

Selective protein kinase inhibitors block head-specific differentiation in hydra

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Abstract

Several studies have suggested that morphogenesis and patterning in hydra are regulated through pathways involving protein kinase C (PKC). Nevertheless, the complete signal system for regeneration in hydra is still not completely understood. Using inhibitors of different signalling pathways we are dissecting this system. We found that sphingosine (2 μ M), staurosporine (0.1 μ M), PP1/AGL1872 (1 μ M) and H7 (25 μ M) were able to inhibit head but not foot regeneration. The inhibition was reversible. When the inhibitor was replaced with hydra medium the animals continue their regeneration in a normal way. The exception was PP1/AGL1872, in this case the animals regenerated only one or two tentacles. These results imply that head and foot regeneration are independent processes and they are not directly related as has been proposed. Sphingosine and PP1/AGL1872 inhibit the transcription of *ksI*, an early regeneration gene, at 24 and 48 h of treatment. Sphingosine 2 μ M arrested the cells on the G1 phase of the cell cycle, but 1 μ M of PP1/AGL1872 did not. The regeneration was not affected if the animals were exposed to inhibitors of human growth factor receptors. We propose that head regeneration in hydra may be regulated at least by two pathways, one going through PKC and the other through Src. The first pathway could be related to cellular proliferation and the second one to cellular differentiation. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Hydra; Head regeneration; Pattern formation; Signal transduction; Protein kinase inhibitors

1. Introduction

Because of its high capacity of regeneration, hydra, a freshwater coelenterate, is a suitable system for studying the morphogenetic processes, not only in developing but also in regenerating animals [1,2]. Hydra are made of a cylindrical structure of two layers of epithelial cells, with the head at one end and the foot at the other. Asexual budding occurs at a fixed region of the lower body column. Two pairs of developmental gradients have been described in hydra, one for the head and one for the foot [3]. If head or foot is removed from the animal, it is able to regenerate either at the appropriate end; even dissociated cells centrifugated into aggregates

can reform the tissue layers, regenerate heads and feet, and will eventually separate into complete animals [4]. Several lines of evidence have implicated a transduction system mediated by protein kinase C (PKC) for head regeneration in hydra. If PKC activators (e.g., the tumour promoter, 12-O-tetradecanoylphorbol-13-acetate, TPA; diacylglycerol, DAG and arachidonic acid, AA) are applied periodically, the animal will develop supernumerary head structures and will lose the foot regeneration capacity [5–7]. Conversely, long-term exposure of hydra to lithium ions produce ectopic feet [8,9]. The activation of PKC by TPA results in enhanced transcription of a head-specific gene, *ksI*; an increased amount of transcript was detected within 1 h [10]. Two PKC genes have been cloned in hydra, which encode members of the classical (cPKC) and a novel (nPKC) families [11,12]. They found that regeneration is accompanied by an increase in the amount of PKC in both soluble and particulate fractions but that head regeneration is

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specifically associated with an increase in the activity of the membrane-bound enzyme. It is clear that PKC has a central role during regeneration, but it is still not known if other signal transduction pathways are involved. The signals which activate PKC are also unknown. Because of the redundancy and overlap of the different signalling pathways, it could be difficult to assign precise roles to particular components of these pathways. The use of inhibitors has been very useful for the dissection of specific signalling pathways. To know which components are implicated in the transduction system for head development in hydra, we have tested several inhibitors. They include sphingosine, staurosporine and H7, selective PKC inhibitors [13–15]; herbimycin A, a tyrosine kinase inhibitor [16]; PP1-AGL1872, an inhibitor of the Src-family of tyrosine kinases [17]; AGL1296 a PDGFR inhibitor *in vitro* [18]; and AGL1478 an EGFR inhibitor *in vitro* [19]. In these experiments the cell cycle, PKC activity and head-specific gene expression were also evaluated.

2. Materials and methods

2.1. Animals and reagents

Hydra vulgaris (strain Basel) and *Hydra magnipapillata* (wt strain 105) were cultured using standard procedures [20]. Epithelial hydra (derived from 105 by colchicine treatment) were cultured by force-feeding [21].

D-Sphingosine, Herbimycin A and Staurosporine, were from Sigma; H7, AGL1478 and AGL1296 were from Calbiochem. PP1/AGL1872 was synthesized by R. Wetzker. BrdU and diaminobenzidine were from Sigma. Anti BrdU antibody was from Becton-Dickinson. Reagents for *in situ* hybridization were from Boehringer Mannheim (Germany). PKC assay system was from Gibco. Solvents and other common chemicals were from Merck.

2.2. Inhibition experiments

2.2.1. Head and foot regeneration

Ten polyps starved for 48 h were decapitated at 2/3 from the foot and cut at the lower peduncle to remove foot. They were immediately incubated in 5 ml of hydra medium plus inhibitor. Control animals were incubated in hydra medium plus the solvent in which the inhibitors were dissolved. The medium was replaced every 12 h and regeneration was followed microscopically for 3 days, after this time the controls completely regenerated the head. The percentage of inhibition was calculated from the number of animals which did not regenerate at this time. After this time the polyps were incubated in hydra medium for 3 more days to see if the effect of the inhibitor was reversible or not.

2.2.2. Foot staining

Normally, foot regeneration was followed microscopically, but sometimes it was difficult to know only by morphology whether or not the foot was regenerated. The development of a new foot was monitored by diaminobenzidine staining as described by Hoffmeister [22].

2.3. Cell cycle

Epithelial polyps, starved for 48 h, were decapitated, the foot was cut and the animals were incubated for 24 or 48 h in hydra medium with or without inhibitors (2 μ M sphingosine, 1 μ M PP1/AGL1296 or 10 mM HU) [23]. They were then incubated for 4 h with BrdU 5 mM final and the epithelial cells cycle were analyzed by the method described by Holstein *et al.* [24].

2.4. Gene expression

The expression of head-specific genes (*ks1*, *hybra* and *hym-301*) and control (actin) was detected by *in situ* hybridization. 20 polyps starved for 48 h were decapitated and incubated in hydra medium with or without inhibitors for 24 and 48 h. The animals were anesthetized in 2% urethane and fixed in 4% paraformaldehyde, both in hydra medium. The *in situ* hybridization was done as described [25].

