogenous serine proteinases, mediate hormone-like cellular responses. PAR_1 [9, 13], PAR_3 [14] and PAR_4 [15, 16] are targeted not only by the coagulation cascade proteinases including thrombin, factor Xa and activated protein C, but also by other proteinases including cathepsin and matrix metalloproteinase-I [17–19]. PAR_2 [20] can be activated by trypsin, mast cell tryptase, neutrophil proteinase 3, tissue factor/factor VIIa/factor Xa, human kallikrein-related peptidases, membrane-tethered serine proteinase-1/matriptase 1 and by parasite cysteine proteinases, but not by thrombin [2, 3, 21, 22].

The PAR family is able to stimulate a variety of intracellular signaling pathways which can be either overlapping or distinct for the different PARs, depending on the PAR subtype and the phenotype or stage of differentiation of its specific cellular 'host' [e.g. platelets vs. hepatocytes: for reviews see: [4, 22–28]]. Like other 'GPCRs', the PARs signal via a variety of heterotrimeric guanyl nucleotide-binding proteins (G proteins), including G_q, G_i, $G_{12/13}$ but not directly via G_s [7, 29]. In addition, PAR_2 and possibly the other PARs are able to signal via a non-G-protein mechanism that involves the beta-arrestin-mediated internalization of a PAR_2-beta-arrestin signaling scaffold [30–35]. The coupling of the PARs to either the G-proteins or arrestins is driven by ligand-triggered changes of receptor conformation that for other GPCRs is thought to involve the putative transmembrane helices 3 and 6 of the receptor [36, 37]. Of importance, different agonists are in principle capable of driving different conformational changes in the receptor to result in selective interactions with different downstream 'effectors'. This principle was outlined by the 'floating' or 'mobile' receptor paradigm some time ago [38, 39]. More recently, the concept has evolved to encompass the concept of 'biased receptor signaling' or 'functional selectivity' as outlined in detail elsewhere [40]. For G-protein-mediated signaling, the receptor acts as a ligand-triggered guanine nucleotide exchange factor (GEF), stimulating the exchange of GTP for GDP in the G_α subunit of the heterotrimeric G-protein oligomer. This exchange enables the 'release' of the G_α subunit from its tight binding to the $G_{\beta\gamma}$ dimer subunit. Each of the G-protein moieties (G_α-GTP and $G_{\beta\gamma}$) is then independently able to interact with other select downstream signaling effectors like ion channels ($G_{\beta\gamma}$) or phospholipase C-β (G_q). This 'dual effector' signaling resulting in principle from the same PAR-activated G-protein heterotrimer (e.g. G_q $G_{\beta\gamma}$) can converge for complex downstream signaling, for instance leading to NF-κB activation and ICAM-1 transcription by the engagement of parallel G_q/PKC- and G_i/PI3-kinase pathways that converge [41, 42]. Alternatively, via a 'biased signaling' process, PARs can be activated to affect selec-

tively, MAPKinase signaling via a $G_{12/13}$-triggered process, without causing a G_q-mediated calcium signaling event [31]. This kind of selective signaling can depend not only on the agonist per se [e.g. thrombin or activated protein-C (APC)] but also upon the membrane environment in which a PAR is localized. For instance, triggering of PAR_1 localized in the caveolae by APC can signal via a distinct set of downstream effectors that differ from those regulated when thrombin activates PAR_1 in a non-caveolar environment [29]. The PAR_1 signal pathways activated in these distinct membrane environments lead to a diametrically opposed set of responses that either increase or decrease endothelial barrier integrity. Thus in principle, it is possible to activate and/or inhibit selectively one or other of the downstream signaling pathways activated by PARs (e.g. calcium vs. MAPKinase signals).

