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REVIEW

From GTP and G proteins to TRPC channels: a personal account

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Abstract By serendipity and good fortune, as a postdoctoral fellow in 1967, I landed at the right place at the right time, as I was allowed to investigate the mechanism by which hormones activate the enzyme adenylyl cyclase (then adenyl cyclase) in Martin Rodbell's Laboratory at the NIH in Bethesda, Maryland. The work uncovered first, the existence of receptors separate from the enzyme and then, the existence of transduction mechanisms requiring guanosine-5′-triphosphate (GTP) and Mg^{2+}. With my laboratory colleagues first and postdoctoral fellows after leaving NIH, I participated in the development of the field "signal transduction by G proteins," uncovered by molecular cloning several G-protein-coupled receptors (GPCRs) and became interested in both the molecular makeup of voltage-gated Ca channels and Ca2+ homeostasis downstream of activation of phospholipase C (PLC) by the Gq/11 signaling pathway. We were able to confirm the hypothesis that there would be mammalian homologues of the Drosophila "transient receptor potential" channel and discovered the existence of six of the seven mammalian genes, now called transient receptor potential canonical (TRPC) channels. In the present article, I summarize from a bird's eye view of what I feel were key findings along this path, not only from my laboratory but also from many others, that allowed for the present knowledge of cell signaling involving G proteins to evolve. Towards the end, I summarize roles of TRPC channels in health and disease.

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Introduction

I was invited to present my personal views on "Half a century of cell signaling" and highlight what we have learned. Personal views start at the time one becomes aware that one had dropped into a relevant field and lucked out. This was when Martin Rodbell with whom I was post-doctoring (1967–1971) asked whether epinephrine, ACTH, and glucagon, which stimulate lipolysis in adipose tissue by raising 3′5′-cylic AMP (cAMP), act via a single adenylyl (then adenyl) cyclase or whether each hormone acts via its own cyclase, further whether an effect of insulin to lower cAMP increased in cells by epinephrine by inhibiting adenylyl cyclase (Butcher et al., 1966 [1]). Rodbell left me with the problem while he went on a sabbatical leave to Geneva. The epinephrine-, ACTH-, and glucagon-stimulated activities were not additive, settling this question: a common pool of adenylyl cyclases was stimulated by a separate pool of receptors (Birnbaumer & Rodblell, 1969 [2]). Which led to the question of how?

The mechanism of activation of adenylyl cyclase by hormone receptors became a major theme. I had been trained as an enzymologist and performed experiments on the kinetics of activation and on the similarity between the mechanism used by hormone receptors and the fluoride ion, which Sutherland's group had found to be an activator of adenylyl cyclase. The upshot of these studies was that hormone and fluoride affected the requirement of the cyclase system for Mg ion (Mg^{2+}) in a similar manner. The role of Mg^{2+} was by no means simple: the substrate of the enzyme was Mg^{2+}-ATP. In addition, there was a regulatory site where Mg^{2+} by itself could stimulate the activity. And there was the curious thing that hormonal stimulation and fluoride stimulation were much "better" when

ATP concentrations were at >10× the concentration of the apparent K_m of the enzyme for ATP. This was nicely seen in a "matrix experiment" in which I changed both the concentrations of ATP and Mg^{2+} (Fig. 1). This also allowed me to build a Hill plot (Fig. 2) showing the action of the hormone ACTH and of fluoride to lower the requirement for Mg^{2+} (Birnbaumer et al., 1969 [3]).

We never found an effect of insulin (it stimulates phosphodiesterase), but, in a roundabout way, changing from fat cell membranes to liver membranes after Rodbell's return from Geneva, incorporation of Steve Pohl and Michiel Krans into the group, and development of a glucagon receptor-binding assay with 125I-labeled glucagon, we became aware that hormone binding was affected by ATP and still better by 1000× lower concentrations of guanosine-5′-triphosphate (GTP) (Rodbell et al., 1971 [4]). This led to the discovery that activation of liver adenylyl cyclase by glucagon required GTP (Rodbell et al., 1971 [5]). The high levels of ATP I had seen to be needed to get good stimulations by hormone and fluoride were to bring in the contaminating GTP.

The concept of discriminator-transducer-amplifier was coined by Rodbell and presaged the existence of the third player in signaling: a component (protein) that would bind GTP and transduce hormone binding to the discriminator (receptor) into activation of adenylyl cylcase (amplifier) and thus amplify the signal of one discriminator into thousands of cAMP molecules. Within a few years (1971–1978), it became clear that the GTP requirement was a general property of hormone stimulated adenylyl cyclases (reviewed in Birnbaumer, 1990 [6]).

A conceptual breakthrough came with Daniel Cassel's and Joseph Selinger's findings in Israel. The Israelis were able to measure stimulation of a turkey membrane GTPase by isoproterenol and found that this GTPase was inhibited by cholera toxin. They next correlated this effect of cholera toxin, with cholera toxin's action to activate adenylyl cyclases, and postulated, correctly, the existence of an active adenylyl cyclase-GTP complex, which is transient until GTP is hydrolyzed (Cassel & Selinger, 1977 [7]). It agreed with Rodbell's finding that a non-hydrolyzable GTP analog, GMP-P(NH)P, activated adenylyl cyclase to the same level as hormones, fluoride, and cholera toxin did. As GTP did not, Rodbell hypothesized that the GTP GMP-P(NH)P difference might reside in the non-hydrolysable nature of the analog, thus implicating the existence of a GTPase activity (Londos et al., 1974 [8]).