2.5. PKC activity

Thirty polyps for each assay were decapitated and incubated with the inhibitor for 4 h. The PKC activity was measured with a PKC assay system from Gibco according to the instructions from the providers.

3. Results

3.1. Inhibition experiments

The development of hydra seems to be regulated by two pairs of morphogenetic gradients, one that leads to head formation at one end and the other that leads to foot formation at the opposite end. The molecular basis of such gradients is largely unknown, but current data point to protein kinase C (PKC) as a key regulator of the head formation. To investigate additional signals which may control the pattern, we used different kind of inhibitors. They can be grouped in three classes, inhibitors against: (1) human growth factor receptors, (2) PKC and (3) Src tyrosine kinase.

First, to obtain the minimal inhibitory concentration (IC) for head or foot regeneration we tested several concentrations of the chemicals. Once the IC for each inhibitor was obtained, hydras were treated for 3 days. After that, the animals were further cultured for 3 more days in medium without inhibitors to examine if the effect was reversible.

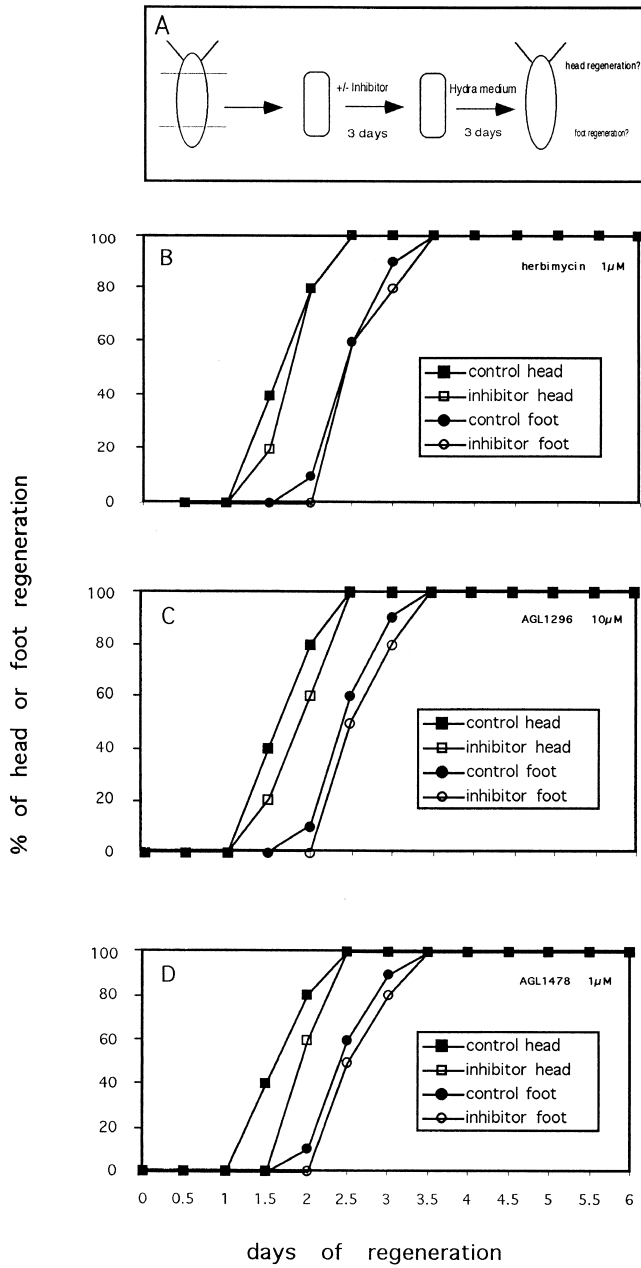


Fig. 1. (a) The animals were allowed to regenerate in medium plus inhibitor for 3 days and another 3 days without it. Effect of herbimycin 1 μ M (b) AGL1296 10 μ M (c) and AGL1478 1 μ M (d) on head and foot regeneration of hydra.

3.1.1. Human growth factor receptor inhibitors did not have an effect on regeneration of hydra

We tested three different inhibitors, herbimycin A, which inhibits the receptor associated tyrosine kinase, from 100 nM to 10 μ M; AGL1296, a PDGFR inhibitor, from 1 μ M to 50 μ M; and AGL1478, an EGFR inhibitor, from 500 nM to 10 μ M. In Fig. 1 we show a typical example for the three inhibitors. None of them were able to inhibit or delay the regeneration of hydra. The

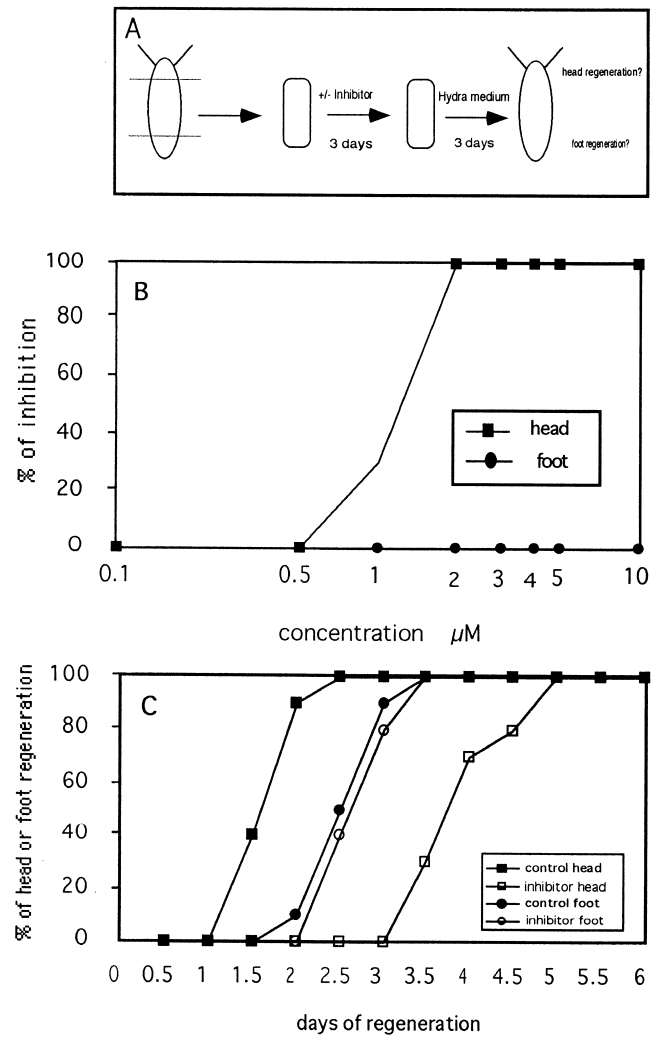


Fig. 2. Effect of sphingosine on head and foot regeneration of hydra. (a) The animals were allowed to regenerate 3 days in medium containing different concentrations of the inhibitor (b) or with 2 μ M for 3 days and then 3 days without inhibitor (c).

treatment with AGL1478 seems to produce a delay of 12 h on head regeneration (Fig. 1d), but in other experiments (even using high concentrations) this behaviour was not always reproducible.