PARs Are Involved in Cancer Progression

Local and systemic coagulation is a hallmark of cancer [review: [43]]. In this complicated scenario, tissue factor (TF) induces the formation of the complex TF-VIIa. Both the complex TF-VIIa-Xa and thrombin (factor IIa) can activate proteinase activated receptors. Thrombin can activate PAR_1 and PAR_4 [44], whereas the binary TF-VIIa enzyme complex is able to activate PAR_2 but not PAR_1 [45, 46]. However, as a TF-VIIa-Xa complex, factor Xa efficiently cleaves PAR_2 as well as PAR_1 [47, 48]. In addition, a variety of other proteinases may also be important in the tumour microenvironment, where both stromal and tumour-derived cells can produce PAR-regulating proteinases. Such enzymes can either, like tumour-derived tissue kallikreins [49–51], activate PAR_2, or alternatively proteinases of tissue origin can 'dis-arm' a PAR, by cleaving downstream of the 'tethered ligand' domain (Fig. 45.1, left), thereby silencing a PAR from activation by its target proteinase (e.g. disarmed PAR_1 can no longer respond to thrombin). Moreover tumour-derived proteinases like matrix metalloproteinase-I can cleave the N-terminal domain of a PAR to unmask a 'non-canonical' tethered activating sequence different from the one revealed by serine proteinases [19, 52]. The ability of thrombin to act via PARs was highlighted by the demonstration of the ability of PAR_1 to stimulate tumour invasion [53, 54] by its expression in carcinosarcoma and melanoma cells [55]. The extensive work in this field related to tumour tissue done over the past decade has therefore focused primarily on PAR_1 for which the expression and signaling at the cellular level have been characterized in tumour cells from different tumour entities including cancers of the larynx [56], pancreas [57], glioma [58, 59], glioblastoma [60, 61], meningioma [62], prostate [63] and colon [64]. In addition, PAR_1 activation has been observed to cause (I) increased tumour cell adhesion to the endothelium, extracellular matrix and platelets, (II) enhanced metastatic capacity of tumour cells, (III) activated cell growth and (IV) increased angiogenesis [65–67]. In breast and pancreatic carcinoma cells, the level of PAR_1 expression has been correlated with the degree of invasiveness [54, 68]. Furthermore, transfection of B16F10 melanoma cells with PAR_1, compared with non-transfected cells, leads to a 2.5-fold

enhanced thrombin-induced tumour cell adhesion to fibronectin and a 39-fold increase in pulmonary metastasis [69]. At present there is substantial evidence that thrombin acting via PAR_1 contributes to the metastatic process of certain epithelial tumours including breast [53, 54, 70], colon [64], kidney [71] and liver [72]. However, PAR_1 is not the only functional receptor for thrombin in tumour cells since several reports have demonstrated that PAR_1 can cooperate with the other thrombin target, PAR_4, to act as a 'dual receptor system' in human astrocytoma cells [73] and in cells from liver cancer [72]. In addition to PAR_1, PAR_2 is also known to be expressed in a variety of epithelial tumour cells from different origins [32, 74–82] and to act as an upstream activator of promigratory signaling pathways [34, 75, 80, 83] resulting in an enhancement of tumour progression.