The G protein, a tale of two continents

A. In Würzburg, Thomas Pfeuffer capitalized on the effect of GMP-P(NH)P, built an affinity matrix linking GMP-P(NH)P's γ phosphate to the matrix, exposed the matrix to detergent-solubilized pigeon erythrocyte membranes and eluted with GTP or GMP-P(NH)P. Although the adenylyl cyclase in

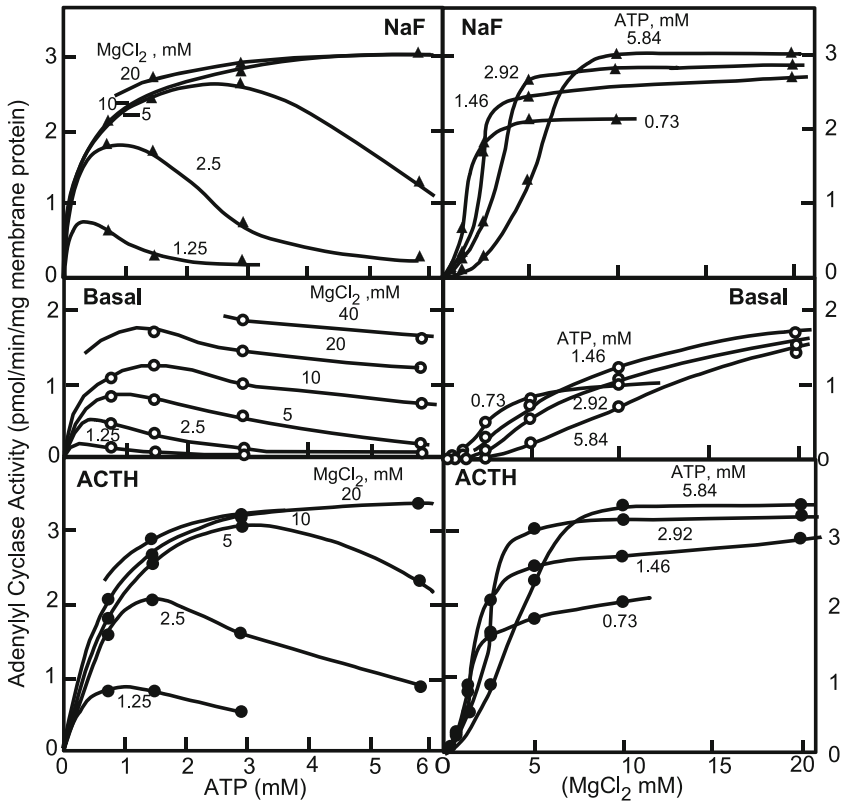

Fig. 1 Changes in the complex interplay between ATP and Mg^{2+} upon stimulation of fat cell membrane adenylyl cyclase: Hormone- (ACTH) and fluoride-stimulated activities show similar dependence on Mg^{2+}. Adapted from Birnbaumer, Pohl and Rodbell, 1969 [3]

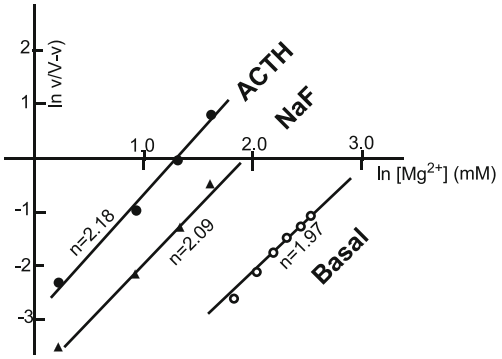

Fig. 2 Hormonal and fluoride stimulation of adenylyl cyclase shift the requirement of the enzyme system for Mg^{2+}. Adapted from Birnbaumer, Pohl and Rodbell, 1969 [3]

detergent solubilized membranes had lost hormonal stimulation, it had retained activation by GMP-P(NH)P and fluoride. This was also lost after incubation with the affinity matrix. The GTP-eluate reconstituted activation by GMP-P(NH)P and fluoride and contained two protein fractions that could be labeled with an photoactivatable GTP analog: one of 42 kDa (now Gs-alpha) and another at 23 kDa (now a mixture of small GTPases including rho, rac, and the rab group of small GTPases) (Pfeuffer, 1977 [9]).

B. In Virginia, Al Gilman's group discovered that the originally labeled "AC-minus" S49 cell variant isolated in San Francisco by Bourne and Coffino was defective in a regulatory component required for enzymatic adenylyl activity in the presence of Mg^{2+} and characterized it as the component responsible also for GMP-P(NH)P and fluoride stimulation. Originally called G/F (for its activity), it is now Gs—published in 1977, in the same issue of the *Journal of Biological Chemistry* in which Pfeuffer's work was published (Ross and Gilman, 1977 [10]). A reconstitution assay of S49 cell adenylyl cyclase in AC^-minus membranes (now cyc^- ("minus") membranes, was used by Gilman's group to purify G/F. It was an αβ dimer. The reconstitution assay was also used by us (Juan Codina) to purify Gs from human erythrocyte membranes.

