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COMMENTARY

SEROTONIN AS A GROWTH FACTOR

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A recent commentary article published in this journal dealt with the vasoactive effects of growth factors [1]. Here we will discuss the mitogenic effects of serotonin (5-HT), essentially known as a neurotransmitter and vasoactive agent, which may be representative of a class of molecules promoting cell growth by interacting with G protein coupled receptors. As Berk and Alexander pointed out [1], vasoactive agents and some growth factors seem to activate common signal transduction mechanisms, among which activation of phosphoinositide (PI) breakdown has received much attention. Whereas it can be taken as established that hydrolysis of PIs plays a major role in smooth muscle contraction due to its effects on Ca^{2+} homeostasis, this signalling pathway may, however, be only of minor importance in mediating the cell proliferation response, as results with serotonin in our laboratory have suggested.

Serotonin is not very well known as a growth factor; during the last few years, only a few publications have dealt with this aspect [2–4]. Nevertheless, the well-developed pharmacology of serotonin receptors makes it a very useful model for studies of the relationship between early biochemical signals and the mitogenic response. The positive effect of serotonin on cell proliferation was discovered in atherosclerosis research. Serotonin is stored in large amounts in blood platelets and is released, together with known growth factors (PDGF†, TGFα), upon platelet activation following interaction of platelets with a damaged vessel wall. In the case of an intact artery, the liberated serotonin may interact with the vascular endothelial cells, inducing them to produce "endothelial derived relaxing factors," which will lead to relaxation of the smooth muscle cells surrounding the endothelium. However, if the endothelium is damaged, serotonin gains direct access to the smooth muscle cells, causing them to contract (see Ref. 5 for review). Nemecek *et al.* [2] showed that, in addition to these vasoactive effects, serotonin stimulates the proliferation of vascular smooth muscle cells in synergy with PDGF and insulin, suggesting that it may play an important role not only in wound healing but also in pathological situations like the formation of atherosclerotic plaques. Therefore, depending on the target cells, serotonin can act as a neurotransmitter, vasoactive agent and growth factor.

Neurotransmitters and vasoactive agents: a new class of growth factors acting through G protein coupled receptors

Serotonin is not the only hormone showing this surprising range of biological activities. In recent years, a number of neuropeptides have been shown to promote cell growth: often cited substances are bombesin, bradykinin and vasopressin [6, 7]. An essential factor in the blood clotting process, thrombin, is a potent mitogen for fibroblasts, smooth muscle and endothelial cells [8–10]. These molecules have in common the ability to activate phosphoinositide breakdown, and/or inhibit adenylate cyclase (AC) [7, 11–18]. Recent evidence suggests that activation of PI specific phospholipase C (PLC) and inhibition of AC are mediated by different receptors [19, 20] coupling differentially to regulatory G proteins, which are supposed to control PLC [21] or AC [22] activity. Hormone receptors interacting with these regulatory proteins seemingly have a common structure, resembling the rhodopsin "prototype" [23]: they are large monomeric molecules of about 50 kD thought to integrate into the plasma membrane through seven membrane spanning α-helices. The primary structures of the three serotonin receptors cloned so far (5-HT_{1c}, 5-HT_2, 5-HT_{1a}) fit exactly into this family [24–26].

On the other hand, the "classical" growth factors EGF, PDGF, FGF and IGF_1 act through receptor tyrosine kinases [27]. They do not activate or inhibit AC and in most cell systems they do not induce significant hydrolysis of PIs [28]. Although a stimulatory effect of PDGF on PI breakdown has often been reported (which is probably at the origin of the vascular effects of the growth factor [1]), it seems to be mediated by a mechanism different from the one employed by G protein coupled receptors [29, 30].

On the basis of our present knowledge, growth factors can therefore be divided into two classes utilizing different strategies of transmembrane signalling.

† Abbreviations: PDGF, platelet derived growth factor; TGFα, transforming growth factor-α; IGF_1, insulin-like growth factor 1; EGF, epidermal growth factor; FGF, fibroblast growth factor; PT, pertussis toxin; PGE_1, prostaglandin E_1; BW501C, *N*-[2-(3-chlorophenoxy)propyl]-2-phenylaminoethanimidamide; Compound 32-550, 9,10-dihydro-2-β-isopropyl-5′ α-*sec*-butyl-6′-desoxo-ergopeptin; and Compound 21-009, 4(3-*tert*-butyl-amino-2-hydroxy-propoxy)indol-2-carbonic acid isopropylester.