Multiple Effects of PAR Activation on Cancer Cells

Studies dealing with a variety of tumour-related cells have observed important effects of PAR activation, several examples of which will be outlined in this paragraph. Seminal work from the Bar-Shavit laboratory has demonstrated the key role that PAR_1 may exhibit in breast cancer cell invasion [53, 54, 70] and recently Gonda et al. provided impressive data showing movements of breast cancer cells and PAR_1 during metastasis *in vivo* using a highly sophisticated nano-imaging technique [84]. In breast carcinoma cells PAR_1 mediates both migratory and invasive effects [85]. These PAR_1-mediated actions occur in cooperation with alpha-vbeta 5 integrin [53] and with the involvement of increases in intracellular calcium [86]. In 1321N1 astrocytoma cells, Blum and colleagues demonstrated that PAR_1-stimulated ATP release is Ca^{2+}-dependent and that concurrent Rho signaling markedly potentiates this effect [87]. In keratinocyte-related HaCaT cells, PAR_2 activation by matriptase, a membrane-tethered serine proteinase released from the cell surface, has been shown to induce intracellular calcium mobilization and to inhibit proliferation. Based on this information, a role for PAR_2 signaling in skin cancer has been suggested [82]. A substantial amount of data also exist pointing to a role for PARs in colon cancer. In cells from this tumour entity, PAR_1 and PAR_2 have been demonstrated to signal via $[Ca^{2+}]_i$ and to induce migratory and proliferative effects that also involve both activation of p42/p44-MAPKinase and trans-activation of the receptor for epidermal growth factor (EGFR) [64, 74, 88, 89]. In addition, PAR_4 has recently surfaced as a new important player in the regulation of colon tumour-derived cells. In colon carcinoma cells activation of PAR_4 has been found to be involved in stimulating mitogenesis. This stimulation is observed to occur in the setting of PAR_4-induced increases intracellular calcium and activation of p42/p44 MAPKinase along with trans-activation of ErbB-2, a member of the epidermal growth factor receptor B-2 receptor family, but not via trans-activation of the EGF-Erb-B1 homodimer receptor itself [90]. Since PAR_4 does not mediate an increase in cytoplasmic free Ca^{2+} in hepatocellular carcinoma cells [72], but does so in colon carcinoma cells, the ability of PAR_4 to stimulate increases in intracellular calcium appears to be dependent

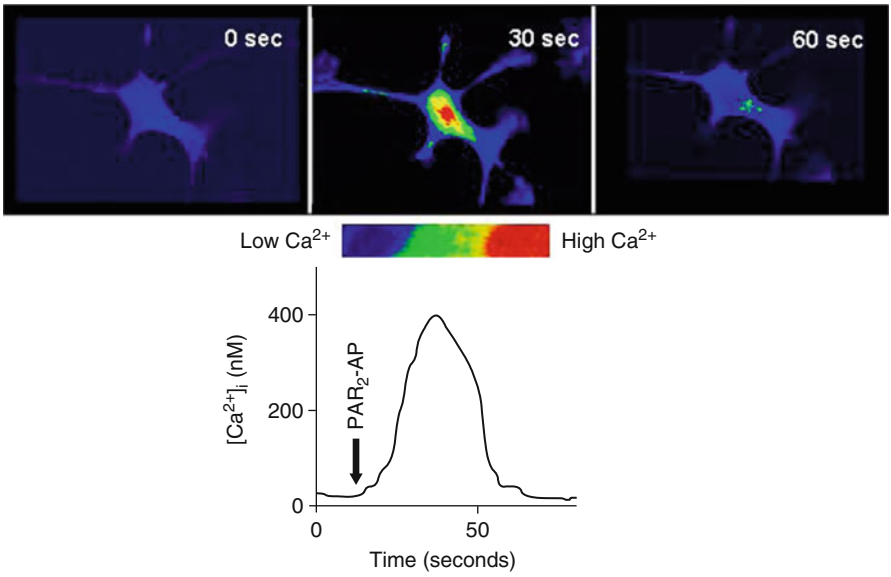

Fig. 45.2 PAR_2 mediates $[Ca^{2+}]_i$ increase in LX-2 hepatic stellate cells. LX-2 cells grown on Lab Tek chambered borosilicate coverglass were loaded with fluo-4-AM (0.5 μM). For calcium measurements, an inverted confocal laser scanning microscope LSM 510 was used. Fluorescence was monitored at 488 nm. *Upper part:* Fluorescence images, in pseudocolor, from an individual LX-2 cell preloaded with fluo-4-AM dye and stimulated with the PAR_2-activating peptide, 2-furoyl-LIGRLO-NH_2 (10 μM). The time sequence of three panels shows a transient fluorescence increase 30 s after PAR_2-AP addition (0 s: time of addition of PAR_2-AP), with a return to baseline fluorescence at 1 min. *Lower part:* Time course of calcium response induced by the PAR_2-activating peptide, 2-furoyl-LIGRLO-NH_2 (10 μM). The intracellular calcium concentration was calculated using the equation $[Ca^{2+}]_i = 345\ (F–F_{min})/(F_{max}–F)$ [104]. The Ca^{2+} affinity of fluo-4 (Kd) is 345 nM [105]. Fmax was obtained by addition of 10 μM ionomycin (+6 mM $CaCl_2$), Fmin by addition of 10 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). Data represent the mean ± SE from calcium measurements in 20 single cells. (LX-2 cells were a gift from Prof. Scott L. Friedman, Division of Liver Diseases, Mount Sinai School of Medicine, New York)

upon the cellular context in which the receptor is expressed. Thus, different tumours with their unique expression of GPCR-regulated effectors have the potential to respond to PAR activation in a unique way that may or may not depend on calcium signaling.