3.1.2. PKC inhibitors repress head but not foot regeneration

Three different PKC inhibitors were used, sphingosine, the natural inhibitor, from 0.1 μ M to 10 μ M; staurosporine from 1 nM to 100 nM and H7 from 0.5 μ M to 100 μ M. The three inhibitors were able to block selectively the head regeneration and they did not have any effect on foot formation, this can be seen in Fig. 2b for sphingosine. The IC that we found was 2 μ M for sphingosine (Fig. 2b), 100 nM for staurosporine and 25 μ M for H7. When higher concentrations were used the animals disintegrate between 12 and 24 h. During the

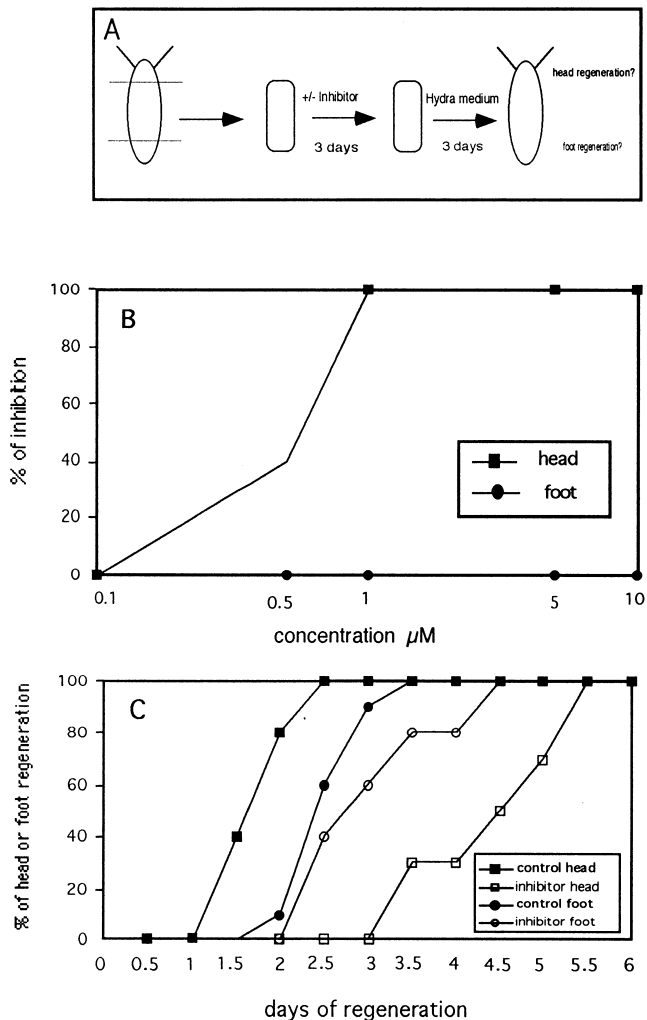


Fig. 3. Effect of PPI/AGL1872 on head and foot regeneration of hydra. (a) The animals were allowed to regenerate 3 days in medium containing different concentrations of the inhibitor (b) or with 1 μM for 3 days and then 3 days without inhibitor (c).

time of incubation of the animals with the inhibitors, they looked fine, it was not possible to see any difference with the controls (Fig. 4a) and we did not find any ectopic structure or different number of tentacles. After the removal of the chemical, the animals regenerate in a normal way indicating the effect of the three inhibitors was completely reversible (Fig. 4b), and the kinetics was similar to the one shown by the controls. This can be seen on Fig. 2c for sphingosine, but the behaviour of the other two compounds was the same.

Sphingosine 2 μM was able to inhibit head development during 3 days of treatment. It is the natural inhibitor of PKC and competes with DAG for a place in the enzyme. Next we examined how long it was able to inhibit head regeneration. We treated decapitated animals for several days, replacing the inhibitor every 12 h.

We found that sphingosine could inhibit head regeneration up to 84 h more or less, since after 96 h of treatment the hydras begin to develop the new head (data not shown). Interestingly, sphingosine does not inhibit bud formation.

If PKC is really involved in the pathway of head regeneration, there must be a specific period at which the enzyme is inhibited. After this period, the presence of the inhibitor must not have any effect. Animals were incubated with 2 μM sphingosine after 0, 2, 4, 6, 8, 10, 12 and 24 h post decapitation. Under this condition only the hydras incubated at time 0 with the inhibitor were completely inhibited. The animals treated between 2 and 8 h showed a delay on regeneration of around 24 h and the ones treated after 10 h were almost not affected (data not shown).

The direct effect of sphingosine on PKC activity was observed by measuring the activity of the enzyme after 4 h of regeneration under inhibitory conditions (2 μM) and in non-treated animals. We measured particulate and soluble PKC activity and we found that particulate PKC activity was reduced 48% in hydras treated with sphingosine. But there was no difference in the soluble PKC activity between control and treated hydras (data not shown). The level of phosphorylation was also measured on proteins and phospholipids. A diminution of 34% in ^{32}P incorporation to total phospholipids was detected in treated animals. There was also an important diminution in total protein phosphorylation due to the sphingosine treatment (data not shown).

3.1.3. Src protein kinase inhibitor

Hydra has a *src* gene and three *ras* genes [26,27, Guaderrama et al. in preparation]. The gene *ras2* is regulated developmentally through a head signal [27], suggesting that the Src-Ras signal transduction pathway could be involved in head formation. We tested this possibility using the Src inhibitor PPI/AGL1872.