A pertussis toxin sensitive mitogenic signalling pathway

Depending on the cell system and the agonists studied, PLC activation was found to be totally [31], partially [13] or not at all [32] inhibited by pertussis toxin (PT). This bacterial toxin was originally discovered to enhance insulin secretion in pancreatic islets, therefore its second name "islet activating protein" [33]. The effect on islets turned out to be correlated with the toxin-induced ADP-ribosylation of "G_i", the inhibitory G protein of AC, leading to its functional uncoupling from the cyclase [34, 35]. As PT affected PLC activation in some cells, it was assumed that the postulated G protein regulating PI breakdown was in these instances a PT substrate [21]. Given the presumed importance of the PLC signalling system, the effect of PT on cell proliferation was investigated in our laboratory and in the laboratory of Dr L. T. Williams.

Both groups independently reported similar results: the mitogenic potential of either thrombin [36] or bombesin [37] was strongly antagonized by PT treatment, whereas the actions of insulin, EGF, FGF [36] and PDGF [37] remained unaffected. These data clearly demonstrated the physiological importance of G proteins in growth factor action (unless the PT effect was assumed to be G protein independent). The fact that the growth factors activating receptor tyrosine kinases proved to be independent of PT treatment elegantly confirmed the concept of the two classes of receptors using distinct mechanisms of signal transduction.

The case of serotonin

To most of us it seemed obvious that the PT-sensitive step in the action of growth factors like thrombin and bombesin was the activation of PI breakdown. However, experiments with serotonin carried out with our cell system confronted us for the first time with results indicating that PLC activation and stimulation of DNA synthesis might be separable events.

The mitogenic effect of serotonin on hamster lung fibroblasts (CCL39 cell line) was discovered during a routine screening of potential PLC agonists. The substance turned out to be a weak but reproducible activator of inositol phosphate release and soon we established that it stimulated DNA synthesis in quiescent cells if applied together with growth factors of the "tyrosine kinase class" (insulin, EGF, FGF) [4]. The effect of serotonin on cell proliferation was inhibited completely by PT, whereas PLC activation was inhibited by only 50%. Serotonin did not stimulate DNA synthesis on its own, which seemed in accordance with the relatively weak effect on PLC, when compared to thrombin. The latter is the strongest pure mitogen for CCL39 cells and its effect seemed to correlate well with PLC activation [10, 38]. Interestingly, low concentrations of thrombin behaved much like serotonin: there was no or only a weak stimulation of PI breakdown and no mitogenic effect detectable if thrombin was administered alone at concentrations below 0.1 nM, though the same doses elicited a strong synergistic DNA synthesis response if added together with FGF [38].

Whereas the thrombin receptor(s) remains mysterious, important progress has been made concerning the identification of pharmacologically distinct serotonin receptors and their coupling to second messenger systems [39, 40]. In an attempt to prove the importance of PLC activation for the effect of serotonin on cell proliferation, we used the known 5-HT_2 receptor antagonist ketanserin [41] to inhibit serotonin-induced PI breakdown, assuming that the 5-HT_2 receptor was responsible for this effect [42]. Indeed, ketanserin efficiently inhibited inositol phosphate formation, but to our surprise it did not interfere with the stimulation of DNA synthesis. Therefore, we took a closer look at the serotonin receptor pharmacology and found that the effect of serotonin on reinitiation of DNA synthesis in quiescent fibroblasts correlated with the activation of 5-HT_{1b} receptors [4], which inhibit AC via a PT-sensitive G_i protein [4, 43].

cAMP, rising like a phoenix?

About 15 years ago, cyclic AMP (cAMP) was indeed considered to be an important regulator of cell proliferation. In many cell types, agonists that increased cAMP formation were found to inhibit cell growth, whereas any decrease of cAMP seemed to favour proliferation [44]. However, the concept was quickly abandoned when it was realized that the contrary was true for other cell types like Swiss 3T3 mouse cells and various epithelial cells in culture [45, 46]. It was difficult to imagine that nature employed completely opposing strategies to regulate cell proliferation in different cell lines.

CCL39 fibroblasts, like bovine aortic smooth muscle cells [47], belong to the first category of cells: agents stimulating AC strongly inhibit cell proliferation independently of the growth factor used to stimulate it [48]. Therefore, the effect of serotonin on AC could have significance: it might counteract a "tonic inhibition" exerted by basal cellular cAMP levels. Unfortunately, it is not easy to test this hypothesis by artificially decreasing the cAMP content of the intact cell, without using hormones.