PARs Are Relevant in Different Cells from the Tumour Microenvironment

Relatively recently, oncologists have begun to focus on the tumour microenvironment as a major contributing factor to the development of cancer. Thus, in the setting of a tumour, both the resident non-tumour cells as well as the tumour cells can

engage in signaling cross-talk (tumour cell to stromal cell and back) that changes the phenotype of the stromal cells and alters the growth and metastatic potential of the tumour cell [91–93]. This cross-talk communication between tumour and stromal cells is mediated by a variety of hormone-like regulators, including secreted growth factors and proteinases [94–96]. PAR expression and function in different cell types found in the stromal elements of the tumour microenvironment, including fibroblasts, inflammatory leukocytes, platelets, macrophages, endothelial cells and smooth muscle cells has been documented in other contexts [reviewed: [1–4]]. Thus, the potential function of PARs in these stromal bystander cells is directly relevant to the malignant process and is currently under close scrutiny [97–102]. For example, in the setting of hepatocellular carcinoma (HCC), one of the leading malignancies worldwide, recently published data suggest that activated stromal hepatic stellate cells (HSCs) in the tumour microenvironment may contribute to the promotion of HCC tumorigenicity [103]. As illustrated in Fig. 45.2 PAR_2 mediates calcium signaling in HSCs that could readily occur in the setting of a hepatic tumour.

Intracellular Calcium – A Key Secondary Messenger in Cancer and a Potential Target for Therapy

Ca^{2+} is a ubiquitous intracellular signaling molecule that is involved in the regulation of almost all cellular functions including gene transcription, metabolism, proliferation and apoptosis [reviewed: [106–110]]. Since cancer growth is based on increased proliferation, decreased differentiation and decreased apoptosis, all of which processes involve a regulation of intracellular calcium concentrations, Ca^{2+}-homeostasis has become an important topic in current cancer research. Apart from the 'calcium-sensing receptor' [111, 112], G-protein-coupled receptor mechanisms involving G_q-stimulated phospholipase C_β and growth factor receptor mechanisms that trigger phospholipase C_γ represent key receptor mechanisms that regulate intracellular calcium. These mechanisms are in addition to the voltage-regulated and other ion channel mechanisms that regulate the entry of calcium from the extracellular environment. The current knowledge in this area is well documented by several detailed and comprehensive review articles that are cited in the following text. Here, only a brief overview is provided that is relevant for understanding the rationale for targeting PAR-mediated Ca^{2+} signaling as a possible therapeutic option for the treatment of cancer.

It is well known that an elevation of cytoplasmic $[Ca^{2+}]_i$ can result either from Ca^{2+}-influx from the extracellular space through a variety of plasma membrane ion channels or from Ca^{2+}-release from intracellular stores. More specifically, voltage- and ligand-gated Ca^{2+} channels in the plasma membrane, along with intracellular ryanodine receptors and inositol (1,4,5)-triphosphate ($InsP_3$) receptors in the endoplasmic reticulum as well as mitochondrial voltage-dependent anion channels and calcium ion exchangers provide fluxes of Ca^{2+} to the cytoplasm [106–108].