The dose-response curve for this inhibitor is shown in Fig. 3b. We chose 1 μM to carry out our inhibition experiments, since at this concentration it was able to inhibit head but not foot development (Fig. 3c). It is possible that at this concentration the inhibitor could have effects other than head inhibition, because the animals were contracted all the time the inhibitor was present (Fig. 4c) but we did not see any other effect microscopically; ectopic structures were never detected, for example. Concentration lower than 1 μM did not have any effect (100 nM) or produce a delay on head regeneration (500 nM). At 3 μM or higher concentrations the inhibitor was toxic for the animals. The effect of the inhibitor was not completely reversible, after the treatment the animals regenerate heads with less tentacles than the controls (Fig. 4d). Also, the kinetics of regeneration after the treatment was different (Fig. 3c).

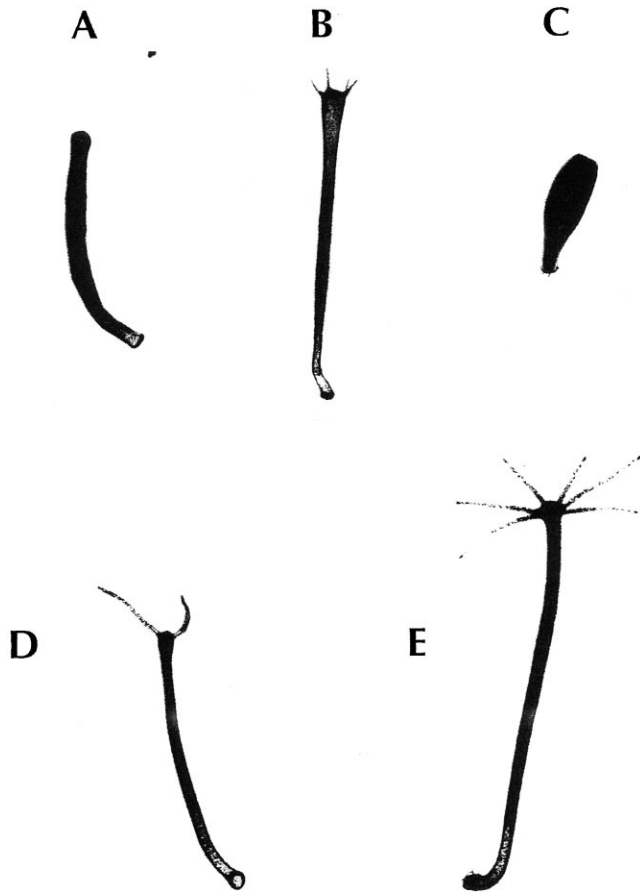


Fig. 4. Effect of the inhibitors on head regeneration of *H. vulgaris* polyps. Photographs of regenerating animals treated 72 h with 2 μ M sphingosine (a) or 1 μ M AGL1872 (c) and control animals (e). After the treatment the hydras were allowed to regenerate for 72 h in inhibitor-free medium (b and d).

This inhibitor also blocks bud formation (data not shown).

3.2. Effect of the inhibitors on the regeneration of epithelial polyps

Hydra has approximately eight well-defined cell types, but with only two of them (ectoderm and endoderm) it is able to reproduce asexually and to regenerate. The analysis of the signalling pathways working on head regeneration should be easier on these animals. From the inhibitor results it is possible that there are at least two signal transduction pathways working during head regeneration, one sensitive to sphingosine and the other to AGL1872. We tested whether they are working in the same way on epithelial animals.

Epithelial polyps were treated with sphingosine 2 μ M and AGL1872 1 μ M, as control, the parental strain 105 was used. Sphingosine and AGL1872 inhibited head regeneration on control animals (Fig. 5b and c). Interestingly, sphingosine did not have an effect on the regener-

ation of epithelial hydras (Fig. 5e), but AGL1872 did (Fig. 5f).

3.3. Effect of the inhibitors on the cell cycle

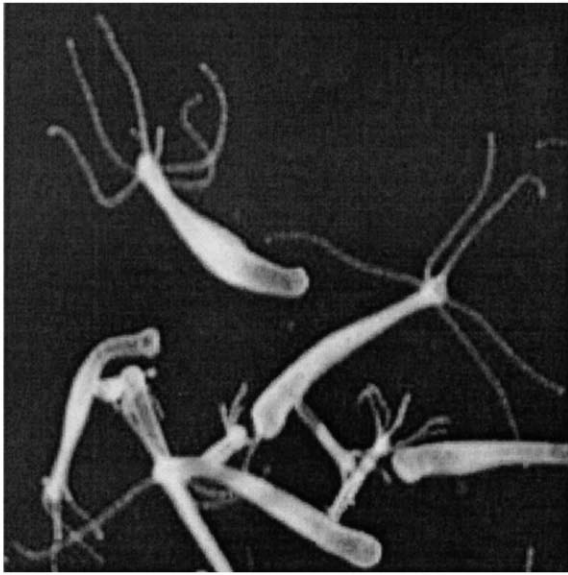
PKC regulates the cell cycle at the G1/S transition [28, 29]. It was expected that sphingosine blocks head regeneration in hydra arresting the cells at G1, as well as other effects. Hydroxyurea had been used to arrest the cells on the S phase in hydra and demonstrated that under such conditions the animals are able to regenerate [23, 30].

BrdU has been used to label hydra cells on the S phase in whole mounts and macerates [31]. We tested whether one of the effects of AGL1872 could be to block the cell cycle as sphingosine does. We found no difference in the incorporation of BrdU between control and AGL1872 treated on epithelial hydras (Fig. 6a–c and d–f), but the animals treated for 48 h with HU had almost no cells labelled (Fig. 6h). We found the same effect on foot development (data not shown). The lack of effect of AGL1872 on the cell cycle was more evident when the number of labelled cells were counted. We did not find significant differences between control and AGL1872-treated animals, but the HU-treated hydras lost almost 50% of the label, even after 24 h of treatment (data not shown).

