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Opinion

Paradigm Shift is the Normal State of Pharmacology

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Paracelsus, who died in 1541, is often credited with founding pharmacology and toxicology. Luckily, the field that is ~500 years old never got petrified. Both pharmacology and toxicology are still young and full of surprises. The era of mechanistic molecular studies was expected to yield more objective data and therefore longer-lived theories. If anything, in the last 40+ years these studies accelerated the rate of paradigm shifts, making the field even more exciting.

Here we illustrate this by focusing on the subfield of signaling by G protein-coupled receptors (GPCRs). Today's students often have an impression that GPCRs were known since times immemorial. In fact, the idea that there is a family of rhodopsin-like receptors, that share key mechanisms of activation and signaling emerged after the cloning of b2-adrenergic receptor in 1986 [1], which revealed its structural similarity with rhodopsin sequenced a few years earlier, particularly the presence of seven trans-membrane (TM) domains [2]. As both rhodopsin and b2-adrenergic receptor coupled to their respective G proteins, this was the birth of the GPCR concept. The idea of the similarity of rhodopsin and other GPCRs turned out to be very fruitful. Observed selective binding to active rhodopsin of its specific kinase and other proteins [3], as well as the observation that rhodopsin phosphorylation greatly enhances the biding of the "48 kDa protein" (as visual arrestin-1 was called at the time) suggested that there must be homologous players in other GPCR-driven signaling systems. Indeed, the cloning of the b2-adrenergic receptor kinase [4] (modern name GRK2) that selectively phosphorylates only the active form of this receptor, as well as only light-activated rhodopsin [5], suggested the existence of the whole family of GPCR kinases. Now we know that most mammals have seven GPCR kinases (systematic names GRKs 1 through 7 [6]), which appeared in evolution even before Metazoa (true animals) [7].

Receptor phosphorylation is part of the desensitization process, whereby the cell blocks the response to a persistent or too strong stimulus. In case of rhodopsin it was shown that phosphorylation simply prepares the receptor for the binding of a desensitizing protein [8], whereas arrestin binding actually blocks further signaling by precluding receptor coupling to a cognate G protein [9]. The mechanism turned out to be similar in case of b2-adrenergic receptor: phosphorylation by b2-adrenergic receptor kinase reduced G protein coupling only marginally, suggesting than an arrestin analog must do the rest of the job [10]. That analog was soon cloned by homology with the visual arrestin-1 and termed b-arrestin, because it showed preference for the b2-adrenergic receptor over rhodopsin [11,12]. The demonstration that arrestin and G protein simply compete for the receptor [13,14], cemented the paradigm of two-step desensitization: receptor phosphorylation followed by tight arrestin binding, which blocked further G protein coupling [15,16]. Recent crystal structures of the receptor-G protein [17,18] and receptor-arrestin [19] complexes clearly showed the structural basis of this competition: both proteins engage the same inter-helical cavity that opens on the cytoplasmic side of the seven TM bundle upon GPCR activation [20].

Accumulating evidence challenged this paradigm as soon as it seemed well established. First, arrestins were found to interact with clathrin, promoting GPCR internalization [21]. This still could be interpreted as a further addition to the desensitization paradigm: arrestins facilitate receptor removal from the cell surface, whereas internalized receptors were believed not to signal. However, the list of non-receptor arrestin binding partners rapidly expanded, and receptor-bound arrestins were shown to initiate a number of signaling

event, including the activation of c-Src [22], JNK3 [23], and ERK1/2 [24]. As arrestins were found to turn on so many signaling pathways [25,26], they were recognized as signaling, rather than shut-off, proteins, in parallel with G proteins. Thus, the paradigm of desensitization morphed into a different one, a signaling switch. Some ligands of certain receptors were found to preferentially activate the signaling mediated by the G proteins or arrestins, which lead to a new paradigm, biased signaling [27-29]. An obvious corollary of that was the idea that drugs might have "on-target" side effects: the therapeutic action can be mediated by one of these branches of signaling, whereas unwanted consequences by the other [30,31]. In practical terms, this suggested that potential drugs have to be screened twice, once for their G protein-mediated effects, and once for arrestin-mediated ones. Yet the promise for better therapies fully justifies this additional effort and expense.