It has become evident that during the multistage process of carcinogenesis, the transformation of a normal cell into the malignant state is associated with a major change in the organization and expression of Ca^{2+} pumps, Na/Ca exchangers and Ca^{2+} channels. These changes occur in a setting that leads to the enhanced proliferation and impaired ability of the cancer cell to die [109]. In addition, work in this area done over the past decade has shown that altered intracellular Ca^{2+} signaling stimulated by G-protein coupled receptors via G_q [113–115] and involving tumour-associated changes in calcium release depots like the ryanodine receptor [116] can play a role in various tumourigenic pathways [117–120]. Thus, modulation of $[Ca^{2+}]_i$ signaling is a potential therapeutic option in cancer. In this regard, strategies can include specific blockade of membrane-localized calcium channels and targeting calcium release mechanisms via the $InsP_3$ and ryanodine receptors. Since many of these targets are expressed in a large number of cell types and organs where they may have essential functions, targeting specific Ca^{2+} channels or pumps with restricted tissue distribution, altered expression in cancer and/or a role in the regulation of tumourigenic pathways are a potential way to specifically disrupt intracellular Ca^{2+} homeostasis in cancer cells wherein different pharmacological strategies are possible [117–120]. One approach makes use of a bystander enzyme mechanism that results in the metabolic conversion of a pro-drug to an active moiety specifically at a site of restricted expression of that enzyme. For instance, since the human kallikrein-related serine peptidase-3 (KLK3, also known as 'prostate-specific antigen') is highly restricted in its expression to prostate tissue, it has proved possible to target the conversion of a thapsigargin 'prodrug' for activation in prostate cancer tissue, where the released thapsigargin can block the sarcoplasmic/endoplasmic Ca^{2+} pump. This 'smart-bomb' targeting method has the ability to induce cell death in prostate cancer cells [121]. A second pharmacological approach involves the direct targeting of specific isoforms of Ca^{2+} channels or pumps associated with a specific cancer type. There is yet another aspect in Ca^{2+} signaling that makes Ca^{2+} channels and pumps highly attractive as therapeutic targets in cancer. While the Ca^{2+} signal in differentiated non malignant cells is spatially and temporally highly regulated [106–110], in tumour cells there is a shift to a more global elevation of intracellular calcium with a sustained elevation of intracellular calcium. Therefore, cancer cells and their calcium-regulated signaling pathways may be more susceptible than normal cells to modulation of their Ca^{2+} channels and pumps [117–120]. Taken together the information obtained over the past decade, including quite recent data [122–124] suggest that the intracellular calcium-regulating machinery may represent a promising target for cancer therapy.

$[Ca^{2+}]_i$ Is Involved in PAR Signaling in Cancer

As outlined above, one of the main cell signaling pathways triggered by activation of distinct PARs is the $G_{q/11}$–mediated activation of phospholipase C_β. This activation, leads to the formation of inositol (1,4,5)-triphosphate and diacylglycerol that

in turn cause the elevation of intracellular Ca^{2+} (illustrated for LX-2 hepatic stellate cells in Fig. 45.2) and activation of protein kinase C. Indeed, the ability of the PAR_1 receptor for thrombin to mobilize intracellular calcium was instrumental in its cloning via an oocyte expression system [9].

This $G_{q/11}$ calcium signaling pathway activated by PARs has been observed in a variety of cancer cell types as seen by the activation of calcium signaling by thrombin in glioma cells ostensibly via PAR_1 [125]. The documentation of PAR-mediated calcium signaling in cancer-derived cells was greatly facilitated by the use of PAR subtype selective peptide agonists based on the sequences of the revealed PAR tethered ligands and PAR_1-targeted antagonists (see Table 45.1 for PAR-selective agonists and antagonists).

The presence of a specific PAR in a target cancer cell and its ability to increase intracellular calcium can be established using a receptor cross-desensitization protocol with PAR-selective agonists and appropriate PAR-inactive 'control' peptides [126]. This approach that uses fluorimetric methods to monitor calcium transients with different calcium sensitive fluorescence dyes has documented PAR-mediated increases in $[Ca^{2+}]_i$ in cells from various malignancies including those from brain [53, 56, 57, 109], colon [64, 74], pancreas [127], kidney[128], breast [19], larynx [56], prostate [112] and liver [72]. Although all of PARs 1, 2 and 4 can couple with G_q to elevate intracellular calcium in all PAR-expressing cells so far examined, the precise downstream consequences of elevated calcium *per se* have not been established in any detail. Further, as already mentioned, upon enzyme or peptide agonist activation the PARs can activate multiple G-proteins leading not only to elevations of intracellular calcium but also to (I) a G_i-mediated inhibition of adenylyl cyclase, (II) activation of MAPKinase [both G_i-dependent as well as G-protein independent *via* beta-arrestin interactions: review: [35]] and (III) a $G_{12/13}$-mediated activation of Rho and its downstream targets. Thus, singling out the PAR-triggered signal pathways that are uniquely calcium-mediated represents a considerable challenge.