If one assumes cAMP or rather decreased cAMP like the second messenger of serotonin action on cell growth in CCL39 fibroblasts, one encounters an important conceptual problem: upon serotonin addition, total cellular cAMP content measured by radioimmunoassay decreases by not more than 15%.* This variation seems insufficient to serve as a biochemical signal; however, in cellular subcompartments, variations may be more significant. It is noteworthy that maximal light-stimulation of retinal rod outer segments leads to a similar (10–15%) reduction of total cGMP measured in these organelles as a whole, yet it is established that cGMP regulates the Na^+ channels determining the cell response, acting locally on a very small membrane segment [49].

However, even though cAMP may be the second messenger of serotonin action in CCL39 cells, we tend to favor the idea that another, so far unknown messenger system is activated by a PT-sensitive G_i.

* I. Magnaldo, unpublished observations, cited with permission.

The measured effect of serotonin on AC would be another correlate, but not at the origin of the mitogenic response. In the platelet [50] and in pancreatic β-cells [51] it has been established that agonists (e.g. epinephrine) causing inhibition of AC do not exert their biological effects through a decrease in cAMP.

Interestingly, all the growth factors activating PLC and stimulating DNA synthesis also inhibit AC in the different cell systems where they are active [14], including Swiss 3T3 cells [18]. These cells, which respond positively to any increase in cAMP content, may be very useful to demonstrate the existence of an effector system different from AC: intracellular cAMP could be clamped to relatively high levels using PGE_1; if a pure G_i-agonist remained mitogenic under these conditions, a signal transduction mechanism different from cAMP would need to be postulated.

Several different biochemical systems in addition to PLC and AC are known or are supposed to be regulated by G proteins. Ionic channels and other phospholipases, as well as tyrosine kinases, have received attention recently [18, 52–54]. Part of our future work will be aimed at an evaluation of these different possibilities.

Serotonin action in non-rodent cells

The $5\text{-}HT_{1b}$ receptor stimulating DNA synthesis in hamster fibroblasts seems to be specifically expressed in rodents. In bovine or human cells, it was never observed. However, a pharmacologically different but functionally equivalent receptor ($5\text{-}HT_{1d}$ subtype) may be expressed at its place [55]. This receptor has been shown to inhibit AC [56], and it is tempting to speculate that it mediates a cell proliferation response analogous to the one elicited by $5\text{-}HT_{1b}$ receptors. Indeed, the relative potencies of the two antagonists used by Nemecek *et al.* to interfere with serotonin mitogenicity in bovine aortic smooth muscle cells [2] support this hypothesis. BW501C is a very potent antagonist of $5\text{-}HT_{1c}$ and $5\text{-}HT_2$ receptors exhibiting IC_{50} values in the nanomolar range, whereas it is much less potent on $5\text{-}HT_{1d}$ sites (IC_{50} above 1 μM). Compound 32-550, on the other hand, shows micromolar affinities for all three receptor subtypes.* Therefore, the fact that 32-550 is more potent than BW501C in inhibiting [^{3}H]thymidine incorporation [2] is in agreement with $5\text{-}HT_{1d}$ receptors mediating the proliferative response to serotonin in the bovine aortic smooth muscle cells studied.

Extrapolations

From our studies on the mitogenic effect of serotonin in hamster fibroblasts two important questions emerged: first, would any agonist stimulating G_i give rise to a serotonin-like stimulation of DNA synthesis? Second, what is the role of PLC activation in growth factor signal transduction; does strong activation (thrombin in CCL39 cells) stimulate DNA synthesis, though weak activation (serotonin) does not?