There is more than one G protein

Adenylyl cyclases are not only stimulated by hormone receptors but also inhibited by another set of receptors. This inhibitory effect was also found to be dependent on GTP. The existence of a Gi was inferred from studies by my laboratory, first in Chicago (Birnbaumer, 1973 [11]) and then in Houston (Hildebrandt et al., 1983 [12]), by Günter Schultz and Karl Heinz Jakobs in Heidelberg (Jakobs et al., 1978 [13]) and in Rodbell's laboratory at the NIH in Bethesda, Maryland (Londos et al., 1978 [14]). Michio Ui in Japan found that pertussis toxin (PTX) inhibits inhibitory regulation of adenylyl cyclase. In 1982, he discovered that PTX is an ADP-ribosyltransferase (Katada & Ui, 1982 [15]) which ADP-ribosylates, a 40 kDa protein, which, in my laboratory, co-purified with Gs (Codina et al., 1983 [16]). Thus, there is a Gi as opposed to a GTPase-activating protein. Another game changing discovery was that Gs, Gi, and Go (the "other" PTX substrate, expressed abundantly in the brain, discovered in the laboratories of Eva Neer at Harvard and Al Gilman in Dallas) were all αβ dimers that dissociate upon binding and activation by non-hydrolyzable GTP analogs. A GTPase-driven, activation-deactivation, subunit dissociation, and re-association cycle had evolved for the original transducer (Fig. 3). In 1981, Lubert Stryer coined the name of transducin for an αβγ GTPase that transduces the light-activated rhodopsin signal into activation of phosphodiesterase in retinal rod cells, which had been characterized biochemically by Bitensky at Yale and Herman Kühn in Jülich, Germany. In 1984, my laboratory (John Hildebrandt and Juan Codina) showed that Gs, Gi, and Go are also αβγ trimers (HiIdebrandt et al., 1984 [20]). In collaboration between my laboratory and that of Robert Lefkowitz, Gs was shown to be a beta-adrenergic receptor activated GTPase. This represents the convergence of adenylyl cyclase related G protein research and visual signal transduction. The parallelism had been completely missed by the adenylyl cyclase nerds, us included.

Pure Gi (then Ni) was also found to be a GTPase (Teresa Sunyer), requiring Mg^{2+} with an apparent K_m 10–15 nM (Sunyer et al., 1984 [21]). This brought the specter of the existence of a Mg^{2+}-binding site in the system to the forefront: on α or on βγ?

Receptors mediating effects of many hormones, many neurotransmitters, and many autacoids, around 750 in humans and 1700 in mice, were all found to regulate cellular responses using the GTPase-G protein signal transduction mechanism. The name G-protein-coupled receptor or G-protein-coupled receptor (GPCR) was coined. Rather than affecting adenylyl cyclase activity, many of these receptors stimulated phosphoinositide mobilization and formation of IP_3 and DAG from PIP_2 by a GTP-dependent activation of phospholipase Cβ (PLCβ). Three different and independent approaches led to the identification the G proteins that stimulate PLCβ with α subunits of ca. 42 kDa. Paul Sternweis, in Dallas, purified proteins that bound to a Gβγ affinity matrix (Pang & Sternweis, 1989 [22]). John Exton's laboratory at Vanderbilt University purified a low abundance protein doublet that stimulated PLCβ activity (Taylor et al., 1990 [23]). Mel Simon at CalTech in California used the new and powerful cDNA cloning approach and "fished" for homologues of Gs/Gi/Go/transducin α subunits, an approach he had pioneered, and we all had started to use and he cloned several homologues. Naming of cloned Gα subunits became difficult. Naming αt, αs, αi, and αo by function was OK, and when more than one existed, such as rod and cone αt, they became αtr and αc, or when three αi's were found to exist they became αi1, αi2, and αi3, but when novel α's were cloned with as yet unknown

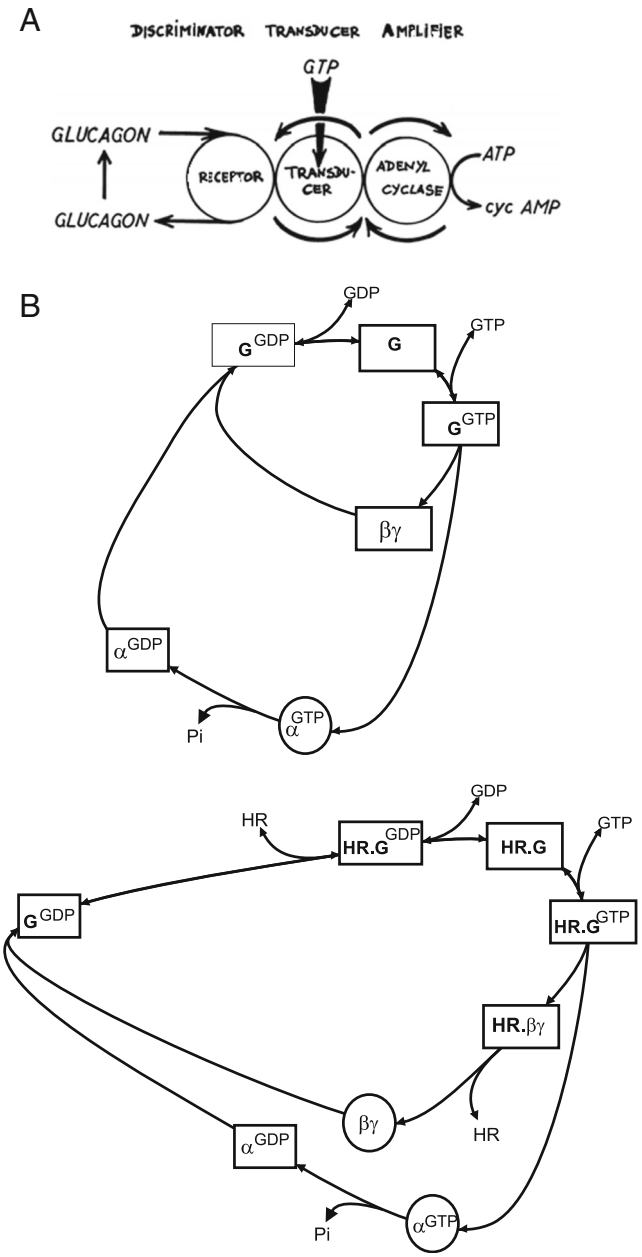

Fig. 3 **a** One of several drawings depicting the discriminator-transducer-amplifier concept proposed by us in 1970. Adapted from Birnbaumer, 1990 [17] and Birnbaumer and Zurita, 2010 [18]. **b** The GTPase-driven double activation- subunit dissociation deactivation-subunit reassociation cycle that is the core of the signal transduction by G proteins mechanism. **b** *Top*: the cycle in the absence of hormonal stimulation. Adapted from Birnbaumer, 1993 [19]; **b** *bottom*, the cycle under hormone receptor stimulation. Although the βγ is shown as dissociating together with HR, this has not been experimentally addressed. Adapted from Birnbaumer, 1990 [6] and 1993 [19]