$Ca^{2\pm}$ and PAR_2-Triggered p42/p44 MAPKinase Signaling

Increases in intracellular calcium result in a complex signaling network that includes p42/p44 MAPKinases as an intracellular effector system critically related to cell growth and transcriptional regulation [129, 130]. For prostate cancer PC3 cells it has been shown that kallikrein related peptidase 4 (KLK4), one of the 15 members of the human KLK family and a trypsin-like prostate cancer-associated serine protease, initiates Ca^{2+} signaling via PAR_1 and PAR_2. Stimulation of PAR_2 by KLK4 also results in p42/p44 MAPKinase activation [131]. Very recently, for hepatocellular carcinoma where altered Ca^{2+} signaling contributes to cancer development and progression [132], a PAR_2 dependent calcium-p42/p44 MAPKinase signaling axis was defined [133]. Since p42/p44 MAPKinases are established key players in HCC progression and invasive growth [134–137], and more specifically, since they contribute to a PAR_2-mediated effect on HCC cell invasion, the results

Table 45.1 PAR tethered ligand sequences, activating peptides, control inactive peptides and antagonists

IUPHAR recommended name	PAR_1	PAR_2	PAR_3	PAR_4
PAR tethered ligand sequences	SFLLRN (h), SFFLRN (m,r) PAR_1 AP, activates PAR_1 and PAR_2	SLIGKV (h), SLIGRL (m,r) PAR_2 AP only activates PAR_2	TFRGAP (h) PAR_3 AP only activates PARs 1 and 2	GYPGQV (h) GYPGKF (m) PAR_4 APs don't activate other PARs
PAR activating peptides	TFLLRN-NH_2, selective for PAR_1	SLIGRL-NH_2 2-furoyl-LIGRLO-NH_2		AYPGKF-NH_2
Control PAR-inactive peptides	RLLFT-NH_2 FTLLR-NH_2	LRGILS-NH_2 LSIGRL-NH_2 2-furoyl-LRGILS-NH_2		YAPGKF-NH_2
PAR antagonists	Trans-cinnamoyl-parafluoro-Phe-paraguanidino-Phe-Leu-Arg-Arg-NH_2 Mercaptopropionyl-Phe-Cha-Arg-Lys-Pro-Lys-Pro-Asn-Asp-Lys-NH_2 Non-peptide antagonists: RWJ56110 and RWJ58259 SCH 530348	Non-peptide antagonist: ENMD 1068 (N1-Methylbutyryl-N4-6-aminohexanoyl-piperazine) Peptide antagonist: K-14585 Palmitoyl-RSSAMDENSEKKRKSAIK (P2pal-18S)		Trans-cinnamoyl-YPGKF-NH_2

suggest a role for both Ca^{2+} and p42/p44 MAPKinase-driven signaling as an invasive axis in HCC cells. What is difficult to sort out is the signaling route whereby MAPKinase is activated in the HCC cells. Activation of p42/p44 MAPKinase could be (I) directly downstream of Ca^{2+} signaling as a consequence of the activation of protein kinase C, (II) independent of $G_{q/11}$ Ca^{2+} signaling, via a $G_{12/13}$-Rho kinase mechanism or (III) via a G-protein-independent mechanism triggered by a beta-arrestin-internalized signal scaffold [35]. In principle, all three mechanisms could result in the activation of MAPKinase signaling pathways in cancer cells. However, it is likely that the downstream effects of MAPKinase activation by these three distinct mechanisms will be found to differ (e.g. increase in transcription vs. activation of cytosolic phospholipase-A2 or changes in cell motility). Thus, identifying those events that result uniquely from elevations in intracellular calcium will be of much interest in the setting of tumour cell behaviour. To sum up, although Ca^{2+} plays a central role in regulating cancer cell behaviour, it has not yet proved possible to single out the impact on tumourigenesis of blocking Ca^{2+} signaling selectively, without affecting other PAR-triggered signaling events.