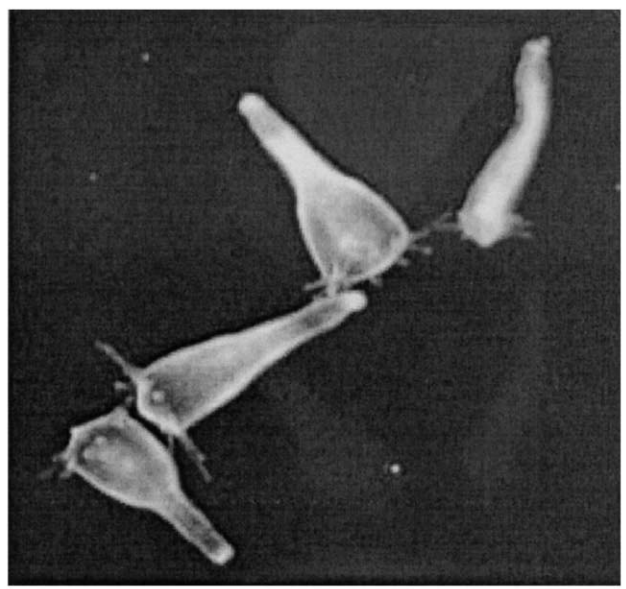
3.4. Sphingosine and AGL1872 inhibit the expression of head-specific genes

It has been shown that PKC activators, such as TPA or DAG, change the expression patterns of head-specific genes [10,32]. The main effects are an increase in the expression of the genes in 5–10 fold, but most importantly, the activators induce the expression on body regions where they normally were not expressed.

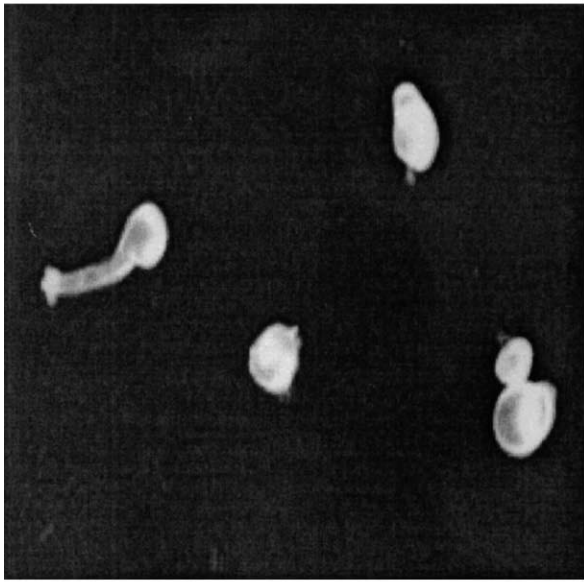
To know if the inhibitors were able to hamper head-specific gene expression we made *in situ* hybridization for *ks1*, *hybra-1* and *hym301*. *ks1* is an early marker of head formation [10], *hybra-1* is also an early gene of head formation but restricted to the hypostome of the adult [33] and *hym301* is a gene expressed in the tentacle forming zone and around the tentacles in the adult (Hatta *et al.*, unpublished observation). Fig. 7 shows the expression of *ks1* during head regeneration and it can be seen that sphingosine 2 μ M (Fig. 7c) and AGL1872 1 μ M (Fig. 7d) were able to inhibit *ks1* expression after 48 h of treatment. Fig. 8 shows the effect of AGL1872 on the expression of other two head specific genes, *hybra-1* (Fig. 8a and b) and *hym301* (Fig. 8c and d), and it is clear that besides the inhibition of Src there is a strong inhibitory effect on the expression of both genes. Any of the inhibitors had an effect upon *actin* gene expression (data not shown).