function, it became more difficult. Although the first such α with unknown function was named αq, the next unambiguous letter of the alphabet after the "o" of αo (p had been used for PLC-stimulating G protein and the cDNAs had as yet an unknown function), the subsequent αs were numbered by Mel Simon 11, 12, 13, 14 and 15/16 (Strathman & Simon, 1990 [24]). Sternweis's Gβγ binding αs and Exton's PLCβ stimulating proteins were encoded in the q/11 cDNA clones (Smrcka et al., 1991 [25]; Taylor & Exton, 1991 [26]; Wu et al., 1992 [27]). Gα12 and Gα13 were eventually shown to stimulate GTP exchange factors specific for Rho, Rac, and Cdc42 with roles in cytoskeletal assembly and remodeling.

Gβγ is also a signaling arm of trimeric G proteins

So had been the discovery of G protein signaling mediated by their α subunits. But in 1990, Monserrat Camps, a student in the Heidelberg laboratories working with Peter Gierschik, showed activation of PLCβ by Gβγ (Camps et al., 1990 [28]). Confirmed by Simon's laboratory at CalTech (Katz et al., 1992 [29]), this became the first of many mammalian effector systems regulated by Gβγs instead of Gαs. PLCβs and adenylyl cyclases are regulated by both Gαs and by Gβγs, and, while Gsα is always stimulatory and Giαs are always inhibitory, Gβγs are either stimulatory or inhibitory, depending on which of nine adenylyl cyclase subtypes one is considering (Taussig et al., 1994 [30]). At the level of voltage-gated calcium channels, first shown to be under G protein regulation by Jürgen Hescheler, Walter Rosenthal, Wolfgang Trautwein, and Gunter Schultz (Hescheler at al., 1987 [31]), Gβγs are stimulatory (Logothetis et al., 1987 [32]).

Whereas by 1992 "Signal transduction by G proteins" had become a field of its own (more fully reviewed in [33, 34]), the mechanism by which G proteins were activated by GPCRs was still a matter of conjecture. It was clear that GPCRs promoted exchange of GTP for GDP. But what drove the reaction forward? Two types of study with Mg^{2+} indicated that there should

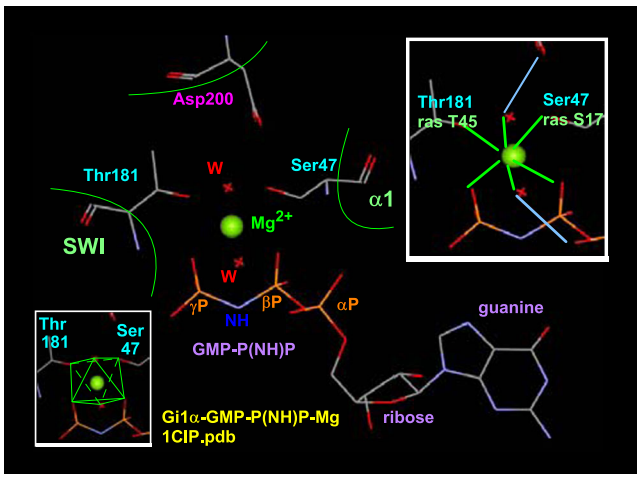

Fig. 4 Atomic model of how the β and γ phosphate oxygens of GTP and the γ subunit's Ser and Thr hydroxyls coordinate Mg^{2+} with the consequence that the GTPase fold is locked into its signaling competent conformation. The coordinates of the crystal structure are those of the crystallized Mg^{2+} GMP-P(NH)P-Gi1γ complex (accession number 1CIP.pdb). Adapted from Birnbaumer and Zurita, 2010 [18]

be a Mg^{2+}-binding site other than GTP (Kd 60 *M). The substrate ATP (Kd also 60 μM) could not be it, because of the following: (1) the apparent Km for Mg^{2+} of the intrinsic GTPase activity mentioned above was 10–15 nM, and (2) the Km for Mg^{2+} for activation of liver membrane Gs by GTPγS, as measured in the absence and presence of glucagon by Ravi Iyengar in 1982, was of about 15 μM, also lower than the two known Mg^{2+}-binding components in the activation reaction (Iyengar et al., 1982 [35]).

The crystals

The GTPγS-Mg^{2+}-transducin α complex was crystalized in 1994 (1TND.pdb), that of the nucleotide- and Mg^{2+}-free transducin αβγ complex (1GOT.pdb) in 1996, and that of the Mg^{2+}-free GDP-Gi1αβγ complex (1GP2.pdb) in 1996. No indication of where an allosteric site for Mg^{2+} could be. At one point, I had placed it on the Gβγ dimer. In 2006, I was referred by David Siderovski to a paper from Steven Sprang's group in which binding of Mg^{2+} to purified nucleotide GTP-Goα complexes increased the intrinsic fluorescence of the Trp of the switch II region of the GTPase fold and did so with an apparent Kd of 8 nM (Raw et al., 1997 [36]). This placed the Mg^{2+} site on the α subunit. Gazing at crystals, I came to realize that Mg^{2+} was held into place by four coordinating oxygens: the β and γ hydroxyls of GTP and one hydroxyl each from the equivalent of ras Se16 and Thr35 (Gs Ser54 and Thr204, and Goα Ser47 and Thr182). The octahedral coordination shell of the Mg^{2+} is completed by the oxygens of two coordinating water molecules held in place by hydrogen bonds of the α phosphate of GTP and the