The idea of biased signaling is becoming generally accepted. At its core is the logic that it must be either-or, G protein- or arrestin-mediated branch of signaling. In recent years quite a few reports challenged the notion that internalized GPCRs do not signal via G proteins: receptors in the endosomes were shown to couple to G proteins and elicit responses [32-38]. These data directly challenged the idea that receptor internalization is the last act of desensitization. Thus, the core assumption of the either-or logic of biased signaling was shown to be faulty: at least in some cases a GPCR can simultaneously couple to a G protein and an arrestin. Recently GPCRs were visualized in complex with a G protein and an arrestin at the same time, which suggested that they might signal via both pathways in parallel [39]. This discovery is likely to drive yet another paradigm shift in GPCR pharmacology, the scope of which is hard to foresee.

The original reports of arrestin-mediated signaling implied that it is initiated by the arrestin-receptor complex [22-24]. While arrestin-mediated activation of ERK1/2 was found to depend on receptor activation [40,41], further studies showed that the activation of JNK3 does not: it can be activated by mutants impaired in receptor binding, while some "pre-activated" arrestins that are more likely to bind GPCRs turned out to be ineffective in this regard [41,42]. Subsequent in vitro experiments with purified proteins proved beyond reasonable doubt that arrestin-dependent activation of JNK3 [43,44], as well as more ubiquitous JNK1 and JNK2 [45], does not require receptors. Free arrestins effectively act as simple scaffolds, bringing together the kinases of JNK activation cascade [43-45]. Interestingly, an arrestin mutant that binds all the kinases in the pathway but does not promote JNK3 phosphorylation was shown to act as a dominant-negative silent scaffold that suppresses JNK activity in cells, apparently by recruiting the kinases away from productive scaffolds [41]. The most convincing evidence regarding the difference between JNK and ERK family kinases came from experiments in the same cells expressing different types of arrestin-3 mutants, where the endogenous receptor was treated with a conventional agonist, presumed inverse agonist (which turned out to be arrestin-biased agonist), or no drug at all. The level of ERK1/2 phosphorylation depended on receptor activation, whereas JNK phosphorylation did not: it only depended on the version of the arrestin-3 expressed in cells [41].

Receptor-binding residues of all arrestins are localized on the concave sides of the two domains [46], as was shown using mutagenesis [47-49], several biophysical methods [50-52], and the crystal structure of the arrestin-1 complex with rhodopsin [19]. Interestingly, a short arrestin-3-derived peptide carrying none of the receptor-binding elements was shown, using purified proteins in vitro, to bind all kinases in the ASK1-MKK4/7-JNK3 cascade and facilitate JNK3 phosphorylation in cells [53]. This peptide, in addition to being the smallest MAP kinase cascade scaffold known, proved that *in vitro* or in cells receptor binding of arrestin is not necessary for its ability to scaffold JNK activation cascade. It should be noted that this is not the only function of arrestins shown to be receptor-independent: caspase-generated arrestin-2-(1-380) fragment was found to play a role in the core mechanism of programmed cell death [54], and both non-visual arrestins were shown to regulate focal adhesion dynamics, a function that depended on their ability to bind clathrin, but not GPCRs [55].

Let us reiterate the highlights. The existence of the family of rhodopsin-like receptors that couple to G proteins (GPCRs) was first appreciated ~30 years ago, in 1986. The next paradigm was that these receptors are desensitized (the coupling to their cognate G proteins blocked) by a two-step mechanism: receptor phosphorylation by one or more of the kinases of the GRK family, followed by arrestin binding, which successfully outcompetes G proteins when the receptor is phosphorylated. The next paradigm was that the arrestin-receptor

complex initiates the second, G protein-independent, wave of GPCR signaling. The next paradigm was that certain ligands promote biased signaling, preferentially via G protein- or arrestin-dependent pathways. Newest findings suggest that some GPCRs can actually signal via both G protein and arrestin at the same time. In parallel, it became clear that arrestins can perform many functions, including signaling, independently of their interactions with receptors. Since all these changes in our models occurred within the brief ~30-year period, it is hard to predict what would remain true and what would have to be changed in coming years. This constant shifting of our ideas how cell signaling works provide never-ending excitement, making centuries-old field of pharmacology as young, fresh, and full of surprises, as ever.

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