To approach these problems, we recently

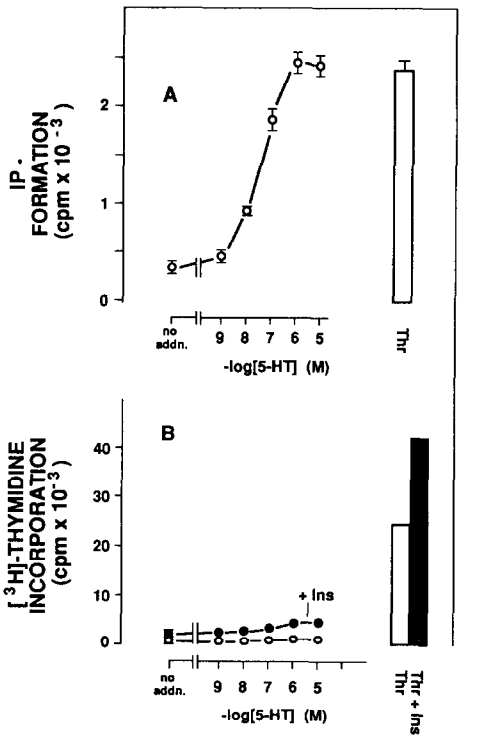

Fig. 1. Stimulation of inositol phosphate formation (A) and DNA synthesis (B) by serotonin (○, ●) and thrombin in CCL39 cells transfected with $5\text{-}HT_{1c}$ receptors. The experiments were carried out as described in Ref. 4. In A, incubation times in the presence of agonists were 3 min. Thrombin (Thr) was used at a concentration of 10 nM, and insulin (B: ●) at 5 μg/mL. Error bars indicate the range (N = 2) or the standard error of the mean (N = 3). (Experiments depicted in figure were performed by C. Kahan, Centre de Biochimie du CNRS, and included with her permission.)

expressed human α_2-adrenergic receptors ($H\alpha_2$; [57]), negatively coupled to AC, and $5\text{-}HT_{1c}$ receptors [24], stimulating PLC, in CCL39 fibroblasts. As anticipated, the α_2-agonist clonidine behaved like serotonin in cells expressing functional $H\alpha_2$ receptors; we observed inhibition of AC, only a weak stimulation of PLC, and synergistic interaction with FGF in the DNA synthesis reinitiation assay (Seuwen K, Magnaldo I, Kobilka B, Lefkowitz RJ and Pouysségur J, unpublished observations). On the other hand, functional expression of $5\text{-}HT_{1c}$ receptors did not lead to a significant increase of the proliferative response to serotonin. Some data demonstrating the effects of $5\text{-}HT_{1c}$ receptor expression in CCL39 cells shall be discussed briefly. The potencies of serotonin and thrombin to stimulate PLC and DNA synthesis reinitiation in quiescent CCL39 cells transfected with $5\text{-}HT_{1c}$ receptors are compared in Fig. 1. Although serotonin elicited a PLC response as strong as thrombin in these cells, it did not stimulate significantly DNA synthesis and, even when tested in synergy with Insulin, [^{3}H]thymidine incorporation remained well below that obtained with thrombin alone. The non-effect of serotonin on cell proliferation compared to thrombin apparently cannot be attributed

* D. Hoyer, unpublished data, cited with permission.

to a qualitatively or quantitatively different PLC response. Both agents led to a similar production of IP_2 and IP_3 inside cells as well as to Ca^{2+}-release from intracellular stores (data not shown). When tested together with FGF in the DNA synthesis reinitiation assay, serotonin evoked the same synergistic effect that is already known from untransfected CCL39 cells [4]. However, whereas in wild type fibroblasts this response is entirely blocked by the 5-HT_{1b} antagonist 21-009 [4], here a small fraction remained 21-009 insensitive but inhibitable by the 5-HT_{1c} antagonist mianserine (Kahan C, Seuwen K and Pouysségur J, unpublished observations). This indicates that strong activation of PLC can indeed generate a proliferative response which is small but qualitatively comparable to the one elicited by activation of 5-HT_{1b} receptors.

Our data suggest that the known G protein-mediated biochemical effects elicited by thrombin, activation of PLC and inhibition of AC [13, 14], both mimicked by serotonin in the 5-HT_{1c} receptor transfected CCL39 cells, are not sufficient to explain its mitogenicity. Therefore, thrombin does probably use yet other transmembrane signalling mechanisms. At present, we cannot exclude the possibility that it activates a receptor tyrosine kinase or a G protein-activated kinase.

Studies carried out in our laboratory on CCL39 cells transfected with the human muscarinic M1 receptor (HM1; [58]) yielded results qualitatively identical to the ones presented above concerning the functional expression of the 5-HT_{1c} receptor. In these cells, carbachol proved to be a more potent stimulator of PI hydrolysis than thrombin; however, only very weak effects on cell proliferation could be observed (Seuwen K, Kahan C and Pouysségur J, unpublished observations).