Table 1 G protein-coupled receptor (GPCRs) families constitute the largest gene family in the mammalian genome. Human and mouse GPCR families[a]

Receptor	H. sapiens	M. musculus
1.Rhodopsin (Class A)	**659**	**1337**
Non-Olfactory	**271**	**300**
alpha (Opsin Family – DRY)	*101*	*105*
Opsin		
Prostaglandin		
Amine (NE, DA, 5HT)		
Melatonin		
Melacortin		
beta	*43*	*46*
gamma (Peptides)	*64*	*67*
MCH		
Chemokine		
delta	*63*	*82*
MAS-related		
Glycoprotein /LH, FSH, TSH)		
Purine		
Olfactory	**388**	**1037**
Olfactory Pseudogenes	~450	?
2. Secretin (Class B)	**15**	**15**
3. Glutamate (Class C)	**22**	**79**
4. Adhesion	**33**	**31**
5. Frizzled	**11**	**11**
6. Taste type 2 (TAS2)	**25**	**34**
7. Vomeronasal type 1 (VR1)	**3**	**165**
8. Other	**23**	**25**
Total	**791**	**1697**

* from Bjarnadottir et al (2006) [40]

[a] Number of GPCRs identified in the human and mouse genomes by subclasses. The number of orthologues in the two genomes is very close except for olfactory receptors of which there are many more in mouse than man, and also V1R-type pheromone receptors of the vomeronasal system of the mouse, which upper primates and man have lost. The data summarized in the table were taken from Bjarnadóttir et al., 2006 [40]

carbonyl group of a conserved aspartate. Thus, the allosteric site was the coordinating α subunit itself. Further, changing viewpoints, Mg^{2+} acts as a keystone locking GTP into place and the GTPase in its active signaling conformation. The only way for Mg^{2+} to exit is hydrolysis of GTP, at which point GDP is also free to exit. The site would then be refilled by GTP or GDP, in proportion to their relative abundance in the cytosol, which is GTP to GDP 10:1. This of course could only happen after the occluding Gβγ dissociates to allow GTP to GDP exchange to happen. GPCRs therefore promote GTP to GDP exchange by promoting the dissociation, total or partial, of Gβγ to open the path for nucleotide exchange. Entering GTP, but not GDP, is again locked into place by Mg^{2+}. And so the cycle proceeds until the GPCR ligand leaves. The geometry of the coordination of Mg^{2+} by GTP and Gi1α is illustrated in Fig. 4. For further reading, see Birnbaumer and Zurita, 2010 [18].

GPCRs

Although the first GPCR with known structure and sequence was rhodopsin, the existence of structural homologues became evident only after the cloning of the various adrenergic receptors in Robert Lefkowitz's laboratory using amino acid sequence information derived from purified proteins (Brian Kobilka and lab colleagues). Cloning by nucleotide sequence homology soon led to an explosion of structural homologues (to which also my laboratory contributed; cf. Liao et al., 1989 [37]; Levy et al., 1992 [38, 39]) that formed the superfamily of GPCRs as we know it today (reviewed in Bjarnadóttir et al., 2006 [40]; summarized in Table 1).

By 1991, it had also become evident that there were many more subtypes of GPCRs than ligands. Presaged by pharmacology and confirmed by structural analysis of the abovementioned cloning work. Thus, acetylcholine (Ach) activates five muscarinic receptors, norepinephrine, and epinephrine interact with nine adrenergic receptors, glutamate interacts with five metabotropic GPCRs (Mg^{2+} luRs), serotonin interacts with seven GPCRs (Fin Olav Levy and Thomas Gudermann cloned two, while in my lab). In all cases, the signaling of the ligand through Gs, Gi, or Gq/11 depends on the GPCR it encounters on its different target cells. For neurotransmission, ligands also interact with non-GPCR ionotropic receptors, i.e., ion channels: nicotinic for ACh, 5HT3 for serotonin, GluR's (NMDA, AMPA) for glutamate, GABA-A (for γ-aminobutyric acid).

Calcium signaling

My laboratory became interested in Ca^{2+} regulation by G proteins when in 1989 my graduate student Ching-Fong Liao cloned the fifth muscarinic receptor (Liao et al., 1989 [37]) and found it to activate the pathway Gq/11→PLCβ→IP3→IP3R→Ca^{2+} release from the endoplasmic reticulum (ER) store, thus triggering changes in cytosolic Ca^{2+} levels. This brought together in my laboratory two signaling fields: GPCR-Gs/Gi proteins and phosphoinositide turnover by Gq/11-coupled GPCRs causing Ca^{2+} release from the ER through activation of the IP3 receptor. The initial observations leading to the understanding of this signaling pathway had been made by the Hokins in the early 1950s (Hokin & Hokin, 1953 [41]). The signaling pathway had been worked out over the years with the participation of the laboratories of Bob Michell (reviewed in Michell, 1992 [42], of Michael Berridge and Robin Irvine that led to the discovery of inositol-trisphosphate (IP3 as a second messenger (Streb et al., 1983 [43]; reviewed in Berridge and Irvine, 1989 [44]); of Katsuhito Mikoshiba (characterization of the IP3 receptor (cerebellar P400, now IP3R, Miyawaki et al. [45]); the cloning of three IP3Rs by Salomon Snyder in collaboration with Axel Ulrich at Genentech (Ross et al. [46]); and many others which remain unnamed.