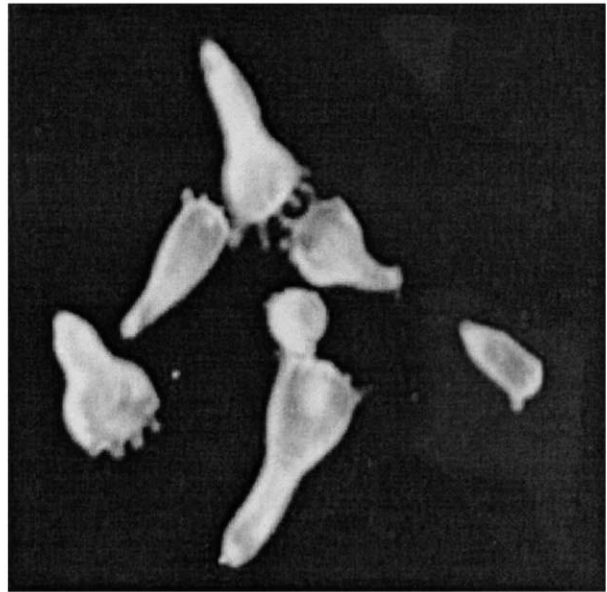
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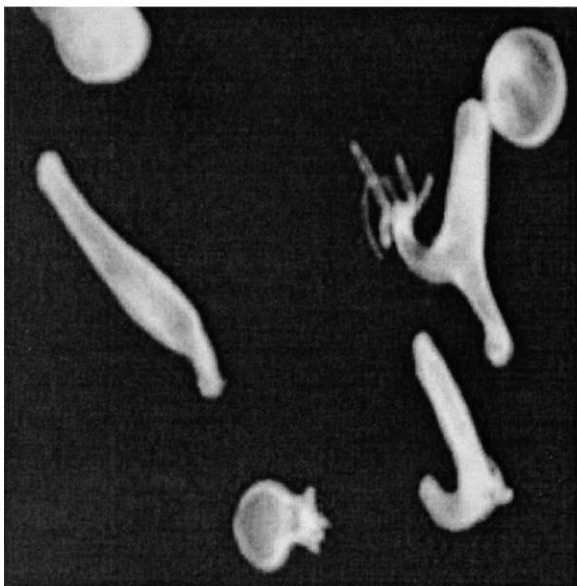
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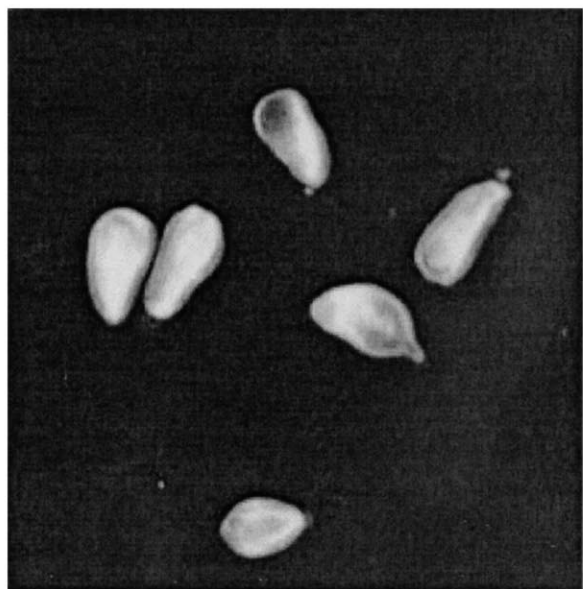
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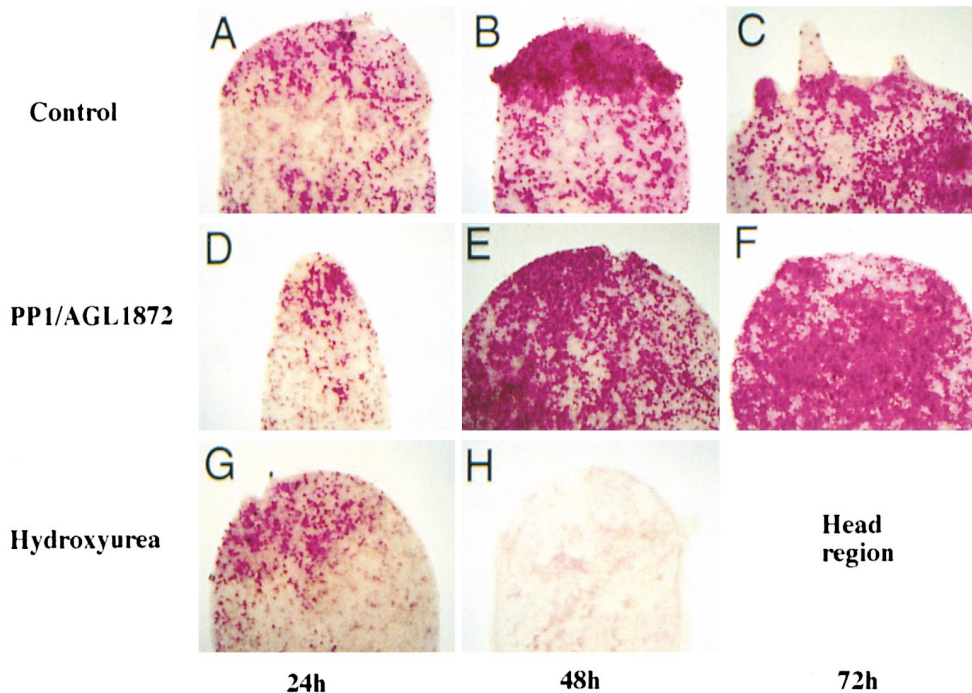


Fig. 6. Polyps of *H. magnipapillata* strain 105 epithelial were decapitated and allow to regenerate in inhibitor-free medium (a–c), plus 1 μ M AGL1872 (d–f) or 10 mM HU (g and h) during 24 h (a, d, and g), 48 h (b, e, and h) or 72 h (c and f) and labelled with BrdU as described in material and methods.

4. Discussion

Several results suggest PKC as key mediator in head regeneration and on pattern formation in hydra [5–7]. Three genes encoding PKC enzymes have been cloned and the pattern of expression and changes in the activity of the enzymes suggest their active participation during head formation [11,12]. Our results demonstrate that, using specific inhibitors of PKC, head regeneration is inhibited. At least for sphingosine, the low concentration used and the specificity of the compound exclude almost completely other possible targets which could produce side effects and they were responsible of the inhibition. The participation of PKC on head regeneration was clearly shown by our results which showed that the inhibition of regeneration by sphingosine reduces almost by 50% the amount of membrane-bound activity of PKC after 4 h of treatment. Hassel *et al.* [12] found an identical correlation. The decrease in protein phosphorylation also implies a signal transduction pathway regulated by PKC like the MAPKs.

Fig. 5. Effect of the inhibitors on head regeneration on normal and epithelial polyps. *Hydra magnipapillata* strain 105 (a) and 105 epithelial (d) were decapitated and allow to regenerate in media plus 2 μ M sphingosine (b and e) or 1 μ M AGL/1872 (c and f) during 72 h. Panels b and c corresponds to strains 105 and e and f to 105 epithelial, respectively.

The level at which PKC regulates is not easy to define because of the complexity of the process we are studying. From the known effects of it, cell cycle regulation seems to be an obvious target of inhibition. In fact, there

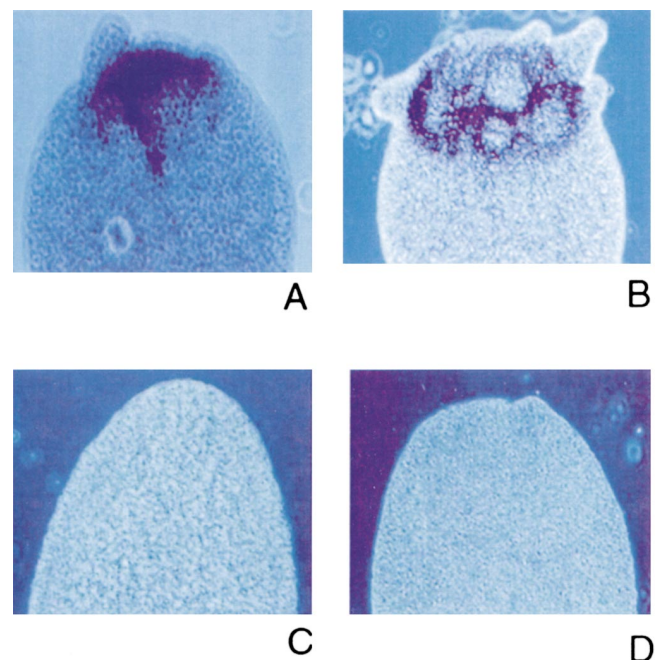


Fig. 7. *In situ* hybridization of hydra polyps. The animals were decapitated and incubated for 24 and 48 h in inhibitor-free medium (a, b), or 48 h with 2 μ M sphingosine (c) or 1 μ M AGL1872 (d). After incubation the expression of *ks1* was examined.