Our studies on $H\alpha_2$ and 5-HT_{1c}/HM1 transfected fibroblasts have confirmed that activation of G_i, as measured by its interaction with AC, gives rise to an important mitogenic signal amplifying the response of CCL39 cells to growth factors that activate receptor tyrosine kinases. On the other hand, we conclude that the role of PLC in growth factor signal transduction may have been overestimated in recent years. It seems quite possible that this enzyme is specialized to mediate rapid responses like contraction and exocytosis, but plays only a minor role in the regulation of normal cell proliferation. Work carried out in other laboratories, again on serotonin, support this hypothesis. In NRK cells, serotonin strongly activates PLC [59], but does not stimulate DNA synthesis.* Kavanaugh *et al.* [3] recently reported that PLC activation by serotonin in bovine aortic smooth muscle cells is independent of PT treatment, whereas the DNA synthesis response is blocked completely under the same conditions. Clearly, differences between cell types may exist. In CCL39 cells, phorbol esters added alone or in combination with growth factors do not stimulate DNA synthesis (unpublished), though in Swiss 3T3 [60] cells they are active. In the latter cells, PLC stimulation may therefore be mitogenic due to activation of the protein kinase C.

G protein coupled receptors as oncogenes

Several viral oncogenes are known to code for aberrant growth factor receptors. Without exception, all belong to the class of receptor tyrosine kinases [27]. So far, no viral transforming gene coding for a G protein coupled receptor has been identified, suggesting that the introduction of a receptor of this kind into a target cell is usually not a successful strategy to trigger its proliferation. However, using a very sensitive cell transformation assay, an "oncogene" (called *mas*) has been isolated from human tumor cell DNA [61], which was later found to encode an angiotensin receptor [62] activating PLC. Unlike in the case of other oncogenes like *ras*, the transforming capacity of *mas* is not dependent on one or more mutations in its primary structure, but seems to be correlated with strong expression [61]. In spite of the growth-deregulating properties of *mas*, however, angiotensin is not a strong growth factor for NIH 3T3 cells expressing the gene [62]. Interestingly, a comparable situation was encountered by Julius *et al.* [63], who studied the effects of 5-HT_{1c} receptor expression in NIH 3T3 cells. Serotonin-dependent focus formation was observed, and focus-forming cells injected into nude mice readily gave rise to tumors. Tumor cells isolated after *in vivo* passage, however, formed foci independently of the presence of serotonin. This result suggests that the presence of the receptor is important during an initiation stage of tumor development, whereas *in vivo* tumor growth requires additional growth-deregulating lesions, rendering the cells largely autonomous. Although it is likely that PLC activation plays an important role during this initiation phase, the possibility cannot be excluded at the moment that other G protein controlled effector systems become affected under conditions of receptor overexpression. Whatever the exact mechanism of action, the results obtained with the *mas* gene and the 5-HT_{1c} receptor show that strong expression of these PLC coupled receptors in a permissive context (e.g. NIH 3T3 cells) can deregulate cell growth, although their ligands do not act as strong growth factors. It will be interesting to compare the transforming potential of receptors negatively coupled to AC, like 5-HT_{1b}, in the NIH 3T3 cell system.

Conclusions

The well-developed pharmacology of serotonin receptors has allowed the demonstration that serotonin-induced stimulation of DNA synthesis in fibroblasts can take place in the absence of a detectable activation of PI breakdown. The receptor responsible for the mitogenic effect in hamster fibroblasts ($5HT_{1b}$) is negatively coupled to AC. We hypothesize that 5-HT_{1d} receptors represent the functional analogs of 5-HT_{1b} receptors in human cells. The coupling of these receptors to G_i proteins explains the sensitivity of serotonin-induced DNA synthesis to PT treatment. We are convinced that the importance of this "G_i-pathway" will be confirmed in the near future by studies on other growth factors acting through G proteins in a variety of cell types.

* Y. Takai, personal communication, cited with permission.

In the hamster fibroblasts we have studied, even strong activation of the PLC signalling pathway is not sufficient to trigger a notable proliferative response, suggesting that the mitogenic potential of a number of growth factors activating PI turnover, notably thrombin, must be attributed to other, so far undefined biochemical events. Therefore, an important aspect of future work will be to establish the exact nature of the signalling pathway(s) activated by (PT-sensitive) G proteins. Several distinct G protein α subunits have been identified by molecular cloning [64, 65] and so far their specificity of interaction with receptors on the one hand and effector enzymes on the other hand is largely unknown. Serotonin will certainly continue to play an important role in these studies.

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