After cloning of the M5 muscarinic receptor (an example of the power of molecular biology), my laboratory was faced with the question: how does it signal? We used the Dowex1 chromatography system developed by Robin Irvine and Michael Berridge to asses stimulation of PLCβ's activity to hydrolyze PIP2 to PIP+IP3 in intact cells pre-labeled with [3H]*myo*-inositol (Irvine et al., 1982 [47]) and determined that it was a Gq/11-coupled GPCR. Interestingly, upon testing other GPCRs for their assumed G protein specificity, we found this parameter to be more of a selectivity than a specificity. Even typical Gs-coupled GPCRs, such as the luteinizing hormone receptor (Gudermann et al., 1992 [48]) and the beta-

Fig. 5 The first TRPC-less mouse (ID tag 4537-7) after having delivered 8 pups, four of which were TRPC-less HeptaKO and four were Trpc3+/-HexaKO

adrenergic receptor (Zhu et al., 1994 [49]) were found to also be able to activate the PLCβ system.

The visualization of changing cytosolic Ca^{2+} levels with ratiometric fluorescent Ca^{2+} indicator dyes, invented by Roger Tsien (Grynkiewicz et al., 1985 [50]), had opened our minds to the dynamic aspects of signaling triggered by Gq-coupled GPCRs. The newly cloned M5 muscarinic receptor turned out to be a potent activator of the Gq/11 signaling pathway and my laboratory's focus became the mechanism by which cells refill Ca^{2+} stores after they are emptied by IP3-IP3R (Liao et al., 1990 [51]). One hypothesis prevailing at that time was that refilling might be mediated by functional homologues of the Drosophila ion channel mutated in the *trp* fly (suggested to me first by Reinhold Penner but also proposed by others in the literature). Did such a homologue exist in the mammalian genome? Between 1995 and 1996, we succeeded in finding not one but six such homologues in the mammalian genome by molecular cloning (Zhu et al., 1994; 1995 [52, 53]). They were initially called TRP (for transient receptor potential, the phenotype of the Drosophila mutant), but parallel, unrelated research lines discovered the existence of other more distant structural homologue of Drosophila trp, such as the capsaicin and menthol receptors, the polycystic kidney disease gene product, one of the mucolipidosis-causing genes. The nomenclature was changed for our TRPs to transient receptor potential-canonical (TRPC) channels, for classic or canonical. A seventh TRPC was found in Yasuo Mori's laboratory 1 year later (Okada et al., 1996 [54]).

Reinhold Penner is the discoverer of an inward Ca^{2+} current activated at the same time as Ca^{2+} refilling starts of stores that are being depleted (Hoth & Penner, 1992 [55]). He called it the calcium release-activated current (Icrac). Icrac is the electrophysiologic correlate to store-depletion activated or store-operated Ca^{2+} entry (SOCE), hypothesized by him, us and others, including Veit Flockerzi, to proceed through the TRPC channels. Indeed, independent of our efforts, Flockerzi's laboratory cloned two TRPCs naming them CCE1and CCE2 (now TRPC4 and TRPC5; Philipp et al, 1996; 1998 [56, 57]). Definitions change as new knowledge accumulates and at present, Icrac channels are activated by store depletion without apparent PLC activation, as occurs when ER Ca^{2+} pumps are inhibited.

Though fulfilling many criteria, TRPCs are not the molecular correlate to Icrac channels, ORAIs however are. ORAIs of which there are three, were discovered in 2006 and are tetra-spanning plasma membrane proteins that form tetrameric (Penna et al, 2008 [58]; Thompson & Shuttleworth, 2013 [59]) or hexameric (Hou et al., 2013 [60]) channels which upon expression form Icrac channels, which TRPCs do not.

Two questions offered themselves: are TRPC channels activated by store depletion? And, what do TRPCs do? These are questions are what my laboratory has been addressing during the last years, much in collaboration with investigators studying phenotypes that develop in TRPC knock-out mice. The results obtained and questions they generated will conclude my review.

Are TRPC channels activated by store depletion?

The answer is that one TRPC, TRPC1, is activated by store depletion (Zitt et al., 1996 [61]; Zeng et al. 2008 [62]; Shi et al., 2012 [63]). The connecting element between store depletion and TRPC1 activation is STIM1, the ER Ca^{2+} sensor. The executing arm of STIM1 is its cytosolic C-terminus. It is easier to develop the STIM1-TRPC1 interaction model by describing the current model that best describes activation of the CRAC channel formed of ORAI molecules.

STIM, ORAI, and their interactions (except when noted STIM refers to STIM1 and ORAI refers to ORAI1)

STIM is a single pass transmembrane molecule with a cytosolic C-terminus. The ER portion has a classical double EF-hand Ca^{2+}-binding domain. Ca^{2+}-occupied STIM is believed to be monodispersed. Upon dissociation of Ca^{2+} due to store depletion, STIM molecules cluster and the clustered C-termini "instruct" the assembly of the CRAC channel from ORAI dimers located in the plasma membrane (PM). Junctional complexes form between ER and PM, connected by clustered STIMs (Wu et al., 2006 [64]; Luik et al., 2006 [65]). Ca^{2+} enters through the CRAC channel. An approximately 100-aa-long region of STIM called crac-activation domain (CAD) or STIM ORAI activation region (SOAR) is the executing region of STIM responsible for activating the CRAC channel made up of ORAI molecules.

ORAI is a tetra-spanning PM protein with cytosolic N- and C-termini. ORAI's N-terminus occludes access of SOAR to its interaction site on ORAI's C-terminus. To open the access to SOAR, the C-terminus of STIM "pushes/pulls" the N-terminus of ORAI out of the way.