PAR-Mediated Increases in Cytoplasmic Free $Ca^{2\pm}$: Involvement of Both Extracellular and Intracellular Calcium

For numerous GPCRs it has been shown, as outlined above, that receptor-triggered increases in free intracellular calcium ion concentration can result from both influx of Ca^{2+} across the plasma membrane and the release of Ca^{2+} from intracellular stores [106, 138]. For PAR_2 this dual mechanism has been suggested for hepatocellular carcinoma cells, where PAR_2-stimulated increases in intracellular calcium can be reduced either by removing extracellular Ca^{2+} with the use of EGTA or by depletion of internal Ca^{2+} stores with thapsigargin [133]. This ‘dual mechanism’ for calcium signaling very likely also occurs for PARs 1 and 4. Thus, to block calcium signaling completely in cancer cells, it may be necessary to inhibit not only the G_q-triggered calcium signal that involves intracellular stores but also the receptor-mediated calcium entry process that occurs via receptor-regulated channels.

Intracellular Calcium Oscillations in Cancer-Derived Cells

Most of the knowledge about the effects of receptor agonists on $[Ca^{2+}]_i$ has come from studies on cell suspensions. In such experiments, the estimated $[Ca^{2+}]_i$ value represents the average value of $[Ca^{2+}]_i$ in all cells in the sample being explored. That response is represented by a peak of intracellular calcium that occurs within a minute of cell activation and a return to baseline calcium concentrations over a 2–5-min time frame, as calcium is first released and then rapidly taken back up into intracellular stores. However, at the single cell level, agonists can also trigger persistent

oscillations in intracellular calcium ion concentrations that wax and wane with time. Agonist-induced oscillations in intracellular calcium concentrations have been observed in many excitable and non-excitable cells, wherein a number of mechanisms have been proposed [for reviews see e.g.:[107, 138–140]]. As an example, such oscillations have been observed in response to PAR_1 activation in glioblastoma cells. The oscillatory response was observed after treatment with either thrombin or by the dual PAR_{1-2} activating peptide, SFLLRN-NH_2 [60]. The relevance of these oscillating intracellular calcium concentrations to tumour cell behaviour has yet to be determined.

Can PAR-Mediated Calcium Signaling Be Selectively Blocked?

Given that PAR-triggered calcium signaling can be of importance for the oncogenic process, an important question to deal with is: Can PAR-mediated calcium signaling be selectively blocked? Studies with human PAR_2 have identified a C-terminal domain that is directly involved in the ability of this receptor to stimulate elevations in intracellular calcium [141]. Thus, when activated by trypsin, a mutant PAR_2 missing a key C-terminal domain was able to stimulate MAPKinase and JNK, but not an elevation in intracellular calcium. In principle, this region of PAR_2 can thus be targeted as a 'calcium regulating domain' for the development of receptor-selective antagonists that will potentially affect calcium transients only in PAR_2-expressing tumour cells. A similar situation was found for the activation of PAR_1. It has been shown that the C-terminal part of PAR_1 is a critical site for receptor coupling to phospholipase C activation and thus for Ca^{2+}-signaling, while the third intracellular loop of PAR_1 is implicated in PAR_1 coupling to MAPkinase activation. Therefore, a strategy specifically targeting Ca^{2+} signaling might be possible not only for PAR_2 but also for the other PAR subtypes [142].