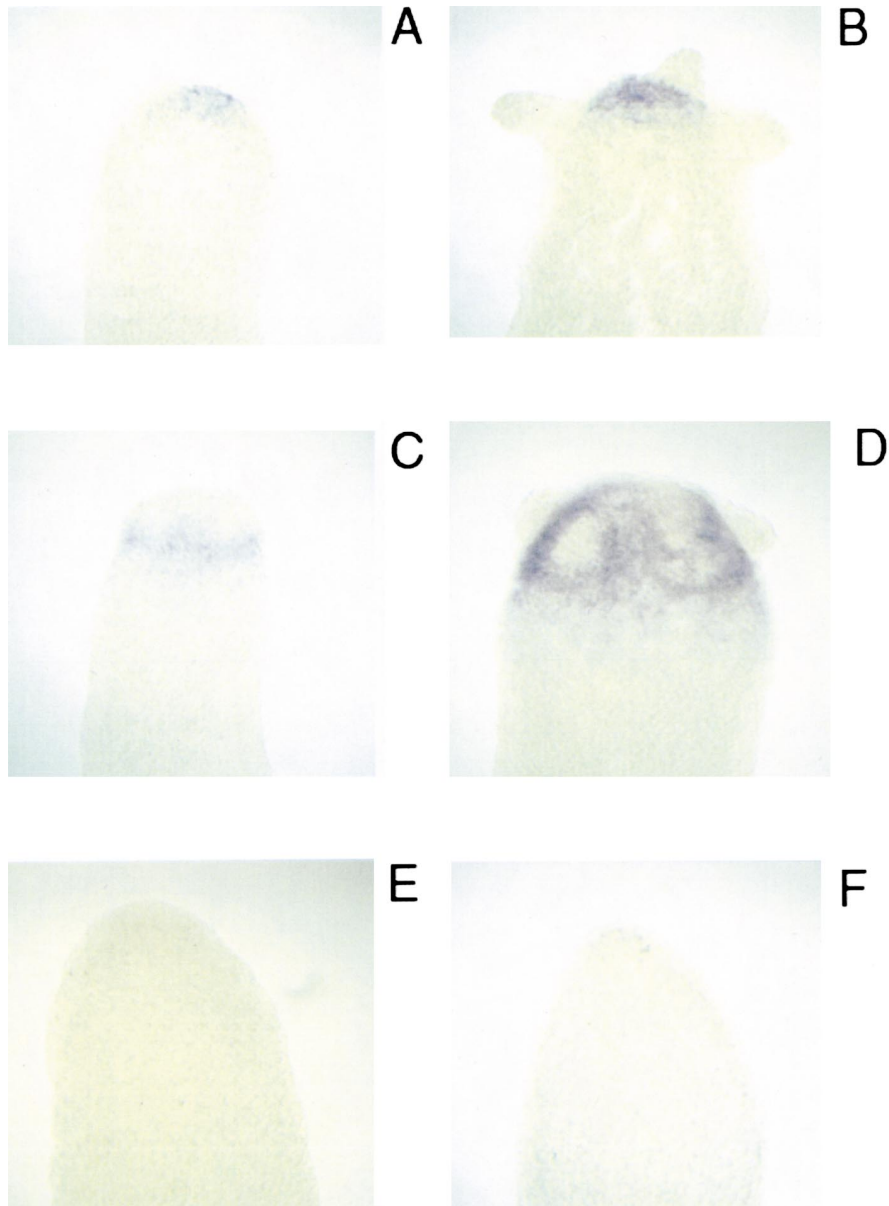


Fig. 8. *In situ* hybridization of hydra polyps. The animals were decapitated and incubated for 24 and 48 h in inhibitor-free medium (a–d) or 1 μ M AGL1872 (e, f). After incubation the expression of *hybrial* (a, b, e, and f) and *hym-301* (c and d) was examined.

is a cell cycle arrest in treated animals. However, this arrest alone can not explain the inhibition of regeneration because arresting the animals with HU did not inhibit head or foot regeneration. Obviously, there must be at least another effect outside the cell cycle arrest. The incapability of sphingosine to inhibit regeneration on epithelial hydra suggest that on these animals the other target is not inhibited. Another possibility could be that their metabolism is modified and their levels of DAG, for example, are elevated, so the amount of sphingosine used was ineffective to inhibit the enzyme. The accumulation of metabolites such as DAG could also explain why after 84 h of incubation in the presence of sphingosine, the inhibitory effect is lost and the ani-

mals regenerate following a kinetics similar to the one shown by the controls. A similar effect was demonstrated in yeast; cells treated with sphingosine were inhibited for almost 12 h. After this time the cells begin to grow and bud. The resumption of the growth was preceded for a peak of DAG (Alvarez and Cerbon, submitted).

Another evidence that PKC must have another effect comes from its ability to inhibit the expression of an early gene of head formation, *ks1*. This effect and the decrease in the levels of protein phosphorylation suggest a pathway similar to the MAPKs, at the end of it there are transcription factors which turn on genes having AP1 sites on their promoter regions [34,35]. This path-

way has not been demonstrated in hydra, but *ksI* can be activated by TPA and has at least two AP sites on its promoter region [10,36].

We have strong evidence to believe that in hydra there is another signalling pathway regulating head regeneration. This is mediated by Src since its inhibitor, AGL1872 at 1 μ M, was able to block head regeneration. It could be possible that the two signals (PKC and Src) converge at any point on the MAP kinases. This seems not to be the case, however, because AGL1872 does not produce cell cycle arrest and epithelial hydras are only inhibited by the Src inhibitor. One interesting possibility could be that the signal through Src directs differentiation and the one through PKC proliferation and perhaps differentiation. That is why both inhibitors block the expression of early and head-specific genes. This model with two signals is in accord with the fact that for head regeneration cell proliferation is necessary but dispensable, because hydra in presence of HU can regenerate even if they are arrested. It explains why the epithelial hydras can have different sensitivity to the inhibitors. It suggests also that the key signal for the development comes through Src, if it is blocked neither head regeneration nor budding can proceed.