Full length ORAI is not activated by the 100-aa-long SOAR; ORAI without part of its N-terminus is activated by SOAR (Yuan et al., 2009 [66]).

In a strict sense, only TRPC1 is activated by store depletion. Akin to ORAI, the activation of TRPC1 occurs as a consequence of two protein-protein interactions with the same regions of STIM that are involved in activating ORAI: (1) STIM1's positively charged KK C-terminus interacts with a negatively charged DD doublet located downstream of TRPC1's TRP box to gate channel opening (Zeng et al., 2008 [62]; Kim et al., 2009 [67]) and (2) STIM1's CAD/SOAR sequence disrupts an inhibitory interaction between

Table 2 Thirty-two reports on roles of TRPC channels deduced (mostly from phenotypes)

-Thirty two reports on roles of TRPC channels deduced (mostly) from phenotypes developed in single and double Trpc KO mice	
A. Deduded from single gene disruption	
Cardiac hypertrophy induced by transverse aorta constriction (TAC) **(Seth et al., 2009 [73])**	TRPC1
Exocrine secretion (saliva) **(Liu et al., 2007 [74])**	
Neurotoxin induced ER stress response and ER calcium homeostasis **(Selvaraj et al., 2012 [75])**	
Pro-inflammatory in murine allergic asthma - **(Yildirim et al., 2012 [76])**	
Prevention of muscle fatigue **(Zanou et al., 2009 [77])**	
Pheromone signal transduction in vomeronasal sensory neurons– **(Stowers et al., 2002 [78])**	TRPC2
Lost between new world and old world monkeys & primates (**Liman and Innan, 2003 [79]**)	
Synaptic transmission and motor control; slow EPSCs (**Hartmann et al., 2008 [80]**)	TRPC3
Static stretch response of endothelial cells. Stretch-ATR1-Gq-TRPC-Ca-ET1-ANP-GCA-cGMP-PKG-zyxin-> gene transcription (**Suresh Babu et al., 2012 [81]**)	
Efferocytosis and survival signaling in macrophages **(Tano et al., 2011 [82])**	
Endothelial cell NO-independent EDH (endotheium dependent hyperpolarization- vascular smooth muscle relaxation **(Senadheera et al., 2012 [83])**	
Sound transduction and auditory neurotransmission **(Wong et al. 2012 [84])**	
Calcium toxicity in secretory epithelia **(Kim et al., 2011 [85])**	
Endothelial cell NO/EDRF (endothelium derived relaxing factor) generation vascular smooth muscle relaxation (**Freichel et al., 2001 [86]**)	TRPC4
Agonis-induced Ca mediated neurotransmitter release from dendrites (**Munsch et al., 2003 [87]**)	
Plateau potentials in hippocampal CA1 pyramidal neurons **(Tai et al., 2010 [88])**	TRPC5
Cold transduction in the peripheral nervoussystem **(Zimmermann et al., 2011 [89])**	
Control of vascular tone (**Welsh et al., 2003 [90]; Dietrich et al., 2005 [91]**)	TRPC6
Albuminuria associated with Ang II induced cardiac hypertrophy **(Eckel et al., 2009 [92])**	
Wound healing (lysoPC, fibroblast transdifferentiation **(Davis et al., 2012 [93])**	
Innate immunity (LPS induced NFkB activation) - **(Tauseef et al., 2012 [94])**	
Ischemia-Reperfusion-induced ROS-mediated Lung Edema) -**(Weissmann et al., 2012 [95])**	
Initiation of epileptic seizures **(Phelan, 2014 [96])**	TRPC7
B. Deduded from doble gene disruption	
Neuronal excitotoxicity **(Phelan et al., 2012 [97])**	TRPC1-C4*
Epileptogenic postsynaptic regenerative plateau potemtials **(Phelan etal., 2012 [97])**	
Short term post synaptic memory, burst firing-induced afterdepolarization **(Phelan et al., 2012 [97])**	
Pathologic cardiac remodeling **(Camacho Lodono et al. [98])**	
Neuronal afterdepolarization **(Stroh et al., 2012 [99])**	TRPC1+TRPC4
Light entrainment of Circadian Rhythms by ipRGCs (melanopsin signaling) (**Xue et al., 2011 [100]**)	TRPC6-C7*
Normal touch **(Quick et al., 2012 [101])**	TRPC3-C 6*
Platelet activation by thrombin plus collagen related peptide PS exposure **(Harper et al., 2013 [102])**	
Cardiac hypertrophy induced by Ang **(Onohara et al., 2006 [103])**	TRPC3+TRPC6
Pathologic Cardiac Hypertrophy **(Seo et al. 2014 [105])**	
Intestinal motility regulation by vagus **(Tsvilovskyy et al., 2009 [105])**	TRPC4+TRPC6

* Most likely operating as heterotetramers

TRPC1's N-terminal and C-terminal-coiled coil domains to allow for the gating function of STIM1's KK-terminus to proceed (Lee et al., 2014 [68]). An elegant recent study from Luis Vaca's laboratory showed that two molecules of SOAR are required to activate what presumably is a tetrameric TRPC1 channel (Asanov et al., 2014 [69]).

The final word, as to what the molecular makeup of a store operated channel may be, is still to be written, as, for example, the store-depletion activated TRPC1 appears to require presence of ORAI (Kim et al., 2009 [67]) and, as my laboratory has published, there are functional interactions between several TRPCs and ORAI, such as TRPC-dependent enhancement of thapsigargin-activated Ca^{2+} entry by ORAI (Liao et al., 2007 [70]) and silencing in resting cells of spontaneous activity of overexpressed TRPC3 and TRPC6 by ORAI (Liao et al., 2008 [71]; reviewed in Liao et al., 2014 [72]).