One may readily ask: How might such signal-selective antagonists be developed? The answer lies in making use of (I) the concept of 'biased' signaling and (II) cell-penetrating peptides. For instance, the PAR_2 antagonist, K-14585 can block PAR_2-stimulated elevations of intracellular calcium and a concurrent activation of p42/44 MAPKinase, but cannot block increases in p38 MAPKinase activation [143]. This compound therefore exhibits 'biased' antagonism for PAR_2. In principle more potent antagonists of this kind can be developed to block calcium signaling selectively. The concurrent blockade of both MAPkinase and calcium signaling may be particularly attractive in terms of targeting cancer cells.

"Pepducins" are cell-penetrating palmitoylated peptides based on sequences of the intracellular loops of G protein-coupled receptors. Due to the ability of their lipid moiety to anchor to the lipid bilayer of the plasma membrane these lipopeptides are thought to act by being internalized and then targeting the receptor-G protein interface [144, 145]. "Pepducins" based on the third intracellular loop of proteinase activated receptors have been successfully used for inhibition of PAR-mediated effects on signaling and cellular level [146–149]. The 'pepducin GPCR

antagonist' approach provides an excellent platform technology for the design of a variety of other PAR inhibiting cell-penetrating peptide variants corresponding to sequences of the intracellular receptor domains that are important for G protein coupling of GPCRs [150, 151]. It is known that for GPCRs, the C-terminus appears to be only of modest relevance for interacting with some G proteins [152–154]. However, as outlined above, a sequence in the C-terminus of PAR_2 has been shown to be important for calcium signaling [141]. This C-terminal domain can be a target for palmitoylation that results in a potential '8th helix' and a 'fourth intracellular loop' in G-protein-coupled receptors. Of particular note, a synthetic pepducin, termed jF5, targeted to this domain of GPCRs, including PAR_1 and the alpha-2A adrenoceptor, can selectively block GPCR-triggered calcium signaling, but not signaling via $G_{\alpha 12}$ [155]. It can be predicted that jF5 would also affect PAR_2 calcium signaling, which is dependent on a homologous sequence that can be a target for palmitoylation [141]. Finally, this 'lipopeptide concept' could also be expanded in principle to target PAR sequences within the transmembrane helical domains 3 and 6 that are also known to regulate GPCR G-protein coupling [36, 37].

Possible Impact of PAR-Triggered Calcium Signaling in Cancer Therapy

Data describing the PAR-induced effects in cancer published over the last 15 years clearly highlight PARs as possible targets in cancer treatment [156]. Given that PAR_1 is an attractive therapeutic target for thromboembolic disease, a number of receptor-targeted antagonists have been developed. Two PAR_1-targeted antagonists, SCH 530348 and E5555 or Atopaxar are currently in Phase III clinical trials for treating acute coronary syndrome [157–159]. Whether these antagonists will prove of value in the clinic for cardiovascular disease is yet to be determined. The compounds may, however be considered for use in the prevention of cancer metastasis and invasion. In addition, novel PAR_2 antagonists containing nonpeptidic moieties have been developed very recently [160]. Their therapeutic potential should also be tested for epithelial carcinoma. Since PAR stimulation does activate calcium signaling and because calcium signaling *per se* can affect cancer cell migration and invasion, agents that also target intracellular Ca^{2+}-signaling like those used in cardiovascular disease [for reviews see e.g.: [161–164]] may prove of value in the setting of cancer along with PAR antagonists. This possibility has yet to be considered.

Over the past decade there has been substantial success in targeting signal transduction pathways for treating cancer [165–167]. Impressive success can be seen in the use of the Abl-kinase-targeted imatinib-like inhibitors and their analogues, and a 'multitarget drug' that affects a number of signal pathways, sorafenib (BAY-43-9006), a bis-aryl urea-type inhibitor that blocks several kinases involved in tumour proliferation and angiogenesis. This inhibitor can affect Raf, vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) signaling [168]. Data from several patient studies indicate that sorafenib

seems to be a promising drug for the treatment of various epithelial cancers including those from breast, colon, kidney and liver [for review see e.g.: [169]]. Since targeting multiple signal pathways rather than a single enzyme may be advantageous in treating cancer, it can be suggested that in combination with other therapeutic agents, the selective blockade of PAR-mediated calcium signaling may be worthy of consideration for dealing with epithelial carcinoma.

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