One easy way to explain the pattern formation in hydra suggests that head and foot formation are antagonistic processes. Several studies have shown that when head regeneration is induced the foot regeneration capacity is reduced. And therefore that both events are dependent [2,37–39]. In our experiments, when head regeneration was inhibited no ectopic foot was induced. These results strongly suggest to us that both events are independent and that both the signals and transduction pathways involved must be different.

Any of the inhibitors against the growth factor receptors tested were able to inhibit head or foot regeneration. This implies that the signal for head and foot regeneration must be endogenous. This sounds logical if we think that hydra medium is composed of water and some salts. Even if, when the hydra is decapitated, some molecules are released to the medium, they are diluted. So, the possibility that this could induce a cell signal pathway is very unlikely or it will take a lot of time to reach the quantity of molecules binding to receptors to transduce the signal. After decapitation the first event that must occur is the seal of the injury, which takes between 1 and 4 h. After this, the animal knows what must be done and the expression of early genes begins. Next the pattern is established and the gradients form again. In our opinion, the new interactions between ectoderm and endoderm cells at the hypostome region mark this region as an organizer. If GAP junctions are closed, therefore the cell–cell communication is blocked, by an antibody, there is an alteration of the patterning process in hydra [40]. Then, this kind of interaction could generate the first signals for head formation

without extracellular signalling molecules. Between 0 and 2 h post decapitation is possible to inhibit head formation with sphingosine, between 4 and 8 h we can only delay the process and after 10 h sphingosine has no effect. Similar results were obtained with AGL1872. These results agree with our previous suggestion and also with the transplantation experiments done by MacWilliams [37], who reports that there is a critical time of about 6 h for head regeneration. Under this hypothesis and for the signaling system, one of the immediate targets seems to be PKC because its central role demonstrated by the use of both activators [5–7] and inhibitors (this work). One provocative suggestion which comes from our results is that an increase of the levels of DAG is an event necessary but perhaps not sufficient to direct the development of hydra. But, under certain conditions (e.g., sphingosine treatment), this endogenous metabolic signal should be enough for the animals to regenerate perhaps using the MAPKs pathway.

Acknowledgments

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References

- [1] Gierer A. *Scient Am* 1974;231:44–59.
- [2] Müller W. *Trends Gen* 1996;12(3):91–6.
- [3] Bode PM, Bode HR. Patterning in hydra. In: Malacinski GM, Bryant SV editors. *Pattern formation. A primer in developmental biology*. New York: MacMillan Publishing Co., 1984. pp. 213–41.
- [4] Gierer A, Berkin GS, Bode H, David C, Flock K, Hansmann G, et al. *Nature New Biol* 1972;239:98–101.
- [5] Müller W. *Development* 1989;105:309–16.
- [6] Müller W. *Differentiation* 1990;42:131–43.
- [7] Müller W. *Roux's Arch Dev Biol* 1993;202:70–6.
- [8] Hassel M, Berking S. *Roux's Arch Dev Biol* 1990;198:382–8.
- [9] Hassel M, Albert K, Hofheinz S. *Dev Biol* 1993;156:362–71.
- [10] Weinziger R, Salgado LM, David CN, Bosch TCG. *Development* 1994;120:2511–7.
- [11] Dev HM. *Genes Evol* 1998;207:489–501.
- [12] Hassel M, Bridge D, Stover N, Kleinholz H, Steele R. *Dev Genes Evol* 1998;207:502–14.
- [13] Nishikawa M. *Life Sci* 1986;39:1101–7.
- [14] Watson P. *Biochem J* 1988;249:345–50.
- [15] Hunn Y. *J Biol Chem* 1994;269:3125–8.
- [16] Hidaka H. *Bioch* 1984;23:5036–41.
- [17] Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, et al. *J Biol Chem* 1996;271:695–701.
- [18] Kovalenko M, Gazit A, Böhrer A, Rorsman C, Rönstrand L, Heldin CH, et al. *Cancer Res* 1994;54(23):6106–14.
- [19] Levitzki A, Gazit A. *Science* 1995;267:1782–8.

- [20] Sugiyama T, Fujizawa T. *Dev Growth Differ* 1977;19:187–200.
- [21] Nishimiya-Fujizawa C, Sugiyama T. *Dev Biol* 1993;157:1–9.
- [22] Hoffmeister S. *Roux's Arch Dev Biol* 1985;194:453–61.
- [23] Cummings S, Bode HR. *Roux Arch Dev Biol* 1984;194:79–86.
- [24] Holstein T, Hobmayer E, David CN. *Dev Biol* 1991;148:602–11.
- [25] Grens A, Gee L, Fisher D, Bode H. *Dev Biol* 1996;180:473–88.
- [26] Bosch TCG, Unger TF, Fisher DA, Steele RE. *Mol Cell Biol* 1989;9:4141–51.
- [27] Bosch TCG, Benitez E, Gellner K, Praetzel G, Salgado LM. *Gene* 1995;167:191–5.
- [28] Takuwa N, Zhou W, Kumada M, Takuwa Y. *Biochem Biophys Res Comm* 1992;188(3):1084–9.
- [29] Frey MR, Saxon ML, Zhao X, Rollins A, Evans SS, Black JD. *J Biol Chem* 1997;272(14):9424–35.
- [30] Dübel S, Schaller CJ. *Cell Biol* 1990;110:939–45.
- [31] Plickert G, Kroiher M. *Development* 1988;103:791–4.
- [32] Martinez DE, Jamrich M, Disken ML, Bode PM, Steele R, Bode HR. *Dev Biol* 1997;192:523–36.
- [33] Technau U, Bode HR. *Development* 1999;126:999–1010.
- [34] Seger R, Krebs EG. *FASEB J* 1995;9:726–35.
- [35] Whitmarsh AJ, Davis RJ. *J Mol Med* 1996;74:589–607.
- [36] Endl I, Lohmann JU, Bosch TCG. *Proc Natl Acad Sci USA* 1999;96:1445–50.
- [37] MacWilliams HK. *Dev Biol* 1983;96:217–38.
- [38] Forman BJ, Javois LC. *Dev Biol* 1999;210:351–66.
- [39] Dev MW. *Biol* 1995;167:159–74.
- [40] Fraser SE, Green CR, Bode HR, Gilula NB. *Science*. 1987;237:49–55.