We asked ourselves whether ORAI could form CRAC channels independent of a TRPC, as ORAI had not been expressed in a cell devoid of TRPCs (all mammalian cells express at least two, most express three, and many express four and some even five TRPCs) and store depletion activates not only ORAI channels but also TRPC1. One approach to convince the world and ourselves as to whether ORAI channels can operate independently of a TRPC was to generate a TRPC-null cell line. Initiated in 2008, using five TRPC KO alleles from my laboratory and those of Catherine Dulac at Harvard (TRPC2 KO) and Veit Flockwrzi and Marc Freichel at the University of the Saarland (TRPC4 KO), we combined by breeding the KO alleles. To my utmost surprise, 5 years later, in May 2013, a female live TRPC-null mouse was born in our animal care facility in North Carolina: a HeptaKO (Fig. 5). It was possible to expand this line and generate embryonic fibroblasts. TRPC-null fibroblast generates an unaltered Tg-induced store operated Ca^{2+} entry (unpublished). Thus, one part of the question has been answered: ORAI channels can operate in the absence of TRPCs. Whether TRPCs can operate in the absence of an ORAI is open for discussion.

What do TRPCs do?

TRPCs turned out to have an amazing array of roles in both health and disease. These roles vary for the individual TRPCs. Among them are as follows:

- TRPC1 intervenes in cardiac hypertrophy development
- TRPC6 is both proinflammatory and required for wound healing;
- TRPC3 plays roles in macrophage's efferocytosic activity and pathologic endothelial cell remodeling;
- Two TRPCs are responsible for endothelium-dependent vascular smooth muscle relaxation: TRPC4 is required for formation of endothelium-derived relaxing factor (EDRF) or NO, TRPC3 is critical for development of endothelium-derived hyperpolarization (EDH);
- TRPC3 is also required for Purkinje cell's slow excitatory postsynaptic currents (sEPSCs), for sound transduction and auditory neurotransmission;
- TRPC5 plays a critical role in development of plateau potentials in CA1 neurons of the hippocampus;
- TRPC7 intervenes in initiation of epileptic seizures;
- TRPC6 and TRPC7 are required for intrinsically photosensitive retinal ganglion cells (ipRGC) to generate an action potential in response to day/night signaling by melanopsin; and
- TRPC2 is the transduction channel in vomeronasal sensory neurons

Table 2 is an expanded list of phenotypes found in TRPC knockout mice.

Given the multiple roles inferred from single and double TRPC knockout studies (Table 2), one cannot but wonder how is it possible that the hepta knockout not only lives but strives? These and other questions remain for future studies to be answered. Figure 6 depicts our current understanding of signal transduction by G proteins as applied to phosphoinositide mobilizing Gq/11-coupled GPCRs and the resulting activation of Ca influx by ORAI and TRPC channels. My closing statement: *the molecular nature of and interplay among none of the elements shown in the figure were known 50 years ago*.

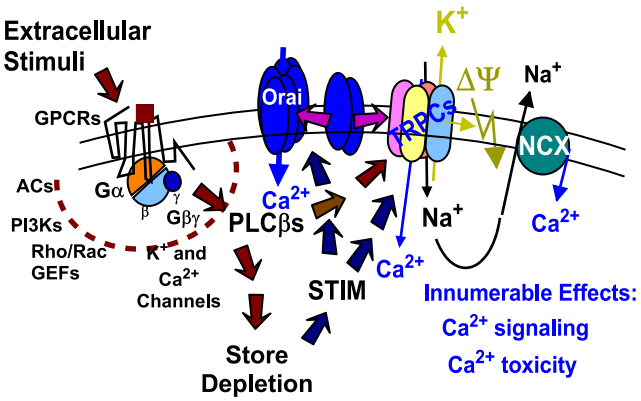

Fig. 6 Signal transduction by G proteins, activation of PLCβ by Gq-coupled GPCRs, and Ca2+ entries that ensue. PLCβ-generated signals activate TRPCs by DAG and as yet undefined signal(s), and trigger Ca2+ release from stores by IP3; store depletion activates STIM which in turn both, assembles ORAI-based CRAC channels from ORAI dimers, and activates TRPC1 (and possibly others). Na+ entering through TRPCs activated by PLCβ signal(s) drives reverse mode exchange of the Na+-Ca2+ Exchanger (NCX) causing more Ca2+ to enter. Activation of TRPCs also causes membrane depolarization to varying degrees depending on cellular context and may activate voltage-gated ion channels of the cell under consideration. In a cardiomyocyte, this would activate voltage gated L-type Ca2+ channels. Ca2+ entering through Orai and Ca2+ entering through TRPC may activate different cellular functions (Cheng et al. 2011 [106]. The direct regulation of TRPCs by ORAI dimers is proposed

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My assignment was: "Half a century of cell signaling; What did we learn?" This was a clear invitation to reminisce while highlighting the important steps in our transit through the world of science, steps that made a difference in our understanding of signal transduction by G proteins and to describe how we branched out from there to other fields: first, the closely related field of GPCRs, then to that of ion channels, and finally, to how cells manage Ca signaling triggered by many of the GPCRs. This I have tried to do. Space here, as was time at the symposium, is limited, and I did not touch on voltage-gated Ca channels in which I was helped by Franz Hofmann and his crew, of which Franz and Martin Biel were in attendance, or the work of my "CaCh cloning Team" Ed Perez-Reyes, Antonio Castellano, Haeyoung Suh Kim, and Toni Schneider, nor the exploration the biophysics of calcium channels with Enrico Stefani and his people, or those from my lab that interacted with his laboratory in Los Angeles.

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